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Contents

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Contents

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Volume 12 **Number** 21 **June 7**, 2006

Contents

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EDITORIAL

Role of receptor tyrosine kinases in gastric cancer: New targets for a selective therapy

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Abstract

Receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor family participate in several steps of tumor formation including proliferation and metastatic spread. Several known RTKs are upregulated in gastric cancer being prime targets of a tailored therapy. Only preliminary data exist, however, on the use of the currently clinically available drugs such as trastuzumab, cetuximab, bevacizumab, gefitinib, erlotinib, and imatinib in the setting of gastric cancer. Preclinical data suggest a potential benefit of their use, especially in combination with "conventional" cytostatic therapy. This review summarizes the current knowledge about their use in cancer therapy as well as new approaches and drugs to optimize treatment success.

Key words: Gastric carcinoma; EGFR; Gefitinib; Trastuzumab; Cetuximab; Imatinib; Erlotinib; Bevacizumab

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INTRODUCTION

Gastric cancer is a common, but difficult to treat disease entity. Apart from potentially curative surgery, chemotherapy as well as radiochemotherapy may be applied, but do not cure the disease[1]. Thus, improvement of gastric cancer therapy will depend on novel therapeutic approaches. Receptor tyrosine kinases (RTKs) are a family of 56 proteins characterized by a transmembrane domain and a tyrosine kinase motif[2]. They function in cell signalling and transmit signals regulating growth, differentiation, adhesion, migration, and apoptosis[3]. Aberrant receptor tyrosine kinase activity was initially described in various epithelial cancers; nowadays, it is well accepted that receptor tyrosine kinases play an important role in almost all types of cancer[4,5]. The mutational activation and/or overexpression of receptor tyrosine kinases transforms cells and often plays a crucial role in the development of cancers.

For this reason, RTKs have become targets of immunological inhibitors, the best known being trastuzumab (Herceptin®, directed against the erb B2 receptor, also known as Her2/Neu oncogene) and cetuximab (Erbitux®, active against the epidermal growth factor receptor [EGFR]). In the case of EGFR, pharmacological inhibitors such as gefitinib (Iresssa®) directed at the kinase domain (EGFR-TKI) of the receptor have been developed; other pharmacological inhibitors of tyrosine kinases include imatinib (Gleevec®) which is licensed for the treatment of leukaemia and gastrointestinal stroma cell tumors (GIST).

As tailored concepts of cancer therapy evolve, RTKs offer prime targets for such an individualized approach to cancer treatment. This review examines the information available on this group of receptors, describes the most relevant subsets that have been found in case of gastric cancer, and summarizes data on the use of their inhibitors in clinical studies. In the end, we present new attempts to optimize the efficacy of already available compounds and promising new drug developments.

Receptor tyrosine kinases

RTKs are membrane bound proteins consisting of a ligand-binding domain at the extracellular surface, a single transmembrane segment, and a cytoplasmic part harboring the protein kinase activity. With the exception of the insulin receptor family of RTKs, all known RTKs form monomers in the cell membrane. Ligand-induced dimerization, resulting in autophosphorylation of their cytoplasmic domains, is the major mode of activation of RTKs.

The known 56 RTKs are divided into 21 families with similar structure and the potential of intrafamilial dimerization; their classification has been reviewed by Robinson *et al*[2]. The best known examples are the epidermal growth factor receptor family (erb B1 to B4), the different vascular endothelial growth factor receptor (VEGFR) subtypes, the

fibroblast growth factor (FGF) receptor family, and the platelet derived growth factor (PDGF) receptor family.

RTKs present in gastric cancer

We have recently analyzed the expression of RTK mRNAs in different human cancers, including 12 samples of gastric cancer[6]. The median of relative gene expression levels of the 56 known RTKs in these cancer specimens are illustrated in Figure 1. Our findings suggest that several RTKs, including those of the EGFR family, the FGFR family, and the different VEGFR subtypes are present in gastric cancer and thus offer potential targets for a selective therapy. In the following, we will summarize findings by others regarding the expression of the RTKs in gastric cancer with emphasis on those for which clinically established modalities of therapeutic intervention exist, namely the EGFR family and VEGF.

EGFR family – EGFR and erb B2

The EGF receptor/ligand system seems to be involved in the regulation of gastric mucosal proliferation and progression of gastric carcinoma. Increased EGFR binding has been observed in gastric carcinomas in comparison to adjacent normal gastric mucosa. Moreover, elevated EGFR levels have been found in gastric carcinoma with worse prognostic factors (T3/4, positive lymph-nodes, G3, diffuse-type). In univariate and multivariate analysis, EGFR levels are an independent indicator of poor prognosis[7,8]. A down-regulation of an inhibitor of EGFR activation, EGFR related protein (ERP) occurs in gastric cancer as well[9]. Changes in the constitutive phosphorylation and activity of tyrosine kinases such as EGFR may contribute to differences in cell adhesion and phenotype of gastric cancer cells[10]. Espinoza *et al* described that gastric adenocarcinomas potentially depend upon the TGFalpha-EGFR autocrine loop for growth and exhibit increased aggressiveness in the presence of aberrant p53[11].

The overexpression of the growth factor receptor oncogene erb B2 in gastric carcinomas was first described by Yonemura *et al* in 1991, who reported a 12% positive rate in 189 gastric cancers[12]. These findings were confirmed by many other studies, which provided further evidence that the highest rate of overexpression was found in patients with advanced disease, and that erb B2 altered expression can be considered as an independent predictor of outcome[13,14]. Ougolkov *et al*[15] found a more frequent overexpression of erb B2 in gastric cancers with concurrent liver metastasis than in those without, and concluded that activated erb B2 may be involved in the process of liver metastasis thus suggesting a role for erb B2 overexpression in identifying gastric cancer patients who are at high risk of developing liver metastasis. In contrast, activating mutations of the erb B2 tyrosine kinase seem to be rare events in gastric cancer[16].

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is expressed in many gastric carcinoma cell lines and may play an important role in cell growth directly. VEGFR is virtually ubiquitous in human tumors and may promote tumor growth and metastasis by participating in both paracrine and autocrine pathways as higher levels of VEGFR have been correlated with more aggressive disease[17]. KDR is the main human receptor responsible for the VEGF activity in both physiological and pathological vascular development, and VEGF-KDR signalling pathway has been validated as a priority target for the development of anti-cancer therapy. A first report on 16 patients with early gastric cancer (pT1-T2, N0) suggested that expression of VEGF may indicate an increased risk of recurrence, making VEGF neutralization an interesting therapeutic target in these subjects[18].

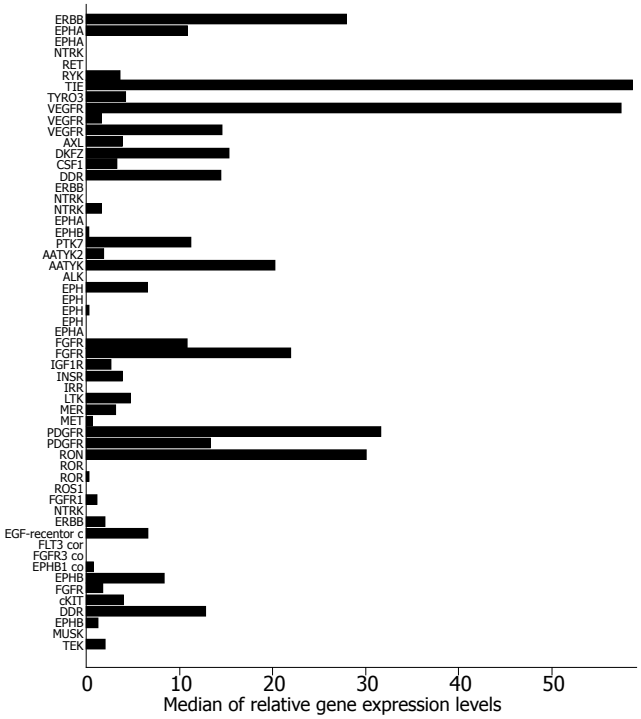

Figure 1 Median gene expression of receptor tyrosine kinases in 12 gastric cancer specimens.

Fibroblast growth factor receptor family

We have previously described an increased expression and autocrine stimulation of fibroblast growth factor (FGF) 2 mRNA in the course of gastric ulcer healing[19]; with respect to gastric cancer, the secretion of growth factors of the FGF family by fibroblasts stimulates proliferation of cancer cells of scirrhous gastric cancer[20]. The expression of FGF2 mRNA (formerly known as basic fibroblast growth factor, bFGF) in gastric cancer specimens is associated with higher microvascular density, tumor progression and a worse prognosis[21]. An orally active inhibitor of FGF-receptor autophosphorylation has been successfully tested in animal models of gastric cancer; further studies are awaited[22].

Platelet derived growth factor receptor

Already more than a decade ago, the expression of platelet derived growth factor (PDGF) and its receptor mRNAs were linked to gastric cancer growth[23]. Later on, the expression of PDGF-A mRNA in biopsy samples of gastric cancer specimens was determined to be a preoperative prognostic marker associated with shorter survival[24]. As

imatinib mesylate as an inhibitor of the PDGFR tyrosine kinase became available, its effectiveness against gastric cancer cells was assessed *in vitro* and *in vivo*. Unfortunately, the agent itself exhibited no cytostatic effect of relevance; yet in combination, imatinib mesylate might serve as an effective chemosensitizer of antitumor drugs, such as 5-FU and paclitaxel for gastric carcinoma, targeting the PDGF/PDGFR-signalling pathway of tumor cells and stromal cells in disease progression and angiogenesis[25].

Leflunomide is a small molecule inhibitor of PDGFR-mediated phosphorylation and inhibits PDGF-mediated cell signalling[26]; it is converted to its main metabolite, SU0020, which interferes with de novo pyrimidine synthesis. At this time, it is not clear whether the mechanism of action of this drug in humans is due to inhibition of PDGF-dependent signalling, inhibition of pyrimidine synthesis, or a combination of both[27-29]. A multi-institutional phase II study in hormone refractory prostate cancer patients with leflunomide found partial responses in 1 of 19 patients, a prostate-specific antigen decline greater than 50% in 3 of 39 patients, and improvement in pain[27].

Hepatocyte growth factor receptor / c-MET

Soman *et al* were able to identify TPR-MET fusion as an event of gastric cancer pathogenesis in certain cases[30]; in addition, c-MET activation seemed to occur[31]. Preliminary data raised in animal models of gastric carcinoma suggest that adenoviral-mediated gene transduction of an HGF antagonist (NK4) results in suppression of tumor growth, invasion, angiogenesis, and metastasis[32,33]. The use of this construct was able to overcome resistance against gefitinib in an animal model of scirrhous gastric cancer[34], yet clinical data is lacking.

USE OF RTK INHIBITORS IN GASTRIC CANCER

Available RTK inhibitors

The inhibitors of tyrosine kinases currently approved by the United States Food and Drug Administration (FDA) for the use in different human malignancies are summarized in Table 1. Substances under development are described in the paragraphs below. If treatment results of RTKIs are to be evaluated, one has to keep in mind that inhibition of a growth factor receptor does not typically aim at killing the cell like "conventional" cytostatics, but stoping proliferation. Therefore, standard assessments of tumor response like complete response, partial response, and stable disease may not reflect the "true" efficacy of such a regime.

Table 1 FDA-licensed tyrosine kinase inhibitors, their targets, modes of action, and clinical use

Agent	Target	Mode of action	Established clinical application
Imatinib mesylate	bcr-abl; PDGFR, c-kit	Kinase inhibitor	CML, GIST, hypereosinophilic syndrome
Gefitinib	erbB1/EGFR	Kinase inhibitor	NSCLC
Erlotinib	erbB1/EGFR	Kinase inhibitor	NSCLC
Cetuximab	erbB1/EGFR	Blocking antibody	Colorectal cancer
Trastuzumab	erbB2/HER	Blocking antibody	Breast cancer
Bevacizumab	VEGF	Neutralizing antibody	Colorectal cancer

Immunological agents

VEGF - Bevacizumab In contrast to the other immunological agents which attach directly to the receptor of the tyrosine kinase, the currently available antibody bevacizumab interrupts the signal cascade by neutralizing the ligand. In a study comprising over 800 patients with metastatic colorectal cancer, the addition of bevacizumab to irinotecan, 5-fluorouracil, and leucovorin as first-line therapy led to a significant increase in survival time[35]. Recent studies concerning the neutralizing antibody bevacizumab, and the small molecule tyrosine kinase inhibitor SU5416, demonstrated that, while unlikely to be effective as monotherapy, incorporation of VEGFR blockade into cytotoxic regimens may increase overall response rates in solid tumors. However, incorporation may also introduce new toxicities, including thromboembolic complications and bleeding[36-38]. Gastrointestinal bleeding, epistaxis and thrombotic events have been seen in 10.3%, 50% and 19.1%, respectively, of the patients receiving bevacizumab alone[39]; these side effects should be taken into consideration when deciding about bevacizumab therapy. There is a phase II trial of bevacizumab, irinotecan, and cisplatin in metastatic or unresectable gastric cancer (National Cancer Institute [NCI] protocol 6447) currently active, of which only the high rate of thromboembolic events in six (25%) of 24 patients (95% CI, 11% to 45%) has recently been reported[40]. However, the incidence of thromboembolic events was not significantly different when compared to a similar irinotecan-based protocol not containing bevacizumab, and there was no difference in the pattern of venous thromboses.

A small molecule inhibitor of VEGFR2 (Vatalanib; PTK787/ZK 222584) has been developed; preliminary data on its use in colorectal cancer exist[41]. With respect to gastric cancer, a Japanese group reported the effects of another VEGFR tyrosine kinase inhibitor (SU6668) in a mouse model of gastric cancer. SU6668 did not directly alter the growth rate of the cancer cells, but inhibited tumor angiogenesis, resulting in the inhibition of tumor dissemination in the peritoneum[42].

Erb B2 / HER-neu – Trastuzumab When considering how EGFR/erb B-targeted therapeutics function, it is important to mention that, in contrast to the small molecule kinase inhibitors, antibodies targeting EGFR and erb B2 have the inherent ability to recruit immune effector cells such as macrophages and monocytes to the tumor through binding of the antibody constant Fc domain to specific receptors on these cells. In xenograft models at least, this mechanism is relevant with the anti-tumor activity of erb B2-targeted trastuzumab[43]. Whether this mechanism has a role in clinical efficacy in cancer patients remains unproven[44]. Trastuzumab (Herceptin) is a humanized monoclonal antibody, which was approved by the United States FDA in 1998 for the treatment of advanced breast cancer. This was the first approval of a monoclonal antibody for use in solid tumor therapy[45]. Recently, it was

investigated whether trastuzumab could affect the growth of HER/neu-overexpressing gastric cancer cells based on the antibody-dependent cell-mediated cytotoxicity (ADCC). It has been demonstrated that these cells could be killed by trastuzumab-mediated ADCC and this correlated with the degree of HER-2/neu expression[46]. However, the trastuzumab-mediated ADCC was significantly impaired when tested in peripheral blood mononuclear cells from patients with advanced disease as compared to those with early disease. Moreover, natural killer cells purified from patients with advanced disease showed less trastuzumab-mediated ADCC in comparison to those from healthy donors. Consequently it has also been postulated that some treatment modalities, such as those involving the use of interleukin 2, could contribute to reverse NK dysfunction, which may be necessary for successful trastuzumab treatment of gastric cancer. Funato *et al*[47] used MKN-7 and KATO-III gastric cancer cells, which express the erb B2 oncogene, to study the mechanism of resistance to cisplatin. They found that erb B2 expression in gastric cancer is related to cisplatin sensitivity, and that anti-erb B2 antisense oligonucleotides could induce increased sensitivity to this drug. Experimental therapy utilizing a different anti-HER mouse-human chimeric monoclonal antibody named CH401 documented efficacy in an *in vivo* model of gastric cancer, yet clinical data are lacking[45].

Epidermal growth factor receptor (EGFR) – Cetuximab

Cetuximab has been approved by the FDA for use in colorectal cancer; cetuximab and other molecules inhibiting the EGFR pathway in colorectal cancer have been recently reviewed[48,49]. One large trial of cetuximab in colorectal cancer comprising 329 patients with irinotecan-refractory metastatic cancer indicated a delayed median time to progression by combining cetuximab with irinotecan[50]. A known adverse characteristic of anti-EGFR therapy is an acne-like skin rash associated with treatment response, which was observed in about three out of four patients[51]. In contrast to gefitinib, the response of cancer cells to cetuximab occurs independent of the mutational status of the EGFR[52,53]. Unfortunately, there are no relevant clinical data available on the use of cetuximab in patients suffering from gastric cancer. As cetuximab is a chimeric antibody which may cause immunological reactions, humanized anti-EGFR antibodies have been developed, one being matuzumab, which currently undergoes phase II trials including studies in patients suffering from gastric cancer[54].

Pharmacological agents – small molecule tyrosine kinase inhibitors

Imatinib: Imatinib is an established and licensed treatment modality in gastrointestinal stroma cell tumors (GIST)[55], but with respect to gastric cancer, only limited data about its use exist. A single study in an animal model of gastric cancer suggested no independent activity of imatinib, but proved an effective chemo- sensitization of antitumor drugs, such as 5-FU and paclitaxel for gastric carcinoma, targeting the PDGF/PDGFR-signalling pathway of tumor cells and stromal cells in disease progression and angiogenesis[25].

EGFR-inhibitors–Erlotinib and Gefitinib

The first representative of this drug class to be approved for cancer therapy was gefitinib (Iressa®) in the third line treatment of non-small-cell lung cancer[56]. A subsequent phase III trial (Iressa Survival Evaluation in Lung cancer, ISEL) failed to demonstrate survival advantage for those patients when compared with placebo[57]. Furthermore, in other malignancies such as gastric carcinoma, preliminary data indicate that treatment efficacy with this regimen is limited as well[58]. A phase II trial to investigate the efficacy, tolerability and pharmacokinetics of gefitinib in pretreated patients with metastatic gastric carcinoma included 75 subjects who were randomised to receive 250 or 500 mg/d gefitinib orally. The authors found that gefitinib monotherapy was generally well tolerated in pretreated patients with gastric metastatic adenocarcinoma, with disease control achieved in 18.3% of cases analyzed. The most common drug-related adverse events were diarrhea, rash and anorexia. The only dose-related adverse events were rash (25.0% at 250 mg/d *vs* 44.7% at 500 mg/d) and anorexia (8.3% at 250 mg/d *vs* 15.8% at 500 mg/d). Rojo *et al*[59]evaluated immunohistochemically the percentage of tumor cells expressing EGFR, pEGFR (the activated phosphorylated form), pMAPK, pAkt (phosphorylated Ser473) and Ki67, before and after treatment with gefinitib. Prior to treatment EGFR expression was found in 62.5% of tumors, whereas pEGFR levels were significantly reduced after the treatment. However, a decreased proliferation was observed only in those tumors with low levels of pAkt, suggesting a role for the PI3k-Akt pathway in gefinitib resistance. Recent studies suggested that clinical response to gefitinib in lung cancer depends on the presence of somatic mutations of the EGF receptor in the tumor which enhance the responsiveness of the receptor to EGF ligand and increase its sensitivity to inhibition by gefitinib[60-62]. In the case of gastric cancer, no such mutations are known. Data on the use of erlotinib (Tarceva®; OSI-744) in gastric cancer is limited to a single study of 70 patients having either gastric cancer (n = 26) or gastroesophageal junction cancer (GEJC) (n = 44). No patient in the gastric cancer cohort presented an objective response, but five patients in the GEJC cohort did so, one being a complete response. An overall response rate was 11%[63]. The best known therapeutics targeting members of the EGFR family which are currently available or under investigation are summarized in Table 2.

Attempts to optimize treatment efficacy of RTKIs

As resistance against single agents may arise and tumor survival may rely on more than one growth factor pathway, several attempts have been made to optimize treatment efficacy, most of them still being in preclinical testing. The following options exist: (1) Combination of inhibitors of the same pathway (e.g. gefitinib and cetuximab) in order to further enhance signal abrogation; (2) To combine inhibitors of different RTK pathways or apply non-selective inhibitors of several pathways in case the cancer cell loses its reliance on one specific pathway; (3) To select cells dependent on growth factor stimulation by use of other cytotoxic drugs or radiation and subsequently eliminate these by an immunological or pharmacological

RTKI.

Enhanced abrogation of one RTK pathway by combining different inhibitors

In 2004, two groups described the use of cetuximab combined with gefitinib and erlotinib as enhanced abrogation of the EGFR signal cascade in *in vitro* as well as *in vivo* models. Matar *et al*[64] utilized an EGFR-dependent human tumor xenograft model and found a synergistic effect on cell proliferation and superior inhibition of EGFR-dependent signalling and induction of apoptosis. Even suboptimal doses of gefitinib and cetuximab given together resulted in a complete and permanent regression of large tumors. In the combination-treated tumors, there was a superior inhibition of EGFR, mitogen-activated protein kinase, and Akt phosphorylation, as well as greater inhibition of cell proliferation and vascularization and enhanced apoptosis. Using cDNA arrays, 59 genes could be identified that were coregulated and 45 genes differentially regulated, including genes related to cell proliferation and differentiation, transcription, DNA synthesis and repair, angiogenesis, signalling molecules, cytoskeleton organization, and tumor invasion and metastasis. Huang *et al*[65] reported similar findings in head and neck tumors and in a model of lung cancer; they observed that gefitinib and erlotinib retained the capacity to inhibit tumor cell growth in case of cetuximab resistance. Again, the combination of antibody and kinase inhibitor resulted in more profound tumor regression and regrowth delay.

Another group applied trastuzumab and an inhibitor of EGFR and erb B2 tyrosine kinases, lapatinib, in erb B2-overexpressing breast cancer cell lines. Only in combination, treatment resulted in a markedly downregulated survivin protein expression and enhanced tumor cell apoptosis thus suggesting a potential improvement in clinical response[66]. But not all findings support the idea of combining inhibitors of the same pathway: in an *in vitro* model using two human epidermoid cell lines the combination of cetuximab and gefitinib demonstrated antagonistic effects. Administration of either drug alone led to a diminution in EGFR levels, while their combination increased the cellular expression of EGFR. These findings suggest that new and tempting treatment strategies on the EGFR target consisting in a double hit with a monoclonal antibody and a TKI must be considered with caution[67]. Data with regard to gastric cancer are currently lacking.

Inhibition of several RTK pathways: combination of different substances, non-selective inhibitors

Cancers possessing complex kinase profiles may respond better to a multimodal therapy tackling several pathways; and such regime may simultaneously reduce the emergence of resistance. Therefore a combination of different agents or a single "unspecific" inhibitor of several pathways may offer advantages over inhibition of a single pathway.

Combinations of different agents: As there is currently no single known inhibitor of all relevant receptor tyrosine kinases, combination of the well evaluated agents offers a straightforward concept of therapy, taking into account that in most cancer therapies different substances are being combined. First data were raised in phase I or II breast cancer trials by combining trastuzumab with small molecule inhibitors of EGFR such as gefitinib or erlotinib indicating that this combination provided a well tolerated targeted therapy with preliminary evidence of antitumor activity[68,69]. As activation of the EGF receptor may induce vessel formation by cellular liberation of VEGF, approaches blocking both pathways possess certain attractiveness. In conditions of limiting VEGF, EGF plays an important role in endothelial cell proliferation, survival, and sprouting of small vessels. Animal data suggest that combined inhibition of EGFR and VEGFR pathways may produce synergistic results and that resistance to EGFR inhibition may be overcome by inhibition of VEGFR tyrosine kinase[70,71]. In an *in vivo* model, the combination of anti-VEGF-R and anti-EGF-R therapies was effective in inhibiting gastric cancer growth whereas the decrease in tumor growth in mice treated with DC101 (an anti-VEGF-R antibody) or cetuximab alone did not reach statistical significance[72]. As mentioned above, the addition of an inhibitor of the hepatocyte growth factor was able to overcome resistance against gefitinib resulting from the interaction of stromal and cancer cells in an animal model of scirrhous gastric cancer[34].

Inhibitors not selective for a single pathway: Agents targeting multiple RTKs are currently undergoing preclinical testing or phase I studies. These include AEE788 (directed against EGFR, erbB2, VEGFR2; Novartis), BAY 43-9006 (Sorafenib; Raf kinase, VEGFR2, PDGF-R beta; Bayer), SU11248 (PDGF-R, VEGFR, c-kit-R, FLT3-R; SUGEN), and ZD6474 (VEGFR and EGFR, AstraZeneca), all designed to inhibit multiple mechanisms of tumor growth in addition to conventional chemotherapy[73-76]. Similar to the combination of anti-EGFR and anti-VEGFR antibodies mentioned above, McCarthy *et al*[77] tested the inhibitor of both tyrosine kinases, ZD6474, in an orthotopic model of gastric cancer. The agent led to marked

Table 2 Therapeutics targeting the EGFR family

Agent	Type	Target	Status
Trastuzumab	Humanized mAb	erbB2	Approved for breast cancer
Pertuzumab	Humanized mAb	erbB2	Phase II trials
Cetuximab	Chimeric mAb	EGFR	Approved for colorectal cancer
Matuzumab	Humanized mAb	EGFR	Phase II trials
Panitumab	Humanized mAb	EGFR	Trials ongoing
Gefitinib	TKI	EGFR	Approved for NSCLC
Erlotinib	TKI	EGFR	Approved for NSCLC
Lapatinib	TKI	EGFR/erbB2	Phase III trial / breast cancer
AEE788	TKI	EGFR/ erbB2/ VEGFR	Phase I trials
CI-1033	Irreversible TKI	EGFR/erbB2	Phase II trials
EKB-569	Irreversible TKI	EGFR/erbB2	Phase II trials
EXEL 7647 /TKI EXEL 0999		EGFR/ erbB2/ VEGFR	Phase I trials

(incomplete list; modified according to ref. 44)

inhibition of tumor growth, tumor cell proliferation, and decrease in microvessel density. The authors concluded that therapies such as ZD6474 that target two distinct aspects of tumor growth, angiogenesis and tumor cell proliferation, warrant further investigation.

Combination of standard chemotherapeutic agents and radiation with inhibition of RTKs

"Conventional" chemotherapy and RTKIs: The combination of antibody based therapies with conventional therapy has become a standard procedure in many cancers. For example, in colorectal cancer, cetuximab or bevacizumab is typically combined with irinotecan-based regimes[35,50]. One of the first reports on the use of an RTKI, trastuzumab, in breast cancer documented the inhibition of DNA repair subsequent to DNA damage by cisplatin by an antibody to Her2/Neu. Therapy with this antibody led to a 35%-40% reduction in repair of cisplatin-DNA adducts after cisplatin exposure and, as a result, promoted drug-induced killing in target cells[78]. Another study using human tumor xenografts found that gefitinib caused growth inhibition of tumors and enhancement of the activity of a number of cytotoxic drugs, but neither was dependent on high levels of EGFR expression[79]. In an animal model of pancreatic cancer, inhibiting phosphorylation of EGFR, VEGFR, and PDGFR by appropriate RTKIs in combination with gemcitabine enhanced the efficacy of gemcitabine alone, resulting in inhibition of experimental human pancreatic cancer growth and significant prolongation of survival[80]. Similar results were obtained in a model of estrogen receptor-positive breast cancer in which successful cooperation of the dual erb B1/B2 inhibitor lapatinib with tamoxifen was evidenced[81]. Yet there are pitfalls of combinatorial therapy: the combination of two substances may not always be beneficial; for example, the combination of tamoxifen and trastuzumab in estrogen-receptor positive breast cancer may be less effective than either substance alone possibly due to an increase in erb B2 signalling pathways that occurs when tamoxifen is added to trastuzumab[82]. In case of gefitinib, addition of this RTKI to platin-based chemotherapy in non-small cell lung cancer (INTACT 2 trial) showed no added benefit in survival, TTP, or RR compared with standard chemotherapy alone. This large, placebo-controlled trial confirmed the favorable gefitinib safety profile observed in phase I and II monotherapy trials[83]. Currently only data from animal or *in vitro* testing exist with respect to gastric cancer; Park *et al*[84] investigated the effect of gefitinib combined with oxaliplatin, 5-fluorouracil, or paclitaxel in a gastric cancer cell line, SNU-1. This study demonstrated the antitumor activity and a significant cell cycle arresting effect induced by gefitinib in SNU-1 human gastric carcinoma cells, and its synergistic interaction with oxaliplatin and paclitaxel. There are preliminary data on a multicenter phase II study of irinotecan, cisplatin, and bevacizumab in gastric or gastroesophageal adenocarcinoma which indicate an excellent disease control rate of 13/15 cases; in a subset of ten patients with measurable disease who had received at least two cycles of therapy, 5 partial responses, 4 minor responses (15%-29% reduction) and 1 stable disease were observed[85]. Other, less toxic substances have been assessed as well. Thus it has previously been documented that gastric tissue exposed to acetylsalicylic acid (ASA) expresses high levels of EGFR[86,87]. A positive loop regulation between COX and erbB2[88] as well as EGFR[89] has been postulated. Since nonsteroidal anti-inflammatory drugs (NSAIDs) might be a tool of carcinoma prevention in the gastrointestinal tract[90] we investigated the mechanisms of a potential synergism of simultaneous cyclooxygenase (COX)- and EGFR-inhibition. It has previously been described that simultaneous administration of COX- and EGFR-inhibitors exerts tumor preventive effects in nude mice[91]. A combination of these two substances with a protein kinase A (PKA) antisense oligonucleotide was able to eliminate tumors in more than half of the animals treated[92]. Preliminary data from our group indicate that acetylsalicylic acid may modulate the expression and activation of EGFR in gastric cancer cells rendering them more susceptible to gefitinib treatment[93].

Radiation therapy and RTKIs: Radiation causes cell death by induction of cellular injury which may rely on subsequent growth factor/receptor tyrosine kinase activation for repair, the combination of radiation and RTKIs seems to be obvious. Currently, however, only data with respect to experimental therapy, especially in animal models, exist. E.g., She *et al*[94] examined the effect of addition of gefitinib to radiation therapy in a nude mouse model of different cancers, including lung and breast cancer. Gefitinib significantly enhanced the antitumor action of radiation therapy against the test tumors without significant adverse effects, increasing the therapeutic selectivity of ionizing radiation in these model systems. In a similar model, PTK787/ZK222584, a specific inhibitor of VEGFR tyrosine kinases, was tested. Tumors vascularized by radiation-damaged vessels responded to PTK787/ZK222584 with longer latency and slower growth rate than controls, and a trend toward further increase in necrosis, indicating that irradiated tumor vessels are more susceptible to VEGFR inhibition than unirradiated vessels[95].

CONCLUSION

Receptor tyrosine kinases participate in several steps of tumor formation including proliferation and metastasis formation. As several of them are upregulated in gastric cancer, they offer potential prime targets for a tailored therapy. Unfortunately, only preliminary data exist on the use of the currently clinically available drugs such as trastuzumab, cetuximab, bevacizumab, gefitinib, erlotinib, and imatinib in the setting of gastric cancer. However, phase II trials are underway to examine the potential of these drugs in adenocarcinoma of the stomach. As RTK inhibitors with a broad range are being developed, the potential usefulness of this drug class is most likely to further increase as preclinical data in models of gastric cancer already indicate their effectiveness. The approach of combining RTKIs with "conventional" means of tumor therapy such as cytostatics and radiation therapy is most likely to find its way into clinical application in near future. RTKIs alone typically only inhibit tumor growth and do not aim at killing the cancer cell, but they might prove to

be an effective chemosensitizer of other antitumor drugs as they are able to block the process of cellular restitution following injury caused by radiation or "conventional" cytostatics. Different trial designs may be necessary given that some RTKIs might only work in small subsets of patients (for example, the fate of gefitinib in non-small cell lung cancer) and often improve patients' condition without leading to an objective response. Genomic or proteomic analysis of aberrations in individual patients may be necessary to decide about a tailored therapy.

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REVIEW

Antibiotics and probiotics in treatment of inflammatory bowel disease

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Abstract

Many experimental and clinical observations suggest that intestinal microflora plays a potential role in the pathogenesis of inflammatory bowel disease (IBD). Manipulation of the luminal content using antibiotics or probiotics represents a potentially effective therapeutic option. The available studies do not support the use of antibiotics in ulcerative colitis (UC). Antibiotics are effective in treating septic complications of Crohn's disease (CD) but their use as a primary therapy is more controversial, although this approach is frequently and successfully adopted in clinical practice.

There is evidence that probiotic therapy may be effective in the prevention and treatment of mild to moderate UC. In contrast, a lack of successful study data at present precludes the widespread use of probiotics in the treatment of CD.

Both antibiotics and probiotics appear to play a beneficial role in the treatment and prevention of pouchitis and further trials are warranted to fully quantify their clinical efficacy.

Key words: Inflammatory bowel disease; Intestinal microflora; Antibiotics; Probiotics

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INTRODUCTION

The rationale for using antibiotics and probiotics in the treatment of inflammatory bowel disease (IBD) is based on convincing evidence implicating intestinal bacteria in the pathogenesis of the disease[1].

The distal ileum and the colon are the areas with the highest bacterial concentrations and represent the sites of inflammation in IBD. In addition, pouchitis, the non-specific inflammation of the ileal reservoir after ileo-anal anastomosis, appears to be associated with bacterial overgrowth and dysbiosis. Furthermore, pouchitis does not occur prior to closure of the ileostomy.

Patients with Crohn's disease (CD) consistently respond to diversion of faecal stream, with immediate recurrence of inflammation after restoration of intestinal continuity or infusion of luminal content into the bypassed ileum[2,3]. Moreover, the composition of the enteric flora is altered in patients with IBD, and enteric bacteria or their products have been found within the inflamed mucosa of patients with CD[4]. Increased number of aggressive bacteria such as *Bacteroides,* adherent/invasive *Escherichia coli* and enterococci, and decreased number of protective lactobacilli and bifidobacteria have been observed in IBD[5].

However, the most compelling evidence that intestinal bacteria play a role in IBD has been derived from animal models. Although there is a great diversity in genetic defects and immunopathology, a consistent feature of many transgenic and knockout mutant murine models of colitis is that the presence of normal enteric flora is required for full expression of inflammation[6]. Indeed, there is evidence that immunological tolerance to commensal bacteria is lost in patients with IBD[7,8]. These findings have led to the proposal that manipulation of intestinal microbiota flora, either with antibiotics or probiotics, may be therapeutic in IBD. Some suggested mechanisms of action of antibiotics and probiotics are shown in Table 1.

There is a growing body of evidence from animal studies and clinical trials that antibiotics and probiotics have therapeutic effects in ulcerative colitis (UC), CD and pouchitis.

ANTIBIOTICS

Animal model studies

In several rodent models the use of broad-spectrum an-

Table 1 Suggested mechanisms of action of antibiotics and probiotics

Antibiotics	Probiotics
Eradication of bacterial antigenic triggers	Inhibition of pathogenic enteric bacteria by:
Elimination of bacterial overgrowth	decreasing luminal pH
Reduction of pro-inflammatory bacterial toxins	secretion of bacteriocidal proteins
Potential immunosuppressive properties of antibiotics	resisting colonization
	blocking epithelial binding
	Improvement in epithelial and mucosal barrier function by:
	production of short-chain fatty acids
	enhancing mucus production
	increasing barrier integrity
	Alteration of immunoregulation by:
	increasing interleukin-10 and TGFβ, and decreasing TNF levels
	increasing IgA production

tibiotics can both prevent and treat experimental colitis, whereas metronidazole and ciprofloxacin can only prevent experimental colitis but cannot reverse the established disease[9-13]. Broad-spectrum antibiotics are effective in almost all models of acute and chronic colitis[13-16], but they have only a transient efficacy in HLA-B27 transgenic rats[17]. Interestingly, ciprofloxacin and metronidazole have selective efficacy in different colonic regions of interleukin-10 (IL-10) knockout mice, suggesting that different bacteria cause inflammation in different colonic segments[15]. These studies suggest that most clinical forms of IBD may respond to a specific combination of broad-spectrum antibiotics.

Ulcerative colitis

Only a few trials on the use of antibacterial agents have been carried out in UC and the results are controversial. Most clinicians have used antibiotics as an adjuvant therapy for severe UC. Dickinson *et al*[18] carried out a double-blind controlled trial on the use of oral vancomycin as an adjunct for acute exacerbations of idiopathic colitis and found that there is no significant difference between the two treatment groups, with only a trend towards a reduction in the need for surgery in patients treated with vancomycin[18].

Intravenous metronidazole used in conjunction with corticosteroids, is as effective as placebo in inducing remission in patients with severe UC[19]. In a double-blind, placebo-controlled trial in patients with acute relapse of UC, Burke *et al*[20] randomized 84 patients to receive corticosteroids plus oral tobramycin or placebo and found that after 1 wk of treatment, 74% of patients in the tobramycin treatment group and 43% in the placebo group ($P < 0.003$) achieve complete symptomatic remission. However, the combination of tobramycin and metronidazole does not have any beneficial effect when compared with a standard steroid treatment in severely acute UC[21].

Mantzaris *et al*[22] investigated ciprofloxacin in a randomised, placebo-controlled study and randomized 70 patients with mild to moderate active UC to receive either 250 mg ciprofloxacin twice a day or placebo for 14 d and found that 70.5% of patients in the ciprofloxacin group and 72% in the placebo group achieve remission. Moreover, a short course of intravenous ciprofloxacin is not effective as an adjunctive treatment to corticosteroids in severe UC[23]. In contrast, some efficacy of ciprofloxan has been observed in a more recent randomised placebo-controlled trial when ciprofloxacin is administered for 6 mo to patients with active UC poorly responding to conventional therapy with steroids and mesalazine[24]. At the end of the study, the treatment-failure rate was 21% in the ciprofloxacin-treated group and 44% in the placebo group ($P < 0.002$). This difference was detected using clinical criteria; while endoscopic and histological findings showed differences only at 3 mo but not at 6 mo.

The non-absorbable broad-spectrum antibiotic, rifaximin, was investigated in a small controlled study to evaluate its efficacy and systemic absorption in patients with moderate to severe active UC refractory to steroid treatment. Twenty-eight patients were randomised to receive either rifaximin 400 mg twice daily or placebo for 10 d as an adjunct to standard steroid treatment. Although there is no significant difference in the clinical efficacy score between the two treatments, only rifaximin determines a significant improvement in stool frequency, rectal bleeding and sigmoidoscopic score[25].

Whilst rifaximin does not permanently alter the colonic microbiota, resistant *Bifidobacterium* species have been found after 3 intermittent courses in patients with UC[26].

Crohn's disease

Broad-spectrum antibiotics are widely used to treat CD[27], but large controlled trials have not yet been performed (Table 2).

Metronidazole has been the most investigated agent. In 1978, Blichfeldt *et al*[28] found that there is no difference between metronidazole and placebo-treated patients in a placebo-controlled, double-blind, crossover trial. However, a positive trend in favour of metronidazole is observed when only the colon is involved[28]. In the National Cooperative Swedish study, metronidazole has been compared with sulphasalazine as a primary treatment for Crohn's disease. Although no significant difference is found between the two groups, metronidazole is effective in patients who fail to respond to sulphasalazine[29]. In

Table 2 Antibiotics trials in active Crohn's disease

Study	Patients (*n*)	wk	Main outcome	Study design	Treatment schedules
Blichfeldt *et al*[28]	22	8	Improvement (clinical/lab score)	DB crossover study	MZ (+ SASP/CS) Placebo (+ SASP/CS)
Ursing *et al*[29]	22	16	Change in CDAI and orosomucoid	DB crossover study	MZ SASP
Ambrose *et al*[30]	72	4	Improvement (Clinical/lab.score)	DB RCT	MZ CO, MZ/CO, placebo
Sutherland *et al*[31]	99	16	Change in CDAI from baseline	DR RCT	MZ (10/20 mg/kg) Placebo
Prantera *et al*[32]	41	12	Clinical remission (CDAI < 150)	NB RCT	MZ + Cipro Steroids
Colombel *et al*[34]	40	6	Clinical remission (CDAI < 150)	NB RCT	CIPRO 5-ASA
Arnold *et al*[35]	47	24	Change in CDAI	NB RCT	CIPRO (+ conc drugs) Placebo (+ conc drugs)
Steinhart *et al*[33]	134	8	Clinical remission (CDAI < 150)	DB RCT	MZ+CIPRO (+ bud 9 mg) Placebo (+ bud. 9 mg)

CDAI: Crohn's disease activity index.

another study, metronidazole was used either as a single therapy or in combination with cotrimoxazole and compared to cotrimoxazole alone and a double placebo in patients with a symptomatic relapse of CD, which shows that after four weeks of treatment there is no difference in response among the three treatment groups[30]. In a Canadian randomised, placebo-controlled trial, Sutherland *et al*[31] demonstrated that treatment with metronidazole for 16 wk significantly decreases the Crohn's disease activity index (CDAI), but no difference is found in the rates of remission compared with placebo. As in the Swedish study[29], the Canadian study found that metronidazole is effective for colonic and ileocolonic CD, but not for ileitis. Unfortunately, metronidazole has numerous side-effects including nausea, anorexia, dysgeusia, dyspepsia and peripheral neuropathy, which limit its use in approximately 20% of patients.

An antibiotic combination was used in an Italian randomised controlled study[32] in which 250 mg metronidazole four times daily plus 500 mg ciprofloxacin twice daily were compared to a standard steroid treatment for 12 wk. No significant differences were reported in the rates of remission between treatments (46% with ciprofloxacin plus metronidazole and 63% with methylprednisolone), suggesting that this antibiotic combination is a potential alternative to steroid treatment in the acute phase of CD[32]. In another trial[33], this combination of metronidazole and ciprofloxacin was supplemented with budesonide (9 mg/d) for active CD. No difference was registered compared to placebo, but the overall response in the two groups was lower than that in previous studies using budesonide, suggesting that antibiotic treatment is more effective in colonic disease than in isolated small bowel disease.

Ciprofloxacin (1 g/d) was compared to mesalazine (4 g/d) in a controlled study[34] of mild to moderate active CD for 6 wk. The results suggest that ciprofloxacin is as efficacious as mesalazine (remission observed in 56% and 55% of patients treated with ciprofloxacin and mesalazine respectively), thus offering a potential alternative treatment for active CD. Furthermore, ciprofloxacin has been shown to be effective when used in combination with standard treatment in patients with resistant disease [35].

Other antibiotics have also been investigated. Shafran *et al*[36] carried out an open-label study on the efficacy and safety of rifaximin (600 mg/d) for 16 wk in the treatment of mild to moderate active CD, and found that at the end of the study, 59% of patients are in remission (CDAI < 150) with a significant reduction of the mean CDAI score compared to baseline ($P < 0.0001$). Leiper *et al*[37] reported that 64% patients have an impressive positive response to clarithromycin, many of whom are unresponsive to other treatments.

Many studies have tried to evaluate the efficacy of antimycobacterial drugs in patients with CD, pursuing the possibility that a strain of *Mycobacterium* might be an aetiological agent in CD. Borgaonkar *et al*[38] performed a meta-analysis of all randomised controlled trials in which antimycobacterial therapy was compared with placebo and found that antimycobacterial therapy is only efficacious in the maintenance of remission after a combined treatment of corticosteroids and antimycobacterial agents. However, the investigator emphasised that because of the high incidence of side-effects and the small number of studies included in the meta-analysis, the data are inconclusive and should be interpreted with caution.

The same antibiotics used to treat luminal CD have been reported to be beneficial for the treatment of perianal CD, but no controlled trials are available[39]. Metronidazole (20 mg/kg) can close 62%-83% fistulae[40,41]. The combination of metronidazole and ciprofloxacin results in an improvement in 64% of patients with closure of fistulae in 21%[42]. Unfortunately, fistulae tend to recur in most patients following cessation of treatment. Although the results of these uncontrolled studies are inconclusive, metronidazole and ciprofloxacin alone or in combination, are used by most clinicians as first-line treatments for patients with perianal disease in conjunction with surgical drainage of abscesses.

The use of antibiotics in the prevention of post-

operative disease recurrence has also been investigated. Rutgeerts *et al*[43] have assessed the efficacy of metronidazole at 20 mg/kg per day in a placebo-controlled double-blind study. In their study, sixty patients were randomised to receive either metronidazole or placebo for 12 wk and endoscopic relapse was evaluated by Rutgeerts score at the end of the treatment. They found that metronidazole significantly decreases the incidence of severe endoscopic relapse (grade 3 or 4) and the clinical recurrence rate. More recently, ornidazole used continuously for 1 year, has been shown to be significantly more effective than placebo in the prevention of clinical and endoscopic recurrence in the neoterminal ileum[44].

Pouchitis

The awareness of the crucial importance that faecal stasis and bacterial overgrowth may play a role in the pathogenesis of acute pouchitis has led clinicians to treat patients with antibiotics.

Antibiotics have become the mainstay of treatment for pouchitis, although controlled trials are not available. Metronidazole is the first-line treatment, and most patients with acute pouchitis respond quickly to its administration of 1-1.5 g/d[45,46]. A double-blind, randomised, placebo-controlled, crossover trial was carried out by Madden *et al*[47] to assess the efficacy of 400 mg of metronidazole three times daily *per os* for two weeks in 13 patients (11 completed both arms of the study) with chronic, unremitting pouchitis. The found that metronidazole is significantly more effective than placebo in reducing stool frequency (73% and 9%), even without improvement in endoscopic appearance and histological grade of activity. However, a significant proportion of patients (55%) may experience side-effects while using metronidazole, including nausea, vomiting, abdominal discomfort, headache, skin rash and metallic taste. Recently Shen *et al*[48] compared the efficacy and side-effects of ciprofloxacin and metronidazole in treating acute pouchitis in a randomised clinical trial. Seven patients received ciprofloxacin (1 g/d) and nine patients received metronidazole (20 mg/kg per day) for 2 wk. The results of this study have shown that both ciprofloxacin and metronidazole are efficacious in the treatment of acute pouchitis. Both reduce the total pouchitis disease activity index (PDAI) scores and lead to a significant improvement in symptoms as well as endoscopic and histological scores. However, ciprofloxacin leads to a greater reduction in PDAI scores as well as improvement in symptoms and endoscopic scores. Furthermore ciprofloxacin is better tolerated than metronidazole (33% of metronidazole-treated patients reported adverse effects, compared with none in the ciprofloxacin group).

Given the management difficulties posed by chronic refractory pouchitis, the use of combined antibiotic treatment has been explored. In an open trial, 18 patients with active pouchitis not responding to standard therapy (metronidazole or ciprofloxacin) for 4 wk, were treated orally with rifaximin (2 g/d) plus ciprofloxacin (1 g/d) for 15 d. Symptom assessment, endoscopic and histological evaluations were performed at screening and after 15 d using PDAI scores. The results indicate that 16 out of 18 patients (88.8%) improve ($n = 10$) or go into remission ($n = 6$) with the median PDAI score before and after therapy being 11 and 4 respectively ($P < 0.002$)[49].

More recently, 44 patients with refractory pouchitis received metronidazole (800 mg to 1 g/d) and ciprofloxacin (1 g/d) for 28 d. The results reveal that 66 patients (82%) go into remission with the median PDAI score before and after therapy being 12 and 3 respectively ($P < 0.0001$), and the patients' quality of life is significantly improved after the treatment (median IBD Questionnaire score increased from 96.5 to 175)[50].

Table 3 Organisms associated with probiotic activity

Most commonly	Other bacterial strains	Yeast
Lactobacilli	Enterococci	Saccharomyces boulardii
Bifidobacteria	Non-pathogenic *E. coli*	
Streptococci		

PROBIOTICS

The use of probiotics for the purpose of health maintenance and disease prevention is first proposed by Elie Metchnikoff, the Russian Nobel prize winner[51], who at the turn of the last century suggested that a high concentration of lactobacilli in the intestinal flora is important for the health and longevity of humans. Probiotics are defined as "living organisms, which upon ingestion in a certain number exert health benefits beyond inherent basic nutrition"[52].

A number of bacteria are associated with probiotic activity (Table 3). For clinical application, probiotic strains need to be resistant to acid and bile and the ability to be metabolically active within the luminal flora where they should ideally survive but not persist in the long term. They should be antagonistic to pathogenic bacteria and safe for human use while maintain their viability and beneficial properties during the manufacturing processes[53].

Animal model studies

Encouraging results of probiotic therapy have been obtained in experimental colitis. Administration of *Lactobacillus reuteri* can significantly reduce inflammation in acetic acid-and methotrexate-induced colitis in rats[54,55]. More recently *Lactobacillus sp.* has been shown to be able to prevent the development of spontaneous colitis in IL-10 deficient mice[56], and continuous feeding with *Lactobacillus plantarum* improves an established colitis in the same knockout model[57]. A strain of *Lactobacillus salivarius* (subsp. *salivarius*) reduces the rate of progression from inflammation to dysplasia and colonic cancer in IL-10 deficient mice[58], and *Bifidobacterium infantis* and of *Lactobacillus salivarius* are able to attenuate inflammation and reduce the ability to produce Th1-type cytokines in the IL-10 knockout model[59].

VSL#3 is characterised by a very high bacterial concentration (each packet containing 450 billion viable bacteria) and the presence of a cocktail of eight different bacterial species. This product contains viable lyophilised bacteria of four strains of lactobacilli (*L. casei, L. plantarum, L. acidophilus, L. delbrueckii* subsp. *bulgaricus*), three strains of

bifidobacteria (*B. longum, B. breve, B. infantis*) and one strain of *Streptococcus salivarius* subsp. *Thermophilus*. Rachmilewitz and colleagues[60] found that VSL#3 results in a significant attenuation of inflammation with a decrease of myeloperoxidase and nitric oxide synthase activity of the iodoacetamide-induced colitis. Madsen and colleagues[61] have reported a significant improvement in inflammation together with a reduction in mucosal levels of pro-inflammatory cytokines and normalisation of colonic barrier integrity in IL-10 knockout mice.

Ulcerative colitis

Promising results of probiotics have been found in the treatment of UC. In 3 recent trials involving the non-pathogenic strain of *Escherichia coli* Nissle 1917, similar efficacy has been observed to that of mesalazine in the maintenance treatment of UC[62-64].

We carried out a pilot study using the probiotic cocktail, VSL#3, as maintenance treatment for patients with UC in remission, allergic or intolerant to sulphasalazine and mesalazine, to assess its impact on the faecal flora. Twenty patients received 6 g a day of VSL#3 (1800 billion bacteria) for 12 mo and were assessed clinically and endoscopically at baseline, at 6 and 12 mo, and in the event of a relapse. Stool culture and determination of faecal pH were also performed at different intervals[65]. Microbiological determination showed a significant increase in concentration of lactobacilli, bifidobacteria and *Streptococcus thermophilus,* evident after just 20 d, which persisted throughout the treatment period, and returned to basal levels within 15 d after treatment. Faecal concentration of *Bacteroides*, enterococci, coliforms, *Clostridia* and total anaerobes and aerobes was not affected, but faecal pH was significantly reduced by the treatment. Fifteen of the twenty patients (75%) remained in remission throughout the treatment period[65].

Furthermore, VSL#3 at very high dosage (3600 billion bacteria/d) can induce remission in 63%, with a positive response in a further 23% of patients with active mild to moderate disease[66].

In addition, an open uncontrolled 4-wk study found that the yeast *Saccharomyces boulardii* could induce remission in 71% of patients with mild to moderate UC[67]. These studies highlight the wide range of organisms that may be beneficial as probiotic therapy for UC.

Crohn's disease

Campieri *et al*[68] performed a randomised trial to evaluate the efficacy of a combination of rifaximin and the probiotic preparation, VSL#3, in the prevention of post-operative recurrence of CD. Rifaximin (1.8 g/d) for 3 mo, followed by VSL#3 (6 g daily) for 9 mo, was compared with mesalazine (4 g/d) for 12 mo in 40 patients after curative resection for CD. After 3 mo of treatment, the antibiotic-probiotic combination resulted in a significantly lower incidence of severe endoscopic recurrence compared to mesalazine [2/20 (10%) *vs* 8/20 (40%)]. This difference was maintained throughout the study period [4/20 (20%) *vs* 8/20 (40%)][68].

No such clinical effect was seen in a study by Prantera *et al*[69] who reported that the probiotic *Lactobacillus GG* could not prevent post-operative disease recurrence in an 1-year double-blind, placebo-controlled trial. Similar negative results have been recently reported by the GETAID French group. A randomised double-blind, placebo-controlled study showed that *Lactobacillus johnsonii* LA1 ($4x10^9$ cfu/d) is not superior to placebo in preventing endoscopic recurrence of CD[70].

In a small pilot study[71], treatment with capsules containing *E.coli* Nissle 1917 was compared to placebo in the maintenance of steroid-induced remission of colonic CD. Twelve patients were treated with *E.coli* Nissle and 11 with placebo. The results showed that at the end of the 12-wk treatment period, the relapse rate is 33% in the *E.coli* group and 63% in the placebo group. Unfortunately, because of the small number of patients treated, this difference did not reach statistical significance.

However, a small comparative open study[72] showed that the combination of *Saccharomyces boulardii* (1 g/d) and mesalazine (2 g/d) is significantly superior to mesalazine (3 g/d) in maintenance of remission, suggesting that probiotic treatment in CD may be beneficial. More recently, a double-blind trial showed that *Lactobacillus* GG is not superior to placebo in prolonging remission in children with CD when given as an adjunct to standard therapy[73].

Pouchitis

Although probiotics are less widely used in clinical practice than antibiotics, they may be efficacious in the prevention and treatment of pouchitis. We have compared the efficacy of VSL#3 with placebo in the maintenance and treatment of chronic pouchitis[74]. Forty patients who obtained clinical and endoscopic remission after 1 mo of combined antibiotic treatment (rifaximin 2 g/d + ciprofloxacin 1 g/d) were randomised to receive VSL#3, 6 g daily (1800 billion bacteria/d) or a placebo of identical appearance for 9 mo. Clinical assessment was carried out every month, endoscopic and histological assessments were performed at entry and subsequently every two months. Stool samples were cultured before and after antibiotic treatment and subsequently every month during maintenance treatment. Relapse was defined as an increase of at least 2 points in the clinical portion of the PDAI and confirmed endoscopically and histologically. Whilst all 20 patients treated with placebo had a relapse during the 9 mo follow-up period, 17 of the 20 (85%) patients treated with VSL#3 remained in remission at this point. Interestingly, all these 17 patients had a relapse within 4 mo after the active treatment. Faecal concentrations of lactobacilli, bifidobacteria and *Streptococcus salivarius* subsp. *thermophilus* were significantly increased within 1 mo after VSL#3 treatment, and remained stable throughout the study. However, this increase did not affect the concentration of the other bacterial groups, suggesting that the beneficial effect of treatment is not mediated by suppression of endogenous luminal bacteria.

A recent study examining the maintenance of remission in patients with refractory or recurrent pouchitis showed that remission is achieved in 85% of patients treated with VSL#3, 6 g/d (1800 billion bacteria/d) and 6% in

the placebo group after 1 year of treatment[75]. In addition, continuous administration of VSL#3 results in a significant increase in IL-10 tissue levels, a significant decrease in tissue levels of the pro-inflammatory cytokines (TNF alpha, IL-1 and IFN gamma) and a decrease in matrix metalloproteinase activity[76]. This may aid our understanding of the mechanisms of action by which VSL#3 maintains remission in pouchitis. In contrast, *Lactobacillus GG* is ineffective in preventing relapse in patients with chronic pouchitis[77].

We have also carried out a double-blind, placebo-controlled trial to evaluate the efficacy of VSL#3 in preventing pouchitis onset following ileal-anal anastomosis for UC[76]. Forty patients were randomised to receive VSL#3, 3 g per day (900 billion bacteria/d) or an identical placebo for 12 mo. Patients were assessed clinically, endoscopically and histologically at 1, 3, 6, 9 and 12 mo according to the PDAI. The results indicate that patients treated with VSL#3 have a significantly lower incidence of acute pouchitis compared with those treated with placebo during the first year of ileostomy closure (10% *vs* 40%; $P < 0.05$). Moreover, the IBD Questionnaire score is significantly improved in the group treated with VSL#3, and the median stool frequency in patients not developing pouchitis, is significantly less in the VSL#3 group compared with the placebo group[78].

CONCLUSION

There is strong evidence that enteric commensal bacteria are involved in the pathogenesis of IBD. Therefore, modification of the gut bacterial flora by antibiotics and probiotics may be effective in treating UC, CD and pouchitis.

Antibiotics are a well established, efficacious treatment option for various manifestations of IBD. Antibiotics play an essential role in treating the septic complications of CD, including intra-abdominal and perianal abscesses and perianal fistulae, although their use in CD as a primary therapy is poorly documented. There is good evidence that ciprofloxacin, metronidazole or their combination is effective in Crohn's colitis and ileocolitis, though not in isolated ileal disease. Large controlled trials are needed to define optimal antibiotic regimens. In addition, their use in UC is not supported by the available studies and large trials with broad-spectrum agents are required. Although proper controlled trials have not yet been conducted, the use of antibiotics in pouchitis is largely justified.

Probiotics provide an attractive alternative to antibiotics in the treatment of IBD as trials to date have shown that they safe and have no side-effects. Promising results have been obtained from studies using probiotics, in both the prevention of relapse and the treatment of mild to moderate attacks of UC. Studies using probiotics in the treatment of CD are less clear due to conflicting and limited data. There is also considerable evidence that the highly concentrated cocktail of probiotics, VSL#3 is efficacious in preventing pouchitis onset and relapse.

Studies have highlighted the importance of selecting a well characterised probiotic preparation for treatment. In fact, viability and survival of bacteria in many available preparations are unproven. It should be remembered that the beneficial effect of one probiotic preparation does not imply efficacy of other preparations containing different bacterial strains, because each individual probiotic strain has its unique biological properties.

There is a need to improve our understanding of the composition of the enteric flora and the relationship between intestinal physiology and the luminal ecosystem. Only then can we truly optimise the bacteria-modifying treatments now available to effectively treat IBD.

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REVIEW

Consensus of primary care in acute pancreatitis in Japan

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Abstract

The incidence of acute pancreatitis in Japan is increasing and ranges from 187 to 347 cases per million populations. Case fatality was 0.2% for mild to moderate, and 9.0% for severe acute pancreatitis in Japan in 2003. Experts in pancreatitis in Japan made this document focusing on the practical aspects in the early management of patients with acute pancreatitis. The correct diagnosis of acute pancreatitis and severity stratification should be made in all patients using the criteria for the diagnosis of acute pancreatitis and the multifactor scoring system proposed by the Research Committee of Intractable Diseases of the Pancreas as early as possible. All patients diagnosed with acute pancreatitis should be managed in the hospital. Monitoring of blood pressure, pulse and respiratory rate, body temperature, hourly urinary volume, and blood oxygen saturation level is essential in the management of such patients. Early vigorous intravenous hydration is of foremost importance to stabilize circulatory dynamics. Adequate pain relief with opiates is also important. In severe acute pancreatitis, prophylactic intravenous administration of antibiotics at an early stage is recommended. Administration of protease inhibitors should be initiated as soon as the diagnosis of acute pancreatitis is confirmed. A combination of enteral feeding with parenteral nutrition from early stage is recommended if there are no clear signs and symptoms of ileus and gastrointestinal bleeding. Patients with severe acute pancreatitis should be transferred to ICU as early as possible to perform special measures such as continuous regional arterial infusion of protease inhibitors and antibiotics, and continuous hemodiafiltration. The Japanese Government covers medical care expense for severe acute pancreatitis as one of the projects of Research on Measures for Intractable Diseases.

Key words: Fluid resuscitation; Protease inhibitor treatment; Antibiotic treatment; Continuous regional arterial infusion; Contrast-enhanced computed tomography

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INTRODUCTION

There are several guidelines for the treatment of acute pancreatitis, mostly based on published evidence[1-4]. If, however, there were no published evidence, data would not be adopted in guidelines even if the therapy is employed in clinical practice. There is little evidence for the treatment of acute pancreatitis in critically early stage, and thus it is not mentioned in the guidelines.

In July 2003, the Japanese Society for Abdominal Emergency Medicine, the Japan Pancreas Society and the Research Committee of Intractable Diseases of the Pancreas supported by the Japanese Ministry of Health, Labour, and Welfare published "Evidence-Based Clinical Practice Guidelines for Acute Pancreatitis"[2,5]. The working party of the Research Committee of the Intractable Diseases of the Pancreas made the present document for the management of acute pancreatitis in the early stages after onset[6], which was not mentioned in the guidelines[2,5]. The management of patients with acute pancreatitis is complicated by an unpredictable outcome[7]. Many patients initially identified as having mild disease progress to severe disease indolently over the initial 48 h. This document is the consensus of experts in pancreatitis in Japan and focuses on the practical aspects in the early management of patients with acute pancreatitis and decisions needed within the first 48 h of care, introducing literatures on the treatment of acute pancreatitis published in Japanese.

This consensus guideline on acute pancreatitis is a review of literatures and experts' opinions in Japan intended for general clinicians in primary care and emergency department, and gastroenterologists who do not have expertise in pancreatitis. However, this consensus by experts is only an example of standard practical medical care for patients with acute pancreatitis in Japan. Physicians need not necessarily follow this consensus but have to determine measures depending on hospital and patient specifics.

ESSENTIALS OF PRIMARY CARE OF ACUTE PANCREATITIS

All patients diagnosed with acute pancreatitis should be managed in the hospital. Monitoring of blood pressure, pulse and respiratory rate, body temperature, hourly urinary volume, and blood oxygen saturation level (SpO_2) by pulse oximetry is essential. Early vigorous intravenous hydration is of foremost importance. The correct diagnosis of acute pancreatitis and severity stratification should be made in all patients using the criteria for the diagnosis of acute pancreatitis and the multifactor scoring system proposed by the Research Committee of Intractable Diseases of the Pancreas[8] as early as possible. Following the correct diagnosis of acute pancreatitis, severity stratification should be performed promptly and repeatedly after the onset, in particular for the first 48 h.

It is not possible to distinguish mild from severe acute pancreatitis during the early stages. Serum levels of amylase and lipase do not reflect the severity of acute pancreatitis[9,10]. Even patients who appeared to be mild acute pancreatitis at the first hospital visit (within 48 h after onset) often deteriorate. All patients with severe acute pancreatitis must be monitored closely for the development of organ failure and should be managed in, or referred to, an intensive care unit (ICU) with full monitoring and systems support. The mortality rate of patients with mild acute pancreatitis within the first 24 h who later showed exacerbation during 24-48 h was reported to be 11%[11].

When the diagnosis of severe acute pancreatitis was made, systemic administration of antibiotics and protease inhibitors, and special measures such as continuous regional arterial infusion (CRAI) of protease inhibitors and antibiotics, continuous hemodiafiltration (CHDF), and selective decontamination of the digestive tract (SDD) should be considered.

Patients with severe acute pancreatitis and those requiring interventional radiological, endoscopic, or surgical procedures should be managed in, or referred to, a specialty unit. Principles of management of acute pancreatitis are summarized in Table 1 and Figure 1.

DIAGNOSIS OF ACUTE PANCREATITIS

Acute pancreatitis should be diagnosed according to the diagnostic criteria for acute pancreatitis proposed by the Research Committee of Intractable Diseases of the Pancreas[8]. Clinical criteria for the diagnosis of acute pancreatitis are (1) acute abdominal pain and tenderness in the upper abdomen, (2) elevated pancreatic enzyme levels in blood, urine, or ascitic fluid, and (3) radiologic abnormalities characteristic of acute pancreatitis. Acute pancreatitis can be diagnosed when two or more of the above criteria are fulfilled and other causes of acute abdominal pain are excluded. Acute exacerbation of chronic pancreatitis is included in acute pancreatitis.

The incidence of acute pancreatitis in Japan appears to be increasing[12] and ranges from 187 to 347 cases per million populations. Approximately 4.9% of patients who visited hospitals complaining of acute abdominal pain were found to have acute pancreatitis[13]. Thus, acute pancreatitis should be considered as a differential diagnosis for cases with signs and symptoms of digestive diseases. A history of the present illness and life-style, physical examination, blood tests such as amylase and lipase, and imaging studies such as abdominal plain X-ray, ultrasonography (US) and computed tomography (CT) should be undertaken to assist in the diagnosis of acute pancreatitis. Therefore, every hospital that receives acute admissions should have facilities for acute pancreatitis to be diagnosed at any time. In addition, physicians should be aware of the presence of acute pancreatitis patients but without abdominal pain or

Table 1 Principles of treatment for patients with acute pancreatitis at initial and early stage

Management	Physical check lists	Examination	Treatment
Within 24 h after onset			
Initial vigorous intravenous hydration Severity stratification Assessment of etiology All patients with severe acute pancreatitis should be transferred to a high special unit or intensive therapy unit.	Consciouness status Abdominal findings Blood pressure Pulse rate Respiratory rate Body temperature Urinary volume Pulse oximetry (SpO_2)	CBC and blood chemistry Chest and abdominal x-ray Abdominal US Abdominal CT and/or MRI Arterial blood gas analysis	Secure and maintain venous route Initial fluid resuscitation (60-160 mL/kg body weight/day) For the first 6 h, fluid resuscitation of about 1/2-1/3 of the amount required for the first 24 h Analgesics and oxygen, as required Protease inhibitors Antibiotics for severe cases and infection of the bile duct Consider CRAI CHDF in severe cases Urgent therapeutic ERCP or EST in patients with cholangitis or with disturbed bile flow (refer to a specialist unit where facilities and expertise are available for ERCP and EST)
From 24 to 48 h after onset			
Re-evaluation of severity All patients with severe acute pancreatitis should be transferred to a high special unit or intensive therapy unit.	Consciouness status Abdominal findings Blood pressure Pulse rate Respiratory rate Body temperature Urinary volume Pulse oximetry (SpO_2)	CBC and blood chemistry Chest and abdominal x-ray Arterial blood gas analysis Abdominal CE-CT, as required	Similar to the above-mentioned treatment In addition Correction of fluid resuscitation Enteral feeding in patients without clear signs and symptoms of ileus and gastrointestinal bleeding
After 48 h of onset			
Fundamental conservative therapy in moderate and mildcases All patients with severe acute pancreatitis should be transferred to a high special unit or intensive therapy unit.	Consciouness status Abdominal findings Blood pressure Pulse rate Respiratory rate Body temperature Urinary volume Pulse oximetry (SpO_2)	CBC and blood chemistry Chest and abdominal x-ray Arterial blood gas analysis Abdominal CE-CT, as required	Similar to the above-mentioned treatment In addition Correction of fluid resuscitation Enteral feeding in patients without clear signs and symptoms of ileus and gastrointestinal bleeding

SpO_2: Blood oxygen saturation level, CBC: Complete blood count; US: Ultrasonography; CT: Computed tomography, MRI: Magnetic resonance imaging; CE-CT: Contrast-enhanced computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography; EST: Endoscopic sphincterotomy; CRAI: Continuous regional arterial infusion of protease inhibitors and antibiotics; CHDF: Continuous hemodiafiltration.

signs and symptoms.

The initial treatment should be started as early as possible following the diagnosis of acute pancreatitis.

PRIMARY CARE OF ACUTE PANCREATITIS (WITHIN 48 H OF DIAGNOSIS)

Severity stratification

Following the correct diagnosis of acute pancreatitis, severity stratification should be performed promptly and repeatedly after the onset, in particular for the first 48 h. Severity of acute pancreatitis should be evaluated and scored by the multifactor scoring system proposed by the Research Committee of Intractable Diseases of the Pancreas (JPN severity scoring system) (Table 2)[8]. We recognize that the Ranson, modified Glasgow, and APACHE II systems for evaluating acute pancreatitis are well established, but they might be outdated and are not used in Japan. Pathology-specific systems such as Ranson's and the modified Glasgow require 48 h of data collection before the severity can be evaluated. JPN severity scoring system contains variables that represent different organ systems and can be consistently recorded at the time of admission to facilitate immediate management decisions.

When the correct diagnosis of acute pancreatitis is made, it should be considered to perform contrast-enhanced CT (CE-CT) and/or CE-magnetic resonance imaging (CE-MRI)[14-17], systemic administration of antibiotics[18] and protease inhibitors[19], and special measures such as CRAI of protease inhibitors and antibiotics[20,21], CHDF[22], and SDD[23]. Patients with severe acute pancreatitis and those requiring interventional radiological, endoscopic, or surgical procedures should be managed in, or referred to, a specialty unit.

There is no simple and reliable index to predict aggravation of acute pancreatitis in the early stages. Despite the importance of recognizing severe disease early in the course, many patients initially identified as having mild disease progress to severe disease indolently over the initial 48 h[11]. Severity stratification should be made repeatedly, particularly within the first 48 h after admission. Early recognition of severe disease and application of

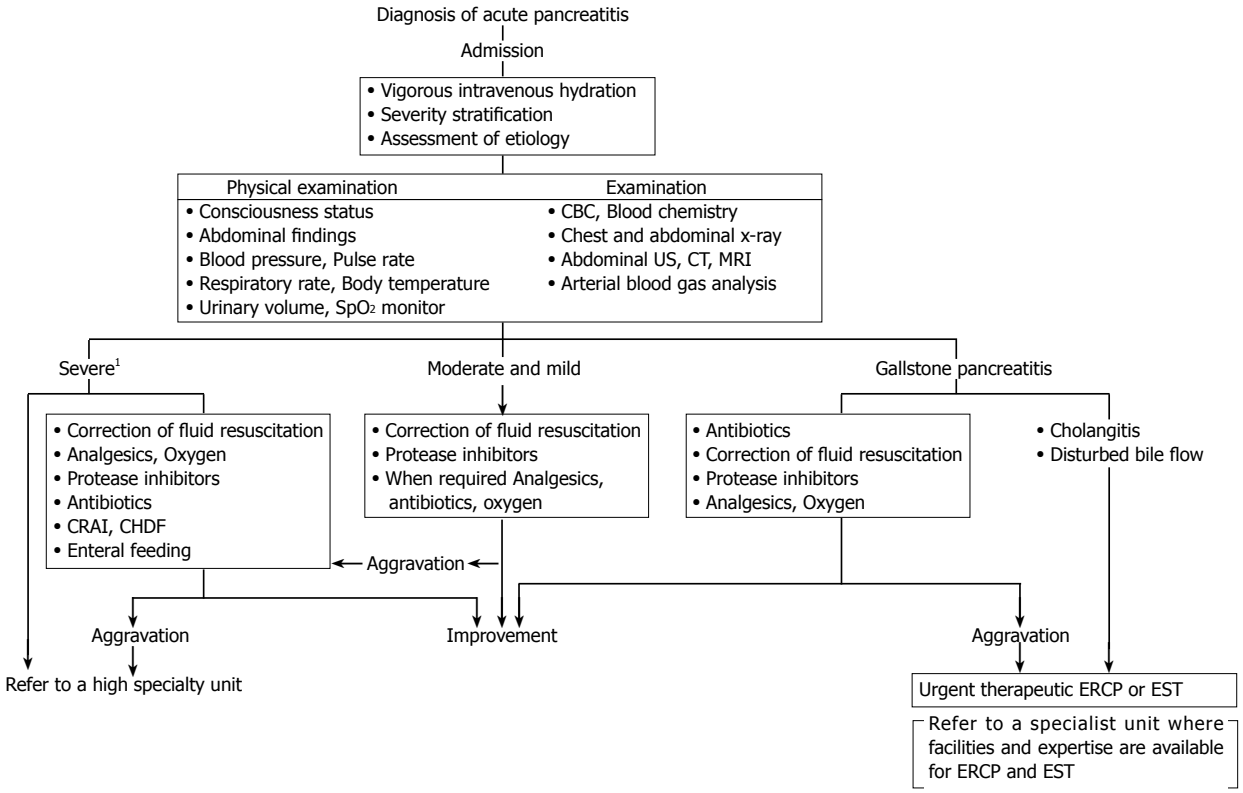

Figure 1 Algorithm of the primary management of acute pancreatitis at an early stage. [1]Hospitals that cannot provide appropriate monitoring and adequate systemic support should transfer all patients with severe acute pancreatitis to a high special unit or intensive therapy unit. SpO_2: Blood oxygen saturation level by pulse oximetry, CBC: Complete blood count, US: Ultrasonography, CT: Computed tomography, MRI: Magnetic resonance imaging, ERCP: Endoscopic retrograde cholangiopancreatography, EST: Endoscopic sphincterotomy, CRAI: Continuous regional arterial contrast infusion of protease inhibitors and antibiotics, CHDF: Continuous hemodiafiltration.

Table 2 Criteria for grading the severity of acute pancreatitis

Prognostic factors	Clinical signs	Laboratory data
Prognostic factor I (2 points for each positive factor)	Shock Respiratory failure Mental disturbance Severe infection Hemorrhagic diathesis	BE ⩽ -3 mEq/L Ht ⩽ 30% (after hydration) BUN ⩾ 40 mg/dL or creatinine ⩾ 2.0 mg/dL
Prognostic factor II (1 point for each positive factor)		PaO_2 ⩽ 60 mmHg (room air) FBS ⩾ 200 mg/dL Total protein ⩽ 60 g/L LDH ⩾ 700 IU/L Ca ⩽ 7.5 mg/dL Prothrombin time ⩾ 15 s Platelet count ⩽ 1×10^5 /mm^3 CT grade IV or V[1]
Prognostic factor III	SIRS score ⩾ 3 (2 points) Age⩾ 70 yr (1 point)	

BE: Base excess, Ht: Hematocrit, BUN: Blood urea nitrogen; FBS: Fasting blood sugar; LDH: Lactate dehydrogenase; SIRS: Systemic inflammatory response syndrome. [1]CT grade IV or V: Presence of diffuse and uneven density in the pancreatic parenchyma or the presence of inflammatory changes extending beyond the border of the pancreas. Severity score: Sum of the points for the positive prognostic factors is defined as the severity score. Standardized criteria: Severe: Presence of more than one prognostic factor I, and/or the presence of more than two prognostic factor II (severity score ⩾ 2 points). Moderate: Presence of one prognostic factor II (severity score = 1 point). Mild: Acute pancreatitis without prognostic factor I or II (severity score = 0 point).

Table 3 Stage classification of acute pancreatitis and mortality rate in nationwide survey in 2003 in Japan

Stage	Severity score	Severity	No of patients (%)[1]	Died[2]	Mortality rate
Stage 0	0 point	Mild	943 (53.3)	1	0.10%
Stage 1	1 point	Moderate	280 (15.8)	2	0.70%
Stage 2	2-8 points	Severe I	455 (25.7)	17	3.70%
Stage 3	9-14 points	Severe II	63 (3.6)	16	25.40%
Stage 4	⩾ 15 points	Most severe	27 (1.5)	16	59.30%
Total			1768 (100)	52	2.90%

[1]In 2004, nationwide survey of patients with acute pancreatitis who visited the hospitals in the year 2003 (from January 1 to December 31) was performed by stratified random sampling method. From the first survey, the total number of patients treated for acute pancreatitis in Japan in the year 2003 was estimated as 35 300 (95% confidence interval, 30 500-40 000). Clinical records of 1768 patients with acute pancreatitis were obtained in the second survey for analysis of etiology and outcome.
[2]Number of patients who died of acute pancreatitis or related complications.

appropriate therapy require vigilance as decisions regarding management need to be made shortly after admission, often within the first 24 h.

Case fatality in acute pancreatitis in Japan in 2003 according to the JPN severity score was 0.2% (3/1223) with 0-1 point (Stage 0, 1), 3.7% (17/455) with 2-8 points (Stage 2), and 35.6% (32/90) more than 9 points (Stage 3, 4) (Table 3)[12].

Assessment of etiology (diagnosis of acute gallstone pancreatitis)

The etiology of acute pancreatitis should be entertained

early, especially in patients with gallstone pancreatitis. Although the vast majority of stones that cause acute pancreatitis rapidly pass out of the common bile duct (CBD)[24], emergency endoscopic retrograde cholangiopancreatography (ERCP) with or without endoscopic sphincterotomy (EST) in selected patients with severe gallstone pancreatitis, who have evidence of biliary obstruction, cholangitis, and an elevated serum bilirubin levels within 24 h of admission, is reported to decrease the incidence of biliary sepsis[25]. Although abdominal US is considered the gold standard for the diagnosis of cholelithiasis, it is not sensitive for the evaluation of choledocholithiasis in the setting of acute pancreatitis. Abdominal US can detect gallstones in the gallbladder in 60%-80% of cases, and in the bile duct in 25%-90% of cases of gallstone pancreatitis in the early stage[26]. Gallstones may be present in the CBD even in the absence of biliary ductal dilatation on abdominal US[27]. Laboratory testing may assist in the early identification of CBD stones. In patients with serum alanine aminotransferase (ALT) (or glutamic-pyruvic transaminase [GPT]) values greater than 150 IU/L at admission and without a history of high quantities of alcohol intake, gallstone pancreatitis cannot be denied even in the absence of bile duct dilatation[27,28]. Increasing serum levels of bilirubin or transaminases within 24-48 h of admission for acute pancreatitis predicts a persistent CBD stone[27]. It is reported that combination of abdominal US and laboratory parameters enables to diagnose gallstone pancreatitis with a sensitivity of 94.9% and a specificity of 100%[24]. When the diagnosis of gallstone pancreatitis is in doubt, repeated imaging studies such as abdominal US, CT, MR cholangiopancreatography (MRCP)[29], or endoscopic ultrasound (EUS)[30], together with serum biochemical tests such as ALT, alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP) and total bilirubin may provide good evidence for the presence or absence of gallstone pancreatitis[28].

Urgent therapeutic ERCP or EST should be performed in acute pancreatitis patients with suspected or proven gallstones[24,31,32]. All patients with severe acute gallstone pancreatitis should be managed in, or referred to, a specialist unit where facilities and expertise exist for ERCP at any time for CBD evaluation followed by EST and stone extraction or stenting, as required.

Abdominal CT

Although it is not clear how soon the full extent of the necrotic process occurs and only a quarter of acute pancreatitis patients develop necrosis, it is current practice in Japan to perform CE-CT on admission for severity stratification and correct evaluation of the range of poorly perfused area. A low-density area (poorly perfused area) on CE-CT suggests ischemic change with vasospasm or necrosis in and around the pancreas[33]. The presence of pancreatic necrosis and its range, infiltration of inflammatory changes are closely related with severity and prognosis[15,34]. In addition, CE-CT is required for the decisions on special measures such as CRAI of protease inhibitors and antibiotics, and CHDF. However, since anxieties persist over the potential for extension of necrosis and exacerbation of renal impairment following the use of intravenous contrast media[35,36], vigorous intravenous hydration for the purpose of intravascular resuscitation is important during and after CE-CT.

In severe cases, CT evidence of a low-density area suggesting ischemic change with vasospasm or necrosis in and around the pancreas, free intraperitoneal fluid and extensive peripancreatic fluid collection are observed since an early stage. Since the full extent of the necrotic process occurs at least 4 d after the onset of symptoms[16] and an early CT may therefore underestimate the final severity of the disease, it is desirable to perform CT on admission and repeated CT for reevaluation 2 or 3 d later.

Evaluation and monitoring of circulatory dynamics and fluid resuscitation

Monitoring index: As the inflammatory process progresses early in the course of the disease, there is an extravasation of protein-rich intravascular fluid into the peritoneal cavity resulting in hemoconcentration. The reduced perfusion pressure into the pancreas leads to microcirculatory changes resulting in pancreatic necrosis[37]. At first, for appropriate fluid resuscitation, initial evaluation and monitoring of circulatory dynamics is important.

In all cases, status of consciousness, blood pressure, pulse and respiratory rate, oxygen saturation, body temperature, and hourly urinary volume should be monitored appropriately and chronologically.

The most important aim of fluid resuscitation is to stabilize circulatory dynamics, largely to keep the blood pressure and pulse rate at levels identical to those before onset of acute pancreatitis, and secure reasonable urinary volume. Since the urinary volume is closely related to appropriate circulatory plasma volume and blood pressure, maintenance of urinary volume is easy to monitor; 1 mL/kg body weight is the minimal hourly urinary volume that should be maintained.

In addition to the above-mentioned monitoring, cardiothoracic ratio (CTR) by chest X-ray, blood gas analysis, serum electrolyte, and hematocrit should be determined to perform appropriate fluid resuscitation. Despite sufficient initial fluid resuscitation, aggravation of consciousness status, and the occurrence and progression of metabolic acidosis require re-examinations of other factors such as mesenteric ischemia and reevaluation of circulatory dynamics by determining central venous pressure (CVP) and monitoring pulmonary-artery catheter pressure. In cases of unstable circulatory dynamics, transfer to ICU with full monitoring and systems support should be considered.

Fluid resuscitation: (1) The amount of fluid resuscitation. Extravasation of protein-rich intravascular fluid into the peritoneal cavity early in the course of acute pancreatitis causes hemoconcentration and intravascular hypovolemia leading to impaired microcirculation in the pancreas. The decreased blood flow leads to stasis, thrombosis and subsequent pancreatic necrosis. Vigorous intravenous hydration can prevent the development of pancreatic necrosis. Fluid resuscitation for acute pancreatitis should be started by extracellular fluid such as acetate or lactate Ringer solution from a peripheral vein. Healthy adults require 1500-2000 mL fluid (30-40 mL/kg body weight),

whereas patients with acute pancreatitis require 2-4 times (60-160 mL/kg body weight) the volume administered to healthy adults.

The severity of acute pancreatitis changes at every moment even if it appears to be mild on admission. Although acute pancreatitis typically results in significant intravascular losses, patients with acute pancreatitis are often given suboptimal fluid resuscitation. Since it is rare for the administration of large fluid volumes to be problematic in mild cases, aggravation by insufficient fluid resuscitation must be avoided. In particular, a large amount of fluid resuscitation (about 1/2-1/3 of the amount required for the first 24 h) is necessary in the first 6 h. Approximately 6 h after initiation of treatment, blood pressure, pulse rate, hourly urinary volume and the above-mentioned index must be re-evaluated to make plan for subsequent fluid resuscitation. In a mild case of acute pancreatitis, the amount and rate of fluid resuscitation should be reduced.

If shock or a pre-shock is present on arrival at the hospital, rapid intravenous fluid resuscitation of 500-1000 mL must be performed. Patients of 60 kg body weight with moderate to severe acute pancreatitis require about 1200-4800 mL fluid resuscitation for the first 6 h and 3600-9600 mL for the first 24 h. If the patient does not recover from circulatory failure by fluid resuscitation, administration of catecholamine and transfer to ICU with full monitoring and systems support should be considered.

We have reported that the average amount of fluid resuscitation on the first hospital day in patients who died of acute pancreatitis was 2788 ± 246 mL (n = 52, mean ± standard deviation) and that the amount of fluid resuscitation in 78.9% of these fatal cases for the first 24 h after admission was less than 3500 mL[38]. Indeed, all patients with inadequate fluid resuscitation (rehydration less than 4.0 liters) as evidenced by persistence of hemoconcentration at 24 h developed necrotizing pancreatitis[39].

The amount of fluid resuscitation required on the first and the following hospital day in moderate acute pancreatitis to maintain 120 mmHg or higher systolic blood pressure and hourly urinary volume of more than 1 mL/kg body weight was 4873 ± 2280 mL/d and 2000-2500 mL/d, respectively, while that in severe acute pancreatitis was 7787 ± 4211 mL/d and 4000-5000 mL/d, respectively[40]. Based on abdominal CT findings, Takeda *et al*[41] reported that the amount of fluid resuscitation on the first hospital day in patients with acute pancreatitis with inflammatory changes extending to anterior pararenal space, mesentery of the colon and the retroperitoneum beyond inferior pole of the kidney was approximately 4000 mL, 6000 mL, and 8000 mL, respectively. In some cases, body weight may increase more than 10 kg within a few days after onset of acute pancreatitis. It will take 1-2 wk for diuresis to occur resulting from re-filling to decrease body weight[41].

A large amount of fluid resuscitation is necessary until an adequate urinary volume is attained. Administration of diuretics before achievement of adequate fluid resuscitation may lead to a deterioration of the situation. Restriction of the amount of fluid resuscitation and excessive diuresis by diuretics because of fear of aggravation of pulmonary and systemic edema by a large amount of fluid resuscitation at this time may exacerbate the dysfunction of important organs including the kidney. Fluid resuscitation should be performed to promote prompt recovery from a potential shock state even if respiratory care under intratracheal intubation is required as a result of a large amount of fluid resuscitation. (2) Route of infusion. It is desirable to secure and keep the central venous route to maintain long-term fluid resuscitation and continuous infusion of protease inhibitors in moderate and severe acute pancreatitis, while the central venous route is redundant in mild cases. In severe acute pancreatitis, a central venous route should also be secured to monitor CVP chronologically and to determine the amount and rate of fluid resuscitation.

In the early stages of acute pancreatitis, vascular permeability often increases, and thus circulatory volume decreases resulting in a tendency for collapsed vascular lumen. Since access of the central vein is difficult in such cases, adequate levels of fluid should be given from a peripheral vein as the initial therapy. However, the femoral vein should be avoided if possible to prevent deep venous thrombosis and infection, and to securely obtain the route for CHDF and CRAI therapy. (3) Caution of fluid resuscitation. Since excessive amounts and a high rate of fluid resuscitation may cause pulmonary edema in patients with acute pancreatitis complicating heart, lung and renal dysfunction, and in elderly people, it is important to control the amount and rate of infusion according to the above-mentioned indexes such as blood pressure, pulse rate, urinary volume and CVP. However, it is thought that shock and aggravation of acute pancreatitis at an early stage tend to be due to insufficient amounts of the initial fluid resuscitation. It should be kept in mind to give adequate amount of fluid resuscitation at an early stage.

Serum levels of electrolyte and calcium should be measured and corrected appropriately, especially when tetany occurs due to low serum calcium concentrations.

There is no evidence to support the usefulness of administration of albumin preparation and fresh frozen plasma (FFP) for primary care within 48 h of onset. Administration of colloid fluid at the acute stage may retard improvement of pulmonary edema in the re-filling period.

Analgesics: Since acute pancreatitis often causes extreme abdominal pain, which may disturb cardiopulmonary functions, adequate pain relief is required.

The following drugs should be given; buprenorphine (0.1-0.2 mg intramuscular or intravenous injection, or 0.3 mg/h intravenous drip infusion) or pentazocine (7.5-15 mg intramuscular or intravenous injection, or 15-30 mg intravenous drip infusion). Since there is a possibility that these drugs induce spasms of the sphincter of Oddi and disturb the outflow of bile and pancreatic juice when given frequently, administration of atropine sulphate in combination with analgesics is recommended. In addition, these analgesics must be given with caution, due to the risk of drug dependence when used continuously. However, neither buprenorphine nor pentazocine is approved by health insurance in Japan for pain of acute pancreatitis. Intramuscular or subcutaneous injection of pentazocine and buprenorphine is only approved for pain control

of carcinoma and postoperative state, while intravenous injection of pentazocine is approved for anesthesia and buprenorphine for myocardial infarction and anesthesia.

A mixture of opium alkaloids and atropine (1.0 mL contains 20 mg opium alkaloid hydrochloride and 0.3 mg atropine sulphate, 1.0 mL subcutaneous injection), and a mixture of morphine and atropine (1.0 mL of this solution contains 10 mg morphine hydrochloride and 0.3 mg atropine sulphate, 1.0 mL subcutaneous injection) can also be given. Pain relief by epidural anesthesia is also possible in a high specialty unit. Since both reagents may cause respiratory depression, intensive monitoring of cardiopulmonary function is required after administration.

For mild pain in acute pancreatitis, non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac suppository and indomethacin suppository can be used. However, these agents should be used with caution due to associated adverse effects such as pernicious anemia, hemolytic anemia, granulocytopenia, thrombocytopenia, liver damage, acute renal failure, asthma, interstitial pneumonia, and peptic ulcer hemorrhage. These drugs are usually not used in moderate and severe cases of acute pancreatitis. In particular, NSAIDs are contraindicated in patients in pre-shock status.

Antibiotics: In mild and moderate cases of acute pancreatitis, prophylactic administration of antibiotics is unnecessary because the incidence of fatal infection of the pancreas and the exudates around the pancreas is low.

In severe acute pancreatitis, prophylactic intravenous administration of antibiotics at an early stage is recommended[18,42,43]. Antibiotics with a broad antibacterial spectrum and good penetration into the pancreas[44] such as imipenem[18,42] and meropenem[43] are desirable. If infection of the biliary tract is suspected, administration of the second or later generation cephalosporins is also recommended. Clinicians should always take microbial substitution and fungal infection into consideration when using antibiotics.

The maximum dose of antibiotics that is approved by the Japanese Government should be used to attain sufficient concentrations in and around the pancreas. For example, intravenous infusion of 1-3 g/d imipenem or meropenem dividing into 3 times is recommended (1.0 g of imipenem or meropenem can be dissolved in 100 mL physiologic saline or 5% glucose solution, and infuse for more than 30 min). However, special attention has to be paid to the patient's liver and renal function. In particular, in severe cases, it is necessary to re-evaluate the dose of antibiotics every day because liver and renal function changes chronologically day by day. If possible, it is recommended to monitor serum concentrations of antibiotics. Moreover, clinicians should be concerned about the possibility of developing fungal infections.

In cases with advanced inflammation in and around the pancreas, SDD is often undertaken to prevent bacterial translocation[23].

Protease inhibitors: Although the effect of protease inhibitors has some controversy, experts of pancreatitis in Japan recommend to administer protease inhibitors as soon as the diagnosis of acute pancreatitis is confirmed[19,45]. Recent meta-analysis of 10 articles of randomized controlled trials evaluating the effects of protease inhibitors for acute pancreatitis has revealed that treatment with protease inhibitors does not significantly reduce mortality in patients with mild acute pancreatitis, but may reduce mortality in patients with moderate to severe acute pancreatitis[46]. Table 4 shows examples of protease inhibitors used for acute pancreatitis in Japan[47].

Table 4 Recommended doses of protease inhibitors for acute pancreatitis[47]

	Mild and moderate	Severe
Protease inhibitor	FOY FUT UTI	FOY + UTI FUT + UTI
Initial dose[2] (during the first 12 h)	Maximum usual one-day dose[1] by continuous intravenous infusion	Maximum usual one-day dose[1] by continuous intravenous infusion
Until d 3[2]	Above dose for 24 h	Above dose for 24 h
First wk[2]	Gradually reduce the above dose or intermittent administration	Above dose for 24 h
Second wk[2]	Reduce the dose or cease	Maintain or gradually reduce the above dose
Third wk[2]	Reduce the dose or cease	Maintain or gradually reduce the above dose

FOY: Gabexate mesilate, FUT: Nafamostat mesilate, UTI: Ulinastatin. [1]Administer the one-day dose during the initial 12 h. [2]Severity stratification should be made repeatedly, and the doses of protease inhibitors must be increased or decreased depending on the severity. The usual daily dosage for acute pancreatitis of FOY, FUT and UTI approved by the health insurance is 200-600 mg, 10-60 mg and 50 000-150 000 units, respectively. Since, however, severe acute pancreatitis often complicates disseminated intravascular coagulation (DIC) and shock, it is recommended to give these reagents in doses that are approved for these disorders in severe acute pancreatitis (daily dosage for severe acute pancreatitis of FOY, FUT and UTI is 20-39 mg/kg body weight/d, 1.44-4.8 mg/kg body weight per day, and 10 000-300 000 units /d, respectively).

Usual doses of gabexate mesilate (FOY), nafamostat mesilate (FUT) and ulinastatin (UTI) for acute pancreatitis are 200-600 mg/d, 10-60 mg/d and 50 000-150 000 units/d, respectively. However, since severe acute pancreatitis is often complicated by disseminated intravascular coagulation (DIC) and shock, it is recommended that these reagents should be given in doses approved for these disorders in severe acute pancreatitis. The guidelines for acute pancreatitis in 1991 from the Research Committee of Intractable Diseases of the Pancreas recommended combination therapy with FOY or FUT together with UTI when severe pancreatitis is predicted[47].

FOY and FUT have several adverse effects that clinicians must be aware of; FUT increases serum potassium (K) concentration[48,49], and FUT and FOY may cause phlebitis, and inflammation, ulcer and necrosis of the skin with extravasation. In addition, there are reports of anaphylactic shock by repeated administration of FOY and FUT[50,51].

Enteral feeding: Based on the fact that enteral feeding stimulates pancreatic secretions[52], classical management recommends that patients presenting with acute pancreatitis be fasted until symptoms begin to resolve. However, there is no sufficient evidence to support the concept of gut rest (prohibiting enteral intake) in acute pancreatitis. Acute pancreatitis results in hypermetabolic and hyperdynamic situation creating a high catabolic stress state[53]. Failure to achieve a positive nitrogen

balance is associated with increased mortality in patients with severe acute pancreatitis[54]. Traditionally, total parenteral nutrition (TPN) has been advocated in these patients in order to supply adequate nutrients and prevent hypercatabolic states avoiding stimulation of exocrine pancreatic secretions. However, TPN in patients with acute pancreatitis has not been shown to be beneficial[55]. Early enteral nutrition through a nasojejunal tube is associated with a significantly lower incidence of infections, reduced surgical interventions, and a reduced hospitalization period compared with TPN[56]. Enteral nutrition is as safe and efficacious as TPN and may be beneficial during the clinical course of this disease. Even in severe acute pancreatitis, a combination of enteral feeding with parenteral nutrition from early stage is recommended if there are no clear signs and symptoms of ileus and gastrointestinal bleeding[58].

Although the majority of studies have reported enteral feeding via a nasojejunal tube, a recent pilot study of early nasogastric feeding in patients with severe acute pancreatitis proved that this approach might be feasible in up to 80% of cases[59]. However, caution should be used when administering nasogastric feeding to patients with impaired consciousness due to the risk of aspiration of refluxed feed.

Other treatments: Patients with acute pancreatitis often have deficiency of vitamin B1 due to alcohol abuse and malnutrition or abnormal and irregular dietary habits. Exacerbation of lactic acidosis or shock in such patients may suggest a deficiency of vitamin B1 and require corrective measures.

TRANSFER TO ANOTHER HOSPITAL

Transfer time to a high specialty unit

All patients when diagnosed with acute pancreatitis should be managed in a hospital. Patients with suspected acute pancreatitis should be examined at hospitals with facilities for blood examination and imaging studies. Depending on the severity, full monitoring and systemic support in high care unit (HCU) or ICU is necessary. Hospitals that cannot provide appropriate monitoring and adequate systemic support, should transfer all patients with severe acute pancreatitis to ICU as early as possible.

Evidence-based therapeutic guidelines for severe acute pancreatitis[2,5] published by the Japanese Society for Abdominal Emergency Medicine, the Japanese Pancreas Society and the Research Committee of Intractable Diseases of the Pancreas recommends transferring acute pancreatitis patients with the JPN severity score 2 points or above[8]. Severe or aggravating cases should be transferred to ICU as early as possible to perform the CRAI of protease inhibitors and antibiotics or CHDF.

Instructions of transfer

During transfer, cardiopulmonary function such as blood pressure, pulse and respiratory rate, and oxygen saturation must be monitored. In addition, a venous route must be secured by continuing fluid infusion to be able to cope promptly with a sudden change. In severe cases, a doctor and a nurse should accompany the patient during transfer.

Before deciding to transfer a patient, it should be considered about the influence of street transportation for long time on the patient's clinical condition when vigorous fluid resuscitation is necessary at early period of illness.

Local core hospitals require facilities that can deal with severe acute pancreatitis.

SPECIAL MEASURES

Continuous regional arterial infusion of protease inhibitors and antibiotics (CRAI)

To obtain high concentrations of protease inhibitors and antibiotics in the pancreas, these reagents are continuously infused into the artery through a catheter with tip placed at a portion where the bloodstream irrigates the inflamed area of the pancreas. Concentrations of protease inhibitors and antibiotics in pancreatic tissue after CRAI are approximately 5[60] and 5-10 times[61], respectively, higher than those after intravenous infusion. This procedure is therefore expected to directly inhibit inflammatory processes in the pancreas by strongly inhibiting activity of proteases that might aggravate pancreatic damage and inhibit most of the pathogens present in pancreatic infections[8,20,21,62]. Since protease inhibitors used in Japan also have anticoagulant and anticomplement activities [63], it is possible that these reagents inhibit the development of pancreatic necrosis by preventing the formation of microthrombi in pancreatic vessels and evolution of ischemia into pancreatic necrosis.

This procedure is indicated in severe acute pancreatitis cases with a low density area of the pancreas on CE-CT on admission, which corresponds with ischemic change and vasospasm on angiography or necrosis in and around the pancreas[33]. Takeda *et al*[21] have demonstrated that CRAI of protease inhibitors and antibiotics is effective in reducing mortality and preventing the development of pancreatic infection when initiated within 48 h after the onset of severe acute pancreatitis. FUT or FOY is used for this procedure, because these agents are synthetic low-molecular-weight protease inhibitors that inhibit a number of serine-proteases, and C1r and C1s esterases[63]. In addition, FOY and FUT might easily penetrate into the pancreatic acinar cells due to their low molecular weight and inhibit the inflammatory process in the pancreas. With regard to antibiotics, imipenem is used because of its broad spectrum and good penetration into pancreatic tissue[44]. Since CRAI of protease inhibitors and antibiotics for the treatment of acute pancreatitis is still debatable and not covered by health insurance in Japan, informed consent must be obtained from the patient before performing this procedure.

Continuous hemodiafiltration (CHDF)

CHDF is a combination of continuous hemofiltration with hemodialysis developed as a continuous renal replacement therapy for patients with severe conditions. It is usually performed continuously for 24 h, and for 3-14 d. This procedure can be used to control fluid balance in patients with excessive fluid resuscitation but inadequate diuresis. However, in some hospitals this procedure is positively performed even if there is sufficient urinary volume, because CHDF can remove excess humoral mediators

including cytokines during the hypercytokinemic state of systemic inflammatory response syndrome such as severe acute pancreatitis[22,64,65].

When this procedure is started early after onset of acute pancreatitis, reduction of chemical mediators and improvement of respiratory function is obvious, while the incidence of multiple organ failure is low compared to cases in which the procedure is initiated at a late stage[64]. Health insurance in Japan has approved CHDF as one of insurance-applicable treatment modalities for severe acute pancreatitis.

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GASTRIC CANCER

Relationships between mucinous gastric carcinoma, MUC2 expression and survival

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Abstract

AIM: To investigate the expression of the four secreted gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) in a series of gastric carcinomas, classified according Laurén's, Mulligan's, WHO and Goseki's classifications, with special attention to all the different components (major and minor) present in tumors and to follow up clinical data.

METHODS: Expression of MUC2, MUC5AC, MUC5B and MUC6 was investigated using immunohistochemistry and *in situ* hybridization.

RESULTS: Expression of secreted gel-forming mucins in gastric carcinoma was particularly complex, each mucin being not restricted to any histopathological type even considering all components (major and minor) present in a given tumor. There was a worst survival in patients with a higher content of mucus (Goseki II or IV) and high positive MUC2 expression.

CONCLUSION: Complexity of mucin gene expression patterns in gastric cancer may reflect a precise state of differentiation at the cell level not recognized in used morphologic classification systems. High expression of MUC2 was nevertheless associated with mucinous subtype of the WHO classification and with group II of Goseki's classification identified by the major component of a particular tumor. The quantity and quality of mucus were related to survival.

Key words: Gastric cancer; Secreted gel-forming mucin; MUC; Immunohistochemistry; *in situ* hybridization; Survival

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INTRODUCTION

Gastric cancers constitute a highly heterogeneous group of tumors with respect to epidemiology, genetics, histopathology and biological behavior. Numerous histopathological classifications have been proposed to identify the morphological variability in a reproductible manner and to correlate to prognosis. These different classifications include Laurén's, Mulligan's, World Health Organization (WHO), and Goseki's classifications, which all are based at least in part on the quantity of mucus present in an individual tumor[1-3]. The Goseki's classification combines two distinct criteria, e.g. the tubular differentiation and the intra-cellular mucin content[3]. It was shown to be the only classification with prognostic value additional to TNM staging[4]. All histopathological classifications are based on the identification of the major component of the tumor, neglecting the various minor components. However, in pathological practice, assessment of a histopathological type of gastric carcinoma is difficult due to the considerable variation frequently present within an individual tumor.

Epithelial mucins consist of at least thirteen different types that are usually subdivided into secreted gel-forming mucins and membrane-bound mucins[5-7]. The first class includes the four large mucins MUC2, MU-

C5AC, MUC5B and MUC6 whose genes are clustered on chromosome 11p15.5 and which are involved in the mucus gel formation. MUC5AC and MUC6 are the major mucin components in normal gastric mucosa. MUC5AC is highly expressed in mucous cells of the superficial and foveolar epithelium, whereas MUC6 is present in mucous neck cells and in mucous glands of cardia and antrum[8-11]. MUC5AC and MUC6 are also expressed in some gastric carcinomas[12-15]. Moreover, MUC2 and MUC5B which are absent or barely detectable in normal gastric tissues have been reported in gastric carcinoma[12-16]. MUC2 expression has been related more specifically to the mucinous carcinomas of WHO classification and to group II of Goseki' s classication (high tubular differentiation and high mucin content) whereas MUC5AC has been associated with the group IV of Goseki's classification (poor tubular differentiation and high mucin content)[14,15,17]. However, coexpression of multiple mucins is frequently observed in gastric carcinomas[9,13,15]. Such a complex pattern of mucin expression may reflect the variety frequently observed in gastric carcinomas.

In an effort to bring additional information in understanding the histopathological diversity of gastric carcinomas, we investigated the expression of the secreted gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) in a series of gastric carcinomas with special attention to all the different components (major and minor) present in tumors and to follow up clinical data.

MATERIALS AND METHODS

Tissue samples and histopathological study

A series of 31 gastric adenocarcinomas was obtained from institutional files. Cases included 7 women and 24 men with a mean age of 65 years-old, from 39 to 89 years. The surgical specimens were quickly immersed in fresh 4 g/L neutral formaldehyde solution (pH 7.4) in phosphate buffer and then were processed for paraffin embedding. Each gastric carcinoma was classified according to each of the following histopathological classifications; i.e. Laurén's classification, Mulligan's classification, WHO classification and Goseki's classification. These classifications are based on the identification and classification of the major component of the tumor on hematoxylin-eosin-saffron (HES) coloration. The Goseki's classification was realized on an adjacent tumor slide with Periodic Acid Schiff and Alcian Blue combined coloration. The Goseki's classification combines two distinct criteria, e.g. the tubular differentiation and the intracellular mucin content[3]. Four group are defined: group I, high tubular differentiation and poor mucin content; group II, high tubular differentiation and high mucin content; group III, poor tubular differentiation and poor mucin content; group IV, poor tubular differentiation and high mucin content[3]. The classification proposed by Mulligan recognizes three forms of gastric carcinoma: the intestinal cell type, the mucous cell type and the pylorocardiac gland cell type. In this classification the intestinal and mucous cell types are similar to the intestinal and diffuse types defined by Lauren. The pylorocardiac gland cell type carcinoma is a distinct group of gastric carcinoma characterized by large cells with clear cytoplasm [2]. The differentiation of each tumor was assessed on the basis of the degree of gland formation as well, moderate, poorly differentiated or undifferentiated. When the tumor presented with a major and one or more minor components, a sample where the different components were present was chosen to further perform the immunohistochemical and *in situ* hybridization studies on serials sections (4-μm thick).

Immunohistochemistry

Immunohistochemistry was performed using an automated immunostainer (ES; Ventana medical systems, Strasbourg, France). Primary antibodies were directed against MUC2 (polyclonal, LUM2-3, 1/1000)[18], MUC5AC (monoclonal, 1/2) (Novocastra, New Castle, UK), MUC5B (polyclonal, Lum5B-2, 1/1000)[19], MUC6 (monoclonal, 1/5) (Novocastra). The pretreatment was realized by a steamer for MUC2 (for 1 min 30) or by a microwave for MUC5AC, MUC5B and MUC6 (for 20 min), in citrate buffer (pH 6.0). For polyclonal antibodies, the sections were incubated for 32 min with normal goat serum to block the non-specific antibody-binding sites and endogenous peroxidase activity was suppressed by first incubating the specimen in 30 mL/L hydrogen peroxide. The immunohistochemistry method used a 3-step indirect process based on the biotin-avidin complex. Slides were counterstained with hematoxylin. Negative controls were run by omission of the primary antibody and positive controls with the appropriate tissue (normal colonic mucosa for MUC2, normal gastric mucosa for MUC5AC and MUC6, normal respiratory submucosal glands for MUC5B). The immunohistochemical results were estimated on the whole tumor and on the different components. The immunostaining was scored semiquantitatively as: 0, no immunostained cells; 1, less than 30% of immunostained cells; 2, more than 30% and less than 60% of immunostained cells; 3, strictly more than 60% of immunostained cells.

In situ hybridization

In situ hybridization was performed as described previously using four ^{35}S-labeled antisense oligonucleotide probes corresponding to each tandem repeat domain of *MUC2*, *MUC5AC*, *MUC5B*, and *MUC6*[20]. Briefly, tissue sections were deparaffinised, rehydrated, incubated with 2 mg/L proteinase K (Roche Diagnostics, Meylan, France) for 15 min and fixed again in 40 g/L paraformaldehyde in PBS for 15 min. Sections were then immersed in 0.1 mol/L triethanolamine (Sigma, L'Isle d'Abeau Chesnes, France) containing 2.5 ml/L acetic anhydride for 10 min. Sections were prehybridized in 4 × SSPE, 1×Denhardt' s buffer for 45 min, and hybridized overnight at 42 ℃ in 20-100 μL of a 4 × SSPE solution containing 500 mL/L formamide, 1 g/L *N*-lauroylsarcosine, 1.2 mol/L sodium phosphate (pH 7.2), 1 × Denhardt's buffer, 3 g/L yeast tRNA, 20 mmol/L dithiothreitol, and 125 MBq/L of ^{35}S-labeled oligonucleotide. After post-hybridization washes, slides were dipped in LM-1 emulsion (Amersham, Les Ulis, France), developed 1-3 wk after exposure, and counterstained with methyl green pyronin (Sigma). The following controls were performed: (1) competition studies by treatment of tissue sections with

Table 1 MUC expression in 31 gastric carcinomas stratified according to Laurén's classification, Mulligan's classification, WHO classification and Goseki's classification *n* (%)

Parameter	*n*	MUC2		MUC5AC		MUC5B		MUC6	
		+ IHC[1]	+ ISH	+ IHC	+ ISH	+ IHC	+ ISH	+ IHC	+ ISH
Laurén									
Intestinal	14	3 (21)	13 (93)	8 (57)	7 (50)	12 (86)	5 (36)	6 (43)	6 (43)
Diffuse	12	1 (8)	6 (50)a	9 (75)	8 (67)	9 (75)	3 (25)	5 (42)	3 (25)
Unclassified	5	3 (60)	4 (80)	3 (60)	1 (20)	3 (60)	1 (20)	1 (20)	2 (40)
Mulligan									
Intestinal cell	18	5 (28)	16 (89)	11 (61)	8 (44)	15 (83)	6 (33)	7 (39)	7 (39)
Mucous cell	13	2 (15)	7 (54)	9 (69)	8 (62)	9 (69)	3 (23)	5 (38)	4 (31)
Pylorocardiac	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
WHO									
Papillary	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Tubular	14	3 (21)	13 (93)	8 (57)	7 (50)	12 (86)	5 (36)	6 (43)	6 (43)
Mucinous	3	3 (100)[a]	3 (100)	2 (67)	0 (0)	2 (67)	0 (0)	0 (0)	1 (33)
Signet-ring cell	12	1 (8)	6 (50)[a]	9 (75)	8 (67)	9 (75)	3 (25)	5 (42)	3 (25)
Undifferentiated	2	0 (0)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)
Goseki									
Group I	11	2 (18)	10 (91)	5 (45)	4 (36)	9 (82)	2 (18)	5 (45)	4 (36)
Group II	5	4 (80)[b]	5 (100)	4 (80)	2 (40)	4 (80)	2 (40)	1 (20)	2 (40)
Group III	9	0 (0)	4 (44)	6 (67)	5 (56)	5 (56)	3 (33)	3 (33)	4 (44)
Group IV	6	1 (17)	4 (67)	5 (83)	5 (83)	6 (100)	2 (33)	3 (50)	1 (17)

[1] MUC2 positivity in more than 30% of tumoral cells. IHC: Immunohistochemistry; ISH: *In situ* hybridization. [a]$P<0.05$, [b]$P = 0.01$ *vs* other groups.

a large excess of unlabeled oligonucleotide identical to or distinct from the ^{35}S-labeled-probe; (2) verification of the absence of background by careful examination of non-epithelial structures (vessels, muscle, and connective tissue). The intensity of the hybridization signal was scored semiquantitatively as: 0, absent; 1, weak (visible at magnification ×200); 2, moderate (visible at magnification × 100); 3, strong (visible at magnification ×40).

Statistical analysis

Comparisons of the percentages in the different classifications were performed using chi-square and Fischer's exact tests. Survival was determined from the date of surgery to the time of death using the Kaplan–Meier method. Statistical significant of differences in survival curves was evaluated using the log-rank test. All statistical analyses were carried out by the SPSS 10.0 (SPSS, Chicago, IL, USA) software system. $P < 0.05$ were considered to indicate significant differences.

RESULTS

Histopathological results

Among the 31 gastric carcinomas, 2 were well differentiated, 11 moderately differentiated, 16 poorly differentiated and 2 undifferentiated. 14 carcinomas were classified as intestinal type in the Laurén's classification, these tumors being mainly classified as intestinal cell type in the Mulligan's classification, as tubular in WHO classification and as group I or II in the Goseki's classification. 12 tumors were classified as diffuse type in the Laurén's classification, being classified as mucous cell type in the Mulligan's classification, as signet-ring cell in the World Health Organization (WHO) classification and as group III or IV in the Goseki's classification. 3 carcinomas, not individualized in the Laurén's and Mulligan's classifications, corresponded to the mucinous type of the WHO classification and were classified in group II of Goseki's classification (Table 1).

Relationship between histopathological classifications and MUC expression

All MUCs were observed in each subtype of gastric carcinomas classified either in Laurén's classification, Mulligan's classification, WHO classification or Goseki's classification (Table 1, Figure 1). MUC2 was the major secreted gel-forming mucin detected in our series of gastric carcinomas. It was detected in 81% of the cases by immunohistochemistry and in 74% of the cases by *in situ* hybridization. MUC2 immunoreactivity was observed in the cytoplasm, in intracellular mucus vacuoles of tumoral cells, and also in extracellular mucus (Figure 1). Detection of MUC2 was not associated with a particular subtype of gastric carcinoma whatever the classification. However, high MUC2 immunostaining (in more than 30% of the tumoral cells), was significantly associated with the mucinous subtype of WHO classification ($P=0.015$) and with the group II of the Goseki's classification ($P=0.01$, Table 1). Although less significant, the absence of *in situ* hybridization signal for MUC2 was more frequent in the signet-ring subtype of the WHO classification ($P=0.04$) and in the diffuse subtype of Laurén's classification ($P=0.04$, Table 1). MUC5AC was detected in 65% of the cases by immunohistochemistry and in 52% of the cases by *in situ* hybridization. MUC5AC immunoreactivity was localized in the cytoplasm of tumoral cells, without staining of intracellular mucus vacuoles (Figure 1). MUC5AC detection either by immunohistochemistry or *in situ* hybridization was not associated with a particular subtype of gastric carcinoma classified by Laurén's, Mulligan's, WHO or Goseki's classifications (Table 1). MUC5B was

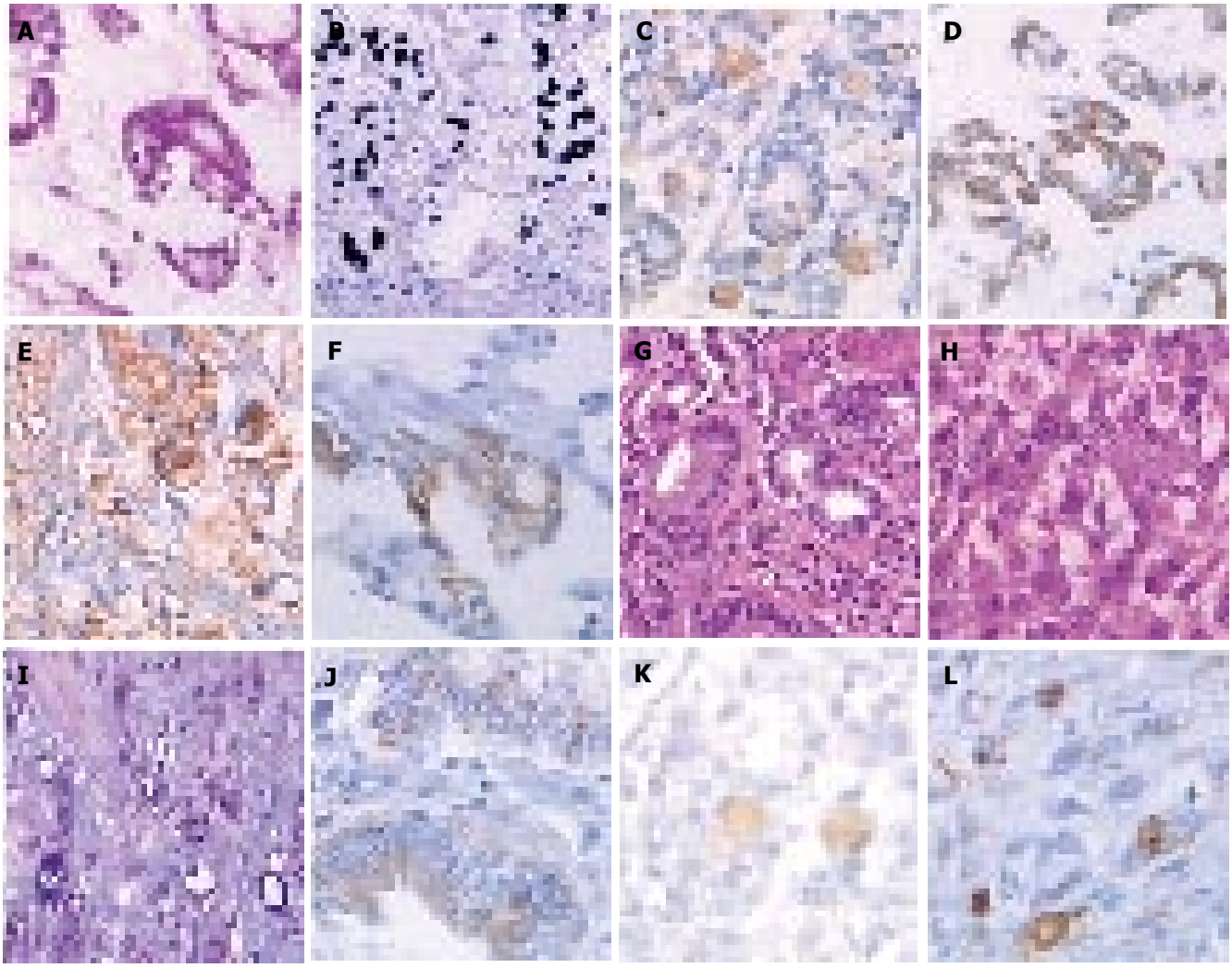

Figure 1 Expression of the secreted gel-forming mucins in gastric cancers. **A**: Mucinous subtype in WHO classification, group II in Goseki's classification (HE-safron × 125); **B:** Strong MUC2 in mucinous subtype (*in situ* hybridization × 40); **C**: MUC2 in intracellular mucus vacuoles in mucinous subtype (SABC × 125); **D:** MUC5AC in the cytoplasm of tumoral cells in mucinous subtype (SABC × 125); **E:** MUC5B in the cytoplasm, within intracellular mucus vacuoles and in extracellular mucus in mucinous subtype (SABC × 125); **F**: MUC6 in the cytoplasm of tumoral cells in mucinous subtype (SABC × 200); **G:** Gastric cancer classified group I in Goseki's classification (HE-safron × 125); **H:** Gastric cancer classified group III in Goseki's classification (HE-safron, × 200); **I:** Gastric cancer classified group IV in Goseki's classification (PAS and Alcian blue × 200); **J:** MUC2 in the cytoplasm of tumoral cells in group I gastric cancer Goseki's classification (SABC × 200); **K:** MUC2 in intracellular mucus vacuoles in group III gastric cancer in Goseki's classification (SABC × 200); **L:** MUC2 in intracellular mucus vacuoles in group IV gastric cancer in Goseki's classification (SABC × 200).

detected in 77% of the cases by immunohistochemistry and in 29% of the cases by *in situ* hybridization. MUC5B immunoreactivity was observed in the cytoplasm, in intracellular mucus vacuoles of tumoral cells and in extracellular mucus (Figure 1). MUC5B detection either by immunohistochemistry or *in situ* hybridization was not associated with a particular subtype of gastric carcinoma whatever the classification (Table 1). The discrepancies between the immunohistochemical and *in situ* hybridization results for MUC5B and at a lesser extent for MUC5AC corresponded mostly to cases which presented less than 30% of positive cells by immunohistochemistry and were negative by *in situ* hybridization. MUC6 was detected in 39% of the cases by immunohistochemistry and in 35% of the cases by *in situ* hybridization. The detection was observed in the cytoplasm of the tumoral cells, without staining of intracellular mucus vacuoles (Figure 1). Its expression was not associated with a particular subtype of gastric carcinoma whatever the classification (Table 1). No significant difference was observed for immunohistochemical or *in situ* hybridization detection of MUC2, MUC5AC, MUC5B or MUC6 between well/moderate differentiated carcinomas and poorly/undifferentiated carcinomas. The coexpression of at least two MUCs in the same tumor was frequent either identified by immunohistochemistry (83% of the gastric carcinomas) or *in situ* hybridization (54%). The coexpression of three MUCs in the same tumor was also remarkable either by immunohistochemistry (64%) or *in situ* hybridization (38%). In the series, 22% of the cases presented with the four MUCs detected by immunohistochemistry and 13% by *in situ* hybridization. The coexpression of at least two MUCs in the same tumor was not associated with a particular subtype of the

Table 2 MUC expression in each component of gastric carcinoma on the basis of Goseki's classification *n*(%)

Goseki,	*n*	MUC2		MUC5AC		MUC5B		MUC6	
		+ IHC	+ ISH	+ IHC	+ ISH	+ IHC	+ ISH	+ IHC	+ ISH
Group I	21	14 (67)	17 (81)	12 (57)	8 (38)	12 (57)	7 (33)	6 (29)	6 (29)
Group II	12	10 (83)	10 (83)	7 (58)	7 (58)	8 (67)	5 (42)	5 (42)	7 (58)
Group III	10	2 (20)	1 (10)	4 (40)	3 (30)	4 (40)	1 (10)	1 (10)	1 (10)
Group IV	13	9 (69)	6 (46)	7 (54)	5 (38)	7 (54)	2 (15)	4 (31)	2 (15)

IHC: Immunohistochemistry; ISH: *In situ* hybridization.

Goseki's classification.

Relationship between the different components and MUC expression

Among this series of 31 gastric carcinomas, we observed an histopathological heterogeneity in the majority of individual tumors. Indeed, 61% of the series presented with more than one component (one major component and one or more minor components). Given the difficulty to demonstrate a constant correlation between MUC expression and histopathological classifications, we further evaluated the expression of the four secreted gel-forming mucins independently in all the different components, major or minor, classified according to the Goseki's classification, frequently present in a same gastric carcinoma.

When the immunohistochemical labeling was studied separately in each component of each gastric carcinoma classified on the basis of the Goseki's classification, MUC2, MUC5AC, MUC5B and MUC6 were observed in all the different groups (group I to IV, Table 2). In particular, MUC2 was not restricted to the components classified as group II of Goseki (83% of histopathological components of group II were positive for the immunohistochemical detection of MUC2), but was also detected in components classified as group I (67%), group III (20%) or group IV (69%). Similarly, MUC5AC, MUC5B and MUC6 were detected in components classified as group I, group II, group III or group IV. The comparison between the tumors which presented with a component of Goseki group II (major or minor) and the tumors which presented without this component was not statistically different.

Relationship between TNM and MUC expression

Among this series of 31 gastric carcinomas, 5 were T1, 20 were T2, 4 were T3 and 2 were T4, 2 were Nx, 6 were N0, 15 were N1, 5 were N2 and 3 were N3. The immunohistochemical and *in situ* hybridization detection of MUC2, MUC5AC, MUC5B or MUC 6 was not associated with T1-T2 tumors when compared to T3-T4 tumors, and was not different between N0-N1 tumors in comparison to N2-N3 tumors. Moreover, the detection of two or three MUCs in the same tumor, either by immunohistochemistry or *in situ* hybridization, was not significantly associated with T1-T2 or T3-T4 tumors, nor with N0-N1 or N2-N3 tumors.

Relationships between TNM, Goseki classification, MUC2 expression and survival

Follow up data showed that there was a significant difference in mean survival time decreasing between the carcinoma patients with T1, patients with T2, patients with T3 and those with T4 tumors (Log rank 9.63, $P=0.022$). There was also a significant difference in mean survival time decreasing between the carcinoma patients without lymph node metastasis (N0) and those with lymph node metastasis (N+) (Log rank 9.29, $P=0.0023$). Patients with Goseki II or IV classes had worst survival (mean 24 mo) than those with Goseki I or III classes (mean 51 mo) $P=0.0036$, Figure 2. Patients with positive immunohistochemical MUC2 expression more than 30% had a worst survival (mean 20 mo) than those with positive MUC2 expression less than 30% (mean 39 mo) $P=0.0434$ and those without MUC2 expression 0% (mean 72 mo) $P=0.0391$. Patients with positive immunohistochemical expression of MUC2 less than 30% had worst but not statistically significant, survival than those without MUC2 expression ($P=0.0951$, Figure 3).

DISCUSSION

In the present study, we looked for a correlation between the expression of the secreted gel-forming mucins MUC2, MUC5AC, MUC5B, MUC6 and histopathological classifications (WHO, Laurén, Mulligan and Goseki) in a series of 31 gastric carcinomas with a special attention to all components (major or minor) present in an individual tumor. Expression of MUC2 was frequently observed in all subtypes of gastric carcinoma whatever the classification. However, the high expression of MUC2 was significantly associated with the mucinous subtype of the WHO classification and with the group II of Goseki's classification (high tubular differentiation and high mucin content). A relationship between gastric mucinous carcinomas and group II of their classification has been previously reported by Goseki *et al*[3]. The WHO and the Goseki's classifications are more precise than the Laurén's and the Mulligan's classifications to classify gastric carcinoma, individualizing the mucinous subtype and group II respectively. Moreover, the Goseki's classification, that combine two distinct criteria, e.g. the tubular differentiation and the intra-cellular mucin content was shown to be the only classification with prognostic value additional to TNM staging[3,4]. The asso-

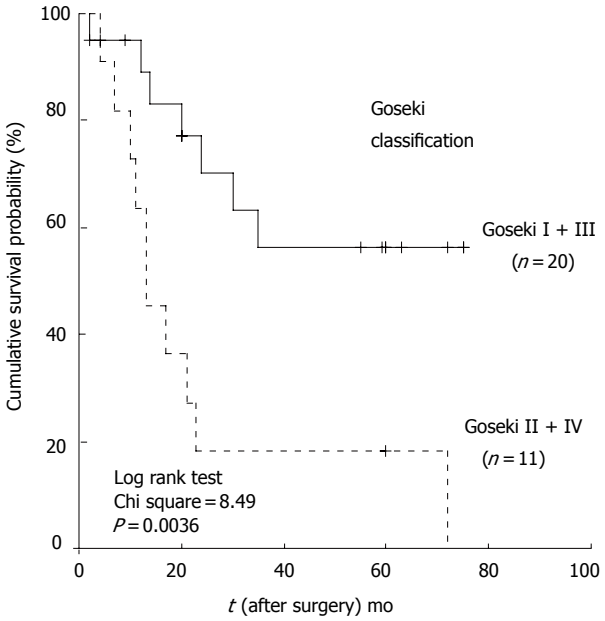

Figure 2 Survival curves for patients divided according to their Goseki classification.

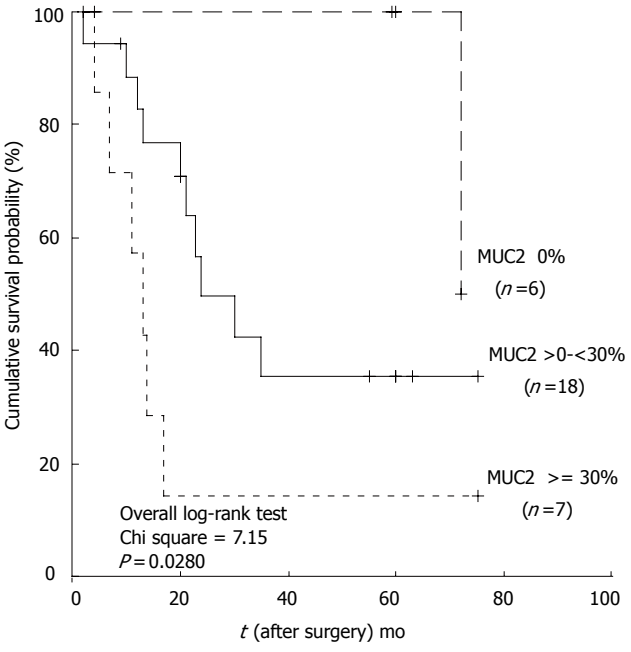

Figure 3 Survival curves for patients divided according to their immunohistochemical MUC2 expression.

ciation between expression of MUC2 and mucinous carcinoma has been previously reported in stomach, as well as in other organs, e.g. colon, breast, pancreas, ovary and pseudomyxoma peritonei[14-15,21-23]. Surprisingly, expression of MUC2 was not significantly associated with the intestinal subtypes of Laurén's or Mulligan's classifications ($P \geq 0.07$), although it is abundantly expressed in small intestine and colon as well as in intestinal metaplasia[8,24].

MUC2 is not the only secreted gel-forming mucin expressed in gastric carcinoma. Some gastric carcinomas expressed MUC5AC and/or MUC6, which are present in normal gastric mucosa[8,11]. Moreover, some gastric carcinomas expressed MUC5B, which was shown previously to be expressed during the stomach embryogenesis[25]. A significative association between the tumoral expression of MUC5AC and diffuse gastric carcinoma as defined in Laurén classification has been observed by some authors in comparison to unclassified carcinomas[14,26]. However, this association was controversed by others using the same MUC5AC antibody but comparing the diffuse subtype to the intestinal and unclassified carcinomas of the Laurén's classification[12,15]. MUC5AC has been also found more frequently expressed in group IV of Goseki's classification, which corresponds in part to the diffuse type of the Laurén's classification[3,15]. In our series, the expression of MUC5AC was frequent in the diffuse subtype of the Laurén's classification (75% of the cases in this subtype), in the group IV of Goseki' s classification (83%) and in the signet-ring subtype of the WHO classification (75%) but the comparison with the other subtypes did not reach the significant level. Moreover, the expression of MUC6 in our series was not associated with any histological type of the gastric carcinomas, whatever the classification, in accordance with previous studies [12-15]. In addition, expression of MUC5B, which before this study had never been extensively studied in gastric carcinoma using a morphological approach, was not found to be associated with particular subtypes of the Laurén's, Mulligan's, WHO or Goseki's classifications.

Given the difficulty to demonstrate a constant correlation between MUC expression and histopathological classifications, we further evaluated the expression of the four secreted gel-forming mucins independently in all the different components, major or minor, frequently present in a same gastric carcinoma. In our series, 39% of the tumors presented one component, whereas 61% presented at least two different components further classified according to the Goseki's classification. With this approach, none of the gel-forming mucin was restricted to any group of Goseki. In particular, MUC2 was not restricted to the components classified as group II but was also expressed in components classified as group I, III or IV. Furthermore, the comparison between the tumors which presented with a component of Goseki II (major or minor) and the tumors which presented without this component was not statistically different. Thus the association between MUC2 and the group II of Goseki was not reinforced by the study of each component. The best correlation between MUC expression and histopathological classifications was achieved for MUC2 and mucinous carcinomas of WHO classification and group II of Goseki's classification, these classifications being based on the identification of the major component of the carcinoma.

In this study, we also looked for a correlation between TNM and MUC expression. We did not found any association between expression of MUC (individual MUC or several MUCs) and T or N stade. More particularly, although increased mucin gene heterogeneity has been described in advanced gastric carcinomas[9], this was not confirmed in our series. A correlation between the

expression of gastric mucins, i.e. MUC5AC and MUC6 and a poorer prognosis has been reported by some authors[27]. Nevertheless, these results were not confirmed by others[17,28]. As expected, we observed a significant difference in mean survival time between the carcinoma patients according to the TNM stage. We observed a shorter mean survival time for patients with gastric carcinoma classified as Goseki II or IV in comparison to patients with Goseki I or III tumors. Interestingly, we also observed a shorter mean survival time for patients with positive MUC2 expression (in more than 30% of the cells) in comparison to patients without MUC2 immunohistochemical detection. Thus the quantity and quality of mucus present in gastric carcinoma were related to survival. This association between MUC2 expression and poorer outcome was controversed in the literature[17,29,30].

In conclusion, expression of secreted gel-forming mucins in gastric carcinoma is particularly complex. Complexity of mucin gene expression patterns in gastric cancer may reflect a precise state of differentiation at the cell level not recognized in the used morphologic classification systems. Nevertheless, this study showed a correlation between a high expression of MUC2 and mucinous subtype of gastric carcinoma that is achieved by considering the major component of the tumor. We also observed a shorter mean survival time in patients with a higher content of mucus (Goseki II or IV) and high positive MUC2 expression.

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LIVER CANCER

Reversing multidrug resistance by RNA interference through the suppression of MDR1 gene in human hepatoma cells

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Abstract

AIM: To reverse the multidrug resistance (MDR) by RNA interference (RNAi)-mediated MDR1 suppression in hepatoma cells.

METHODS: For reversing MDR by RNAi technology, two different short hairpin RNAs (shRNAs) were designed and constructed into pGenSil-1 plasmid, respectively. They were then transfected into a highly adriamycin-resistant HepG2 hepatoma cell line (HepG2/ADM). The RNAi effect on MDR was evaluated by real-time PCR, cell cytotoxicity assay and rhodamine 123 (Rh123) efflux assy.

RESULTS: The stably-transfected clones showed various degrees of reversal of MDR phenotype. Surprisingly, the MDR phenotype was completely reversed in two transfected clones.

CONCLUSION: MDR can be reversed by the shRNA-mediated MDRI suppression in HepG2/ADM cells, which provides a valuable clue to make multidrug-resistant hepatoma cells sensitive to anti-cancer drugs.

Key words: Multidrug resistance; ShRNA; MDR1; Hepatocellular carcinoma

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide, especially in Asia and Africa, with an estimated 0.5 million new cases and around 1 million deaths annually[1]. MDR is the most common impediment to successful chemotherapy for a variety of cancers[2], especially HCC. Classic MDR characterized by cross resistance to antineoplastic drugs, is caused by over-expression of MDR1 gene encoding P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) transporter superfamily. In liver, the expression of P-gp on the apical side of various cell membranes enhances the secretion of drugs into bile[3]. A disruption of P-gp-mediated drug extrusion results in a resensitization of tumor cells to treatment with antineoplastic agents, and may allow a successful drug treatment of the multidrug resistant cancer cells.

Most efforts at reversing MDR during the past two decades have been focused on compounds that modulate p-gp activity. In clinical trails, the efficiency of these drugs are difficult to access, mainly because of inherent adverse pharmacokinetic side effects[2], such as hypotension, heart failure, hyperbilirubinemia, and immunosuppression by cyclosporin A. Moreover, tumor cells can acquire resistance to the applied chemosensitizers, a so-called tertiary resistance. Consequently, it is necessary to develop alternative, less toxic and more efficient strategies to overcome MDR. It may, therefore, be more appropriate to target MDR1 gene expression. Indeed, MDR1 transcription has been targeted with ecteinascidin 743 in pre-clinical studies[4] and more recently by modulation of the orphan nuclear receptor SXR[5]. Strategies involving antisense and transcriptional decoy[6] and the use of anti-MDR1 mRNA hammerhead ribozymes have also been suggested [7].

RNAi is a conserved cellular mechanism by which double-stranded RNA (dsRNA) silences the corresponding homologous gene[8]. These 21-25 nucleotides with long, double-stranded small interfering RNA (siRNA) molecules can direct degradation of eukaryotic mRNAs in a sequence-specific manner. Two major discoveries have highlighted the potential of RNAi application in gene therapy. The introduction of synthetic siRNA of 21 nucleotides into mammalian cells can result in efficient gene silencing[9], and shRNAs expressed from RNA promoters within expression vectors also trigger RNAi[10, 11]. Recently, modulation of MDR by transfection of synthetic siRNAs has been reported [12, 13]. However, complete reversal to the drug-sensitive phenotype of parental cells has not been

obtained in any study. This is probably due to two reasons. Firstly, the transfection of synthetic siRNAs causes only transient suppression of target genes, which is often limited to the cell lines that are easy to be transfected. Secondly, the P-gp has a long half life time[14]. Thus, for achieving stable long-term RNAi effects, shRNA is developed[10].

In this study, we constructed two shRNAs targeting different coding regions of human MDR1 gene, because not all siRNA target sequences are equally potent[15]. After two shRNAs were respectively introduced into a multidrug resistant cell line HepG2/ADM derived from HepG2, efficiency of MDR1 gene suppression and whether MDR was reversed were evaluated.

Table 1 Sequences of shRNA oligonucloetides

Oligonucloetides	Sense + loop + antisense DNA template
MDR-A	5′-GATCCGCTTTGGCTGCCATCATCCAttcaagacgTGGATGATGG CAGCCAAAGTTTTTTGTCGACA-3′; 3′-GCGAAACCGACGGTAGTAGGTaagttctgcACCTACTACCGT CGGTTTCAAAAAACAGCTGTTCGA-5′
MDR-B	5′-GATCCGGCCTAATGCCGAACACATttcaagacgATGTGTTCGGCATTAGG CCTTTTTTGTCGACA-3′; 3′-GCCGGATTACGGCTTGTGTAaagttctgcTACACAAGCCGTAATCCGGAA AAAACAGCTGTTCGA-5′

The underlined parts are loops of shRNA oligonucleotides.

MATERIALS AND METHODS

Cell lines and cell culture

The HepG2 cells (Chinese Center for Type Culture Collection) were cultured in DMEM (Gibco BRL, Gaithersburg, MD, USA) contianing 10% of heat-inactivated FCS (Gibco BRL, Gaithersburg, Md. USA). Cells were incubated at 37 ℃ in a 50 mL/L CO_2 air incubator with saturated humidity. The establishment of a multidrug-resistant cell line HepG2/ADM was described previously[16]. The HepG2/ADM cells could survive a 24 h-treatment with adriamycin at a concentration of 5.0 mg/L, and are able to keep growing in the medium containing 1.0 mg/L adriamycin. To maintain MDR phenotype, HepG2/ADM cells were cultured with DMEM supplemented with adriamycin at 1.0 mg/L (Pharmacia, Uppsala, Sweden).

Design of siRNAs and cloning of siRNA hairpin loops

Two different siRNAs targeting different parts of the MDR1 gene (accession number: M14758) were designed: MDR-A (5'-AACTTTGGCTGCCATCATCCA-3', targeting nucleotide 586-606 of MDR1 mRNA sequence) was described previously [17]; MDR-B (5'- AAGGCCTA-ATGCCGAACACAT-3', targeting nucleotide 3494-3514 of MDR1 mRNA sequence) was designed according to the recommendation from the website http://sirna.qiagen.com. The two sequences were individually incorporated into a pair of oligonucleotides (produced by Wuhan GenSil Biotechnology, China. Table 1) where the target sequence appears as antisense followed by sense orientations separated by a 9-nucloetide spacer sequence and flanked at either end by Hind III or BamH1 restriction enzyme sites. The annealed oligonucleotides were cloned into pGenSil-l (Wuhan GenSil Biotechnology, China) according to the manufacturer's recommendations. The transcribed shRNA of MDR-A was 5'ATCCAttcaagacgTG-GATGATGGCAGCCAAAGU- -U-3', and the transcribed shRNA of MDR-B was 5'-GGCCTAATGCCGAACA-CATttcaagacgAT- -GTGTTCGGCATTAGGCCUU-3'. The underlined parts were loops of the two shRNAs.

Transfection and selection of stably transfected cell clones

The HepG2/ADM cells were transfected with 2μg of pGenSil-1/MDR-A, pGenSil-1/MDR-B, or empty vector pGenSil-1. Transfection was performed in 50%-60% confluent cells in 6-well plates using 9 μL of Lipofectamine 2000™ (Invitrogen, USA). Forty-eight hours after transfection, cells were grown in 10 cm cell plates with medium containing G418 (400 μg/mL) but without adriamycin. After 3 wk of culture, visible colonies were picked up and expanded. The stably transfected clone cells were observed to show green fluorescence under microscope and the clones without expression of the transfected gene did not show green fluorescence (Figure 1).

Detection of P-gp Function

Rh123 accumulation is sensitive and specific for indicating the transport function of P-gp[18, 19]. To observe the RNAi efficiency in the transfected cells, Rh123 (Sigma, MO, USA) was chosen to detect P-gp functions. The assay was performed as described by Broxterman *et al* [18]. Briefly, cells were cultured in 6-well plates. When the cells reached 70%-80% confluence, Rh123 was added to cells at a final concentration of 0.25 μg/mL and incubated at 37 ℃ for 1 h. The cells were washed 3 times with 4 ℃ PBS and resuspended at $5\text{-}10 \times 10^5$ cells/mL in 4 ℃ PBS. Rh123 fluorescence was analyzed with a FACStar flow cytometer (Becton Dickinson Immune Cytometry Systems) equipped with an argon laser. The blast population was gated by forward- and side-scatter characteristics. Rh123 fluorescence of 10 000 cells was measured logarithmically through a 530 nm bandpass filter at an excitation wavelength of 488 nm. HepG2 cells without incubation with Rh123 served as a negative control. Rh123 efflux was measured by counting cells in the M1 region of the plot and calculated as percentages of cells in the M1 region of the plot. The bigger percentage of cells in M1 region indicated the greater cellular Rh123 efflux and also a higher P-gp function. All 146 clones were checked.

Real-time reverse transcription chain reaction

Human MDR1 real-time PCR primers used in experiments are 5R-TGGTTCAGG TGGCTCTGGAT-3R and 5R-CTGTAGACAAACGATGAGCTATCA -CA-3R. Human GAPDH primers for real-time PCR used have been previously described[20]. Total RNA was isolated from the cells by TRIzol (Invitrogen, USA) 60-70 d after cell transfection. All RNA samples were stored at -80 ℃ until use. RNAs were reversely transcribed into cDNAs using RevertAid-TM first strand cDNA synthesis kit (Fermentas, Inc.) according to the manufacturer's instructions. Real-time PCR and data collection and result analysis were performed on

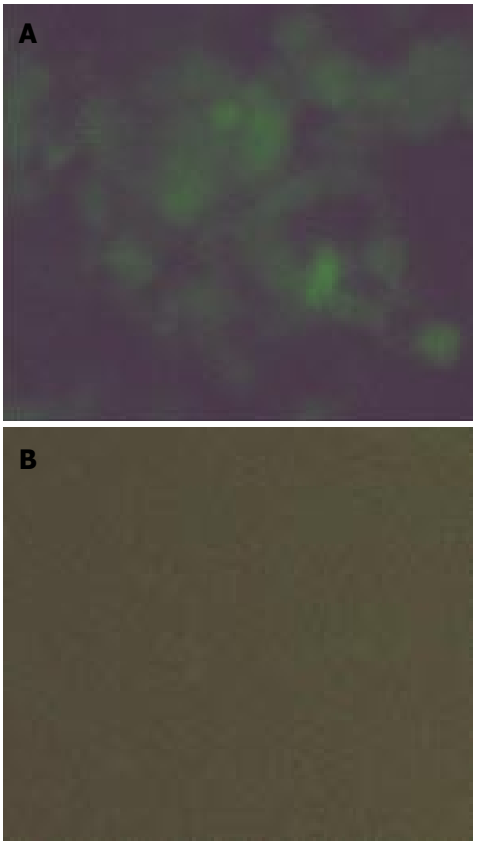

Figure 1 Stably transfected cells showing green fluorescence under a fluorescence microscope (**A**) and cells without stable expression of the transfected gene (**B**).

ABI PRISM®7000 sequence detection system (Applied Biosystem, USA). All quantitations were normalized to an endogenous control GAPDH. The relative quantitation value of each target gene was analyzed using a comparative C_T method. The following formula was used to calculate the relative amount of the transcript in the sample and normalized to an endogenous reference (GAPDH): $2^{-\triangle\triangle CT}$, where $\triangle C_T$ is the difference in C_T between the gene of interest and GAPDH, and $\triangle\triangle C_T$ for the sample = $\triangle C_T$ of the actual sample -$\triangle C_T$ of the lowest expressing sample (used as calibrator). The MDR1/GAPDH ratio in drug sensitive HepG2 cells was set at 1, and $L0g_{10}1 = 0.00$.

Cell cytotoxicity assay

The cytotoxicity assay and ID50 determination of anticancer drugs on the different clone cells were analyzed by the microculture methyl tetrazolium (MTT) method as described previously [21]. Briefly, in each experiment 10 000 cells per well were seeded in 96-well plates and cultured with DMEM or DMEM complemented with adriamycin in dilution series for 48h. Four repeated wells were used for each concentration. The total medium volume of each well was 200 μL. After 48 h of culture, 50 μL of (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, MO, USA) at 2 mg/mL was added to each well and incubated for 4 h at 37 ℃. Plates were centrifuged at 800r/min for 6 min, the medium was aspirated from plates, leaving about 30 μL of medium in each well. One hundred and fifty μL of dimethyl sulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany) was added to each well and the plates were shaken for 10 min to solubilize the formazan crystals. The plates were read immediately at 540 nm on a scanning multi-well spectrophotometer (Titertek, Alabama, USA). Absorbance from the cells treated with adriamycin was corrected against the absorbance from the untreated control cells. The ID50 value was defined as the dosage of drugs at which 50% of cells died after 48 h of treatment, which was calculated from 3 independent experiments for each clone. Since the basal growth rates of the cell lines had no significant difference in the first 2 d (data not shown), the errors caused by that were ignored.

Statistical analysis

For statistical analysis of P-gp function and cell cytotoxicity assay, the unpaired *t*-test was performed on SPSS 11.0 software. The real-time PCR data were analyzed using the SDS software on the ABI PRISM®7000 sequence detection system, the confidence limit was set at 95%.

RESULTS

ShRNAs decreased P-gp function

The RNAi efficiency was evaluated by measuring specific P-gp activity. P-gp-mediated transport indicated by intracellular decrease of Rh123 fluorescence was studied using flow cytometry. The P-gp activities of 58 pGenSil-1/MDR-A-transfected clones and 78 pGenSil-1/MDR-B-transfected clones were analyzed. Meanwhile, 10 HepG2/ADM control clones transfected with an empty pGenSil-1 vector were also analyzed. Two of the pGenSil-1/MDR-B-transfected clones showed a complete suppression of the P-gp function. More than 60% of the pGenSil-1/MDR-B-transfected clones (49 of 78 clones) showed an intermediate decrease of the P-gp function, whereas 8 clones did not show any significant changes of P-gp function. None of the pGenSil-1/MDR-A-transfected clones showed a complete suppression of P-gp activity, although different suppressions of P-gp function were detected. None of the control clones exhibited any significant alterations of P-gp function. Compared with HepG2 cells, the P-gp functions of HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 cells were at a low level near to that of the HepG2 cells, the P-gp functions of the HepG2/ADM cells and control HepG2/ADM/vec clone cells were much higher (Figure 2).

ShRNAs decreased MDR1 mRNA level

To further confirm the data from P-gp function study with Rh123 fluorescence, real-time RT-PCR was performed to quantify the RNAi-mediated suppression of MDR1 mRNA. The MDR1 mRNA expressions of parental cells, HepG2/ADM cells, HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 cells and the HepG2/ADM/vec control clone cells were measured. Expression of MDR-B shRNA in HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 reduced MDR1 mRNA expressions by 93% and 92%, respectively. MDR1 mRNA expression was reduced by 40% in the HepG2/ADM/vec control clone (Figure 3).

ShRNAs reversed multidurg-resistant phenotype

The reversal of MDR-mediated by shRNA was measured

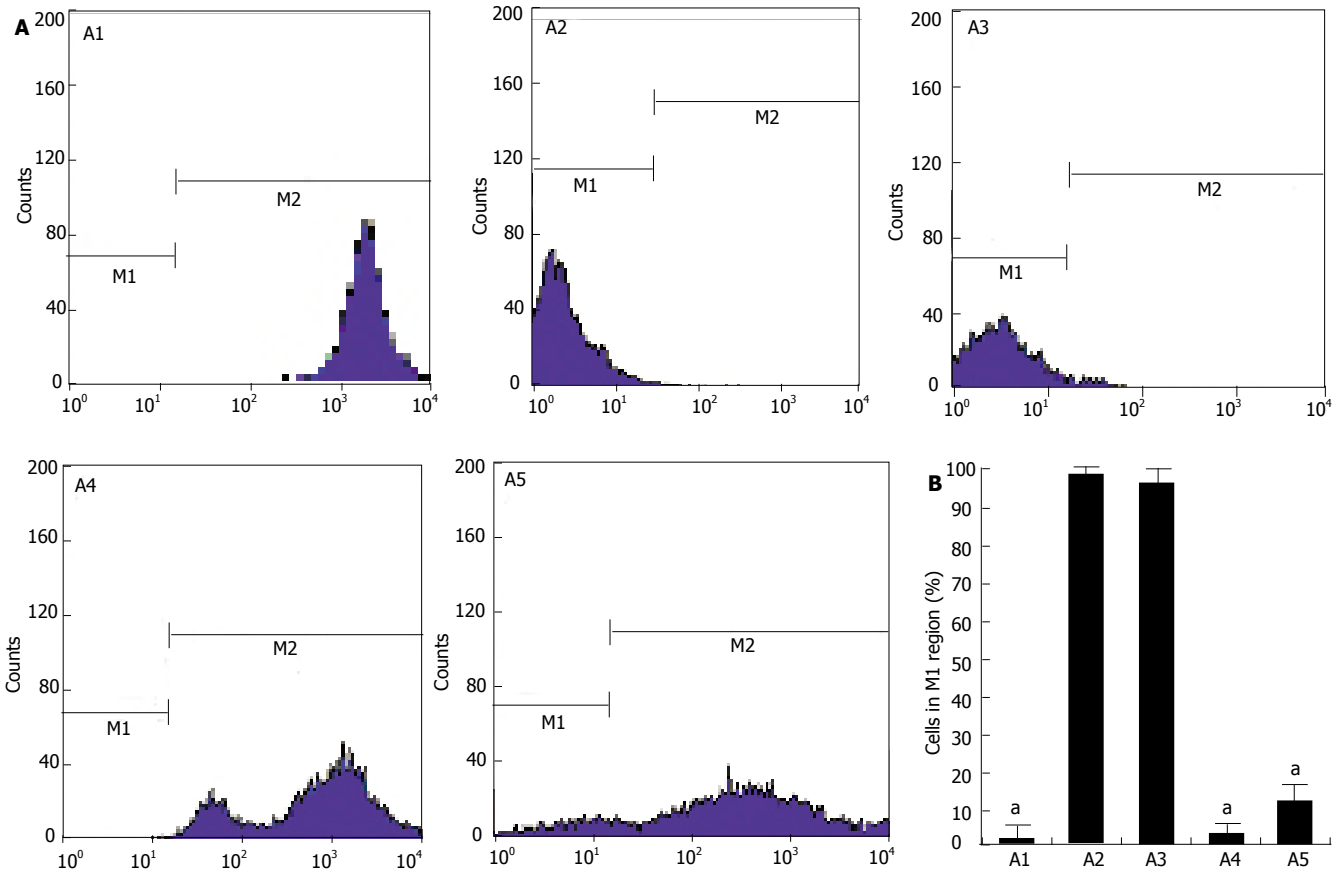

Figure 2 P-gp activity in HepG2 cells (A1), HepG2/ADM cells (A2), HepG2/ADM/vec control clone cells (A3); HepG2/ADM/pGenSil-l/MDR-B clone 1 cells (A4); HepG2/ADM/pGenSil-l /MDR-B clone 2 cells (A5) Rhodamine123 efflux was measured by counting cells in the M1 region of the plot. The marker bar M1 was set to indicate the cells with high Rhodamine123 efflux; the marker bar M2 was set to indicate the cells with low rhodamine123 efflux. Data were presented as mean ± SD. $^aP < 0.05$ *vs* untreated multidrug-resistant cell line HepG2/ADM.

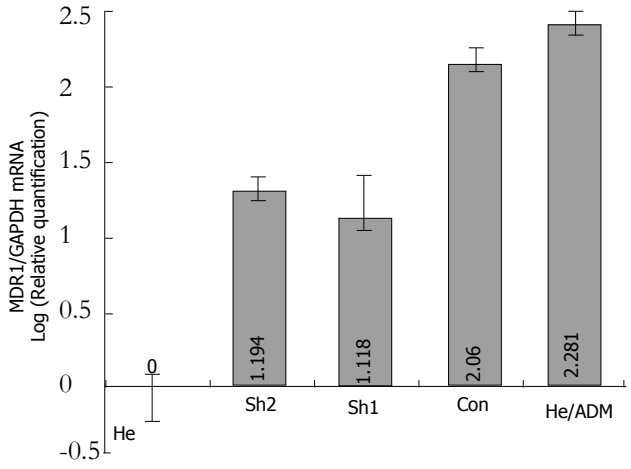

Figure 3 shRNA-mediated suppression of MDR1 mRNA. He, sh1, sh2, con and He/ADM represent HepG2, HepG2/ADM/pGenSil-l/MDR-B clone 1, HepG2/ADM/pGenSil-l/MDR-B clone 2, HepG2/ADM/vec control clone and HepG2/ADM, respectively. The relative expression values were the mean of triplicate real-time PCR reactions. Bars indicate SE.

using cell cytotoxicity to access adriamycin-specific ID50 levels. The resistance of HepG2/ADM to adriamycin was 102-fold stronger than that of the parental cell line at ID50 (Table 2). The HepG2/ADM/vec control clone had a similar resistance to HepG2/ADM cells. The resistance

Table 2 Effect of stable expression of shRNAs-mediated drug resistance to adriamycin

Cell line	Adriamycin IC50 (mg/L)	Resistance index (RI)
HepG2	0.141 ± 0.008	1
HepG2/ADM	14.394 ± 0.263[a]	102.085
Con	13.275 ± 0.353[a]	94.149
sh1	0.269 ± 0.035	1.908
sh 2	0.467 ± 0.617[a]	3.312

$^aP < 0.05$ *vs* ID50 value of HepG2. The data are presented as mean ± SD. Sh1, sh2 and con represent HepG2/ADM/pGenSil-l/MDR-B clone 1, clone 2, and the control HepG2/ADM/vec clone, respectively.

of HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 cells was decreased to a very low level near to that of the drug-sensitive HepG2 cells.

DISCUSSION

To improve the effect of RNAi-mediated gene silencing, some vector plasmids have been developed to express siRNAs as inverted repeats showing similar potency to initial RNAi. These siRNA-like molecules are commonly designated as shRNAs. For generating shRNAs, these vectors have been designed to be able to obtain the stable expression of shRNAs using strong RNA polymerase-II-depen-

dent promoters like CMV or EF1a, or RNA polymerse-III promoters like U6 or H1[10, 22-27]. Since experiences with antisense and ribozyme technology indicate that RNA polymerase-III expression systems can offer a great potency for maintaining stable expression of short RNA molecules *in vitro* as well as *in vivo*[28], the U6-driven expression vector pGenSil-1 was applied in this study. The size of the loop structure of shRNA has a considerable influence on the gene suppression activity. Brummelkamp *et al* [10] reported that a 9- nucleotide loop shows more effects than a 7-nucleotide loop, whereas a 5-nucleotide loop structure shows only moderate effects. Therefore, the two shRNAs were designed with a 9-nucleotide loop in our study.

The two shRNAs in this study showed different RNAi efficiencies. PGenSil-1/MDR-A was selected as previously described by Yague *et al* [17]. In his report, MDR-A shRNA constructed in pSUPER plasmid had an excellent RNAi effect on KD30 cell line, a multidrug resistant cell line derived from K562 leukemia cells. On the contrary, MDR-A shRNA did not show any significant RNAi effect on HepG2/ADM cells. One reason may be that the KD30 cell line is generated by a single-step selection in a low concentration of adriamycin (0.0174 mg/L)[17], while the HepG2/ADM cells are resistant to a high concentration of adriamycin. Another reason may be that the same siRNA sequence may have different RNAi efficiency in different tumor cells[12]. The different vector and loop structure of shRNA may also affect RNAi effects. Our study indicated that the pGenSil-1/MDR-B had a significant RNAi effect on HepG2/ADM cells. The MDR1 mRNAs were reduced by 93% and 92% respectively in HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 by expressions of MDR-B shRNA. The remaining MDR1 mRNA in the two clones was probably due to transgenic deletion, incomplete or inappropriate integration effects. The two clones showed a complete reversal of multidrug-resistant phenotype, indicating that the MDR-B siRNA might be very useful in designing new strategies for treatment of hepatoma in the future.

The empty pGenSil-l vector-transfected clone showed a slight decrease of MDR1 gene expression, which was suggested by real-time RT-PCR, Rh123 efflux test and cell cytotoxicity assay. That may be due to the withdrawal of adriamycin from the cell culture medium for a relatively long time[29].

The general obstacle to cancer gene therapy is the delivery of a therapeutic gene. Additional problems of clinical P-gp inhibition by anti-MDR1 siRNAs may arise from the physiological expression of this ABC transporter in several epithelial and endothelial cells. For example, P-gp is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain[30], suggesting that this efflux pump plays an important role in the blood-brain barrier and is crucial for limiting the potential neuro-toxicity of many anticancer drugs. Thus, in clinical practice the application of P-gp-inhibiting shRNA expression vectors should be restricted to P-gp expressing cancer cells. One promising strategy for cancer cell-specific delivery of siRNAs targeting MDR1 is to develop vector systems delivering siRNA in a cell-type specific manner. Lentiviral vectors could offer some advantages in upcoming preclinical studies[31]. A "replication-defective" E1A-mutant adenoviral vector that efficiently and selectively replicates in "classic" multidrug-resistant cells has been reported[32]. These viral vectors may contribute to developing more useful vectors for the MDR reversal gene therapy in multidrug-resistant cancers.

In conclusion, the highly adriamycin-resistant HepG2/ADM cells can be completely reversed to an adriamycin-sensitive phenotype by the shRNA-mediated RNAi. The target sequence can be integrated into gene therapy vectors for the potential application in clinic.

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VIRAL HEPATITIS

Matrix-derived serum markers in monitoring liver fibrosis in children with chronic hepatitis B treated with interferon alpha

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Abstract

AIM: To evaluate prospectively 4 selected serum fibrosis markers (tenascin, hyaluronan, collagen VI, TIMP-1) before, during and 12 mo after IFN treatment of children with chronic hepatitis B.

METHODS: Forty-seven consecutive patients with chronic hepatitis B (range 4-16 years, mean 8 years) underwent IFN treatment (3 MU tiw for 20 wk). Fibrosis stage and inflammation grade were assessed in a blinded fashion before and 12 mo after end of treatment. Serum fibrosis markers were determined using automated assays.

RESULTS: IFN treatment improved histological inflammation but did not change fibrosis in the whole group or in subgroups. Only hyaluronan correlated significantly with histological fibrosis(r = 0.3383, P = 0.021). Basal fibrosis markers did not differ between responders (42.5%) and nonresponders(57.5%). During IFN treatment only serum tenascin decreased significantly in the whole group and in nonresponders. When pretreatment values were compared to values 12 mo after therapy, TIMP-1 increased in all patients and in nonresponders, and hyaluronan decreased in all patients and in responders.

CONCLUSION: Tenascin reflects hepatic fibrogenesis and inflammation which decreases during IFN treatment of children with chronic hepatitis B. TIMP-1 correlates with nonresponse and hyaluronan with histological fibrosis.

Key words: Biopsy; Collagen VI; Fibrogenesis; Fibrosis; Hepatitis B virus; Hyaluronan; Serum marker; Tenascin; TIMP-1

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INTRODUCTION

The potential of interferon α (IFNα) to normalize aminotransferase activity, eliminate serum HBV DNA and HBeAg and reduce liver necroinflammation in patients with chronic hepatitis B is widely acknowledged[1-3]. In children with chronic hepatitis B treatment with IFN leads to long-term serological and biochemical remission in less than 50%[4-6]. Nonetheless, several reports suggested that IFN treatment for hepatitic C can halt or even reverse liver fibrosis[7-10], while its antifibrogenic potential in chronic hepatitis B needs to be confirmed[11]. Histological staging of liver fibrosis plays a central role in the liver pathological assessment since progressive fibrosis may lead to cirrhosis, the most important predictor of decompensated liver diseases and death[3, 12].

Liver biopsy is the standard method to assess fibrosis stage which allows to semiquantify the extent of fibrosis, yielding a static view[13]. However, biopsy has significant disadvantages for the assessment of fibrosis and fibrosis progression. Thus despite minor histological fibrosis, progression might be fast, since the accumulated extracellular matrix is the result of a dynamic process characterized by changes in matrix synthesis (fibrogenesis) and removal (fibrolysis)[14]. Furthermore, liver biopsy is invasive and histological scoring systems are not sensitive enough to detect small changes in fibrosis stage. Finally, bioptical sampling error can reach 25%-33% for a difference in one stage, when using the METAVIR system which ranges from 0 (no fibrosis) to 4 (cirrhosis)[15,16]. Therefore, noninvasive markers that may reflect overall hepatic fibrogenesis and fibrolysis in chronic hepatitis would be of great clinical benefit,

allowing repeated assessment of progression or therapeutic interventions, especially in children with chronic hepatitis B or C who are treated with IFN or other antiviral or potential antifibrotic agents[17-19].

The aim of this study was to investigate the clinical usefulness of selected matrix-derived serum markers (tenascin, hyaluronan, collagen VI, tissue inhibitor of metalloproteinase 1 or TIMP-1) in a long-term follow-up of children with chronic hepatitis B treated with IFN α.

MATERIALS AND METHODS

Patients

The study was carried out prospectively in 47 children (mean age 8 years, range 4-16, 31 boys and 16 girls) with serologically and biopsy-verified chronic hepatitis B. The children were positive for HBs and HBe antigens and had increased serum activity of HBV DNA polymerase for at least 1 year. Patients with autoimmune hepatitis or HCV coinfection were excluded from the study. None of the children was treated with antiviral and immunomodulating drugs during the 12-month period before inclusion into the study. Informed consent was obtained from all patients' parents and the protocol was approved by the local ethical committee of the Medical University of Bialystok. Serum samples were evaluated at three time points: at the start and the end (5 mo) of IFN α treatment, and 12 mo after end of treatment. Serum samples were stored at -70 ℃ until use. Standard liver tests were measured by validated automated methods and included total bilirubin, albumin, alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (gamma-GT). HBsAg and HBeAg were determined by MEIA (IMx, Abbott).

IFN treatment and definition of response

IFN (IFN α 2a: 30 children or IFN α 2b: 17 children) was applied at the dose of 3 MU tiw subcutaneously for 20 wk according to the schedule approved by the Polish Interferon Study Group[20]. HBeAg/antiHBe seroconversion and lack of HBV DNA polymerase activity 1 year after end of treatment was considered as the criterion of treatment response.

Measurement of serum fibrosis markers

Monoclonal antibodies were used to detect tenascin, collagen VI and TIMP-1 in sandwich immunoassays performed in an automated analyzer employing fluoresceine-labelled capture antibodies and alkaline phosphatase labeled detection antibodies. Hyaluronan was determined using biotinylated cartilage link protein. The immune complexes were separated from serum using magnetic particles covered with monoclonal anti-fluoresceine (anti-biotin in case of hyaluronan). The assays were developed for the BAYER IMMUNO 1 immunoassay system and validated in several cohorts of liver patients and healthy individuals[21-23].

Histological analysis

Percutaneous liver biopsies were obtained before treatment and 12 mo after IFN α discontinuation. The liver specimens were fixed in buffered formalin and embedded in paraffin. Histological sections were stained using hematoxylin-eosin, Masson-Goldner, Masson's trichrome and reticulin stains. Fibrosis stage and inflammation grade were assessed in a blinded fashion by a single pathologist according to the method of Batts and Ludwig[24].

Table 1 Baseline characteristics of the group of children with chronic hepatitis B

Data of the patients	Mean	SD	Minimum	Maximum
Age (yr)	8	3.51	4	16
HBV infection (mo)	44	30	10	144
ALT (nkat/L)	1600	1567	350	9902
AST (nkat/L)	1367	1117	567	7718
GGT (nkat/L)	250	150	50	1150
Bilirubin (μmol/L)	8.5	4.3	3.4	23.9
Albumin (g/L)	65.6	4.2	57.2	74.3
Hyaluronan (μg/L)	34.3	21.5	14.6	113.5
Tenascin (μg/L)	748.8	265.9	295.6	1480.2
TIMP-1(μg/L)	558.1	121.2	342.2	852.3
Collagen VI (μg/L)	5.8	2.2	2.3	14.2
Staging	2.0	0.6	1	3
Grading	1.6	0.7	1	3

Normal ranges: AST: 167-667 nkat/L; ALT: 167-667 nkat/L; GGT: 150-583 nkat/L; Bilirubin: 1.7-18.8 μmol/L; Albumin: 58.8-69.6 g/L.

Statistical analysis

Results were expressed as means ± SD. Statistical analysis was performed with the Wilcoxon rank-sum test for independent samples and Wilcoxon signed rank test for paired samples. The relationship between the serum fibrosis markers and liver histology scores was analysed by the Spearman rank-correlation test for nonparametric data and by the Pearson method for parametric data. Tests were considered statistically significant at $P < 0.05$.

RESULTS

Patient characteristics

The baseline characteristics of the 47 children are presented in Table 1. They were classified into responders (n = 20; 42.5%) and nonresponders (n = 27; 57.5%). There were no significant differences between the groups regarding age, gender, body mass, duration of HBV infection, the levels of bilirubin and albumin, the activity of GGT, levels of baseline liver fibrosis markers (tenascin, hyaluronan, collagen VI, TIMP-1) and grade of inflammation. However, treatment responders displayed a significantly higher activity of ALT (2184 ± 2217 *vs* 1150 ± 550 nkat/L) and AST (1717 ± 1567 *vs* 1100 ± 500 nkat/L) (P = 0.0453 for both) and a higher fibrosis score according to Batts and Ludwig (2.3 ± 0.5 *vs* 1.8 ± 0.6; P = 0.004).

Basal levels of serum fibrosis markers

We arbitrarily defined mild/moderate fibrosis as stage ⩽ 2 (n = 39) and advanced fibrosis as stage = 3 (n = 8) accor-

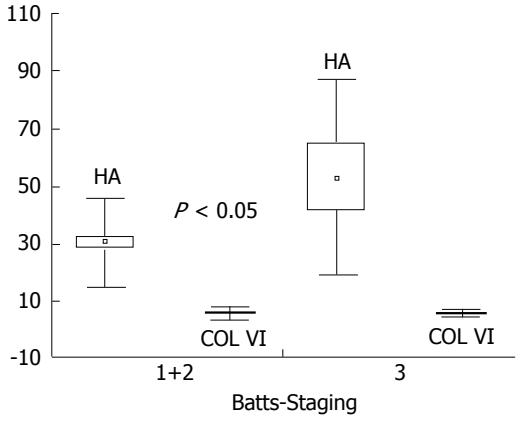

Figure 1 Correlation of serum fibrosis markers with histological staging (mean ± SD).

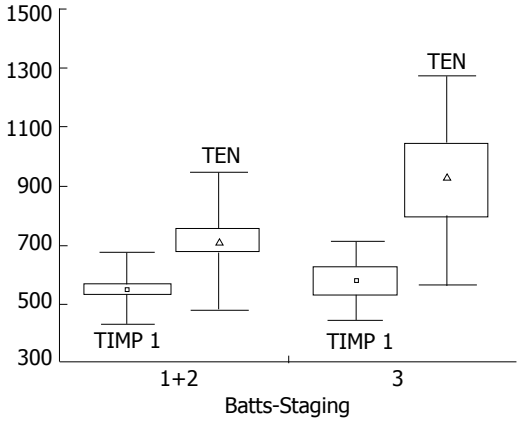

Figure 2 Correlation of serum fibrosis markers with histological staging (mean ± SD).

Table 2 Effect of IFN alpha on serum fibrosis markers in children with chronic hepatitis B (mean ± SD)

Patients	Marker (μg/L)	Before IFN (1)	After IFN (2)	12 mo after IFN (3)	*P* 1 *vs* 2	*P* 1 *vs* 3	*P* 2 *vs* 3
All	TIMP-1	558.1 ± 121.2	569.2 ± 134.8	636.9 ± 125.8	NS	b	NS
n = 47	CollagenVI	5.8 ± 2.2	6.2 ± 1.8	Nd	NS	-	-
	Tenascin	748.8 ± 266.0	641.5 ± 216.8	764.0 ± 250.0	b	NS	b
	Hyaluronan	34.3 ± 21.5	40.0 ± 30.9	28.7 ± 19.3	NS	b	b
Responders	TIMP-1	544.1 ± 120.3	555.4 ± 109.3	608.0 ± 134.9	NS	NS	NS
n = 20	CollagenVI	5.6 ± 1.85	6.7 ± 1.8	Nd	NS	-	-
	Tenascin	766.2 ± 289.9	686.2 ± 225.8	765.5 ± 249.9	NS	NS	NS
	Hyaluronan	40.3 ± 28.1	36.8 ± 18.0	30.7 ± 17.2	NS	a	a
Non	TIMP-1	567.9 ± 123.1	581.2 ± 155.0	657.2 ± 118.3	NS	b	NS
Responders	CollagenVI	6.0 ± 2.4	5.7 ± 1.7	Nd	NS	-	-
n = 27	Tenascin	736.6 ± 252.7	602.6 ± 205.6	762.9 ± 254.9	b	NS	b
	Hyaluronan	29.6 ± 13.6	42.7 ± 39.1	27.2 ± 20.9	NS	NS	b

Nd: Not determined; NS: Not significant; $^{a}P < 0.05$, $^{b}P < 0.01$.

ding to Batts and Ludwig. There were no significant differences in mean serum levels of tenascin, collagen VI and TIMP-1 between children with mild and advanced liver fibrosis, while levels of hyaluronan were higher in the latter group (30.3±15.8 *vs* 53.1±34.3 μg/L; $P = 0.0266$). There was a trend for increased tenascin and TIMP-1 in children with advanced fibrosis (Figure 1, Figure 2). We also arbitrarily defined mild inflammation as grade 1 ($n = 20$) and severe inflammation as grade ⩾2 ($n = 27$) according to Batts and Ludwig. There were no significant differences in mean concentrations of all serum fibrosis markers between children with mild and severe hepatic inflammation.

Effect of IFN on serum fibrosis markers

At end of treatment there were no significant changes in serum fibrosis markers in responders, while in nonresponders only tenascin decreased significantly ($P = 0.0074$). Twelve months after end of treatment serum hyaluronan was significantly lower than before treatment ($P = 0.0076$), while serum TIMP-1 was increased ($P = 0.0072$). In responders only hyaluronan decreased significantly ($P = 0.0304$), while in nonresponders the level of TIMP-1 increased ($P = 0.0064$, Table 2). Tenascin reached pretreatment levels in both responders and nonresponders.

Effect of IFN on liver histology

There were no significant changes in fibrosis stage after IFN therapy in the whole cohort, 2.0 ± 0.6 *vs* 2.1 ± 0.6, according to Batts and Ludwig and in subgroups, responders: 2.3 ± 0.5 *vs* 2.2 ± 0.7; nonresponders: 1.8 ± 0.6 *vs* 2.0 ± 0.6. Histological inflammation improved significantly in the whole group, 1.6 ± 0.7 *vs* 1.2 ± 0.7, $P = 0.0373$.

Correlation between serum fibrosis markers, histology and biochemical parameters

There were no significant correlations between baseline levels of the 4 serum fibrosis markers with liver fibrosis or inflammation according to Batts and Ludwig, or with AST, ALT, GGT, albumin or bilirubin. Only hyaluronan correlated significantly with histological fibrosis ($r = 0.3383$, $P = 0.021$).

DISCUSSION

Liver biopsy has been considered the gold standard for the assessment of hepatic fibrosis. Current recommen-

dations suggest that this procedure precede antiviral treatment in most patients with chronic hepatitits B or C[25]. However, liver biopsy is invasive with the potential for complications, such as bleeding which occurrence ranges from 0.3% to 0.5%[26,27], and mortality up to 0.1%[26,28]. In addition, since the biopsy core only represents 1/20 000 to 1/50 000 of the liver, biopsy is prone to sampling error, and variations in fibrosis staging may be high among different pathologists[15,16,21,29]. For these reasons, especially in children, non-invasive detection of histological liver damage, paticularly of fibrosis, is needed. Ideally serum markers of fibrosis should be applicable to patients with chronic hepatitis to either diagnose the stage of liver fibrosis, potentially replacing liver biopsy for this purpose, or to monitor progression of fibrosis or fibrogenesis, particulary during treatment [17,30]. Markers of the dynamics of fibrogenesis and fibrolysis are urgently needed, e.g. for short-term assessment of antifibrotic drug effects, but difficult to validate.

In this study we evaluated the changes of 4 serum fibrosis markers derived from the extracellular matrix (tenascin, hyaluronan, collagen VI and TIMP-1) before, at the end of and 12 mo after treatment of children with chronic hepatitis B with IFN. Our results showed a significant decrease of hyaluronan in responders and increased TIMP-1 in nonresponders, when levels before and 12 mo after interferon α treatment were compared. While falling during treatment, serum tenascin reached pretreatment levels in both responders and nonresponders. There were no significant changes in histological liver fibrosis 12 mo after the 5-mo course of IFN in all patients or in the subgroups of responders and nonresponders. This was expected, since the rate of fibrosis progression or regression in patients with chronic hepatitis B or C was usually slow. Assuming that IFN has at least some antifibrotic activity, as suggested before in large retrospective analyses of patients with chronic hepatitis C[31, 32], the histological scoring systems are obviously not sensitive enough to detect small changes in liver fibrosis and (modest) antifibrotic treatment effects. Nonetheless, the course of serum fibrosis (fibrogenesis) markers in our small but well defined group of children suggests that IFN indeed has antifibrogenic activity, especially in responders. This antifibrotic effect seems to be transient, as exemplified by serum tenascin which was depressed only during IFN treatment.

Prior to our study there had been no longitudinal, prospective studies of serum fibrosis markers in children with chronic hepatitis B, and only few studies analysed the effect of IFN therapy on the stage of liver fibrosis in children. Our findings are consistent with our previous study[33] and with those of others, who did not observe improvement of liver fibrosis by antiviral treatment in children, when biopsy was performed before and immediately after[34] or 9-12 mo after end of treatment[35, 36], while Gregorio *et al*[37] found significant improvement in staging in responders. However, these reports included small numbers of patients (≤24). It has been demonstrated that fibrosis stage changes more slowly than inflammation grade[7, 38]. This explains why we did not observe a significant improvement in fibrosis, while inflammation was clearly suppressed by IFN treatment.

We found that hyaluronan was the best serum marker to predict advanced liver fibrosis, since its level correlated significantly with histological fibrosis and was significantly higher in children with advanced *vs* mild/moderate liver fibrosis. These data are in keeping with previous results in patients with chronic viral hepatitis[39-43]. Thus the ability of this test to differentiate patients with extensive liver fibrosis from those with mild liver fibrosis was stronger than that of other markers, i.e., PIIINP, collagen IV, MMP-1, MMP-2 and TIMP-1[44] and laminin, collagen IV, PIIINP and TGF β1[45].

In children, up to now serum hyaluronan had only been studied in biliary atresia and cystic fibrosis[46-48], and there had been no data on collagen VI in chronic viral hepatitis or on TIMP-1 or tenascin in childhood liver diseases in general. Previous studies indicated that most serum fibrosis markers are influenced by body growth, especially PIIINP[49,50]. Thus healthy children have higher PIIINP levels than adults, excluding its use as reliable fibrosis marker for pediatric patients. Hyaluronan appears to be a useful marker of fibrosis stage also in children due to its short biological half life of only a few minutes and a prominent uptake by sinusoidal endothelial cells[51]. Similarly, tenascin and TIMP-1 are applicable to children with chronic hepatitis B as markers of fibrogenesis/inflammation and of fibrogenesis, respectively.

Our data suggest that serum hyaluronan, tenascin and TIMP-1 could be useful fibrosis markers in future studies of children with chronic viral hepatitis.

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BASIC RESEARCH

Identification of biomarkers of human pancreatic adenocarcinomas by expression profiling and validation with gene expression analysis in endoscopic ultrasound-guided fine needle aspiration samples

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Abstract

AIM: To compare gene expression profiles of pancreatic adenocarcinoma tissue specimens, human pancreatic and colon adenocarcinoma and leukemia cell lines and normal pancreas samples in order to distinguish differentially expressed genes and to validate the differential expression of a subset of genes by quantitative real-time RT-PCR (RT-QPCR) in endoscopic ultrasound-guided fine needle aspiration (EUS-guided FNA) specimens.

METHODS: Commercially dedicated cancer cDNA macroarrays (Atlas Human Cancer 1.2) containing 1176 genes were used. Different statistical approaches (hierarchical clustering, principal component analysis (PCA) and SAM) were used to analyze the expression data. RT-QPCR and immunohistochemical studies were used for validation of results.

RESULTS: RT-QPCR validated the increased expression of *LCN2* (lipocalin 2) and for the first time *PLAT* (tissue-type plasminogen activator or *tPA*) in malignant pancreas as compared with normal pancreas. Immunohistochemical analysis confirmed the increased expression of *LCN2* protein localized in epithelial cells of ducts invaded by carcinoma. The analysis of *PLAT* and *LCN2* transcripts in 12 samples obtained through EUS-guided FNA from patients with pancreatic adenocarcinoma showed significantly increased expression levels in comparison with those found in normal tissues, indicating that a sufficient amount of high quality RNA can be obtained with this technique.

CONCLUSION: Expression profiling is a useful method to identify biomarkers and potential target genes. Molecular analysis of EUS-guided FNA samples in pancreatic cancer appears as a valuable strategy for the diagnosis of pancreatic adenocarcinomas.

Key words: Pancreas; Colon; Adenocarcinoma; Gene expression profiling; Endoscopic ultrasonography; Ultrasound; Fine needle aspiration

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INTRODUCTION

Pancreatic cancer remains one of the most deadly tumor types. The 5-year survival rate after diagnosis is less than 3.5%[1]. So far, the difficulties persist to diagnose pancreatic cancer early. Indeed, the molecular mechanisms underlying pancreatic oncogenesis remain partially understood.

Several fundamental studies suggest the implication of a number of molecules involved in cell cycling, apoptosis or signal transduction. Unfortunately, the clinical relevance of these molecules is still pending. For example, the screening of the activating mutations of the proto-oncogene *K-ras*, which are the most frequent alterations observed to date in pancreatic cancer, is not thought to be sufficiently robust as a diagnostic or prognostic marker[2]. The combination of several genetic alterations such as those found in tumor suppressor genes *p16, DPC4, p53* did not improve the sensitivity and specificity of K-*ras* mutation test for the diagnosis[3]. The development of new diagnostic tools is hence crucial for the detection of pancreatic cancer at an early stage.

Large-scale analysis of gene expression has been widely proposed as a powerful method for malignancy diagnosis, predicting invasion and metastasis through the identification of biomarkers. Pancreatic cancer has previously been the focus for such studies[4-8]. However, a rather low concordance between different studies was found in a meta-analysis of several of these studies[9]. There are several possible explanations; one of them being differences in probe design (oligonucleotides or cDNA), support (nylon membranes or glass slides) or the underlying detection technology (fluorescence or radioactivity). No matter what the reason is, there are controversies about the need of multiple well-defined and validated approaches to find a transcriptional "consensus" for a given tissue or cell type.

In this study, we used commercially dedicated macroarrays containing 1176 genes, selected on their functional implication in cancer biology, to study the expression profiles in pancreatic adenocarcinomas (surgical specimens and cell lines). We compared these profiles with those of normal pancreas, other adenocarcinoma cell lines of colon origin as well as with a non-adenocarcinoma leukemia cancer cell line. This array has never been used in expression profiling studies of pancreatic adenocarcinomas.

An important issue is the limited access to pancreatic tissue specimens. Being a minimally invasive technique for patient exploration, endoscopic ultrasound-guided fine needle aspiration (EUS-guided FNA) is now largely used for pancreatic tumor diagnosis[10]. Therefore, to support the clinical relevance of these studies, we determined whether quantification of these markers would be feasible in EUS-guided FNA specimens for prognostic or molecular diagnosis procedures.

MATERIALS AND METHODS

Cell and tissue samples

All cell lines were of human origin and were grown at 37℃, 50 mL/L CO_2 in the presence of 10 mL/L fetal calf serum, penicillin/streptomycin (Invitrogen Inc. Carlsbad, CA, USA) and 2 mmol/L L-glutamine (Invitrogen). ASPC-1 (ATCC: CRL-1682), Capan-1 (ATCC: HTB-79), Capan-2 (ATCC: HTB-80), NP29 (kindly provided by Dr. Gabriel Capellá, Barcelona, Spain), HCT 116 (ATCC: CCL-247) and K562 (ATCC: CLL-243) cells were grown in RPMI 1640 medium (Invitrogen). PANC-1 (ATCC: CRL-1469), B × PC-3 (ATCC: CRL-1687), SW480 (ATCC: CLL-228), SW620 (ATCC: CLL-227) and MIAPaCa-2 (ATCC: CRL-1420) cells were grown in Dulbecco's modified Eagle medium (DMEM), 1.0 g/L glucose. HT-29 cells (ATCC: HTB-38) were maintained in DMEM with 4.5 g/L glucose. Caco-2 cells (ATCC: HTB-37) were grown in RPMI 1640 in the presence of 10 g/L non-essential amino acids. Pancreatic ductal adenocarcinoma specimens were obtained from patients undergoing pancreaticoduodenectomy, after written consent and in accordance with French ethical guidelines. Pancreatic cancer samples were obtained through EUS-guided FNA in patients who have given their written consent. The protocol was approved by the Ethical Committee from Midi-Pyrénées CPPRB-1. Briefly, FNA was performed using GF-UC 30p ultrasound endoscope (Olympus, Rungis, France). Samples of pancreatic cancer tissue were obtained from each patient. The core biopsies were then transferred in Dubosq-Brazil medium and the cellular material remaining in the needle was immediately put in RNA later (Ambion, Woodward Austin, TX, USA). All cases of pancreatic cancer were diagnosed based on histological features. One of the normal pancreatic specimens was obtained from an organ donor. The other two normal pancreatic RNA samples were from Clontech (Palo Alto, CA, USA), each a pool from two individuals.

RNA extraction, cDNA labeling and membrane hybridization

Cells were rinsed in PBS and total RNA was extracted with the RNeasy mini-kit (QIAGEN, Valencia CA, USA). Adenocarcinoma tissues were first grinded mechanically in liquid nitrogen with a pestle in a mortar. Cellular samples obtained by EUS-guided FNA were temporarily stored at -25℃ in RNA later (Ambion), and total RNA purified using the RNeasy micro-kit (QIAGEN). The quality and the quantity of the RNA were systematically determined with an Agilent Bioanalyzer 2100. Atlas Pure Total RNA Labeling System (Clontech) and α^{33}P-dATP (-92.5 TBq/mmol) (GE-Amersham, Saclay, France) were used for cDNA target synthesis of 25-40 μg of total RNA. Only targets with a total activity superior to 1 million cpm were used. The membranes (Atlas Human Cancer 1.2, Clontech) were hybridized overnight, washed according to the manufacturer's recommendations and exposed on phosphor storage screens for generally three days. The screens were then scanned in a phosphorimager. All cell lines were analyzed at least twice, i.e. independent RNA extraction, labeling and hybridization procedures.

Expression data analysis

The scanned images were analyzed with the ImaGene software (V.4.0) (Biodiscovery Inc. Los Angeles, CA). Spots having an intensity inferior to the negative spots (foreign DNA) on the membrane (mean + 2SD) in more than 50% of the experiments in each category were eliminated. By this procedure, 871 genes were retained and used for further analysis. The gene names used are respecting the nomenclature proposed by HUGO. Functions and tissue expression distribution came from the database SOURCE available at http://source.stanford.edu or from

Table 1 Sense (S) and antisense (AS) primers used in quantitative RT-PCR analyses

Primer	Exon	Sequence (5′-3′)	Amplicon
LCN2 (Lipocalin2) S	1	TGATCCCAGCCCCACCT	74 bp
LCN2 (Lipocalin2) AS	2	CCACTTCCCCTGAATTGGT	
PLAT (tPA) S	2	TGGAGAGAAAACCTCTGCGAG	72 bp
PLAT (tPA) AS	3	CCATGATTGCTTCACAGCGT	
KRT7 (Keratin7) S	7	CTCTGTGATGAATTCCACTGGTG	72 bp
KRT7 (Keratin7) AS	8	CCCATGGTTCCCCCGA	
18S S		AAACGGCTACCACATCCAAG	155 bp
18S AS		CCTCCAATGGATCCTCGTTA	

the relevant references as indicated. The Genesis software (V1.5.0)[11] was used for clustering analysis. For principal component analysis (PCA) with biplot representation [12] the R software (V.1.9) was used (http://www.r-project.org/). FDR analysis was performed with the Significance Analysis of Microarrays (SAM) software (V1.21): http://www-stat.stanford.edu/~tibs/SAM/.

Real-time RT-QPCR

For quantitative real-time RT-PCR (RT-QPCR) analysis, 3 μg (tumor samples, cell lines, and normal pancreas) or 5-10 ng (cell aspiration) of total RNA were reversely transcribed using Superscript II RNase H$^-$ reverse transcriptase (Invitrogen) and random hexamers using standard conditions. Generally, 5% of the RT reaction was used as template for the subsequent RT-QPCR reactions using the SYBR Green technology (Applied Biosystems Foster City, CA, USA) in a GeneAmp 5700 sequence detector system (Applied Biosystems). The expression levels were normalized to 18S ribosomal RNA levels. The sequence, orientation and corresponding exon localization of the primers used are shown in Table 1 as well as the length of the amplicon.

Immunohistochemical analysis

Paraffin-embedded tissue sections were dewaxed and permeabilized with citrate buffer, pH 6, for 3 min ×5 min in a microwave oven. The slides were rinsed 3 min× 5 min in PBS at room temperature and treated for 10 min with Dako protein block solution (Dako, Glostrup, Denmark). The sections were then incubated with a monoclonal mouse anti-human lipocalin 2 antibody (clone 211-01) (The Antibody Shop, Denmark), (1:100 in PBS, 10 g/L BSA) over night at 4℃. After rinsing (3 min × 5 min in PBS), the sections were treated with 30 mL/L H_2O_2, 100 mL/L methanol in PBS for 15 min followed by two rinses (2 min × 5 min) in PBS, 10 g/L BSA, and the incubation with an HRP-coupled rabbit anti-mouse IgG antibody (P0161; Dako) (1:50 in PBS, 10 g/L BSA) for 1 h at 20℃. The slides were then rinsed 3 min × 5 min in PBS + 10 g/L BSA and incubated 1 h (20℃) with an HRP-coupled goat anti-rabbit IgG antibody (P0448; Dako) (1:50 in PBS, 10 g/L BSA) and finally rinsed 2 min × 5 min in PBS + 1% BSA prior to development with one drop of AEC + substrate chromogen solution (Dako) for 15 min. The slides were washed in running cold water for 10 min and counterstained with Mayer's hemalun solution (Merck, Darmstadt, Germany) and mounted with Dako glycerol mounting medium. Pictures were taken with the Visiolab 2000 software using a Nikon eclipse E400 microscope.

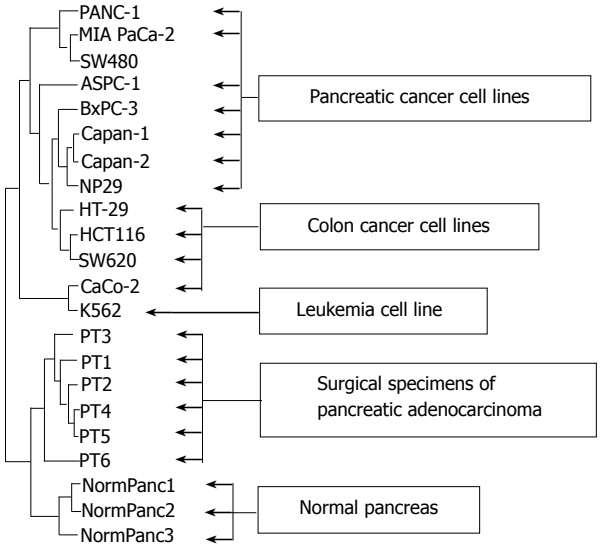

Figure 1 Hierarchical clustering analysis of sample expression profiles. Dendrogram of centered mean expression data using euclidian distance with average linkage clustering, showing relationships in gene expression profiles of samples. Closely related samples are found in the same branch of the tree and a reduced branch height represents a closer relationship between groups.

RESULTS

Expression profiling

Using Clontech's Human Cancer 1.2 macroarrays, the expression profile of 1176 genes associated with cancer was studied in 7 human pancreatic adenocarcinoma cell lines (PCL), PANC-1, MIA Paca-2, ASPC-1, BxPC-3, Capan-1, Capan-2, NP29, and 6 tumor samples, PT1-PT6, obtained through surgical resection of pancreatic adenocarcinomas. In order to assess the specificity of the expression of pancreatic tumor cells, 5 human colon cancer cell lines (CCL), SW480, HT-29, HCT116, SW620, Caco-2, 1 leukemia cell line (LCL), K562 and 3 normal human pancreatic tissue samples, NormPanc1-3, were included in the study. After screening of the expression data as described in the material and methods section, 871 genes were retained for further analysis. The expression data are available at the following URL: http://ifr31w3.toulouse.inserm.fr/microArrayPancreas/DataSummary.xls. In a hierarchical cluster analysis of samples based on centered mean expression data, two main clusters could be identified, corresponding to cell lines (up) and tissue samples (down), respectively (Figure 1). Among the clusters identified within the cell lines, one was purely pancreatic (BxPC-3, Capan-1, Capan-2 and NP29) and one comprised only colon cancer cell lines (HT-29, HCT116 and SW620). At the top of the dendrogram, a colon cancer cell line SW480 clustered with two pancreatic cancer cell lines, PANC-1 and MIA PaCa-2. In a hierarchical clustering analysis of the 871

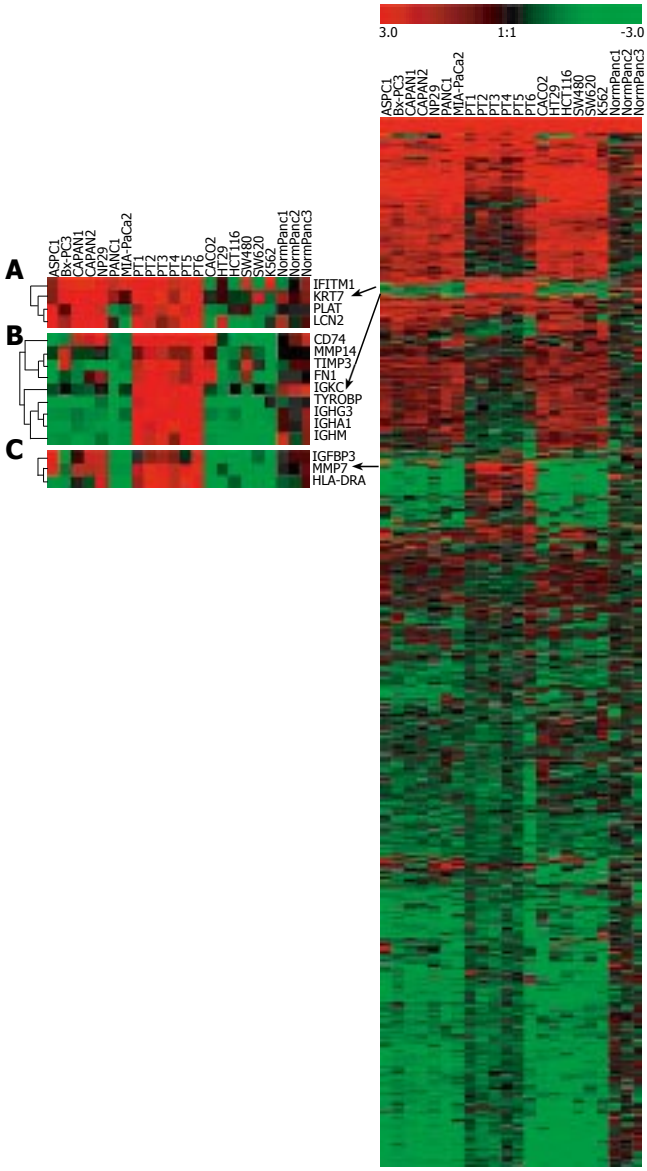

Figure 2 Hierarchical clustering analysis of gene expression profiles. Right: Expression profile of the 871 genes retained after data-filtering. Left: Three magnified subclusters (**A**-**C**) showing the genes overexpressed in most of pancreatic tumors. Red color indicates high expression levels; green color indicates low expression levels.

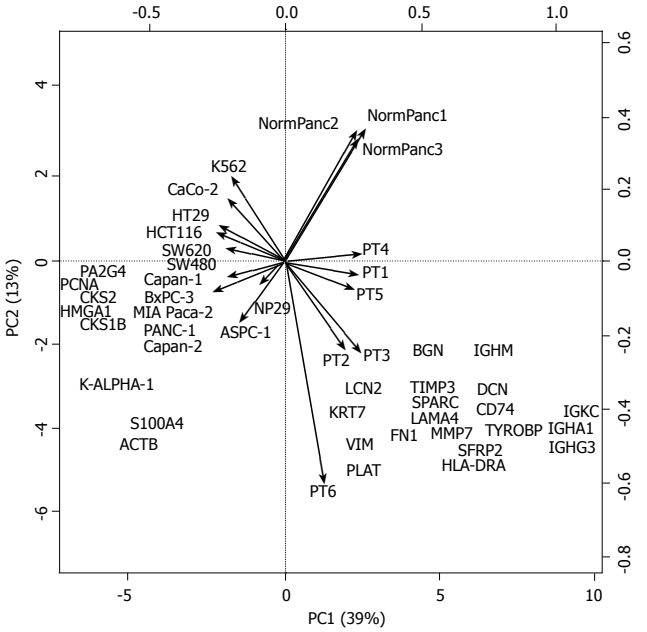

Figure 3 Principal component analysis (PCA) of gene expression data. Biplot resulting from a PCA of the line and column centered data containing 871 genes (individuals) in lines and 22 samples in columns (variables). The 28 genes contributing the most to the total variability are shown. The two principal components (PC1 and PC2) contribute to more than 50% (39% and 13%) of the total variability and resolve four biological sample categories: PC1 on the horizontal x-axis distinguish between cell lines (left) and tissue samples (right) whereas PC2 on the vertical y-axis distinguish between malignant pancreas samples (bottom) and other sample categories (top).

genes retained after filtering, three subclusters (A, B and C) contained genes with an elevated expression in pancreatic tumor samples (PT) and a lower expression in normal pancreas and non-pancreatic cancer cell lines (NormPanc, CCL and LCL) (Figure 2). Subcluster A containing four genes, *IFITM1*, *KRT7*, *PLAT* and *LCN2*, exhibited an expression profile particularly specific for neoplastic pancreas. These four genes were overexpressed in the majority of PCL and in PT1 to 6, but not in non-pancreatic cell lines and in normal pancreas. Subclusters B and C showed genes overexpressed in all PT, in some pancreatic cancer cell lines but not in normal pancreas.

In order to estimate the variability of the experimental procedures, several biological and technical replicates were performed. In a hierarchical clustering analysis, these replicates either clustered directly together or regrouped within clusters of the respective cell line validating our experimental protocols and data acquisition procedures (data not shown). We used the SAM software to detect the genes that were significantly over-expressed in malignant pancreas, referring to the mean expression in pancreatic tumors (PT) and pancreatic cancer cell lines (PCL) as compared with the other groups normal pancreas (NormPanc), colon cancer cell lines (CCL) and leukemia cell line (LCL). Among the genes selected by SAM (FDR < 2%), the ones with an at least 2-fold higher expression level in malignant pancreas are shown in Table 2. In the same table are also represented other expression fold ratios, i.e. the comparison between pancreatic and colon cancer cell lines (column PCL/CCL) as well as the fold ratio between pancreatic tumors and normal pancreas (column PT/NormPanc). The five genes with the highest expression ratios were *PLAT*, *KRT7*, *CD74*, *MMP7*, and *LCN2*. The ratio of mean expression level of these genes in PT as compared with that in NormPanc, was found to be equal to or above 3.36, indicating that they discriminate well between adenocarcinoma and normal tissues. The ratio of the gene mean expression level in PCL as compared with that in CCL discriminates between pancreatic and colon cancer cells, which is important to differential diagnosis.

Although clustering methods are the most widely used technique to group genes based on expression patterns, PCA is rapidly gaining acceptance in the field of transcriptome analysis. PCA permits data visualization of complex multidimensional datasets by projecting data into a sub-space with 2 or 3 dimensions. Moreover, a biplot representation of PCA results (Figure 3) provides

Table 2 Significantly overexpressed genes (over 2-fold) in malignant pancreas

Gene symbol	Gene description	Mean fold expression ratios		
		Malignant Panc/others	PCL/ CCL	PT/ NormPanc
PLAT	Plasminogen activator, tissue-type	6.22	4.36	6.74
KRT7	Keratin 7	5.72	4.92	3.97
CD74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	5.44	1.61	8.00
MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	5.36	3.73	5.70
LCN2	Lipocalin 2 (oncogene 24p3), NGAL	5.25	8.32	3.36
HLA-G	HLA-G histocompatibility antigen, class I, G	4.04	1.57	4.32
IGHG3	Immunoglobulin heavy constant gamma 3	3.95	0.94	11.81
HLA-DRA	Major histocompatibility complex, class II, DR alpha	3.70	1.57	7.01
TIMP1	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	3.50	1.61	9.20
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	3.48	3.00	2.40
ITGB4	Integrin, beta 4	3.36	1.16	4.34
KRT19	Keratin 19	3.27	1.34	2.98
CTSD	Cathepsin D (lysosomal aspartyl protease)	3.21	2.75	2.89
CASP4	Caspase 4, apoptosis-related cysteine protease	3.10	2.43	1.80
IGFBP3	Insulin-like growth factor binding protein 3	3.08	3.53	1.87
PLAU	Plasminogen activator, urokinase	3.08	2.83	2.01
MMP11	Matrix metalloproteinase 11 (stromelysin 3)	2.96	1.11	7.22
DTR	Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)	2.84	1.74	3.33
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.76	1.65	3.17
LAMA4	Laminin, alpha 4	2.74	2.14	3.30
ITGB8	Integrin, beta 8	2.59	2.31	4.44
IFITM1	Interferon induced transmembrane protein 1 (9-27)	2.41	5.47	7.10
AXL	AXL receptor tyrosine kinase	2.35	1.63	2.91
ITGAE	Integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide)	2.31	1.58	3.56
CD59	CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344)	2.26	2.11	2.95
KRT10	Keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	2.09	2.18	1.60
CYR61	Cysteine-rich, angiogenic inducer, 61	2.03	2.41	1.22
PTGES	Prostaglandin E synthase	2.03	2.84	1.11
HIF1A	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	2.01	1.24	2.87

a powerful visual overview of the relationships between genes and samples. The sub-space determined by PCA captures the highest amount of the total variability. In our line and column centered data matrix, the first (PC1) and second (PC2) principal components captured 39% and 13% of the total variability, respectively (Figure 3). In the biplot, samples are shown as arrows, and the genes are shown by their HUGO ID symbols. Arrows of similar length, pointing in the same direction display a higher degree of correlation. As observed in the hierarchical clustering analysis (Figure 1), SW480 was more associated to pancreatic cell lines than to other colon cell lines. Also in agreement with figure 1, the leukemia cell line K562 was correlated with the colon cell line Caco-2. An analysis of the PC1 (horizontal x-axis) revealed a clear distinction between tissue samples and cell lines, with a positive x-coordinate value for all tissue samples and negative for all cell lines. More interestingly, the same analysis of PC2 (vertical y-axis) discriminated malignant pancreas (pancreatic tumors and pancreatic cell lines) from the other samples categories studied, with the exception of SW480.

To improve the interpretation, only the 28 genes contributing most to the total variability of the data set are shown in the biplot. The co-localization between a gene and a sample type signifies an overexpression in the given sample type. Accordingly, genes such as *PCNA*, *HMGA1*, *PA2G4*, *CKS2* and *CKS1B* were specifically overexpressed in cell line samples, whereas *IGHM*, *IGKC*, *IGHA1* and *IGHG3*, all immunoglobulins, were highly associated with tumor samples. The extreme position of these genes on the horizontal axis in the biplot indicates they are involved in the distinction between cells and tissue samples. The overexpression of genes of the immune response is a characteristic of cancer tissue samples versus cell lines. Furthermore, a set of genes, including *PLAT*, *VIM*, *HLA-DRA*, *IGHG3*, *ACTB*, *S100A4*, and others located in the lower half of the biplot, was involved in the discrimination between malignant pancreas and all other samples. Among them, *LCN2*, *KRT7*, *VIM* and *PLAT*, appeared to be highly correlated with both pancreatic tumors and pancreatic cell lines. However, in the third principal component (PC3), *VIM* was positioned far from the three other genes (data not shown). Interestingly, *CTRL* (chymotrypsin-like), was found to be specifically expressed in normal pancreas and absent in PT or PCL. CTRL is a poorly characterized serine protease with chymotrypsin- and elastase-2-like activities, which is expressed as a pro-protease in normal pancreatic tissue[13]. The absence of *CTRL* expression in adenocarcinoma samples is in agreement with the absence of other serine

Table 3 Relative mRNA levels presented as $2^{[Ct(18S)-Ct(gene\ of\ interest)]}$ (mean x 10^{-2} ± SEM)

Gene	*n*	KRT7	LCN2	PLAT
Pancreatic tumors	6	1.14. ± 0.47	3.58 ± 0.65[a]	2.34 ± 0.89[a]
Pancreatic cell lines	6	79.62 ± 90.97	188.99 ± 81.13[a]	13.51 ± 4.67[a]
Normal pancreas	3	0.42 ± 0.23	1.05 ± 0.85	0.14 ± 0.08

[a]$P < 0.05$, *vs* normal pancreas.

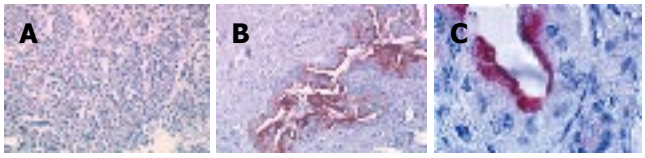

Figure 4 Lipocalin 2 protein expression in pancreatic cancer tissue Immunohistochemical analysis by AEC + substrate chromogen staining. **A** (x 40): normal pancreas; **B** (x 200), **C** (x 1000): pancreatic adenocarcinomas.

protease digestive enzyme, such as chymotrypsin and trypsin, present in normal pancreatic tissue and known to be down-regulated in pancreatic cancer.

Differentially expressed genes in neoplastic pancreas

Taken together, the three statistical methods used in our study (hierarchical clustering, Figure 2, the SAM method, Table 2, and PCA, Figure 3) retained seven genes as differentially overexpressed in malignant pancreas. Among these genes, 3 are involved in the immune response: *CD74*, *HLADRA*, and *IGHG3*, and 4 genes previously suggested as key proteins in pancreatic cell biology or oncogenesis**:** *KRT7*, *MMP7*, and *PLAT* and *LCN2*. Among them, three genes *LCN2*, *KRT7* and *PLAT* were selected for further validation by real-time RT-QPCR. This choice was based on the highly specific expression profile in malignant pancreas (Figure 2; subcluster A) and elevated mRNA levels in the corresponding samples (Table 2) as well as on their specific positioning in the PCA analysis, that is in the interface between the pancreatic tumors and pancreatic cancer cell lines. These studies confirmed significantly elevated levels of *LCN2* and *PLAT* mRNA in malignant pancreas samples, tumors and cancer cell lines (Mann-Whitney test: $^{a}P < 0.05$) (Table 3). However, they were unable to confirm the macroarray results concerning *KRT7* expression levels.

The PLAT protein is known to be overexpressed in pancreatic cancer tissues[14]. However, even though several studies have reported elevated *LCN2* mRNA levels in pancreatic cancer[15], and LCN2 protein in human pancreatic juice in patients with pancreatic cancer[16], the presence of the LCN2 protein in pancreatic adenocarcinoma is less well characterized. Therefore, in order to evaluate the expression of the lipocalin 2 protein in pancreatic tumors, we performed immunohistochemical analysis using a monoclonal anti-lipocalin 2 antibody. As shown in Figure 4A, little or no labeling was observed in normal pancreas ($n = 3$). However, in sections of pancreatic adenocarcinomas ($n = 5$), a strong apical labeling was detected in ducts invaded by carcinoma (Figure 4B and C).

LCN2, KRT7 and PLAT transcripts in EUS-guided FNA samples

We investigated the possibility to use EUS-guided FNA as a source of pancreatic sample to study the expression of *KRT7*, *LCN2* and *PLAT* genes as diagnostic biomarkers. The presence of these mRNAs was therefore examined by RT-QPCR analysis in 12 samples, originating from different individuals, obtained through EUS-guided FNA from patients with pancreatic adenocarcinoma. Compared with normal pancreas, the expression level of the *KRT7*, *LCN2* and *PLAT* transcripts were 26.7 ± 18.16, 20.6 ± 7.6 and 70.8 ± 35.4 (mean ± SE), respectively. In each case, 18S ribosomal RNA was used for normalization. Even though 10 out of 12 patients presented elevated levels of expression for *KRT7* and *LCN2* and 11 for *PLAT,* only the levels of *PLAT* and *LCN2* expression were significantly different from normal pancreas (unilateral Student *t* test: $P < 0.05$). These results were thus perfectly concordant with those obtained from pancreatic cancer cell lines and tumors.

DISCUSSION

Large scale analysis of gene expression has been widely proposed as a powerful method for identification of molecular markers of neoplasia and for the generation of novel taxonomies for cancer. Accordingly, several studies, have been conducted to characterize the expression profiles in human pancreatic adenocarcinomas[15, 17]. However, to the best of our knowledge, none of these studies report the comparison between pancreatic and colon cancer cell lines. Moreover, this study reports, for the first time, about the use of the dedicated Atlas Human Cancer 1.2 cDNA macroarray containing 1176 cancer-associated genes to study expression profile in pancreatic carcinoma. We report the expression profiles of pancreatic adenocarcinoma cell lines and tumors and compared with that of normal pancreas specimens, colon cancer cell lines and one hematopoietic cell line K562. This was done to differentiate pancreatic and non-pancreatic cancer gene profile in view of differential diagnosis. The fact that several groups have previously reported elevated expression levels of *LCN2* mRNA in pancreatic cancer[18-21], was reassuring for the validation of our study design. However, reports validating this overexpression at the protein level are very scarce[22]. Lipocalins are small extracellular proteins with important role in cell proliferation and differentiation, possessing protease inhibitory properties and/or carrying lipophilic ligands such as retinoids and fatty acids into the cells[23]. The localization of LCN2 protein at the apical side of cells from ducts invaded by the tumoral process might be of pathophysiological importance and supports further analysis for a better understanding of the role of this protein in cancer.

Interestingly, the overexpression of *PLAT* in pancreatic adenocarcinoma has not been reported in previous expression profiling studies. PLAT was recently shown to play a critical role in tumor angiogenesis and in the devel-

opment of exocrine pancreatic cancer[24], contributing to an invasive phenotype[14,25]. The vast majority of pancreatic adenocarcinomas express KRT7[26,27] and it has previously been indicated as overexpressed in pancreatic cancer by gene expression profiling studies[28]. However, the KRT7 protein is suggested as a marker of normal pancreatic duct epithelial cells playing a role in cell differentiation[29]. The reported expression of KRT7 in both normal and malignant pancreas could, at least in part, explain why we were unable to observe a significant difference in expression levels between both sample types. Among the seven genes retained on our study, three are highly implicated in the immune response: *CD74*, *HLADRA, IGHG3*. *IGHG3* was only overexpressed in tumor samples suggesting an immune cell origin. In contrast, elevated levels of *CD74* and *HLADRA* were found in tumor samples and also in some pancreatic cancer cell lines. Elevated levels of *CD74*, and *HLADRA* were observed in several types of cancers, including gastric cancer and renal epithelial neoplasms[30,31].

The quantitative RT-PCR on EUS-guided FNA samples of *PLAT* and *LCN2*, validated the overexpression found by the macroarray analysis. Thus, the quality and the amount of cellular sampling using pancreatic EUS-guided FNA allow the extraction of sufficient quantities of RNA to perform RT-QPCR analysis as a new tool for early diagnosis, as described recently for lymph node metastasis[32]. Furthermore, when performed directly on resected tumor pieces, fine needle aspiration has been shown to produce a relative enrichment of cancer cells, in comparison to tumor samples. This enrichment has been attributed to the capability of epithelial cancer cells to be aspirated more easily than stromal cells[33]. To the best of our knowledge, no gene expression had been evaluated with EUS-guided FNA biopsies from patients with pancreatic adenocarcinoma. EUS-guided FNA is a safe method for patient exploration. Morbidity rate ranges from 1% to 3% when performed by experienced endosonographers[34]. Therefore, identification and quantification of potential molecular markers for pancreatic cancer on cellular samples obtained by EUS-guided FNA could be a promising approach for the diagnosis of solid pancreatic masses. Future studies are needed to prospectively evaluate new molecular biomarkers using this procedure, in order to increase the accuracy of current standard histological and cytological analyses, that was only 80 to 85% in pilot studies[35,36].

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BASIC RESEARCH

Adiponectin deficiency exacerbates lipopolysaccharide/D-galactosamine-induced liver injury in mice

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Abstract

AIM: To examine the effects of adiponectin on the functions of Kupffer cells, key modulators of lipopolysaccharide (LPS) -induced liver injury.

METHODS: D-galactosamine (GalN) and LPS were injected intraperitoneally into adiponectin-/- mice and wild type mice. Kupffer cells, isolated from Sprague-Dawley rats, were preincubated with or without adiponectin, and then treated with LPS.

RESULTS: In knockout mice, GalN/LPS injection significantly lowered the survival rate, significantly raised the plasma levels of alanine transaminase and tumor necrosis factor-α (TNF-α) and significantly reduced IL-10 levels compared with wild type mice. TNF-α gene expression in the liver was which higher and those of IL-10 were lower in knockout mice than in wild type mice. In cultured adiponectin-pre-treated Kupffer cells, LPS significantly lowered TNF-α levels and raised IL-10 levels in the culture media and their respective gene expression levels, compared with Kupffer cells without adiponectin-pre-treatment.

CONCLUSION: Adiponectin supresses TNF-α production and induces IL-10 production by Kupffer cells in response to LPS stimulation, and a lack of adiponectin enhances LPS-induced liver injury.

Key words: Adiponectin; Lipopolysaccharide; Kupffer cell; TNF-alpha; IL10

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INTRODUCTION

Obesity is currently a serious medical problem world wide. It is an independent risk factor of non-alcoholic steatohepatitis (NASH) and alcoholic liver injury[1,2]. The pathogenesis of these diseases involves lipopolysaccharide (LPS)[3-5]. The complex of LPS and LPS-binding protein (LBP) activates Kupffer cells to secrete tumor necrosis factor (TNF)-α, which plays an important role in liver injury[6,7]. The control of the response of Kupffer cells to LPS is thought to be critical in the prevention of LPS-induced liver injury. Indeed, the selective inhibition of Kupffer cells by the administration of either gadolinium chloride or methyl palminate results in the abrogation of liver injury with an inhibition in TNF-α secretion[8]. Interleukin (IL)-10, which is secreted by Kupffer cells in the liver after LPS stimulation[9], has strong anti-inflammatory effects in the liver, and prevents liver fibrosis[10,11]. Interferon (IFN)-γ activates the signal transducer and activator of transcription (STAT-1) in hepatocytes and promotes apoptosis in hepatocytes after administration of D-Galactosamine (GalN)/LPS[12]. In GalN/LPS induced liver injury, the modulation of the production of these cytokines from Kupffer cells is important.

Adiponectin, a 30 ku adipocyte complement-related protein (Acrp30), is an adipocyte-specific plasma protein (normal level, 5-30 mg/L), and is paradoxically decreased in obesity and in cases of alcoholic fatty liver[13,14]. Adiponectin contains two types of receptors, AdipoR1 and AdipoR2. Whereas AdipoR1 is expressed ubiquitously and abundantly in skeletal muscle, AdipoR2 is most abundantly expressed in the liver and is an intermediate-affinity receptor for full-length and globular adiponectin[15]. Adiponectin improves the insulin sensitivity, and has anti-

atherogenic and anti-inflammatory effects. In the liver, it has anti-fibrogenic effects and regulates hepatic stellate cells[16].

We recently reported that adiponectin inhibits the phagocytosis of human macrophages and LPS-induced TNF-α release[17], and induces IL-10 gene expression in human macrophages[18]. We hypothesized that adiponectin has anti-inflammatory effects on Kupffer cells, key modulators of LPS induced liver injury, by regulating the release of cytokines. However, the effects of adiponectin on LPS-induced liver injury and Kupffer cells remained poorly understood. In the present study, to clarify the effects of adiponectin on LPS-induced liver injury, we investigated the effects of adiponectin on GalN/LPS-induced liver injury using adiponectin knockout mice. We also examined the effects of adiponectin on cytokine production from Kupffer cells in primary cultures.

MATERIALS AND METHODS

All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine. Recombinant mouse full-length adiponectin was prepared as described previously[16]. The method of disruption of the mouse adiponectin gene was described previously[19].

Murine model of acute hepatitis

To clarify the role of adiponectin in LPS-induced liver injury, experiments were conducted using adiponectin-/- mice and wild-type (WT) control C57B6 mice (28-32 g body mass; 10-12 wk old)[19]. Each mouse was simultaneously injected intraperitoneally with 700 mg/kg of GalN and 10 μg/kg of LPS. The doses of GalN and LPS were determined in previous studies and our preliminary study[12]. LPS from *E. coli* O55:B5 and D-galactosamine (GalN) were purchased from Sigma (St. Louis, MO). Groups of 10 mice were treated to determine the survival curve. Groups of 6 mice were treated to measure the plasma levels of alanine aminotransferase (ALT), TNF-α, IL-10, and IFN-γ. Groups of 6 mice were sacrificed as follows: before, 0.5, 1, and 4 h after the administration to measure the gene expressions of TNF-α IL-10, IFN-γ in the whole liver and IFN-γ in the spleen, assessed by means of real-time polymerase chain reaction (PCR).

LPS stimulation on Kupffer cells

Male Sprague-Dawley (SD) rats (10 wk old, $n = 6$) were anesthetized with pentobarbital sodium, and the portal vein was cannulated. The liver was perfused with a ethylene glycol bis (beta-aminoethyl ether)-N, N, N', N' -tetraacetic acid (EGTA) solution, and digested with a 0.5 g/L collagenase solution. Differential centrifugation on Nycodenz (Pharma, Oslo, Norway) density gradients was performed as described before[20]. We evaluated the purity of the isolated Kupffer cell population by counting CD68 positive cells, ranging from 88.5% to 93.0%. Kupffer cells from each rat were maintained and treated separately. The Kupffer cells were maintained at 37°C under 50 mL/L CO_2 in Dulbecco's modified Eagle medium containing 100 mL/L fetal calf serum with or without adiponectin (10 mg/L) for 24 h. After a 24 h pretreatment with and without adiponectin, LPS was added to the culture medium at a concentration of 10 mg/L. The doses of adiponectin and LPS were determined in previous studies and our preliminary study[6,17]. TNF-α, IL-10, IFN-γ concentrations in the culture medium were measured 0 and 4 h after the commencement of LPS stimulation. Total RNA of Kupffer cells was isolated at 0, 0.5, 1, and 4 h after the commencement of LPS stimulation.

Measurement of plasma concentrations of ALT and cytokines

Plasma ALT concentrations were measured by using a transaminase CII-test kit according to the protocol provided by the manufacturer (Wako Pure Medical, Osaka, Japan). Circulating levels of TNF-α, IL-10, and IFN-γ were assessed using commercial enzyme-linked immunosorbent assay (ELISA) kits for mice (Biosource Int., Camarillo, CA). Their concentrations in the culture media of Kupffer cells were quantified using ELISA kits for the rat (Biosource Int.).

Quantification of gene expression levels

Total RNA from whole liver, spleen and Kupffer cells were extracted using a Qiagen (Hilden, Germany) QIAshredder and an RNA-easy Mini kit according to the instructions provided by the manufacturer. The reverse-transcription polymerase chain reaction (RT-PCR) was performed as described previously[21]. The Quantitect PCR probe kit and Quantitect gene assay kit for mice TNF-α, IL-10, and IFN-γ were purchased from Qiagen and used in a real-time PCR analysis of the mouse samples. Primers for rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH), TNF-α, IL-10, IFN-γ, and mice GAPDH were designed using the computer program Primer Express (Applied Biosystems, Foster city, CA). Dynamo SYBR Green qPCR kit (Finzymes, Espoo, Finland) was used for the real-time PCR analysis of rat TNF-α, IL-10, IFN-γ, GAPDH and mice GAPDH. Real-time PCR was performed using a DNA Engine Opticon 2 real-time PCR Detection System (MJ Research, Waltham, MA). Relative gene expression was quantified using GAPDH as an internal control. The expressions of adiponectin receptors, AdipoR1 and AdipoR2 were assessed with RT-PCR as previously published[22].

TUNEL assay

Quantification of apoptotic hepatocytes in liver sections was performed by counting the number of TUNEL-positive cells. The data were expressed as the average number of TUNEL-positive cells per five high power fields (HPF) (×100). The TUNEL assay was performed based on the instructions provided by the manufacturer (In Situ Apoptosis Detection Kit; TaKaRa, Shiga, Japan, and Liquid DAB Substrate kit; Zymed Lab. Inc., South San Francisco, CA).

Statistical analysis

The results are presented as the mean ± SE. Analysis of variance (ANOVA) for the groups was performed by the

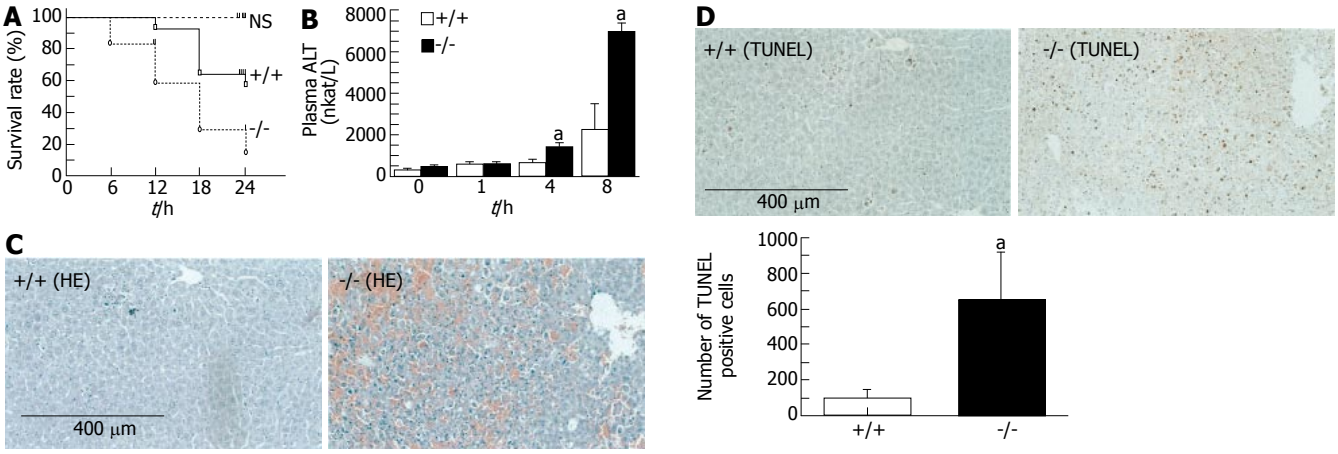

Figure 1 GalN/LPS induced changes in mice. **A:** Survival rate (n = 10); **B:** Plasma ALT (mean ± SE, n = 6). $^aP < 0.05$, $^bP < 0.01$, *vs* WT; **C:** Histology of the liver. Massive liver injury in adiponectin-/- mice. (Hematoxylin-eosin × 100); **D:** Large numbers of apoptotic hepatocytes in adiponectin-/- mice (TUNEL × 100). Scale bar = 400 μm. $^aP < 0.05$, *vs* WT mice.

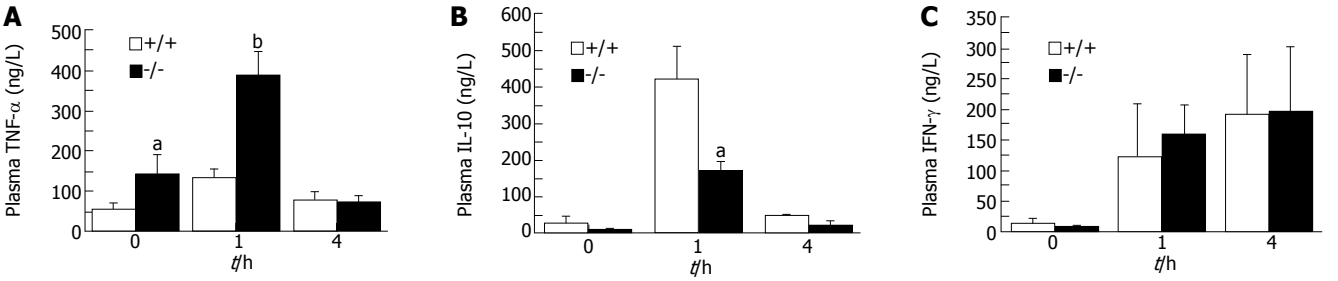

Figure 2 Plasma concentrations of TNF-α, IL-10 and IFN-γ in mice after GalN/LPS administration. (mean ± SE, n = 6). $^aP < 0.05$, $^bP < 0.01$, *vs* WT.

Mann Whitney test, followed by Scheffé's test for multiple comparisons to allow pairwise test for significant differences between groups. The statistical significance of the lethality rates was determined by a Log-rank test. Statistical significance was defined as $P < 0.05$.

RESULTS

GalN/LPS induces severe liver injury in KO mice

The survival rate of adiponectin-/- mice after GalN/LPS administration was significantly lower than that of WT mice 24 h after the administration ($P = 0.041$; log-lank test; Figure 1A). GalN/LPS administration resulted in an elevation of plasma ALT concentrations in both WT and adiponectin-/- mice, although increases at 4 and 8 h after administration were significantly higher in adiponectin-/- mice than in WT mouse (at 4 h; WT, 693.5 ± 472.8 *vs* adiponectin-/-, 1458.6 ± 541.8 nkat/L; $P < 0.01$, at 8 h; WT, 2255.5±3242.3 *vs* adiponectin-/-, 6988.1 ± 978.5; $P < 0.05$; Figure 1B). A histological examination revealed that GalN/LPS administration induced massive liver injury in adiponectin-/- mice mice (Figure 1C). The number of TUNEL-positive hepatocytes in adiponectin-/- mice livers was significantly higher than that in WT mouse livers 8 h after GalN/LPS administration (WT, 95 ± 118 *vs* adiponectin-/- mice, 683 ± 729 apoptotic cells/HPF; $P < 0.05$; Figure 1D).

Effects of GalN/LPS on plasma TNF-α, IL-10 and IFN-γ

Prior to GalN/LPS administration, plasma TNF-α concentrations were significantly higher in adiponectin-/- mice than in WT mice ($P < 0.05$; Figure 2A). Plasma TNF-α concentrations increased reaching peak levels 1 h after the administration, and were significantly higher in adiponectin-/- mice mice than in WT mice at their peak ($P < 0.01$; Figure 2A). Plasma IL-10 concentrations also increased, reaching peak levels 1 h after the administration of GalN/LPS. In adiponectin-/- mice, IL-10 plasma concentrations were significantly lower than those of WT mice at their peak ($P < 0.05$; Figure 2B). Plasma IFN-γ concentrations increased continuously up to 4 h after GalN/LPS administration, and no significant difference between adiponectin-/- mice and WT mice was found (Figure 2C).

Effects of GalN/LPS on liver TNF-α, IL-10, IFN-γ and splenic IFN-γ mRNA expression

The TNF-α mRNA expression level in the liver increased, reaching a peak level 1 h after GalN/LPS administration, and this level was significantly higher in adiponectin-/- mice than in WT mice 1 h after the administration ($P < 0.01$; Figure 3A). The IL-10 mRNA expression level in the liver also increased, reaching a peak level 4 h after GalN/LPS administration, and the levels at 1 and 4 h were significantly higher in WT mice than in adiponectin-/- mice (at 1 h;

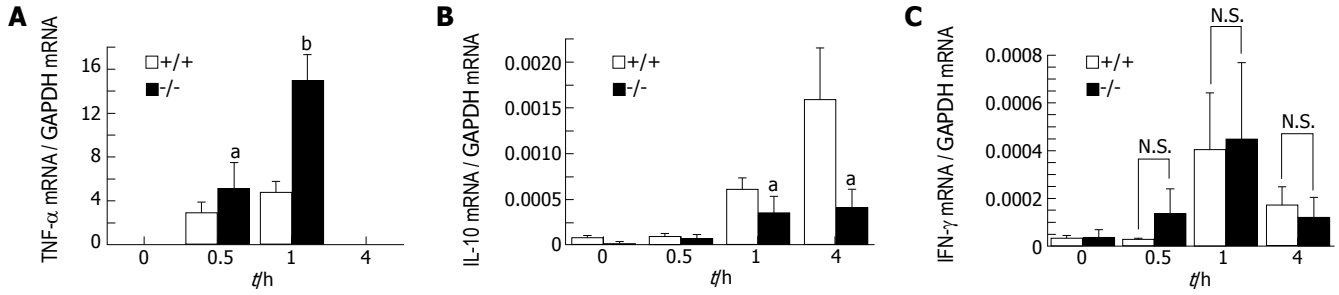

Figure 3 TNF-α, IL-10 and IFN-γ in the liver (**A-C**) of mice following GalN/LPS administration. (mean ± SE, $n = 6$). [a]$P < 0.05$, [b]$P < 0.01$, *vs* WT (Scheffé's test).

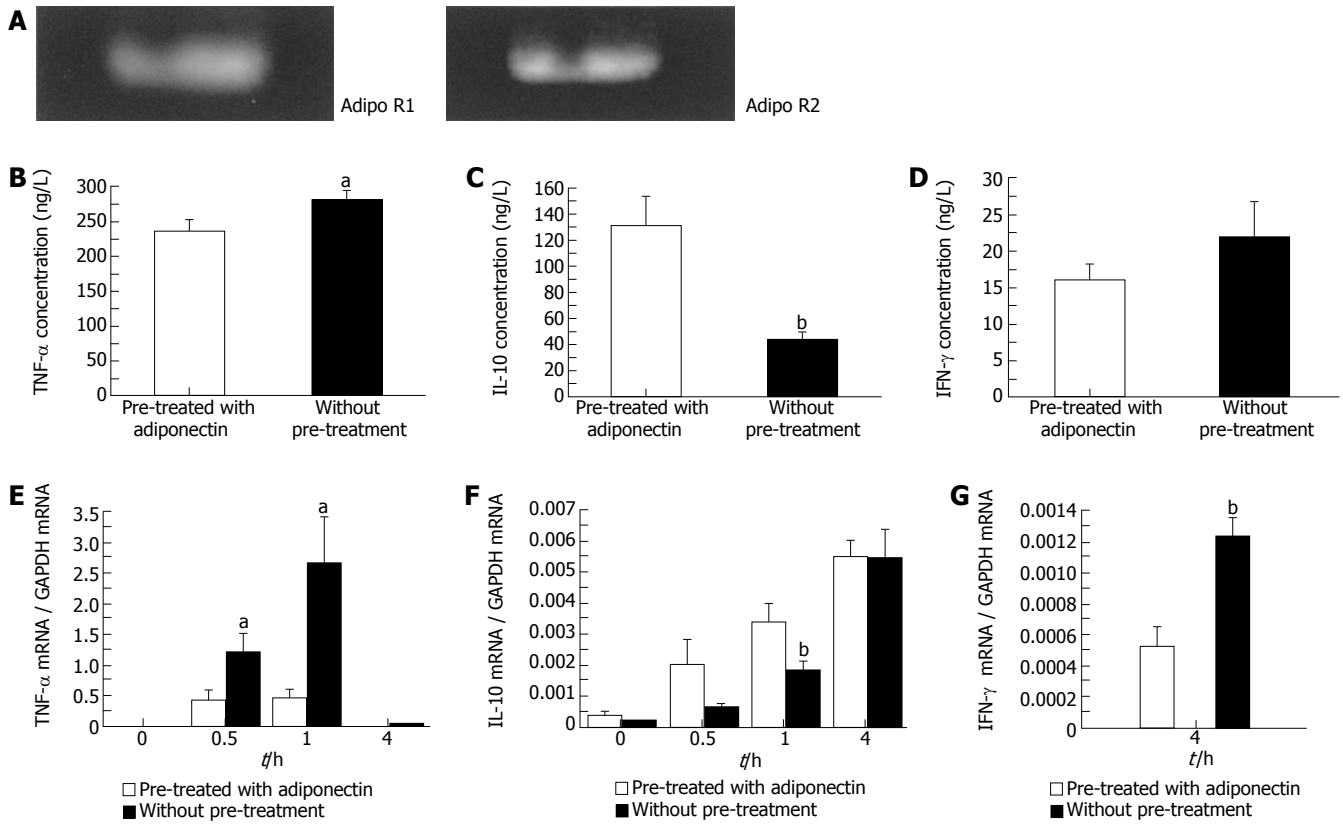

Figure 4 The expressions of adipoR1 and AdipoR2 in Kupffer cells (**A**). Effect of adiponectin on TNF-α, IL-10 and IFN-γ production by LPS-stimulated Kupffer cells *in vitro* (**B-D**). Pre-treated with or without adiponectin (10 mg/L) for 24 h followed by the stimulation with LPS (10 mg/L). TNF-α, IL-10 and IFN-γ mRNA expression in Kupffer cells after LPS stimulation (**E-G**). (mean ± SE, $n = 6$). [a]$P < 0.05$, [b]$P < 0.01$ (Scheffé's test).

$P < 0.05$; Figure 3B). The IFN-γ mRNA expression level in the liver increased, reaching peak levels 1 h after GalN/LPS administration, but no significant difference between adiponectin-/- mice and WT mice was found (Figure 3C). In the spleen, the IFN-γ mRNA expression levels also reached peak levels at 1 h after the administration, but there was no significant difference between adiponectin-/- mice and WT mice (data not shown).

Adiponectin receptors expression in Kupffer cell

We comfirmed the expressions of AdipoR1 and AdipoR2 in rat Kupffer cells using RT-PCR (Figure 4A).

Adiponectin inhibits LPS-induced TNF-α production in Kupffer cells

Pretreatment of Kupffer cells with adiponectin led to a reduction in the levels of TNF-α released in response to LPS stimulation. TNF-α levels in the culture media of adiponectin-pretreated Kupffer cells were significantly lower than those for untreated cells 4 h after LPS stimulation ($P < 0.05$; Figure 4B). The LPS-induced TNF-α mRNA expression level in Kupffer cells was markedly suppressed by adiponectin pre-treatment. The expression level of TNF-α mRNA in Kupffer cells increased, reaching peak levels 1 h after LPS stimulation, but the levels at 0.5 and 1 h after the commencement of LPS stimulation in culture media of adiponectin-pretreated Kupffer cells was significantly lower than in those of untreated cells (at 0.5 h; $P < 0.05$, at 1 h; $P < 0.05$; Figure 4E).

Adiponectin increases LPS-induced IL-10 production in Kupffer cells

Pretreatment of Kupffer cells with adiponectin increased the release of IL-10 in response to LPS stimulation.

IL-10 concentrations in the culture media of adiponectin-pretreated Kupffer cells were significantly higher at 4 h after LPS stimulation than those without adiponectin pre-treatment ($P < 0.01$; Figure 4C). The expression level of LPS-induced IL-10 mRNA in Kupffer cells was markedly increased as a result of adiponectin pre-treatment. The expression level of IL-10 mRNA in Kupffer cells was significantly higher in adiponectin-pretreated Kupffer cells 1 h after LPS stimulation than in untreated cells ($P < 0.05$; Figure 4F).

Lack of effect of adiponectin on LPS-induced IFN-γ production in Kupffer cells

Pretreatment of Kupffer cells with adiponectin had no significant effect on IFN-γ concentrations in the culture media (Figure 4D). But the expression level of LPS-induced IFN-γ mRNA in Kupffer cells was significantly higher in adiponectin-pretreated Kupffer cells 4 h after LPS stimulation than in untreated cells. IFN-γ gene expression in Kupffer cells at 0 and 1h after LPS stimulation could not be determined quantitatively by RT-PCR ($P < 0.01$; Figure 4G).

DISCUSSION

The major findings of the present study were that a lack of adiponectin accelerates LPS-induced liver injury, and that adiponectin has an anti-inflammatory effect on Kupffer cells. Our results demonstrate that adiponectin-pretreated Kupffer cells produced less TNF-α and more IL-10 as a result of LPS treatment. These results suggest that adiponectin has an anti-inflammatory effect on LPS-treated Kupffer cells. An intraperitoneal injection of GalN/LPS in mice resulted in a more serious liver injury that was associated with a significantly higher mortality in adiponectin-/- mice than in WT mice. The plasma levels of TNF-α were significantly increased and those of IL-10 were significantly decreased in adiponectin-/- mice compared to WT mice, and TNF-α gene expression in the liver was significantly higher and those for IL-10 were lower in adiponectin-/- mice than in WT mice. These results indicate that a lack of adiponectin enhances LPS-induced liver injury and that the altered production of cytokines in Kupffer cells caused by a lack of adiponectin affect the severity of LPS-induced liver injury.

TNF-α causes liver cell apoptosis in GalN-sensitized mice[7,23]. Mice that are deficient in TNF-α receptors are protected against GalN/LPS-induced liver damage[24]. In our study, plasma TNF-α levels increased significantly in adiponectin-/- mice after GalN/LPS administration compared with WT mice, suggesting that a lack of adiponectin causes an over response of TNF-α production. Indeed, TNF-α gene expression in the liver increased significantly in adiponectin-/- mice after GalN/LPS administration compared with WT mice. Sennello *et al.* recently reported that adiponectin protects hepatocytes from TNF-α induced cell death[25]. Our results are in agreement with this conclusion. We previously reported that, in peripheral macrophages, adiponectin inhibits LPS-induced TNF-α production possibly through the suppression of TNF-α-induced IκB-α-NF-κB activation via the cAMP-dependent pathway[17,26]. In the present study, adiponectin pre-treated Kupffer cells showed a lower response to LPS than the controls. The TNF-α concentration in the culture media of adiponectin pre-treated Kupffer cells showed a significantly lower elevation than those of untreated cells, and TNF-α gene expression levels after the LPS stimulation of adiponectin pre-treated Kupffer cells were significantly lower than those of untreated cells. The TLR4 receptor system plays an important role in innate immunity[27]. Kupffer cells of mice express TLR4 mRNA and respond to LPS. The LPS-binding protein (LBP) complex associates with the CD14, and via TLR4, activates Kupffer cells to secrete TNF-α[7]. Adiponectin may inhibit this receptor system directly by suppressing IκB-α-NF-κB activation, although further studies will be needed to confirm this conclusion.

IL-10 exhibits a hepatoprotective role in GalN/LPS-induced liver injury by inhibiting the release of TNF-α[10,11,28]. In our study, plasma IL-10 levels were significantly diminished in adiponectin-/- mice after the administration of GalN/LPS compared with WT mice. GalN/LPS treatment of adiponectin-/- mice significantly reduced IL-10 gene expression in the liver compared with WT mice. Kupffer cells are known to release substantial amounts of IL-10 following LPS stimulation[9]. In our study, IL-10 concentrations in the culture medium of adiponectin pre-treated Kupffer cells showed a significantly higher response to LPS stimulation than the control cells. We recently reported that adiponectin increases IL-10 gene expression in human macrophages[18]. IL-10 gene expression in adiponectin pre-treated Kupffer cells was also significantly higher than in untreated cells. These data show that physiological concentrations of adiponectin are sufficient to induce IL-10 production in Kupffer cells in response to LPS stimulation, and that a lack of adiponectin resulted in a lower IL-10 response to LPS stimulation in Kupffer cells. These results suggest that adiponectin inhibits TNF-α production directly as well as indirectly through the induction of IL-10. Further studies will be required to elucidate the precise mechanism of this cross-talk.

IFN-γ is also involved in the toxic effects of LPS on the liver[29], and IFN-γ monoclonal antibodies reduce LPS-induced mortality in mice[30]. The overexpression of IFN-γ activates STAT-1 in the liver and promotes hepatocyte apoptosis following GalN/LPS administration[12]. IL-10 is reported to inhibit IFN-γ[31]. However, Sennelo *et al.* recently reported that, in a model of Concavaline A induced hepatitis, there was no significant difference in serum IFN-γ levels between lipodystrophic aP2-nSREBP-1c transgenic mice, with higher serum adiponectin, and *ob/ob* mice, with lower serum adiponectin levels[25]. Our study found no significant differences in plasma levels of IFN-γ between adiponectin-/- mice and WT mice, or IFN-γ gene expression levels in the liver and splenic lymphocytes between adiponectin-/- mice and WT mice. We found no significant difference in IFN-γ concentration in the culture media of adiponectin pre-treated Kupffer cells and untreated Kupffer cells. However, 4 h after the LPS stimulation, IFN-γ gene expression levels of adiponectin pre-treated Kupffer cells were significantly lower than

those of untreated cells. Further studies will be required to elucidate the effect of adiponectin on IFN-γ production in Kupffer cells.

Obesity is an independent risk factor for the development of chronic liver diseases such as NASH and alcoholic liver disease[1,2]. Serum adiponectin levels are decreased in obesity, and alcohol decreases the expression of adiponectin[13,14]. Adiponectin has been reported to confer protective effects against alcoholic liver injury, and hypoadiponectinemia is involved in the progression of non-alcoholic fatty liver disease (NAFLD) to steatohepatitis[32,33]. Our results suggest that the hypoadiponectinemia in obesity induces an altered response of Kupffer cells to LPS, and is possibly involved in the development of alcoholic liver disease and NASH. Adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) have been cloned by expression cloning[15]. AdipoR1 is expressed abundantly in skeletal muscle, and is also expressed in the liver. AdipoR2 is predominantly expressed in the liver. Thakur V *et al* showed the expressions of adipoR1 and R2 in Kupffer cells and we also found these expressions in Kupffer cells[34]. Recently Wolf AM *et al* reported the up-regulation of adiponectin in liver in ConA mediated acute liver failure[35]. In GalN/LPS induced acute liver injury, the expressions of adiponectin in the liver could not be determined quantitatively by RT-PCR (data not shown). To elucidate the effect of adiponectin produced in the liver in this model, further studies will be needed. Masaki *et al* reported that adiponectin prevents LPS-induced hepatic injury in a KK-Ay obese mice model[36]. KK-Ay obese mice show not only hypoadiponectinemia but also hyperglycemia and insulin resistance. Hyperglycemia and hyperinsulinemia should have an effect on the response of inflammatory cytokines to endotoxemia[37]. We used adiponectin-/- mice to rule out the effects of such other factors. In this model, we were able to clarify the direct effect of adiponectin deficiency on LPS-induced liver injury *in vivo*.

In conclusion, a deficiency of adiponectin could enhance LPS-induced liver injury possibly through modulation of cytokine production by Kupffer cells. The development of adiponectin receptor agonists or molecules that can induce adiponectin secretion might be helpful in controlling LPS-related liver diseases such as NASH and alcoholic liver injury.

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BASIC RESEARCH

Effect of solanine on the membrane potential of mitochondria in HepG2 cells and $[Ca^{2+}]_i$ in the cells

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Abstract

AIM: To observe the effect of solanine on the membrane potential of mitochondria in HepG2 cells and $[Ca^{2+}]_i$ in the cells, and to uncover the mechanism by which solanine induces apoptosis.

METHODS: HepG2 cells were double stained with AO/EB, and morphological changes of the cells were observed using laser confocal scanning microscopy (LCSM). HepG2 cells were stained with TMRE, and change in the membrane potential of mitochondria in the cells were observed using LCSM. HepG2 cells were double stained with Fluo-3/AM, and change of $[Ca^{2+}]_i$ in the cells were observed using LCSM. HepG2 cells were double stained with TMRE and Fluo-3/AM, and both the change in membrane potential of mitochondria and that of $[Ca^{2+}]_i$ in the cells were observed using LCSM.

RESULTS: Cells in treated groups showed typical signs of apoptosis. Staining with TMRE showed that solanine could lower membrane potential; staining with Fluo-3/AM showed that solanine could increase the concentration of Ca^{2+} in tumor cells; and those of double staining with TMRE and Fluo-3/AM showed that solanine could increase the concentration of Ca^{2+} in the cells at the same time as it lowered the membrane potential of mitochondria.

CONCLUSION: Solanine opens up the PT channels in the membrane by lowering the membrane potential, leading to Ca^{2+} being transported down its concentration gradient, which in turn leads to the rise of the concentration of Ca^{2+} in the cell, turning on the mechanism for apoptosis.

Key words: Solanine; Hepatocarcinomatic cell; Ca^{2+} in the cell; Membrane potential; Laser confocal scanning microscopy

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INTRODUCTION

Solanine is found mainly in the tuber of the potato (Solanum tuberosum L.)[1-5] and in the whole plant of the nightshade (Solanum nigrum Linn) of the family Solanaceae. The content of this substance is rather high in the green peel and the sprouts of potato and is the main toxic substance[6]. The whole plant of the nightshade contains many steroid alkaloids, including solamargine, solasonine, and solanine, as well as saponin and other substances. It can be used for anti-tumor purposes, with a strong inhibitory effect on tumors in animals and a clearly toxic effect on tumor cells[7]. Its ethanol extract is capable of inhibiting the growth of breast cancer and induce apoptosis in tumor cells[8]. The extract from the nightshade also has a strong anti-inflammatory effect because it can facilitate the formation of antibodies[9]. The anti-tumor effect of solamargine has been reported[10], but there is as yet no report about any anti-tumor effect of solanine. From our past experience in both in vivo and in vitro experiments, we have found that solanine is cytotoxic to cells, especially for the hepatocarcinomatic cell HepG2. Through morphological observation, as well as DNA ladder and flow cytometry, we discovered that solanine exerted its anti-tumor effect by inducing apoptosis in HepG2.

The concept "apoptosis" was officially proposed by Kerr in 1972[11]. In the three decades since it was proposed, it has always been the focus of biological researches. Especially since 1990s, the study of apoptosis has suddenly been propelled to the frontier of life science, leading to an upsurge of research activities involving almost all the fields of biomedical studies. As a result, a great deal of literature accumulated in a few years about the

morphological characteristics of apoptosis, biochemical changes in apoptotic cells, genes regulating apoptosis, and various factors that can lead to apoptosis, as well as the relationship between apoptosis and health conditions. Membrane potential of mitochondria[12,13] and $[Ca^{2+}]_i$ in the cells[14,15], two factors closely associated with apoptosis, are themselves supplementary to and closely related with each other.

Rapid and continuous increase in the concentration of calcium ion is the earliest detectable biochemical change in cells undergoing apoptosis. The fact that the use of calcium-free culture medium and other methods capable of inhibiting the rise in the concentration of Ca^{2+} in cells can help to inhibit apoptosis[16] also shows that the rise in the concentration of Ca^{2+} in cells is an important condition for apoptosis to take place. A typical sign of apoptosis is the appearance of apoptotic bodies, but it is only when the concentration of Ca^{2+} has been raised that endogenous nuclease is activated, cutting the DNA chain between nucleosomes into discontinuous fragments (or apoptotic bodies), the basic unit of which is 180-200 bp long.

The mitochondrion is the storehouse for calcium in the cell, and change in its membrane potential can lead to Ca^{2+} and other ions being released from the mitochondrion or flowing into it, and the release and influx of Ca^{2+} and other ions are also the direct causes for changes in the membrane potential of mitochondrion. The essence of membrane potential change is the opening of the PT channels in the membrane. The opening of these channels precedes the release of Ca^{2+}, which is followed by changes in the membrane potential.

There are many approaches to studying apoptosis. In the present study, we tried to explicate the mechanism by which solanine induced apoptosis of tumor cells by focusing on the mitochondria and by observing the effect of solanine on the membrane potential of $HepG_2$ and $[Ca^{2+}]_i$ in the cell(Figure 1).

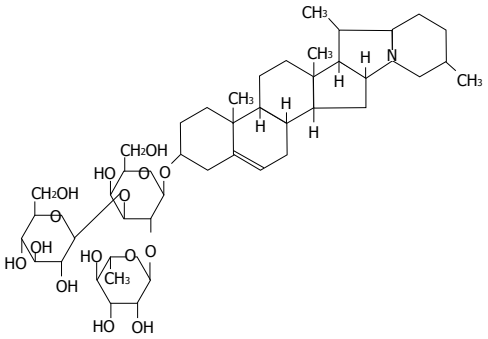

Figure 1 Molecular structure of solanine.

MATERIALS AND METHODS

Materials

The cell line: $HepG_2$ human hepatocarcinomatic cell lines were purchased from the Institute for Cancer Research, Heilongjiang Cancer Hospital.

Test drugs: Solanine with a purity of 99% was provided by Heilongjiang Institute For Drug Control.

Chemical reagents: RPMI 1640 culture medium was supplied from HyClone Laboratories; fetal bovine serum from Hangzhou Sijiqing Biological Engineering Materials Co.,Ltd; pancreatin from Gibio; ethidium bromide (EB) from AMRESCO; acridine orange (AO) from AMRESCO; Fluo-3/AM from Biotium, US; TMRE(molecular probe) from Biotium, US; HEPES from AMRESCO.

Preparation of the reagents: Fluo-3/AM 0.1 mg/stick was diluted to suitable concentration with DMSO, and then separately loaded and stored in the dark. TMRE 25 mg/stick was made into 1 mg/mL mother solution using anhydrous alcohol, separately loaded and stored in the dark. These were diluted with buffer solution for laboratory use and added to achieve the needed final concentrations before they were used in experiments.

Apparatuses: CO_2 incubator (CO-150, NBS, US); inverted microscope (CKX-41-32, Olympus, Japan); superclean bench (SW-CJ-2F, Suzhou Purification Equipments of the Sujing Group, Suzhou); laser confocal scanning microscope (SP2, Leica, Germany) were used.

Methods

Cell culture and treatment: $HepG_2$ cell culture was incubated in RPMI 1640 medium containing 10% fetal bovine serum at 50 mL/L CO_2 and 37℃, and transfer of culture was performed once every 3-4 d. When the cells grew steadily in the phase of logarithmic growth, 0.25% pancreatin was used to digest the cells. Digested cells were re-suspended using RPMI 1640 medium containing 10% fetal bovine serum and counted, and the concentration of cells was adjusted to 1×10^4 /mL. Cell suspension was added to the round troughs of 35 mm Petri dishes at 200 μL/dish, and the dishes were divided into 2, 0.4, 0.08, 0.016, and 0.0032 μg/mL treatment groups, the control, and the positive control (to be treated with camptothecine). After being incubated for 24 h at 50 mL/L CO_2 and 37℃, the treatment groups were treated with solanine of different concentrations so that their final concentrations were 2, 0.4, 0.08, 0.016, and 0.0032 μg/mL, respectively; an equal volume of RPMI 1640 was added to the control, while the positive control was treated with camptothecine, with a final concentration of 0.08 μg/mL. The dishes were then incubated for another 48 h at 50 mL/L CO_2 and 37℃ in CO_2 incubator.

Observation of Solanine-induced morphological changes of $HepG_2$ nuclei using LCSM: After 48 h, the cells were taken out of the incubator. The culture solution in the Petri dishes was sucked out, and the cells were rinsed 3 times with PBS at 5 min/time, double stained with 200 μL of acridine orange (AO)/ethidium brumide (EB) so that the final concentration was 5 μg/mL, incubated for 5-10 min at 37℃, and rinsed 3 times with PBS, 5 min/time. Then 200 μL of PBS was added, and LCSM was used to observe the morphology of the cells. Dual-channel activation was used, with an excitation wavelength of 488 nm and emission wavelength of 500-520 nm for PMT1(AO) and an excitation wavelength of 543 nm and emission wavelength of 600-700 nm for PMT2(EB)[17]. Other devices included Objective APO CS40 × /1.25 oil, zoom > 1, pinhole 1.5 Airy, mode XYZ, format 512 × 512.

Observation of Solanine-induced change in $[Ca^{2+}]_i$

in HepG2 cells using LCSM: After 48 h, the cells were taken out of the incubator. The culture solution in the Petri dishes was sucked out, and the cells were rinsed 3 times with HEPES, and 4 μg/mL Fluo-3/AM (Molecular Probes) was added, 200 μL/dish. After the cells were incubated at 37℃ for 50 min and rinsed with HEPES for 3 times, 200 μL of HEPES culture medium was used to cover all the cells in the troughs, and then LCSM was used to observe the fluorescence intensity (FI) of the cells, with an excitation wavelength of 488 nm and emission wavelength of 555 ± 15 nm. Other devices included objective APO CS40 ×/1.25 oil, zoom > 1, pinhole 1.5 Airy, mode XYZ, format 512 × 512.

Observation of Solanine-induced change in the membrane potential of the mitochondria in the cells using LCSM: After 48 h, the cells were taken out of the incubator. The culture solution in the Petri dishes was sucked out, the cells were rinsed 2-3 times with PBS, and 200 μL of tetramethyl rhodamine ethyl ester (TMRE, Molecular Probes) was gently added to the dishes, so that the final concentration was 2 μmol/L (1.03 μg/mL). The cells were then incubated for 30 min and, after the staining solution was sucked out, gently rinsed 3 times with PBS. 200 μL PBS was used to cover all the cells in the troughs, and then LCSM was used to observe the fluorescence intensity of the cells, with an excitation wavelength of 543 nm and emission wavelength of 570 ± 20 nm. Other devices included objective APO CS40 ×/1.25 oil, zoom > 1, pinhole 1.5 Airy, mode XYZ, format 512 × 512.

Simultaneous observation of changes in the morphology of cells, membrane potential, and the concentration of calcium in the cells: Experiment was performed on 2 groups of HepG2 cells simultaneously. Cell incubation, treatment, and operation procedures were all the same as above, with the same amount of time and conditions for cell incubation. One group was marked with AO/EB and the effect of solanine on its HepG2 cells was observed using LCSM; while HepG2 cells in the other group were marked with TMRE and Fluo-3/AM, and changes in membrane potential of the mitochondria and $[Ca^{2+}]_i$ in the cells were observed using LCSM. Methods for AO/EB double staining and measurement were as before, while the procedures for double staining with Fluo-3/AM and TMRE and measurement are as follows: (1) Staining after 48 h, the cells were taken out of the incubator. The culture solution in the Petri dishes was sucked out, the cells were rinsed 3 times with RPMI1640, and 150 μL of tetramethyl rhodamine ethyl ester (TMRE, Molecular Probes) working fluid with a concentration of 2 μmol/L (1.03 μg/mL) was pipetted onto the dishes. The cells were then incubated for 30 min at 37℃, and after the staining solution was sucked out, rinsed 3 times with RPMI1640. Finally 150 μL of Fluo-3/AM (Molecular Probes) with a concentration of 4 μg/mL was added. The cells were then incubated for 50 min at 37℃ and rinsed 3 times with RPMI1640, and 200 μL of RPMI1640 was added to cover all the cells in the troughs, whereupon the Petri dishes were mounted for measurement. (2) Observation LCSM was used to examine the fluorescence intensity (FI) of the cells. Dual-wavelength excitation was used, with excitation wavelengths of 488 nm and 543 nm, respectively. The emission wavelength used for PMT1 was 555 ± 15 nm, while that for PMT2 was 570 ± 20 nm. Other devices included Objective APO CS40 × /1.25 oil, zoom > 1, pinhole 1.5 Airy, mode XYZ, format 512 × 512. Sequential scanning was used to eliminate interference due to spectral overlap.

Statistical analysis

t test was performed on data obtained from the experiments, with the results expressed in the format mean ± SD. $P < 0.05$ was taken as significant.

RESULTS

Effect of Solanine on the morphology of HepG2 cells

After the DNA-specific fluorochromes AO and EB were used to stain HepG2 cells, LCSM was used to observe the morphological changes of HepG2 cells treated with solanine. From Figure 2A, it can be seen that the control was morphologically normal. The nuclei of different cells were of similar sizes, regularly shaped, and evenly stained. The more deeply colored parts of the nuclei represented heterochromatin which did not take part in transcription under normal circumstances. With solanine treatment, however, the cells showed marked morphological changes. In the groups treated with 0.0032 μg/mL and 0.016 μg/mL of solanine (Figures 2B and 2C, respectively), the cells were wrinkled, and the chromatin was concentrated and marginalized. In the group treated with 0.08 μg/mL of solanine (Figure 2D), cells with fragments and apoptotic bodies appeared, a typical sign for apoptosis. In the groups treated with 0.4 μg/mL and 2 μg/mL of solanine (Figures 2E and 2F, respectively), the number of cells containing apoptotic bodies was significantly increased. At the same time, it could be seen that high dosage of solanine was lethal to tumor cells, because with increasing dosage, the number of viable cells decreased significantly. Apoptotic bodies also appeared clearly in the positive control treated with camptothecin with the final concentration of 0.08 μg/mL.

Change induced by Solanine in $[Ca^{2+}]_i$ in HepG2 cells in the process of apoptosis

As shown in Figure 3 the effect of different dosages of solanine (0.0032, 0.016, 0.08, 0.4, and 2 μg/mL) on $[Ca^{2+}]_i$ in HepG2 cells was observed using LCSM with staining with the fluorescent probe Fluo-3/AM. On the left side of each group is the photograph (green) showing $[Ca^{2+}]_i$ taken under the confocal microscope, with the depth of the color representing the fluorescence intensity (FI). On the right is the 3D configuration reconstructed using LCSM for the group, with different colors reflecting different FI values, which indirectly reflect the concentrations of $[Ca^{2+}]_i$ for the test group in question. It is shown from these photographs that the $[Ca^{2+}]_i$ for the control in Figure 3A was very low, while those for the groups treated with solanine (Figures 3B-F) were all increased, in a concentration dependent manner. The positive control camptothecin did not increase $[Ca^{2+}]_i$. Statistics on FI obtained through LCSM for the various groups in Table 1 showed the same results.

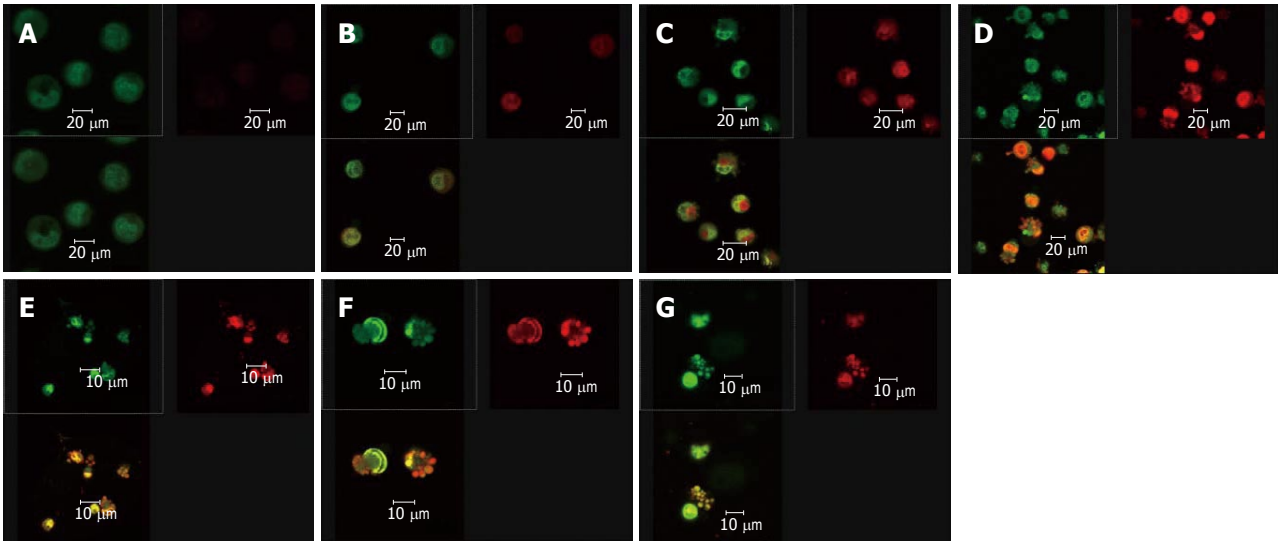

Figure 2 Effect of solanine on the morphology of HepG2. **A**: Control group, PMT1 nuclear chromatin was stained green and showed normal structure, while PMT2 showed no or only weak red fluorescence; **B**: Group treated with Solanine with final concentration of 0.0032 μg/mL: The cells show slightly pyknotic and crumb-shaped structures. Permeability of cell membrane has increased, so that AO and EB can enter the cells, resulting in the cells showing both green and red fluorescence; **C**: Group treated with Solanine with final concentration of 0.016 μg/mL: The cells show slightly pyknotic and crumb-shaped structure. Fragments or apoptotic bodies appear in the nuclei of some of the cells; **D**: Group treated with solanine with final concentration of 0.08 μg/mL: Cell structure is further damaged. The cells are not only stained with AO and EB, but morphologically the nuclei have become deeply stained fragments or apoptotic bodies; **E**: Group treated with solanine with final concentration of 0.4 μg/mL: Cell structure is damaged even further. Apoptotic bodies have definitely appeared; **F**: Group treated with solanine with final concentration of 2 μg/mL: Definite apoptotic bodies can be seen, while the number of cells in sight has decreased; **G**: Group treated with camptothecin with final concentration of 2 μg/mL: The appearance of apoptotic bodies is obvious in HepG2 cells.

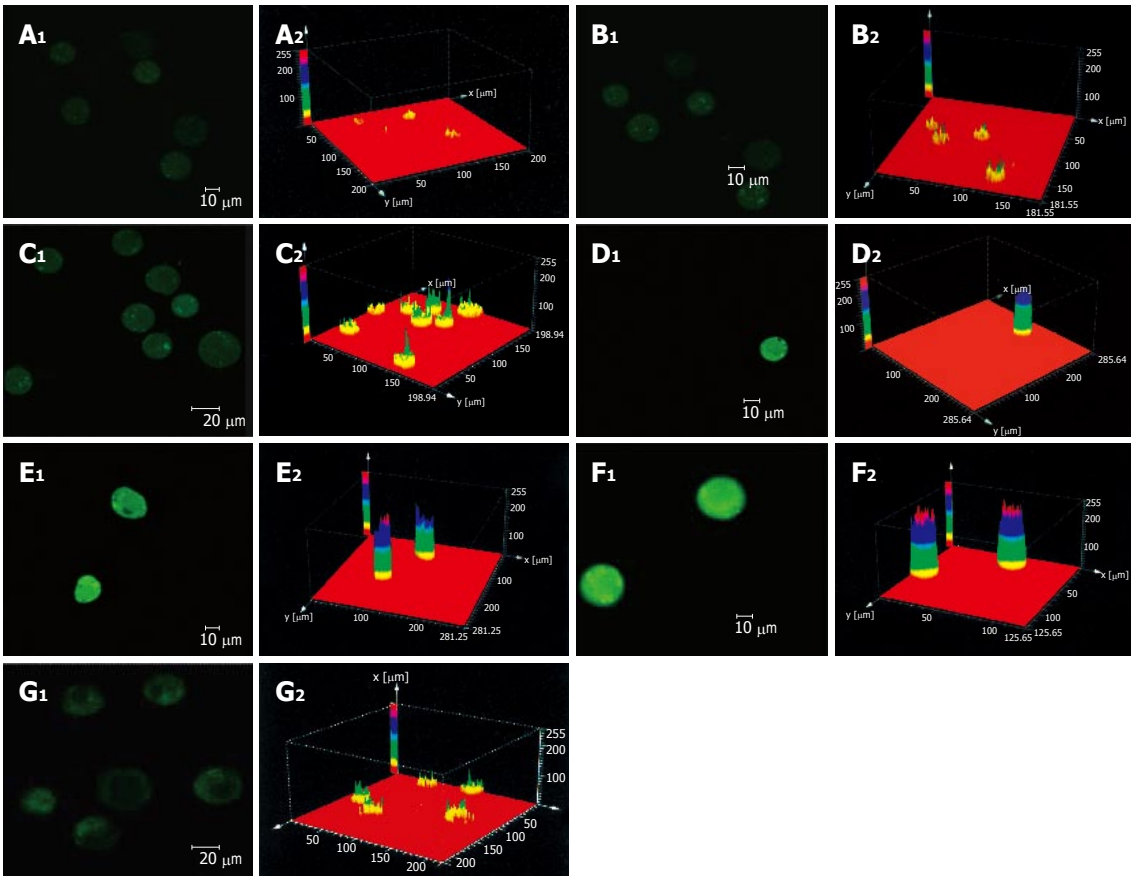

Figure 3 Effect of Solanine on $[Ca^{2+}]_i$ in HepG2 cells. **A**: Control; **B**: 0.0032 μg/mL solanine; **C**: 0.016 μg/mL solanine; **D**: 0.08 μg/mL solanine; **E**: 0.4 μg/mL solanine; **F**: 2 μg/mL solanine; **G**: 0.08 μg/mL camptothecin.

Change induced by Solanine in the membrane potential of mitochondria in HepG2 cells in the process of apoptosis

The effect of different dosages of solanine (0.0032, 0.016, 0.08, 0.4, and 2 μg/mL) on the membrane potential of mitochondria in HepG2 cells was observed using LCSM with staining by the fluorescent probe TMRE (Figure 4). The brightness of the coloration reflected different values of FI, thus indirectly indicating the membrane potential of mitochondria in the cells for these different groups. From those photographs, it could be seen that the membrane

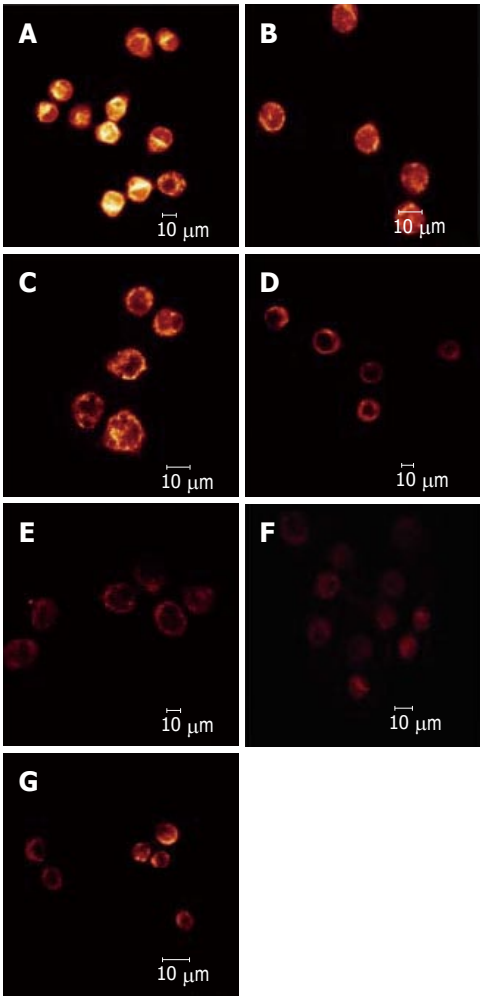

Figure 4 Effect of Solanine on membrane potential of mitochondria in $HepG_2$ cells. **A**: Control; **B**: 0.0032 μg/mL solanine; **C**: 0.016 μg/mL solanine; **D**: 0.08 μg/mL solanine; **E**: 0.4 μg/mL solanine; **F**: 2 μg/mL solanine; **G**: 0.08 μg/mL camptothecin.

Table 1 Effect of Solanine on $[Ca^{2+}]_i$ in $HepG_2$ cells (mean ± SD)

Group	Final concentration (μg/mL)	Number of cells (*n*)	FI
Control	-	18	23.98 ± 10.06
Camptothecin group	0.08	16	26.92 ± 8.20
Solanine groups	0.003	25	40.31 ± 10.15[b]
	0.016	37	59.31 ± 17.56[b]
	0.08	17	91.01 ± 23.19[b]
	0.4	19	158.75 ± 14.52[b]
	2	20	209.85 ± 10.86[b]

Comparison with blank: [b]$P < 0.01$.

Table 2 Effect of solanine on the membrane potential of mitochondria in $HepG_2$ cells (mean ± SD)

Group	Final concentration (μg/mL)	Cell number (*n*)	FI
Control	-	53	75.70 ± 31.24
Camptothecin group	0.08	16	47.17 ± 15.98
Solanine groups	0.003	66	70.04 ± 17.83
	0.016	90	63.21 ± 16.36[b]
	0.08	144	52.26 ± 26.46[b]
	0.4	37	32.50 ± 19.99[b]
	2	33	28.49 ± 9.71[b]

Comparison with blank: [b]$P < 0.01$.

potential of mitochondria in the cells was relatively high for the control in Figure 4A, while those for the groups treated with solanine (Figures 4B-F) were all increased, in a dosage dependent manner. Statistics on FI obtained through LCSM for the various groups in Table 2 showed that the membrane potential of mitochondria in the cells was significantly lowered ($P < 0.01$) in groups treated with 0.016, 0.08, 0.4, and 2 μg/mL of solanine. The membrane potential in the 0.0032 μg/mL group was also lowered, but the difference from the control was not significant. The membrane potential of mitochondria in $HepG_2$ cells of the group treated with 0.08 μg/mL of camptothecin was also significantly lowered.

Simultaneous observation of changes induced by solanine in cell morphology, $[Ca^{2+}]_i$ in cells, and mitochondrial membrane potential in $HepG_2$ cells in the process of apoptosis

Double staining with AO/BE and with Fluo-3/AM and TMRE were simultaneously used in this experiment (Figure 5). Changes in cell morphology, $[Ca^{2+}]_i$ in cells, and membrane potential of mitochondria in the same cell were simultaneously observed using LCSM for groups treated with different concentrations of solanine, so as to examine simultaneous changes in cell morphology, $[Ca^{2+}]_i$ in cells, and membrane potential of mitochondria in cells at the same time points in the apoptosis process induced by solanine of different concentrations and, moreover, to explore the physiological mechanisms of $[Ca^{2+}]_i$ and membrane potential of mitochondria in the cells and the relationship between them. As shown in Figure 5A for the control group, the cells were normal morphologically, and the $[Ca^{2+}]_i$ in the cells was relatively low, while the corresponding mitochondrial membrane potential was relatively high. Results for the groups treated with solanine (with final concentration of 0.0032, 0.016, 0.08, 0.4, and 2 μg/mL, respectively) are shown in Figures 5B-F. It could be seen that with the dosage increased, the shape of the cells gradually became more and more irregular, and typical apoptotic cells such as irregularly shaped cells and apoptotic bodies appeared, with apoptotic bodies, characteristic of apoptosis appearing first in the group treated with 0.08 μg/mL of solanine (Figure 5D). As the dosage increased, the percentage of apoptotic bodies gradually increased, and $[Ca^{2+}]_i$ in the cells also gradually increased, while the membrane potential of mitochondria in the cells gradually decreased for the corresponding groups. The group treated with camptothecin showed no change in $[Ca^{2+}]_i$ but it did show a significantly decreased membrane potential of mitochondria, results consistent with those obtained with single staining. It also showed that solanine induced apoptosis in $HepG_2$ cells by a

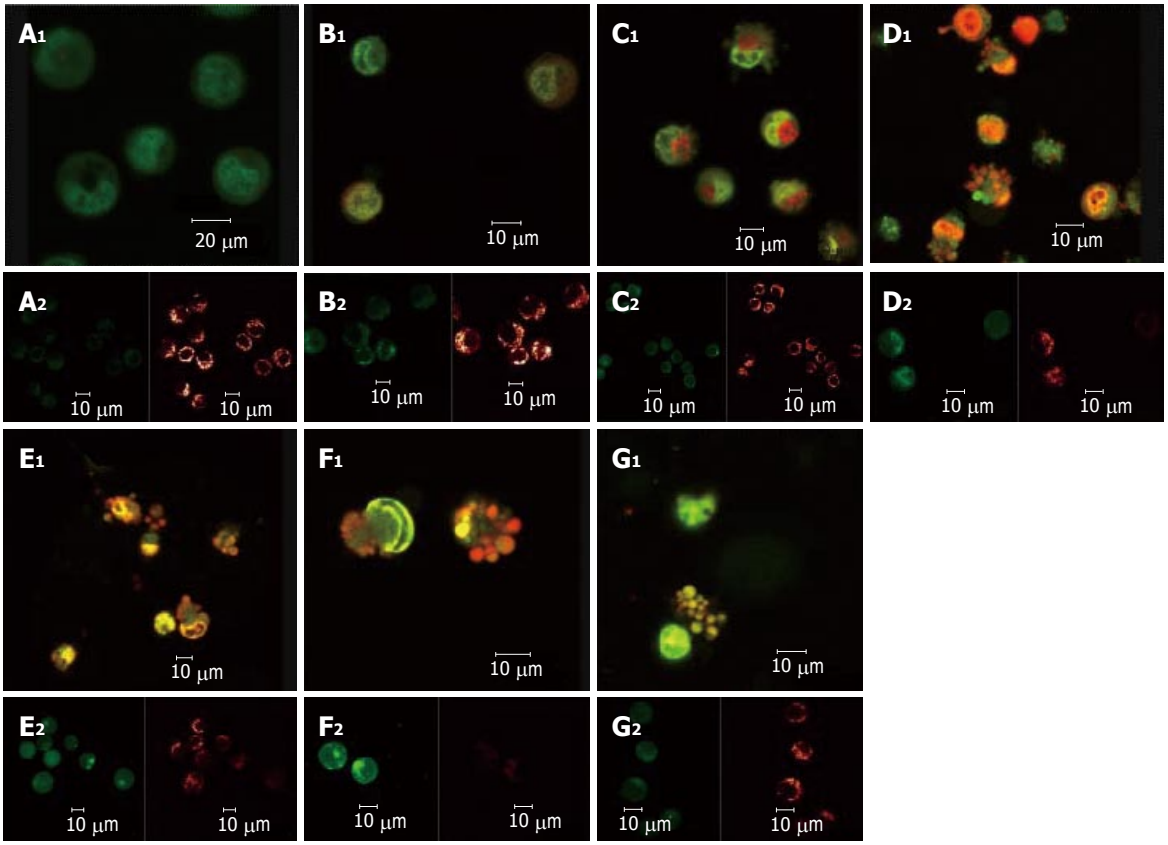

Figure 5 Changes induced by solanine in cell morphology, $[Ca^{2+}]_i$ in cells, and membrane potential of mitochondria in $HepG_2$ cells in the process of apoptosis. **A**: Control; **B**: 0.0032 μg/mL solanine; **C**: 0.016 μg/mL solanine; **D**: 0.08 μg/mL solanine; **E**: 0.4 μg/mL solanine; **F**: 2 μg/mL solanine; **G**: 0.08 μg/mL camptothecin.

Table 3 Effect of solanine on $[Ca^{2+}]_i$ in $HepG_2$ cells and mitochondrial membrane potential (mean ± SD)

Group	Final concentration (μg/mL)	Number of cells	Fluo-3/AM-FI	TMRE-FI
Control	-	111	24.96 ± 4.03	51.85 ± 19.18
Camptothecin group	0.08	21	28.80 ± 7.01	25.50 ± 5.76[b]
Solanine groups	0.003	49	33.85 ± 9.30[a]	39.52 ± 15.41[b]
	0.016	33	34.37 ± 14.18[a]	35.01 ± 11.12[c]
	0.08	36	42.73 ± 14.75[b]	28.13 ± 4.31[d]
	0.4	39	52.80 ± 11.07[b]	23.21 ± 12.22[d]
	2	37	58.62 ± 16.04[b]	13.09 ± 4.71[d]

Comparison with blank: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.005$; [d]$P < 0.001$.

different mechanism from camptothecin. Detailed changes of different groups are shown in Table 3.

Solanine-induced changes in $[Ca^{2+}]_i$ and membrane potential of mitochondria in $HepG_2$ cells in the process of apoptosis and their distribution in the cells

When the images for the double staining with Fluo-3/AM and TMRE were recombined using the image analyzing capacity of the laser confocal scanning microscope, it could be seen that Ca^{2+} was widely distributed in the whole cell, but with different concentrations in different regions of the cell. In contrast, membrane potential of mitochondria was distributed only in certain parts of the cell (Figure 6). Superimposing the two images, it can be seen that regions where Ca^{2+} was more concentrated were basically also those where membrane potential was distributed.

DISCUSSION

The mitochondrion is an important organelle of the eukaryotic cell and the main site for the production of ATPs in animal cells[18,19]. NADH is produced from the tricarboxylic cyclophorase series in mitochondrial matrix through the dehydroxidization of the substrate, and then oxidized through the electron transport chain on the inner membrane of the mitochondrion. This leads to the transmembrane transfer of protons, resulting in a transmembrane proton gradient (or transmembrane potential). The ATP synthetase on the inner membrane of the mitochondrion synthesizes ATPs using the energy derived from the proton gradient. Through the ADP/ATP carriers on the inner membrane of mitochondria, ATPs synthesized this way are exchanged for ADPs from the cytoplasm, thus entering the cytoplasm to take part in various processes requiring energy.

In recent years, reports have been made that the dissipation of the membrane potential of mitochondria precedes the activation of nuclease[20], and that it precedes the exposure of phosphatidylserine on the surface of the cell. When the transmembrane potential of mitochondria is dissipated, the cell would enter the irreversible apoptotic process. The uncoupled respiratory chain of the mitochondrion would produce a large amount of active oxygen, oxidizing the cardiolipin on the inner membrane of the mitochondrion. Experiments have shown that the decoupling reagent mClCCP can lead to the apoptosis of

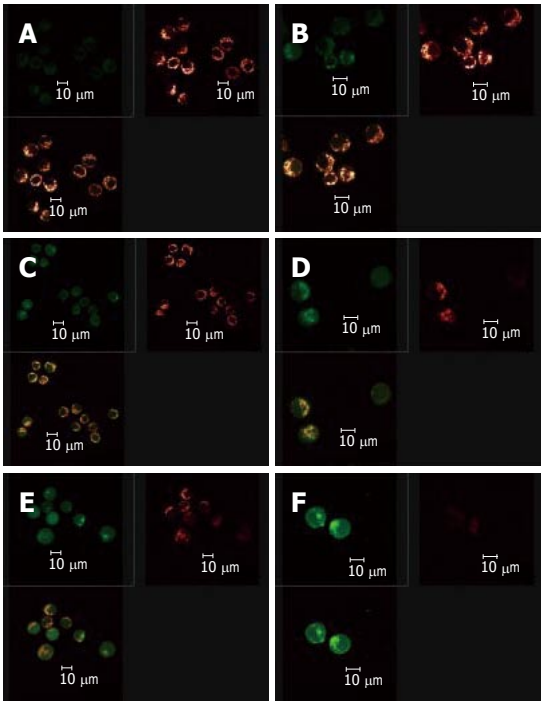

Figure 6 Distribution of solanine-induced changes in $[Ca^{2+}]_i$ and membrane potential of mitochondria in the $HepG_2$ cells in the process of apoptosis. **A**: Blank; **B**: 0.0032 μg/mL solanine; **C**: 0.016 μg/mL solanine; **D**: 0.08 μg/mL solanine; **E**: 0.4 μg/mL solanine; **F**: 2 μg/mL solanine.

lymphocytes[21,22], whereas if the transmembrane potential of mitochondria can be stabilized, apoptosis would be prevented. Therefore, the dissipation of transmembrane potential of mitochondria is closely related to apoptosis.

The dissipation of transmembrane potential of mitochondria in the process of apoptosis is mainly caused by changes in the permeability of the inner membrane of mitochondria, which is due to the formation of dynamic pores for permeability transition (PT) (each composed of several proteins) at points of contact between the outer and inner membranes of the mitochondrion[23]. PT channels are made up of proteins from various parts of the mitochondrion and those from the cytoplasm[24,25]. They include a protein in the cytochylema (hexokinase)[25], proteins on the outer membrane of the mitochondrion (peripheral benzodiazepine receptors and voltage-dependent anion channels), a protein in the interstice between the inner and outer membranes of the mitochondrion (creatine kinase)[26], a protein on the inner membrane of the mitochondrion (the ADP-ATP carrier)[27], and a protein in the mitochondrial matrix (cyclophilin D)[28]. Any substance, such as protoporphyria IX, the ligand for benzodiazepine receptors, that can specifically induce the formation of PT channels in mitochondria could cause apoptosis[29-32]. PT channels are a kind of channels with high electric conductivity. The lowering or even disappearance of membrane potential would suggest that PT channels are open.

Results from earlier experiments show that solanine can induce the apoptosis of $HepG_2$ cells. In the present study, double staining with AO/EB and solanine (0.0032, 0.016, 0.08, 0.4, and 2 μg/mL) carried by TMRE were used to treat $HepG_2$ cells, and LCSM was used to observe changes in the membrane potential of mitochondria as solanine induced the appearance of apoptotic bodies[33]. As solanine induced the appearance of apoptotic bodies and caused the apoptosis of cells, the membrane potentials of mitochondria in cells in groups treated with different dosages of solanine (0.0032, 0.016, 0.08, 0.4, and 2 μg/mL) were all decreased in comparison with the control (Figures 2 and 4, and Table 2). The amount of decrease was dosage-dependent, and the difference from that of the control was significant for the groups treated with 0.016, 0.08, 0.4, and 2 μg/mL of solanine, respectively, suggesting that solanine can lead to the lowering of the membrane potential of mitochondria in $HepG_2$ cells. However, the lowering of the membrane potential of mitochondria is a manifestation of the opening of the PT pores of the mitochondria[34], so solanine can lead to the opening of the PT pores of the mitochondria.

The opening of PT pores can lead to the occurrence of two events[35]: (1) the intra-membrane and extra-membrane ion concentrations tend toward equilibrium, the transmembrane H^+ gradient disappears, and the respiratory chain is uncoupled; (2) the flow of intra- and extra-membrane ions toward equilibrium leads to a hypertonic mitochondrial matrix, resulting in change in the volume of the mitochondrion. Since the surface area of the folded inner membrane of mitochondrion is larger than that of the outer membrane, this would lead to the rupture of the outer membrane.

These two events lead respectively to two consequences. Since a large number of Ca^{2+} have accumulated in mitochondria, the concentration of Ca^{2+} in mitochondria is much higher than that in the cytoplasm. The occurrence of the first event would lead directly to the flow of Ca^{2+} from the mitochondria to the cytoplasm, resulting in rapid increase in the concentration of Ca^{2+} in the cytoplasm. In the present study, the Ca^{2+}-specific molecular probe Fluo-3/AM was used to carry solanine at different concentrations to treat $HepG_2$ cells, and LCSM was used to observe changes in $[Ca^{2+}]_i$ in the cells after the treatment. In groups treated with different dosages of solanine (0.0032, 0.016, 0.08, 0.4, and 2 μg/mL), the concentrations of Ca^{2+} in $HepG_2$ cells were all increased in a dosage-dependent way (Figure 3 and Table 1). This verifies the inference we have drawn, namely, by opening up PT channels, solanine leads to the release of Ca^{2+} from the mitochondria, resulting in the increase of $[Ca^{2+}]_i$ in the cell. In order to obtain more powerful evidence for our inference, LCSM with double staining with Fluo-3/AM and TMRE was used to observe simultaneously the change in membrane potential and the change in $[Ca^{2+}]_i$ in the cell after the treatment. The results showed that as the dosage increased, the rate of apoptosis gradually increased, the membrane potential gradually decreased, and $[Ca^{2+}]_i$ in the cell gradually increased (Figure 5 and Table 3). These results were consistent with that obtained with single staining.

At the same time, the data collected using LCSM was analyzed and observation was made on the distribution of $[Ca^{2+}]_i$ and the membrane potential of mitochondria in the cells in the apoptotic process of $HepG_2$ induced by solanine. The image overlay function of LCSM was used

to superimpose and compare the images of $[Ca^{2+}]_i$ and the membrane potential of mitochondria in the cells. Results showed that membrane potential was mainly distributed on the inner membrane of the cells, with no distribution in the center of the cells; but the inner membrane was precisely where mitochondria were concentrated (Figure 6). $[Ca^{2+}]_i$ was distributed throughout the whole cell, but the distribution was uneven. This can be observed in the photograph with green fluorescence in Figure 6, where the brightness of the fluorescence depends on the region of the cell. When the image for $[Ca^{2+}]_i$ distribution (green) was superimposed on that for membrane potential (red), it can be seen that regions where Ca^{2+} was concentrated overlaps with where membrane potential was concentrated (as shown by the yellow regions in the third photograph in each group in Figure 6), suggesting that the distribution of Ca^{2+} in the mitochondria was high. In addition, the concentration of Ca^{2+} in both the mitochondria and the cytoplasm continued to rise in the different groups, suggesting that as $[Ca^{2+}]_i$ increased in the cytoplasm, Ca^{2+} flew out not only from the mitochondria, but also from some other source(s) as well, e.g., an inflow of Ca^{2+} from outside the cell.

In conclusion, solanine can facilitate the opening of the PT channels in mitochondria, leading to release of Ca^{2+} from these organelles. This results in an increase in the concentration of Ca^{2+} in the cell, thus triggering the mechanism for apoptosis and the occurrence of apoptosis. However, to uncover how the PT channels are opened and which enzymes and genes are involved in this process, further research is needed.

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BASIC RESEARCH

Dynamic functional and ultrastructural changes of gastric parietal cells induced by water immersion-restraint stress in rats

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Abstract

AIM: To investigate the dynamic functional and ultrastructural changes of gastric parietal cells induced by water immersion-restraint stress (WRS) in rats.

METHODS: WRS model of Sprague-Dawley (SD) rats was established. Fifty-six male SD rats were randomly divided into control group, stress group and post-stress group. The stress group was divided into 1, 2 and 4 h stress subgroups. The post-stress group was divided into 24, 48 and 72 h subgroups. The pH value of gastric juice, ulcer index (UI) of gastric mucosa and H^+, K^+-ATPase activity of gastric parietal cells were measured. Ultrastructural change of parietal cells was observed under transmission electron microscope (TEM).

RESULTS: The pH value of gastric juice decreased time-dependently in stress group and increased in post-stress group. The H^+, K^+-ATPase activity of gastric parietal cells and the UI of gastric mucosa increased time-dependently in stress group and decreased in post-stress group. Compared to control group, the pH value decreased remarkably (P = 0.0001), the UI and H^+, K^+-ATPase activity increased significantly (P = 0.0001, P = 0.0174) in 4 h stress subgroup. UI was positively related with stress time (r = 0.9876, P < 0.01) but negatively with pH value (r = -0.8724, P < 0.05). The parietal cells became active in stress group, especially in 4 h stress subgroup, in which plenty of intracellular canalicular and mitochondria were observed under TEM. In post-stress group, the parietal cells recovered to resting state.

CONCOUSION: The acid secretion of parietal cells is consistent with their ultrastructural changes during the development and healing of stress ulcer induced by WRS and the degree of gastric mucosal lesions, suggesting gastric acid play an important role in the development of stress ulcer and is closely related with the recovery of gastric mucosal lesions induced by WRS.

Key words: Stress ulcer; Gastric parietal cells; Ultrastructure; H^+, K^+-ATPase activity; Sprague-Dawley rat

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INTRODUCTION

Stress ulcer is a common complication of severe brain trauma and psychosis in clinical practice. Critically ill patients are at increased risk of developing stress-related gastric mucosal lesions and gastrointestinal bleeding[1,2]. But the pathogenesis of stress-related gastric mucosal lesions is not fully elucidated. The development and healing of stress ulceration are a complex process affected by many factors. It had been shown that gastric acid plays an important role in the development of stress-induced ulcer and is the most common endogenous destructive factor[3-5].

It is reported that water immersion-restraint stress (WRS) may lead to changes of gastric acid secretion. Some studies indicate that WRS increases acid secretion[6,7], while other studies showed that gastric acid secretion decrease during WRS[8,9]. Gastric acid secretion is intimately related with the ultrastructure of parietal cells, but the dynamic ultrastructure change of parietal cells during WRS has not been reported yet.

Moreover, changes of parietal cells ultrastructure and gastric acid secretion during the healing of stress-induced ulceration are poorly understood so far. The purpose of the present study was to investigate the dynamic ultrastructural and functional of gastric parietal

cells induced by WRS in order to demonstrate the role of gastric acid in the development of stress ulcer and in the healing of gastric mucosal lesions.

MATERIALS AND METHODS

Animals and water immersion-restraint stress model

Fifty-six male SD rats, weighing 180-220 g were purchased from Xipuer-Bikai Experimental Animal Co. LTD, Shanghai, China. The animals were fasted for 24 h with free access to water and deprived of water 1 h before the experiment. Animals were randomly divided into control group (n = 8), stress group (n = 24) and post-stress group (n = 24), and exposed to various periods of stress and immersed in 19 ± 1℃ water as previously described[10]. Rat in control group were sacrified after lightly anesthetized with ether. Rats in stress group were killed after 1, 2 and 4 h of WRS respectively. Rats in post-stress group were killed after 24, 48 and 72 h of WRS respectively[11].

Measurement of gastric juice pH

All rats were anesthetized with pentobarbital sodium (30 mg/kg *ip*). A midline laparotomy was performed and the stomach was exposed. The pylorus and cardia were ligated, and the pH electrode of pH/mV meter (59 002-00, Cole-Parmer Instrument Company, USA) was inserted into stomach lumen to measure the pH value of gastric juice[12].

Evaluation of gastric mucosal ulcer index (UI)

The ulcer index (UI) of gastric mucosal lesions was evaluated by the score system reported by Nie SN *et al*[13]. Briefly, after measurement of the pH value of gastric juice, the stomach was opened along the greater curvature and rinsed with 0.1 mol/L ice-cold phosphate-buffered saline (PBS). The stomach was then examined with a 10 × magnifier to observe erosions and make scores as 1-5: 1: small round hemorrhagic erosion, 2: hemorrhagic erosion < 1 mm, Grade 3: hemorrhagic erosion = 1-2 mm, 4: hemorrhagic erosion = 2-3 mm, 5: hemorrhagic erosion > 4 mm. The score was multiplied by 2 when the width of erosion was larger than 1 mm.

H+, K+-ATPase assay

The oxyntic mucosa was scraped with glass slides, immediately frozen in liquid nitrogen and stored at -80 ℃ for H^+, K^+-ATPase assay, at which time they were thawed at 4℃. Microsomal gastric H^+, K^+-ATPase was prepared after homogenization of gastric mucosa and sucrose-Ficoll gradient centrifugation[14,15]. H^+, K^+-ATPase was assessed with a kit (Nanjing Jiancheng Biotechnology Company), and protein was quantified using a UV/spectrophotometer (PE)[16]. The gastric mucosal H^+, K^+-ATPase activity was expressed as μmol Pi/mg protein/h (U/mg prot).

Electron microscopy

Tissue specimens of the oxyntic mucosa for electron microscopy, were immediately fixed in a solution containing 1% glutaraldehyde and 3% formaldehyde buffered at pH 7.4 with 0.1 mol/L sodium PBS for 2 h at 4℃. Secondary fixation was carried out in 1% osmium tetroxide solution buffered at pH 7.4 with 0.1 mol/L sodium phosphate buffer for 1 h at 4℃. The specimens were then dehydrated in graded acetone solutions and embedded in epoxy resin (Epon 812). Ultrathin sections (60-80 nm) were made for double staining with aqueous uranyl acetate and lead citrate, and observed under transmission electron microscope[17]. Parietal cells were recognized by the presence of secretory canaliculi and/or tubulovesicles. Secreting parietal cells were defined as cells having secretory canaliculi. Non-secreting cells had tubulovesicles but no secretory canaliculi[18].

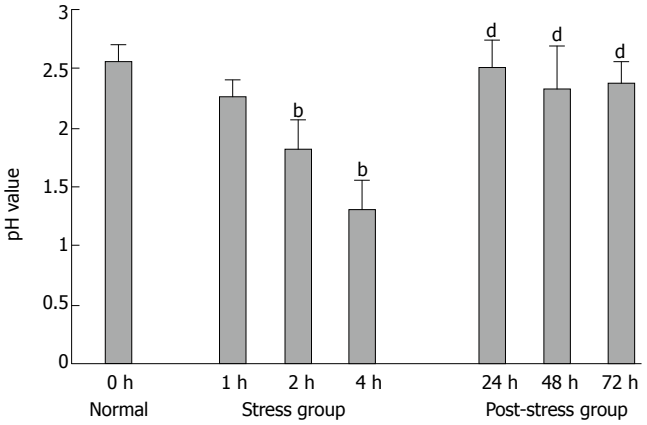

Figure 1 pH value of gastric juice in all groups. $^bP < 0.01$ *vs* normal group; $^dP < 0.01$ *vs* stress 4 h group.

Statistical analysis

All data were expressed as mean ± SD. The statistical differences between different groups were analyzed by Student's *t*-test. The relationship between two variants was analyzed by linearly relevant analysis. $P < 0.05$ was considered significant.

RESULTS

Change of gastric juice pH value

The pH value of gastric juice was 2.56 ± 0.14 in normal group and gradually declined after 1, 2 and 4 h of WRS in rats (2.26 ± 0.04, 1.81 ± 0.25 and 1.31 ± 0.24, respectively) (Figure 1). The pH value was significantly lower (2.0-fold) in stress 4 h group than that in normal group ($P < 0.001$). The pH values of gastric juice after 24, 48, 72 h of WRS in post-stress groups was significantly higher (1.9-fold, 1.8-fold and 1.8-fold, respectively) than that in stress group ($P < 0.001$), but there was no statistical significance compared to normal group ($P > 0.05$, Figure 1). These data demonstrated that the pH value of gastric juice decreased time-dependently in stress group and increased in post-stress group.

Change of gastric mucosal ulcer index (UI)

There was no gastric mucosal lesion in normal group. Scattered spot or lineal erosions, hemorrhage and ulcers were observed in oxyntic mucosa in stress group. The gastric mucosal UI gradually increased after 1, 2 and 4 h of WRS in rats (9.5 ± 2.98, 22.5 ± 3.16 and 35.0 ± 3.93) (Figure 2). The UI decreased significantly (2.0-fold and 4.7-fold) after

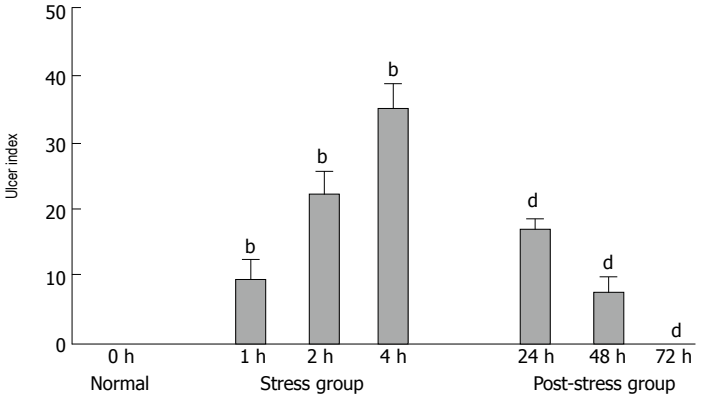

Figure 2 Ulcer index (UI) of gastric mucosa in all groups. [b]$P < 0.01$ *vs* normal group; [d]$P < 0.01$ *vs* stress 4 h group.

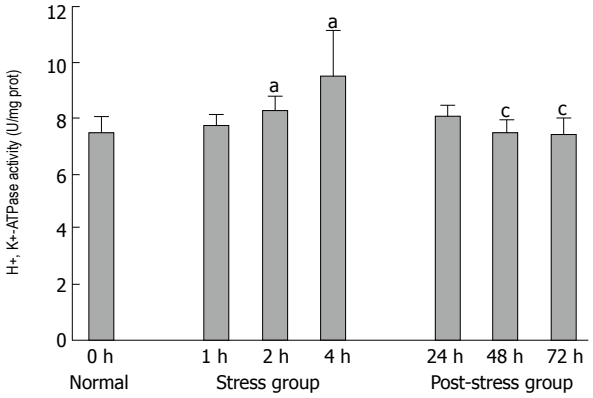

Figure 3 H^+, K^+-ATPase activity of parietal cells in all groups. [a]$P < 0.05$ *vs* normal group; [c]$P < 0.05$ *vs* stress 4 h group.

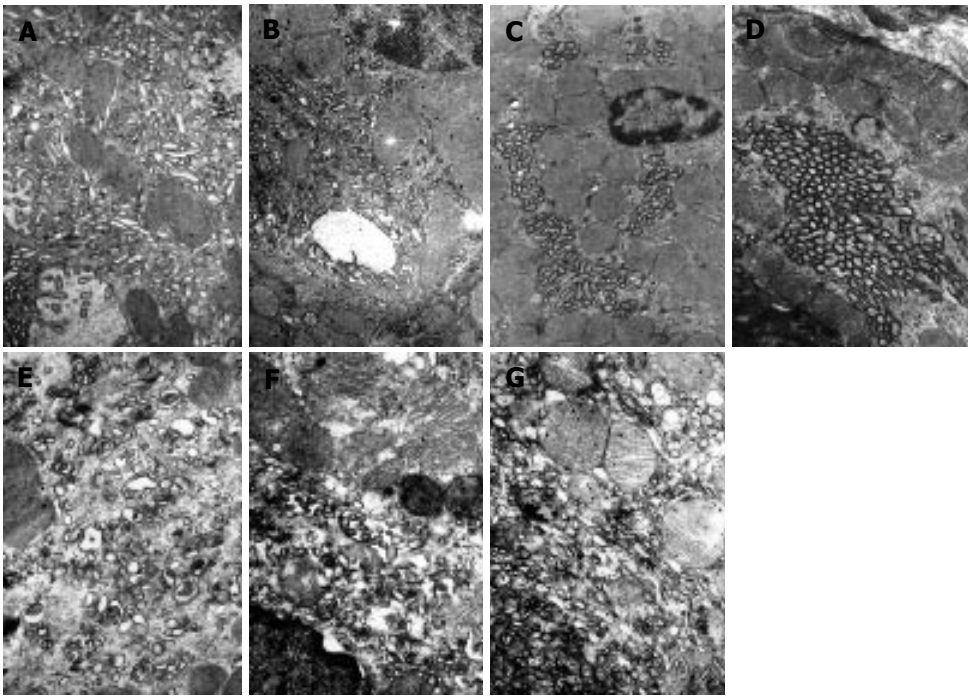

Figure 4 Ultrastructural change of parietal cells. **A:** Gastric parietal cells presented resting state, abundant tubulovesicles and few intracellular canaliculi lined with rare microvilli could be found in the cytoplasm in normal group (×12 000); **B**: Parietal cells presented pre-secreting state, intracellular canaliculi lined with short microvilli were observed, plenty of vesicles were located around the canaliculi and accumulated on the apical plasma membrane in 1 h WRS group (×12 000); **C:** Parietal cells presented secreting state with decreased vesicles and increased intracellular canaliculi lined with elongated microvilli in 2 h WRS group; **D:** Parietal cells presented active secreting state and cytoplasm was almost devoid of vesicles and intracellular canalicular lumen contained numerous elongated microvilli in 4 h WRS group (×12 000); **E - G:** at 24, 48 and 72 h at the end of WRS for 4 h, Parietal cells returned to resting state with abundant tubulovesicles and few canaliculi in cytoplasm in post-stress group swollen mitochondria, vacuolar degeneration, crista-fragmentation, concentrated karyotin, roughness of nuclear envelopes and dilated perinuclear space were observed (×15 000).

24 and 48 h of WRS in post-stress group compared to that in stress group ($P < 0.001$). No lesion occurred in the oxyntic mucosa of rats 72 h after WRS (Figure 2). These data demonstrated that the UI of gastric mucosa increased time-dependently in stress group and decreased in post-stress group. UI was positively related with stress time ($r = 0.9876$, $P < 0.01$) but negatively with pH value ($r = -0.8724$, $P < 0.05$).

H+, K+-ATPase activity

The H^+, K^+-ATPase activity of gastric parietal cells was 7.48 ± 0.59 U/mg prot in normal group and gradually increased after 1, 2 and 4 h of WRS in rats (7.72 ± 0.41, 8.28 ± 0.52 and 9.50 ± 1.63 U/mg prot, respectively) (Figure 3). The H^+, K^+-ATPase activity was significantly higher (1.1-fold and 1.3-fold) in stress group than in normal group ($P < 0.05$). The H^+, K^+-ATPase activity decreased in post-stress group, but there was no statistical significance ($P > 0.05$). The H^+, K^+-ATPase activity was significantly lower (1.3-fold, respectively) after 48 and 72 h of WRS in post stress group than in stress group ($P < 0.05$, Figure 3). These data demonstrated that the H^+, K^+-ATPase activity of gastric parietal cells increased time-dependently in stress group and decreased in post-stress group.

Ultrastructural change of parietal cells

As shown in Figure 4A, gastric parietal cells in normal group presented resting state, a large number of tubulovesicles and few intracellular canaliculi lined with rare microvilli could be found in cytoplasm. As shown in Figure 4B, parietal cells in 1 h WRS subgroup presented pre-secreting state, intracellular canaliculi lined with short microvilli were observed, and plenty of vesicles in the cytoplasm were located around the canaliculi and accumulated on the apical plasma membrane, and the secretory surface was more elaborate than that of the non-secreting cells. As shown in Figure 4C, parietal cells in 2 h WRS subgroup presented secreting state, where decreased vesicles and increased

intracellular canaliculi lined with elongated microvilli were observed. The remaining vesicles were in the region adjacent to the surface and frequently fused with the plasma membrane. In addition, there were abundant mitochondria in the cytoplasm. As shown in Figure 4D, parietal cells in 4 h WRS subgroup presented active secreting state. The cytoplasm was almost devoid of vesicles, whereas the intracellular canalicular lumen contained numerous elongated microvilli. The cytoplasm in these secreting cells was extremely dense owing to the close packing of mitochondria. As shown in Figure 4E-G, in 24, 48 and 72 h WRS subgroups, the parietal cells returned to resting state, abundant tubulovesicles and few canaliculi were observed in the cytoplasm in post-stress group after 24, 48 and 72 h WRS. Besides, Swollen mitochondria, vacuolar degeneration and crista-fragmentation were observed in some parietal cells. Concentrated karyotin, roughness of nuclear envelopes and dilated perinuclear space were observed in other cells, which might be due to the damaged parietal cells induced by stress.

DISCUSSION

Stress ulcers are the superficial mucosal lesions located predominantly in fundus of the stomach. They occur mainly in severe trauma and sepsis as well as in acute severe psychological stress. It has become apparent that the physiological stress of critical illness increases the risk of developing stress-related mucosal injury and gastrointestinal bleeding. The pathogenesis of stress ulcers is not completely understood. however, recent studies indicate that the origin may be multifactorial, and there is an imbalance between protective and destructive factors[19-21]. Major destructive factors include acid, pepsin, bile, reperfusion injury and free oxygen radicals[22-24]. Protective factors include adequate mucosal blood flow, mucus-bicarbonate layer, epithelial cell renewal and prostaglandins[25-27]. It is generally agreed that mucosal ischemia is the major inciting event in the pathogenesis of acute stress ulceration of the stomach and that the presence of luminal acid and pepsin is required in the development of overt ulceration[28]. Back-diffusion of acid occurs in the absence of overt disruption of the mucosal barrier, and is closely related to the formation of ulcers.

In this study, we found that gastric mucosal lesions gradually aggravated after 1, 2 and 4 h of WRS in rats. Gastric mucosa lesions progressed to apparent erosions and hemorrhage in 4 h WRS group. There positive relevance between the ulcer index UI was significantly related with the exposure time to WRS. We also found that gastric juice pH value decreased and H^+, K^+-ATPase activity of parietal cells gradually increased after 1, 2 and 4 h of WRS, indicating that gastric acid secretion increases with the exposure time to WRS. The pH value was significantly lower (2.0-fold) and the H^+, K^+-ATPase activity was significantly higher (1.3-fold) in stress group than in normal group. UI was negatively related with pH value. These results strongly suggest that gastric acid can mediate acute gastric mucosal lesions induced by WRS.

The present study assessed the dynamic ultrastructural change of gastric parietal cells in rats induced by WRS. Gastric parietal cells also known as oxyntic cells, have two systems in the cytoplasm under transmission electron microscope: intracellular secretory canaliculus system and tubulovesicular system[29]. In normal group, abundant tubulovesicles and few intracellular canaliculi lined with rare microvilli were found in cytoplasm and the gastric parietal cells presented resting state. Tubulovesicles gradually decreased while intracellular canaliculi lined with elongated microvilli gradually increased after exposed to WRS for 1, 2 and 4 h. In 4 h WRS group, parietal cell cytoplasm was almost devoid of vesicles and the intracellular canalicular lumen contained numerous elongated microvilli. Our observation showed that parietal cells presented a series of states from resting, pre-secreting, secreting to active secreting after 0, 1, 2 and 4 h of WRS. Our study also showed that gastric mucosal lesions induced by WRS paralleled to time-course changes of ultrastructure of gastric parietal cells. These results indicate that WRS can time-dependently activate parietal cells and induce gastric acid secretion.

The mechanism of the recovery of gastric mucosa after stress is not fully understood. the healing of stress ulcer is a very complex process affected by multi-factors. It has been reported that the mucosal integrity and repair involved enhancement of gastric mucosal blood flow and increased mucosal cell proliferation mediated by epidermal growth factor (EGF) and transforming growth factor alpha (TGFα)[30,31]. The importance of gastric acid in the process of restructuring gastric mucosa after WRS has not yet been evaluated. In present study, we found that the injured gastric mucosa was gradually restructured after the stress was removed and no lesion was observed after 72 h at the end of WRS for 4 h. We also found the pH value increased significantly at 24, 48 and 72 h of WRS. These results strongly demonstrate that the decrease of gastric acid is beneficial for restructuring injured gastric mucosa.

In the present study, we found that the parietal cells returned to resting state while swollen mitochondria, vacuolar degeneration, crista-fragmentation, concentrated karyotin, roughness of nuclear envelopes and dilated perinuclear space were observed after 24, 48 and 72 h of WRS, suggesting that damaged parietal cells induced by WRS.

The major founding of our study is that the ultrastructural changes of parietal cells is consisitent with the change of H^+, K^+-ATPase activity in the development and healing of stress ulceration induced by WRS. Moreover, the recovery of ultrastructural and functional changes of parietal cells was earlier than the restructuring of damaged gastric mucosa after WRS, suggesting that the ultrastructural and functional changes of parietal cells may be an important pathophysiologic mechanism in restructuring stress ulceration.

In conclusion, ultrastructural and functional changes of gastric parietal cells are induced by WRS in rats. Increased gastric acid secretion induced by WRS is consistent with the ultrastructural changes of parietal cells and is significantly correlated with the degree of gastric mucosa lesions. Gastric acid not only plays an important role in the development of stress ulcer, but also is closely related to the recovery of gastric mucosal lesions induced by WRS.

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BASIC RESEARCH

Cloning and molecular characterization of Δ^{12}-fatty acid desaturase gene from *Mortierella isabellina*

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Abstract

AIM: To clone Δ^{12} -fatty acid desaturase gene of *Mortierella isabellina* and to functionally characterize this gene *in vitro* and *in vivo*.

METHODS: Reverse transcriptional polymerase chain reaction (RT-PCR) was used to clone the open reading frame of Δ^{12}-fatty acid desaturase gene (D12D) of *Mortierella isabellina*. Plasmids pEMICL12 and pYMICL12 were constructed with it. pEMICL12 was transformed into *Escherichia coli* (*E.coli*) strain BL21 using $CaCl_2$ method for expression after induction with IPTG. pTMICL12 was transformed into *Saccharomyces cerevisiae* strain INVSc1 using lithium acetate method for expression under the induction of galactose. Northern blotting method was used to investigate the effect of temperature on the transcriptional level of this gene in *S.cerevisiae* strain INVSc1.

RESULTS: Recombinant plasmids pEMICL12 and pTMICL12 were successfully constructed and transformed into *E.coli* and *S.cerevisiae* separately with appropriate method. After induction with IPTG and galactose, it was found that expression of Δ^{12}-fatty acid desaturase genes in *E.coli* and *S. cerevisiae* under appropriate conditions led to the production of active Δ^{12}-fatty acid desaturase, which could convert 17.876% and 17.604% of oleic acid respectively to linoleic acid by GC-MS detection *in vitro* and *in vivo*.

CONCLUSION: Cloning and expression of *M.isabellina* D12D gene in *E.coli* and *S.cerevisiae* is successfully completed.

Key words: *Mortierella isabellina*; Δ^{12}-fatty acid desaturase; *In vitro* expression

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INTRODUCTION

As an economically important filamentous fungi, *M isabellina* which belongs to *Mucor sp.* can produce linolenic acid(GLA) that plays very important roles in regulating the hormone level and fatty acid metabolism in human. In addition, recent study indicated that GLA can alleviate the symptoms of many kinds of diseases such as neurodegeneration[1,2] , adipogenesis[3], cancer[4,5] , Protofibrils[6-8] , cardiovascular disease[9-11], diabetes[12] and psoriasis[13]. Being a kind of nutritionally and pharmaceutically important substance, GLA has become the hotspot both in academic and applied fields. *M isabellina* has been a widely used strain in industry to produce GLA by fermentation, in which GLA accounts for 8 percent of total fatty acids. Further efforts to enhance the percentage of the GLA by traditional mutation or by changing fermentation conditions such as medium and temperature have been made, but the extent is relatively small. Therefore, we attempted to reconstruct this strain with genetic engineering.

In most eukaryotic cells, GLA is biosynthesized by two desaturation processes, using stearic acid C18:1 as initial substrate[14-17] . Linoleic acid (LA) C18:2 is synthesized from C18:1 by the reaction catalyzed by Δ^{12}-fatty acid desaturase and then LA can be converted to GLA by Δ^{6}-fatty acid desaturase. LA can also be converted to α-linolenic acid (ALA) by ω3 fatty acid desaturase. LA and ALA are so-called essential fatty acids (EFAs)[18,19] to human bodies because of their inability to synthesize these compounds *de novo*. Linoleic acid is the precursor of polyunsaturated fatty acids (PUFAs) in both routes of n-6 and n-3[20]. Therefore, Δ^{12}-fatty acid desaturase is the key enzyme for producing LA and is also the speed-limiting enzyme for routes of n-6 and n-3. Cloning and characterization of Δ^{12}-fatty acid desaturase gene will help understand the biosynthetic pathway of GLA and further enhance the percentage of GLA in total fatty acids.

Our research interest focuses on PUFAs having 18

carbon atoms or more such as linoleic acid and linolenic acid in fungus. In this paper, we report the isolation of cDNA of D12D from *M isabellina*. The cDNA was expressed in a classical system, *S.cerevisiae* and *E.coli*.

MATERIALS AND METHODS

Materials and culture conditions

Mortierella isabellina M_{6-22} was grown at 28℃ for 2 d in liquid medium containing 2% glucose, 1% bacto-yeast extract, 0.2% KH_2PO4 and 0.1% $MgSO_4$, with pH adjusted to 6.0. *S. cerevisae* strain INVScI was used as recipient strain in transformation experiments and was grown at 30℃ in complex medium containing 1% bacto-yeast extract, 2% bacto-peptone and 2% glucose. *E.coli* strain DH5a was grown at 37℃ in Luria–Bertani medium (LB) supplemented with 100 mg/L of ampicillin. *E.coli* strain BL21 and the expression vector pET21a were purchased from Novegon(Novegen Inc., Madison, WI).

Restriction endonucleases, and the other DNA-modifying enzymes were obtained from TaKaRa Bio. Dalian, China Co. Ltd. Other chemicals were purchased from Sangon Bio. Shanghai, China Co. Ltd.

RNA extraction

Mycelia were harvested by filtration and washed with phosphate buffer. The extraction of total RNA was done according to the guanidinium thiocyanate method as described by Chomczynski and Sacchi[21] and extracts were stored at -70℃ for future use.

Cloning of Δ^{12}-fatty acid desaturase gene by RT-PCR

A pair of primers was designed according to the reported sequence of Δ^{12}-fatty acid desaturase gene from *Mortierella alpine*[22] to clone the open reading frame (ORF) of the *M isabellina* D12D gene, P1: 5'-TAC CTC ***GAA TCC*** **ATG** GCA CCT CCC AAC ACT ATC GAT GCC-3'; P2: 5'-AAC CGT ***CTC GAG*** **TTA** CTT CTT GAA AAA GAC CAC GTC TCC-3', which corresponded to the nucleotide sequences of start and stop codons (in boldface) of the Δ^{12}-fatty acid desaturase gene, respectively. The 5'-end of both primers contained an *Eco*R Ⅰ and an *Xho* Ⅰ site(underlined and in boldface), respectively that were underlined to facilitate subsequent manipulation. PCR was performed in a Biometra T-gradient thermal cycler using the following program: at 94℃ for 1 min, 53℃ for 1 min, 72℃ for 2 min for 30 cycles, followed by extension for 10 min at 72℃. PCR fragments were subcloned into pGEM-T vector (Promega, Madison, WI) to produce recombinant plasmid pTMICL12, which was then transformed into *E.coli* DH5a. Subsequently, nucleotide sequences were determined (TaKaRa Bio, Dalian, China). Analysis of the sequences was done with DNAMAN software (version 4.0, Lynnon BioSoft, Quebec).

Plasmid construction

The ORF of Δ^{12}-fatty acid desaturase from *M isabellina* was digested from pTMICL12 with *Eco*R Ⅰ and *Xho* Ⅰ and subcloned respectively into *S.cerevisiae* expression vector pYES2.0 and *E.coli* expression vector pET21a. Positive transformants were screened on the LB plate with ampicillin resistance. The resultant positive plasmid was validated by PCR and restriction enzyme digestion. The construction sketch of pYMICL12 is shown in Figure 1. The resultant plasmids, pYMICL12 and pEMICL12 were stored and propagated in *E.coli* strain DH5a.

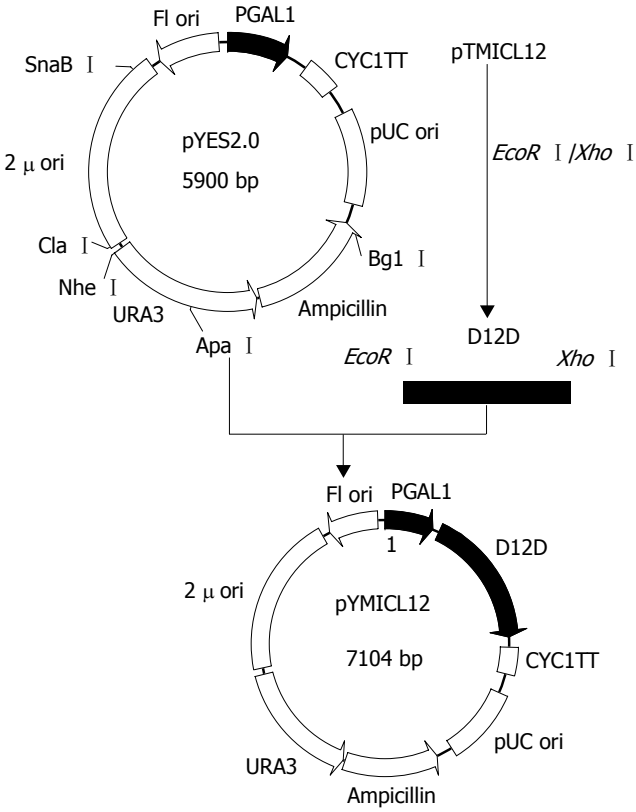

Figure 1 Construction sketch of pYMICL12.

Yeast transformation and inducement of transgenic yeast

The recombinant plasmid pYMICL12 was transformed into *S.cerevisiae* INVSc1 using the lithium acetate method [23]. Positive transformants were selected by plating on complex synthetic minimal medium agar lacking uracil (SC-Ura) and grown at 30℃ for 2-3 d. The putative Δ^{12}-desaturase gene was heterologously expressed in yeast, which was induced under transcriptional control of the GAL1 promoter. Yeast cultures were grown to logarithmic phase at 30℃ in synthetic minimal medium containing different concentrations of galactose(1%, 2%, 3%, 4% respectively), and 0.67% yeast nitrogen. Subsequently, cells were harvested by centrifugation followed by washing in sterile water for three times. The cells were dried and ground into fine powders for determination of fatty acid composition by gas chromatography and for gas chromatography–mass spectrometry (GC-MS) analysis.

Inducement of the transgenic E.coli cells

Transformant containing plasmid pETMICL12 was cultured in M9 medium at 37℃ overnight. Five hundred milliliter M9 medium was inoculated into 2.8 L Fernbach flask with aliquot from the overnight culture until the light density reached 0.5. IPTG was added until the final concentration reached 0.4 mmol/L. After 4 hours' induction,

cells were pelleted by centrifugation at 5000 r/min for 10 min at 4℃ and stored at -70℃ for future use.

Preparation of cell membrane

Cells defrosted at room temperature were suspended in 30 mL buffer A (containing 10 mmol/L $MgCl_2$, 50 mmol/L MOPS-NaOH, 1 mmol/L EGTA, 10 μg/mL DNase) and then were treated with supersonic 450 Hz for 40 cycles, 4 s per cycle. Intact cells and cell debris were pelleted by centrifugation at 5000 r/min for 10 min at 4℃. Supernatants containing cell membrane were pelleted by super-centrifugation at 150 000 r/min for 30 min at 4℃ and then were re-suspended in 200 μL buffer A.

SDS-PAGE

Ten percent of the gel was used to separate the protein bands, and stained by Coomassie Blue R-250[24].

In vitro assay of activity of Δ^{12}-fatty acid desaturase

Sodium salt was added into the membrane suspension in buffer A to reach the final concentration of 150 μmol/L and then the mixture was incubated at 25℃ in water bath for 30 min after oleic acid was added. Soon after that, the mixture was extracted with chloroform methanol and water (volume ratio 2:1:2). The extraction was methyl-esterified with 5% HCl/methanol at 85℃ for 150 min. Fatty acid methyl ester was solubilized with hexane[25,26].

Detection of desaturase activity expressed in transgenic yeast

Total fatty acid was extracted from the cells by treating 100 mg yeast powder with 5 mL 5% KOH in methanol for saponification at 70℃ for 5 h. pH of the product was adjusted to 2.0 with HCl (6 mol/L) before the fatty acid was methyl-esterified with 4 mL 14% boron trifluoride in methanol at 70℃ for 1.5 h. Then, fatty acid methyl esters (FAME) were solubilized with hexane after addition of saturated sodium chloride solution. FAMEs were analyzed by gas chromatography (GC; GC-9A, Shimadzu, Kyoto, Japan) and identified by the comparison of their peaks with that of standards (Sigma). Qualitative analysis of FAME was performed by GCMS using an HP G1800A GCD system (Hewlett–Packard, Palo Alto, CA, USA). Both analyses were carried out with the same polar capillary column (HP, 5.30 mU, 0.25 mm in internal diameter). The mass spectrum of a new peak was compared with that of the standard for identification of fatty acid.

Analysis of mRNA level in transgenic yeast after induction at different temperature

Northern blotting was used to investigate the effect of temperature on the transcriptional level of MID12. Total RNA of transgenic yeast after induction at different temperatures was extracted by UNIQ-10 column (Sangon Corp.) and digoxingenin (Roche Diagnostics Corp.) was used to label the DNA probe (PCR of MID12)

RESULTS

Target gene obtained

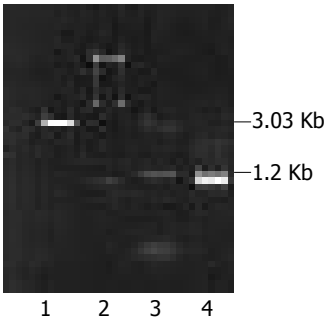

Figure 2 Identification of the recombinant plasmid, pTMICL12 by restriction enzyme. 1: pGEM-T/*Eco*R Ⅰ/*Xho* Ⅰ; 2: DNA size marker λ DNA/*Bst* Ⅱ; 3: pTMICL12/*Eco*R Ⅰ/*Xho* Ⅰ; 4: PCR product of D12D DNA.

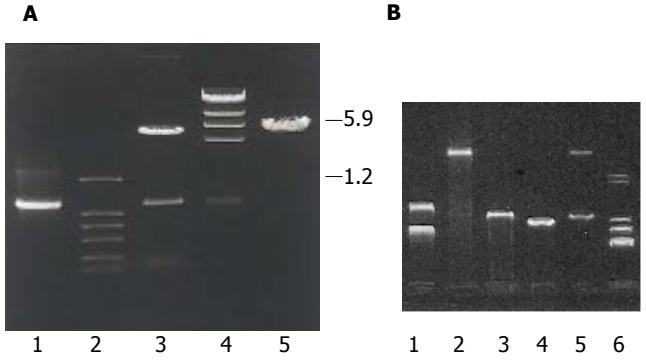

Figure 3 **A**: Identification of pYMICL12. 1: PCR product of D12D DNA; 2: DNA size marker DL2000; 3: pYMID12/*Eco*R Ⅰ/*Xho* Ⅰ; 4: DNA size marker λDNA/Hind Ⅲ; 5: pYES2.0/*Eco*R Ⅰ/*Xho* Ⅰ. **B**: Restriction analysis of recombinant plasmids pEMICL12. 1: Plasmid pEMICL12; 2: PCR product of D12D; 3: pET21a/ *Eco*R Ⅰ; 4: pMAGL12/ *Xho* Ⅰ; 5: pEMICL12/ *Eco*R Ⅰ + *Xho* Ⅰ; 6: λDNA/HindⅢ marker.

PCR product about 1.2 kb long was gel-purified by electrophoresis and cloned into pGEM-T vector. Positive clones were selected on LB plate by color reaction. PCR and enzyme restriction were used to analyze the resultant transformant named pTMICL12. After digestion with *Sac*Ⅱ and *Pst* Ⅰ, a 3.03 kb fragment and a 1.2 kb target gene were obtained as shown in Figure 2. Subsequently, nucleotide sequences were determined (TaKaRa Bio, Dalian, China)

Nucleic acid sequence of Δ^{12} -fatty acid desaturase in *M. isabellina* has been deposited in Genbank database and assigned the accession No. AF417245. Comparison of the DNA sequence with other Δ^{12}-fatty acid desaturase gene showed that it has the highest identity with Δ^{12}-fatty acid desaturase gene from *M. alpina* (about 99.92%) and higher identity with other D12D from fungi[27-30] (more than 40%). Therefore, this sequence encoded a putative Δ^{12}-fatty acid desaturase gene.

Expression vector construction for S. cerevisiae and E coli

In order to investigate the function of this sequence, target gene was subcloned into YES2.0 and pET21a to construct two recombinant plasmids pYMICL12 and pEMICL12 which were transformed into *E.coli* DH5a for propagation. After digested by *Eco*R Ⅰ and *Xho* Ⅰ, both of the plasmids released a 1.2 kb fragment (Figure 3), indicating that the recombinant plasmids were successfully reconstructed.

Detection of desaturase activity in transgenic yeast

After inducement, the total fatty acid in transgenic yeast

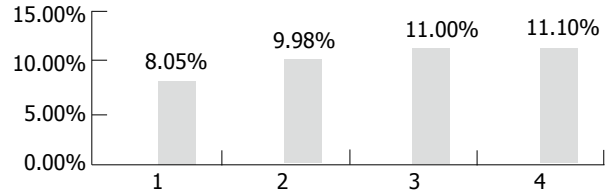

Figure 4 Percentage of linoleic acid in transgenic yeast induced by different concentration of galactose. 1: 1% galactose; 2: 2% galactose; 3: 3% galactose; 4: 4% galactose.

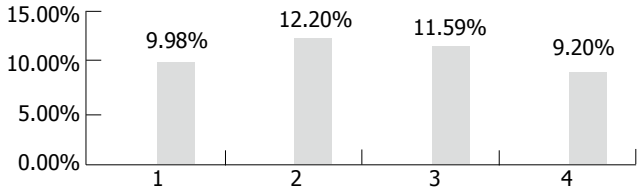

Figure 5 Percentage of linoleic acid in transgenic yeast induced for different durations. 1.12 h; 2. 24 h; 3.36 h; 4. 48 h.

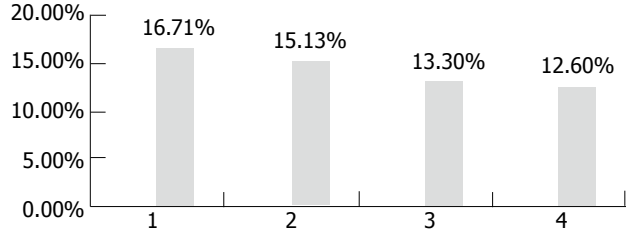

Figure 6 Percentage of linoleic acid in transgenic yeast induced at different temperature. 1: 15℃; 2: 20℃; 3: 25℃; 4: 30℃.

harboring plasmid pYMICL12 was extracted and analyzed by GC. It was found that under different conditions, the expressed desaturase showed different activities.

First, the transgenic yeast was induced at 30℃ for 36 h, with different concentrations of galactose: 1%, 2%, 3%, 4%. The result of GC showed that percentage of LA in transgenic yeast was the highest when it was induced with 3% galactose (Figure 4).

Then, different duration times were used to induce the transgenic yeast by 3% galactose at 30℃ for 12, 24, 36, and 48 h respectively. The result of GC showed that the optimal induction time was 24 h as shown in Figure 5.

Thirdly, the transgenic yeasts were induced by 3% galactose for 24 h at different temperatures of 15℃, 20℃, 25℃, and 30℃ respectively. It was found that the lower the temperature, the higher the activity of the expressed enzyme. The highest percentage of LA reached 16.706% of total fatty acid in induced yeast harboring plasmid pYMICL12 when the temperature was 15℃ (Figure 6).

Therefore, the optimal condition for expressing D12D from *M isabellina* was: 3% galactose induction for 24 h at 15℃. GC analysis of the fatty acid composition of the transgenic yeast containing plasmid pYMICL12 indicated that a novel peak that had the same retention time with that of LA standard appeared. That specific peak was absent from the control transformant (Figure 7).

Northern blot

Northern blot was performed to check if there was an increase of mRNA after induction by low temperature which resulted in the higher activity of Δ^{12}-fatty acid desaturase in transgenic yeast, INVSc1(pYMICL12).

After induction at different temperatures, total RNA was extracted. In the formaldehyde-denaturalization electrophoresis, 30 mg RNA was used for each sample. The result of the Northern blot is shown in Figure 8.

Any visible change of the level of RNA could not be found on the photo of Northern blot. Analysis by VDS-scan also showed no obvious difference between the samples, implying there were no changes of the mRNA level of D12D in the transgenic yeast at different temperature. The level of transcription was not the key cause for the change of LA level in INVSc1 (pYMICL12) under cold condition. The increasing activity of Δ^{12}-fatty acid desaturase might come from the protection of the enzyme activity by low temperature, which even prolonged the half life of Δ^{12}-fatty acid desaturase mRNA.

Detection of Δ^{12}-fatty acid desaturase expressed in E coli by SDS-PAGE

Transformants harboring plasmid pEMICL12 were cultured in M9 medium and induced by IPTG to express Δ^{12}-fatty acid desaturase from *M isabellina*. Cell membranes were pelleted by super-centrifugation. Analysis with SDS-PAGE showed that a specific protein band that had the molecular weight of approximately 43 kd existed on the gel, whereas cell membrane from control transformant harboring the plasmid pET21a did not express the specific protein. In addition, the molecular weight of the specific protein band was nearly identical to other reported Δ^{12}-fatty acid desaturase. These results suggested that this specific band putatively was Δ^{12}-fatty acid desaturase (Figure 9).

In vitro assay of activity of Δ^{12}-fatty acid desaturase

In order to test whether this protein band could convert OA to LA, *in vitro* assay with membrane protein was done. *E coli* can not produce OA that is the most commonly used substrate of Δ^{12}-fatty acid desaturase. Therefore, OA must be added into the reaction mixture to be converted to LA by Δ^{12}-fatty acid desaturase. GC analysis of the mixture after reaction indicated that transformant containing plasmid pETMICL12 exhibited Δ^{12}-fatty acid desaturase activity and that the membrane protein could convert 17.604% of the OA to LA(indicated by arrow), whereas cell membrane protein from transformant containing empty plasmid pET21a did exhibit Δ^{12}-fatty acid desaturase activity. Also, the retention time of the specific peak in Figure 10 was identical to standard LA from Sigma. These results indicated that the specific band had Δ^{12}-fatty acid desaturase activity.

DISCUSSION

M isabellina strain $M_{6\text{-}22}$ is a mutant strain in which total fatty acids account for 60 percent of the dry weight of the mycelium. Also, it synthesizes PUFAs only up to 18-carbon with c isomer of linolenic acid (GLA), which make the subsequent purification relatively easy. Thus, *M isabellina*

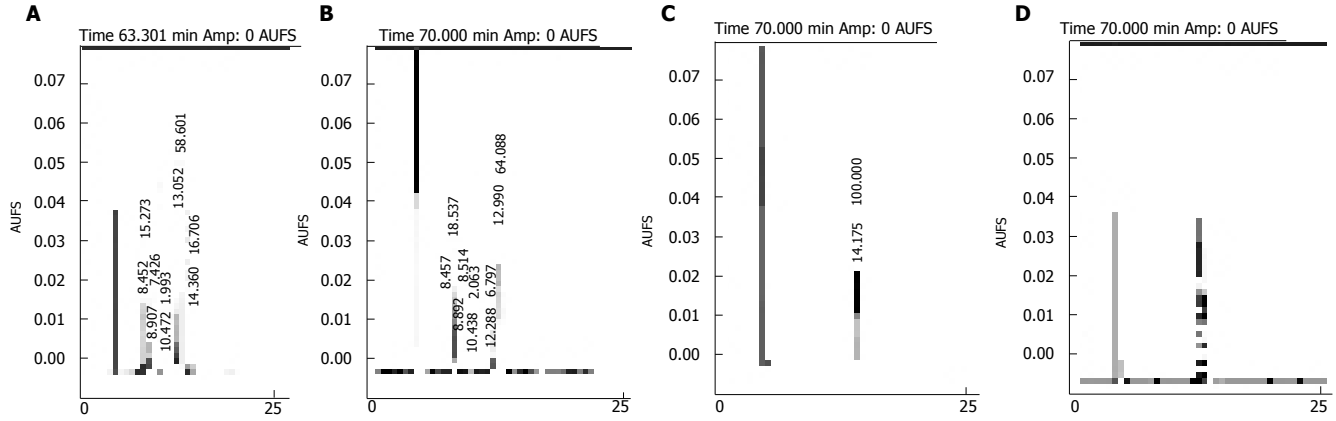

Figure 7 GC analysis of the total fatty acid in transgenic yeast, INVSc1 (pYMICL12). **A**: Total fatty acid in transgenic yeast, INVSc1 (pYMICL12) after induction by 3% galactose at 15℃ for 24 h; **B**: Total fatty acid in transgenic yeast, INVSc1 (pYES2.0) after induction by 3% galactose at 15℃ for 24 h; **C**: Linoleic acid; **D**: Oleic acid.

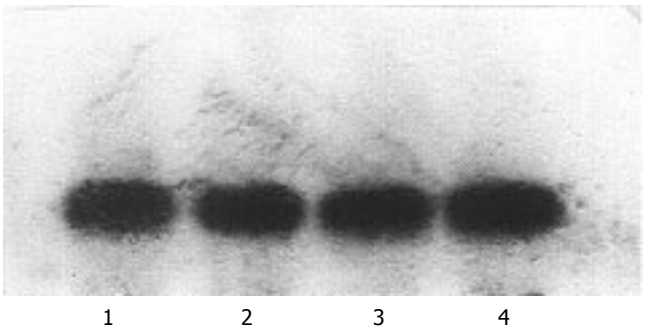

Figure 8 Northern blot of total RNA of transgenic yeast induced at different temperature. 1: 15℃; 2: 20℃; 3: 25℃; 4: 30℃.

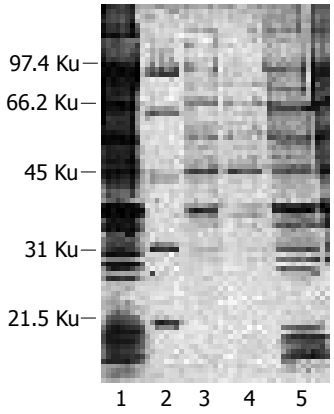

Figure 9 SDS-PAGE of membrane protein in transgenic *E.coli*.1.Total protein of BL21; 2: low mass marker; 3: Total protein of BL21/pMICL12; 4: Total soluble protein of BL21/pMICL12 after supercentrifugation; 5: Membrane protein of BL21/pMICL12.

strain $M_{6\text{-}22}$ is a promising producer of GLA and also a potential strain to be used to produce other nutritionally and pharmaceutically important PUFAs. However, increasing the percentage of GLA meets great challenges, which made us to turn to other methods including genetic engineering. As we all know, cloning the genes encoding corresponding enzymes is the first thing we should do. In our previous work, we have successfully cloned and characterized Δ^6-fatty acid desaturase gene from *M isabellina*[31]. In order to further elucidate the biosynthetic pathway of polyunsaturated fatty acid, we decided to clone the enzyme genes that are ahead of the Δ^6-fatty acid desaturase gene in reaction chains.

As *M isabellina* is very near to *M alpine* in phylogeny, we adopted the strategy of direct cloning the gene by designing the primer identical to the reported *M alpine* Δ^{12}-fatty acid desaturase gene. Using this method, a fragment of proximately 1.2 kb was obtained. Sequence comparison indicated it has the highest identity with *M alpine* Δ^{12}-fatty acid desaturase gene. In order to functionally identify this nucleic acid sequence, two expression systems were applied: yeast and *E.coli*. In both systems, the heterologously expressed proteins exhibited Δ^{12}-fatty acid desaturase activity, indicating that this sequence encoded a functional desaturase. The high identity between the two sequences suggests that *M alpine* and *M isabellina* belong to the same species. But there indeed exist great differences between the two species. *M alpine* can produce many kinds of PUFAs, but *M isabellina* can only synthesize PUFAs up to 18-carbon. This indicates that other factors may contribute to the difference.

Protein expression in yeast is influenced by many factors such as temperature, induction time. In this work, different temperatures were used. In a certain extent (15-30℃), the higher the temperature, the lower the desaturase activity. At even lower temperature, the yeast INVSc1 (pYMICL12) could achieve more LA. However, a temperature lower than 15℃ would hinder the growth of the mycelium, which reduced the total activity of Δ^{12}-fatty acid desaturase.

Although desaturase activity increased as the temperature decreased, northern blotting analysis indicated that the increasing of desaturase activity was not due to the corresponding increase of the mRNA level. In our study, no significant difference existed among the mRNA level at different temperatures. Other researchers also have reported the same result[28]. This indicates that under certain conditions, mRNA level can not be influenced by temperature. The most important factor that can change it may be the promoter activity. Does the increased activity of Δ^{12}-fatty acid desaturase, however, come from the influence of coldness on mRNA level or protein level? Or does longer half life of Δ^{12}-fatty acid desaturase even directly influence activity of protein? There are different opinions about this in academia.

In conclusion, this report about cloning and character-

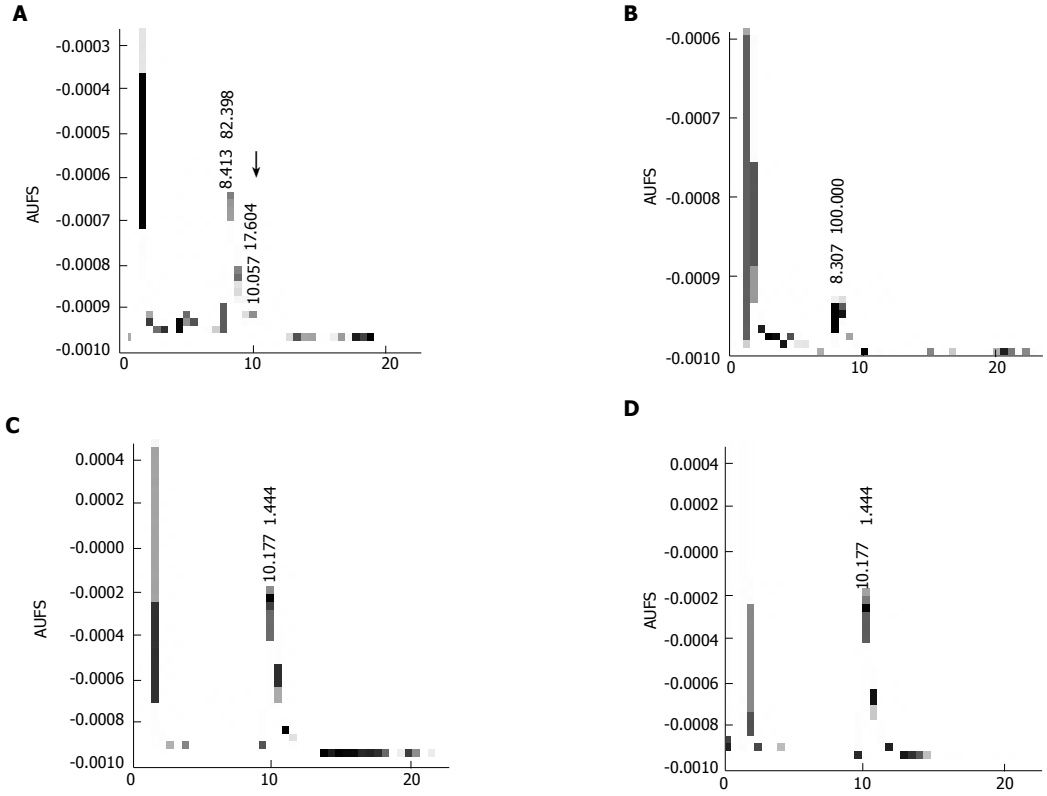

Figure 10 Gas chromatographic analysis of fatty acid methyl esters of lipids catalysed by membrane protein of *E.coli* in system *in vitro*. **A**: Fatty acid components catalysed by membrane protein of BL21/ pMICL12; **B**: Fatty acid components catalysed by membrane protein of BL21/ pET21a; **C**: Linoleic Acid; **D**: Oleic Acid.

ization of the *M isabellina* Δ^{12}-fatty acid desaturase gene will be helpful for us to utilize the industrially used strains.

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CLINICAL RESEARCH

Malnutrition affects quality of life in gastroenterology patients

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Abstract

AIM: To investigate the association between malnutrition and quality of life in patients with benign gastrointestinal disease.

METHODS: Two hundred patients (104 wellnourished and 96 malnourished) were assessed according to the Subjective Global Assessment, anthropometric measurements and bioelectrical impedance analysis. Quality of life was determined with the validated Medical Outcomes Study 36-item Short-Form General Health Survey (SF 36). Muscle function was assessed by hand grip strength and peak flow.

RESULTS: Body mass index, body cell mass, arm muscle area and hand grip strength were significantly lower in the malnourished patients. Quality of life was generally lower when compared to norm values. Seven out of eight quality of life scales (excluding *bodily pain*) were significantly reduced in the malnourished patients. Comparing patients with liver cirrhosis and inflammatory bowel disease (IBD), patients with IBD experienced significantly lower values in the perception of *bodily pain,social functioning* and *mental health*. Malnourished liver cirrhotics suffered reductions in more scales (six out of eight) than malnourished IBD patients did (four out of eight).

CONCLUSION: Quality of life is generally low in benign gastrointestinal disease and is further reduced in patients who are classified as malnourished. It appears that liver cirrhosis patients experience a higher quality of life than IBD patients do, but the impact of malnutrition seems to be greater in liver cirrhosis than in IBD.

Key words: Quality of life; Malnutrition; Gastrointestinal disease; Liver cirrhosis; Inflammatory bowel disease

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INTRODUCTION

Malnutrition is common in chronic and acute gastrointestinal disease affecting both morbidity and mortality. Impairment of nutritional status frequently also decreases muscle function[1,2], which lastly results in an impaired functional status.

Studies have shown a close relationship between malnutrition and decreased quality of life in some populations such as elderly institutionalized patients[3,4], patients with cancer[5-7] and patients on haemodialysis[8-10]. Quality of life is a subjective multidimensional construct reflecting functional status, emotional and social well being as well as general health. Its measurement is based on the patient's perception of well being.

In order to evaluate patients' situation or benefits of new treatment strategies, quality of life has become a new clinically relevant outcome parameter[11-13]. In chronic disease in particular, where recovery from disease is not always achievable, quality of life is essential and its measurement should be integrated as one main intervention target. It is therefore crucial to better understand the relationship between more objective measures such as disease parameters, nutritional status and subjective quality of life.

Studies investigating factors influencing quality of life in benign gastrointestinal (GI) disease have not looked at the impact of nutritional deficiency[14-17]. Hitherto, there are no data on quality of life in relation to nutritional status in patients with benign gastrointestinal disease. Since malnutrition frequently occurs in gastroenterological disease, we explored the impact of malnutrition as identified by the Subjective Global Assessment in patients with benign gastroenterological disease. We also focused the evaluation on subgroups of our study patients with chronic gastrointestinal disease like liver cirrhosis or chronic inflammatory bowel disease (Crohn's disease or ulcerative colitis).

MATERIALS AND METHODS

Patients

A total of 200 patients with benign gastroenterological disease admitted to a gastroenterology ward at our university hospital were recruited for the study. Sixty one patients with liver cirrhosis (n = 33 men and n = 28 women), 69

patients with inflammatory bowel disease (n = 29 men and n = 40 women) as well as 70 patients with various acute and chronic gastroenterological diagnoses (n = 32 men, n = 38 women) were included in the study (Table 1). All patients were considered for inclusion if they had a non-neoplastic disease regardless of disease severity. Exclusion criteria were malignant comorbidity and terminal disease (life expectancy < 3 mo) in order not to bias interpretation of quality of life. Patients with neuro-muscular disease, hemiplegia or rheumatoid arthritis were excluded in order to avoid potential confounders on muscle strength. Furthermore, patients with implanted defibrillator were excluded from the bioelectrical impedance analysis. All measurements were performed within 36 h of admission. Patient recruitment was consecutive until at least 100 well nourished patients with benign gastrointestinal disease were included. Then, only malnourished patients were consecutively included in order to obtain comparable patient groups.

All patients signed written informed consent and this study was approved by the local ethics committee.

Table 1 Clinical diagnoses of the study population

Diagnoses	*n*		*n*		*n*
Liver cirrhosis	**61**	**Others**	**70**		
Alcoholic liver cirrhosis	51	Biliary disease	11	Functional dyspepsia/ IBS	4
Cryptogenic liver cirrhosis	4	Gastritis / reflux disease	11	Ulcer disease	3
Biliary liver cirrhosis	4	Chronic pancreatitis	9	Colon and/or rectal polyps	2
Post hepatic liver cirrhosis	2	Non infectious colitis	7	Malabsorption syndrome	2
Inflammatory bowel disease	**69**	Diverticulitis	8	Viral hepatitis	2
Crohn's disease	46	Fatty liver disease	8	Proctologic disease	2
Ulcerative colitis	23			**Total**	**200**

Quality of life

Quality of life was assessed employing the validated Medical Outcomes Study 36-item Short-Form General Health Survey described in detail elsewhere[18,19]. The questionnaire consists of 36 questions forming 8 multi item scales and is self administered [18,19]. These scales range from 0-100 percent (absolute values), but norm values differ substantially across the scales. In order to facilitate interpretation and to compare the results with norm values obtained in 1998 (1998 SF-36 US population norms), all scales were therefore not only expressed as absolute values but also norm based scored, i.e. each scale was scored to have the same average and the same standard deviation. We employed the standard (US) scoring algorithms, as has been recommended by Ware *et al*[19]. Results for the eight scales can then easily be compared directly since they are all standardized in relation to population norms.

Classification of malnutrition

The Subjective Global Assessment (SGA) was carried out using the protocol developed by Detsky *et al*[20]. It relies on the patient's history regarding weight loss, dietary intake, gastrointestinal symptoms, functional capacity, and physical signs of malnutrition (loss of subcutaneous fat or muscle mass, edema, ascites). Patients were classified as well nourished (A), moderately or suspected of being malnourished (B) or severely malnourished (C). Since subgroups of patients classified as SGA C were too small, comparative analyses were performed between well-nourished patients classified as SGA A and malnourished patients classified as either SGA B or C.

Anthropometry

Body weight was measured on light clothes with a portable electronic scale (seca 910, Hamburg Germany) to the nearest 0.1 kg and height was measured with a portable stadiometer (seca 220 telescopic measuring rod) to the nearest 0.1 cm. Weight and height were used to calculate BMI (weight (kg)/height (m)^2). Mid upper arm circumference (of the nondominant arm) was measured to the nearest 0.1 cm with a nonelastic tape measure and triceps skinfold was measured to the nearest 0.1 mm with a Holtain caliper (Crymych, UK) on the non dominant relaxed arm midway between the tip of the acromion and the olecranon process. Arm muscle area (AMA) and arm fat area (AFA) were calculated applying the formula by Gurney[21].

Body composition

BIA was performed using a BIA 2000M (Data Input GmbH, Darmstadt, Germany) applying alternating electric currents of 800 μA at 50 kHz. Patients were measured in the morning after an overnight fast, in the supine position with arms and legs abducted from the body. Source and sensor electrodes were placed on the dorsum of both hand and foot of the dominant side of the body. Total body water and fat free mass (FFM) were then calculated according to the formula by Kushner and Schoeller, body cell mass (BCM) and extracellular mass (ECM) were calculated as described previously[22]. BCM was also expressed corrected for height (BCM/m^2). ECM/BCM ratio was calculated to detect shifts between BCM and ECM.

Phase angle is a bioimpedance parameter that reflects the contribution between resistance, the pure opposition of a biological conductor to alternating electric current, and capacitance, which is the resisting effect produced by the tissue interfaces (arc tangent of this ratio transformed to degrees). It has been shown to be of clinical relevance in a number of disease settings[23,24].

Muscle function

Hand grip strength was measured both in the nondominant and in the dominant hand with a Digimax electronic dynamometer (Mechatronic GmbH, Germany). The patients performed the test while sitting comfortably with shoulder adducted and neutrally rotated, the elbow supported on a table and flexed to 90 degrees, forearm and wrist in neutral position. The patients were instructed to perform a maximal isometric contraction.

Table 2 Study population and differences between well and malnourished patients

Characteristics	Overall study population (n = 200)	Well nourished (SGA A) (n = 104)	Malnourished (SGA B C) (n = 96)	P
Gender (M/F)	94/106	44/60	50/46	NS
Age (yr)	54.9 ± 17.1	56.7 ± 16.7	52.9 ± 17.3	NS
Albumin (g/L)	37 ± 7.1	39 ± 7.0	35 ± 7.0	0.001
Body mass index (kg/m²)	23.7 ± 4.9	25.7 ± 4.6	21.6 ± 4.2	<0.000
Phase angle (°)	4.9 ± 1.1	5.2 ± 1.0	4.5 ± 1.1	<0.000
BCM/height (kg/m²)	8.1 ± 1.8	8.6 ± 1.7	7.4 ± 1.8	<0.000
ECM/BCM	1.3 ± 0.6	1.2 ± .4	1.4 ± .7	<0.000
Arm muscle area (mm²)	4327.5 ± 1261.8	4713.2 ± 1200.3	3916.1 ± 1200.1	<0.000
Hand grip strength (kg)	29.1 ± 11.0	30.6 ± 10.5	27.3 ± 11.4	0.030
Peak flow (L/min)	346.0 ± 118.4	353.4 ± 113.2	337.6 ± 124.2	NS

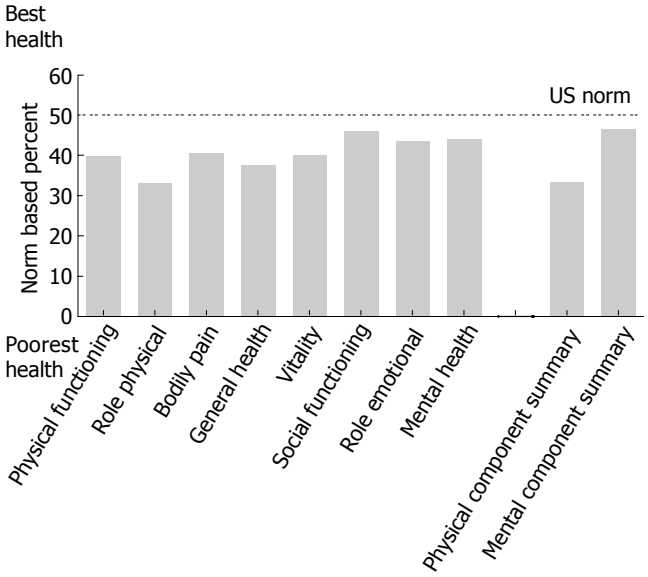

Figure 1 Quality of life in the study population.

Table 3 Study population and differences between liver cirrhosis and IBD patients

Differences	Liver cirrhosis	Inflammatory bowel disease	P
SGA A *vs* BC	34/27	33/36	NS
Gender (M/F)	33/28	29/40	NS
Age (yr)	56.8 ± 12.2	38.5 ± 13.4	< 0.000
Albumin (g/Ll)	33.0 ± 6.0	38.0 ± 7.0	< 0.000
Body mass index (kg/m²)	26.1 ± 4.9	21.2 ± 3.6	< 0.000
Phase angle (°)	4.7 ± 1.0	5.4 ± 1.2	< 0.000
BCM/height (kg/m²)	8.4 ± 1.9	7.9 ± 1.8	NS
ECM/BCM	1.4 ± .5	1.2 ± .8	< 0.000
Arm muscle area (mm²)	4606.3 ± 1485.9	3921.9 ± 1069.8	0.009
Hand grip strength (kg)	30.9 ± 11.6	31.5 ± 11.4	NS
Peak flow (L/min)	328.2 ± 116.9	375.8 ± 112.5	0.048

The test was repeated within 30 s and the highest value was recorded.

Peak expiratory flow was investigated by the Vitalograph© Peak Flow Meter. Patients were instructed to exhale as fast and as forcefully as possible. The test was repeated within 30 seconds and the highest value was recorded.

Statistical analysis

Statistical analysis was carried out using the software package SPSS© (version 11, SPSS Inc. Chicago, IL, USA). All data are given as mean ± SD. The Mann–Whitney U Test was used for comparison between groups. An acceptable level of statistical significance was established a priori at $P < 0.05$.

RESULTS

Patients' characteristics are given in Table 2. Patients classified moderately or severely malnourished (n = 96) by the Subjective Global Assessment were not significantly older than well nourished patients were. As anticipated however, they exhibited lower blood albumin levels, a significantly reduced body cell mass corrected for height and a significantly increased ECM/BCM ratio when compared to the well nourished patients. Body mass index and arm muscle area were also reduced. Moreover, muscle function as assessed by hand grip strength was significantly lower in the malnourished patients (Table 2). Peak flow was not different between well and malnourished patients.

All patients suffered impaired quality of life in all scales when compared with norm values from the US adult population (Figure 1). Characteristics of the liver cirrhosis and IBD patients are given in Table 3. IBD patients were significantly younger than liver cirrhosis patients and had higher serum albumin level. BCM did not differ between the two groups. IBD patients exhibited lower body

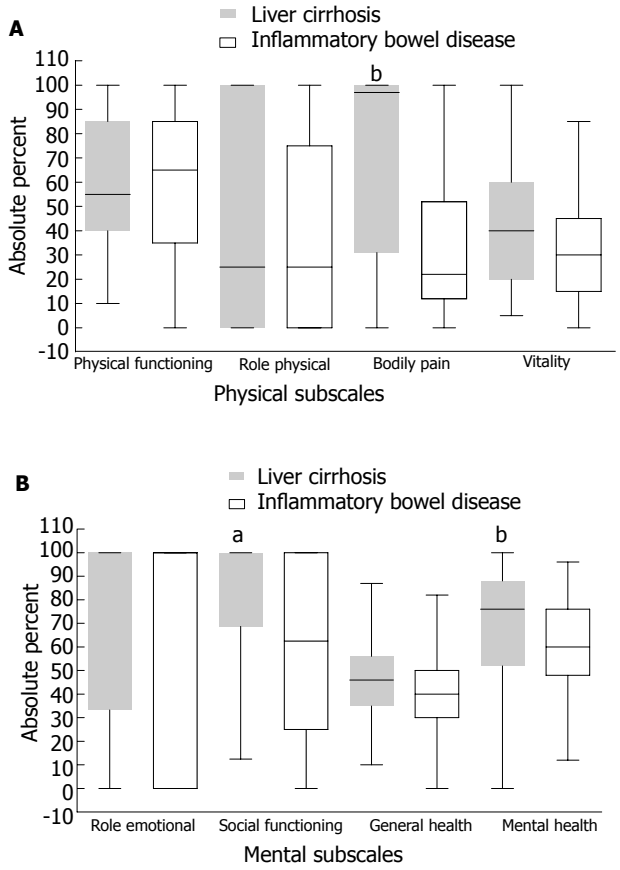

Figure 2 Quality of life ($^{a}P < 0.05$, $^{b}P < 0.01$).

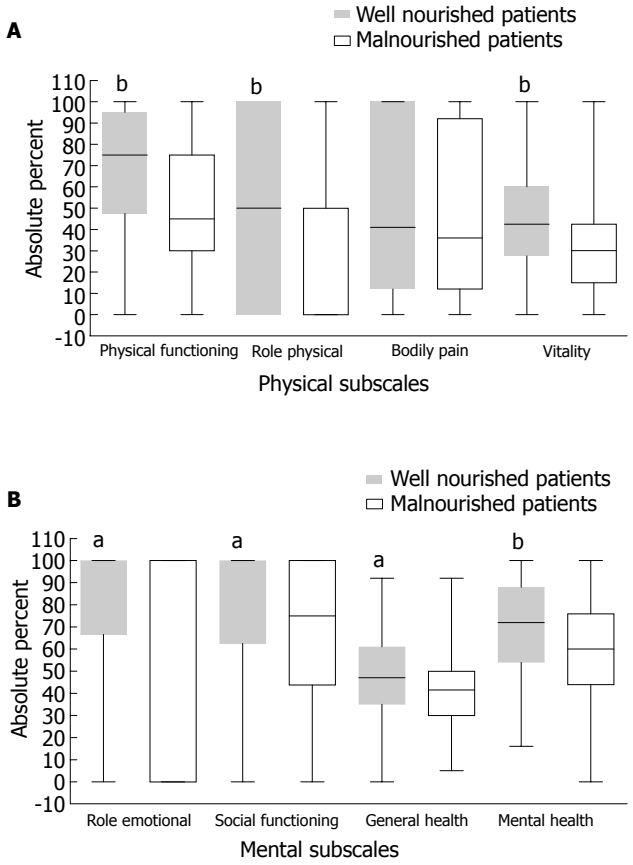

Figure 3 Quality of life in malnourished *vs* well nourished patients ($^{a}P < 0.05$, $^{b}P < 0.01$).

mass index, higher phase angle but reduced arm muscle area. ECM/BCM ratio was significantly also lower in IBD patients than liver cirrhosis patients. Hand grip strength was not different between the two groups but peak flow was higher in IBD patients. When evaluating their quality of life, IBD patients exhibited slightly lower quality of life values than liver cirrhosis patients did. This was significant in the scales concerning bodily pain ($P < 0.00001$), general health ($P < 0.00001$), mental health ($P = 0.031$) and social functioning ($P = 0.016$) (Figure 2).

Impact of malnutrition on parameters of quality of life

Malnourished patients from the overall study group suffered significantly impaired quality of life in both the mental and physical dimension of the quality of life. All subscales except bodily pain were reduced (Figure 3).

Evaluating the two chronic disease groups, malnourished liver cirrhosis patients experienced significantly lower quality of life in six out of the eight scales compared to well nourished liver cirrhosis patients. Malnourished IBD patients showed reduced quality of life in four out of the eight scales (Table 4).

DISCUSSION

In this study we investigated the quality of life of 200 patients with benign acute and chronic gastrointestinal disease in relation to their nutritional status. Quality of life in relation to nutritional status has been investigated in patients on haemodialysis, in institutionalized elderly and in patients with cancer[3-9,25,26]. At present however, there is not sufficient information on quality of life in relation to nutritional status in chronic benign gastroenterological disease, although malnutrition is frequently observed in these patients[27,28] and has well known adverse effects on clinical outcome[29-31]. In order to use quality of life as a clinical outcome parameter in these patients, it is therefore essential to enhance the understanding of quality of life and its association with nutritional status. We employed the SF 36 questionnaire which has been validated in various settings[32-34] and has the advantage of being disease unspecific. We moreover compared the results with values gathered in the 1998 US National Survey of Functional Status. Quality of life was generally impaired in our study population when compared to the norm values. Similar to the findings in other disease settings we also found that quality of life appeared to be compromised in malnutrition. Patients from the overall study group classified as malnourished experienced a significantly lower quality of life than the well nourished patients in all scales except in the perception of pain, leading to impairment of both the mental and the physical dimension of quality of life.

Among our study patients, the largest subgroups were patients with liver cirrhosis or IBD. Since health related quality of life obviously depends on underlying disease we also focused the evaluation on these patients.

Malnourished liver cirrhosis patients suffered statistically significant reductions in all scales except the

Table 4 Quality of life in malnourished *vs* wellnourished patients with either liver cirrhosis or inflammatory bowel disease (absolute percentage)

QOL scales	Liver cirrhosis			Inflammatory bowel disease		
	Wellnourished	Malnourished	*P*	Wellnourished	Malnourished	*P*
Physical functioning	74.0 ± 23.8	44.6 ± 22.1	0.000	66.6 ± 31.6	52.1 ± 28.4	0.036
Role physical	52.3 ± 42.6	22.2 ± 36.9	0.003	49.2 ± 45.1	24.4 ± 34.6	0.024
Bodily pain	88.9 ± 28.5	59.3 ± 47.4	0.007	67.7 ± 41.9	59.1 ± 46.5	NS
General health	82.6 ± 27.4	81.0 ± 28.7	NS	71.9 ± 31.6	51.1 ± 33.1	0.012
Vitality	73.2 ± 38.2	59.0 ± 39.4	NS	35.4 ± 33.5	37.7 ± 33.7	NS
Social functioning	51.8 ± 19.8	41.3 ± 16.1	0.041	42.1 ± 20.2	37.6 ± 13.5	NS
Role emotional	46.8 ± 21.3	31.7 ± 20.0	0.007	39.1 ± 23.7	26.9 ± 18.8	0.027
Mental health	78.1 ± 17.6	56.9 ± 25.9	0.001	64.9 ± 18.9	56.1 ± 21.8	NS

perception of social functioning and bodily pain whereas malnourished IBD patients were compromised in social functioning but mainly in the physical scales such as physical functioning, role physical and vitality.

One difficulty in evaluating our study results was to reliably differentiate between the effects of disease and the effects of malnutrition on quality of life, since they obviously are closely interrelated. Severity of disease is likely to trigger a worsening of nutritional status and malnutrition has well known adverse effects on the clinical outcome. Studies investigating health related quality of life in relation to disease severity do not show consistent findings. Kalantar-Zadeh *et al*[8] observed that quality of life assessed by the SF 36 had a strong association with disease severity judged by prospective hospitalization and mortality in patients on haemodialysis. Bianchi *et al*[10] showed a relationship between the disease severity index Child Pugh Score and the Psychological General Wellbeing Index. In patients with hepatocellular carcinoma however, quality of life was not primarily related to tumour mass or hepatocellular failure, but sleep disorders were shown to be strongly associated with quality of life. Similarly, in primary biliary cirrhosis, no associations between scales of the Nottingham health profile and biochemical liver tests, histological stages or duration of the disease were found[17]. Moreover, Hauser *et al* found psychiatric or active medical comorbidity to be of a larger influence than disease severity in a population with various liver diseases[35].

In this study, reliable data on disease severity were only available in liver cirrhosis and Crohn's Disease. In these groups, malnourished patients also experienced greater disease severity classified by the Child Pugh Score or the Crohn´s Disease Activity Index (data not shown). This clearly makes interpretation of the results more difficult and we consider it a limitation of the study. We cannot reliably conclude whether the observed impairments of quality of life in malnutrition are due exclusively to nutritional deprivation.

Malnutrition however is known to be associated with impaired functional status[1,2], worsened immune system[36], delayed recovery, higher mortality and morbidity[31]. The malnourished patients in our study population exhibited both lower muscle mass (as measured by body cell mass and arm muscle area) and muscle function and these changes were not attributable to age. It is therefore highly probable that malnutrition per se-defined mainly by weight loss in the preceding 6 months-has a measurable impact at least on the physical aspect of quality of life, which in the end is likely to affect the mental dimension as well.

Interestingly, overall quality of life was lower in the patients suffering from IBD than in liver cirrhosis patients, which was due to the perception of general health, mental health, vitality, social functioning and bodily pain. The physical scales physical functioning, role physical and vitality were not significantly higher in the IBD patients despite their younger age. There were no significant differences between patients with ulcerative colitis or Crohn's disease (data not shown). Considering that IBD in general is associated with a better prognosis and longer survival time than liver cirrhosis, these results are somewhat alarming. Our findings indicate that acute disease specific aspects such as diarrhoea and abdominal pain occurring in IBD rather than survival associated disease severity lead to a greater impairment of health-related quality of life. Marchesini *et al*[14] who studied quality of life in an Italian cohort of liver cirrhotics found similar associations between non life threatening symptoms such as muscle cramps and quality of life.

It is evident that subjective perception of quality of life depends on more than nutritional status or disease severity. Cancer patients currently on remission might e.g. experience a better quality of life than their healthy but overworked physician. When deciding on optimal therapy, it must also be taken into account that patients with impaired quality of life must be considered in need of intensified attention and care.

In conclusion, quality of life is impaired in benign gastrointestinal disease and becomes further compromised in malnutrition. It appears that liver cirrhosis patients experience a higher quality of life than IBD patients do, but the impact of malnutrition seems to be greater in liver cirrhosis than in IBD. Further studies are required to investi-

gate and identify therapy strategies that improve quality of life in chronic benign gastroenterological disease. Whether nutritional intervention is successful in enhancing quality of life in these patients remains to be studied.

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CLINICAL RESEARCH

Rectal nitric oxide as biomarker in the treatment of inflammatory bowel disease: Responders *versus* nonresponders

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Abstract

AIM: To explore rectal nitric oxide (NO) as biomarker of treatment response in ulcerative colitis (UC) and Crohn's disease (CD), and examine relationships between rectal NO, mucosal expression of NO synthases (NOS), and pro-inflammatory cytokines.

METHODS: Twenty-two patients with UC and 24 with CD were monitored during steroid treatment. Rectal NO levels were measured and clinical activities were assessed on days 1, 3, 7 and 28. Mucosal presence of NOS and pro-inflammatory cytokines were analyzed by immunohistochemistry and RT-PCR.

RESULTS: Active UC and CD displayed markedly increased rectal NO levels (10950 ± 7610 and 5 040 ± 1 280 parts per billion (ppb), respectively) as compared with the controls (154 ± 71 ppb, $P < 0.001$). Rectal NO correlated weakly with disease activity in both UC and CD ($r = 0.34$ for UC and $r = 0.48$ for CD, $P < 0.01$). In 12 patients, a steroid-refractory course led to colectomy. These patients had only slightly increased NO levels (UC: 620 ± 270 ppb; CD: 1260 ± 550 ppb) compared to those with a therapeutic response (UC: 18860 ± 530 ppb, $P < 0.001$; CD: 10060 ± 3200 ppb, $P < 0.05$).

CONCLUSION: Rectal NO level is a useful biomarker of treatment response in IBD as low NO levels predicts a poor clinical response to steroid treatment.

Key words: Crohn's disease; Cytokines; Nitric oxide; Steroids; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory diseases sharing a clinical course with flares of disease, characterized by an increase of symptoms due to increased inflammatory activity of the intestinal mucosa. Symptom-based clinical activity indices are today's standard methods applied to monitor disease activity in clinical trials, but rarely used in clinical practice. Available indices have been criticized for depending almost exclusively on clinical features that are often subjective. The use of systemic markers of inflammation, such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), platelet count and white blood cell (WBC) count are commonly used in clinical practice, but correlation to ongoing intestinal inflammation is poor[1,2]. An approach of direct assessment of mucosal inflammation has been promising, such as measurements of tumor necrosis factor (TNF)-α and interleukin (IL)-1β[3] and intestinal permeability tests[4]. The implementation of these tests is, however, restricted by a complicated analysis. The development of a more feasible objective marker of mucosal inflammation is, therefore, warranted. Data on local measurements of inflammatory products such as calprotectin, lactoferrin and nitric oxide (NO) in UC and CD have been promising[5].

We use a minimally invasive method employing chemiluminescence to measure rectal NO levels in conditions with inflamed intestinal mucosa[6,7]. Increased NO generation has been demonstrated in both UC and CD[6,8,9]. The excessive formation of NO is elaborated by inducible nitric oxide synthase (iNOS)[10,11]. Some groups have also reported increased endothelial NOS (eNOS) activity in inflamed mucosa in patients with UC[12] and iNOS expression restricted to epithelial cells[13,14]. Other groups have demonstrated iNOS expression also in

macrophages and granulocytes of the lamina propria[10,11].

The activation of iNOS is dependent on the transcription factor nuclear factor (NF)-κB, which is activated by bacterial products (e.g., endotoxins) and pro-inflammatory cytokines, such as TNF-α, IL-1β and interferon (INF)-γ. These pro-inflammatory cytokines act in synergy to stimulate NO production[15]. The aim of the present study was to investigate rectal NO levels as a biomarker of response in UC and CD during glucocorticosteroid (GCS) treatment, using established clinical indices as gold standard for clinical activity. We also assessed mucosal immunoreactivity to the cytokines TNF-α, IL-1β and INF-γ as well as activation of eNOS, iNOS and neuronal NOS (nNOS).

MATERIALS AND METHODS

Patients

Patients treated with prednisolone (0.5-1 mg/kg orally) at the Karolinska University Hospital for active UC or CD were eligible to enter this study. Forty-six consecutive patients, 22 with UC and 24 with CD, diagnosed by conventional endoscopic, radiological and histological criteria of IBD[16] were recruited to this study. Diagnosis of UC or CD was confirmed for all included patients at follow-up one-year after completion of the study. The majority of patients had concomitant medication with aminosalicylates, whereas no patient was on immunomodulators at baseline (Table 1).

The patients were studied at four different occasions during the first month of treatment, before onset of prednisolone treatment (d 1), and at follow-up during ongoing treatment on days 3, 7 and 28. A flexible sigmoidoscopy was performed at baseline, and at all follow-up visits. Disease activity was assessed using the Disease Activity Index (DAI)[17] for the patients with UC. The Harvey-Bradshaw Index (HBI)[18] was used for the patients with CD. The endoscopic classification was done according to the DAI score also for the CD patients.

At the last visit (d 28), the patients were divided into responders (remission) and non-responders (no remission) for further subgroup analysis. Remission was defined as DAI $\leqslant$ 2 in UC, and HBI $\leqslant$ 4 in CD, whereas non-responders were defined as DAI $\geqslant$ 3 in UC and HBI $\geqslant$ 5 in CD, respectively.

Two different control groups were used: the control for immunohistochemistry analyses consisted of six individuals undergone control colonoscopy after prior polypectomy, and the control for rectal NO consisted of 25 healthy volunteers with no history of gastrointestinal symptoms or abdominal surgery.

The study was approved by the Karolinska Institutet Ethics Committee. Written informed consent was taken from all patients.

Determination of rectal NO levels

NO was measured with a chemiluminescence analyzer (CLD 700, *Eco* Physics, Dürnten, Switzerland). The detection limit for NO was 1 part per billion (ppb). The analyzer was calibrated at known concentrations (100-10000 ppb) of NO in nitrogen gas (AGA, Lidingö, Sweden), administered through an electromagnetic flow controller (Environics, Middletown, CT, USA). The chemiluminescence assay is highly specific for NO without interference from other nitrogen oxides[19]. For sampling of rectal gas, we applied an all-silicon catheter (Argyle®, Sherwood Medical, Tullamore, Ireland) inserted into the rectum, using lubrication gel, free of local anesthetics, to a level 10 cm above the anal sphincter[7]. The balloon of the catheter was then inflated with 10 mL of ambient air containing less than 5 ppb of NO, and left for 10 min to equilibrate with gases in the rectum. Thereafter, the gas was withdrawn from the catheter balloon and diluted to a final volume of 50 mL before chemiluminescence analysis with correction for dilution. Analyses were performed within 15 min of sampling[7]. In cases where measurements exceeded the upper detection limit, further dilution steps were made in order to measure NO within the calibrated range.

Table 1 Patient characteristics

	Ulcerative colitis (n = 22)	Crohn's disease (n = 24)
Sex (M/F)	13/9	14/10
Age (yr) (mean and range)	41 (18 – 78)	42 (20 – 69)
Duration (yr) (mean and range)	7.4 (0 – 30)	9 (0 – 28)
Smoking habits (n)		
Active	2	3
Former	6	8
Non-smoker	7	9
Unknown	7	4
Concomitant medication (n)		
Aminosalicylates	18	12
Local steroids	6	2
Site of disease		
Ileocolonic		24
Rectum	3	
Proctosigmoiditis	6	
Extensive/total	13	
Disease activity index (mean and range)	9.4 (5 – 12)	
Harvey-Bradshaw index (mean and range)		14.9 (4 – 29)
Endoscopic score (mean and range)	2.2 (1-3)	2.1 (0-3)

Immunohistochemistry

Mucosal biopsies were sampled at sigmoidoscopy. Biopsy specimens were always taken in the vicinity of lesions or ulcerations. In case of colectomy (n =12), the surgical specimens were additionally used for analyses. Biopsies were kept in Histocon (Histolab, Gothenburg, Sweden) on ice and snap-frozen in liquid nitrogen within 30 min. Approximately 6 μm thick cryostat sections were mounted on gelatin-coated glass slides, and stored at -80 ℃. Before staining, the slides were thawed at room temperature and subsequently fixed in cold 20 g/L formaldehyde in phosphate-buffered saline (PBS). All following incubation and washing steps were performed in PBS supplemented with 1 g/L saponin (Sigma Chemicals, St Louis, MO, USA) to permeabilize cellular membranes[20]. Peroxidase activity was blocked with 3 g/L hydrogen peroxidase and 1 g/L sodium azide in PBS-saponin[21]. Human and goat serum

(Vector Laboratories Inc, Burlingame, CA, USA) and avidin/biotin blocking kit (Vector Laboratories) were used to block unspecific bindings. The sections were incubated overnight at 4℃ with mouse monoclonal antibodies to iNOS (NOS-IN 20 mg/L, Sigma), nNOS (NOS-BI 73 mg/L, Sigma), eNOS (NOS-EI 17 mg/L, Sigma), IL-1β (2D8 1.4 mg/L, ImmunoKontact, Abingdon, Oxon, UK), TNF-α (Mab I 10 mg/L + Mab II 14 mg/L, Pharmigen, San Diego, CA, USA), and to INF-γ (7-B6- 10 mg/L + 1-DIK 10 mg/L, Mabtech AB, Nacka, Sweden). Mouse IgG_1 (28 mg/L, Dako, Glostrup, Denmark) served as an isotype-matched negative control. Biotinylated goat anti-mouse IgG_1 (4 mg/L, Caltag laboratories, Burlingame, CA, USA) was used as secondary antibody, except for the eNOS staining (an IgA antibody) where it was substituted with biotinylated goat anti-mouse immunoglobulin (11 mg/L, Dako). The biotinylated secondary antibodies were followed by horse-radish peroxidase-conjugated avidin/biotin-complex (Vectastain, ABC-elite, Vector Laboratories). Positive peroxidase staining was developed with 3, 3´-diaminobenzidine (DAB peroxidase substrate kit, Vector Laboratories) and counterstained with haematoxylin (Histolab).

Quantification of immunohistochemical staining

All microscopic evaluations were performed by one investigator (T.L.) who was blinded to the clinical data as well as analyzed parameters (eNOS, nNOS, iNOS, TNF-α, IL-1β, INF-γ and IgG_1). To validate the observer's quantification, eight randomly chosen sections were additionally analyzed by a second observer (C. J.). Absolute correlation was seen between the two observers. Sections were analyzed using light microscope (Nikon Ltd, Tokyo, Japan). For each section, three different grid areas rich in positive cells of satisfactory technical quality were chosen for quantitative analysis of NOS and cytokine immunohistochemistry. Due to some background staining of the epithelial cells, we restricted our quantitative analysis to the lamina propria. For each area, the number of positive cells was counted at high-power magnification (× 400), thereafter the total number of positive cells was divided with the total grid area. For each section, the result was expressed as the mean number of positive cells per one grid area. For comparison, the percentage of positive cells in the lamina propria was also calculated. A high correlation between immunohistochemistry quantification expressed as positive cells per grid area and percentage positive cells of total cell number in lamina propria was observed ($r = 0.98$, $P < 0.001$).

Measurement of iNOS gene expression

Colonic biopsies were taken from four patients with CD and two with UC. The biopsies were collected from both normal and inflamed parts of the colon in each patient. Tissue samples were immediately placed in RNAlater (Qiagen, Hilden, Germany), stored for 24 h at 2-8℃ and then at -80℃.

After thawing, total RNA was isolated from the biopsies using the RNeasy mini kit (Qiagen). cDNA was synthesized using oligo $(dT)_{20}$ primers and SuperScript III enzyme (Invitrogen, Carlsbad, CA, USA). Semi-quantitative RT-PCR was performed using cDNA normalized against the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR products obtained with primers specific for the human iNOS gene (sense 5´-CCAAAAGGGTCATCATCTCT-3´; antisense 5´-CCTGCTTCACCACCTTCTTG-3´) (GenBank accession number AF049656) in the linear amplification range (39 to 45 cycles) were electrophoresed on a 15 g/L agarose gel, photographed and bands quantified (Kodak Gel Logic 100 Imaging System, Rochester, NY, USA).

Table 2 Disease activity index, UC patients

t/d	All (n = 22)	Responder (n = 10)	Non-responder (n = 12)	Operated (n = 5)
1	9.4 (5-12)	9.5 (5-12)	9.4 (5-12)	11.2 (10-12)
3	7.4 (2-12)	7.1 (2-10)	7.6 (5-12)	12
7	5.7 (2-9)	4.8 (2-7)	7.2 (5-9)	NA
28	2.5 (0-8)	0.8 (0-2)	5 (3-8)	NA

Responder (DAI 2); non-responder (DAI ⩾ 3).

Table 3 Harvey-Bradshaw index, CD patients

t/d	All (n = 24)	Responder (n = 8)	Non responder (n = 16)	Operated (n = 7)
1	14.9 (4 - 29)	12.8 (4 - 26)	15.9 (9 - 29)	16.9 (11 - 29)
3	11.2 (2 - 22)	7.9 (2 - 13)	12.9 (2 - 22)	16.2 (11 - 22)
7	7.7 (1 - 19)	5.8 (1 - 19)	9.6 (2 - 13)	11.5 (10 - 13)
28	4.2 (1 - 9)	1.9 (1 - 3)	6.9 (4 - 9)	NA

Responder (HBI ⩽ 4); non-responder (HBI ⩾ 5).

Statistical analysis

For statistical analysis and graph plotting, GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used. Data were expressed as mean ± SE, and range where appropriate. Groups of independent data were compared using the Mann-Whitney U test. Intra-group variation was analyzed with the paired Wilcoxon test. Correlation coefficients between different analyses were calculated using the Spearman rank-test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical activity

Baseline DAI or HBI scores did not differ significantly between steroid responding *versus* non-responding patients and did not predict clinical outcome. On d 28, 45% (10/22) patients with UC and 33% (8/24) patients with CD were in remission (DAI ⩽ 2 and HBI ⩽ 4, respectively) (Table 2, Table 3). In 26.08% (12/46) patients (5 UC, 7 CD), colectomy was carried out on d 2-26 due to steroid-refractory severe disease. The DAI and HBI scores in this subgroup did not differ significantly on d 1 between subsequently operated patients and those responding to treatment (DAI: 9.5 ± 0.7 *vs* 11.2 ± 0.5 for responding and operated UC patients; and HBI: 12.8 ± 2.3 *vs* 16.9 ± 2.3

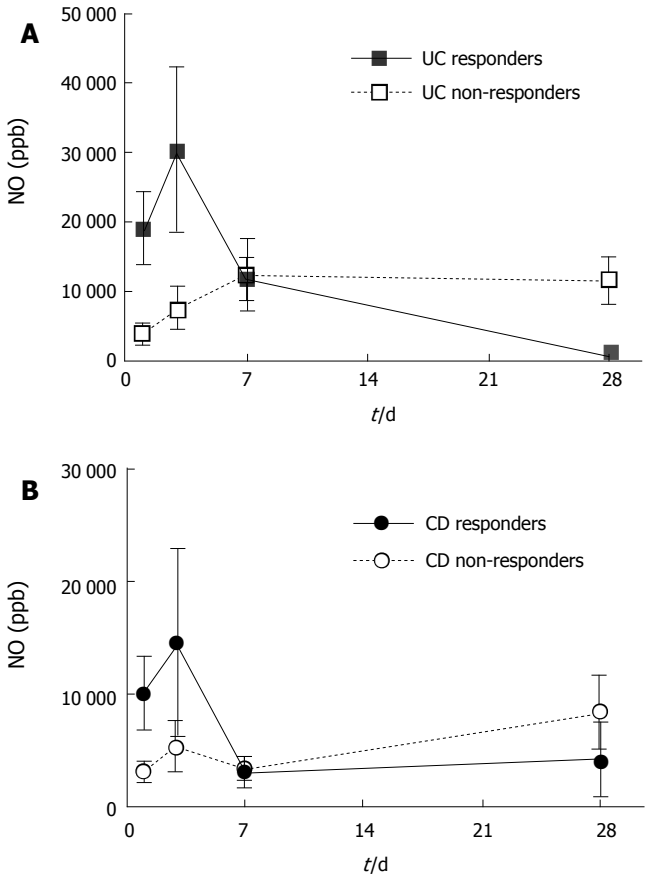

Figure 1 **A**: Rectal NO levels in UC; **B**: Rectal NO levels in CD.

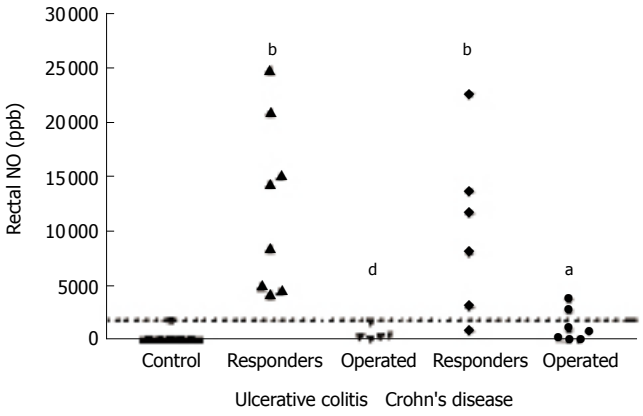

Figure 2 Rectal NO levels in the treatment of UC and CD patients on d 1. $^{b}P < 0.001$ *vs* Control; $^{d}P < 0.01$, $^{a}P < 0.05$ *vs* patients responding to treatment.

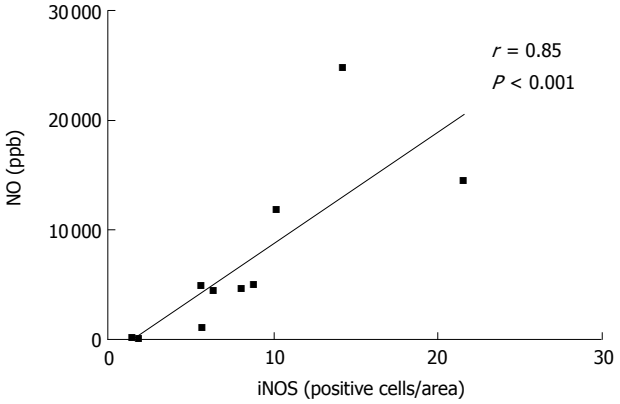

Figure 3 Correlation between mucosal iNOS-expressing cells and rectal NO levels in patients with UC.

for responding and operated CD patients) (Table 2, Table 3).

Rectal NO levels

On d 1, patients with active UC and CD had greatly increased rectal NO levels (10 950 ± 7610 and 5040 ± 1280 ppb, respectively) as compared with the controls (154 ± 71 ppb, all $P < 0.001$). Repeat measurements of rectal NO showed a numerical increase on d 3 compared to d 1, after which a decrease was seen (Figure 1). This pattern of NO release was mainly attributed to the patients responding to steroid treatment, whereas non-responders displayed a less prominent increase on day 3, and showed no subsequent decrease (Figure 1). For steroid-responding patients, rectal NO levels decreased significantly between d 1 and 28 (from 18 860 ± 5390 to 850 ± 450 ppb in UC, and 10 060 ± 3200 to 4130 ± 3380 ppb in CD, $P < 0.001$ and $P < 0.05$, respectively).

Rectal NO levels were correlated weakly with clinical activity scores of the whole study population, DAI for UC ($r = 0.34$, $P < 0.01$), and HBI for CD ($r = 0.48$, $P < 0.01$). However, the association was clear-cut in the group of responders to treatment ($r = 0.72$ for UC and $r = 0.64$ for CD, all $P < 0.001$).

A different rectal NO pattern was seen in the subgroup of patients in whom colectomy was carried out. The rectal NO levels of these patients were significantly lower at baseline (620 ± 270 ppb in UC, and 1260 ± 550 ppb in CD) than corresponding values in the patients with a treatment response ($P < 0.001$ for UC and $P < 0.05$ for CD) (Figure 2). Applying a cut-off level of rectal NO at 2000 ppb in combination with DAI ⩾ 10 for patients with UC identified all five patients with a steroid-refractory disease, leading to colectomy within 7 d, Whereas none of the UC patients responding to treatment combined rectal NO ⩽ 2000 ppb and DAI ⩾ 10. For CD patients, the same cut-off of NO ⩽ 2000 ppb in combination with HBI ⩾ 10 detected 5 of 7 patients subsequently operated, and was seen in 3 CD patients not leading to surgery.

Expression of NO synthases and pro-inflammatory cytokines

The number of iNOS-expressing cells, as judged by immunohistochemistry, was significantly higher in patients with UC and CD on d 1 as compared with the healthy controls (all $P < 0.001$). Furthermore, the number of iNOS-positive cells decreased between d 1 and 28 (Figure 4), reaching a borderline significance by pooling UC and CD patients ($P = 0.064$). The number of iNOS-positive cells was obviously correlated with rectal NO levels in patients with UC ($r = 0.53$, $P < 0.01$). This association was strengthened by analyzing the patients responding to treatment ($r = 0.85$, $P < 0.01$) (Figure 3).

However, no correlation was found between number of iNOS-positive cells and rectal NO levels in patients with CD. The majority of iNOS-positive cells in lamina

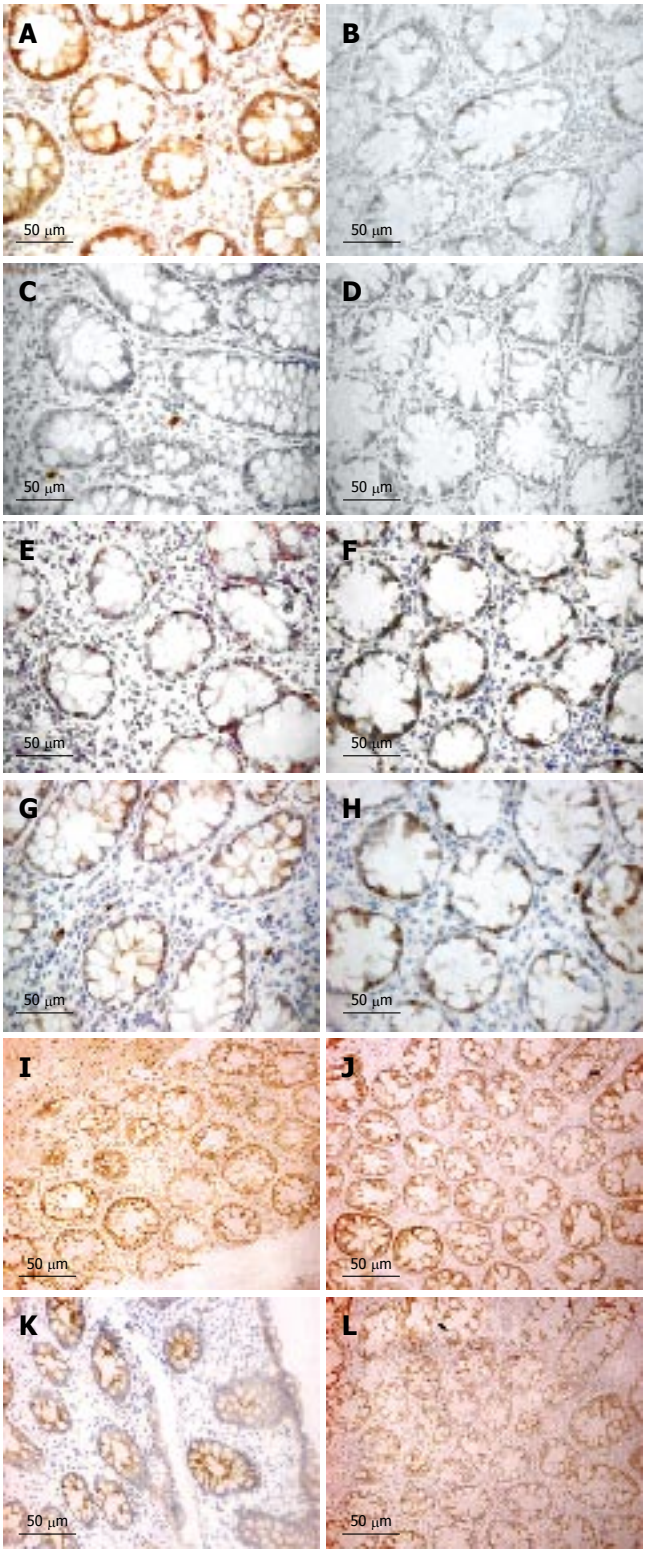

Figure 4 **A** Colonic histology of CD patients (**A**, **C**, **E**, **G**) before and (**B**, **D**, **F**, **G**) after treatment on d 28. Antibodies against iNOS (**A**, **B**), TNF-α (**C**, **D**), IL-1β (**E**, **F**) and INF-γ (**G**, **H**); Colonic histology of healthy control. Antibodies against iNOS (**I**), TNF-α (**J**), IL-1β (**K**) and INF-γ (**L**).

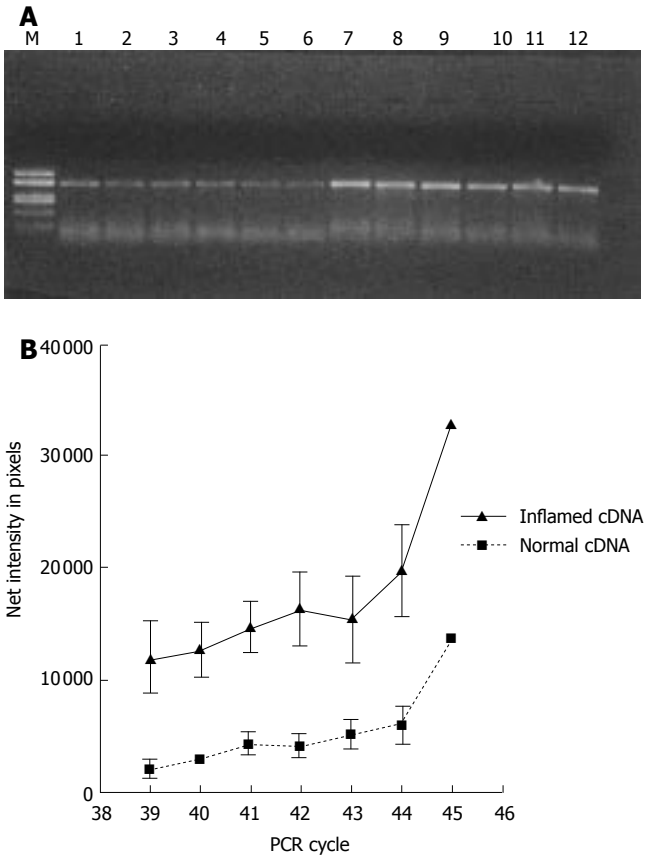

Figure 5 **A**: Semi-quantitative PCR iNOS gene of cDNA from non-inflamed (lanes 1-6), and inflamed (lanes 7-12) colon biopsies from a patient with ulcerative colitis; **B**: Difference in semi-quantitative PCR iNOS gene expression.

propria had the morphology of polymorphonuclear leukocytes (Figure 4).

Semi-quantitative RT-PCR showed over-expression of iNOS mRNA in the inflamed compared to normal parts of the mucosa ($P < 0.05$) (Figure 5). Presence of eNOS-positive cells was abundant in all sections and localized to the epithelium, but showed no temporal relationship with rectal NO levels. Recovery of nNOS staining was slight and, when occurring, mostly seen in the submucosa in nerve cells and randomly in mononuclear cells of the lamina propria.

TNF-α, IL-1β and INF-γ expressions, as judged by immunohistochemistry, were restricted to mononuclear cells in the lamina propria. IL-1β expression was significantly increased on d 1 compared to healthy controls ($P < 0.05$), whereas TNF-α and INF-γ were not. However, both TNF-α and IL-1β decreased numerically between d 1 and 28 for UC and CD (Figure 4). By pooling data from UC and CD patients, a significant reduction was seen ($P < 0.05$ for both TNF-α and IL-1β immunoreactivity). INF-γ did not change between d 1 and 28. A marked increase of iNOS-expressing cells was correlated with TNF-α expression in both UC ($r = 0.46$, $P < 0.05$) and CD ($r = 0.44$, $P < 0.05$). By pooling data for UC and CD, a weak correlation was detected for IL-1β ($r = 0.33$, $P < 0.05$). No correlation was seen between the numbers of iNOS and INF-γ-expressing cells.

DISCUSSION

This study provide evidence suggesting that the greatly increased rectal NO levels seen in active UC and CD are elaborated by high iNOS activity. In our study, a strong correlation was seen between rectal NO levels and clinical

activity indices in patients responding to steroid treatment. A relationship between ongoing inflammatory activity and increased NO production in the gut is well established[6,8]. In agreement with this, we found a correlation with clinical disease activity indices in both UC and CD.

Taking into account the relatively low number of patients recruited, the subgroup analysis have to be interpretated with caution. In the non-responding group, no correlation was seen between rectal NO and clinical activity indices. This is in part explained by the aberrant rectal NO pattern in the 12 patients who subsequently underwent colectomy due to severe disease. The finding that baseline rectal NO $\leqslant$ 2000 ppb in severe UC detected all patients in need of surgery within 7 d due to steroid-refractory disease makes it tempting to suggest rectal NO measurement as a predictive marker of the need of colectomy in active UC. In CD patients, rectal NO seems to be a less strong marker for steroid-response, which is in agreement with previous studies demonstrating a 70% endoscopic remission post-treatment in UC as compared with only 13% endoscopic remission in CD[22,23], as well as a recent study by Costa *et al*[24] showing that fecal calprotectin is a stronger marker for relapse in UC than in CD[24].

Due to the high affinity of NO to hemoglobin[25], one may argue that luminal NO is scavenged by blood in the colon, which should cause falsely low rectal NO levels in the most severe UC and CD cases. This is however unlikely since other severe cases in our group displayed high rectal NO levels in the presence of overt rectal bleeding.

The role of NO in intestinal inflammation is unclear, as both pro-inflammatory and tissue-protective properties have been demonstrated[26]. Our observation that high rectal NO levels at the first visit was associated with a favorable clinical outcome is consistent with the idea that NO may act as an endogenous inhibitor of an aggregated immune response. Tissue-protective properties of NO have been shown in animal models of colitis[27]. The finding that NO production in collagenous colitis, a chronic inflammatory bowel disease without mucosal cell damage[28], might be even greater than in active IBD supports the concept of NO acting as a modulator of inflammatory activity in the gut. An uncontrolled chronic inflammation could, however, lead to an intracellular depletion of L-arginine, during which NOS will produce O_2^- instead of NO, O_2^- will then immediately react with NO to form $ONOO^-$, considered to be highly cytotoxic[29].

Genetic polymorphisms of iNOS might be a plausible explanation for the different rectal NO patterns seen in the group of responding *versus* non-responding patients. iNOS polymorphism has previously been associated with outcome variables in different diagnoses[30-32].

There is substantial evidence pointing towards iNOS-expressing epithelial cells lining the mucosa as a major site of NO production in intestinal inflammation[13-15]. We could not confirm this finding in our present study as we encountered unspecific staining of the epithelial cells, but we consider the correlation between the number of iNOS-positive cells in lamina propria and the production of NO, measured as rectal NO levels, as circumstantial evidence, suggesting the importance of these cells in the production of the increased NO levels seen in active UC and CD. This is supported not only by earlier studies showing iNOS-positive macrophages and granulocytes in the mucosa in IBD[11], but also by our present finding of a molecular activation of iNOS within the tissue. Our morphological examination of iNOS-positive cells ascribes polymorphonuclear leukocytes as a plausible cellular source of NO production.

Whether the constitutive forms of NOS contribute to the increased NO production in IBD is unclear. For eNOS or nNOS, we could not detect any differences in staining between healthy controls, active IBD or IBD in remission. Little information is available on possible changes in eNOS and nNOS in IBD in literature. One previous study reports findings in line with our results[14], i.e. activation of mainly iNOS, whereas another group claims specific changes in eNOS and nNOS expression in UC[12].

Determination of tissue cytokine levels in IBD has resulted in disparate results. In general, pro-inflammatory cytokines (TNF-α, IL-1β and INF-γ) are believed to have a regulatory function in the activated immune response in IBD. Clearly the last years' development has pin-pointed the key role of TNF-α in CD[3,21,33]. In line with some previous studies, we failed to show increased TNF-α expression in IBD compared to controls[34,35]. However, we found a decrease in TNF-α expression as a response to treatment, supporting data demonstrating an association between TNF-α expression and active IBD[3,36,37]. Our data showing increased IL-1β expression in active IBD compared to healthy controls are consistent with earlier studies providing evidence that IL-1β has a central role in the mucosal inflammation as seen in IBD[34,36,37]. In our study, we found no support for INF-γ as a marker of disease activity in IBD.

We found a correlation between TNF-α and IL-1β expression and iNOS expression, supporting the concept that these cytokines may have a role in inducing NO production[15].

In summary, our study shows that active UC and CD are associated with highly increased rectal NO levels. Rectal NO levels decreases in response to steroid treatment, hence offering a plausible and feasible objective method to monitor disease activity in IBD. Low rectal NO levels $\leqslant$ 2000 ppb might be a predictive marker for steroid-refractory IBD requiring acute colectomy. Furthermore, our data suggest that the major part of the increased NO production seen in active IBD is elaborated by the inducible form of NOS.

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CLINICAL RESEARCH

Effects of adacolumn selective leukocytapheresis on plasma cytokines during active disease in patients with active ulcerative colitis

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Abstract

AIM: To investigate the relationship between ulcerative colitis (UC) clinical activity index (CAI) and circulating levels of IL-1ra, IL-10, IL-6 and IL-18.

METHODS: Blood levels of IL-1ra, IL-10, IL-6 and IL-18 were measured in 31 patients with active UC, the mean CAI was 11.1, ranging from 5-25; and 12 healthy individuals as controls. Patients were given granulocyte and monocyte adsorptive apheresis (GMA) with Adacolumn. Leucocytes which bear the FcγR and complement receptors were adsorbed to the column leucocytapheresis carriers. Each patient could receive up to 11 GMA sessions over 8 wk.

RESULTS: We found strong correlations between CAI and IL-10 (r = 0.827, P < 0.001), IL-6 (r = 0.785, P < 0.001) and IL-18 (r = 0.791, P < 0.001). IL-1ra was not correlated with CAI. Following GMA therapy, 24 of the 31 patients achieved remission and the levels of all 4 cytokines fell to the levels in healthy controls. Further, blood levels of IL-1ra and IL-10 increased at the column outflow and inflow at 60 min suggesting release from leucocytes that adhered to the carriers.

CONCLUSION: Elevated blood levels of IL-6 and IL-18 together with peripheral blood granulocytes and monocytes/macrophages in patients with active UC show activative behaviour and increased survival time can be pro-inflammatory and the targets of GMA therapy.

Key words: Interleukin-1 receptor antagonist; Interleukin-6; Interleukin-10; Interleukin-18; Ulcerative colitis

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INTRODUCTION

Factors which initiate and perpetuate ulcerative colitis (UC) are not well understood. However, the condition is often associated with elevated circulating granulocytes and monocytes/macrophages[1-5] that show activative behaviour and increased survival time[6-10]. Another feature of active UC is the increased generation and activities of inflammatory cytokines which can initiate and perpetuate the disease[11-15]. Furthermore, recent clinical and basic research data indicate that the clinical relapse of UC is mediated by an unchecked influx of granulocytes and monocytes/macrophages into the mucosal tissue[16-19]. The level of neutrophils in the mucosa was quantitatively related to the severity of intestinal inflammation and relapse both in UC and Crohn's disease[16].

Granulocytes and monocytes/macrophages are major sources of inflammatory cytokines[20-24] and therefore, it is assumed that high and activated peripheral blood lev-

els of these leucocytes can be a major pro-inflammatory condition. Factors believed to contribute to the elevated neutrophil counts and increased survival time include inflammatory cytokines[8] and paradoxically corticosteroids[9] that are given to most patients with severe active UC. The circulating levels of anti- and pro-inflammatory cytokines are likely to follow leucocyte levels or activities and are potentially related to disease activity.

However, leucocytes produce proinflammatory as well as anti-inflammatory cytokines. Thus, interleukin-6 (IL-6) produced by neutrophils and monocytes/macrophages (as well as other cell types including endothelial cells) is reported to play a major role in neutrophil and monocyte recruitment during inflammation[15]. Similarly, IL-18 produced by monocytes/macrophages is thought to be a potent inducer of interferon-γ (IFN-γ) production, hence contributing to the induction of Th1 responses[23, 24] A similar scenario may be described for anti-inflammatory cytokines. Thus, elevated level of IL-1 receptor antagonist (IL-1ra) is considered to reflect a natural compensatory mechanism to counter the activities of the pro-inflammatory cytokine IL-1β in inflammatory diseases[25-26]. Similarly, during inflammation, production of IL-10 increases and potentially can switch off the production of pro-inflammatory cytokines including IFN-γ, IL-1β, IL-6, IL-8, IL-12 and tissue necrosis factor (TNF-α).

Therefore, there are cytokines which can initiate and perpetuate inflammation and others which mitigate the inflammation. Accordingly, cytokines currently represent the best validated therapeutic targets[25-30]. With this in mind, we studied the circulating levels of 2 major anti-inflammatory and 2 major pro-inflammatory cytokines during active UC and during remission. IL-10 and IL-1ra were chosen as two known anti-inflammatory cytokines while IL-6 and IL-18 were considered to represent two typical pro-inflammatory cytokines. Since peripheral blood granulocytes and monocytes/macrophages are thought to represent major sources of both pro- and anti-inflammatory cytokines[1-6], we targeted these cells using granulocyte and monocyte/macrophage adsorptive apheresis (GMA) with Adacolumn[4,5].

MATERIALS AND METHODS

Study objectives

First, to see the relationship between CAI and circulating levels of the two major anti-inflammatory cytokines IL-10, IL-1ra and two pro-inflammatory cytokines IL-6 and IL-18 during active UC. Second, to investigate the effects of adsorptive granulocytes and monocyte/macrophage apheresis (GMA) on the levels of these cytokines and CAI. This was to provide an insight into the mechanisms of clinical efficacy of GMA with Adacolumn presently and hitherto reported[4,5,31]. Third, to identify predictors of response to GMA.

Patients

The demography of the 31 patients included in this study is presented in the Table 1. There were 17 males and 14 females, mean age 34.3 years, range 14-58 years.

Table 1 Demography of 31 patients with active ulcerative colitis who were investigated in this study

Backgroung	Measurement	
Male/Female	17/14	
Age, yr (range)	34.5-15.3	(14-58)
Age at first attack (yr)	29.1-11.9	(12-54)
Duration of UC (yr)	5.9-7.2	(0.5-26)
Number of relapses	4.3-3.9	(1-15)
Classification of severity		
Mild		4
Moderate		16
Severe		11
Mean (range) clinical activity index (CAI)[33]		11.1(5-25)
Total colitis		24
Left sided colitis		7

CAI was determined according to Rachmilewitz[32]. UC was severe in 11, moderate in 16 and mild in 4. All patients had been treated with conventional medications including 5-aminosalicylic acid (5-ASA) and prednisolone prior to entry. Eighteen of the 31 patients were steroid dependent , 9 were steroid refractory and 4 were steroid naive. The conventional medication was continued and the steroid dose was tapered when CAI score decreased to remission level (4 or less). Twelve healthy individuals of the same age range served as a control group.

Granulocyte and monocyte adsorptive apheresis

GMA was performed with Adacolumn precisely as described previously[4,5,20,31,33]. In Japan, GMA with Adacolumn has been approved by the Ministry of Health for public funding to treat patients with active UC. Adacolumns were purchased from Japan Immunoresearch Laboratories (Takasaki, Japan). Each patient could receive up to 11 GMA sessions. Patients with CAI > 12 received 2 sessions/wk in the first 3 wk and then one session/wk. During wk 6, CAI scores were determined and GMA was continued until patients had received up to 11 sessions. This therapy was added to the patients' ongoing treatment following a relapse or worsening UC symptoms. No additional conventional medication was given during this study.

Assessment of response to therapy

CAI equal or greater than 5 was the entry criteria. Clinical remission was defined as a CAI decrease to 4 or less and mucosal vascular patterns had become visible (at least partly). When CAI had fallen, but was still above 4, the patient was considered to have improved.

Measurement of cytokines

Peripheral blood was sampled directly via a venepuncture in the antecubital vein and also from the Adacolumn inflow at the start of GMA and then at the end of the 60 minutes GMA session from the column inflow and outflow (blood which was returning to patients). IL-1ra, IL-10, IL-6 and IL-18 were measured using enzyme linked immuno-sorbent assays (ELISA). Human IL-1ra ELISA

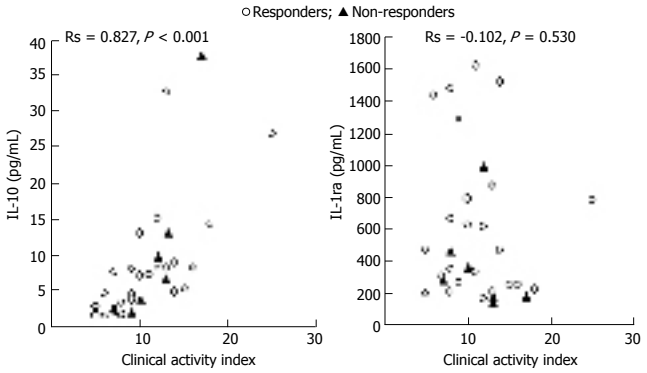

Figure 1 The relationship between blood levels of IL-10 and IL-1ra as measured in serum samples and the ulcerative colitis CAI in 31 patients with UC.

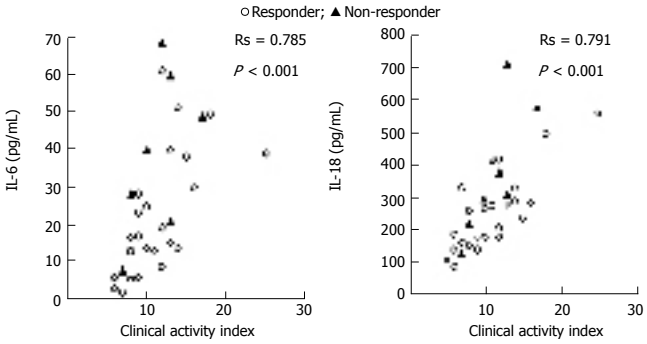

Figure 2 The relationship between blood levels of IL-6 and IL-18 and CAI in 31 patients with UC.

kit was from R&D systems (Minneapolis, USA); the ELISA kit for IL-10 was from Biosource Europe SA (Nivelles, Belgium); human IL-18 ELISA Kit was from MBL (Medical and Biological Laboratories, Nagoya Japan) and the ELISA kit for IL-6 was from FUJIREBIO Inc. (Tokyo). All assays were done at an special research laboratory (SRL) in blind manner and the data were processed by an individual who was unaware of the subjects' clinical conditions or the purpose of the study.

Ethics

As indicated above, Adacolumn is officially approved in Japan for the treatment of patients with active UC. However, when blood samples were required for research other than for routine clinical laboratory, patients were informed of the extra volume of blood to be taken and the purpose of the blood sample. All patients we consulted agreed to donate blood samples for assaying cytokines. They were advised that refusal to donate blood will not jeopardize their future treatment and care.

Statistical analysis

The amounts of IL-1ra, IL-10, IL-6 and IL-18 are presented as individual observations and comparisons are made using the Stat View Software. Statistical tests were the Mann-Whitney U test, Scheffe's test, the Turkey-Kramer test or the Spearman's rank correlation test, indicated in the figure legends. A significance level of 0.05 was used for all statistical tests, and two-tailed tests were applied when appropriate.

RESULTS

Blood levels of anti- and pro-inflammatory cytokines during active UC

The relationship between CAI and blood levels of IL-10 and IL-1ra in 31 patients is presented in Figure 1. The Spearman's rank correlation coefficient (Rs) showed a strong positive relationship between blood levels of IL-10 and CAI, while no correlation was found between blood levels of IL-1ra and CAI. Similarly, the majority of non-responders to GMA (albeit a few) had low blood levels of IL-1ra, while no obvious difference between responders and non-responders to GMA was seen for circulating IL-10 levels. Likewise, Figure 2 shows the relationship between blood levels of IL-6, IL-18 and CAI in the 31 patients. Also for these two pro-inflammatory cytokines, Rs shows a strong positive correlation with CAI. Most of the few non-responders to GMA had high blood levels of IL-6 and IL-18.

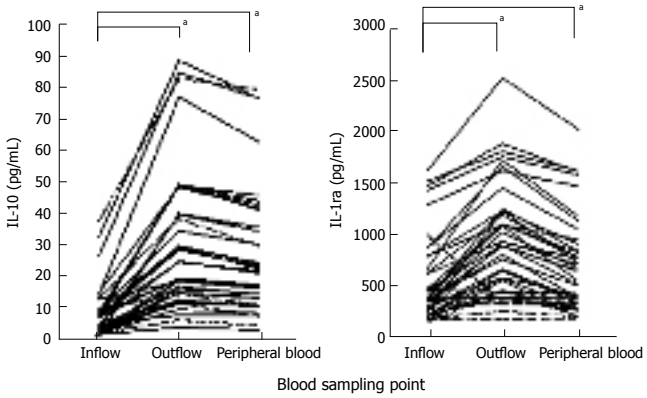

Figure 3 Blood levels of IL-10 and IL-1ra at the Adacolumn inflow (peripheral blood) at the start of GMA therapy and outflow at the end of the 60 min GMA and again peripheral blood just after the completion of 60 minutes GMA therapy in 31 patients with active UC. [a]$P < 0.05$ *vs* inflow by Turkey-Kramer test.

Blood levels of anti- and pro-inflammatory cytokines during GMA

Figure 3 shows the changes in blood levels of IL-10 and IL-1ra at the Adacolumn inflow (peripheral blood) at the start of GMA therapy and outflow at the end of the 60-min GMA and again peripheral blood just after the completion of GMA in the 31 patients. The dotted lines represent patients who did not respond to GMA during the observation period. This figure shows that blood levels of both IL-10 and IL-1ra are significantly higher in the column outflow relative to inflow and this accounts for the elevated levels of these cytokines in the peripheral blood during the GMA procedure. We found strong correlations between ulcerative clinical activity index (CAI) and circulating levels of IL-10, IL-6 and IL-18. In contrast, IL-1ra was high in most patients during active UC, but did not show correlation with CAI. However, the circulating levels of all 4 cytokines were low during remission, similar to the levels in healthy controls. It is inferred that the increments in IL-1ra and IL-10 in the column outflow are due to release from monocytes/macrophages and neutrophils

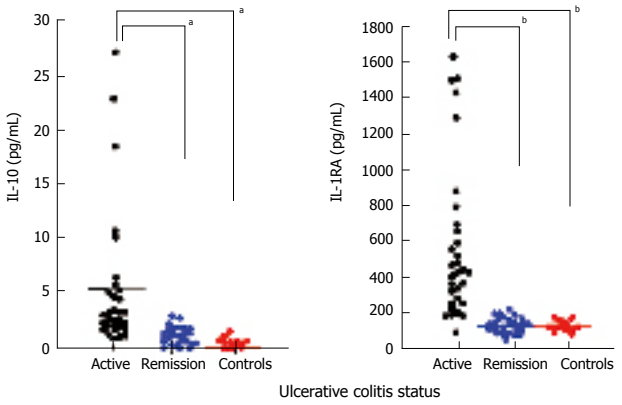

Figure 4 Blood levels of IL-10 and IL-1ra in patients with UC during active disease (n = 31), when in remission (n = 24) and in age matched controls (n = 12). [a]$P < 0.05$, [b]$P < 0.01$ *vs* active by Scheffe's test.

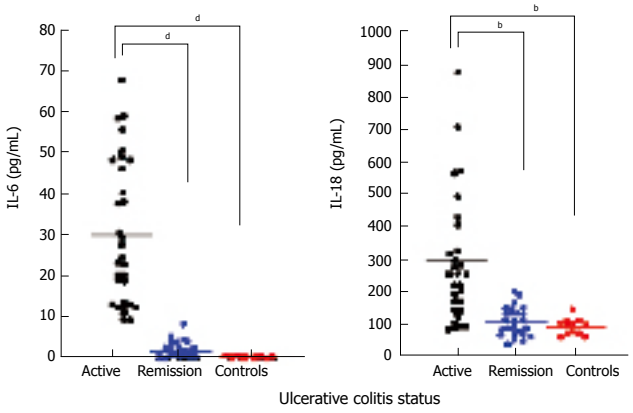

Figure 5 Blood levels of IL-6 and IL-18 (measured in serum samples) in patients with UC during active disease (n = 31), when in remission (n = 24) and in age matched controls (n = 12). [b]$P < 0.01$, [d]$P < 0.001$ *vs* active by Scheffe's test.

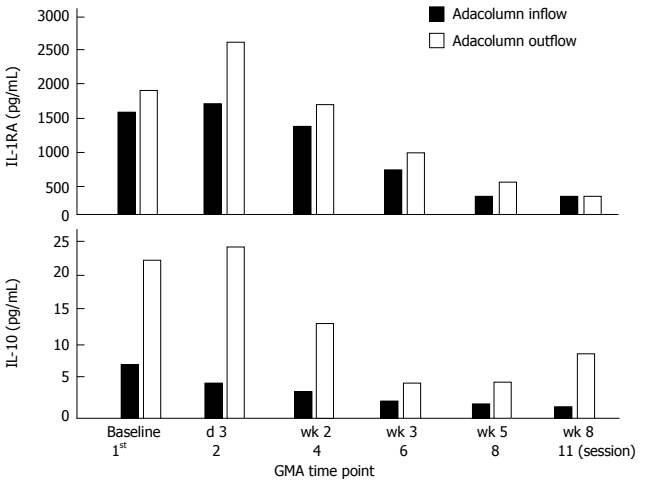

Figure 6 Changes in blood levels of IL-10 and IL-1ra in one typical case during an 8 wk GMA course (up to 11 GMA sessions).

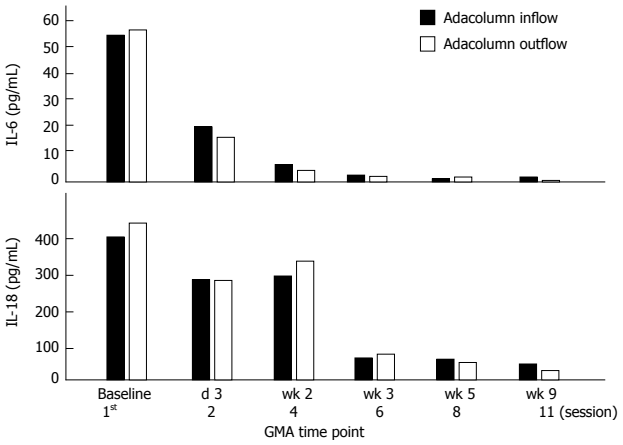

Figure 7 Similar to the data shown in Figure 6, here we see changes in blood levels of IL-6 and IL-18 were seen in one typical case during an 8 wk GMA course.

that adhere to the Adacolumn leucocytapheresis carriers, with additional contributions from activated Th2 and B lymphocytes (IL-10). Blood levels of IL-6 and IL-18 did not change significantly during the 60 min GMA procedure. The likely explanation could be inactivation within the column by a strong flux of proteases and active oxygen derivatives released by adherent leucocytes.

Blood levels of anti- and pro-inflammatory cytokines during active UC and remission

Figure 4 shows blood levels of IL-10 and IL-1ra in patients with UC during active disease (n = 31), when in remission (n = 12) and in age matched controls (n = 12). The results show wide variations in the blood levels of these two anti-inflammatory cytokines during active disease, but for both cytokines, the levels are very low during remission, similar to the levels in healthy controls. The 12 patients in remission were from the 31 patients with active disease who achieved remission following GMA (wk 12).

Blood levels of IL-6 and IL-18 are presented in Figure 5. The data for these two pro-inflammatory cytokines are mirror images of the data in Figure 4 for two anti-inflammatory cytokines. The results show wide variations in the blood levels of these two cytokines during active disease, but for both cytokines, the levels are very low during remission, similar to the levels in healthy controls.

Blood levels of anti- and pro-inflammatory cytokines during GMA course

Figure 6 shows the changes in blood levels of IL-10 and IL-1ra in one typical case during an 8 wk GMA course. Two prominent features can be seen: higher levels during active disease; in the column outflow and decline with the number of GMA sessions. This is a case who responded to GMA and achieved remission during this therapy.

Figure 7 shows IL-6 and IL-18 levels in one typical case during an 8 wk GMA course. Also for these two pro-inflammatory cytokines, the levels were high during active disease and fell rapidly (IL-6) during the course of GMA therapy. However, unlike IL-10 and IL-1ra, the levels of these two cytokines did not increase in the column outflow.

Changes in CAI associated with GMA

Patients received up to 11 GMA sessions to deplete the activated and excessive peripheral blood granulocytes and

monocytes/macrophages. There was a significant ($P < 0.05$) fall in CAI at wk 6 and after 11 sessions, 24 of the 31 patients were in clinical remission (CAI ⩽ 4). The mean CAI value at entry was 11.1, ranging 5-25. The corresponding values one week after the last treatment session were 2.4, ranging 0-15.

Safety of GMA

All patients completed their GMA therapy, compliance was excellent. GMA was safe, no severe side effects were seen during or after the GMA procedures. Transient flushing and mild headache were seen in a small number of patients, similar to the side effects reported in previous GMA studies[4,31,33].

DISCUSSION

Factors which initiate and perpetuate or exacerbate UC are yet to be characterized. The current view is that treatment strategies based on understanding of the factors that initiate or exacerbate UC (targeted therapy) should have greater efficacy margins and less treatment-related side effects[11,19,34,35]. However, UC is an inflammatory disorder reflecting unphysiological activities of pro-inflammatory cytokines and an over-exuberant intestinal inflammatory response[11,34,35]. Hence, it is assumed that cytokines can initiate and perpetuate inflammation and currently represent the best therapeutic targets. With this in mind, we studied their circulating levels during active disease and remission. IL-10 and IL-1ra were chosen as two typical anti-inflammatory cytokines while IL-6 and IL-18 were considered to represent two typical pro-inflammatory cytokines. The circulating levels of all 4 cytokines were high in most patients during active UC, but low during remission, similar to the levels in healthy controls. Further more, most non-responders to GMA had low blood levels of IL-1ra. This study did not measure mucosal tissue levels of these 4 cytokines which might be more appropriate to intestinal inflammation. Since monocytes/macrophages and granulocytes are major sources of both pro- and anti-inflammatory cytokines, this might suggest that these leucocytes contribute to the circulating levels of cytokines. Accordingly, we targeted these leucocytes using GMA with Adacolumn. The column carriers adsorb about 65% of granulocytes, 55% of monocytes and a small fraction of lymphocytes from the blood in the column (FcγR and complement receptors bearing leucocytes). The procedure is followed by a marked increase in peripheral blood lymphocyte counts, notably CD4+ T cells[5] and CD10 negative (immature) neutrophils[36].

The major cellular sources of IL-1ra are known to be monocytes/macrophages and polymorphonuclear granulocytes[37] while the major cellular sources of IL-10 are reported to be monocytes/macrophages, also Th2 cells and B lymphocytes[28]. Similarly, IL-6 is produced by neutrophils, monocytes/macrophages as well as endothelial cells[15] while IL-18 is produced by monocytes/macrophages, epithelial and Kupffer cells[23,24]. Each of these cytokines has a different profile of inflammatory or anti-inflammatory action. Both IL-1ra and IL-10 are reported to have strong anti-inflammatory actions[26,27,29,38-42]. Overproduction of the pro-inflammatory cytokine, IL-1β is thought to be involved in the perpetuation of inflammatory bowel disease, while IL-1ra, the naturally occurring antagonist of IL-1β is believed to counteract the actions of IL-1β; an imbalance of IL-1β and IL-1ra production is thought to be a major factor in the perpetuation of intestinal inflammation[27,38]. The production of IL-1ra is reported to be stimulated by several substances including adherent IgG, certain cytokines like IL-10[26,37,39,42,43]. IgGs adsorb to cellulose acetate carriers of Adacolumn (see below) and this may be one explanation for the elevation of IL-1ra during GMA. Similarly, IL-10 is reported to be a major Th2 cytokine that is known to inhibit the production of IFN-γ, IL-1β, IL-6, IL-8, IL-12 and TNF-α limiting the emergence of Th1 cytokine field[30,44]. In contrast, IL-6 is recently reported to have complex inflammatory actions. Thus it has a major role in leucocyte extravasation by activating endothelial cells and stimulating the production of chemokines including IL-8 and monocyte chemoattractant protein[15]. This indicates that circulating IL-6 can be a major pro-inflammatory factor. IL-18 has been recognized to be a potent inducer of IFN-γ production by T-cells[24,45]. Either independently or in synergy with IL-12, the effects of IL-18 via induction of IFN-γ can lead to a rapid activation of the monocyte/macrophage system with an upregulation of these cells' innate immune capabilities[46,47] that may lead to a Th1 type inflammatory response[23,24]. Hence high levels of circulating IL-18 should be considered pro-inflammatory.

One obvious question which has been drawing our attention in this study was why a consistent increase in IL-6 and IL-18 at the Adacolumn outflow could not be detected as was seen for IL-1ra and IL-10. It is not to say that they were not released from cells which adhere to the column carriers, rather to say that no increase was detected. However, it is logical to view the inside the column as an environment in which very high levels of active oxygen derivatives and proteases (released from adherent leucocytes) prevail. It is likely that the cytokines we have measured have different stability within the column environment and those which survive in appreciable amounts show an increase at the column outflow.

Patients with autoimmune diseases are known to have circulating immune complexes (ICs) in their plasma[49,50]. The Adacolumn adsorptive leucocytapheresis carriers are made of cellulose acetate and cellulose acetate is known to adsorb IgG and ICs from plasma[50]. Upon adsorption, the binding sites on IgG and ICs become available for the Fcγ receptors on neutrophils and monocytes/macrophages[50-53]. Further more, cellulose acetate with adsorbed IgG and ICs generates active complement fragments including C3a, C4a and C5a[5,50,53]. The opsonins, C3b, C3bi C4b, C4bi, C5b and others derived from active complement fragments also adsorb onto the carriers[5,50,53-55] and serve as binding sites for the leucocyte complement receptors, CR1, CR2, CR3 (Mac-1, CD11b/CD18) and CR4[54-57]. Leucocyte adsorption to the cellulose acetate carriers of Adacolumn is governed by the Fcγ receptors (FcγRs) and the complement receptors on the leucocytes.

The expression of these two sets of receptors is common on neutrophils and monocytes/macrophages. In contrast, the expression of complement receptors is not a common feature of lymphocytes except on small subsets of B, T and NK (natural killer) cells[55,58]. Similarly, FcγRs are not widely expressed on lymphocytes except on a small population of $CD19^+$B cells[58] and $CD56^+$NK cells[59]. These basic processes should explain why the carriers selectively adsorb granulocytes and monocytes/macrophages, but not lymphocytes.

In conclusion, elevated blood levels of IL-6 and IL-18 together with peripheral blood granulocytes and monocytes/macrophages which in patients with UC show activative behaviour and increased survival time can be pro-inflammatory and are not counteracted by anti-inflammatory cytokines during active UC. Depletion of these leucocytes by GMA was associated with remission of UC in most of the treated patients and a decline in circulating cytokines. These observations support the assumption that peripheral blood granulocytes and monocytes/macrophages influence the circulating levels of cytokines, hence UC disease and should be appropriate targets of the GMA therapy.

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CLINICAL RESEARCH

Non-alcoholic fatty liver disease may not be a severe disease at presentation among Asian Indians

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Abstract

AIM: To evaluate the clinical and biochemical profile of patients with non alcoholic fatty liver disease (NAFLD) and to assess their histological severity at presentation.

METHODS: Consecutive patients presenting to the liver clinic of All India Institute of Medical Sciences (AIIMS) with raised transaminases to at least 1.5 times upper limit of normal, and histologically confirmed non-alcoholic fatty liver disease were included. Patients who had significant alcohol intake or positive markers of other liver diseases or who were taking drugs known to produce fatty liver were excluded. The clinical, biochemical and histological profile of this group was studied.

RESULTS: Fifty-one patients with NAFLD formed the study population. Their median age and BMI were 34(17-58) years and 26.7(21.3-32.5) kg/m^2 respectively and 46 (90.1%) were males. The majority of the patients had mild inflammation, either grade 1 [32 (63%)] or grade 2 [16 (31%)] and only 3 (6%) patients had severe (grade 3) inflammation. Twenty-three (45%), 19 (37%), 8(16%) and 1(2%) patient had stage 0, 1, 2 and 3 fibrosis respectively on index biopsy and none had cirrhosis. On univariate analysis, triglyceride levels more than 150 mg % (OR = 7.1; 95% CI: 1.6-31.5, P = 0.002) and AST/ALT ratio > 1 (OR = 14.3; 95% CI: 1.4-678.5, P = 0.008) were associated with high grades of inflammation and none was associated with advanced fibrosis. On multivariate logistic regression analysis, hypertriglyceridemia >150 mg% was the only factor independently associated with presence of high grade of inflammation (OR = 1.6; 95% CI: 1.3-22.7, P = 0.02), while none was associated with advanced fibrosis. Triglyceride levels correlated positively with inflammatory grade (r = 0.412; P = 0.003).

CONCLUSION: NAFLD in North Indian patients is a disease of young over-weight males, most of whom are insulin resistant and they tend to have a mild histological disease at presentation.

Key words: Inflammation; Fibrosis; Triglycerides; Non alcoholic steatohepatitis

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INTRODUCTION

With the increasing awareness and early recognition of asymptomatic patients with raised transaminases and the ongoing epidemic of the metabolic syndrome, non alcoholic fatty liver disease (NAFLD) has become a common cause of referral to hepatology clinics[1]. NAFLD can be divided into 4 histological types (types 1 to 4)[2]. Studies from the west have demonstrated that NAFLD, especially NAFLD type 3 and 4 can progress over a variable period, in 25%-40% patients, to cirrhosis and can contribute to liver-related mortality[2,3]. Recently there have been reports linking the development of hepatocellular carcinoma to non alcoholic steatohepatitis (NASH)[4,5]. Studies from Europe and United States have demonstrated that up to 40% patients with NAFLD have advanced fibrosis and a proportion even have cirrhosis on the index biopsy[6,7].

The prevalence of fatty liver in the general population of India has been shown to be as high as 24%[8], which is similar to that reported from some of the western countries, where it parallels the prevalence of obesity[9,10]. Despite the high prevalence of NAFLD in India and rapidly rising incidence of type 2 diabetes mellitus in this country, data on hepatic morphology, to indicate the severity of NAFLD is limited[11-13]. The above-mentioned 3 studies reported demographic and biochemical profile of patients with NAFLD. Histological parameters were studied in only a small number of patients. By now it is clear that histological type 3 and 4 NAFLD are

associated with progressive liver disease and therefore needs therapeutic interventions. The present prospective study was designed to assess the magnitude of advanced liver damage and fibrosis among consecutive patients with biopsy proven NAFLD.

MATERIALS AND METHODS

Study design

It is a cross sectional study to evaluate the clinical, biochemical and histological profile of patients with NAFLD. The study was approved by the ethics committee of the All India Institute of Medical Sciences(AIIMS), New Delhi.

Patients

Inclusion criteria: Consecutive patients presenting to the liver clinic of AIIMS, with raised transaminases to at least 1.5 times upper limit of normal, and histologically confirmed NAFLD, were included.

Exclusion criteria: Patients in whom alcohol intake exceeded 20 grams per day (history of alcohol intake was taken separately from the patients and the closest relatives), patients with positive markers for other liver diseases (hepatitis viruses A through E, autoimmune, Wilson's disease, alpha 1 anti-trypsin deficiency, hemochromatosis) and patients who were on medications known to induce fatty liver such as methotrexate, estrogens, amiodarone and tamoxifen were excluded.

Methods

All patients were evaluated with a thorough history and examination especially to exclude intake of significant amounts of alcohol and to exclude intake of potentially hepatotoxic drugs. Anthropometric measurements were taken at the initial visit. These included the weight, height, waist and hip circumferences. Body mass index (BMI) and waist hip ratios were calculated as weight/height^2. After an overnight fast, 10 mL of blood was collected for a complete blood count and biochemical investigations including a liver function profile, lipid profile, fasting serum insulin and fasting blood glucose.

Hepatitis virus serologies included hepatitis B surface antigen (HBsAg), total antibodies to hepatitis B core antigen (total anti-HBc) and antibodies to hepatitis C virus (anti-HCV). Anti-HCV was done using a sensitive commercial ELISA (Xcyton, Bangalore, India)[12] and the remaining viral serologies were done using commercial ELISA kits (Organon Teknika, Boxtel, The Netherlands). Serum ferritin and 24-h urinary copper estimation were done using conventional biochemical techniques[13,14]. Alpha one anti-trypsin (α1AT) phenotyping was done using isoelectric focusing (PHAST system; Pharmacia Biotech, Uppsala, Sweden)[15]. The anti-nuclear antibodies (ANA), anti- smooth muscle antibodies (ASMA) and anti-liver kidney and microsomal antibodies (anti-LKM1) were carried out using the immunofluorescence technique. Hepatitis C virus RNA was detected using the polymerase chain reaction method as standardized at our laboratory[15] .

Liver biopsy was done after an informed consent in all patients suspected to have NAFLD, using an 18-gauge Menghini's aspiration needle. Each biopsy specimen, after being fixed in 10% formalin, had routine hematoxylin-eosin stains and special stains such as reticulin, Masson's trichrome, Perl's iron stain and Orcein stain for copper and periodic acid Schiff after digestion with diastase. Immunoperoxidase staining to detect HBsAg and hepatitis B core antigen (HBcAg) was carried out in all patients to exclude the presence of chronic viral hepatitis. The classification given by Brunt *et al* was used to grade and stage NASH[16].

An ultrasonography scan of the upper abdomen was done in all patients in order to examine presence of fatty liver and to exclude any obstructive biliary pathology or the presence of any hepatic space occupying lesion.

Fasting serum insulin values were used along with simultaneous fasting blood glucose measurements to calculate HOMA-IR (homeostasis model assessment) according to the formula given below.

HOMA-IR = (fasting serum insulin (μIU/mL)×fasting plasma glucose (mg%))/(22.5 ×18)

Value of HOMA-IR more than 1.64 implied the presence of abnormally high insulin resistance[17].

Definitions

Diabetes was regarded to be present if the fasting venous plasma glucose was more than 126 mg%[18] . Patients with BMI of more than 23 were seen to be overweight and those with a BMI of >25 were labeled as obese (Asian standards)[19]. Hypertriglyceridemia was defined as a fasting serum triglyceride level of more than 150 mg%. Patients who had at least three of the following five components: hyperglycemia (fasting blood sugar >110 mg% or known type 2 diabetes mellitus, central obesity (as defined by a higher waist-hip ratio), hypertension, hypertriglyceridemia (serum triglyceride > 150 mg%), and low HDL cholesterol levels (< 50 for women and < 40 for men), were labeled to have the metabolic syndrome. Since we did not have waist hip ratio in a large number of patients, we used obesity (BMI > 25 kg/m^2) as a surrogate for high waist hip ratio, as one of the component of the metabolic syndrome[1,20].

Statistical analysis

The results were expressed as median (range) for continuous variables and as frequencies (proportions) for categorical variables. Factors suspected to influence the severity of grade and stage were tested in a univariate and multivariate logistic regression analysis. For the univariate and multivariate analyses, the dependent variable was the presence of mild inflammation (grades 0, 1) or severe inflammation (grades 2, 3) for assessing the association with inflammatory grade and mild fibrosis (stages 0, 1) or severe fibrosis (stages 2, 3, 4) for assessing association with fibrosis stage. The independent variables were age>35, sex, BMI > 25 kg/m^2, AST/ALT ratio >1, cholesterol >200 mg%, triglycerides >150 mg%, HOMA-IR >1.64, presence or absence of hypertension and metabolic syndrome. Correlation between histological severity and the above mentioned independent variables was carried out using Spearman's correlation. For skewed data, non-parametric tests were used wherever necessary. All data were analyzed using the SAS 8.0 statistical package.

RESULTS

From January 1999 to June 2005, 51 patients with histologically proven NAFLD were registered at the liver clinic of

AIIMS. This number formed 2.6% of all liver clinic referrals during this time period.

The median age of this cohort of patients was 34 (17-58) years, the BMI was 26.7 (21.3-32.5) kg/m^2 and 46 (90.1%) were males. Median AST/ALT ratio was 0.63 (0.31-2.61). Table 1 gives the baseline demographic and biochemical profile of the patients.

Prevalence of features of metabolic syndrome

Table 2 depicts the prevalence of individual features of the metabolic syndrome in these patients. Eighty percent patients had high HOMA-IR, while 69.4%, 40.8%, 36.4%, 11.8% and 10% had obesity, hypertriglyceridemia, low HDL cholesterol, hypertension and diabetes respectively. All the investigations and clinical features required for diagnosing the metabolic syndrome were available in 43 patients. Of these, 9(20.9%) patients fulfilled the criteria for a diagnosis of metabolic syndrome (presence of 3 or more components of the metabolic syndrome).

Histological severity and factors associated with severe disease

Twenty-eight (55%) patients had NASH (type 3 or 4 NAFLD) whereas the rest had only fatty liver with or without spotty necrosis without evidence of either necrosis or fibrosis (type 1 or 2 NAFLD).

Inflammatory grade: The majority of the patients had mild inflammation, either grade 1 [32 (63%)] or grade 2 [16 (31%)]. Only 3(6%) patients had severe or grade 3 inflammation.

We evaluated factors which could have been associated with moderate to severe inflammation (grade 2 or 3) versus those with mild inflammation (grade 0 or 1). On univariate analysis, triglyceride levels more than 150 mg % (OR = 7.1; 95% CI: 1.6-31.5, $P = 0.002$) and AST/ALT ratio > 1 (OR = 14.3; 95% CI: 1.4-678.5, $P = 0.008$) were significantly associated with high grades of inflammation. Presence of metabolic syndrome was more common among patients who had severe inflammation although it did not reach the significant value (Table 3). On multivariate logistic regression analysis, hypertriglyceridemia more than 150 mg% was the only factor independently associated with presence of high grade of inflammation on index biopsy (OR = 1.6; 95% CI: 1.3-22.7, $P = 0.02$). There was also positive correlation between the triglyceride levels and inflammatory grade ($r = 0.412$; $P = 0.003$) (Figure 1)

Fibrosis stage: Most of the patients had either no or minimal fibrosis. Twenty-three (45%) and 19 (37%) patients had stage 0 and stage 1 fibrosis respectively on liver biopsy. Eight (16%) had stage 2 and 1 (2%) had stage 3 fibrosis. No patient had cirrhosis on index biopsy. On evaluation of factors, which could have been associated with severe fibrosis (stages 2, 3, 4 *versus* stages 0,1), metabolic syndrome (OR = 5.6; 95% CI: 0.7-36.5, $P = 0.04$) and AST/ALT ratio > 1 ($P = 0.09$) were more commonly present among patients with advanced fibrosis but failed to reach significance (Table 4).

Table 1 Baseline demographic and biochemical parameters of the cohort

Parameters	Value
Age (yr)	
n	51
Median	34
(Range)	(16-58)
Sex ratio (males/females)	46/5
Body mass index (kg/m^2)	
n	49
Median	26.7
(Range)	(21.3-32.5)
Bilirubin (mg%)	
n	51
Median	0.8
(Range)	(0.5-13.0)
Aspartate aminotransferase (IU/L)	
n	51
Median	66
(Range)	(29-230)
Alanine aminotransferase (IU/L)	
n	51
Median	98
(Range)	(52-349)
Alkaline phosphatase (IU/L)	
n	51
Median	159
(Range)	(87-372)
Albumin (gm%)	
n	47
Median	4.6
(Range)	(3-5.4)
Cholesterol (mg%)	
n	49
Median	180
(Range)	(96-323)
Triglycerides (mg%)	
n	49
Median	145
(Range)	(48-339)
HDL cholesterol (mg%)	
n	44
Median	41
(Range)	(28-65)
Fasting plasma glucose (mg%)	
n	49
Median	90
(Range)	(70-150)
Fasting serum insulin (μIU/L)	
n	45
Median	10.4
(Range)	(1.6-28.4)
HOMA-IR	
n	45
Median	2.4
(Range)	(0.3-6.0)

HDL: High density lipoprotein, HOMA-IR: Homeostasis model assessment for insulin resistance.

DISCUSSION

Although NAFLD has been demonstrated to be present in almost one quarter of the general population[10] and has been

Table 2 Prevalence of individual components of metabolic syndrome

Component of metabolic syndrome	*n* (%)
Obesity	34 (69.4)
Hypertriglyceridemia	20 (40.8)
Low HDL cholesterol	16 (36.4)
High HOMA-IR	36 (80)
Hypertension	6 (11.8)
Diabetes	5 (9.8)
Metabolic syndrome	9 (20.9)

HDL: high density lipoprotein; HOMA-IR: Homeostasis model assessment for insulin resistance.

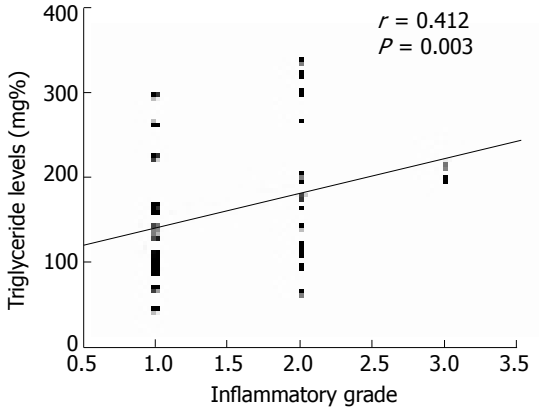

Figure 1 Scatter plot demonstrating positive correlation between triglyceride levels and inflammatory grade. [Coefficient of correlation (*r*) = 0.412; *P* = 0.003 using spearman's correlation].

Table 3 Factors associated with advanced grade of inflammation

Variables	Grade 0/1 (*n*/*N*)	Grade 2/3 (*n*/*N*)	*P*
Male	29/32	17/19	1.0
Age >35 years	13/32	10/19	0.40
BMI >25 kg/m^2	18/30	16/19	0.11
AST/ALT ratio > 1	1/32	6/19	0.008
Cholesterol > 200 mg%	9/30	5/19	1.0
Triglycerides >150 mg%	7/30	13/19	0.002
HOMA-IR >1.64	21/26	15/19	1.0
Hypertension	4/32	2/19	1.0
Metabolic syndrome	3/30	6/19	0.07

BMI: body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HOMA-IR: homeostasis model assessment for insulin resistance; Univariate analyses; *P* value on chi-square or Fisher's exact test.

Table 4 Factors associated with advanced stage of fibrosis

Variables	Stage 0/1 (*n*/*N*)	Stage 2/3/4 (*n*/*N*)	*P*
Male sex	4/42	1/9	1.0
Age >35 years	17/42	6/9	0.26
BMI >25 kg/m^2	26/42	8/9	0.24
AST/ALT ratio > 1	4/42	3/9	0.09
Cholesterol > 200 mg%	13/40	1/9	0.41
Triglycerides >150 mg%	14/40	6/9	0.13
HOMA-IR >1.64	28/36	8/9	0.66
Hypertension	5/42	1/9	1.0
Metabolic syndrome	5/40	4/9	0.04

BMI: body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HOMA-IR: Homeostasis Model assessment for insulin resistance; Univariate analyses; *P* value on chi-square and fisher's exact test.

shown to be responsible for one third of patients with asymptomatic transaminitis in India, little is known about the baseline clinical, biochemical and histological profile of these patients. The present study described the baseline profile in North Indian patients with biopsy proven NAFLD.

Our study demonstrated NAFLD to be a disease of younger age group with a mean age at presentation being 33.3 (8.01) years. More than 90% of the patients were males. This predilection for involvement of males has been seen not only in other studies from Northern India[13-15] , but also in the United States[21]. This is different from what used to be the classical phenotype of NASH patients, where it was described to be a disease of middle-aged females. The reason for this male preponderance has been described as a higher waist-hip ratio in men as compared to women, an indicator of central obesity and insulin resistance. Twenty-one percent of our patients with NAFLD were found to have the metabolic syndrome. The prevalence rate of metabolic syndrome in the general population of India has been reported to be approximately 8% in men older than 20 years[22], but no study has earlier reported the prevalence of the metabolic syndrome in patients with NAFLD from India.

The present study also highlights certain important differences between the phenotype of the cohort of NAFLD as seen in Northern India versus that which has been described classically from the west. This difference would not be surprising, considering the fact that the body fat distribution and risk of cardiovascular complications with similar amount of body fat differs between Asian Indians and their western counterparts. This has even prompted lowering of BMI and waist-hip ratio cut-offs for defining overweight and obesity among Asians[23,24]. Since obesity and fat distribution are integral parts of the insulin resistance syndrome and fatty liver forms part of the spectrum of abnormalities associated with this syndrome, the difference in the baseline profiles of NAFLD patients would be obvious.

The mean age of presentation of our patients, most of whom were detected during workup of asymptomatic transaminitis was about a decade younger than the western patients. The younger age of presentation may suggest increasing health awareness among our population with lower thresholds for health check-ups during which the asymptomatic transaminitis is picked up and investigated. It may also suggest the

epidemic proportion of the metabolic syndrome in India, wherein the population which is fast acquiring western lifestyle gets exposed to the detrimental effects of high calorie diet and physical inactivity at an earlier age. In fact, Indians have been shown to have higher body fat percentage and adverse pattern of body fat distribution including abdominal adiposity even when the BMI is within limits considered as normal for Caucasians[25]. Further, in the age group of 11-17 years, the prevalence of overweight and obesity is 11.63% and 2.38% in urban and 4.7% and 3.63% in rural Indian population respectively[26]. The mean BMI of our patients [26.5 (2.8) kg/m^2] was similar to cohorts described in other studies from North India[13,14], but significantly lower than what has been described in the Caucasian populations, where the mean BMI has always been reported to be above 30[2,3,8,26,27]. As described earlier, Indian patients despite having a lower BMI have similar body fat percentage as the Caucasians. This would mean higher visceral fat distribution, evidenced by higher waist hip ratios, despite lower BMIs. Unfortunately, waist hip ratios were not available in the present study for most of the patients. However, 80% of our patients had high HOMA-IR which is an index of insulin resistance and has been correlated earlier with visceral adiposity and higher waist hip ratios.

Most important finding in the present study was the presence of histologically mild disease in the cohort. Two thirds of the patients had minimal or mild inflammation (grade 0 or 1). Severe (grade 3) inflammation was present in only 3 patients. Hypertriglyceridemia was found to be significantly and independently associated with presence of severe inflammation.

It has already been demonstrated that insulin resistance leads to higher free fatty acid load to the liver, consequently higher triglyceride synthesis and increased secretion of triglyceride rich very low density lipoprotein (VLDL) from the liver[28,29]. In fact, circulating VLDL triglycerides have been correlated with liver fat density[30]. Higher triglyceride and fatty acid deposits may provide substrates for oxidative stress, which is a putative second hit in pathogenesis of progressive NAFLD[31]. High triglyceride levels have been shown to be independently associated with septal or advanced fibrosis by Ratziu *et al*. They also demonstrated that septal fibrosis had a significant positive correlation with liver necroinflammatory activity, thereby indirectly linking hypertriglyceridemia with necroinflammation[9]. In the present study however, we have demonstrated a direct correlation between hypertriglyceridemia and necroinflammatory activity in the liver.

Interestingly, none of our patients had cirrhosis on the index biopsy and only 2% had advanced (stage 3 or 4) fibrosis. Most (> 90%) had minimal or no fibrosis on presentation. Similar mild histology among NASH patients has been reported in other studies from India[13-15] as well as one study from Israel[32]. This is in contrast to the reported series from the west, where advanced fibrosis or cirrhosis has been demonstrated to be present in up to 50% of NAFLD cohorts[7-9,24]. One reason which can be cited for mild fibrosis in our patients is the younger age at presentation, because extent of fibrosis has been shown to increase with increasing age and increasing duration of disease[8,9]. Therefore it is possible that the cohort in the present study happened to have been detected at an early stage and with advancing age the disease might progress. This can only be confirmed later when follow up histology is available in these patients. Another factor which could be responsible for milder disease in these patients is the lower BMI. But this may not be true, because as has been discussed earlier, Asians with lower BMI may also be predisposed to similar risk of developing the metabolic syndrome as Caucasians with higher BMI. Further the effect of BMI or age was not seen on the degree of fibrosis in the univariate analysis. No other factor was found to be associated with severe fibrosis in the present study. The lack of association might be due to small number of patients in the group of severe fibrosis, as only a minority of patients had advanced fibrosis. Studies from the west, however have reported higher BMI, older age, type 2 diabetes mellitus, AST/ALT ratio > 1 and hypertriglyceridemia to be independent risk factors for presence of advanced fibrosis on index biopsy[8,9]. The milder histological disease in the present cohort may also represent a genetic predisposition in our patients, just like their genetic predisposition to develop metabolic syndrome. Whether these patients with mild fibrosis continue to have a mild disease or they progress to a stage of cirrhosis can only be answered once we have an adequate follow up on them.

To conclude, NAFLD in North Indian patients is a disease of young overweight males, most of whom are insulin resistant and they tend to have a mild histological disease at presentation.

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RAPID COMMUNICATION

Perinuclear anti-neutrophil cytoplasmic antibodies (p-anca) in chronic ulcerative colitis: Experience in a Mexican institution

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Abstract

AIM: To assess the prevalence and clinical value of p-ANCA in a sample of Mexican ulcerative colitis (UC) patients.

METHODS: In a prospective, IRB-approved protocol, p-ANCA was determined in 80 patients with UC (mean age, 32 ± 12.9 years). The severity and extension of disease were determined by clinical methods, searching a statistical association with p-ANCA status.

RESULTS: p-ANCA were detected in 41 (51%) patients. Severity of disease was the only clinical variable statistically associated with their presence ($P < 0.0001$; OR = 9; CI 95% = 3.2-24.7).

CONCLUSION: The prevalence of p-ANCA was similar to that reported in other countries. Their presence was associated to UC severity, but offered no more information than the obtained by clinical methods.

Key words: Ulcerative colitis; Inflammatory bowel disease; Perinuclear anti-neutrophil cytoplasmic antibodies

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INTRODUCTION

Anti-neutrophil cytoplasmic antibodies (ANCA) have been described in subjects with different types of vasculitis, and they are essential to establish the diagnosis of Wegener´s granulomatosis, microscopic polyangiitis, Churg-Strauss Syndrome and renal-limited vasculitis[1-4]. Perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA), a subset of ANCA, have been described in patients with inflammatory bowel disease, mainly ulcerative colitis (UC). However, their role in pathogenesis and diagnostic value are still controversial[5]. Previous reports have suggested that p-ANCA might be a sensitive and specific test to diagnose UC[6], to distinguish it from Crohn's disease and other colitides[7], and to provide a prognosis regarding response to medical treatment and risk of pouchitis following the pelvic pouch[8,9].

The prevalence of p-ANCA in patients with UC varied from 40% to 88%[10-13]. High titers have been reported by some authors in subjects with active disease, but its clinical value is controversial because others have not found any correlation between their levels and the activity or extension of disease[14-18].

The value of a diagnostic test, such as p-ANCA, should be evaluated by several practical factors like costs, availability, and usefulness for clinical decisions before accepting them as a standard diagnostic tool.

The aim of this study was to assess the prevalence and clinical usefulness of p-ANCA in a sample of Mexican patients with UC.

MATERIALS AND METHODS

A prospective study was performed at the Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran, which is a referral center for gastrointestinal diseases in Mexico City. The protocol was reviewed and approved by the Institutional Review Board. All patients invited to participate in the study signed an informed consent before inclusion. Eighty consecutive patients with diagnosis of UC were included. In all cases, the diagnosis of UC was based on clinical, endoscopic and histopathologic findings. The extension of disease was determined by total colonos-

copy, and categorized into pancolonic or left-sided colitis. The severity of disease was determined on clinical basis according to the Truelove and Witts criteria[19], and categorized into mild, moderate and severe. Clinical data, including severity and extension of disease were collected by an investigator who was blind to the results of p-ANCA.

p-ANCA determination

Detection of ANCA was done by Indirect Immunofluorescence (IFL), in accordance with the recommendation of the International Workshop[20]. Human neutrophils were prepared from peripheral blood of patients and normal healthy volunteers. The slides were fixed in 98% ethanol at 4℃ for five minutes and dried quickly in air. After using phosphate-buffered saline (PBS) in a dilution of 1:40, the elements where dissolved twofold until it reached 1:320. Following incubation for 1 h r at RT, the slides were washed three times with PBS and bound antibodies were incubated for 30 min and detected with FITC-conjugated rabbit anti-human IgG at RT. Subsequently, the slides were washed three times with PBS and covered with glycerin-phosphate-buffered saline. A titer of 1:20 or higher was considered positive.

The antigen-specific enzyme-linked immunosorbent assay (ELISA) method was used to test the sera for the presence of either mieloperoxidase (MPO) or anti-proteinase-3 antibodies. Human MPO or human proteinase-3 were diluted to 2 mg/L in carbonate buffer (pH 9.6) and 100 μL of each solution was placed in the wells of a 96-well microplate and left for 24 h at 4℃. After plate was washed with PBS three times, 100 μL of diluted sera (1:100 in PBS containing 0.1% tween 20 and 0.1% skim milk) was added to each well, and the plate incubated for 1 h at 37℃. After washing, the enzyme reaction was performed, and color development was measured with a microplate colorimeter. Optical density values > 2 SD more than the mean of the control subjects were considered positive.

Statistical analysis

The student t- -test was used for dimensional variables, and the association between categorical variables was studied by means of the χ^2 with Yates correction. The *P* values were 2-tailed and less than 0.05 was considered statistically significant.

RESULTS

Eighty patients with diagnosis of UC were included. There were 41 men and 39 women, with a mean age of 32 ± 12.9 years. The mean duration of disease was 7.2 ± 6.6 years. Pancolitis was present in 58% and left-sided colitis in 42%. Disease severity was categorized as severe in 20 (25%), moderate in 7 (10%), mild in 15 (18%), and there was no evidence of activity in the remaining 38 (47%) patients. Extraintestinal manifestations were present in 43%; and corresponded to arthritis (23%), primary sclerosing cholangitis (7.5%), erythema nodosum (5%), ankylosing spondylitis (2.5%), pyoderma gangrenosum (2.5%), anterior uveitis (2.5%), and aphthous ulcers (2.5%). Fourteen (25%) patients required surgical treatment due to failure of medical therapy.

Table 1 Clinical features in UC patients with positive and negative p-ANCA

Clinical feature	p-ANCA + *n* = 41	p-ANCA - *n* = 39	*P* value	OR	CI 95%
Pancolitis	22	24	0.62	0.72	(0.29-1.76)
Left-sided	19	15			
Colectomy	7	7	0.84	0.94	(0.29-2.98)
No colectomy	34	32			
EIMs present	14	17	0.52	0.67	(0.27-1.65)
EIMs absent	27	22			
Severe	31	10	< 0.0001	9	(3.2-24.7)
No Severe	10	29			

OR = odds ratio; CI 95% = Confidential interval 95%; *n* = Number of patients.

The p-ANCAs were detected by IFL in 41 (51%) and c-ANCA in 4 (5%) subjects. Titers ranged from 1:20 to 1:160. The antigenic specificity of p-ANCA tested by ELISA in 33 patients showed a positive reaction for myeloperoxidase in 29 and proteinase-3 in 4. Age, gender and age at diagnosis was similar between patients with or without p-ANCA .

A statistical association was observed between p-ANCA status and the severity of UC (*P* = < 0.0001; OR 9, CI 3.2-24.7). Twenty-two (48%) patients with pancolitis and 19 (56%) with left-sided colitis were positive for p-ANCA (*P* = 0.47; OR = 0.72; 95% CI: 0.27-1.94). Seven out of the 14 (50%) patients who underwent surgical treatment were positive as compared with 34 of 66 (52%) who were not operated upon [*P* = 0.91; OR = 0.94; 95% CI: 0.26-3.34].No association was found between the presence of extraintestinal manifestations and p-ANCA. See Table 1.

DISCUSSION

The determination of p-ANCA could give information in subjects with UC in three different clinical issues: (1) as a marker of genetic heterogeneity; (2) to assess inflammatory activity, and (3) for prognosis regarding response to medical treatment and postoperative outcome (pouchitis).

The prevalence of p-ANCA in our series was 51%, within the range published by other authors in different parts of the world[9-13]. It supports that not all patients with UC exhibit the same immunologic pattern, a fact against a pathogenic role of ANCA's in subjects with UC. On the other hand, the wide range of prevalence reported in the literature that goes from 40 to 88% could be rather explained by ethnic or genetic variations than by technical difficulties since both, IFL and ELISA, are simple, sensible, and reproducible assays, as observed in the present study[14,21].

Ethnic and genetic variability have been consistently reported in patients with IBD[22,23]. In a previous study, the authors found that Mexican patients with UC had an increased frequency of HLA-DR1 (DRB1*0102 and 0103) and HLA-DR2 (DRB1*15) when compared with healthy controls. HLA-DRB1*0103 and *0102 were associated

with more severe disease and necessity of surgical treatment[24]. Yang *et al* observed a linkage between p-ANCA positive UC patients and HLA-DR2[25]. The clinical usefulness of p-ANCA as a genetic marker was suggested many years ago by Shanahan who found a higher prevalence of p-ANCA in first-degree relatives of patients with UC[26]. This finding was confirmed by Lee[27] *et al*, in a different ethnic group. So, p-ANCA determination could be useful as a marker of genetic predisposition. However, today there is no information about predictive values of p-ANCA in this setting, and more research is necessary to answer this important question.

Several studies, although not all, have demonstrated a correlation between serum levels of p-ANCA and severity of UC[5,12,15,29-31]. In the present study, an association between the presence of p-ANCA and a subset of patients with severe UC was found but, as other studies, there was no correlation between levels of p-ANCA and the activity of disease evaluated by clinical and biochemical methods. From the point of view of the authors, a sophisticated laboratory exam such as the p-ANCA determination may not currently have a role in the evaluation of severity because it can be evaluated by simple and reproducible methods.

Fleshner *et al*, reported high pre-operative levels of p-ANCA in subjects who developed chronic pouchitis after the ileo-anal pouch[8]; however, it should be noted that some patients with acute and chronic pouchitis were p-ANCA negatives. The follow-up of patients in our series is still too short to analyze this aspect.

In conclusion, the prevalence of p-ANCA in this series was similar to that reported in other countries. Their presence was associated to severity of UC, but offered no more information than the obtained by clinical methods. Future research may clarify their role as a screening test for the first-degree relatives of UC patients, or for patients requiring an ileo-anal pouch.

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RAPID COMMUNICATION

Experimental obstructive jaundice alters claudin-4 expression in intestinal mucosa: Effect of bombesin and neurotensin

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Abstract

AIM: To investigate the influence of experimental obstructive jaundice and exogenous bombesin (BBS) and neurotensin (NT) administration on the expression of the tight junction (TJ)-protein claudin-4 in intestinal epithelium of rats.

METHODS: Forty male Wistar rats were randomly divided into five groups: I = controls, II = sham operated, III = bile duct ligation (BDL), IV = BDL+BBS (30 μg/kg per d), V = BDL+NT (300 μg/kg per d). At the end of the experiment on d 10, endotoxin was measured in portal and aortic blood. Tissue sections of the terminal ileum were examined histologically and immunohistochemically for evaluation of claudin-4 expression in intestinal epithelium.

RESULTS: Obstructive jaundice led to intestinal barrier failure demonstrated by significant portal and aortic endotoxemia. Claudin-4 expression was significantly increased in the upper third of the villi in jaundiced rats and an upregulation of its lateral distribution was noted. Administration of BBS or NT restored claudin-4 expression to the control state and significantly reduced portal and aortic endotoxemia.

CONCLUSION: Experimental obstructive jaundice increases claudin-4 expression in intestinal epithelium, which may be a key factor contributing to the disruption of the mucosal barrier. Gut regulatory peptides BBS and NT can prevent this alteration and reduce portal and systemic endotoxemia.

Key words: Obstructive jaundice; Tight junctions; Claudin-4; Intestinal permeability; Intestinal barrier; Regulatory peptides; Bombesin; Neurotensin

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INTRODUCTION

Patients with obstructive jaundice, especially when exposed to the additional stress of an invasive diagnostic or therapeutic procedure, are prone to septic complications and renal dysfunction contributing to high morbidity and mortality rates[1]. Systemic endotoxemia appears to play a key role in the development of these complications[2]. Experimental and clinical studies have shown that obstructive jaundice increases intestinal permeability permitting the escape of endotoxin from the gut lumen[3,4]; however, little is known of the molecular events leading to intestinal permeability alterations.

Tight junctions (TJs) are located at the apical part of lateral membranes of polarized epithelial and endothelial cells forming a barrier that regulates the permeability of ions, macromolecules and cells through the paracellular pathway[5,6]. Up to now, three groups of macromolecules are considered as bona fide integral components of the tight junction: occludin, claudins and junctional adhesion molecule[6]. We have recently demonstrated using immunohistochemistry that intestinal mucosal barrier dysfunction in obstructive jaundice is associated with regional loss of occludin expression in the intestinal epithelium, observed mainly in the upper part of the villi[7]. Our findings were recently confirmed by other

investigators using immunoblotting[8].

Claudins comprise a multigene family consisting of 24 members believed to be major functional elements of TJ[9]. TJ strands are copolymers of heterogeneous claudin species and occludin, and heterogeneous claudin species constitute the backbone of TJ strands *in situ*[10]. The localization and contribution to barrier function of each member of the claudin family vary among tissues. Claudin-4 is highly expressed in rat intestine[11], with a preferential localization in the epithelium overlying the tips of intestinal villi and the follicle associated epithelium (FAE) of gut associated lymphoid tissue (GALT)[12]. We focused on this molecule because in obstructive jaundice loss of occludin expression was observed mainly at the upper part of the villi where claudin-4 is preferentially expressed.

Regulatory peptides bombesin (BBS) and neurotensin (NT), with a wide spectrum of biological actions on gastrointestinal tissues (influencing intestinal growth and adaptation, intestinal motility, blood flow, secretion, nutrient absorption and immune response), exert a protective role in preserving gut barrier integrity after various injurious insults[13-18]. Beyond their potent enterotrophic action we have shown that BBS and NT fully restored intestinal occludin expression in bile duct ligated rats, thus preventing endotoxin translocation. It suggests a role of these peptides as molecular modulators of TJs[7].

The present study was undertaken to investigate the effect of experimental obstructive jaundice on intestinal claudin-4 expression and examine the potential effect of BBS and NT on this parameter of intestinal mucosal barrier.

MATERIALS AND METHODS

Animals

Forty male albino Wistar rats, weighing 250-320 g, were used in the study. They were housed in stainless-steel cages, three rats per cage, under controlled temperature (23℃) and humidity conditions, with 12-h dark/light cycles, and maintained on standard laboratory diet with tap water *ad libitum* throughout the experiment, except for an overnight fast before surgery.

The experiments were carried out according to the guidelines set forth by the Ethics Committee of Patras University Hospital, Patras, Greece.

Experimental design

Animals were randomly divided into five groups: I (n = 8), controls; II (n = 8), sham operated; III (n = 8), bile duct ligation (BDL); IV (n = 8), BDL and BBS treatment and V (n = 8), BDL and NT administration. All surgical procedures were performed under strict sterile conditions, and light ether anesthesia. Rats from groups II, III, IV and V underwent laparotomy on d 0. *Via* a 1 cm upper midline incision, the gastroduodenal ligament was isolated and the common bile duct was mobilized. In groups III, IV and V, the common bile duct was further doubly ligated in its middle third with a 4-0 silk suture and was transected between the two ligatures. The abdominal incision was closed in two layers with chromic 4-0 cat gut and 4-0 silk.

For the 10 subsequent days, the animals of groups IV and V were treated daily with BBS (10 μg/kg, subcutaneously, three times a day) and NT (300 μg/kg, intraperitoneally, once a day) respectively, while the animals of groups I, II, and III were divided to receive daily either three subcutaneous or one intraperitoneal injection of 0.5 mL normal saline. All injections were given after topical application of an antiseptic solution of povidone iodine 10%. Previous studies have shown that the way of saline administration does not affect the results[7]. On the 10th d, all animals were operated (group I) or reoperated (groups II, III, IV and V). Samples were obtained according to the experimental protocol, after which the rats were sacrificed by exsanguination.

Peptides preparation

Bombesin: A stock solution of BBS (Sigma Chemical Co, St. Louis, Missouri, USA) was prepared by first dissolving the amount of peptide needed for the study in 1 mL sterile water containing 0.1% (w/v) bovine serum albumin and then diluted with normal saline containing 1% (w/v) bovine serum albumin, so that the amount of BBS needed for each injection to be contained in a volume of 0.1 mL. The solution was divided into equal aliquots of 0.1 mL which were stored in plastic tubes at -20℃. At the time of administration, in order to prolong absorption, each aliquot was mixed with 0.4 mL of a solution 8% (w/v) hydrolyzed gelatin (Sigma Chemical Co, St. Louis, Missouri, USA). A final volume of 0.5 mL, containing 10 μg BBS/kg body weight, was injected subcutaneously three times daily. Selection of dose and route of administration was based on previous reports[7].

Neurotensin: A stock solution of NT (Sigma Chemical Co, St. Louis, Missouri, USA) was prepared by first dissolving the amount of peptide needed for the study in 1 mL sterile water containing 0.1% (w/v) bovine serum albumin and then diluted with normal saline containing 0.1% (w/v) bovine serum albumin, so that the amount of BBS needed for each injection to be contained in a volume of 0.1 mL. The solution was divided into equal aliquots of 0.1 mL which were stored in glass vials at -20 ℃. At the time of administration, each aliquot was further diluted with 0.4 mL sterile saline to a final volume of 0.5 mL and was given intraperitoneally through a bolus injection containing 300 μg NT/kg body weight. Selection of dose and route of administration was based on previous reports[7].

Bilirubin and endotoxin measurements

For the determination of total bilirubin, 0.5 mL of blood was collected from all animals, by transecting the tip of their tail. Then a laparotomy was performed and in all groups, the portal vein and the abdominal aorta were punctured and samples of 1 and 2 mL of blood, respectively, were obtained for estimation of endotoxin. Bilirubin concentrations were assayed using a standard biochemical technique and expressed in mg/dl. Endotoxin concentration was determined by the quantitative chromogenic Limulus Amebocyte Lysate test (QCL-1000, BioWhittaker, Walkersville, USA) and expressed in EU/mL. Samples were processed according to the manufacturer's instructions. By this test it is possible to measure concentrations of endotoxin $\geqslant$ 0.01 EU/mL.

Immunohistochemistry for claudin-4

In tissue sections of the terminal ileum from all rats a standard immunohistochemical technique was applied to detect claudin-4. Deparaffinization, rehydration and microwave antigen retrieval were performed in Trilogy™ solution (Cell Marque, Hot Springs, AK). Then slides were incubated for 1 h at room temperature with a commercially available mouse monoclonal anti-claudin-4 antibody (1:40, ZYMED Laboratories, San Francisco, CA), followed by antigen-antibody detection using the DAKO ChemMate EnVision detection kit (DAKO A/S, Glostrup, Denmark). Colour was developed with diaminobenzidine (Sigma Fast DAB tablets, D-4293, St. Louis, Mo) and counterstained with Harris hematoxylin. All procedures took place at room temperature and between steps sections were washed in TBS. In negative control slides, the primary antibody was substituted by mouse serum. Twenty randomly selected adjacent perpendicularly sectioned villi from each case (n = 8 cases per group) were evaluated. The frequency of claudin-4 immunohistochemical expression in the upper third of the villi was recorded by dividing the number of claudin-4 positive enterocytes by the total number of enterocytes in the upper third of each villous.

Statistical analysis

The results are expressed as mean (SD). Comparisons among multiple groups were performed using the one-way ANOVA, followed by Dunnette's T3 post hoc test. Variance equality was tested by Levene statistical analysis. Differences were considered significant when $P < 0.05$.

RESULTS

Animal outcomes

All animals survived and were in good health throughout the experiment. Bile duct ligated rats were clinically jaundiced within three days. At reoperation on d 10 it was found that the ligation and division of the common bile duct in groups III, IV and V had been successful in all cases and resulted in dilatation of the common bile duct remnant proximal to the ligature without signs of bile leakage.

Bilirubin and endotoxin concentrations

Bile duct ligation led to significantly raised total bilirubin levels in groups III, IV and V compared with control and sham groups ($P < 0.001$). Figure 1 demonstrates endotoxin values, measured in blood collected from portal vein and aorta. Jaundiced animals (group III) presented significantly elevated endotoxin concentrations in portal and aortic blood compared with groups I and II ($P < 0.001$). When ligation of common bile duct was followed by either BBS or NT treatment, endotoxin values were significantly reduced both in portal vein ($P < 0.001$, respectively) and aorta ($P < 0.001$, respectively).

Evaluation of histology and claudin-4 immunostaining

Overall, the ileal architecture remained intact and epithelial continuity was retained, in all specimens studied, while no significant pathology was observed. Obvious alterations of claudin-4 immunohistochemical expression in the intestinal epithelium were observed among experimental groups. In control and sham operated rats claudin-4 was expressed in a few villous surface epithelial cells (Figure 2A), while occasionally unstained villi were observed. Claudin-4 was detected as membranous immunostaining, stronger on the basolateral sides than on the apical sides, with weak cytoplasmic staining (Figure 2A). In jaundiced rats, claudin-4 was expressed in most epithelial cells lining throughout the upper third of the villi, and this staining was observed in every villous (Figure 2B). Also the lateral expression of claudin-4 was upregulated in jaundiced rats (Figure 2D). In BBS or NT-treated rats claudin-4 expression was restored to the control state (Figure 2C). The frequency of claudin-4-positive cells in the upper third of the villi was significantly increased in jaundiced rats as compared with control and sham operated ($P < 0.001$), whereas it was reduced in rats administered BBS or NT ($P < 0.001$ as compared to BDL) (Figure 3).

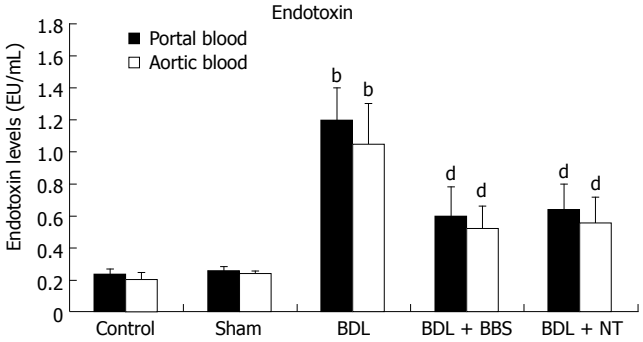

Figure 1 Portal and aortic endotoxin concentrations. Values are mean ± SD, $^{b}P < 0.001$ *vs* sham; $^{d}P < 0.001$ *vs* BDL. BDL, bile duct ligation; BBS, bombesin; NT, neurotensin.

DISCUSSION

Increased intestinal permeability has been postulated to be a key factor contributing to bacterial and endotoxin translocation and the pathogenesis of the so called "gut derived sepsis"[19]. In obstructive jaundice, increased intestinal permeability has been demonstrated not only in animal models but in patients as well and has been positively correlated with severity of biliary obstruction[3,4]. The present study, using an experimental model of biliary obstruction for 10 d in rats, demonstrated significant portal and aortic endotoxemia, a clinically important marker of ineffective intestinal barrier, accompanied by altered immunohistochemical expression of the TJ-protein claudin-4 in intestinal epithelium.

The molecular mechanisms responsible for permeability alterations in obstructive jaundice have not been adequately investigated until now. Previously we had shown for the first time that intestinal mucosal barrier dysfunction in obstructive jaundice is associated with regional loss of expression of the TJ-protein occludin in the intestinal epithelium, observed mainly at the upper part of the villi[7]. Our immunohistochemical observations were confirmed with immunoblotting method by other investi-

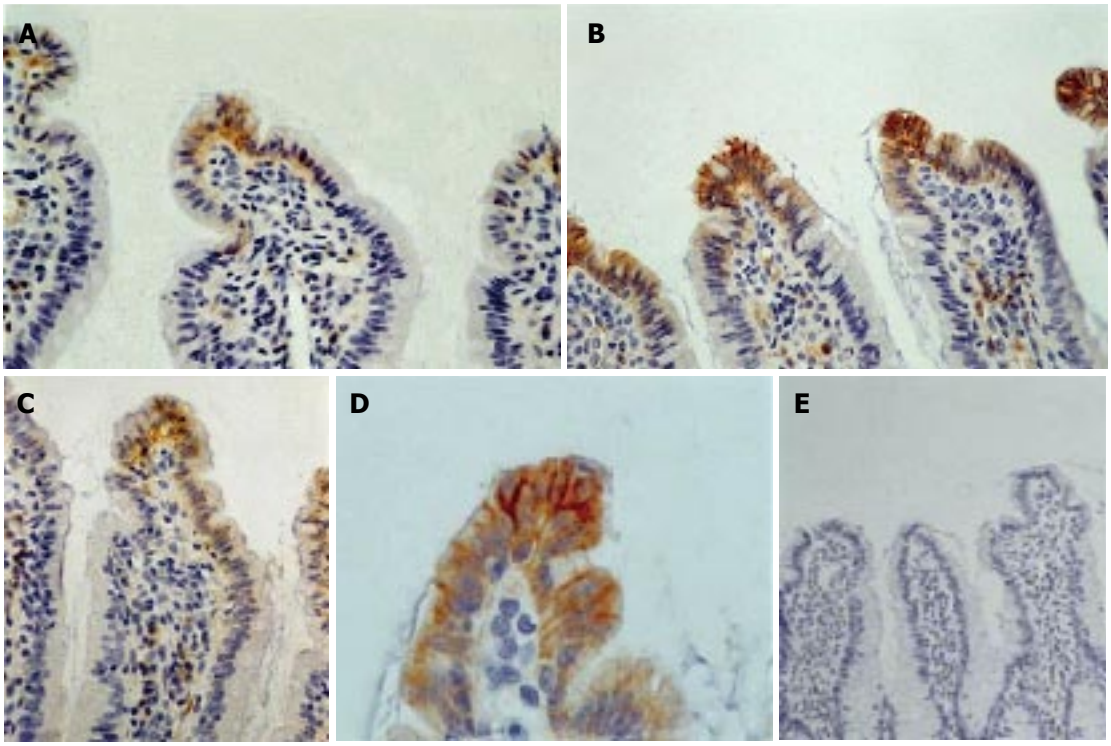

Figure 2 Immunohistochemical expression of claudin-4 in the mucosa of terminal ileum: In control and sham operated rats claudin-4 is expressed on the basolateral sides of a few villous surface epithelial cells (**A**: original magnification x 400). In obstructive jaundice, immunoreactivity for claudin-4 is stronger and this molecule is expressed by most epithelial cells lining throughout the upper third of the villi and not only in the tip (**B**: original magnification x 400). At higher magnification we can see the enhancement of the lateral distribution of claudin-4 in obstructive jaundice (**D**: original magnification x 1000). In bombesin or neurotensin-treated rats claudin-4 expression is restored to the control level (**C**: original magnification x400). Substituting anti-claudin-4 antibody by mouse serum no staining is observed (**E**: original magnification x 200).

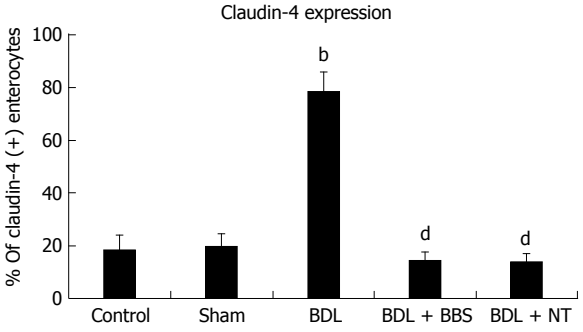

Figure 3 Frequency of claudin-4 (+) enterocytes in the upper third of the villi. Values are mean ± SD, $^{b}P < 0.001$ *vs* sham; $^{d}P < 0.001$ *vs* BDL. BDL, bile duct ligation; BBS, bombesin; NT, neurotensin.

gators, who additionally showed that obstructive jaundice led to decreased mucosal expression of the TJ-associated protein zonulin-1 as well[8]. The present study offered further insight into TJs alterations in the intestinal mucosa in obstructive jaundice, demonstrating upregulation of claudin-4 expression in the upper part of the villi.

The results obtained by the present study may initially seem paradoxical and a question raised is how increased expression of a TJ-protein may lead to increased permeability. Claudins are the only known variable elements in TJs and different expression, combination and mixing ratios of various members of the claudin family are essential in the regulation of barrier properties of TJs[20]. Overexpression of some claudins is often associated with the expected reduction of permeability, e.g. claudin-1 overexpression in Madin-Darby canine kidney (MDCK) cells increases transepithelial resistance[21]. On the other hand, increased blood-brain barrier permeability in inflammatory pain is associated with significant increase of claudin-3 and -5 expression, while occludin expression is decreased[22]. Regarding the functional role of claudin-4 in the intestinal epithelium, it may be associated with loosening of intercellular junctions and opening of the paracellular route, as indicated by findings of previous studies[12]. For instance, it has been shown that there is a close association between the locations of claudin-4 and TUNEL-positive apoptotic cells in intestinal villi and the FAE of GALT, suggesting that claudin-4 may be involved in the process of peeling off epithelial sheets of apoptotic enterocytes. In addition, claudin-4 is preferentially expressed at the apices of intestinal villi and the FAE, which represent important antigen-sampling sites. Given that both apoptosis and antigen capturing are associated with looseness of intercellular junctions, Tamagawa *et al* suggested that claudin-4 expression contributes to the formation of relatively loose intercellular junctions[12]. The present study provides support to the above proposal. Furthermore, taking into consideration our recent findings of decreased intestinal occludin expression in obstructive jaundice[7] and the results of the present study showing increased intestinal claudin-4 expression, one could hypothesize the existence of a novel "switch mechanism" by which claudin-4 may replace occludin and lead to increased permeability of tight junctions. The key role of claudin-4 and occludin in obstructive jaundice-associated intestinal permeability alterations is further evidenced by improvement of gut mucosal barrier

after restoration of their expression by regulatory peptides administration.

Which is the critical factor inducing these alterations in obstructive jaundice? The present study cannot answer this question due to the interplay of several factors in the intact organism. However, a recent *in vitro* study has shown that bile is crucial for maintenance of intestinal TJs integrity[8]. Beyond absence of intraluminal bile other factors such as systemic endotoxemia, cytokinemia and intestinal oxidative stress may contribute to intestinal barrier failure in obstructive jaundice[5,23-25]. Oxidative stress, attracting a growing research interest, has been recognized as an important modulator of TJs. It has been shown that oxidative stress increases epithelial permeability through modulation of the assembly, localization, expression and function of the molecular components of the TJ structural complex[24]. We have recently shown that experimental obstructive jaundice induces intestinal oxidative stress, demonstrated by increased lipid peroxidation, protein oxidation and oxidation of non-protein and protein thiols[25]. Therefore, oxidative stress may be implicated in altered occludin and claudin-4 expression in the intestinal mucosa of jaundiced rats.

The present study also showed that when jaundiced rats were treated with either BBS or NT, claudin-4 expression in the intestinal epithelium was restored to the control state and endotoxin levels in portal and aortic blood were significantly reduced. These regulatory peptides may have restored claudin-4 expression via a direct receptor-mediated mechanism. Animal studies provide evidence for the presence of BBS and NT receptors in intestinal epithelial cells[26,27]. Binding to peptides' specific G protein-coupled receptors activates multiple intracellular signals including protein phosphorylation pathways, accumulation of cyclic-adenosine monophosphate and mobilization of intracellular Ca^{++}[28], which may account for the maintenance of structurally and functionally effective TJs[6,20]. A potential molecular mechanism mediating the effect of BBS and NT on claudin-4 expression is activation of protein kinase C (PKC). PKC plays a central role in transducing neuropeptides' signals[28] and recent studies have shown that PKC activity is required for normal claudin assembly and the integrity of the intestinal epithelial barrier[29]. Also the antiapoptotic effect of BBS and NT on the intestinal epithelium of jaundiced rats[25] may be related to their modulating action on the expression of claudin-4, since there is evidence that intestinal apoptosis and claudin-4 expression may be interconnected[12]. Additionally, indirect mechanisms such as reduction of systemic endotoxemia, improvement of energy supply to enterocytes through splachnic vasodilatation[30,31] and reduction of intestinal oxidative stress[25] may contribute to the regulatory action of BBS and NT on the TJ structure and function.

In conclusion, our results show that enhanced claudin-4 expression in the upper part of the intestinal villi represents an important molecular mechanism for increased intestinal mucosal permeability in obstructive jaundice. Gut regulatory peptides BBS and NT, acting as molecular modulators of TJs, totally restored this alteration thus preventing endotoxin translocation from the gut lumen. These findings offer further insight into the pathophysiology of gut barrier failure in obstructive jaundice and suggest the potential therapeutic efficacy of regulatory peptides. However, further investigations to elucidate the details of the functional mechanisms and possible side effects of BBS and NT are needed before their clinical application.

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RAPID COMMUNICATION

Role of soluble triggering receptor expressed on myeloid cells in inflammatory bowel disease

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Abstract

AIM: To investigate the probable role of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) in the pathogenesis of inflammatory bowel disease (IBD).

METHODS: Fifty-eight patients were enrolled; nineteen healthy volunteers served as controls; 8 patients were diagnosed with Crohn's disease, and 31 with ulcerative colitis. Clinical and endoscopic activity indexes of patients with Crohn's disease and ulcerative colitis respectively were estimated. Upon admission blood was sampled; sTREM-1 and TNFα were measured by an immunoassay and malondialdehyde (MDA) by the thiobarbiturate assay, after passage through an HPLC system.

RESULTS: Median ± SE of TNFα of controls, patients with Crohn's disease and patients with ulcerative colitis were 6.02 ± 3.94, 7.98 ± 5.08 (P = NS *vs* controls), and 8.45 ± 4.15 ng/L (P = 0.018 *vs* controls) respectively. Respective values of sTREM-1 were 53.31 ± 32.93, 735.10 ± 197.17 (P = 0.008 *vs* controls) and 435.82 ± 279.71 ng/L (P = 0.049 *vs* controls). sTREM-1 was positively correlated with Crohn's disease activity index and clinical and endoscopic activity indexes of ulcerative colitis (P = 0.002, 0.001 and 0.009, respectively). sTREM-1 of patients with ulcerative colitis was positively correlated with TNFα (P = 0.001).

CONCLUSION: sTREM-1 seems to behave as a novel mediator in IBD in correlation with the degree of the inflammatory reaction of the intestinal mucosa.

Key words: sTREM-1; Pro-inflammatory cytokines; Malondialdehyde; Inflammatory bowel disease

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INTRODUCTION

Accumulated evidence over the last decade demonstrates that inflammatory bowel disease (IBD) is a heterogeneous group of diseases resulting from different pathogenetic mechanisms with a common symptomatic expression[1]. Dysfunction of the innate and adaptive immune systems associated with gut mucosa might involve impairment of mucosal barrier function and development of localized or systemic inflammatory and autoimmune processes[2,3]. Triggering receptor expressed on myeloid cells (TREM)-1 is a recently discovered receptor expressed on the surface of neutrophils and monocytes in the presence of microbial components. Engagement of TREM-1 has been reported to trigger the synthesis of proinflammatory cytokines, though its expression failed to elevate in several autoimmune diseases[4,5]. Expression of TREM-1 was not identified on cell membranes of macrophages of intestinal lamina propria; its absence was connected with prevention of excessive inflammatory reactions by bacterial flora, and thus, of any tissue damage of the intestine[6].

A soluble form of TREM-1, named sTREM-1, has been found; it is thought to be released from cell membranes as a result of the severity of inflammation. sTREM-1 was found elevated in samples of gastric juice of patients with peptic ulcer disease; its correlation with gastritis score leads to the assumption of a probable implication of sTREM-1 in the pathogenesis of gastritis[7]. Based on the latter findings in gastric diseases, the present study aimed to clarify the significance of sTREM-1, if any, in inflammatory bowel disease.

MATERIALS AND METHODS

Subjects

Fifty-eight patients were enrolled in the prospective

study from January 2001 to June 2001; nineteen healthy volunteers served as controls; eight patients were diagnosed with Crohn's disease; and 31 with ulcerative colitis. Exclusion criteria for the study were (1) gastric or colonic neoplasia, (2) liver cirrhosis, (3) any history of administration of monoclonal anti-TNFα antibodies (infliximab, adalimubab) or soluble TNFα receptors (etanercept), (4) concurrent existence of infectious or ischemic colitis and (5) patients with Crohn's disease type L4B2 and L4B3 classified according to Vienna classification system[8].

Crohn's disease was confirmed by findings on barium radiography, endoscopy, and histopathology, as described by others[9]. The activity of Crohn's disease among patients was defined according to Crohn's Disease Activity Index (CDAI). Patients with CDAI score ranging between 150 and 400 were thought to have mild activity of the disease and patients with CDAI score ranging between 400 and 600 were thought to have severe activity. Remission was considered when CDAI score was less than 150[10].

The diagnosis of ulcerative colitis relied on history, stool examination, the sigmoidoscopic or colonoscopic appearance and the histological assessment of rectal or colonic specimens. Activity of ulcerative colitis was defined by its clinical and endoscopic severity. According to its clinical severity patients were categorized as suffering from disease remission, or mild, moderate, and severe disease as described previously; the latter disease activity was graded between 0 and 3, respectively[11]. According to endoscopic findings a system scaling between 0 and 4 was applied. The latter system is as follows: 0: normal mucosa, 1: loss of vascular pattern, 2: granular mucosa, 3: friability on rubbing and 4: spontaneous bleeding or ulceration of the mucosa[12].

Patients with no clinical, laboratory, histological and endoscopic abnormalities who underwent colonoscopy served as controls. The study was conducted in accordance to the Helsinki Declaration. Upon admission, a total of 20 mL of blood were collected after puncture of one forearm vein. Ten mL was added into 40 mL of trypticase soy broth (Becton Dickinson) for quantitative blood culture. The other 10 mL of blood was collected in a sterile tube and centrifuged; the supernatant was kept at -70℃ until assayed. Serum was applied for the estimation of sTREM-1, TNFα and malondialdehyde (MDA).

Estimation of sTREM-1

Estimation of sTREM-1 was performed by a home-made enzyme immunoassay. Capture antibody of sTREM-1 (R&D Inc, Minneapolis, USA) was diluted to 4000 mg/L and distributed in a 96-well plate at a volume of 0.1 mL per well. Samples of serum were centrifuged at 12 000 g for 10 min and the supernatants were removed. After overnight incubation at 25℃, wells were thoroughly washed with a 0.5 g/L solution of Tween in PBS (Merck) (pH: 7.2-7.4). Then 0.1 mL of standard concentrations of sTREM-1 (15.1-4000 ng/L, R&D Inc) diluted with reagent diluent (10 g/L BSA in PBS, pH 7.2-7.4, 0.2 micron filtered) serving as a buffer or of serum was added in wells. After incubation for two hours, wells were washed thrice, and 0.1 mL of one 400 ng/mL dilution of sTREM-1 detection antibody (R&D Inc) was added per well. The plate was then incubated for two hours, and attached antibodies were signalled by streptavidin. Concentrations of sTREM-1 in each well were estimated by the optical density detected at 450 nm after addition of one 1:1 solution of H_2O_2: tetramethylbenzidine as a substrate (R&D Inc). sTREM-1 concentration was expressed in ng/L. All determinations were performed in duplicate; the inter-day variation of the assay was 5.23%.

Estimation of TNFα

Tumor necrosis factor alpha was measured in serum with an enzyme immunoassay (Amersham, London, UK). Lowest limit of detection was 0.5 ng/L. All measurements were performed in duplicate and cytokine concentrations were expressed as ng/L.

Estimation of malondialdehyde (MDA)

Lipid peroxidation was estimated by the concentration of MDA, as already described[13]. Briefly, a 0.1 mL aliquot of each sample was mixed to 0.9 mL of trichloroacetic acid 200 g/L (Merck) and centrifuged at 12 000 g, 4℃ for 10 min. The supernatant was removed and incubated with 2 mL of thiobarbituric acid 2 g/L (Merck) for 60 min at 90℃. After centrifugation, a volume of 10 μL of the supernatant was injected into a high-performance liquid chromatography system (HPLC, Agilent 1100 Series, Waldbronn, Germany) with the following characteristics of elution: Zorbax Eclipse XDB-C18 (4.6 mm × 150 mm, 5 μm) column below 37℃; mobile phase consisting of a 50 mmol/L K_3PO_4 (pH 6.8) buffer and methanol 990 g/L at a 60/40 ratio with a flow rate of 1 mL/min; fluorometric detection with signals of excitation at 515 nm and emission at 535 nm. The retention time of MDA was 3.5 min and it was estimated as μmol/L by a standard curve created with 1, 1, 3, 3-tetramethoxy-propane (Merck). All determinations were performed in duplicate.

Statistical analysis

Comparison between groups was made by Mann-Whitney U test. Correlations between concentrations of sTREM-1, TNFα and MDA and severity indexes were performed according to Spearman's rank of order. $P < 0.05$ was considered as significant.

RESULTS

Patients' clinical and demographic characteristics are shown in Table 1. Blood cultures obtained from patients of all study groups were sterile. Concentrations (Median ± SE) of sTREM-1 of controls, patients with Crohn's disease, and patients with ulcerative colitis were 53.31 ± 32.93, 735.10 ± 197.17 ($P = 0.008$ *vs* controls), and 435.82 ± 279.71 ($P = 0.049$ *vs* controls) ng/L, respectively (Table 1). Respective concentrations of TNFα were 6.02 ± 3.94, 7.98 ± 5.08 (P = NS *vs* controls), and 8.45 ± 4.15 ($P = 0.018$ *vs* controls) ng/L (Table 1). Respective concentrations of MDA were 0.93 ± 0.23, 1.46 ± 0.56, and 1.49 ± 0.61 ($P = 0.041$ *vs* controls) μmol/L (Table 1). The concentrations

Table 1 Demographic data of IBD patients

Demographic data	Control group	Crohn's disease	Ulcerative colitis
Gender (Male/Female)	12/7	3/5	18/13
Age (mean ± SD, yr)	64.2 ± 14.8	29.4 ± 13.0	43.8 ± 16.4
White blood cells (mean ± SD, x 10^9/μL)	7.9 ± 2.3	9.0 ± 3.0[a]	9.5 ± 3.6[a]
Platelets count (mean ± SD, x 10^9/μL)	237 ± 56	187 ± 38[a]	179 ± 33[a]
Blood cultures	sterile	sterile	sterile
Clinical activity index of UC (mean ± SD)	-	-	1.52 ± 0.26
Endoscopic activity index of UC (mean ± SD)	-	-	1.65 ± 0.45
Crohn's disease activity index (mean ± SD)	-	331.81±117.25	-
TNFα (median ± SE, ng/L)	6.0 ± 3.9	8.0 ± 5.1	8.4 ± 4.2[a]
sTREM-1 (median ± SE, ng/L)	53 ± 33	735 ± 197[b]	435 ± 279[a]
MDA (median ± SE, μmol/L)	0.93 ± 0.23	1.46 ± 0.56	1.49 ± 0.61[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* controls.

Table 2 sTREM-1, TNFα and MDA in IBD patients

Activity index	TNFα (ng/L)	sTREM-1 (ng/L)	MDA (μmol/L)
UC Clinical AI			
Grade 0	4.19 ± 1.52	7.09 ± 4.90	1.74 ± 0.11
1	5.13 ± 0.98	18.91 ± 15.00	1.87 ± 1.05
2	4.98 ± 0.42	288.28 ± 133.82[b]	1.83 ± 0.46
3	9.98 ± 1.23[a]	1596.45 ± 397.38[b]	1.97 ± 0.31
UC endoscopic AI			
Grade 0	4.33 ± 1.51	4.91 ± 3.21	1.36 ± 0.11
1	5.13 ± 0.98	18.91 ± 15.00	1.87 ± 1.05
2	4.86 ± 0.41	288.28 ± 133.82[b]	1.66 ± 0.43
3	8.98 ± 0.42[a]	1108.19 ± 309.95[b]	1.74 ± 0.39
4	21.99 ± 10.50[a]	2281.25 ± 556.77[b]	1.91 ± 0.50
CDAI			
Score < 150	4.88 ± 1.44	4.91 ± 3.21	1.78 ± 0.11
150-400	5.21 ± 0.88	16.03 ± 4.19	1.88 ± 0.67
400-600	5.67 ± 0.91	2281.25 ± 556.77[a]	1.95 ± 0.95

[a]$P < 0.05$, [b]$P < 0.01$ *vs* grade 0 and score < 150.

of sTREM-1 were higher in patients with severe degree of Crohn's disease than in patients in remission of Crohn's disease ($P = 0.032$, Table 2).

Correlation between sTREM-1 and TNFα of patients with ulcerative colitis was significant ($P = 0.001$) (Figure 1). No correlation in patients with ulcerative colitis was found between sTREM-1 and MDA, and TNFα and MDA, consecutively; nor was correlation found in patients with Crohn's disease between sTREM-1 and TNFα, sTREM-1 and MDA and TNFα and MDA.

DISCUSSION

Triggering receptor expressed on myeloid cells (TREM-1) is a recently discovered receptor on cell membranes of neutrophils and monocytes involved in inflammatory processes and activated by microbial components. Engagement of TREM-1 has been reported to trigger the synthesis of proinflammatory cytokines[4]. sTREM-1 is the soluble counterpart of TREM-1 that is increased in gastric juice of patients with peptic ulcer disease[7]. Based on the latter involvement of sTREM-1 in diseases of the gastric mucose, it was hypothesized that sTREM-1 might be implicated in inflammation of intestinal mucosa. Our data revealed that sTREM-1 was increased in the sera of patients with ulcerative colitis and Crohn's disease (Tables 1 and 2). Various hypotheses could be made to explain the mechanism of increase of sTREM-1 based on the role of bacteria and fungi to trigger TREM-1[14]. It might be assumed that sTREM-1 was increased as a consequence of two probable conditions related with a microbial infection; (1) bacteremia as a result of bacterial translocation through the inflamed intestinal mucosa, and (2) infectious colitis. Both mechanisms are unfavorable for the following two reasons: (1) patients with infectious colitis were excluded from the study, and (2) quantitative blood cultures obtained from patients of all study groups were found sterile. Furthermore no differences were found for white blood cell and platelet counts between controls and patients with

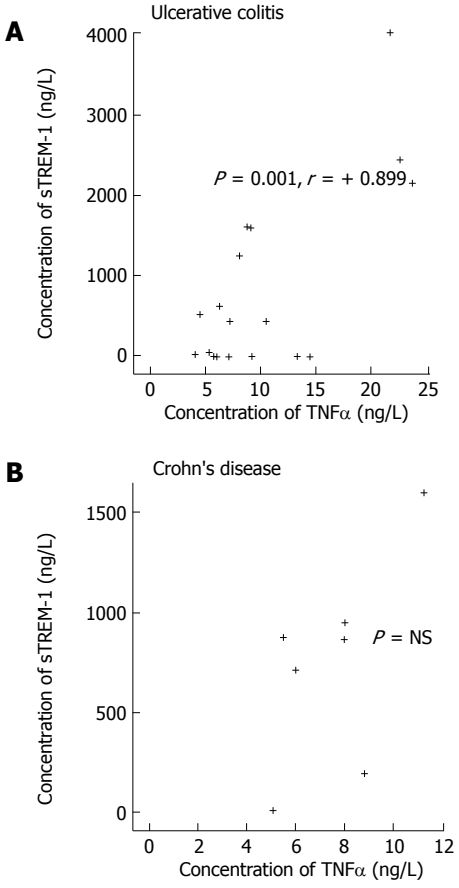

Figure 1 Correlations between sTREM-1, TNFα of IBD patients.

ulcerative colitis as well as between controls and patients with Crohn's disease (Table 2). So, it might be presumed that sTREM-1 increase was not a result of a microbial infection.

The similar kinetics of sTREM-1 and TNFα might indicate the implication of sTREM-1 in the pathogenetic mechanisms leading to IBD. Evolution of IBD seems to

result from the derangement of balance between pro-inflammatory cytokines and mediators with anti-inflammatory activity. The latter imbalance contributes to impairment of mucosal barrier function leading to colonic inflammation[15]. sTREM-1 was increased only in IBD of moderate and severe degree signifying the possibility that sTREM-1 might be an indicator of the severity of the colonic inflammation (Table 2) reflecting the degree of the inflammatory reaction taking place in the intestinal mucosa.

The significance of MDA increase in IBD has already been described by others[16,17]. MDA is a compound functioning as an indicator for the severity of oxidative stress. Oxidative stress seems to be involved in the pathogenesis of several inflammatory processes[16,17]. In the present study, MDA was not correlated with sTREM-1. The latter finding could be explained due to the fact that MDA is an indicator of lipid peroxidation that is not specific to IBD. The present data show for the first time, to our knowledge, that sTREM-1 levels correlate with clinical disease degree and TNFα levels in ulcerative colitis, raising the possibility that it is a mediator in this condition. Further research is mandatory to elucidate the role of sTREM-1 in the evolution from the initial immunologic events occurring in the inflamed intestinal mucosa to the development of inflammatory bowel disease.

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RAPID COMMUNICATION

Prevalence of occult HBV infection in haemodialysis patients with chronic HCV

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Abstract

AIM: To study the prevalence and clinical effects of occult HBV infection in haemodialysis patients with chronic HCV.

METHODS: Fifty chronic hemodialysis patients with negative HbsAg, and positive anti-HCV were included in the study. These patients were divided into two groups: HCV-RNA positive and HCV-RNA negative, based on the results of HCV-RNA PCR. HBV-DNA was studied using the PCR method in both groups.

RESULTS: None of the 22 HCV-RNA positive patients and 28 HCV-RNA negative patients revealed HBV-DNA in serum by PCR method. The average age was 47.2 ± 17.0 in the HCV-RNA positive group and 39.6 ± 15.6 in the HCV-RNA negative group.

CONCLUSION: The prevalence of occult HBV infection is not high in haemodialysis patients with chronic HCV in our region. This result of our study has to be evaluated in consideration of the interaction between HBsAg positivity (8%-10%) and frequency of HBV mutants in our region.

Key words: Chronic HCV; Haemodialysis; Occult HBV infection

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INTRODUCTION

In most cases, occult HBV infection is defined with low existence of HBV infection in HbsAg deficiency. Serum HBV-DNA level in these patients is generally lower than 10^4 copies/mL. It is reported that there is a high prevalence of occult HBV infection in patients with chronic hepatitis C, HCC and haemodialysis patients, criptogenic liver disease, drug injection users and HIV patients, and in those undergoing frequent blood transfusion (those with hemophilia disease, etc.) and blood donors[1-8]. The existing data is limited on occult HBV among patients on long-term dialysis. It has been recently suggested that occult HBV infection might be associated with a lower response of hepatitis C virus (HCV) to standard interferon therapy in patients with normal renal function. The prevalence of occult HBV infection in renal dialysis patients ranges between 0% and 58% in published reports. The aim of this survey is to address epidemiological and clinical significance of occult HBV infection in patients receiving regular dialysis in Turkey.

MATERIALS AND METHODS

A total of 50 patients with chronic renal failure receiving haemodialysis have been chosen with negative HbsAg and positive anti-HCV for at least 6 months and accepted for this study from among the patients of dialysis unit, and from among patients who have applied to Internal Diseases, Faculty of Medicine, Dicle University. These patients were divided into two groups, HCV-RNA positive (22 patients) and HCV-RNA negative (28 patients). Average haemodialysis periods were 4.9 ± 2.3 years in the HCV-RNA positive group[1-9] and 4.6 ± 2.1 years in the HCV-RNA negative group[1-8]. There were 13 male and 9 female subjects in the HCV-RNA positive group, with an average age of 47.2 ± 17.0 years[18-24]. There were 17 male and 11 female subjects in the HCV-RNA negative group, with an average age of 39.6 ± 15.6 years[13-16]. Cirrhosis existence in patients was externalized both clinically and through laboratory tests.

Fifteen ml blood was taken from the patients, centrifuged at 2500 r/min for 5 min, and stored at -25 ℃ with separated serum samples. Hepatitis panel (HBsAg, anti-HBs, anti-HBc IgG) was studied by "Macro Enzyme Immune Assay (Macro EIA) " method in Elecsy 2010 (Roche) device and with similar brand kits; and anti-HCV was studied with the Cobas Core II Immunochemical system (Roche). The "Real-time PCR method" with Robo Gene

(Roboscreen, Leipzig, Germany) kits in ABI PRISM 5700 (Applied Biosystems) device HCV-RNA was used. HBV-DNAs of cases included in the study were quantitatively evaluated by PCR method. Techne Cylogene Thermal Cycler (Techne Cambridge Ltd) Duxford-Cambridge U.K. device was used for HBV-DNA PCR amplification. For PCR amplification, viral DNA was insulated from 100 μL serum in the incubation tampon containing 1 mg/mL Proteinase K at 37℃ through phenol-chloroform extraction and ethanol precipitation following a 3-h incubation.

RESULTS

Patients included in the study were divided into two groups, HCV-RNA positive and HCV-RNA negative. Demographic and clinical characteristics of these patients are shown in Table 1. Thirty (60%) of 50 patients were male, while 20 (40%) patients were female, with an age range of 13-84 years, and averaging 42.9 ± 16.5 years.

HCV-RNA PCR examination was positive in 22 (44%) patients, and negative in 28 (56%) patients. No HBV-DNA (0%) was detected through the high-sensitivity PCR study in both patient groups with positive and negative HCV-RNA. Accordingly, occult HBV infection was detected in none of the 50 patients in our study.

Results in anti-HCV positive and HCV-RNA positive group (22 patients) are as shown in Table 1. Considering both groups together, the results in 50 patients are as follows.

Serum ALT levels are above normal in 12 (24%) of the 50 patients, 8 showed persistent and meaningful ALT level in their six-month monitoring. HCV-RNA was positive in 7 of the 8 patients, and negative in 1 patient. With regard to the duration of haemodialysis, only 6 of the 50 patients were receiving haemodialysis for less than 3 years, 44 patients were receiving haemodialysis treatment for 3 years and over. The minimum haemodialysis duration was 1 year. Twelve (57.1%) out of a total of 21 patients in the two groups with insulated anti-HBs seropositivity received HBV vaccination in their history.

Table 1 Demographic and clinical characteristics of patients

	HCV-RNA Positive (*n* = 22)	HCV-RNA Negative (*n* = 28)	Total (*n* = 50)
Average age (yr) (age range)	47.2 ± 17 (18 - 24)	39.6 ± 15.6 (13-76)	42.9 ± 16.5 (13 - 84)
Sex Male	13 (59.1%)	17 (60.7%)	30 (60%)
Sex Female	9 (40.9%)	11 (39.3%)	20 (40%)
Average Haemodialysis period (yrs) (haemodialysis range)	4.9 ± 2.3 (1 - 9)	4.6 ± 2.1 (1-8)	4.8 ± 2.2 (1-9)
ALT (IU/mL)	52.9 ± 52.8 (4 - 186)	29.3 ± 20.9 (2-88)	39.7 ± 39.7 (2 - 186)
Insulated anti-HBs Seropositivity	7 (31.8)	14 (50%)	21 (42%)
Insulated anti-HBc IgG seropositivity	3 (13.6%)	1 (3.5%)	4 (8%)
Anti-HBs and anti-HBc IgG Seropositivity	10 (45.4%)	6 (21.4%)	16 (32%)
Anti-HBs and anti-HBc IgG Seronegativity	2 (9.1%)	7 (25%)	9 (18%)
HBV-DNA (PCR) Positivity	0 (0%)	0 (0%)	0 (0%)

DISCUSSION

In most cases, occult HBV infection is defined with low existence of HBV infection in HbsAg deficiency. Serum HBV-DNA level in these patients is generally lower than 10^4 copies/mL. It is reported that there is a high prevalence of occult HBV infection in patients with chronic hepatitis C, HCC and haemodialysis patients, in those with criptogenic liver disease, drug injection users and HIV patients, and in those who underwent frequent blood transfusion (those with hemophilia disease, etc) and blood donors[1-8].

As they are similar transmission models, HBV and HCV co-infection are prevalent clinical presentations. Anti-HBc prevalence reported in patients with chronic HCV infection is 50%-55%[1,2,9]. This ratio is a close value, 59% (13/22) in our study. Most studies show that HBV-DNA genome existence is 22%-87%[10,11] in patients with negative HBsAg and positive HCV-RNA. HBV-DNA is seen in 46% of anti-HBc positives, and in 20% of anti-HBc negatives[11]. In chronic HCV related liver diseases, frequency of detectable HBV-DNA is apparently higher than HCV non-related liver diseases[11]. However, variable prevalence of occult HBV infection is seen in the permanence of HBV-DNA PCR[12]. And this makes us think that HBV viremia shows fluctuations during occult HBV infection.

Clinical interaction of HCV and occult HBV infection is still controversial. Some studies report that cirrhosis is seen more frequently in those patients with HCV and occult HBV co-infection than in those with HCV infection alone[11]. However, there are also studies that are not in support of this[13]. Parallel to this, HCV and occult HBV co-infection had a lower incidence in non-cirrhotic hepatitis[14]. Occult HBV co-infection is related to higher ALT levels and histological activity. Despite this, other studies reveal that the incidence of liver related complication in these patients is at a level comparable to those with HCV infection alone[9-13]. Patients with active HBV and HCV co-infection tend not to respond to interferon treatment. Some studies show that such low interferon response exists in patients with HCV and occult HBV co-infection[14-16]. However, other studies reveal that occult HBV co-infection has no effect on the interferon response of HCV infection. Interferon treatment may accelerate HCV clearance in patients with HCV and occult HBV co-infection, but HBV-DNA remains at detectable level. HCV-RNA level is apparently higher in patients with occult HBV co-infection than patients with HCV infection alone. Despite all these, three recent studies show a lower prevalence of chronic HCV and occult HBV co-infection and that the effects of occult HBV infection on chronic HCV infection remained low in these patients[17-19]. While one of these studies report occult HBV co-infection prevalence at 6.7% in patients

with chronic HCV infection, no difference was found in the frequency of occult HBV infection between those with and without a marker for HBV infection in their history[18]. Another study found that occult HBV infection had no effect on the early phase response of chronic HCV infection combined treatment[17]. And the last study states that occult HBV infection has no significant effect on treatment response upon HCV viral titre, liver enzymes, histological parameters and HCV and interferon-α and ribavirin in patients with chronic hepatitis C[19].

In a study on chronic haemodialysis patients, serum in PCR and HBV-DNA in PBMC was studied in 67 patients. While serum HBsAg and HBV-DNA were found negative in all of these patients, HBV-DNA was positive in 5 patients (7.5%) in PBMC[6].

Another study focused on the disease etiology in 107 patients with negative HCC and cirrhosis HbsAg and 192 chronic hepatitis patients as control group with negative HbsAg. In the group with HCC, HCV was blamed as etiological factor in 73 (68%) patients, criptogenic liver disease in 29 (27%) patients and alcohol in 5 (5%) patients. HCV was blamed in 153 (80%) patients of the group with chronic hepatitis HCV, criptogenic liver disease was blamed in 32 (17%) patients and alcohol was blamed in 7 (4%) patients[2]. HBV-DNA PCR was checked in these patients, being positive in 68 (64%) patients with HCC, 63 (33%) patients with chronic hepatitis. In patients with HBV-DNA positive HCC, anti-HBc positivity was found higher than anti-HBc negativity percentage (64% and 56%, respectively). Frequency of occult HBV infection varies in patients with HCC. The prevalence of anti-HBc/Anti-HBs is 43% in such patients, and the HBV-DNA prevalence varies between 5%-80%[2-20].

Although there are strong evidences aimed at the relationship between occult HBV infection and HCC, the role and place of HBV is not clear in this series of incidents. Is it a silent bystander, a cofactor, or criminal alone? There are strong evidences about the etiological relationship between chronic HBV infection and HCC. It is unlikely that occult HBV infection is only a silent bystander in patients with HCC. The high prevalence of occult HBV infection in patients with HCC supports the role of occult HBV infection in the development of HCC compared with the results in patients with cirrhosis or chronic hepatitis. It is quite possible that occult HBV infection plays a role as cofactor in HCC development. Two viruses may mutually interact, causing more painful inflammation and quicker cirrhosis progress. Alternatively, direct oncogenic effect of two viruses may be additive or synergistic[2]. This has been an issue of interest in patients with occult HBV coinfection. Evidences relating to occult HBV infection in patients with HCC related to HCV are being gradually seen more frequently[1,2,4]. In a study made in Spain, in 109 anti-HCV negative and HBsAg negative patients with increased liver enzymes with unknown etiology, 19% HBV-DNA was detected in serum[21]. Again in another study, in 50 patients with criptogenic chronic hepatitis, there was detectable HBV-DNA in 15/50 (30%) patients. Of these 15 patients, 66% had increased liver enzymes and 53% suffered from painful fibrosis and cirrhosis. In their repeated liver biopsies during follow-up, in 2/11 (18.2%) patients, the disease progressed from chronic hepatitis to cirrhosis. Generally considering, clinical results of occult HBV infection after delayed HBsAg clearance vary. These results depend upon the underlying liver disease, duration of active HBV infection and degree of liver damage before HBsAg clearance[1,2].

An increased frequency of occult HBV infection was reported in immune-dominant patients[1]. Lymphoma, aplastic anemia or cythotoxic treatment for leukemia, allongenic or otolog bone marrow transplantation practice in patients with occult HBV infection chronic might cause reactivation of Hepatitis B or development of fulminant hepatic insufficiency[22,23]. However, chronic hepatitis B reactivation with induced treatment is less frequent and painful in those with occult HBV infection before the treatment than those with chronic hepatitis B with positive HBsAg before the treatment[1]. Viral infection progresses with the application of immune-suppressive agent, destruction starts with immune intermediary in hepatocytes infected with HBV after the regaining of immunity with the interruption of this agent.

In patients with fulminant hepatic insufficiency, the pathogenetic role of occult HBV infection has been very well defined. In these patients, positivity of serum HBV-DNA ranges between 0%-47%[24-26]. Recurrence of HBV infection following liver transplantation for fulminant hepatic insufficiency shows possible HBV replication in these patients[24,25]. However, the pathogenetic role of occult HBV infection in patients with fulminant hepatic insufficiency is doubtful and weak. A multi-centered study in the USA shows that occult HBV infection is not related with acute liver insufficiency[26].

Occult HBV infection was found in 81/180 patients (45%) in drug injection users in Baltimore[7]. In a study, 10% occult HBV infection was detected in patients with HIV[8]. In Japan, occult HBV infection was detected in 22 (51.2%) of 43 hemophilia patients[5]. It has been reported that in numerous studies covering wide populations conducted in many different countries, occult HBV infection incidence varied between 0%-15% in blood donors[3].

Most of the relevant authors are mostly unable to provide clear replies to the question what is the responsibility and effect of occult HBV infection in the pathogenesis of diseases where there is a high prevalence of occult HBV infection and where the pathogenetic role of apparent HBV infection is well known. And they are still suspicious on most things. Although there are questions in minds about the subject, pathogenetic role of occult HBV infection is accepted best in HCC.

It is reported that the prevalence of occult HBV infection is parallel with the prevalence of apparent HBV infection in that region[1,2]. For example, occult HBV infection prevalence varies between 7%-19% among blood donors in endemic regions where 70%-90% of the population are exposed to HBV[2]. In Western countries (e.g., there is pre-reported HBV exposure in 5% of the American population), frequency of occult HBV infection ranged between 0%-9%[2]. Prevalence of occult HBV infection can also be affected by incorrect negative and incorrect positive results based on the sensitivity and specificity of the HBV-DNA PCR method. Therefore, the

specificity of the methods has to be verified and cross-contamination has to be prevented. Moreover, in order to positively consider the result in HBV-DNA PCR method, one needs to show at least two different primers from different areas of the HBV genome clone. Most of the studies so far mentioned the sample collection method at very few occasions[1,2,4]. Cross contamination may occur particularly in retrospective studies where the blood samples used in the past are collected.

In a study made by Beşışık *et al*. in 33 patients with chronic hepatitis C and at the same time chronic haemodialysis with negative HBsAg and positive HCV-RNA, serum HBV-DNA PCR study yielded positive results in 12 (36.4%) patients. YMDD mutation was detected in 6 (50%) of these 12 patients[27]. According to this study, there is increased occult HBV infection incidence in patients with chronic hepatitis C treated with chronic haemodialysis and YMDD mutation is the responsible agent in an important part of them. In our study, according to the HCV-RNA condition of the 50 cases chronic haemodialysis with negative HbsAg and positive anti-HCV, the study group was divided into two groups, positive HCV-RNA (22 patients) and patients with negative HCV-RNA (28 patients). None (0%) of the patient groups revealed HBV-DNA in serum by PCR method despite the HCV-RNA positive group has been studied twice in different times. Contrary to the above-mentioned similar study, there is no increased incidence of occult HBV infection in patients with chronic haemodialysis with chronic hepatitis C in our region. In our study, 3 patients showed insulated anti-HBc IgG seropositivity and positive HCV-RNA (13.6%), and 1 patient showed negative HCV-RNA (3.5%). And the percentage of having at least one of the markers of past HBV infection was 89.9% (20 patients) in HCV-RNA positive group and 75% (21 patients) in HCV-RNA negative group. However, no increase was detected in occult HBV infection incidence in our sturdy.

The result of our study has to be evaluated in consideration of the interaction between the HBsAg positivity (8%-10%) for Diyarbakır region and the frequency of HBV mutants in this region. Besides, it has become necessary to review the sensitivity and specificities of still non-standardized HBV-DNA PCR methods and define a common method of diagnosis accordingly.

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RAPID COMMUNICATION

Expression of cyclooxygenase-2 is associated with p53 accumulation in premalignant and malignant gallbladder lesions

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Abstract

AIM: To examine the relationship between cyclooxygenase-2 (COX-2) overexpression and p53 accumulation in gallbladder carcinoma and its precursor lesions.

METHODS: Sixty-eight gallbladder tissue samples comprising 14 cases of normal gallblader epithelium, 27 cases of dysplasia (11 low-grade dysplasia and 16 high-grade dysplasia) and 27 adenocarcinomas were evaluated by immunohistochemistry for COX-2 expression and p53 accumulation. The relationship among COX-2 expression, p53 accumulation and clinicopathological characteristics was analysed.

RESULTS: COX-2 was expressed in 14.3% of normal gallbladder epithelium, 70.3% of dysplastic epitehlium, and 59.2% of adenocarcinomas. When divided into low- and high-grade dysplasia, COX-2 was positive in 5 (45.4%) cases of low-grade and 14 (87.5%) of high-grade dysplasia (P = 0.019). Accumulation of p53 was detected in 5 (31.2%) cases of high-grade dysplasia and in 13 (48.1%) of carcinomas. No p53 accumulation was found in normal epithelium or low-grade dysplasia. COX-2 overexpression was observed in 17 of 18 (94.4%) cases with p53-accumulation in comparison with 20 (40.0%) out of 50 cases without p53 accumulation (P < 0.001).

CONCLUSION: The significant differences in COX-2 expression among normal epithelium, low-grade dysplasia and high-grade dysplasia suggest that overexpression of COX-2 enzyme is an early event in gallbladder carcinogenensis. Furthermore, since accumulation of p53 correlates with COX-2 expression, COX-2 overexpression observed in gallbladder high-grade dysplasia and carcinoma might be partly due to the dysfunction of p53.

Key words: Gallbladder; Carcinogenesis; Cyclooxygenase-2; p53; Immunohystochemistry

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INTRODUCTION

Gallbladder carcinoma is the fifth most common malignancy of the digestive tract. Recent molecular genetic studies have shown that selected proto-oncogenes and tumor-suppressor genes are involved in the development and progression of gallbladder carcinoma, and a different spectrum of molecular genetic changes appears to be responsible for each of the different preneoplastic conditions[1]. An association of gallbladder carcinoma with cholelithiasis or an anomalous arrangement of the pancreaticobiliary duct suggests that long-term inflammation may modulate tumorigenesis as well as the progression of this type of carcinoma[2,3]. Based on the histopathological examination of surgically resected gallbladder, an assumption has been made that epithelial changes found adjacent to invasive carcinomas in the gallbladder wall are premalignant and may progress to carcinoma. The rarity of most presumptive premalignant conditions has made it difficult to obtain better knowledge of the biological characteristics of precursor lesions of gallbladder carcinoma. At present, three major putative pathways of gallbladder carcinogenesis seem to be important: dysplasia, adenoma and anomalous pancreaticobiliary ductal junction. Roa[4] suggested that the period required to transform dysplasia into an advanced carcinoma is about 15 years. However, gallbladder carcinomas are usually diagnosed at an advanced stage, representing a challenge in the management because they respond poorly to therapy and are therefore associated

with a poor prognosis. A postoperative 5-year survival rate between 5% and 13% has recently been reported[5,6]. Since this type of cancer is so difficult to cure by surgery alone, new molecular targets are needed for its prevention and treatment.

Prostaglandins (PGs), a family of lipid-derived autocrine and paracrine mediators, can favor tumorigenesis by altering cell proliferation, differentiation and adhesion, such as by modulating vascular response and immune surveillance. PGs are derived from arachidonic acid. The key step in the conversion of free arachidonic acid to PG is catalysed by the cyclooxygenase enzyme (COX)[7]. Three isoforms of COX (COX-1, COX-2 and COX-3) have been identified. While COX-1 is constitutively expressed as a housekeeping gene in most cells, COX-2 is not detected in normal tissues, except in kidney, liver and pancreatic islands, although it is expressed as an early response to many stimuli, such as inflammatory cytokines, growth factors and oncogens[8]. There is only little information about the recently identified COX-3 isoform. PGs have been shown to mediate gallbladder inflammatory response, since non-steroidal anti-inflammatory drugs (NSAIDs) have been proved to decrease gallbladder inflammation[9]. Accumulating evidences suggest that increased PGs levels via overexpression of the inducible COX-2 isoform are important in the development of human cancer[10]. Though increased levels of PGs and COX-2 expression have frequently been found in malignant growths of the digestive tract (colon, stomach, oesophageal cancer) similar data about biliary tumors are rare[11,12].

Point mutations or deletions of the p53 gene are observed in approximately 50% of cancers. Wild-type p53 is a key molecule in the cellular DNA damage response, causing restriction of cell proliferation by inducing cell cycle arrest and apoptosis. The product of mutant p53 has often been found to be a stable protein with a long half-life, while wild-type p53 has a short half-life and is not generally accumulated to a sufficient high level to be detectable by standard immunohistochemistry. An increase in the p53 protein level can therefore be used as an indication of p53 mutation, although there are still some discrepancies[13]. Immunohistochemically, p53 accumulation was detected in 36%-67.8% of gallbladder carcinomas[14-15]. In gallbladder carcinogenesis, an accumulation of p53 protein was reported in association with premalignant transition to malignancy[16]. p53 and COX-2 are thus two important molecules intimately linked to tumorigenesis. COX-2 has been implicated in positive regulation of growth and tumorigenesis, while the tumor suppressor p53 is a negative regulator of these processes. However, there is evidence of COX-2 expression normally suppressed by wild-type p53, suggesting that loss of p53 function may influence COX-2 overexpression[17]. Conversely, COX-2 can in turn inhibit p53-dependent transcription[18].

The purpose of this study was to evaluate the relationship and potential role of COX-2 and p53 in gallbladder carcinogenesis. Immunohistochemically, we analysed the expression profile of COX-2 in gallbladder dysplasia and carcinoma and its correlation with p53 protein accumulation.

MATERIALS AND METHODS

Subjects

A retrospective analysis was performed on 68 gallbladder tissue specimens, including 14 normal epithelium, 11 low-grade dysplasia, 16 high-grade dysplasia and 27 adenocarcinomas, obtained from patients treated with cholecystectomy in the period from 1998 to 2000. No patient had a history of regular ingestion of selected drugs such as NSAIDs or COX-2 inhibitors. We studied 14 cases of normal gallbladder epithelium without dysplastic and/or carcinomatous epithelial changes and 11 with low-grade dysplasia from elective cholecystectomy because of gallstones. In addition, 16 samples of high-grade dysplasia and 27 adenocarcinoma samples were obtained from patients who underwent cholecystectomy due to gallbladder adenocarcinoma. Dysplasia was defined histologically by varying degrees of pseudostratification, nuclear atypias, loss of polarity, and mitotic figures. Depending on the severity of changes, dysplasia was divided into low- and high-grade types. Adenocarcinomas were graded according to the World Health Organisation classification of gallbladder tumors[19]. There were 8 well differentiated, 4 moderately differentiated and 15 poorly differentiated adenocarcinomas.

Methods

COX-2 and p53 expression were investigated by immunohistochemistry. Formalin-fixed, paraffin embedded specimens were sectioned in series at a thickness of 5 μm. After deparafinisation, the slides were immersed for 30 min in 0.3% hydrogen peroxide/methanol to deplete endogenous peroxidase. Antigen retrieval was achieved using the pressure-cooking method for 4 min in citrate buffer (pH = 6.0). For COX-2 expression, polyclonal anti-human COX-2 antibody (Cayman Chemical Company, Ann Arbor, MI, USA) was used in a dilution of 1:500. For p53 analysis, monoclonal mouse anti-human p53 antibody, DO-7 (DAKO, Glostrup, Denmark) diluted at 1:100 was used. After the application of primary antibodies, the specimens were treated with biotinylated secondary antibody for 30 min. Antigen visualisation was achieved by applying a standard streptavidin-biotin complex (ABC, DAKO, Denmark) for 30 min followed by diaminobenzidin chromogen (DAB, Sigma Chemical CO, Germany) in 0.1% H_2O_2 PBS solution. The specimens were counterstained with haematoxylin. The specificity of the applied antibodies was checked with positive or negative controls. Specimens treated without primary antibodies served as negative controls. For positive controls of p53, specimens of human colon carcinoma with known p53 immunoreactivity were processed in the same way as the gallbladder samples. Vascular endothelial cells and fibroblasts observed in all gallbladder specimens provided internal positive controls for COX-2 staining.

Immunostaining was independently evaluated by two investigators (AC and ML) and a consensus agreement was achieved. COX-2 expression was evaluated according to the percentage of positive cells and the intensity of staining. The percentage of positive cells was scored as 0

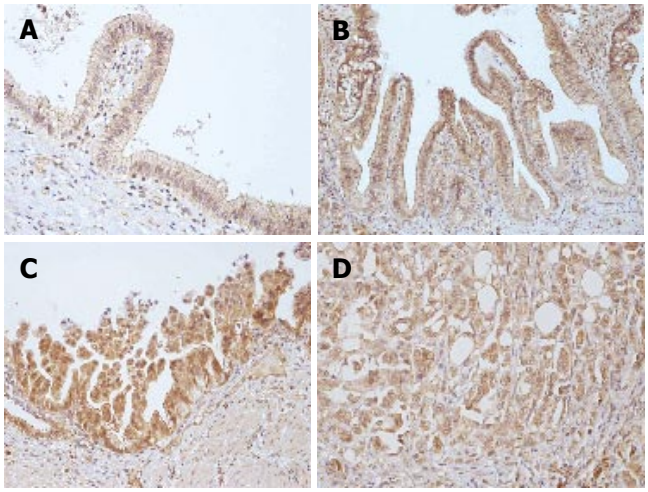

Figure 1 COX-2 expression. **A:** Normal gallbladder epithelium; **B:** Low-grade dysplasia; **C:** High-grade dysplasia; **D:** Gallbladder adenocarcinoma.

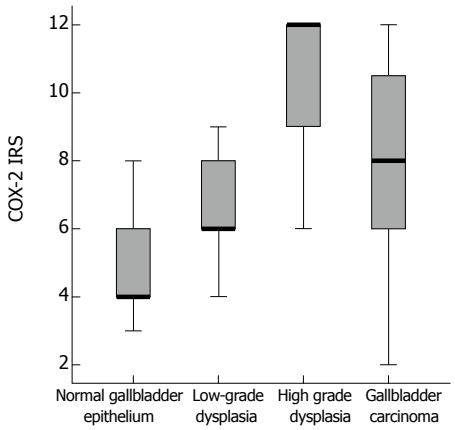

Figure 2 COX-2 – IRS index.

(0%), 1 (< 10%), 2 (10-50%), 3 (51%-80%) or 4 (> 80%). The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). For immunoreactive score (IRS), the percentage of positive cells and the staining intensity were multiplied, resulting in a value between 0 and 12. To separate tumors with weak from those with strong COX-2 expression and to define a cut-off point that might be reproducible for future studies, we united samples with an IRS of 0-6 into one group with negative to weak COX-2 expression (the COX-2 negative group) and those with an IRS of 7-12 (the COX-2 positive group). The minimum requirement for positive IRS was therefore either moderate expression in > 80% of cells or strong expression in > 50% of cells. The expression of p53 was evaluated according to the percentage of positive cell nuclei. The reaction to p53 was considered positive when at least 20% of the nuclei in tumor cells were stained.

Statistical analysis

SPSS 11.0 for Windows (SPSS Inc., Chicago IL) was used. Chi-square tests were used to analyse the correlation between COX-2 overexpression and p53 accumulation and pathological features. The relationship between COX-2 and p53 expression was evaluated by the Mann-Whitney U-test. $P < 0.05$ was considered statistically significant.

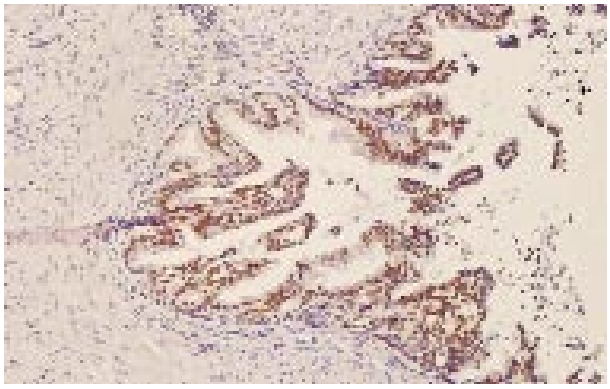

Figure 3 Accumulation of p53 in nuclei of high-grade gallbladder dysplasia.

RESULTS

Surgical specimens of the gallbladder were obtained from 68 patients who had undergone cholecystectomy for clinical chronic cholecystitis or cholelithiasis (n = 51) and primary gallbladder carcinoma (n = 27). There were 43 female and 25 male patients, the median age of patients was 59 years (range from 36 to 78 years). The expression of COX-2 and p53 was analysed by immnohistochemistry in 14 cases of normal gallbladder epithelium, 27 cases of dysplasia and 27 adenocarcinomas. The cytoplasm of gallbladder epithelial cells was stained for COX-2 to various degrees (Figure 1). Additionally, COX-2 immunoreactivity was observed in fibroblasts, endothelial cells, and smooth muscle cells in all specimens. Normal gallbladder epithelium was COX-2 positive (according to the cut-off criteria of the IRS index) in only 2 (14.3%) cases. Of 27 cases of dysplasia, 19 (70.3 %) were COX-2 positive. When we divided this group into low- and high-grade dysplasia, COX-2 was positive in 5 (45.4%) cases of low-grade dysplasia and 14 (87.5%) cases of high-grade dysplasia (P = 0.019). The differences between normal epithelium and low-grade dysplasia were not statistically significant (P = 0.085). COX-2 was positive in 16 (59.2%) of 27 carcinomas.

The greatest positivity for COX-2 was in poorly differentiated carcinomas (73.3%). The differences between high-grade dysplasia and carcinoma were not statistically significant (P = 0.113). COX-2-IRS values detected in normal gallbladder epithelium, dysplasia and carcinoma are shown in Figure 2.

p53 was negative in normal gallbladder epithelium and low-grade dysplasia,. Accumulation of p53 protein was identified in the nuclei of 5 (31.2%) cases of high-grade dysplasia (Figure 3) and 13 (48.1%) carcinomas. Accumulation of p53 protein was the highest (80%) in poorly differentiated gallbladder carcinoma. In the whole spectrum of 68 tissue samples, we found only one COX-2 negative case among 18 p53 positive cases. Of 50 p53 negative cases, 30 (60 %) cases were COX-2 negative ($P < 0.01$).

DISCUSSION

Our analysis of COX-2 and p53 expression in normal, dysplastic (premalignant) and malignant gallbladder epithelium is an attempt to reveal a possible relationship between the two molecules, as well as their role in

gallbladder carcinogenesis. The role of COX enzymes in the process of carcinogenesis has been dramatically emphasized by confirmation that NSAIDs decrease the number of colonic polyps in humans with adenomatous polyposis and lower the incidence of colorectal cancer[20]. Gallbladder cancer has several analogous characteristics with this type of neoplasia. It is preceeded by a lengthy precancerous process taking several years. This process is characterized by chronic inflammation, lithyasis, intestinal metaplasia, and dysplasia in the epithelium of the surrounding mucosa[4]. We observed overexpression of COX-2 in 14 (87.5%) of 16 gallbladder high-grade dysplastic tissues in comparison to 5 out of 11 (45.4%) low-grade dysplasia and 2 (14.3%) of 14 normal gallbladder epithelium. Some slight COX-2 immunoreactivity in normal gallbladder epithelium was not unexpected and could be explained by the well-established connection between COX-2 expression and inflammation[20]. In carcinomas, COX-2 overexpression was found in 16 (59.2%) of 27 gallbladder samples. Our results are consistent with studies by Asano *et al*[11] and Zhi *et al*[21], who found 67.3% and 71.9% gallbladder tumour samples with COX-2 overexpression, respectively. Recently, Tsuchida[3] reported that COX-2 inhibitor meloxicam suppresses carcinogenesis in the gallbladder in an animal model. Our observation that COX-2 overexpression was highest in high-grade dysplasia supports the hypothesis of the role of COX-2 in early carcinogenesis. Liang[22] first suggested that COX-2 expression might be involved in the early proliferative phase but not in the late metastatic phase of the evolution of colorectal cancer. Our study is in agreement with Luzar[23] , who distinguished three histological degrees of gallbladder epithelial changes according to the number of hTERT signals: a) normal and regenerative gallbladder mucosa, b) low-grade dysplasia and c) high-grade dysplasia and adenocarcinoma of the gallbladder. We found no statistical differences in our study in COX-2 expression between normal gallbladder epithelium and low-grade dysplasia ($P = 0.085$), probably due to COX-2 expressed in normal mucosa on account of inflammation. However, the differences were significant between low-grade and high-grade dysplasia. No direct data are yet available about the progression rate of high-grade dysplasia to invasive carcinoma of the gallbladder. For comparison, high-grade dysplasia in the stomach was detected to regress in only 5% of cases, persisted in 14%, and progressed in 81%-85%[24] .

In high-grade dysplasia, p53 protein accumulation was found in 5 (31.2%) cases. Low-grade dysplastic and normal epithelium was p53 negative. Thirteen (48.1%) of 27 carcinomas were p53 positive. The clone against which the p53 antibody was raised (DO-7) results from the recognition of wild type and mutated protein. However, p53 detection was not expected in normal cells. The appearance of a nuclear accumulation of p53 protein might arise from mutation of the protein itself, resulting in failure of its degradation pathway or a mutation of the human mouse double minute (hMDM)-ubiquitin pathway, resulting from the failure of degradation. Accumulation of the wild-type p53 protein owing to a failure of degradation might push the cell into cell cycle arrest and apoptosis. Such cells would occur rather sparsely in tumors because they could not proliferate as malignant clones. It therefore seems very likely that the accumulation of p53 in dysplastic and malignant gallbladder epithelium resulted from p53 mutation[25]. Our results are consistent with some previous reports about p53 gene mutations involved in the carcinogenesis of the gallbladder epithelium[26-27] that p53 overexpression appeard in only 84.6% of gallbladder carcinomas and in 18.7% of patients without carcinoma. Matsubara[28] detected p53 gene mutation in 38.5% of noncancerous biliary epithelial lesions and in 57.1% of adenocarcinomas.

Data about the inter-relationship between p53 and COX-2 are conflicting. Our results are consistent with studies of Shigemasa[29] and Erkinheimo[30]. In both their studies, performed on ovarian carcinoma, a significant positive correlation was found between COX-2 expression and p53 accumulation. The same correlation was accepted in a study of head and neck tumors[31], suggesting that restoration of wild-type p53 expression might interfere with tumour growth by inhibiting the COX-2 pathway. In endometrial carcinoma[32] and colon carcinoma[22], higher COX-2 expression was also associated with p53 accumulation. In contrast, Cho[33] observed no association between COX-2 overexpression and p53 accumulation in renal cell carcinoma. Subbaramaiah[34] demonstrated that wild-type p53 suppressed COX-2 expression by inhibiting its promotor activity. The level of COX-2 protein and mRNA was found markedly decreased in cells expressing wild-type, but not mutant p53[35]. A study of head and neck carcinoma cell lines[36] confirmed that wild-type p53 overexpression has some anti-tumor effects through the suppression of COX-2 gene activity. However, Swamy[18] reported that COX-2 at least in part contributes to the dysfunction of p53 and that celecoxib, a COX-2 selective inhibitor, protects p53 functional activity.

In conclusion, our study is the first to analyse the relationship of COX-2 expression and p53 accumulation in various histological stages of gallbladder epithelial abnormalities and gallbladder adenocarcinoma. Our results suggest that COX-2 might play an important role in gallbladder carcinogenesis. Overexpression of COX-2 appears to be an early event in gallbladder carcinogenesis, already occurring at the stage of preneoplastic epithelial changes (e.g. high-grade dysplasia) and may be, at least in part, related to p53 dysfunction.

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RAPID COMMUNICATION

Glycemic index of cereals and tubers produced in China

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Abstract

AIM: To determine the GI of some cereals and tubers produced in China in an effort to establish the database of glycemic index (GI) of Chinese food.

METHODS: Food containing 50 g carbohydrate was consumed by 8-12 healthy adults after they have been fasted for 10 h and blood glucose was monitored for 2 h. Glucose was used as reference food. GI of food was calculated according to a standard method.

RESULTS: GI of 9 types of sugar and 60 kinds of food were determined.

CONCLUSION: Food GI is mainly determined by nature of carbohydrate and procession. Most of cereals and tubers produced in China have similar GI with their counterparts produced in other countries.

Key words: Glycemic Index; Cereals; Tubers; Carbohydrate

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INTRODUCTION

GI is a useful index initially coined by Jenkins[1] *et al* to describe the extent to which certain food can increase the blood glucose in human. It is an accurate determination of change of blood glucose after consumption, digestion and absorption of certain food. GIs of 60 kinds of food were determined and reported by Jenkins in 1981. Since then, GI of local foods have been reported by Miller and other scientists[2,3]. Up to date, GI of about 2000 foods have been determined and reported around the world.

The study of GI was limited to clinic research and dietary treatment of diabetes in the early stage of GI study. Since the late 1990s, more and more attention has been paid to the application of GI in body weight maintenance and chronic disease controlling and positive links have been established between diet GI and some pathological conditions such as obesity[4], heart disease[5], diabetes[6].

China is an Asian country with more than thirteen billion people of 56 nationalities and various eating habits and foods. With the rapid development of economy, lifestyle of Chinese people is changing also and a cluster of chronic diseases are emerging, some of which have become health threat of the people as seen in the western countries. Therefore, links between dietary habits and chronic diseases should be investigated and strategies should be developed for the prevention, as well as treatment of such diseases. It is urgent and necessary for us to determine the GI values of Chinese foods. The current study was designed to determine the GI of about 150 types of cereals and tubers by a standard method.

MATERIALS AND METHODS

Subjects

Healthy volunteers (aged 20-45 years, BMI in kg/m^2 range 18.5-25) from Beijing (north China), Chengdu (southwest China), Liaoyang (northeast China) and Yinchuan (northwest China) were involved in the study. All subjects were free from obesity, diabetes and other metabolic diseases and postmenopausal women were excluded. All subjects gave their written informed consent and ethical approval for the study was obtained from the Ethical Committee of Chinese Centers for Disease Control and Prevention.

Test foods: All the foods used in this study were purchased from local supermarket and raw foods were cooked by traditional method used in daily-life of

Chinese. The carbohydrate (CHO) content of the test food was calculated based on food composition data of China according to the following formula: weight of test food = 50/CHO proportion of test food. In cases when CHO content of test food was less than 10%, food weight used to determine its GI was reduced to 25 g. Each food was tested in 10-12 volunteers.

Methods

Oral glucose tolerance test (OGTT): About 10-12 volunteers received OGTT each time. After fasting for 10 h, volunteers were required to arrive at the laboratory at 8 o'clock in the morning. Venous blood samples were obtained before and 15, 30, 45, 60, 90, 120 min after consumption of 50 g glucose dissolved in 200 mL drinking water. Blood was collected into a syringe with sodium fluoride.

Test of glucose response of food: Volunteers with normal glucose tolerance were allowed to receive the glucose response test two days later. When volunteers fasted for 10 h arrived at the laboratory, venous blood samples were collected before and 15, 30, 45, 60, 90, 120 min after consumption of food containing 50 g carbohydrate. For each sample, blood glucose was determined. Each test was repeated and each group of volunteers received no more than tests of three foods. Between tests, at least two days was required for wash-out.

Determination of blood glucose: Calculation of GI: Glucose (GI = 100) was used as reference. The calculation was based on the method developed by Wolever[7]: GI = [(area under the 2-h blood glucose curve for food)/(area under the 2-h blood glucose curve for the same amount of glucose)]× 100

Statistical analysis

The values of GI were expressed as mean ± SD. Dixon test was used to evaluate differences between values of the same group. $P = 0.05$ was selected as level of significance.

RESULTS

Nine sugars were selected as test foods. When glucose was used as the reference (GI of glucose: 100), the GI rank of these sugars from high to low was maltose > white sugar > honey > sucrose > lactose > fructose (Table 1). The GI values of maltose of different source were not equal, which maybe was resulted from procession and the difference of purity.

GI of cereals

Cereals refer to a variety of foods rich in carbohydrate, including traditional starchy food such as steamed bread and noodle, and food processed by modern technology such as breakfast cereals and some instant foods. The significant difference of GI of different cereal foods suggested that food procession and food type were factors influencing the value of GI (Table 2).

GI of some tubers

Tubers also include various types of foods, of which potato, yam, taro and lotus are commonly eaten by Chinese people. Their GI values are shown in Table 3.

Table 1 Glycemic index of some sugars (mean ± SD)

Name	*n*	GI
Glucose	10	65.0 ± 6.3
Fructose	10	23.0 ± 4.6
Lactose	10	46.0 ± 3.2
Chocolate	10	49.0 ± 8.0
Maltose	10	105.0 ± 5.7
Maltose 2	10	75.0 ± 20.3
White sugar	10	83.8 ± 12.1
Honey	10	73.5 ± 13.3

DISCUSSION

Carbohydrate is a major source of energy of human being. Nutrition surveillance in China shows that carbohydrate intake is 360 g/d, which provides 60%-70% of total energy of a person. Cereals and food produced from cereals account for 90% of total dietary carbohydrate. It is obvious that cereals are critical for the health of Chinese.

Because people have realized that overtake of fat is associated with a cluster of chronic diseases, increasing the intake of carbohydrate has been the major concern of nutritionists in dietary recommendation. But controversies still remain as to how and what kind of carbohydrate should be increased in diet.

Food GI is a quantitative physiological parameter used to describe the relative glycemic effect of carbohydrate-containing food compared with reference food (glucose or white bread)[8]. After ingestion, all the digestible carbohydrates are conversed to monosaccharide and absorbed in the intestine, then blood glucose is increased and satiety is ensured. Insulin and other hormones secretion is induced by the increased blood glucose and blood glucose level is then reduced toward the basic level, thus homeostasis is maintained. Avoiding dramatic fluctuation of blood glucose is critical to both the healthy and the diabetic persons. A lower postprandial blood glucose peak and slower decreasing rate are seen after intake of low GI food compared with high GI food. It is evident that carbohydrate content of one food does not always reflect its availability. GI provides a new method for the nutritional evaluation of food.

Food is categorized into three groups according to their GI[8], food with GI < 55 is called low GI food, and high GI food means food with GI >75, food with GI ranging between 55-75 is called medium GI food. Consumption of low GI food will ensure a longer lasting satiety and keep more stable blood glucose, at the same time overtake can be avoided easily compared with consumption of high GI food. High GI food is not good for diabetes and people with impaired glucose tolerance.

GI is a concept comprehensively reflecting the digestibility and utility of food. Factors affecting the GI of food such as composition, content, and type of carbohydrate, physical character and procession are all taken into account in this concept[9,10]. Our results showed that different types of carbohydrate have different

Table 2 Glycemic index of cereal products (mean ± SD)

Food name	*n*	GI	Food name	*n*	GI
Cooked rice	12	83.2 ± 3.1	Steamed bread (refined)	9	88.1 ± 20.2
Brown rice (cooked)	10	87.0 ± 5.0	Wheat pancake	10	79.6 ± 11.5
Sticky rice (cooked)	10	87.0 ± 7.0	Wheat dough, deep-fried	8	74.9 ± 21.0
Sticky rice2 (cooked)	9	88.0 ± 5.6	Bread (refined wheat)	10	87.9 ± 10.2
Sticky rice (higher amylose)	10	50.0 ± 6.0	Bread (whole wheat)	10	69.0 ± 10.4
Rice porridge	10	69.4 ± 18.5	Bread (whole wheat with dried fruit)	10	47.0 ± 7.0
Sticky rice porridge	9	65.3 ± 20.6	Wheat Noodle(dried)	10	46.0 ± 5.8
Black rice porridge	9	42.3 ± 9.0	Wheat Noodle(fresh)	8	81.6 ± 19.1
Rice bran porridge	9	19.0 ± 3.0	Dumpling (shallot + meat)	10	28.0 ± 9.9
			Steamed stuffed bun (shallot + meat)	10	39.1 ± 13.0
Rice cake	8	82.0 ± 7.2	Biscuit (thin)	9	81.0 ± 7.5
Instant rice (in hot water 3 min)	10	46.0 ± 8.5	Biscuit	9	72.0 ± 15.5
Instant rice (cooked 6 min)	10	87.0 ± 5.5	Danone biscuit 1	9	47.1 ± 12.4
Corn powder porridge	10	68.0 ± 10.6	Danone biscuit 2	9	39.3 ± 11.8
Corn granule	10	51.8 ± 9.2	Cake crisp	10	59.0 ± 6.0
Corn flake	9	78.5 ± 12.2	Powder (buckwheat)	8	54.0 ± 3.0
Corn flake 2	9	74.0 ± 10.0	Noodle (buckwheat)	9	59.3 ± 3.0
Oatmeal (unpackaged)	9	55.0 ± 6.1	Bread (buckwheat)	8	66.7 ± 6.0
Oatmeal	8	83.0 ± 18.7	Instant noodle (buckwheat)	8	53.2 ± 5.0
Porridge (instant)	8	69.4 ± 7.2	Millet porridge	8	61.5 ± 9.0
Popcorn	6	55.0 ± 2.0	Powder (various cereals)	9	57.9 ± 3.5
Sweet corn (cooked)	10	55.0 ± 5.0	Whole wheat powder	10	42.0 ± 7.5
Oat biscuit	10	55.0 ± 2.5	Nutritional pancake	8	65.7 ± 5.3
Barley flake	7	69.0 ± 7.3	Biscuit	7	70.0 ± 8.3
Rice (S+R) porridge	9	65.5 ± 16.0	WoTao (Corn+wheat)	10	64.9 ± 16.5

influence on blood glucose. For example, rice rich in amylopectin has high absorption rate and consequently high blood glucose because digestive enzyme can easily reach the structure of starch chain. The opposite is true as to the amylose. Possible explanation for GI difference of different food is: first, food rich in dietary fiber, resistant starch or other indigestible carbohydrate has stronger resistance to digestive enzymes and its digestion and absorption in the intestine is slow and incomplete. Under this kind of conditions, low blood glucose is seen. Second, physical properties of food such as the size of granule and maturity are factors determining GI. Third, time and temperature in procession have significant effect on food GI as seen in the case of potato and vermicelli. It is also obvious that procession is a crucial factor in determining food GI as demonstrated by GI values of different corn, rice, potato and wheat products.

Table 3 Glycemic index of tubers (mean ± SD)

Food	*n*	GI
Potato (cooked)	10	66.4 ± 3.8
Potato (steam)	10	62.0 ± 5.7
Potato crisp (oil fry)	10	60.3 ± 7.0
Potato noodle (with meat)	9	16.7 ± 10.4
Cake (Cushawand potato)	8	108.0 ± 13.4
Yam (steam)	10	51.0 ± 12.0
Yam (cooked)	10	54.0 ± 5.5
Potato mashed	10	73.0 ± 9.2
Potato noodle	9	13.6 ± 2.1
Sweet potato (red, cooked)	8	76.7 ± 12.3
Taro (cooked)	7	47.7 ± 12.7
Pill potato+yam powder	9	34.5 ± 11.7
Lotus root powder	8	32.6 ± 17.0

In accordance with literatures, we found that GI of sucrose is not so high as and the GI of starch is not so low as predicted. The GI of sucrose is lower than expected because sucrose is disaccharide consisting of glucose and fructose which is absorbed and directly transferred to the liver where it is ultimately transformed into glucose and therefore has a lower GI (GI = 23). About half of all the sucrose we ingest is glucose, and fructose accounts for another half. Refined sugar is used in most of processed foods which has a GI of about 60, which is the mean of glucose GI and fructose GI. The GI of starchy foods depends on the rate at which the food can be absorbed and transferred into the blood, and the extent to which the food can raise the concentration of blood glucose. The GI of food with rapid digestibility is usually high. Compared with GI data of other countries, we found that GI of foods produced in China has similar GI to that of counterpart foods produced abroad with the exception of maltose, honey and white sugar. In the present study we found that maltose from different source shows different GI (105 *vs* 75); the GI of honey (67) and white sugar (83) are higher than that published by other authors (GI of honey and white sugar were 58 and 70, respectively). The explanation for this discrepancy is the difference of procession and origin of food. For example, maybe honey and white sugar were supplemented with glucose syrup.

Food of low GI often implies lower utilizable energy. In practice, control of quantity of total energy remains a primary concern in dietary prevention and treatment of obesitic diabetes. GI has been proved a useful and simple concept in food selection, diets arrangement and dietary regulation of blood glucose. GI is not only useful in the dietary treatment of diabetes, hypertension patients and obesity, but also can be applied to dietary

management of athletes, food procession and study of relationship between dietary intake and chronic disease[11]. Establishment of GI values of Chinese foods is the first step of GI study in China. More studies should be conducted in this field.

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RAPID COMMUNICATION

A randomized controlled clinical trial: Interruption of intrauterine transmission of hepatitis B virus infection with HBIG

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Abstract

AIM: To evaluate the efficacy of interruption of intrauterine infection of HBV with HBIG in pregnant women with positive HBeAg and HBsAg.

METHODS: A prospective randomized controlled trial was adopted. Sixty cases with positive HBeAg and HBsAg were coincident with the criteria of inclusion, and 8 cases were excluded. Fifty-two cases were analyzed (28 cases in trial group and 24 in control group). All cases in trial group received 200 IU HBIG intravenously every 4 wk for 3 times from the 28th wk. The cases of control group received placebo in the same way. All pregnant women were detected for HBeAg and HBV-DNA at the beginning of the trial and end of the trial (delivery). The cord blood of all newborns were collected for detecting HBeAg and HBV-DNA simultaneously.

RESULTS: For investigation of HBeAg of newborns in trial group, 6 of 28 cases of newborns had positive HBeAg, the HBeAg positive rate being 21.4%, the total rate of 95% CI being 8%-41%. In control group, 19 of 24 cases of newborns had positive HBeAg, HBeAg positive rate was 79.2%, the rate of 95%CI being 5%-93%. By statistical analysis, χ^2 = 17.26, P < 0.01, RR = 0.27, 95% CI (6.3 × 10^{-6}, 8.6 × 10^{-5}). For investigation of HBV-DNA of newborns in trial group, 7 of 28 cases of newborns had positive HBV-DNA, HBV-DNA positive rate being 25%, the total rate of 95% CI being 11%-45%. In control group, 20 of 24 cases of newborns had positive HBV-DNA, HBV-DNA positive rate was 83.3%, the total rate of 95% CI being 63%-95%. By statistical analysis, χ^2 = 17.62, P < 0.01, RR = 0.30, 95% CI (1.5 × 10^{-5}, 1.7 × 10^{-4}). The results indicated that there was significant difference in HBeAg positive rate and HBV-DNA positive rate of newborns between the two groups. In trial group, 7 of 28 newborns had HBV-DNA positive, but the HBV-DNA load of newborns was lower than that of their mothers. In control group, 20 of 24 newborns still had HBV-DNA positive, and the HBV-DNA load of newborns was close to those of their mothers. Statistical analysis indicated that there was no significant difference in HBV-DNA load between postnatal women without HBIG intervention and their filial generations (T = 81.5, P > 0.1).

CONCLUSION: It is effective and safe to prevent intrauterine infection of HBV with HBIG from the 28th wk in pregnant women with positive HBeAg and HBsAg. In clinical application, those pregnant women with negative HBeAg and positive HBV-DNA also need to be interrupted by HBIG.

Key words: Interruption; Intrauterine; Transmission; Hepatitis B virus; HBIG

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INTRODUCTION

Hepatitis B virus(HBV) is highly endemic in China. It has been estimated that there are more than 1.2 million of chronic carriers of HBV nationwide. Most of them are infected by mother-infant transmission. Mother-infant infection of HBV can result in chronic HBV carrier state, chronic hepatitis, cirrhosis, hepatecellular carcinoma and the cycling of mother-infant transmission. So to prevent mother-infant transmission is a key strategy to control hepatitis B prevalence in our country. For a long time, it has been successful to vaccinate the infants of HBV positive mother with hepatitis B vaccine or both with hepatitis B immune globulin (HBIG) after birth. However, 10%-20% of the infants have no response to vaccination[1]. The main reason of failing to immunization is intrauterine

infection of HBV. Therefore, it will be meaningful to interrupt the intrauterine infection of HBV. In this study, we try to explore the effects of interruption of intrauterine infection of HBV with HBIG in pregnant women with positive HBeAg. It will be reported as follows.

MATERIALS AND METHODS

Design of clinical trial

A prospective randomized controlled trial was adopted. Trial group and control group were determined by randomized number table. The study (from January. 1997 to May, 2002) were undertaken by 4 sub-centres, including the First Affiliated Hospital, Xinjiang Medical University, Urumqi Military General Hospital; Maternal and Children' s hospital; Xinjiang Jian Gong Hospital. According to the estimation of sample number, 30 cases were included in each group. With regard to the missing of follow-up, we started the clinical trial based on 40 cases in each group.

Subjects

The criteria for inclusion and exclusion were determined before starting the trial. The criteria for inclusion: the pregnant women given pre-labor checkups at obstetric clinic of above hospitals, with positive HBeAg and better general condition, without threatened abortion or threatened premature labor, and hypertension, with normal liver function, and to deliver at the same hospital. The criteria for exclusion: to stop pregnancy for some reasons, to deliver at other hospitals and lose follow-up, and to be administered HBIG not according the regulation.

Sixty cases of pregnant women were coincident with the criteria for inclusion, but 8 cases were excluded, including , 3 cases who stopped pregnancy; 2 cases who transferred to other hospitals; 2 cases who lost to follow-up for unknown reasons and 1 case who used HBIG not according the regulation. So at last, 52 cases were analyzed, including 28 cases in trial group and 24 cases in control group. There was no significant difference about pregnant age, parity, fetal age, delivery mode and median of HBV-DNA (respectively 4.3×10^8 and 3.98×10^8 copy/mL) between those two groups. There was no history of fetal distress in all newborns of the two groups. Fifty-two pregnant women with positive HBeAg labored 52 newborns. No side effects were observed in trial group.

Methods of treatment

All cases in trial group received 200 IU HBIG intravenously every 4 wk for 3 times (28 wk, 32 wk, 36 wk). And side effects were observed. The cases of control group were followed up for checkups, without any treatment.

HBeAg and HBV-DNA test

All pregnant women were detected for HBeAg and HBV-DNA at the beginning of the trial and the labor day. The cord blood of all newborns were collected, separated for serum and stored at -20℃ for detecting HBeAg and HBV-DNA simultaneously. HBeAg was tested by ELISA (commercial kit from Huamei Biotechnology Company, No 20020605), HBV-DNA was tested by FQ-PCR (commercial kit from Da`an Biotechnology Company, No.s19990003). The procedures of detection were performed according to the instruction of the manufacturers.

Table 1 Comparision of cord blood: HBeAg and HBV-DNA positive rate

Groups	cases	HBeAg(+)		HBV- DNA(+)	
	n	*n*	%	*n*	%
Trial	28	6	21.4	7	25
Control	24	19	79.2	20	83.3

Table 2 HBV-DNA levels in 7 mother-infant pairs in trial group (gene copy/mL)

Pair No.	Pregnant women	Filial generation
1	1.16×10^8	1.88×10^5
2	4.45×10^8	2.84×10^4
3	2.74×10^8	1.83×10^5
4	4.78×10^7	3.56×10^5
5	1.53×10^8	2.66×10^5
6	3.94×10^8	2.92×10^4
7	2.28×10^8	1.68×10^5

Statistical analysis

Relative risk (RR) and 95% CI were calculated, χ^2 test was used to compare the difference of qualitative data, Wilcoxon matched rank test was used to compare the difference of HBV-DNA load.

RESULTS

In trial group, 6 of 28 cases of newborns had positive HBeAg (Table 1). HBeAg positive rate was 21.4%, the total rate of 95% CI being 8% and 41%. In control group, 19 of 24 cases of newborns with HBeAg positive (Table 1), HBeAg positive rate was 79.2%, the rate of 95%CI being 5% and 93%. By statistical analysis, $\chi^2 = 17.26$, $P < 0.01$, RR = 0.27, 95% CI: 6.3×10^{-6} and 8.6×10^{-5}. The results indicated that there was significant difference in HBeAg positive rate of newborns between the two groups. The risk of intrauterine transmission could be reduced by immunoprophylaxis with HBIG. In trial group, 7 of 28 cases of newborns had positive HBV-DNA (Table 1), HBV-DNA positive rate was 25%, the total rate of 95% CI being (11%, 45%), In control group, 20 of 24 cases of newborns had positive HBV-DNA, (Table 1), HBV-DNA positive rate was 83.3%, the total rate of 95% CI being 63% and 95%. By statistical analysis, $\chi^2 = 17.62$, $P < 0.01$, RR = 0.30, 95% CI 1.5×10^{-5} and 1.7×10^{-4}. It indicated that there was significant difference in HBV-DNA positive rate of newborns between the two groups. It was effective to decrease the HBV-DNA load of pregnant women with HBV-DNA positive by application of HBIG.

In 28 cases of postnatal women with HBIG intervention, 21 cases of newborns had negative HBV-DNA, 7 cases had HBV-DNA positive, but the HBV-DNA

load was lower than that of their mothers (Table 2). By Wilcoxon matched rank test (T = 28, $P = 0.02$), it indicated that the HBV-DNA levels of filial generation decreased after intervention with HBIG. In control group, only 4 of 24 cases with negative HBV-DNA, the others were still HBV-DNA positive, and the HBV-DNA load was close to those of their mothers. By Wilcoxcon matched rank test (T = 81.5, $P > 0.1$), it indicated that there was no significant difference in HBV-DNA load between postnatal women without HBIG intervention and their filial generations.

DISCUSSION

The prevention of HBV infection by vaccinating newborns has been previously demonstrated to be extremely efficacious. However, some newborns fail to respond to immunization[2]. Intrauterine infection is regarded as the main reason of failure. It has been reported that the rate of intrauterine infection is up to 10%-44%[3]. It shows that intrauterine infection plays an important role in mother-infant transmission of HBV. The intrauterine infection may occur as early as on the 19th wk of pregnancy, but the main time is possibly in the third-trimester of pregnancy[4]. Recently more and more researchers are trying to interrupt the intrauterine infection by intervention. HBIG is a common method to prevent HBV transmission from mother to baby[5,6].

The mechanism of intrauterine infection of HBV has been studied by some researchers[7,8], the findings proved that the main route of HBV transmission from mother to fetus is transplacental. HBIG is an antibody of IgG type. The nourish cells with Fc recepter in placenta can transmit the IgG antibody from mother to infant since the 20 wk during pregnancy. In one study[9], HBIG was injected frequently to pregnant women with positive HBeAg starting from the 20th wk during pregnancy, the positive rate of anti-HBs of newborns was up to 91.42% and no side effect and birth defect were observed in both mothers and newborns. The result showed that anti-HBs can get into the body of infants via placenta after using HBIG during pregnancy. That is to say the mechanism of preventing intrauterine infection of HBV by HBIG is production of fetal passive immunity[9].

This study is a randomized controlled trial to interrupt intrauterine transmission of HBV by HBIG in pregnant women with positive HBeAg. The results showed that it was effective to waken the effectiveness of mother-infant transmission of HBV after using HBIG frequently. In trial group, a few newborns were still HBeAg positive , but the HBV-DNA load had decreased obviously. It was likely the result of application of HBIG before labor. We presume that HBV-DNA load may decrease further if we keep on using HBIG and hepatitis B vaccine to those newborns with positive HBeAg. This deserves to study in the future. No side effect was found in both mothers and babies during the clinical trial.

Why were there still 7 cases of newborns with HBeAg positive in trial group, in our opinion., one of reasons was that cord serum used for HBeAg test was not better than vein serum, because cord serum is easy to be contaminated by the blood of mother with positive HBeAg. Another reason may be the high load of HBV-DNA in serum of these 7 mothers.

How to improve the effects of interruption of intrauterine transmission, other methods can be taken into consideration. Yue *et al*[10] used HBIG and together with hepatitis B vaccine to interrupt 30 pregnant women every 4 week from the 20th wk until delivery. The results showed that 29 newborn were HBsAg negative, 27 HBV-DNA negative and 10 were anti-HBs positive. In another chinical trial[11], 56 cases were given 200 IU HBIG intramuscularly every 4 wk from the 28th wk, while 43 cases received 100 mg lamivudine orally every day, 52 cases in the control group received no special treatment. The results demonstrated that the risk of HBV intrauterine infection effectively reduced in the 3rd trimester of HBsAg positive pregnant women, and there was no significant difference between HBIG and lamivudine groups. In present study, *Su et al*[12] has reported that 38 cases of pregnant women were given lamivudine before pregnancy, during pregnancy, until after labor. The result showed that none of 38 newborns was chronic carrier of HBsAg. In infant animals, lamivudine does not cause dysplasia however evidence of safety in human infants is limited. So some researchers prefer to use lamivudine from the 16th wk or 24th wk to those pregnant women with positive HBeAg for the safety of infants. We are waiting for the outcomes.

HBeAg is a marker which indicates active duplication of HBV, high concentration of HBV and strong possibility of infection. So we chose HBeAg positive pregnant women as subjects in this study. However it is unsuitable for clinical application to prevent from intrauterine transmission of HBV because the mutation of pre-C region of HBV can result in negative HBeAg , but they still had high load of HBV-DNA[13]. In clinical application, those pregnant women with negative HBeAg and positive HBV-DNA also need to be interrupted by HBIG. It has been reported that there is closed relationship between HBV-DNA load of serum of pregnant women and intrauterine infection[14,15]. Therefore it will be better to guide the application of prevention of mother-infant transmission of HBV by HBV-DNA load test quantitively.

It is clear that intrauterine infection is the main route of HBV transmission. According to present study, there is still a route of paternal-infants transmission of HBV[16]. Zhang *et al*[17]detected HBV markers of sperm of male patients with hepatitis B, HBV markers of peripheral blood of their partners and their newborns, the outcome was: HBV markers of mothers were negative, HBV markers of newborns were positive, situations were same as those of their fathers. It indicated that there was possibility of HBV transmission from farther to infant. Zhao *et al*[18] have detected HBV-DNA of sperma in male patients with hepatitis B, then a capture test of normal sperma was performed. The samples of infants from induced labor were tested for HBsAg and HBV-DNA, the result showed that HBV can cause vertical transmission via sperma. So in order to control intrauterine infection of HBV, we need to consider both mother and father. For noninfected pregnant women, while their partners are HBV carriers, application of pro-

phylaxis also should be considered in these fathers.

The limited number of HbsAG positive women and excluded cases may have produced a bias in our results warranting further study.

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RAPID COMMUNICATION

Study on biological characters of SGC7901 gastric cancer cell-dendritic cell fusion vaccines

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Abstract

AIM: To detect the biological characters of the SGC7901 gastric cancer cell-dendritic cell fusion vaccines.

METHODS: The suspending living SGC7901 gastric cancer cells and dendritic cells were induced to be fusioned by polyethylene glycol. Pure fusion cells were obtained by selective culture with the HAT/HT culture systems. The fusion cells were counted at different time points of culture and their growth curves were drawn to reflect their proliferative activities. The fusion cells were also cultured in culture medium to investigate whether they could grow into cell clones. MTT method was used to test the stimulating abilities of the fusion cells on T lymphocytes' proliferations. Moreover, the fusion cells were planted into nude mice to observe whether they could grow into new planted tumors in this kind of immunodeficiency animals.

RESULTS: The fusion cells had weaker proliferative activity and clone abilities than their parental cells. When they were cultured, the counts of cells did not increase remarkably, nor could they grow into cell clones in culture medium. The fusion cells could not grow into new planted tumors after planted into nude mice. The stimulating abilities of the fusion cells on T lymphocytes' proliferations were remarkably increased than their parental dendritic cells.

CONCLUSION: The SGC7901 gastric cancer cell-dendritic cell fusion vaccines have much weaker proliferative abilities than their parental cells, but they keep strong abilities to irritate the T lymphocytes and have no abilities to grow into new planted tumors in immunodeficiency animals. These are the biological basis for their anti-tumor biotherapies.

INTRODUCTION

With the development of modern immune research, biotherapy has brought new hopes for gastric cancer patients to conquer this kind of malignant tumors[1]. The anti-tumor vaccines based on dendritic cells (DC) play important roles in anti-tumor biotherapies[2]. Researchers obtain the cancer cells' antigens by various methods to irritate the dendritic cells, and increase the antigen presenting and immune stimulating abilities of dendritic cells[3,4]. Researchers usually destroy the cancer cells by frozen or ultrasound[5] methods. The components of the destroyed cancer cells were used as cancer antigens to irritate the dendritic cells. Many other researchers transmitted the cancer cell related mRNA into dendritic cells [6] or mix-cultured the cancer cells and dendritic cells[7]. They also attempted to improve the antigen presenting and immune stimulating abilities of dendritic cells by these methods. Although all these methods could partially enhance dendritic cells' capabilities and anti-tumor immune function of cancer patients, they still have some limitations. In recent years, methods to induce the dendritic cells and cancer cells to be fusioned together have been developed in order to investigate whether the fusion cells have stronger immune stimulating abilities than normal dendritic cells. Gastric cancer cells have no strong and specific tumor antigens, which makes it difficult for gastric cancer biotherapies. If gastric cancer cells and dendritic cells could be fusioned together, the cancer cell antigens might be presented more efficiently and more obvious anti-tumor effects might be achieved. In this research, the gastric cancer cells and dendritic cells were induced to be fusioned together by cell fusion technology. We detected the fusion cells' biological characters so to provide experimental data for gastric cancer biotherapies.

MATERIALS AND METHODS

Reagents

The reagents used included rhGM-CSF (Promega Co. Ltd, USA), rhIL-4 (R&D System Co. Ltd), TNF-a(R&D System Co. Ltd), Polyethylene glycol (Sigma, USA), Hypoxanthine (Sigma, USA), Aminopterin (Sigma, USA), Thymidine (Sigma, USA).

Preparation for suspending SGC7901 gastric cancer cells and dendritic cells

The frozen stored SGC7901 gastric cancer cells were cultured in the DMEM culture medium, and the suspending living cancer cells were prepared for the fusion process. The peripheral blood mononuclear cells were separated from venous blood of gastric cancer patients by use of lymphocytes separating solution. rhGM-CSF (100 ng/mL), rhIL-4 (50 ng/mL) and TNF-α(20 ng/mL) were added into the cultured peripheral blood mononuclear cells to induce them to transform into dendritic cells.

Preparation for SGC7901 gastric cancer cell-dendritic cell fusion vaccines

Suspending living SGC7901 gastric cancer cells and dendritic cells were mix-cultured with the ratio of 10:1. The two kinds of parental cells were induced to be fusioned by polyethylene glycol and selectively cultured by HAT/HT culture solutions. After the culture process, we obtained pure fusion cells as anti-tumor vaccines. Simultaneously, the suspending living SGC7901 gastric cancer cells and dendritic cells were mix-cultured with the same ratios, but were not induced to the fusion process. This group was designed as control group.

Growth curves of fusion cells

The obtained pure fusion cells (cell density 1.2×10^6/mL) were inoculated into the culture media on the culture slabs. During the culture process, we counted the cell numbers daily. After fourteen days, we drew the growth curves of cells. The SGC7901 cancer cells' and dendritic cells' growth curves were drawn at the same time respectively, and these curves were served as control.

Cloning abilities of fusion cells

The suspending living fusion cells (cell density 1×10^4/mL) were inoculated into the agar culture medium on twenty-four holes culture slabs. The cell numbers in each hole were 1000, 2000 and 10 000 respectively. After two weeks' cultivation, the cell clones which included more than 50 cells were counted under the microscopes. At the same time, the clone abilities of SGC7901 gastric cancer cells and dendritic cells were tested by the same methods.

Abilities of fusion cells to stimulate T lymphocyte proliferation

T lymphocytes were separated from peripheral blood of gastric cancer patients by using nylon hair. The cell densities of T lymphocytes, fusion cells and mix-cultured dendritic cells were adjusted to 5×10^6/mL. The fusion cells and mix-cultured dendritic cells were cultured on the culture slabs with 96 holes. T lymphocytes were added. The ratios of T lymphocytes to fusion cells and mix-cultured dendritic cells were 100:1, 50:1, 10:1 and 1:1 respectively. For each ratio, six holes were repeated in order to decrease the systemic errors. After sixty hours' cultivation, ^{3}H-TdR was added into each hole on the slabs (1Ci per hole). Twelve hours later, the CPM values were tested by Beckman liquid scintillation counting. The culture groups which included T lymphocytes only or DMEM culture medium were designed as control groups.

Abilities of fusion cells to grow into new planted tumors in nude mice

The suspending living pure fusion cells (cell density 1×10^9/mL, cell number 5×10^8) were planted subcutaneously into the back of the BALB/c nude mice. The injections were performed every three days for about two weeks. We observed whether the planted fusion cells could grow into new planted tumors in the nude mice, and the percentages of planted tumor cells growing into new planted tumors were tested. At the same time, the SGC7901 gastric cancer cells were planted into other groups of nude mice. These groups were designed as control groups.

Statistical analysis

Data were imported into Systat software (SPSS) for statistical computations and graphing. One-way analysis of variance (ANOVA), independent-samples T test and Chi-Square tests were used to evaluate the differences between groups. $P < 0.05$ was considered as significant.

RESULTS

Growth curves of fusion cells

We obtained the fusion cells after the selective culture process. The fusion cells' phenotypes and morphological characters were examined by flow cytometer and histological methods. The parental SGC7901 gastric cancer cells proliferated fast during their cultivation. After the subculture, the cancer cells stayed in proliferative delitescence for a short period, and then they entered into the logarithmic growth period with cell number increasing quickly. The cancer cells' proliferation slowed down and their number decreased gradually (Figure 1). Dendritic cells had much weaker proliferative abilities than SGC7901 cancer cells. During their cultivation, the cells' number did not increase remarkably. Like the parental dendritic cells, fusion cells also had weak proliferative abilities with a flat growth curve. This indicated that fusion cells' proliferative characters were much similar to parental dendritic cells (Figure 2).

Cloning abilities of fusion cells

Clone abilities effectively reflect the proliferation of single cells. If a kind of cell could grow into numerous cell clones in the agar culture medium, it indicates these cells have strong abilities to proliferate. In our study, we found that SGC7901 gastric cancer cells had strong proliferative abilities. They grew into numerous cell clones fast. Like the parental dendritic cells, fusion cells also had much weaker proliferations. During their cultivation, they could not

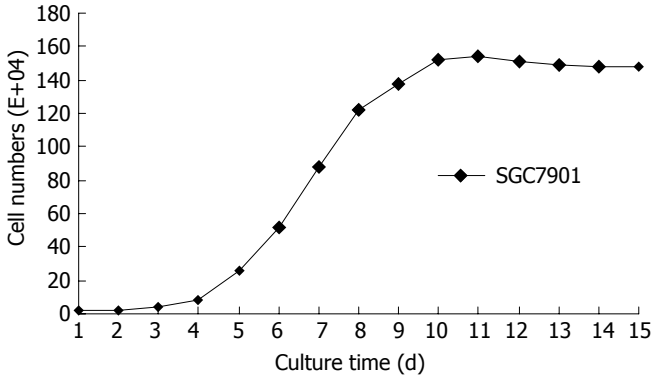

Figure 1 The growth curve of SGC7901 gastric cancer cells.

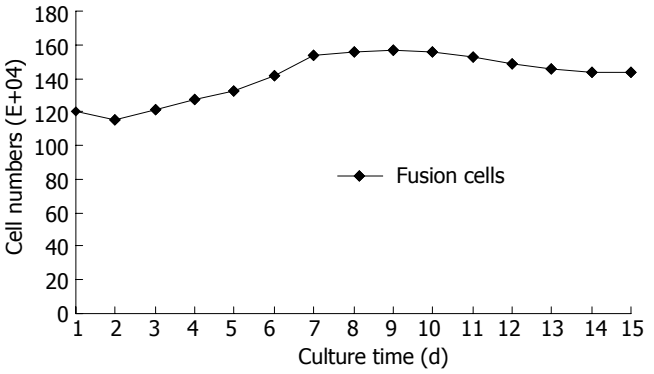

Figure 2 The growth curve of SGC7901-DC fusion cells.

grow into visible cell clones.

Abilities of fusion cells to irritate T lymphocytes' proliferation

The abilities of fusion cells to irritate T lymphocytes were much stronger than the mix-cultured DC group ($P < 0.01$) (Table 1).

Abilities of fusion cells to grow into new planted tumors in nude mice

After injected subcutaneously into the back of the BALB/c nude mice, the SGC7901 gastric cancer cells grew into new planted tumors quickly, whereas the fusion cells could not grow into new tumors in nude mice, even with a higher cell density and injection frequency, suggesting that fusion cells had no abilities to proliferate and grow into new planted tumors in immunodeficiency animals.

Table 1 Abilities of different cells to irritate T lymphocytes' proliferations (mean ± SD, CPM)

Groups	1:1	1:10	1:50	1:100
Fusion vaccine group	1 5083 ± 231[1]	9608 ± 83[2]	4214 ± 135[3]	3020 ± 28[4]
Mix-cultured DC group	5977 ± 272	3019 ± 38	2086 ± 58	1500 ± 41

[1]$P = 0.005$, [2]$P = 0.003$, [3]$P = 0.002$, [4]$P = 0.003$ *vs* Mix-cultured DC group.

DISCUSSION

Characters of fusion cells' growth curves, cloning abilities in vitro and their biological significance

Cell number and growth curves could directly reflect cell proliferative characters[8]. Clone ability is an important criterion to judge cells' growing capabilities[9]. The stronger the growth powers, the more cell clones could be formed. Cancer cells had strong abilities to proliferate. In our research we found that when the SGC7901 gastric cancer cells were subcultured, they quickly grew into the logarithmic proliferation period and the cell number increased remarkably[10]. After a short period the cell number was doubled. They grew into clearly cell clones only after seven days' cultivation. On the contrary, dendritic cells had much weaker proliferative abilities with a flat growth curve. There were no remarkable increases of the cell number during their cultivation, nor did cell clones formed. We found that fusion cells' growth characters were similar to their parental dendritic cells. They also had much weaker proliferative abilities and could not grow into cell clones during their cultivation. The cell number maintained a stable level and the growth curves were just a flat line. The SGC7901-DC fusion cells kept more biological characters of their parental dendritic cells. Different from their parental cancer cells, the fusion cells had no abilities to proliferate fast and grow into cell clones. These indicate that fusion cells were safe to be used as anti-tumor vaccines, which are biological basis for fusion cells research in cancer treatment.

Characters of fusion cells to irritate T lymphocyte's proliferation and their biological significance

After tumor antigens were presented by the dendritic cells, T lymphocytes were irritated and showed anti-tumor biological effects[11]. T lymphocytes *in vivo* can be divided into several sub-groups which have different phenotypes and biological effects. The most important sub-groups are CD4+ T lymphocytes (Th) and CD8+ T lymphocytes (Tc/CTL)[12]. The in-activated pre-Tc cells were irritated and became active Tc cells *in vivo* by the tumor antigens and CD4+ Th cells. These activated Tc cells exerted anti-tumor biological effects by the double signal approaches[13]. The cell number and activities of T lymphocytes directly affected anti-tumor immune responses of cancer patients[9]. Our purposes of modifying dendritic cells as anti-tumor vaccines were to increase their abilities of stimulating T lymphocytes' proliferation and their activities[14,,15]. Some researchers reported that when the tumor antigen related genes were transmitted into dendritic cells T lymphocytes *in vivo* could be strongly irritated to become CD4+ Th cells and CD8+ Th cells [16,17]. In our research, we found that the SGC7901-DC fusion cells could strongly irritate T lymphocytes' proliferation, while the mix-cultured dendritic cells could not. This indicated that fusion cells had much stronger abilities to activate anti-tumor immune responses. So, fusion cells are possible to be used as anti-tumor vaccines.

Abilities of fusion cells to grow into new planted tumors in immunodeficiency animals and their biological significance

New planted tumors in immunodeficiency animals are

perfect models for oncology researches[18]. We injected the SGC7901 cancer cells and SGC7901-DC fusion cells into different groups of nude mice and found that SGC7901 cancer cells could grow into new planted tumors quickly but the fusion cells could not, even when much higher cell densities and more injection frequencies were applied. This implied that SGC7901-DC fusion cells had no abilities to grow into new planted tumor in nude mice. This is the most important difference between the parental SGC7901 cells and the SGC7901-DC fusion cells. This character together with the growth curves and clone abilities indicated that fusion cells were safe when used as anti-tumor vaccines *in vivo* and *in vitro*.

The strong abilities of SGC7901-DC fusion cells to irritate T lymphocytes' proliferation and the characters that the fusion cells could not grow into new planted tumors *in vivo* are the precondition of anti-tumor biological therapy.

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CASE REPORT

Granular cell tumor of stomach: A case report and review of literature

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Abstract

Granular cell tumor (GCT) was described for the first time by Abrikosoff in 1926. It is a relatively rare neoplasm that may occur at many sites, but most commonly in the skin or soft tissues. The occurrence of GCT in the gastrointestinal tract is rare, accounting approximately for 8% of all tumors, among which the most common site is the esophagus, whereas gastric localization is very rare. Gastric GCTs can be solitary or, more frequently, associated with other gastrointestinal localization. Although GCTs are usually clinically and histologically benign, some malignant cases have been reported. Histologically, these tumors consist of polygonal and fusiform cells disposed in compact "nests" and immunohistochemical staining for S-100 protein supports the proposed derivation from Schwann cells. A correct preoperative diagnosis of this tumor can only be made in 50% of all patients and it is always based on endoscopic biopsy. Laparoscopic or conventional wedge resection represents the treatment of choice. In this study, the authors reported a case of a 49-year-old woman with a solitary granular cell tumor of the stomach with infiltrative pattern, successfully treated with surgical resection. A review of literature is also presented with emphasis on diagnostic criteria concerning the malignant form.

Key words: Granular cell tumor; Stomach; Benign; Surgical resection

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INTRODUCTION

Granular cell tumors (GCT) were described for the first time by Abrikosoff in 1926. They are uncommon, usually benign, soft tissue tumors rarely seen in clinical practice. They may occur at many sites, although it affects most frequently skin or subcutaneous tissues of the chest and upper extremities, tongue, breast and female genital region. The onset of this type of tumor in the gastrointestinal (GI) tract is rare. Approximately 8% of all GCTs occur in the digestive tract[1] and among these the most common site is the esophagus, followed by the large intestine[2]. Gastric localization is seldom seen. Yasuda et al[3], reported a case of gastric GCT in 1995 and underlined that this lesion was previously found in the stomach only in 24 patients. Since 1996, five more cases have been described[4-9].

In this study, we report a case of a woman with a solitary GCT of the stomach, incidentally found upon GI endoscopy, which had an infiltrative growth pattern and was successfully treated by surgical resection.

CASE REPORT

A 49-year-old woman was admitted to our Unit for further evaluation of a gastric mass which had been incidentally in an abdominal ultrasound examination for her nonspecific dyspeptic symptoms (Figure 1). She had no remarkable past medical history, and no significant alcohol consumption or history of smoking or drugs. Upon hospitalization, physical examination, biochemical data and tumor markers (CEA, CA-19-9) were all within normal limits. Upper gastrointestinal endoscopy was performed and revealed a hemispherical mass of about 3 cm in diameter, located on the lesser curvature of the gastric antrum and overlaid by normal mucosa. Search for *H pylori* by biopsy urease (Pantorc® *Hp* Test, Byk Gulden Italia S.p.A.) was negative. The esophagus, duodenum and the remaining parts of stomach were normal. Multiple deep (jumbo) biopsies from the gastric mass and from the surrounding gastric mucosa were performed. Histopathological examination of mass biopsies showed a pattern compatible with GCT, whereas other mucosal biopsies were normal. A subsequent CT scan of the abdomen showed a solid mass, with a slight contrast enhancement, of about 3 cm in diameter with well-defined margins located near the gastric angulus, arising from a layer of the gastric wall. Neither further invasion beyond the gastric wall, nor visible metastatic lesions in the liver or enlarged lymph nodes were observed. During laparotomy a tumor mass was found on the anterior wall of the lesser

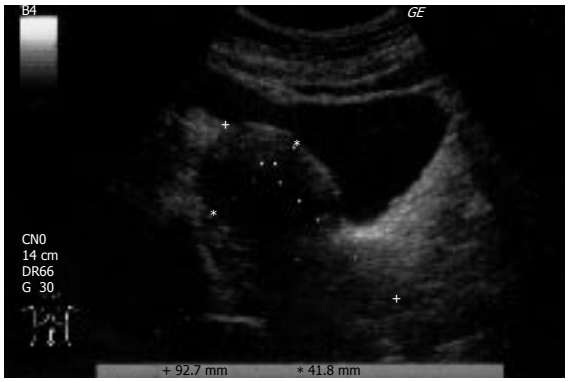

Figure 1 Echo tomography of gastric region. A solid hypoechogenic mass with a diameter of 2.7 cm × 2.2 cm protruding from beyond the anterior wall of the stomach.

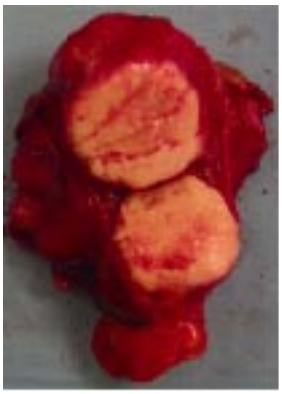

Figure 2 Macroscopic appearance of resected specimen: section of surgical specimen: a soft yellow mass of about 2 cm in diameter.

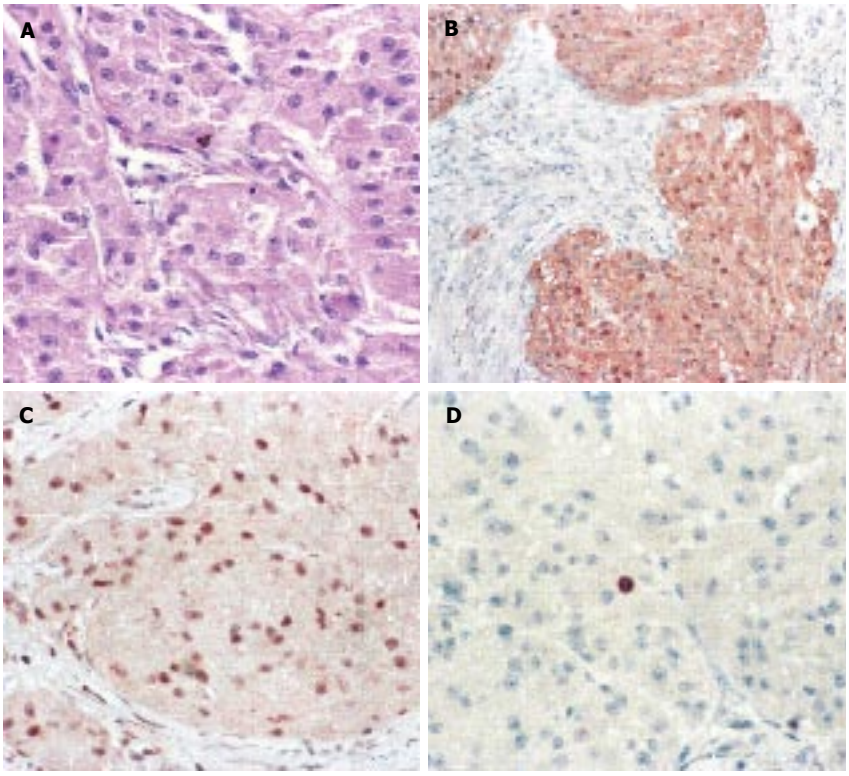

Figure 3 Histological findings of the resected specimen. **A:** hematoxylin–eosin staining. Sheet of large and polygonal cells with round to oval, eccentrically located nuclei and eosinophilic granular cytoplasm with lower mitotic activity; **B**: immunohistochemical staining for S-100 protein. Diffuse and strong expression of S-100 protein; **C**: immunohistochemical staining for p53. Some of the tumor cells are positive for p53; **D**: immunohistochemical staining for Ki-67. Some of the tumor cells are positive for Ki-67.

curvature of the stomach. There were no liver metastases or regional lymph node swelling. A wedge resection, with a gastric section performed at a distance of about 1.5 cm from the macroscopic margin of the tumor, was carried out, obtaining a total removal of the lesion.

In the resected specimen, the tumor measured 2 cm in diameter and appeared as a yellow mass (Figure 2). Microscopically, the tumor was located mainly in the muscle layer of the stomach. It formed solid nests of cells ranging from round to polygonal shape associated with few spindle cells. The nuclei were of different sizes with nucleoli at times evident and finely dispersed chromatin. The cytoplasm was abundant and finely granular. Mitotic activity was very low. The tumor focally infiltrated near muscular layer but did not affect mucosal surface (Figure 3A). Immunohistochemistry showed, S-100 protein present nearly in all cells within the tumor mass (Figure 3B), p53 in 40% of cells (Figure 3C) and Ki-67 in less than 9% of cells (Figure 3D). These histological findings met the criteria for a diagnosis of benign GCT[10].

The postoperative course was uneventful and the patient was discharged on postoperative fifth day. She remained asymptomatic and no recurrent disease was observed after a two year follow-up.

DISCUSSION

From an extensive review of literature about gastric GCT, no differences of tumor incidence was found as regard to gender, whereas fourth through sixth decades of life seem to be more affected. In term of ethnical issue of affected patients, the most reported cases were described in Japanese population. Moreover, in 50% of cases, gastric GCT proved to be associated with esophageal synchronous localization and was rarely associated with other benign or malignant gastric diseases. In only two reported patients,

Table 1 Characteristics of reported gastric granular cell tumors since 1998

Authors (yr)	Country	Age (yr)	Gender	Tumor size (cm)	Gastric site	Simultaneous occurrences of GCT	Simultaneous gastric lesions	Treatment
Sebastian (1998)	Spain	53	M	7	Upper	Esophagous	Ulcers	Surveillance
David (1999)	USA	45	F	2	Upper	Esophagous	No other lesions	Surgical
Eguchi (2002)	Japan	64	M	1.5	Body	No other occurrences	Cancer and lymphoma	Surgical
Maekawa (2003)	Japan	53	M	NR	Upper multiple	Esophagous	No other lesions	Surgical
Sailors (2005)	USA	65	F	1.5	n.r.	Tranverse colon	Cancer	Surgical

NR: non reported; GCT: granular cell tumor.

similarly to our case, gastric GCT did not show multiple localizations or was associated with other gastric lesions[3,11] (Table 1).

These gastric tumors are mostly confined macroscopically to submucosa. Their size may range from few millimeters to some centimeters, never surrounded by capsule. Histologically, gastric GCTs are composed by polygonal and fusiform cells arranged as compact "nests". Immunohistochemical staining for S-100 protein supports the proposed origin of the tumor from Schwann cells[12].

Although GCTs are usually benign clinically and histologically, some malignant cases have been reported. There are less than 30 reported cases of malignant GCTs in world literature[13] and the only documented case of a malignant GCT of the stomach was reported in 1996 by Matsumoto *et al*[9] (Table 1).

Features associated with malignancy include local recurrence, rapid growth to a size greater than 4 cm, cell necrosis, spindling of tumor cells, cytologic atypia and high mitotic activity, vescicular nuclei with large nucleoli, and a high nuclear-to-cytoplasm ratio[5,10]. It has also been reported that, on immunohistochemical staining, positivity rate of more than 50% for p53 and more than 10% for the Ki-67 index respectively, was significantly correlated with malignancy[7,10]. Extent of infiltration[5] or highly developed tumor microvessels[14] or focal pleomorphism[7] have never been considered as a criterion for diagnosis of malignancy. Altogether, these features are suggestive for malignant potential of the tumor. The tumor mass in our patient infiltrated only focally and marginally the muscular layer and did not display high expression of p53 and Ki-67. Furthermore, after the follow-up for two years, no evidence of recurrences was seen.

It is questionable whether these tumors always produce GI symptoms[15], even when they are of small size. It is quite likely that, if small, a change (pre-symptomatic) was found during upper GI endoscopy, X-ray or ultrasound examination performed for nonspecific dyspepsia. When large, gastric GCTs may instead present as gastric outlet obstruction[11], or massive upper GI hemorrhage[16].

A correct preoperative diagnosis of this tumor can be made by endoscopic biopsy. It is generally accepted that the best way to obtain an appropriate sampling of such tumors beneath the mucosa is either by boring or by performing a jumbo biopsy. A definitive diagnosis can only be established by endoscopic biopsy in 50% of the patients[17]. Endoscopic ultrasonography has recently been used more frequently for determining the depth of tumor invasion in the gastrointestinal wall, and may also be useful to evaluate GI tract submucosal tumors[2]. Endoscopic excision of GCT should be strongly considered[3]. If the tumor is attached to muscularis propria, an injection of saline can be useful to increase the distance, so that endoscopic removal can be performed more safely[3]. Therefore, since the gastric GCT is without capsule and focal areas of infiltration of the gastric wall can be present, laparoscopic or conventional wedge resection, including at least one centimeter of normal tissue, is the best therapeutic option. Laparoscopic approach could be difficult due to tumor localization beneath the posterior wall or lesser curvature, or mass size (very large or very small). Almost all gastric GCTs, observed from 1990 to date, were treated with conventional surgical approach.

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CASE REPORT

Primary signet-ring cell carcinoma of the colon at early stage: A case report and a review of the literature

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Abstract

A 67-year-old man, who had undergone surgery to resect multiple gastric cancers 4 years ago, visited our hospital for surveillance colonoscopy. Colonoscopy revealed a discolored, 7-mm in diameter, flat-elevated lesion with central depression in the transverse colon near the splenic flexure. Although the findings of endoscopy and barium enema were suggestive of submucosal invasion, the patient chose to undergo endoscopic mucosal resection. Pathological examination of the resected specimen revealed signet-ring cell carcinoma and a positive surgical margin. A second operation was performed, and no residual tumor or metastasis to lymph nodes was found in the resected specimens. Primary colorectal cancers composed of signet-ring cell carcinoma detected and treated at an early stage are extremely rare. We present a case and review the literature.

Key words: Signet-ring cell carcinoma; Early colorectal cancer; Endoscopic mucosal resection

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INTRODUCTION

Signet-ring cell carcinoma is uncommon in the colon and rectum, with a reported incidence ranging from 0.1% to 0.9%[1,2]. In general, signet-ring cell carcinoma shows the characteristic appearance of "linitis plastica" and behaves more aggressively than carcinoma of other histological types[1,2]. As symptoms usually appeared late, signet-ring cell carcinomas are commonly detected at advanced stages. Therefore, cases detected and treated at early stage are rare. In this case report, we present our experience with a signet-ring cell carcinoma in the transverse colon detected at early stage and review the literature.

CASE REPORT

A 67-year-old man visited our hospital 4 years ago with a history of 4 synchronous primary cancers of the stomach. He underwent total gastrectomy, and the surgical specimen contained 2 early cancers which invaded the mucosa and submucosa, and 2 advanced cancers which penetrated serosa without invasion of adjacent structures. No components of signet-ring cell carcinoma were found on histology. He had a brother and a sister, and the family history included that his father had died of a gastric cancer at the age of 68, and his sister had an advanced sigmoid colon cancer and a uterine cancer at the age of 62 and 65, respectively. The patient's past history included an operation for synchronous gastric cancers, however there was no history of inflammatory bowel disease. He also had a focal cancer in an adenoma with moderate atypia, which had been completely resected endoscopically one year before. Therefore, he underwent surveillance colonoscopy at our department of endoscopy. Findings on laboratory tests were unremarkable. Total colonoscopy, with a magnifying colonoscope (CF-200Z, Olympus Optical Co., Japan) was easily introduced into the cecum without complications. Colonoscopy revealed a discolored flat-elevated lesion with central depression, 7 mm in diameter, in the transverse colon near the splenic flexure (Figure 1A). The tumor bled easily from the depressed area without contact from the endoscope and had convergent mucosal folds suggesting submucosal cancer involvement. After spraying of 0.2% indigo-carmine dye, the depression was seen more clearly (Figure 1B). After chromoendoscopy with 0.2% indigo-carmine dye and 0.05% crystal violet staining, we attempted to observe the surface structure of this

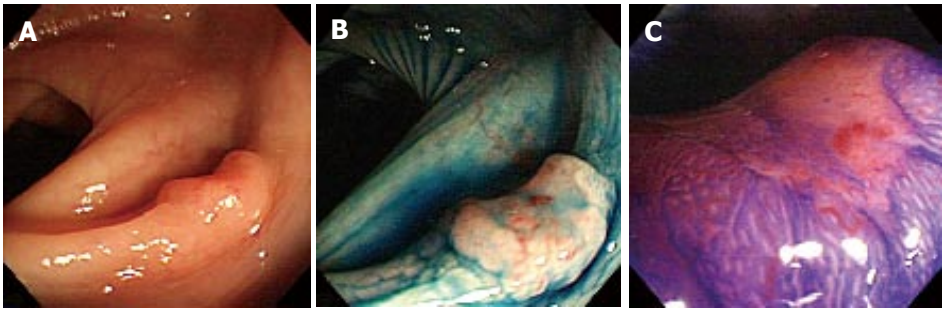

Figure 1 A: Colonoscopy revealed a discolored, flat-elevated lesion with central depression, 7 mm in diameter, in the transverse colon near the splenic flexure; **B:** Chromoendoscopy with 0.2% indigo-carmine dye showed the depressed area more clearly; **C:** Magnifying colonoscopy after 0.05% crystal violet staining was attempted but failed to detect the pit pattern for depth estimation, as the depressed area was covered by dense mucus, which could not be removed by repeated water washing.

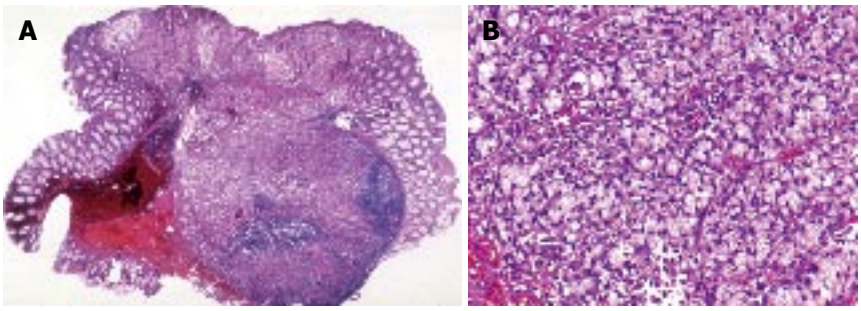

Figure 2 A, B: Histologically, the resected specimen showed a signet-ring cell carcinoma that had deeply invaded into the submucosal layer, and the vertical cut end of the resected tumor was positive for carcinoma cells.

lesion, the so-called "pit patterns", at a higher magnification to estimate the depth of invasion[3,4]. However, the pit patterns could not be detected, as dense mucus coated the surface and could not be removed by repeated washing (Figure 1C)[5]. The findings of barium enema and conventional colonoscopy suggested submucosal cancer invasion, and thus, surgical resection, including node dissection, was recommended for curative treatment. However, the patient refused surgical resection but consented to endoscopic mucosal resection (EMR). The lesion was successfully removed en bloc with EMR without complications. The resected specimen was 7 mm in diameter. The resected specimen was fixed with 20% formalin, and then the mucus on the surface was removed for better staining and observation. The fixed lesion was stained with 0.05% crystal violet and observed stereomicroscopically under water immersion. Stereomicroscopy revealed a type V (non structural) pit pattern in the depressed area. These findings suggested that this lesion had deeply invaded the submucosal layer[3,6]. Histologically, the tumor was composed of signet-ring cell carcinoma, without accompanying adenoma or other types of cancer cells, that had infiltrated deeply into the submucosal layer with invasion of lymphatic vessels (Figure 2A-B). Immunostaining was used to examine the expression of MLH1 and MSH2 of the resected specimen; the tumor was positive for MSH2 but negative for MLH1. A cut end of the resected specimen was positive for carcinoma cells. Systemic diagnostic imaging including abdominal computed tomography showed no evidence of distant metastasis or ascites. Thereafter, the patient consented to undergo an additional laparotomy. The resected specimen revealed no residual carcinoma at the EMR site and showed no metastasis to lymph nodes. The postoperative course was uneventful, and the patient is still alive and in apparent good health 5 years after the operation.

DISCUSSION

More than 96% of signet-ring cell carcinomas arise in the stomach, and the rest occurred in other organs, including the colon, rectum, gallbladder, pancreas, urinary bladder, and breast[7]. The case described here was believed to be a primary lesion of the transverse colon because a thorough workup did not reveal any other sites of involvement. Although the patient's history included surgical resection of an advanced gastric carcinoma, a secondary carcinomatous deposit should also be excluded. Metastasis from gastric carcinoma is probably the most common secondary carcinoma in the colon and rectum[8]. It may present itself as single or multiple strictures mimicking primary colorectal carcinoma or inflammatory bowel diseases[8,9]. Furthermore, multiple flat and depressed lesions or polyps have also been reported as rare presentations of colonic metastasis from gastric signet-ring cell carcinoma[10-13]. Our case presented a solitary depressed lesion without metastasis or lymphatic, hematogenous or peritoneal involvement, and the patient is still alive 5 years after surgery; therefore, on the basis of these findings, this case could be judged as a primary lesion. Crystal violet staining is commonly used to evaluate pit patterns, especially for lesions in which there is the suspicion of submucosal invasive carcinoma. Unfortunately, the pit patterns could not be detected in this case, as dense mucus coated the surface and failed to be removed by repeated washing endoscopically. This might reveal that signet-ring cell carcinoma produces abundant mucus.

Primary signet-ring cell carcinoma of the colon and rectum, as first described by Laufman and Saphir in 1951, is rare[14]. The reported incidence is about 1%[1,2]. The histological appearance of the tumor is characterized by cells with abundant intracytoplasmic mucin and peripherally placed nuclei. Signet-ring cell carcinomas of the colon and

rectum are usually diagnosed at an advanced stage, because symptoms usually develop late. Thereafter, cancers limited to the mucosal and submucosal layers are rarely detected; to the best of our knowledge, only 26 cases, including ours, have been reported[15-20]. The clinicopathological details of the reported cases are summarized in Table 1. The patients included 21 males and 5 females with a mean age of 57.1 years (range of 6-79 years). The mean size of the tumors was 16.0 mm (range, 2 to 45 mm). Macroscopically, 16 (61.5%) cases were flat or depressed. The smallest tumor was a tiny, discolored, flat lesion 2 mm in diameter. Despite the small size, it surprisingly metastasized to lymph nodes[18]. Tsujinaka et al have described the first case of early signet-ring cell carcinoma of the colon without an adenomatous component. It was a depressed lesion that was believed to have arisen via a de novo pathway[15]. This tumor only showed signet-ring cancer cells without accompanying adenoma or other types of cancer cells. It is unknown whether signet-ring cell carcinoma usually arises from a pre-existing adenomatous polyp or as a so-called de novo carcinoma[21]. However, according to the reviewed articles, there are 3 reported cases with an adenomatous component; therefore, an adenoma-signet-ring cell carcinoma sequence also exists[16,17].

The sites of reported cases are mostly in the right-sided colon (50%, 13 cases), but 7 are in the left-sided colon and 6 are in the rectum (Table 1). In our case, the transverse colon was the most commonly involved site. Eight of the reported cases also had multiple synchronous colorectal cancers. The right-sided predilection, histological characteristics, and the tendency to multiplicity are now well documented in hereditary non-polyposis colorectal cancer. However, in the present case, the family history and past history did not fulfill the diagnostic criteria[22,23]. Considering the clinicopathological characteristics of this case, immunostaining was used to examine expression of MLH1 and MSH2 of the resected specimen; the tumor was positive for MSH2 but negative for MLH1. Signet-ring cell carcinomas have been analyzed for microsatellite instability, which is present in approximately 30% of tumors[24]. Furthermore, although not available in our case, mutations of K-ras and p53 gene have been reported in signet-ring cell carcinomas of the colon and rectum; however, the frequency of K-ras gene mutation in signet-ring cell carcinomas is significantly lower than that of well and moderately differentiated carcinomas[24]. These results suggest that the genetic background of signet-ring cell carcinomas might differ from that of well or moderately differentiated carcinomas of the colon and rectum. DNA-replication errors are suggested to be at least partly involved in the carcinogenesis of signet ring cell colorectal carcinoma[24]. Although, our patient did not have inflammatory bowel disease, an association of inflammatory bowel disease and signet-ring cell carcinoma of up to 14% has been reported[25]. Psathakis et al. reported two (14.3%) of 14 patients with signet-ring cell carcinoma, who had a long history of ulcerative colitis[26]. Anthony et al. reported two cases of Crohn's disease that developed signet-ring cell carcinoma[27].

In conclusion, we have reported a rare case of primary signet-ring cell carcinoma of the colon detected and treated at early stage, and reviewed the literature.

Table 1 Clinicopathological details in patients with primary early colorectal signet ring cell carcinomas

Mean age (range) (yr)	57.1 (6-79)
Sex (male/female)	21/5
Mean size (range) (mm)	16.0 (2-45)
Site of lesions	
Right-sided colon (cecum/ascending/transverse)	13 (3/1/9)
Left-sided colon (descending/sigmoid)	7 (3/4)
Rectum	6
Depth of invasion	
Intramucosal	6
Submucosal	20
Macroscopic features	
Polypoid	10
Flat	3
Depressed	13
Lymph node metastasis	
Positive	2
Negative/unknown	17/7

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CASE REPORT

Postoperative bile leakage managed successfully by intrahepatic biliary ablation with ethanol

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Abstract

We report a case of postoperative refractory bile leakage managed successfully by intrahepatic biliary ablation with ethanol. A 75-year-old man diagnosed with hepatocellular carcinoma underwent extended posterior segmentectomy including the caudate lobe and a part of the anterior segment. The hepatic tumor attached to the anterior branch of the bile duct was detached carefully and resected. Fluid drained from the liver surface postoperatively contained high concentrations of total bilirubin, at a constant volume of 150 mL per day. On d 32 after surgery, a fistulogram of the drainage tube demonstrated an enhancement of the anterior bile duct. Endoscopic retrograde cholangiography demonstrated complete obstruction of the proximal anterior bile duct and no enhancement of the peripheral anterior bile duct. On d 46 after surgery, a retrograde transhepatic biliary drainage (RTBD) tube was inserted into the anterior bile duct under open surgery. However, a contrast study of RTBD taken 7 mo post-surgery revealed that the fistula remained patent despite prolonged conservative management, so we decided to perform ethanol ablation of the isolated bile duct. Four mL pure ethanol was injected into the isolated anterior bile duct for ten minutes, the procedure being repeated five times a week. Following 23 attempts, the volume of bile juice reached less than 10 mL per day. The RTBD was clamped and removed two days later. After RTBD removal, the patient had no complaints or symptoms. Follow-up magnetic resonance imaging demonstrated atrophy of the ethanol-injected anterior segment without liver abscess formation.

Key words: Postoperative complication; Bile leakage; Ethanol ablation

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INTRODUCTION

Bile leakage is one of the major postoperative complications that may occur after hepatectomy. The reported incidence of postoperative bile leakage ranged from 4.8% to 7.6%[1-3]. Biliary complications including bile leakage after hepatic surgery are a major cause of morbidity and extended hospital stays. Lo *et al*[4] reported a high mortality rate in patients who required reoperation for biliary complication after liver resection. Biliary drainage is usually important and necessary for the treatment of bile leakage, but sometimes fails to improve bile leakage despite prolonged conservative drainage[5].

We report a case of postoperative refractory biliary leakage managed successfully by intrahepatic biliary ablation with ethanol.

CASE REPORT

A 75-year-old man was referred to our hospital with a diagnosis of hepatic tumor. Abdominal computed tomography confirmed a 63 mm × 59 mm hepatic mass locating mainly in segment I with invasion into segment VI, VII, VIII, and V. This tumor obstructed the posterior branch of the bile duct, and oppressed the anterior branch of the bile duct, middle hepatic vein and infra vena cava (Figure 1A, B). Abdominal angiography revealed a hypervascular tumor in the right lobe at the early enhanced phase (Figure 1C). Serum alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) was 227 ng/mL (normal, < 20) and 62 mAU/mL (normal, < 40), respectively. He had no additional underlying liver disease. We diagnosed him as hepatocellular carcinoma and decided upon surgery for radical treatment. An extended posterior segmentectomy including the caudate lobe and a part of the anterior segment was performed. Consistent

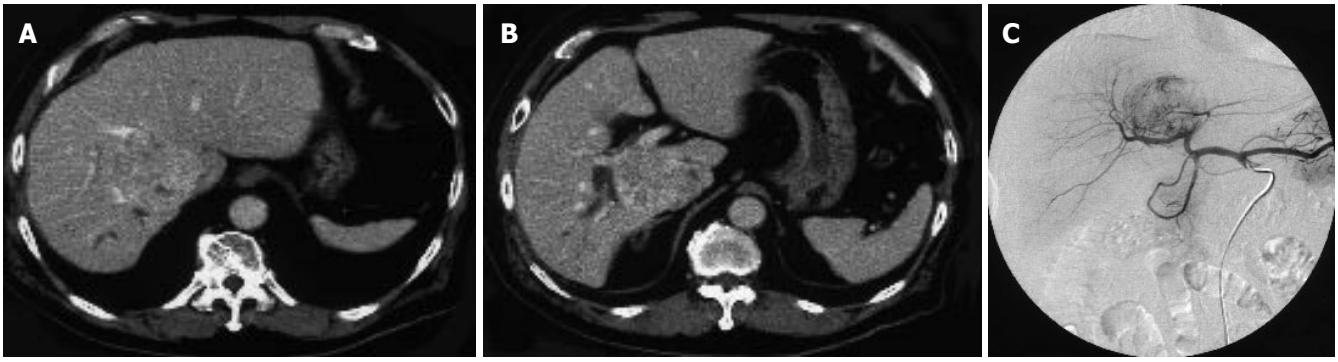

Figure 1 **A**, **B**: Abdominal computed tomography confirmed a 63 mm × 59 mm hepatic mass locating mainly in segment I with invasion into segment VI, VII, VIII, and V. This tumor obstructed the posterior branch of the bile duct, and oppressed the anterior branch of the bile duct; **C**: Angiography revealed a hypervascular tumor in the right lobe at the early enhanced phase.

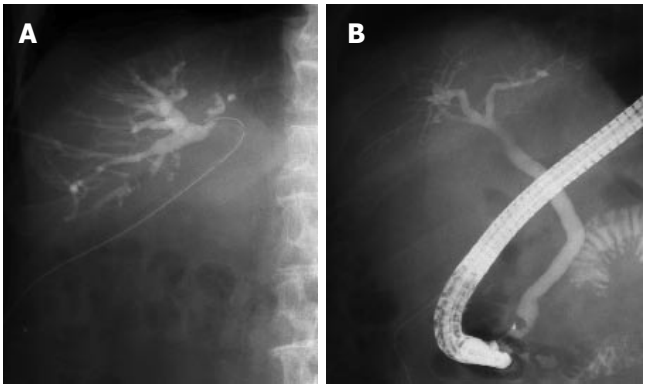

Figure 2 **A**: A fistulogram from the drainage tube demonstrated a connection between the anterior bile duct and fistula; **B**: Endoscopic retrograde cholangiography showed obstruction of the anterior bile duct, and no connection between the common bile duct and the anterior bile duct.

with the findings of the preoperative computed tomography, the tumor was attached to the anterior branch of the bile duct, middle hepatic vein, and inferior vena cava, and was detached carefully from them and resected. During surgery, no particular problems were seen, including bile leakage. Postoperatively, the fluid from a drain locating in the resected liver surface contained total bilirubin (61.2 mg/dL), the volume of which was constant at 150 mL per day without decrease. This was thought to be caused by bile leakage. On d 32 after surgery, a fistulogram obtained from the drainage tube demonstrated a conduit between the anterior bile duct and fistula (Figure 2A). Endoscopic retrograde cholangiography performed on d 39 after surgery demonstrated obstruction of the anterior bile duct (Figure 2B). Taken in sum, these examinations suggested a hole in the distal part of the anterior bile duct with its proximal obstruction. We tried to insert a biliary drainage tube into the anterior branch by percutaneous and endoscopic procedures to decrease the intraductal pressure and close the fistula, but failed. On d 46 after first surgery, a retrograde transhepatic biliary drainage (RTBD) tube was inserted into the anterior bile duct under open surgery. We waited to close the fistula in the outpatient follow-up, however a contrast study of the RTBD taken 7 mo post-surgery showed that the fistula remained patent. Because external biliary drainage continued despite prolonged conservative management, we decided to perform ethanol ablation of the isolated bile duct.

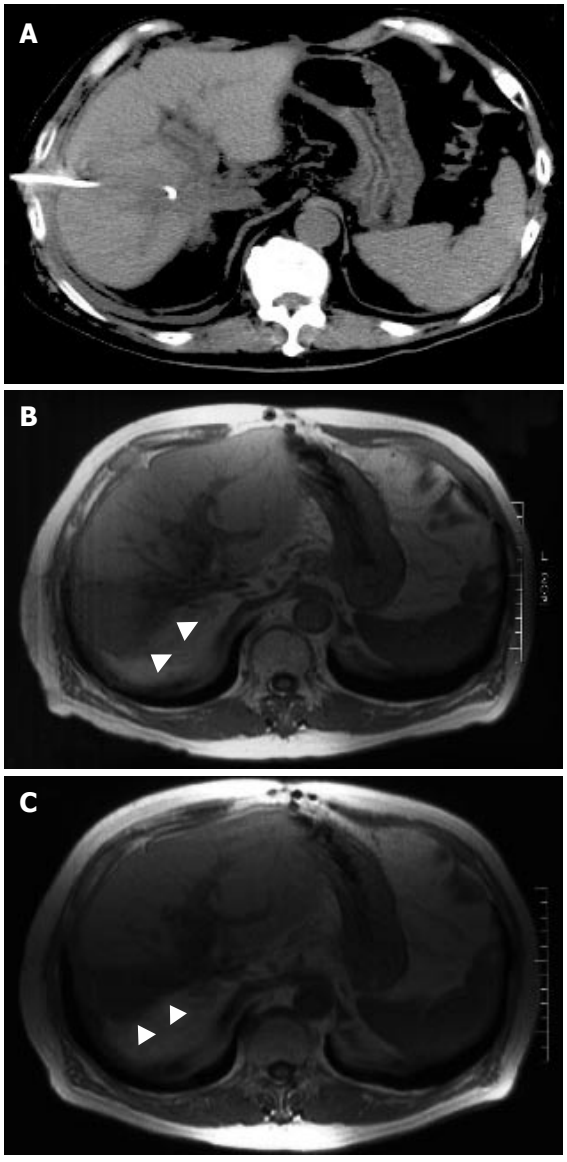

Figure 3 Follow-up computed tomography taken before ethanol injection (**A**), magnetic resonance imaging 3 mo (**B**) and 15 mo (**C**) after injection. Arrows indicate the atrophy of the ethanol-injected anterior segment.

A 5 Fr balloon occlusion catheter was placed in the

anterior bile duct through an RTBD tube. After the balloon was inflated to prevent leakage of the injected ethanol from the bile duct to the fistula, 4.0 mL pure ethanol was injected for ten minutes. Ethanol injection over 4.0 mL caused upper abdominal discomfort, so we decided that 4.0 mL was the appropriate volume. The ethanol injection was repeated five times a week. The volume of bile drainage was diminished to less than 100 mL after five injections, and the injections were continued thereafter. On the 23rd attempt, the volume of bile juice dropped to below 10 mL per day, and then the RTBD was clamped and removed two days later. Total bilirubin concentration of the drainage was also decreased from 61.2 mg/dL pre-injection to 6.9 mg/dL on the 11th attempt, and to 3.9 mg/dL on the 20th attempt. After RTBD removal, the patient had no complaints or symptoms. Compared with computed tomography taken before ethanol injection (Figure 3A), magnetic resonance imaging demonstrated atrophy of the ethanol-injected anterior segment without liver abscess formation (Figure 3B-C).

DISCUSSION

This report indicates that ethanol ablation may be effective in unfortunate cases of refractory biliary leakage after hepatectomy. However, this method can be performed in only biliary fistula without communication with the bile duct of the other remaining liver, because ethanol affects the remaining bile duct and causes irreversible damage. From this point of view, the reported case is thought to be one particularly suitable for ethanol biliary ablation.

Ethanol has been generally used as an injected agent percutaneously or angiographically for the treatment of various tumors because of its highly destructive properties, which lead to cell death by causing cell membrane lysis and protein denaturation[6]. Percutaneous transhepatic ethanol injection to the biliary tract has been reported. Controversy has surrounded chemical ablation of the gallbladder as an alternative to cholecystectomy for over a decade[7-11]. Recently, selective intrahepatic biliary ethanol injection as an alternative to preoperative portal vein embolization[12] or postoperative bile leakage[13] has been introduced. Kyokane *et al* reported that selective intrahepatic biliary ethanol injection destroyed the biliary epithelium, permeated parenchyma, induced hepatocyte denegeration, and resulted in compensatory hypertrophy of the non-injective hepatic lobe in an animal study. Compared with portal embolization, biliary ethanol injection was reported not to cause such severe side effects. In biliary ethanol injection, the hepatocyte denegeration with preservation of hepatic blood supply may prevent clinical liver necrosis or abscess[12]. Li *et al*[14] also demonstrated that biliary injection using phenol plus cyanoacrylate could achieve the effect of chemical hepatectomy.

Although this patient complained of abdominal pain during excessive ethanol ablation, this symptom was promptly relieved when the injection was stopped and the ethanol was removed from the bile duct via an RTBD tube. We were able to find the optimal injected ethanol dose through the patient's complaint and fistulography, after which the patient did not complain of abdominal pain. In this method, the decision on the optimal injected dose may be important to prevent side effects[15].

Unfortunately, biliary leakage is sometimes encountered, and it can be complicated when it does occur[3,5,16]. Ethanol ablation of the bile duct provides a management option in cases in which conservative treatment has failed and a surgical approach is relatively difficult.

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CASE REPORT

Synchronous anorectal melanoma

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Abstract

Anorectal melanoma is a very rare tumor with poor prognosis. Rectal bleeding is the most frequent symptom and surgical treatment ranges from local excision to radical abdominoperineal resection. We report a case of a 75-years-old male patient who presented with a history of recurrent rectal bleeding, and whose histopathological diagnosis was melanoma. Macroscopically, we found two distinct tumors in anorectal region, 0.5 cm and 1.5 cm from dentate line. The first one was pedunculated, on a thin stalk, measuring 1 cm in greatest diameter, and the second one was sessile and nodular measuring up to 2.8 cm in largest diameter. Microscopic examination and immunohistochemical analysis of both tumors confirmed the diagnosis of melanoma. This case represents multiple synchronous primary melanoma of the anorectal region, with a possibility that one of the lesions is primary melanoma and the second one is a satellite lesion.

Key words: Melanoma; Anorectal region; Satellite lesions; Synchronous melanomas

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INTRODUCTION

Anorectal melanoma is a rare condition accounting for 0.2%-3% of all melanoma cases, and 0.1%-4.6% of all malignant tumors of the rectum and anus[1,2,3]. Melanoma is defined as "rectal" when it occurs in the rectum above the anorectal junction[4], while anal canal is below anorectal line. Rectal bleeding was the most common reported symptom, and sometimes the importance of the bleeding is underestimated as it was assumed to be a sign of hemorrhoid disease causing a delay in making the correct diagnosis and starting treatment[4,5]. Surgical treatment of this tumor ranges from the radical abdominoperineal resection with bilateral inguinal lymphadenectomy, to the conservative local excision alone[4]. Long-term survival is very low; approximately 6.7% to 12% of patients were reported to be free of disease five years after their operations[6,7]. Most recent studies did not find correlation between tumor necrosis and blood vessel invasion and survival rate, while correlation of survival and tumor thickness is controversial. In the most studies, tumors evaluated by flow cytometry that showed aneuploidy correlated with poorer prognosis. S-phase fraction higher than 15% also correlated with a worse prognosis[4,5].

CASE REPORT

A 75-year-old male patient presented with a history of recurrent rectal bleeding which had become more frequent and severe during the previous year. Colonoscopy revealed only one polipoid and fragile mass in the rectum, and punch biopsy showed pigmented melanoma. Computed tomography (CT) scan and ultrasound did not find enlargement of regional lymph nodes.

Pathologic examination of the received specimen revealed two distinct tumorous masses; the first one that was pedunculated, on a thin stalk, 0.5 cm above dentate line, measuring 1 cm in greatest diameter. The second one was sessile and nodular, 1.5 cm above dentate line, measuring up to 2.8 cm. Both tumors were bluish brown on cut sections (Figure 1A).

After surgery, all specimens were formalin fixed, paraffin embedded, cut at 5 μm and routinely stained with hematoxylin and eosin. Deparaffinisation and immunohistochemical staining was performed following Microwave Streptavidin ImmunoPeroxidase (MSIP) protocol on DAKO TechMate™ Horizon automated immunostainer. We used primary antibodies to HMB-45 and S-100 (purchased from DAKO, Copenhagen, Denmark). Microscopically, both tumors were composed of atypical spindle cells with prominent nucleoli and highly pigmented. First tumor, the smaller one, was covered with squamous epithelium, partially ulcerated, infiltrating mucosa and submucosa but without extension to the

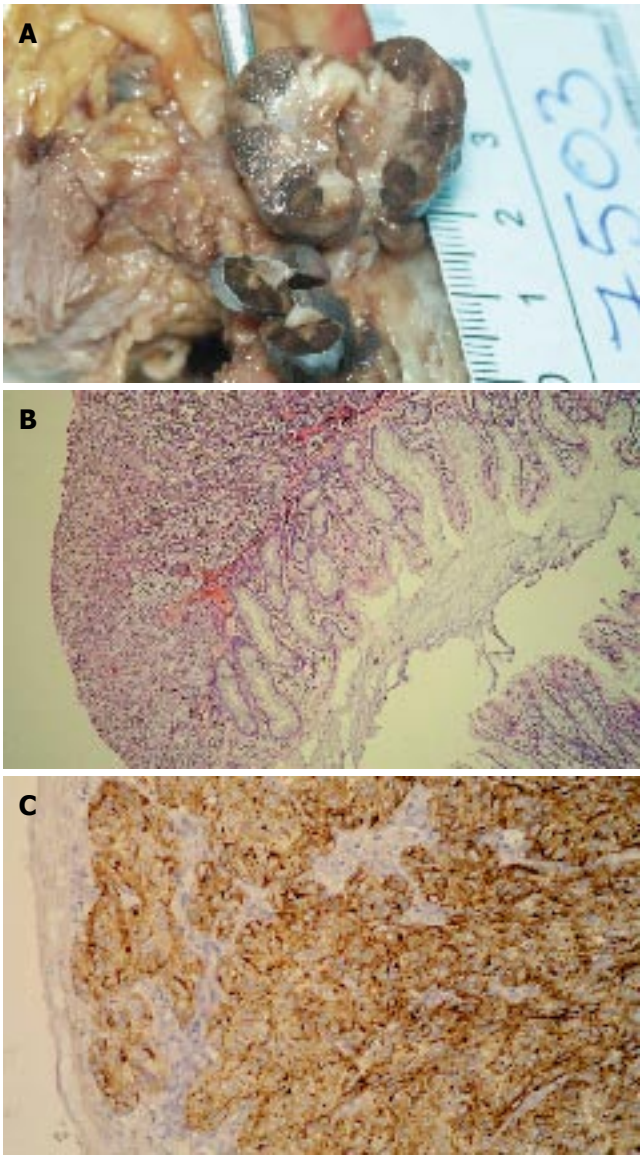

Figure 1 **A**: Macroscopical appearance of anorectal region with two melanomas; **B**: Photomicrograph of rectal melanoma covered with columnar epithelium (HE, original magnification x 100); **C**: Photomicrograph of anal melanoma covered with squamous epithelium showing strongly positive immunostaining for HMB-45 (original magnification x 200).

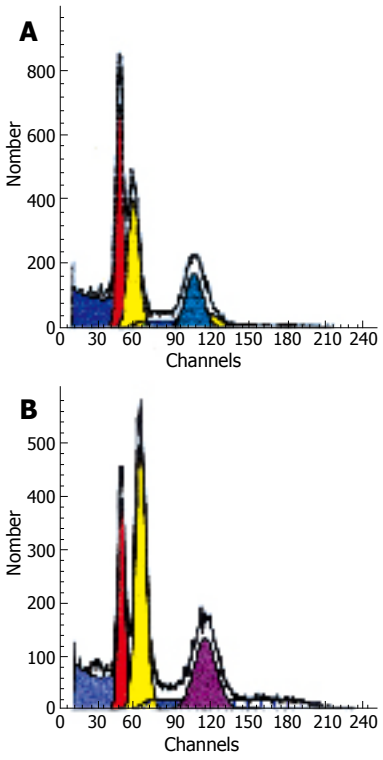

Figure 2 DNA histograms. **A**: Smaller tumor with two aneuploid peaks: left peak-hypodiploid population with DNA index of 1.22; right peak-hyperdiploid population with DNA index of 2.22. Middle peak-the diploid population of non tumoral cells; **B**: Larger tumor with two aneuploid peaks: left peak-hypodiploid population with DNA index of 1.32; right peak-hyperdiploid population with DNA index of 2.33. Middle peak-the diploid population of non tumoral cells.

muscle layer. Mitotic rate was 10/10 HPF. The larger tumor was covered with partially ulcerated columnar epithelial cells, without muscle layer infiltration (Figure 1B). Mitotic rate was 13/10 HPF. Tumor thickness, measured by computer program, was 5.75 mm for the smaller tumor, and 9.11 mm for the larger one. Both tumors showed immunohistochemically positive reaction for HMB-45 and S-100 (Figure 1C).

Paraffin embedded specimens were processed for the flow cytometry. Cellular DNA content and proliferative activity were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using excitation wavelength at *A*488 nm and 15-mW argon ion laser. DNA histograms were generated on at least 20 000 nuclear events with a Mod-FitLT V2.0 (Verity Software House Inc.,Topshame, ME and Becton Dickinson).

DNA flow cytometry analysis showed two aneuploid peaks (hypodiploid and hyperdiploid populations) in both tumors, but with different DNA indexes and S-phase fractions (Figure 2A, 2B). S-phase fraction for the polipoid tumor, covered with squamous epithelium was 11.84%, and for the nodular one, covered with columnar epithelium was 26.82%.

DISCUSSION

Anorectal melanoma is a very rare neoplasm, and in the reviewed literature we did not find any similar case of anorectal melanoma, either with satellite lesion or with multiple melanomas in anorectal region. We found only one reported case of metachronous melanoma in the upper digestive system, involving esophagus and stomach[8]. The case presented in this paper is specific by reporting two, synchronous melanomas, of which one of the tumors was found in anal canal, and it was partially covered by ulcerated squamous epithelium, while the other one was in rectum, covered with partially ulcerated columnar epithelium. Between described tumors there was an area of normal rectal mucosa without tumor involvement. For almost 30 years patient had recurrent bleeding because of hemorrhoid disease, which probably caused delay in making a correct diagnosis. Although some authors did not find difference in overall survival between patients treated with local excision and those with abdominoperineal resection[2,9], the study from the Memorial Sloan Kettering Cancer Center by *Brady et al.* suggested abdominoperineal resection as a better approach for patients without advanced disease, because patients treated with abdominoperineal resection were more commonly associated with better long-term survival than those treated with other procedures[4,6]. After physical, CT scan and ultrasound examination which did

not find any signs of metastatic disease, patient was treated by radical abdominoperineal resection.

The most important doubt in this case is the nature of both lesions. Are they both primary anorectal melanoma or is one of the two tumor primary, and which one, and the second one is probably a satellite lesion?

In skin melanomas, satellite lesions are defined as tumor nests or nodules within 2 cm of the primary tumor, while in-transit metastasis involves skin or subcutaneous tissue more than 2 cm from the primary tumor, but not beyond the regional lymph nodes. The significance of their presence is seen in survival studies, where lymph node metastasis is one of the most important predictor factors of survival in melanoma patients, and presence and number of involved lymph nodes is more important than actual gross dimension of the involved lymph node. In new melanoma staging system, patients with in-transit metastases or satellite lesions without lymph node disease have a similar prognosis to those with positive lymph node metastases. In the revised staging system, these patients are now upstaged as having N2c disease compared with the 1997 staging system in which these patients were considered as having T4 disease[10].

In this case, it is likely that one of the lesions is primary tumor and the second one is the satellite lesion. Concerning colonoscopy, which was performed few months before surgery, and which found only one polipoid tumor, it is possible that the smaller tumor is primary, and the second one is a satellite lesion. Hypothesis that one of the tumors is a satellite lesion is also supported with histological findings, because both tumors are composed of the same histological type. However, the surface of both tumors is ulcerated, making it very difficult to establish connection of the tumor cells with the surface, and concerning different S-fases on DNA flow cytometry there is still a possibility that this is the case of a multiple synchronous primary melanoma in anal canal and rectum.

Importance of distinguishing exact nature of the found lesions, if we could apply the same rules as for staging cutaneous melanoma, is better staging and predicting prognosis.

This case represents the multiple synchronous primary melanoma of the anorectal region, with a possibility that one of the lesions is primary melanoma and the second one is a satellite lession.

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Meetings

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107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology
Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

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Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4–5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6–7 May 2006
Berlin
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ILTS 12th Annual International Congress
3–6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15–18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
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Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3–7 June 2006
Seatlle - Washington
www.fascrs.org

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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