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EDITORIAL

Colorectal cancer screening: The role of CT colonography

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Abstract

Computed tomography colonography (CTC) in colorectal cancer (CRC) screening has two roles: one present and the other potential. The present role is, without any further discussion, the integration into established screening programs as a replacement for barium enema in the case of incomplete colonoscopy. The potential role is the use of CTC as a first-line screening method together with Fecal Occult Blood Test, sigmoidoscopy and colonoscopy. However, despite the fact that CTC has been officially endorsed for CRC screening of average-risk individuals by different scientific societies including the American Cancer Society, the American College of Radiology, and the US Multisociety Task Force on Colorectal Cancer, other entities, such as the US Preventive Services Task Force, have considered the evidence insufficient to justify its use as a mass screening method. Medicare has also recently denied reimbursement for CTC as a screening test. Nevertheless, multiple advantages exist for using CTC as a CRC screening test: high accuracy, full evaluation of the colon in virtually all patients, non-invasiveness, safety, patient comfort, detection of extracolonic findings and cost-effectiveness. The main potential drawback of a CTC screening is the exposure to ionizing radiation. However, this is not a major issue, since low-dose protocols are now routinely implemented, delivering a dose comparable or slightly superior to the annual radiation exposure of any individual. Indirect evidence exists that such a radiation exposure does not induce additional cancers.

Key words: Computed tomography colonography; Colon neoplasms; Colon polyps; Colorectal cancer screening; Computed tomography colonography safety; Computed tomography colonography accuracy; Computed tomography colonography radiation exposure; Computed tomography colonography cost-effectiveness

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INTRODUCTION

It is now 16 years since Vining *et al*[1] presented the first virtual images of the colon at the 1994 meeting of the Society of Gastrointestinal Radiologists. This marked the birth of "virtual colonoscopy" (VC), an intriguing name useful for marketing to patients, providers and the public, or "CT colonography" (CTC), the name most radiologists prefer.

When discussing the role of CTC in colorectal cancer (CRC) screening, it is necessary to separate the present and existent role from the potential one.

The present role is, without any further discussion, the integration into established screening programs as a replacement for barium enema (BE) in the case of incomplete colonoscopy (CC). In fact, since 2006 the American Gastroenterological Association (AGA) Clinical Practice and Economics Committee has endorsed CTC as the method of choice for colon investigation in cases of incomplete colonoscopy[2] and numerous evidence exists in the literature showing a clear superiority of CTC over BE in the detection of CRC and polyps[3-5]. It is also clear that performing CTC in patients with a positive Fecal Occult Blood Test (FOBT) would not be an efficient triage technique in terms of cost-effectiveness, due to the high prevalence of clinically relevant colonic lesions[6,7].

The potential role of CTC is to act as a first-line CRC screening modality. In this regard, it was in March 2008 that CTC obtained its major success: the American Cancer Society (ACS), the US Multi-Society Task Force on Colorectal Cancer and the American College of Radiology (ACR) released consensus guidelines on CRC screening for average-risk individuals. These guidelines distinguished diagnostic tests into two groups: those able to detect CRC, thus potentially reducing mortality (FOBT; Fecal Immunochemical stool Testing, FIT; and stool DNA testing), and those able to detect both polyps and cancer, thus potentially reducing both the incidence of and the mortality from CRC. This latter group of tests includes CC, sigmoidoscopy, BE and - for the first time - also CTC, with the recommendation that it be performed every 5 years starting at 50 years of age[8]. Unfortunately, this position did not remain unequivocal: in fact, the US Preventive Services Task Force (USPSTF) considered the evidence insufficient because of the unknown impact of both extra-colonic findings and radiation exposure, the poor data on cost and cost-effectiveness, and the still unsolved problem of ideal bowel preparation[9]. Other associations, such as the Asia Pacific Working Group on Colorectal Cancer[10] and the American College of Gastroenterology[11], consider CTC a second-line screening test for those unwilling or unable to undergo CC and for those in whom CC was incomplete. Furthermore, in 2008 in the USA, the Centers for Medicare and Medicaid Services denied the reimbursement of screening exams done with CTC[12].

These facts mean on one hand that CTC is considered useful by clinicians, but, on the other hand, that radiologists have much to do until this imaging method is implemented for screening, as testified by a survey conducted among US primary care physicians (PCP)[13]. When asked which diagnostic tests they perceive as being very effective in reducing CRC mortality, 22% answered CTC *vs* 95% CC; and when asked which test they would recommend for CRC screening, most respondents indicated CC (95%) and FOBT (80%), but only about 5% answered CTC.

Keeping in mind these facts, the potential role of CTC in CRC screening will be discussed in the next paragraphs, taking into consideration that an ideal screening test is not yet available and that any screening test is a compromise among four major variables: efficacy, compliance, safety and cost.

EFFICACY

The issue of diagnostic accuracy of CTC for CRC and polyps has been debated for a long time, because of the conflicting results in some of the papers published in the literature[14-16]. This has been recently confirmed by a meta-analysis showing that "CTC is highly specific for the detection of colorectal polyps and tumors" and that "some studies reported high sensitivities, but the results of the studies were highly heterogeneous, while the studied variables explained only part of this discrepancy"[17].

These results led researchers to design three important studies: two large, multicenter trials testing the performance of CTC in comparison with CC in respectively asymptomatic subjects at average risk, i.e. a typical screening population [the American College of Radiology Imaging Network (ACRIN) trial performed in the USA][18] and in a mixed population of asymptomatic subjects at risk higher-than-average and in patients referred for a positive FOBT [Italian Multicenter Polyps Accuracy CTC study (IMPACT) trial][19]; and one multicenter trial [Special Interest Group in Gastrointestinal and Abdominal Radiology (SIGGAR) trial run in the UK] conducted on symptomatic patients with the aim to detect CRC[20]. In particular, the ACRIN trial tried to minimize the variables possibly affecting CTC performance. For this reason only $\geqslant$ 16-row MDCT scanners were used, patients were administered oral contrast agent for stool tagging together with cathartic agent and training of the radiologists observing the images was an important component of the study. In particular, CTC readers were obligated to have read at least 500 cases, or to have attended a 1.5-d training course, and all had to pass a certified exam in which they detected at least 90% of adenomas 1 cm or larger in 50 cases. More than half of the readers had to undergo additional training in order to pass the certified exam initially and, with additional training, all the readers eventually passed.

Results from ACRIN and IMPACT have been recently published[18,19], whereas those from SIGGAR[20] are still under data analysis. Both the ACRIN and IMPACT trials reported per-patient sensitivity of 90% for polyps > 10 mm and 78%-84% for polyps larger than 6 mm; per-patient specificity was extremely high as well, over 85% independently of lesion size (Table 1). The major drawback of ACRIN was represented by the poor positive predictive value (PPV) (23% for polyps $\leqslant$ 10 mm), which might negatively affect a screening program, leading to useless CC, with patient discomfort, embarrassment of radiologists, potential risk of complications and increased costs. Unfortunately there is no explanation for these data, unless one would claim a psychological attitude to overcall in order to reach the threshold of 90% for clinically significant polyps despite a loss in specificity. A definitely better PPV was documented in the IMPACT trial (62% for lesions larger than 6 mm) as well as in studies obtained

Table 1 Results from major trials on a per-patient basis: sensitivity, specificity, positive and negative predictive values

Multicenter trials	All polyp size	Polyps (≥ 5 mm)	Polyps (≥ 6 mm)	Polyps (≥ 7 mm)	Polyps (≥ 8 mm)	Polyps (≥ 9 mm)	Polyps (≥ 10 mm)
Per patient sensitivity							
ACRIN	N/A	65%	78%	84%	87%	90%	90%
IMPACT	N/A	N/A	85%	86%	88%	91%	91%
Munich	84%	91%	N/A	N/A	N/A	N/A	92%
Per patient specificity							
ACRIN	N/A	89%	88%	87%	87%	86%	86%
IMPACT	N/A	N/A	88%	87%	86%	85%	85%
Munich	47%	93%	N/A	N/A	N/A	N/A	98%
Per patient PPV							
ACRIN	N/A	45%	40%	35%	31%	25%	23%
IMPACT	N/A	N/A	62%	N/A	N/A	N/A	N/A
Munich	48%	N/A	70%	N/A	N/A	N/A	79%
Per patient NPV							
ACRIN	N/A	95%	98%	99%	99%	99%	99%
IMPACT	N/A	N/A	96%	N/A	N/A	N/A	N/A
Munich	84%	N/A	98%	N/A	N/A	N/A	99%

Results are categorized according to polyp size. PPV: Positive predictive value; NPV: Negative predictive value; N/A: Not assessable.

in high-experience centers, the University of Wisconsin (PPV, 91.5%) and a group of Korean hospitals (PPV, 69% for lesions > 6 mm and 92% for those > 10 mm)[21,22].

However, the negative predictive values in both the ACRIN and the IMPACT trials was rather high, approaching 100%; this is extremely important in order to reassure negative patients about the significance of the examination.

Excellent results were also obtained in the Munich Colorectal Cancer Prevention Trial[23], a single-center study where around 300 asymptomatic subjects underwent low-dose CTC in comparison with other screening tests (CC, sigmoidoscopy and FOBT).

It is noteworthy to mention that in a screening project offered by the University of Wisconsin[24], after 2 years of recruitment over 3000 subjects of two different, non-randomized groups underwent CTC and CC. The detection rate for advanced adenomas was 3.2% for CTC and 3.4% for CC (difference not statistically significant), with the advantage of a large reduction in the number of polypectomies in the CTC group without any complication as opposed to seven perforations which occurred in the CC group.

Despite the good results there are still some open issues under debate within the radiological as well as the gastroenterological communities. These are the significance of diminutive (< 6 mm) polyps, the management of intermediate (6-9 mm) lesions, the detection rate for non-polypoid, flat lesions and the impact of the extra-colonic findings.

According to a very recent systematic review[25] of published studies reporting the distribution of advanced adenomas in asymptomatic screening cohorts, diminutive polyps have a minimal clinical impact. In fact, the frequency of advanced lesions among patients whose largest polyp was ≤ 5 mm, 6-9 mm, < 10 mm, and > 10 mm in size was 0.9%, 4.9%, 1.7%, and 73.5%, respectively (Figure 1). As a consequence, a 6-mm polyp size threshold for polypectomy referral would identify over 95% of subjects with advanced adenomas, whereas a 10-mm threshold would identify 88% of cases. From a cost-effectiveness point of view, detection and removal of all polyps including those smaller than 5 mm, would be very inefficient, with a cost per year of life gained > $460 000[26], absolutely unacceptable in terms of cost-effectiveness. It is also true that this approach, not removing diminutive polyps, necessitates an extensive education of patients and PCP. In fact, according to a recently published survey[27], the majority of patients, PCP and gastroenterologists would not choose to follow up small polyps identified by CTC with CC because of the fear of missing precancerous lesions.

The management of intermediate (6 to 9 mm) lesions is also under debate, despite the fact that today any polyp 6 mm or larger should be preferably referred for CC and polypectomy, also according to ACS CRC screening guidelines[2]. However, evidence does exist from cost-effectiveness[28] as well as follow-up[29] studies indicating that, for the future, polyp follow-up might be an alternative to referral for CC and polypectomy. These data have been recently reinforced by studies conducted with CC and subsequent polypectomy, where the rate of advanced adenoma in 6-9 mm polyps was demonstrated to be 6.6%[30].

A potential disadvantage of CTC would be the possible impaired ability to detect non-polypoid, flat lesions (Figure 2). This issue deserves some consideration. First of all, flat lesions represent a subset of sessile polyps, and according to a recent publication[31] the overall prevalence in a screening population is around 5.8% (if flat lesions are defined as those with a height not exceeding 0.5 of the diameter[32]). However, within this definition, slightly elevated lesions are also included, which, in some cases, may be classified as sessile. For this reason, an elevation not higher than 3 mm is often used, especially for small lesions not larger than 2-3 cm. The other important remarks are that "completely flat lesions are exceedingly

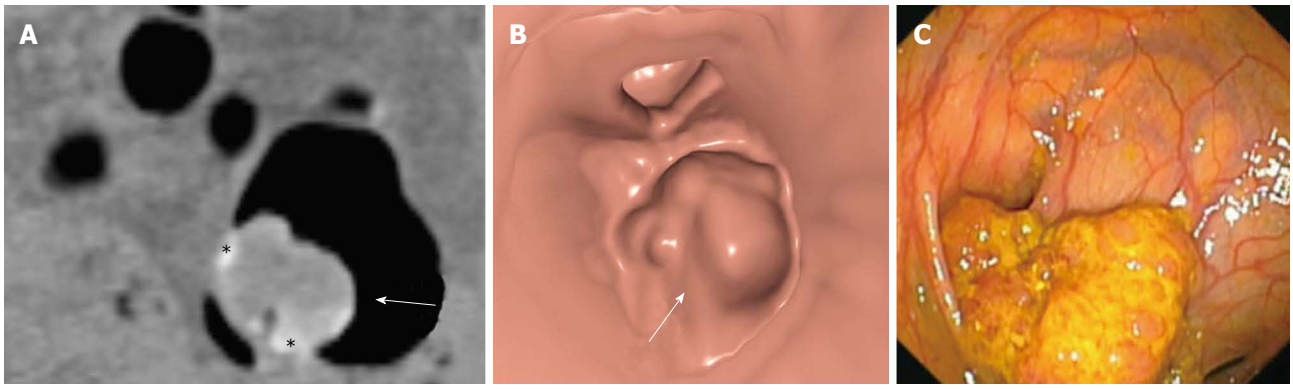

Figure 1 Sessile polyp: adenoma with low-grade dysplasia. A: Coronal reformatted image showing a polypoid lesion (arrow), partly surrounded by tagged fluid (asterisks); B: The same lesion as shown on 3D endoluminal view (arrow); C: Conventional colonoscopy.

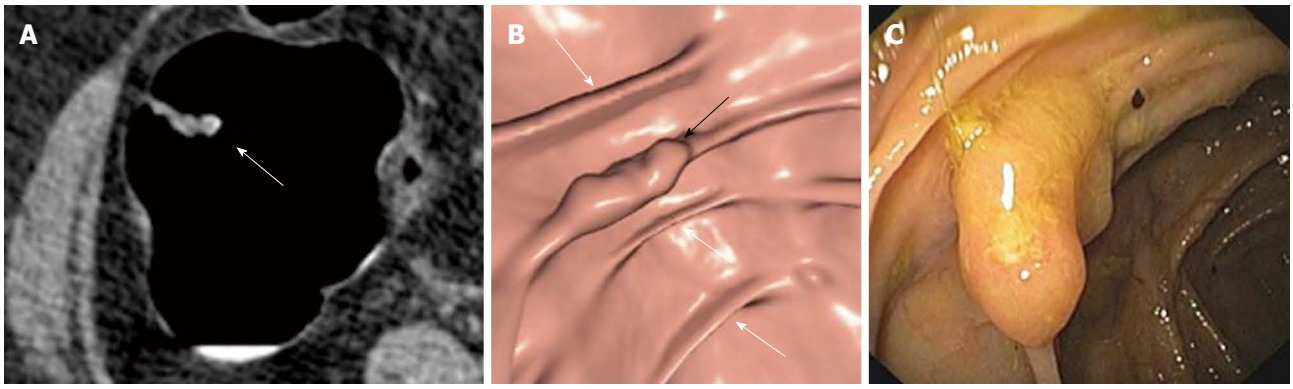

Figure 2 Non-polypoid (flat) lesion: advanced adenoma. A: On 2D axial computed tomography image an irregularly thickened fold (arrow) is detected; B: On 3D endoluminal image the lesion is better appreciated (black arrow), in particular if compared with the normal adjacent colonic folds (white arrows); C: The same lesion at colonoscopy before removal.

rare" and that depressed lesions are less than 1% of all colorectal lesions and only a quarter of those are observed in a screening population. This means that the majority of non-polypoid lesions are at least slightly elevated and this works in favor of the possible detection with CTC. Unfortunately, at the moment only few and conflicting data about the sensitivity of CTC for flat lesions are available. At the beginning disappointing results were published[33,34], because of technical limitations as well as readers' experience. More recently, better results were reported, with sensitivity in the range of 80%-90% for flat adenocarcinomas[32,35]. These results seem to be confirmed by a recent retrospective analysis of the data from the ACRIN trial showing a sensitivity of 89% for flat adenomas ≤ 6 mm (at the prospective analysis, sensitivity was 68%) (Fidler J, presented at ACRIN meeting, October 1, 2009).

The impact of extra-colonic findings will be discussed in the paragraph concerning costs.

COMPLIANCE (ADHESION TO A SCREENING PROGRAM)

Compliance means adhesion of healthy individuals to a screening program. This is a very complex issue, long debated in the literature and presently without a solution, since virtually in any Western country a high proportion of adults 50 years of age or older have not undergone any CRC screening test[36]. Colonoscopy, considered to be the most effective screening method, suffers from a very low participation rate. Very recent data from Italy[37] underline the difficulties and barriers in implementing a CC population screening, at least through primary care. Reported participation in a CC screening arm was extremely low in Southern Italy (2.8%), and higher, but still disappointing, in North-Central Italy (12.4%).

To increase screening uptake is extremely important, since the adhesion rate of the invited population directly affect the efficiency of the program. For example, if we consider that the efficacy of CC in reducing the incidence of CRC is around 76%[38], the efficiency with regard to CRC prevention rate, considering an adhesion rate of 20%, drops to 15% (76% × 20% = 15%).

The question is whether CTC would be able to increase screening uptake. The three most common deterrents that patients expressed about receiving CC were bowel preparation, embarrassment and fear of discomfort[39,40]. The advantage of CTC is the use of a gentler preparation or unprepped (laxative-free) examination[41]. Furthermore, the pain related to colon distension by air may be minimized by the use of carbon dioxide delivered

by an electronic pump. The use of carbon dioxide is also associated with a faster absorption, making the patient more comfortable immediately after the examination[42].

Unfortunately, only few data are available regarding adhesion rate and CTC. In a study conducted in Western Australia[43], 2000 people were invited and 28.4% accepted to undergo CTC for screening, with 62% of them preferring CTC over optical colonoscopy. The fact remains that we need data resulting from real screening experiences.

SAFETY

CTC is a safe test, definitely safer than colonoscopy. The results of different surveys show a perforation rate associated with CTC ranging between 0.06% and 0.08%[44,45], even lower in screening[46], compared with 0.1%-0.2% for diagnostic colonoscopy[47]. It should also be noted that the comparison between CTC and CC is very difficult, with the risk of overestimating the clinically significant perforations at CTC, because of the much higher sensitivity of CT in the detection of even tiny air bubbles. In fact, most of the perforations reported in a UK survey[44] were treated conservatively, without surgical intervention. CTC complications in most of the cases are due to technical factors, such as the use of a rigid catheter for bowel distension (now replaced by thin rubber devices), manual distension with air (now minimized by the use of an electronic pump delivering carbon dioxide and able to control pressure and volume), inexpert personnel and incorrect patient selection.

Other complications occasionally reported have been vasovagal reactions due to colonic overdistention[48].

The main potential drawback of screening with CTC is the exposure to ionizing radiation[49] and the consequent theoretical risk of inducing cancer. The risk is theoretical because there are still many uncertainties with regard to the true effects of ionizing radiation at low doses, such as those used in diagnostic radiology[50,51]. According to the Health Physics Society[52], "below 5-10 rem (50-100 mSv) (which includes occupational and environmental exposures), risks of health effects are either too small to be observed or are nonexistent"; and the French Academy report[53] stated that the linear no-threshold (LNT) hypothesis for assessing the risk associated with low doses is not based on scientific evidence. However, in contrast, the International Commission on Radiological Protection (ICRP)[54] and the Biological Effects of Ionizing Radiation (BEIR) Ⅶ report[55] considered LNT as the best method to assess low dose exposures, in order to be more conservative and more protective towards patients because of the multiple uncertainties. Unfortunately even if this hypothesis may be true it cannot be proved since we have little direct evidence of harm below 100 mSv.

Even if the LNT hypothesis is considered the most accurate, the problem of radiation exposure of patients undergoing CTC seems to be minimal. In fact, the mean exposure in the case of a screening examination has been calculated in a recent survey[56] to be around 5-6 mSv, which is twice the normal background radiation exposure in the US (2.5-3.0 mSv per year)[57]. In addition we have to consider, in a screening scenario, that CTC should be repeated every 5 years, not earlier. For a radiation dose of around 5-8 mSv at age 50 years, the lifetime risk of death from cancer varies between 0.02% and 0.03%[58]. If we think about cost/benefit of the examination, this minor risk should be compared with the theoretical risk of CRC in average risk individuals, which is around 5%[59].

A more precise idea about the amount of radiation exposure comes from a comparison with other categories of workers continuously exposed to low dose radiation, such as, for example, airline crews and nuclear workers. As an example, the value of 5-6 mSv, to be received in a screening scenario every 5 years, should be compared with radiation exposure of airline crews, who are submitted to an average of 5 mSv per year every single year of their activity, with a long life exposure close to 80 mSv. A recent survey of airline pilots from eight different European countries has shown no increase in mortality from radiation-induced cancers over a 30-year period of time[60]. Similar data were observed in nuclear workers in two recently published experiences[61,62].

COSTS

Cost analysis is a very difficult task, especially in the absence of real data and based only on mathematical models. Among the studies published in the literature[63-69] 5 out of 7 are in favor of CC and only two were able to demonstrate a better cost-effectiveness of CTC. A recent review of the literature[70] has pointed out the profound differences among the models as well as the weakness of such an approach, where a minimal variation of a single input may completely alter the final results. As an example, if the cost ratio between CTC and CC is ⩽ 0.7, the model is usually in favor of CTC, but if the cost of CTC is higher than 80% of the cost of CC, it will be CC which is the most cost effective method. Another very important issue, adhesion rate, is never taken into account in the models, where it is considered to be equal among the different screening tests, although this is probably not the case. In addition, differences in healthcare systems, reimbursement, cost of the equipment and personnel are other important variables affecting the final outcome.

When considering cost, the issue of extra-colonic findings should be taken into account. The detection of extra-colonic findings can be considered a potential advantage of CTC, since previously unknown life-threatening diseases, which are not insignificant[71], can be diagnosed and treated, with a clear impact on patients' life expectancy. However, the major problem is the extra time necessary for reporting these findings and the cost induced by unnecessary investigation of common benign abnormalities, especially because of their high prevalence[72,73]. In a recent publication[74], the mean cost per patient was $31.02 for nonsurgical and $67.54 for surgical work-up procedures. Although extra-colonic findings have been traditionally

regarded as an additional cost, they have been recently considered as a potential benefit (i.e. detection of unsuspected abdominal aortic aneurysm or renal cancer), able to improve CTC cost-effectiveness[75].

CONCLUSION

In conclusion, CTC has a present role in CRC screening programs, i.e. the replacement of BE in the case of incomplete colonoscopy. The potential role is the proposal of CTC as a first-line CRC screening modality. In this setting, CTC has clear advantages, such as accuracy, safety and subject acceptance. Further research should be warranted to clarify, in particular, two aspects: the uptake rate of the general population and the real cost and benefits derived from a CTC screening program.

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EDITORIAL

Essential role of monocytes and macrophages in the progression of acute pancreatitis

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Abstract

Acute pancreatitis (AP) is an inflammatory condition of the pancreas caused by an imbalance in factors involved in maintaining cellular homeostasis. Earliest events in AP occur within acinar cells accompanied by other principal contributors to the inflammatory response i.e. the endothelial cells, immunocytes (granulocytes, monocytes/macrophages, lymphocytes) and neutrophils. Monocytes/macrophages are important inflammatory mediators, involved in the pathophysiology of AP, known to reside in the peritoneal cavity (in the vicinity of the pancreas) and in peripancreatic tissue. Recent studies suggested that impaired clearance of injured acini by macrophages is associated with an altered cytokine reaction which may constitute a basis for progression of AP. This review focuses on the role of monocytes/macrophages in progression of AP and discusses findings on the inflammatory process involved.

Key words: Acute pancreatitis; Monocytes; Peritoneal macrophages; Alveolar macrophages; Kupffer cells

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INTRODUCTION

Inflammation is a complicated progressive process which is initiated by the body in response to tissue injury or infection. Inflammation proceeds *via* the sequential release of mediators that leads to vasodilatation and increased blood flow, increased vascular permeability, causing the accumulation of a fluid exudates, and the activation of neurosensory pain fibers giving rise to the classical signs of acute inflammation i.e. heat, redness, swelling, and pain[1]. Acute inflammation is associated with high levels of polymorphonuclear cells, particularly neutrophils, whereas chronic or adaptive immune inflammation has higher levels of mononuclear cells, macrophages, T- and B-lymphocytes.

Regulated release of chemokines and expression and activation of cellular adhesion molecules recruits leukocytes at the site of inflammation. Leukocyte recruitment is a critical step in the inflammatory process[2]. Membrane bound vascular cell adhesion molecule-1 (VCAM-1), intracellular cellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 and E-selectin are expressed on endothelial cells, smooth muscle cells, and tissue macrophages. These adhesion molecules in coordination with others, for example, selectins, allow binding of ligands on leukocytes to mediate rolling, firm attachment and transendothelial migration. Endothelial cells being the interface between the tissue and circulation play

an important role in inflammatory and immune-relevant cells[3,4].

Acute pancreatitis (AP) is an inflammatory condition of the pancreas that involves peripancreatic tissue and remote organs. Excessive systemic inflammatory response syndrome (SIRS) in AP leads to distant organ damage and multiple organ dysfunction syndrome (MODS), which is the primary cause of morbidity and mortality in this condition. Mild AP is self limiting but up to 25% of the patients suffer a severe attack and around 30% of these will die. Approximately half of the deaths in AP occur within the first 2 wk of illness and are generally attributed to organ failure. The rest of the deaths occur weeks to months later, characterized by extensive retroperitoneal pancreatic necrosis and septicemia[5]. AP involves a complex cascade of events initializing in pancreatic acinar cells. An unknown trigger within the pancreas leads to conversion of digestive proenzymes into their active form, initiating auto digestion of the gland causing hemorrhage, necrosis, edema and complete destruction of pancreatic parenchyma. Intrapancreatic activation of trypsinogen by lysosomal hydrolases is an early triggering event in AP[6]. Interestingly both pharmacological and genetic deletion of lysosomal hydrolases like cathepsin B can reduce the severity of pancreatitis[7]. Other pharmacological agents which block trypsinogen activation can also modulate the outcome of AP[8,9].

Immune cells involved in elaborating the inflammatory mediators in AP are the pancreatic acinar cells, endothelial cells, neutrophils, lymphocytes, monocytes and macrophages. Inflammatory mediators believed to participate in the pathophysiology of this condition include: tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1β), interleukin-6 (IL-6), platelet activating factor (PAF), ICAM-1, IL-8, growth related oncogene-a/cytokine-induced neutrophils chemo attractant (GRO-α/CINC), monocyte chemotactic protein-1 (MCP-1), IL-10, complement component C5a, substance P (SP), hydrogen sulfide (H_2S), and neutral endopeptidase (NEP)[10]. In recent years, it has become clear that the signaling molecule nuclear factor κB (NF-κB) plays a central role in the initiation and progression of AP[11]. The emerging body of evidence suggest that blocking NF-κB activation can markedly reduce the severity of AP[12,13]. These findings have opened a window of opportunity for the use of selective NF-κB inhibitors in regulating the inflammatory process in AP. The expression levels of various proinflammatory mediators like TNF-α and IL-1β in AP are positively regulated by NF-κB[14,15]. Systemic amplification of AP is associated with excessive release of these inflammatory mediators from local tissue and systemically. This systemic amplification is responsible for most of the mortality associated with AP[16]. Studies indicate that both pancreatic and extra pancreatic (lung, liver, monocytes, macrophages and endothelial cells) activation of NF-κB is associated with development of MODS in AP[17,18].

In this review we discuss the recent advances, till date, pointing towards the fundamental role of different monocyte/macrophage populations in the progression of AP.

MONOCYTE/MACROPHAGE SYSTEM

Macrophages are among the most versatile cells of the body. Compelling evidence has emerged in recent years with respect to their roles in innate immunity, inflammation, and tumor progression. Macrophages are released from bone marrow into the bloodstream as promonocytes which develop into monocytes and migrate to tissue and undergo final differentiation into specific types of tissue resident macrophages[19]. The phenotypes of these tissue resident macrophages vary markedly within tissues, including Kupffer cells in liver, alveolar macrophages in lungs, osteoclasts in bone, microglial cells in brain, Langerhans cells in skin.

Monocyte and macrophage in AP

Since macrophages orchestrate both the initiation and the resolution of inflammation, they are an interesting target for designing a therapeutic strategy focused on the control of systemic effects of AP. Macrophage activation constitutes a key component of the immune response and several proinflammatory cytokines and bacterial products participate actively in the triggering of this process.

The degree of macrophage activation might be one of the important factors that determine the severity of AP[40]. Besides acinar cells, monocytes/macrophages are the main inflammatory cell population involved in the pathogenesis of AP (Figure 1). In AP, the inflammatory process starts with the migration of monocytes and neutrophils in the circulation into the pancreatic interstitial space, mediated by adhesion molecules on leukocytes. These infiltrating cells assist the production of different cytokines and various inflammatory mediators. As a result, amplification of non-infectious inflammation initiated in the pancreas to specific distant organs such as the lung, liver and spleen occurs[20]. The CC chemokines such as MCP-1, macrophage inflammatory protein (MIP)-1α and RANTES (CCL5) are believed to activate primarily monocytes, whereas the CXC chemokines, such as IL-8, tend to preferentially activate neutrophils. Bhatia *et al*[21] have shown that administration of bindarit, a blocker of MCP-1 synthesis (prophylactically or therapeutically), significantly reduces the severity of AP suggesting that MCP-1 may be an early inflammatory mediator in AP.

CD14, is a glycosyl-phosphatidylinositol anchored cell surface molecule expressed on the cell surface of immune effector cells of myeloid and monocytes hematopoietic lineage, which acts in concert with mammalian Toll-like receptors (TLR). It exists in 2 forms - soluble and membrane bound - and the soluble form has both immune stimulatory and inhibiting activities. Increased soluble CD14 expression and expansion of the proinflammatory CD14/CD16 monocytes subset has been reported in AP, suggesting that soluble CD14 receptor may serve as a key mediator of the systemic endothelial response, and its targeted disruption with anti-CD14 monoclonal antibodies may afford some therapeutic benefit in preventing the development of MODS and septic complications associated with AP[22]. Li *et al*[23] demonstrated that during early stages of

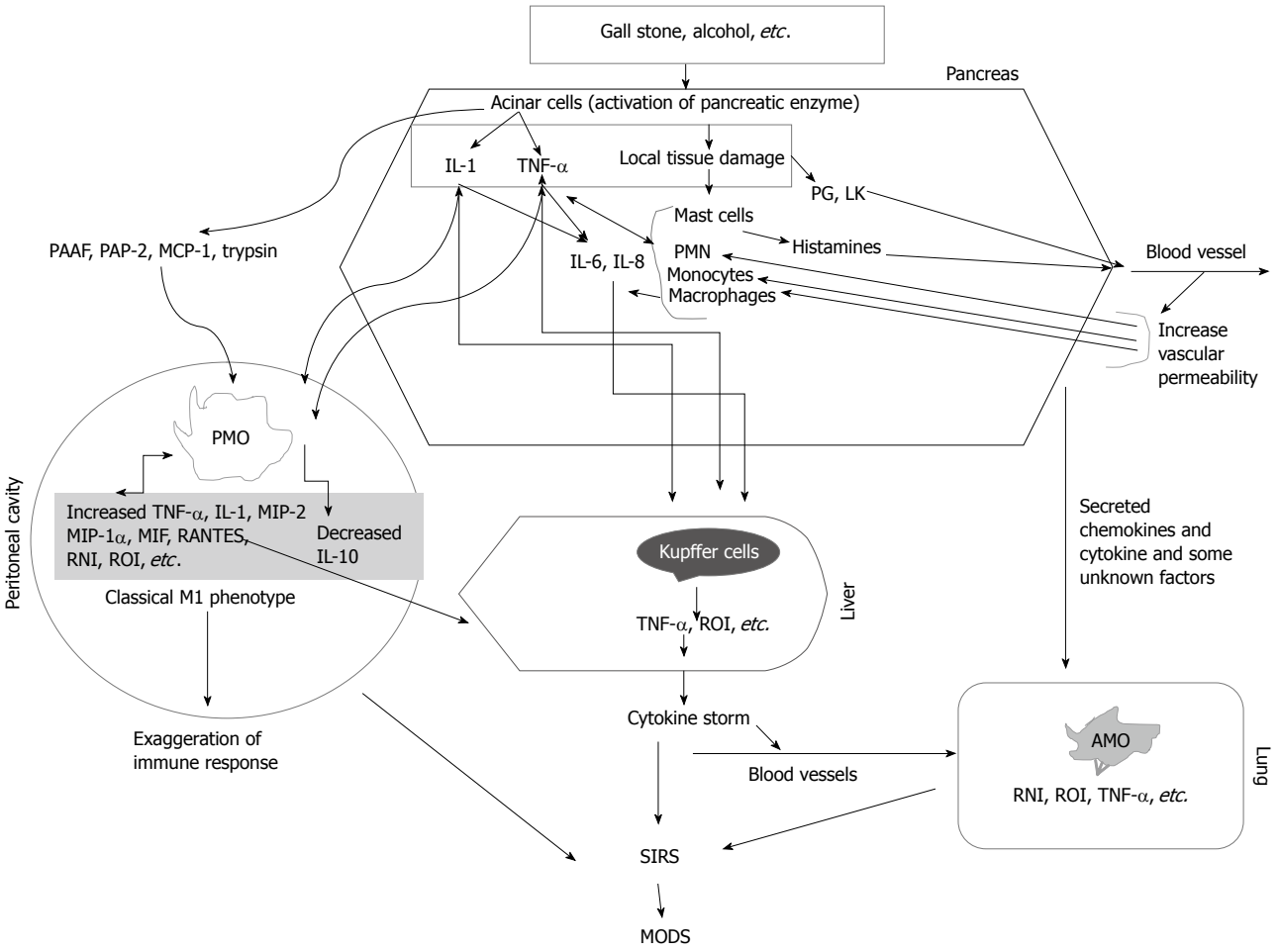

Figure 1 Our current understanding of the role of monocytes and macrophages in the pathogenesis of acute pancreatitis. Local inflammation in the pancreas leads to secretion of pro-inflammatory cytokines and some unknown factors. These inflammatory mediators activate peritoneal macrophages (PMO), Kupffer cells, and alveolar macrophages (AMO), which if uncontrolled can cause multiple organ dysfunction syndrome (MODS). PMO display a classical M1 type activation in acute pancreatitis thus supporting the inflammatory process. TNF: Tumor necrosis factor; RNI: Reactive nitrogen intermediates; ROI: Reactive oxygen intermediates; PAAF: Pancreatitis associated ascitic fluid; PAP: Pancreatitis-associated protein; MCP: Monocyte chemotactic protein-1; PG: Prostaglandins; LK: Leukotrienes; SIRS: Systemic inflammatory response syndrome; PMN: Polymorphonuclear leukocytes; MIP: Macrophage inflammatory protein.

AP, expression of TLR4 is upregulated in peripheral blood mononuclear cells (PBMC) with a simultaneous increase in TNF-α expression suggesting an important role of TLR-4 in pathogenesis of AP. TNF-α and IL-1β are regarded as most prominent "first-line" cytokines. Studies have indicated that during the course of pancreatitis, initially IL-1β and TNF-α are produced in the pancreas by acinar cells, in a process mediated by leukocytes, and organs such as liver, lungs and spleen which have large populations of macrophages, serve as subsequent sources of production of pro-inflammatory cytokines[24]. The pancreas itself contains an unknown proportion of resident macrophages. The liver and lung are susceptible to injury and organ failure during AP. Peritoneal macrophages, Kupffer cells and alveolar macrophages play a pivotal role in controlling the progression of AP, due to their ability to generate pro- and anti-inflammatory mediators.

Peritoneal macrophages

Peritoneal inflammatory cells play an important role in the production of chemical mediators and in the defense against infection of the abdominal cavity. Macrophages are the major resident immune cells within the pancreas and in the peritoneal cavity. Severe AP (SAP) and early multi system organ failure are associated with the sustained release of both pro- and anti-inflammatory cytokines in the ascitic fluid, the thoracic lymph and the systemic circulation. The peritoneal compartment which is in the close vicinity to the site of pancreatic inflammation and necrosis is the site for net proinflammatory reaction. Release of IL-1β and TNF-α by peritoneal macrophages in early stages of AP induces a cascade of other inflammatory cytokines, activation of neutrophils, and induction of the pro-inflammatory response whereas the anti-inflammatory response is mainly systemic in nature. Preventing the activity of both cytokines concurrently has no additional effect on the degree of pancreatitis but does attenuate the systemic stress response and is associated with an additional but modest decrease in mortality[10]. Thus, in AP, peritoneal macrophages act as a principal contributor to the acute systemic inflammatory response that in turn determines the severity of disease. Peritoneal macrophages isolated from rats with SAP showed over production of nitric oxide. Increased nitric oxide secretion is implicated in progression of AP[25].

Abnormal trypsin activation in the pancreas contributes to the pathogenesis of AP. Lundberg *et al*[26] showed that trypsin stimulates the production of cytokines in peritoneal macrophages and that injection of trypsin into the peritoneal cavity induces lung injury. Hori *et al*[27] demonstrated that TGF-β produced by peritoneal macrophages induces hepatocellular injury *via* apoptosis in the rat SAP model.

Studies demonstrate that pancreatitis associated ascitic fluid (PAAF) of SAP affects peritoneal macrophage functions thereby contributing to the pathologic course of disease. They showed that incubation of peritoneal macrophages with PAAF leads to rapid and prolonged activation of NF-κB and TNF-α production[28]. The major site of TNF-α gene transcription in AP is the pancreatic activated macrophages[29]. Their deactivation in the early course of AP increases survival and decreases the severity of disease. Liu *et al*[30] suggested that activation of NF-κB and p38 MAPK in monocytes/macrophages from animals with AP might play a role in transcription and biosynthesis of TNF-α and IL-6. Animal experiments indicated that sterile ascites without cytokines from AP can stimulate production of cytokines from macrophages derived from spleen and lung *in vitro* and can induce cytokine production systemically *in vivo*[31].

Mikami *et al*[32,33], by depleting peritoneal macrophages using liposome encapsulated dichloromethylene biphosphonate, suggested that peritoneal macrophages extend inflammation from the pancreas to the peritoneal cavity and subsequently induce lung injury leading to SIRS and multiple organ failure opening the possibility that therapeutic modification of peritoneal macrophages may be a new therapeutic approach in patients with AP. The immune response in AP depends to a larger extent on macrophages as they represent about a third of infiltrating mononuclear cells. Macrophages are the main source for the production of inflammatory mediators; their presence in pancreatitis might contribute to the amplification of the immune response during disease progression.

With the progression of AP, there is a change in number and ratio of CD4+ and CD8+ lymphocytes indicating the possible involvement of T-lymphocytes. Observation by Demlos *et al*[34] demonstrated that CD4+ T cell depletion reduces the severity of pancreatitis. CD4+ T cells act as a costimulator for macrophage activation *via* antigen presentation and proinflammatory cytokine release[35].

Activation and trafficking of inflammatory cells involves chemokines and their receptors. Interestingly deletion of the receptor for MIP-1α and RANTES, the CCR1 receptor, was associated with protection from pulmonary inflammation but did not reduce the severity of cerulein-induced pancreatitis. This protection from lung injury is associated with decreased levels of TNF-α in a temporal sequence indicating the critical role of CCR1 receptor in the extension of pancreatic injury to the systemic response. The authors underline these findings by suggesting that CCR1 may be activated on either peritoneal or lung macrophages, leading to an autocrine process whereby increased levels of TNF-α drive further induction of both α and β chemokines, resulting in recruitment of inflammatory cells to the pancreas and lung[36]. However, in the same model, inhibition of CXCL2 (MIP-2) protected partially against both pancreatic and lung injury[37]. Attenuation of pancreatic injury in this study may be due to the fact that CXCL2 (MIP-2), in contrast to CCL5 (RANTES) and CCL3 (MIP-α), also attracts neutrophils in addition to monocytes. Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine released by macrophages and lymphocytes, is emerging as an important player in the pathogenesis of AP. Pre-treatment with anti-MIF antibodies improved the survival in rats with AP[38]. Recently, work by Sorli *et al*[39] suggested that peritoneal macrophages showed a classical M1 phenotype in AP, characterized by high expression of TNF-α and lack of changes in the mannose receptor. TNF-α can in turn activate macrophages for release of other macrophage derived inflammatory cytokines thus exaggerating the inflammatory response. Their study also indicates that peritoneal macrophages could be reprogrammed *in vitro* to reparative alternatively activated (M2) macrophages by IL-4 and IL-13 thus opening the possibility that therapeutic modulation of macrophage activation can help in the treatment of AP[39].

In any immune response dying cells are normally phagocytosed by macrophages; this elimination of dying cells is associated with anti-inflammatory cytokine switching. Recent observations by Liang *et al*[40] have suggested that there is a close relation between modes of pancreatic acinar cell death, the release of cell contents and the inflammatory reaction of peritoneal inflammatory cells.

In recent years, neuropeptide SP has gained considerable attention as a mediator of neurogenic regulation of inflammation. Earlier work by Sun *et al*[41] has suggested that neuropeptide SP is a pro-inflammatory mediator involved in pancreatitis and during acute inflammation, SP induces chemokine release from macrophages infiltrating into local and distant damaged tissues. Mice lacking NK-1Rs which bind to SP are protected against cerulein induced pancreatitis and associated lung injury. Macrophages have receptors for SP. Recently we have also shown the effect of SP on the murine macrophage cell line RAW264.7, as well as isolated primary macrophages, indicating that SP, at nanomolar concentrations, elicited selective chemokine production from murine macrophages[42]. Indeed, unpublished observations from our lab indicate that macrophages isolated from mice lacking NK-1R receptors show M2 phenotype displaying an anti-inflammatory cytokine switch. These observations led us to conclude that SP, acting *via* NK-1R on macrophages, plays an important role in regulating the severity of pancreatitis and associated lung injury.

Pancreatitis-associated proteins (PAP) are members of the *Reg* gene family; these 14-17 kDa proteins have been shown to be strongly induced during AP[43]. Although originally identified during AP, they have been reported in other inflamed pathologic organ systems including Crohn's disease, inflammatory bowel disease, liver injury, neuronal, ovarian, and cardiac tissue damage[44,45]. Recent investigation suggests that PAP2 is a potential mediator of early

inflammation in AP, its act specifically by orchestrating the macrophage inflammatory response, and may do so by working in concert with other PAP isoforms[46]. They also demonstrated that macrophages may express a potential PAP2 receptor[46].

Kupffer cells

Liver macrophages or Kupffer cells are the most abundant mononuclear phagocytes in the body. They are a predominant source of inflammatory cytokine released in the systemic circulation. Cytokine release from the liver represents about 50% of total cytokine release from the body[47]. Recent studies have shown the involvement of the liver in complex networks of events triggering the multiorgan dysfunction associated with AP. During AP the inflamed pancreas generates soluble inflammatory mediators. Once pancreatic mediators reach the liver, they strongly activate Kupffer cells (resident macrophages), they then greatly amplify the release of cytokines into the blood stream and thus contribute to the systemic manifestation of AP. Activated Kupffer cells release different inflammatory mediators, immunoregulatory and inflammatory cytokines, reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI), hydrogen peroxide, *etc.* which play a significant role in progression of pancreatic inflammation into a systemic process[48]. TNF-α released from the pancreas triggers the early events in AP, as pancreatitis progresses TNF-α reaches the liver, further manifesting the disease outcome by causing a cytokine storm. This cytokine is considered as one of the most important mediators of the systemic complications associated with AP[49]. The observation that in native pancreatitis the systemic manifestation of AP is more severe as compared to graft pancreatitis (as the mediators released by pancreas in graft pancreatitis are sent directly through the iliac vein) suggested that the liver plays an active role in the development of lung injury secondary to AP[50]. Gloor *et al*[51,52] further confirm this observation by preventing the passage of blood coming from pancreas to liver. Interestingly it has been shown that blocking the activity of Kupffer cells by gadolinium chloride in a sodium taurocholate AP model significantly reduces the increased TNF-α in serum during pancreatitis to its control levels, followed by reducing the inflammatory response in the lung, and in turn reducing the systemic complications associated with AP and improving the survival rate in mice. The authors confirm their observation *in vitro*, by using cultured Kupffer cells[53,54]. Additionally, inhibiting the Kupffer cell activity before induction of AP significantly diminished the associated lung injury with a decrease in NF-κB activity[55]. Altogether, these results show a link between the local inflammatory process in the pancreas and its manifestation by liver cells, especially Kupffer cells, causing the appearance of systemic organ dysfunction secondary to AP.

Alveolar macrophages

Patients with AP may develop acute lung injury, manifest clinically as the adult respiratory distress syndrome (ARDS). Most patients who die during the early stages of SAP die either with or as a result of this lung injury. However, the events that link AP to acute lung injury are not fully understood. It has been postulated that alveolar macrophages (AM) are involved in development of acute local disorders as a consequence of extra pulmonary stimuli like pancreatitis, peritonitis, or trauma. AM have the capacity to secrete a very large number of inflammatory mediators, including lipid mediators, chemokines, cytokines, growth factors and reactive oxygen and nitrogen species. They may therefore play multiple roles in the respiratory tract and may be pro-inflammatory or anti-inflammatory. They may be activated by several stimuli, including cigarette smoke, pro-inflammatory cytokines, endotoxin and immune stimuli. Their capacity to release multiple chemokines leads to the recruitment of several cell types from the circulation, including monocytes, neutrophils and T lymphocytes. Polymorphonuclear neutrophils play an important role in the development of ARDS and activation of the complement system, and generation of AM derived factors promotes neutrophil aggregation into lungs. The capacity of AM to mobilize a large amount of leukocyte and to release secretory products such as RNI and ROI suggests that these cells can be involved in lung damage associated with AP. The activation of AM seems to be regulated by cytokines and inflammatory mediators, which are generated during the course of AP. Sailai *et al*[56] suggested that the inhibition of NF-κB activation may reverse the lung injury of acute necrotizing pancreatitis (ANP) by inhibiting the release of inflammatory mediators by AM. Nitric oxide (NO) is an important marker of oxidative injury in the lung. High TNF-α and NO released by activated AM in ANP suggest a role for AM in inducing lung injury associated with ANP[57]. Studies have suggested that, during the early phase of AP, AM-derived NO contributes to lung injury. Administration of the NOS inhibitor L-NMMA prevented lung injury in this model. In conclusion, this study shows that lung damage induced by experimental AP develops with AM activation. The liver plays an active role in the activation of AM in this experimental model[58]. In addition, neutrophil recruitment into the lungs during AP seems to be mediated by chemotactic mediators (TNF-α and MIP-2) released by activated AM.

In AP, endothelial cells, polymorphonuclear neutrophils and macrophages release PAF, which has been implicated as a key mediator in the progression of AP, leading to complications and unacceptably high mortality rates. PAF participates in the occurrence and development of AP and administration of platelet-activating factor receptor antagonists (PAF-RAs) could significantly reduce local and systemic events after AP[59]. BN52021 (PAF-RA) prevented the labilization of the lysosomal membrane of AM thus suggesting that complications of AP could be dependent on the stabilization of AM lysosomes[60]. Another PAF antagonist (TCV-309) prevented hyperactivity of AM by reducing cytokine induced neutrophil chemoattractant expression by AM[61]. These studies further point to the use of PAF antagonist in reducing the secondary complications associated with AP.

Hsp72 induction is associated with the early stages of

lung neutrophil infiltration. In a study using a 5% sodium taurocholate model of AP it was found that Hsp72-was over expressed in bronchial epithelium, alveolar macrophages, infiltrating neutrophils, blood vessels and blocking of P-selectin activity diminished the expression of Hsp72 in lungs thus suggesting that Hsp72 induction is mediated by neutrophil infiltration into the lungs[62].

Macrophages generate ROI and therefore contribute to the increased oxidative stress. Increased levels of pancreatic phospholipase A2 (PLA2) are detected in the systemic circulation and bronchoalveolar lavage fluid from patients with lung injury in AP. Reports indicate that PLA2 regulates the cytokine production of monocyte/macrophages and the phagocytosis and superoxide (O2-) generation of neutrophils[63]. AMs are activated by PLA2 and produce a large amount of NO that contributes to lung injury in AP. Blocking of PLA2 activity prevented the lung injury associated with pancreatitis[64].

The secretion of multiple inflammatory proteins by AM is largely a result of increased expression of inflammatory genes orchestrated by proinflammatory transcription factors, such as NF-κB and AP-1. Sphingosine-1-phosphate (S1P), a biological active lipid generated by numerous cell types, significantly reduced the NF-κB activation of the AM, ameliorating pulmonary injury[65], thus suggesting that AM could be a therapeutic target of S1P for combating pulmonary injury associated with pancreatitis.

CONCLUSION

Though the role of monocytes/macrophages has long been considered as evidence for a host response against the inflammatory process, recent findings suggest that monocytes/macrophages are active players in the progression of AP. Over the past few years understanding of molecular mechanisms underlying the progression of AP has improved considerably. A better understanding of regulation and function of different macrophage populations involved in AP will help to establish more therapeutically effective novel therapies for AP management. Strategies targeting molecules (NF-κB and STAT-3) and mechanisms supporting M2 type macrophages will further help in restraining the inflammatory process.

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FRONTIER

Lymphangiogenesis: A new player in cancer progression

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Abstract

Lymph node metastasis is the hallmark of colon cancer progression, and is considered one of the most important prognostic factors. Recently, there has been growing evidence that tumor lymphangiogenesis (formation of new lymphatic vessels) plays an important role in this process. Here, we review the latest findings of the role of lymphangiogenesis in colorectal cancer progression, and discuss its clinical application as a biomarker and target for new therapy. Understanding the molecular pathways that regulate lymphangiogenesis is mandatory to pave the way for the development of new therapies for cancer. In the future, tailored treatments consisting of combinations of chemotherapy, other targeted therapies, and anti-lymphangiogenesis agents will hopefully improve patient outcomes. This progression to the clinic must be guided by new avenues of research, such as the identification of biomarkers that predict response to treatment.

Key words: Colorectal neoplasms; Angiogenesis; Lymphangiogenesis; Lymphatic vessels; Lymphatic metastasis; Vascular endothelial growth factor; Monoclonal antibody D2-40; Therapy-related neoplasms; Biomarkers

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INTRODUCTION

Colorectal cancer (CRC) is the third most common type of cancer to develop and to cause death in the United States[1]. Although the pattern of spread of CRC may vary, the initial step involves lymphatic invasion and metastasis to regional lymph nodes[2]. Patients with lymphatic invasion have a less favorable outcome, and lymph node metastasis is one of the most important prognostic factors in CRC[3]. In fact, both the Dukes and TNM staging systems, which have been the most widely used staging systems for CRC, are based on the assessment of lymph node metastasis in addition to the extent of primary tumor and distant metastatic disease[4,5]. Patients with an early stage tumor without evidence of lymph node metastasis (Dukes A, TNM stage Ⅰ) have an excellent post-operative prognosis and a 5-year survival rate of 80%-90%, while patients with advanced tumors with regional lymph node disease (Dukes C, TNM stage Ⅲ) have a 5-year rate of 25%-60%. Furthermore, patients with distant metastatic disease (Dukes D, TNM stage Ⅳ) have a 5-year rate of less than 10%[6-8]. In addition, the number of nodes with metastatic disease has an important impact on the prognosis of patients with CRC. In fact, the influence of lymph node metastatic

disease on prognosis in CRC is so great that there is not only a difference between N1 and N2 (1 to 3 nodes *vs* 4 or more nodes), but based upon separate analyses by the Surveillance Epidemiology and End Results program, there is also a significant prognostic difference within each of these groups. Accordingly, N1 is subdivided into N1a (1 node involvement) and N1b (2-3 node involvement), and N2 is subdivided into N2a (4 to 6 node involvement) and N2b (7 or more). This new TNM staging system lymph node sub-categorization is based on survival[8], which is in further agreement with the importance of lymph node metastatic disease in CRC patient outcome. Moreover, detailed analysis of lymph node status allows for accurate staging, which is now shown to be associated with better outcomes[8].

While local or regional CRC can be controlled with complete surgical resection, combination therapy is required to treat systemic disease. Among patients with newly diagnosed CRC, 25% will first present with metastatic disease[9]. Even among patients who present with localized, resectable disease, 30% will have a recurrence with metastatic disease[9]. There has been remarkable progress in the treatment of metastatic CRC during the last decade in the fields of surgery, radiation, chemotherapy, and targeted therapy[9-11]. Over the last decade, a better understanding of the processes involved in tumorigenesis and cancer metastasis has led to the development of a new category of systemic drugs called targeted therapies. The term targeted therapy refers to drugs that selectively target specific molecular pathways involved in tumorigenesis and/or tumor metastatic progression[11]. In CRC, two targets have been intensively investigated and are currently under Phase Ⅲ clinical trials: the vascular endothelial growth factor (VEGF) pathway that controls angiogenesis, the phenomenon where new blood vessels are formed to feed the enlarging tumor and develop access to the blood stream; and the epidermal growth factor pathway that controls cell survival and proliferation. The former is targeted by the anti-VEGF monoclonal antibody, bevacizumab (Avastin™), and the latter is targeted by the anti-EGFR monoclonal antibodies, cetuximab or panitumumab[9,12]. Recently, there has been growing evidence that not only angiogenesis, but also lymphangiogenesis, the formation of new lymphatic vessels, is important in CRC metastatic progression[2,13-23]. This article will review the latest reports on lymphangiogenesis not only in experimental models, but also in clinical studies, and also review its clinical application as a biomarker and as a new targeted therapy.

WHY IS ANGIOGENESIS IMPORTANT FOR CANCER PROGRESSION AND METASTASIS?

In order for cancer to progress and metastasize, the primary tumor must have access to the systemic circulation, either through blood or lymphatic vessels. Angiogenesis is defined as the process whereby new blood vessels are formed from existing vessels, and as such, is a natural physiological process. Under normal physiologic conditions, angiogenesis only occurs in adults during menstruation, gestation and wound healing. At other times, anti-angiogenic factors maintain the endothelial cells that form blood vessels in a quiescent state[24]. The theory that angiogenesis could support tumor metastatic progression and therefore be a target for cancer therapy was proposed by Folkman *et al*[25,26] in the 1970s. He hypothesized that cancer requires angiogenesis to "feed" the cancer enabling it to grow beyond a certain size, and to allow for systemic spread. After 2 decades of developing this theory, modern molecular and cell biology techniques verified the role of angiogenesis in cancer growth *via* animal tumor models and clinical trials of bevacizumab, a humanized monoclonal antibody that neutralizes VEGF[27,28].

Tumors secrete multiple angiogenic factors and/or down-regulate angiogenesis inhibitors to induce tumor angiogenesis. VEGF-A is one of the key factors responsible for stimulation and maintenance of the disorganized, leaky, and torturous tumor vasculature. Other factors include members of the platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor, angiopoietin, and hepatocyte growth factor (HGF) families[29]. Conversely, blockade of VEGF function inhibits angiogenesis and suppresses tumor growth *in vivo*[30]. While the discovery of factors important for angiogenesis has not yet led to a new cure for cancer, understanding that this process is essential for tumor metastasis has revealed several possibilities for targeted therapy. Applying this research approach to lymphangiogenesis can produce new potential targeted therapies.

WHY IS LYMPHANGIOGENESIS IMPORTANT FOR CANCER PROGRESSION AND METASTASIS?

The importance of lymph node metastasis in the progression of CRC has been well established and has a great impact on prognosis[8]. For cancer metastasis to the lymph node to occur, the cancer cells must access the lymphatic vessels to reach the regional lymph nodes. Applying approaches similar to the ones used to understand angiogenesis is expected to identify molecular mechanisms that control the processes related to lymphangiogenesis[31]. Therefore, understanding how lymphatic vessels are formed under physiologic and non-cancerous pathologic conditions can help provide an understanding of lymphangiogenesis in cancer in order to provide new avenues for targeted therapy development.

Although the ancient Greeks had already described aspects of the lymphatic system, the lymphatic vasculature was only properly considered to be a distinct circulatory system in 1622 by Asselli[32]. The lymphatic vasculature forms a vessel network that drains interstitial fluid from tissues and returns it to the blood circulation *via* the thoracic duct. Lymphatic vessels are also known to be an essential part of the body's immune defense. Descrip-

tions of the metastatic spread of cancer can be found as far back as the 14th century[33], and the involvement of the lymphatic system in metastatic progression has been described since the 18th century[34]. The traditional theory was that tumor cells metastasized to lymph nodes by utilizing pre-existing lymphatic vessels, and that lymphatic vessel entry occurred by permeation or embolization, not through the creation of new lymphatic vessels in response to cancer. Although the regeneration of lymphatic vessels was observed by Clark and Clark in 1932, cancer metastasis and the concept of lymphangiogenesis were not linked until the last two decades[35]. Despite an acceptance for centuries of the important role of the lymphatic system as the primary pathway for the metastatic spread of tumor cells to regional lymph nodes, and possibly even also to distant organs, the exact mechanism of this process has remained unclear until recently[36].

Over the past few years, understanding of the cellular and molecular aspects of physiologic lymphangiogenesis and tumor-induced lymphangiogenesis has advanced after the discovery of VEGF-C and its function to promote the growth of lymphatic vessels[37]. Initially, the study of lymphangiogenesis largely focused on the primary site of tumor growth and adjacent tissues, which is known as "tumoral lymphangiogenesis"[38,39]. However, lymphangiogenesis was also observed around regional lymph nodes, in particular the sentinel nodes where tumor cells first metastasize, a phenomenon now known as "lymph node lymphangiogenesis"[6,40]. Lymph node lymphangiogenesis and increased lymph flow through tumor-draining lymph nodes are speculated to actively promote metastasis *via* the lymphatics[41]. Recent evidence indicates that tumor cells can also induce lymph node lymphangiogenesis - even before they metastasize - and that metastatic tumor cells continue to induce lymphatic vessel growth within sentinel lymph nodes, theoretically promoting their further metastatic dissemination[42,43].

As expected, the majority of studies point to a positive correlation between tumor-induced lymphangiogenesis and lymphatic metastasis[6,13-17,44,45]. Because the physiologic role of the lymphatic system is to collect interstitial fluid from peripheral tissues and return it to the systemic blood circulation, it is hypothesized that tumor-induced lymphangiogenesis occurs in order to drain interstitial fluid away from the tumor. Therefore, targeting this process provides a potential avenue for cancer therapy[46]. In fact, experimental inhibition of this process in animal models suggested that lymphangiogenic growth factors facilitate the metastatic spread of tumor cells *via* the lymphatics[14,18-20]. The results highlight the key role that lymphangiogenic growth factors and new lymphatic vessels play in tumor metastatic progression. These early studies indicate that targeting lymphangiogenic growth factors in tumors could be a strategy for restricting the metastatic spread of cancer[31].

IN VIVO AND *IN VITRO* MODELS OF LYMPHANGIOGENESIS

The recent discovery of the key lymphangiogenic factors

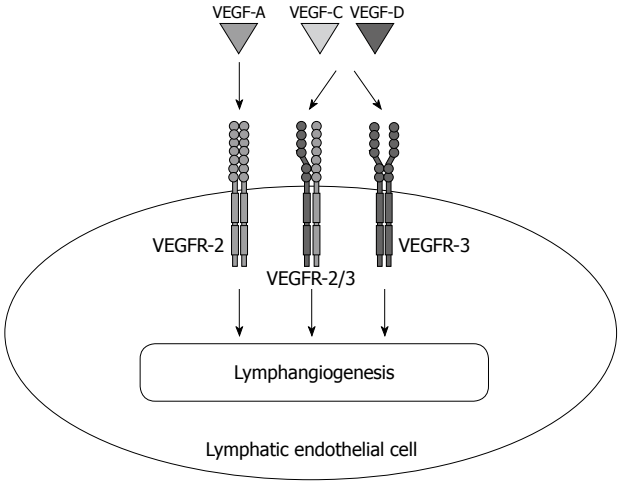

Figure 1 Lymphangiogenic growth factors and their receptors expressed by lymphatic endothelium. Vascular endothelial growth factor (VEGF) receptor (VEGFR)-3 is a member of the fms-like tyrosine kinase family and specifically binds VEGF-C and VEGF-D, but not VEGF-A. Recent studies also indicate an important role for the VEGF-A/VEGFR-2 signaling pathway in lymphangiogenesis.

VEGF-C and VEGF-D, other proteins related to these factors, and their receptor VEGF receptor (VEGFR)-3, have provided novel insights into how the lymphatic vessels and blood vessels coordinately grow and affect human disease[47]. In fact, these factors are associated with a number of human tumor types[31]. These secreted glycoproteins largely signal *via* the cell surface tyrosine kinase receptor VEGFR-3/Flt4 present on the surface of lymphatic endothelial cells (LECs), and VEGFR-3 activation promotes LEC proliferation, migration, and survival, which result in lymphatic vessel proliferation *in vitro* and *in vivo*[6]. Furthermore, recent studies indicate that the VEGF-A/VEGFR-2 signaling pathway plays a major role not only in angiogenesis, but also in lymphangiogenesis[48,49] (Figure 1).

In vitro techniques to study lymphangiogenesis have evolved with the development of methods to isolate and culture LECs. LECs have been isolated from lymphatic vessels or skin followed by enzymatic digestion and flow cytometric cell sorting using markers specific to LECs. Several LEC markers have been recently identified, including: lymphatic vessel endothelial hyaluronan receptor-1[50]; glomerular podocyte membrane mucoprotein, podoplanin (D2-40)[51]; the homeobox gene product, Prox-1[52,53]; and VEGFR-3[54,55]. Although these markers have aided in the purification of LECs, the limited quantity of cells obtained and the reduced growth potential of these cells have posed a challenge. To address the challenge of only having a limited quantity of cells after the purification of LECs, immortalization with SV40 large T antigen[56] or transformation with human telomerase reverse transcriptase[57] have been utilized to extend the life span of LECs, and transgenic mice have been developed to harvest immortalized LECs[58]. Protocols for the isolation of LECs from microlymphatic vessels in different tissues in rats have recently been established[59,60]. In most experimental assays, LECs are seeded as monolayers on culture plates or onto the surface of matrix-coated plates. While 2-D cul-

tures cannot undergo all of the steps of lymphatic vessel formation, these culture systems allow the analysis of each step individually, using various assays of cell activity (e.g. gene expression profiling), cell proliferation, apoptosis, adhesion, migration (wound scratch assay, Boyden chamber assay), and morphogenesis (tubulogenesis)[61].

Numerous *in vivo* models to study the growth of lymphatic vessels have utilized the same techniques as those used for blood vessel growth. The growth of vessels into the avascular cornea in response to specific factors or inflammation has been historically utilized as a model to study lymphangiogenesis[62-64]. Another extensively used model is the development of lymphedema and lymphangiomas. Lymphedema is swelling due to the failure of fluid drainage by the lymphatics which occurs as a result of obstruction or secondary changes impairing lymph flow. Several mouse models carrying mutations or chromosomal aberrations recapitulate this phenotype[65,66], and surgical ablation of lymphatic vessels can induce lymphedema and subsequently lymphangiogenesis[67,68]. Lymphangiomas, characterized as benign malformations of the lymphatic system, have been induced by injection of incomplete Freund's adjuvant, either into the mouse ear[69] or intraperitoneally[70], causing lymphatic vessel hyperplasia leading to inflammation and lymphangiogenesis[71].

Breast, gastric or CRC cells over-expressing VEGF-C implanted into transgenic mice induced tumor-associated lymphangiogenesis in orthotopic mouse models[13,72,73]. Skin carcinogenesis models in transgenic mice over-expressing VEGF-A or C showed that tumors in these mice were significantly more likely to metastasize[40,74]. As compared to VEGF-A, VEGF-C did not increase the size of the primary tumors, but induced the expansion of metastatic networks within the lymph nodes and promoted metastasis to distant sites such as distant lymph nodes and the lungs[74]. In addition to VEGF, PDGF-BB, FGF-2, HGF and angiopoietin enhance lymphangiogenesis[75-79]. Sphingosine-1-phosphate (S1P) also stimulates lymphangiogenesis in both *in vitro* and *in vivo* models[80,81]. S1P is generated by the action of two sphingosine kinases, sphingosine kinase 1 and 2[82,83]. Tumor cells, which are characterized by high levels of sphingosine kinase 1 expression, can release S1P into the extracellular space[84], which in turn can lead to paracrine-induced angiogenesis and lymphangiogenesis[81]. Interestingly, LEC-specific deletion of sphingosine kinase 1 in the sphingosine kinase 2 knockout mouse inhibits lymphatic vessel maturation[85].

In vivo models demonstrate that lymphangiogenesis promotes CRC metastasis, suggesting new avenues for the development of targeted therapy and prognostic markers. VEGF-C and VEGF-D, which are up-regulated in CRC, appear to drive tumor lymphangiogenesis through the VEGF-C/VEGF-D/VEGFR-3 pathway, while other growth factors, such as VEGF-A, have modulatory effects on this process[48,49]. VEGF-A expression levels significantly correlate with metastasis to the lymph nodes in CRC[48,49]. Orthotopic implantation of VEGF-C over-expressing DLDA colon cancer cells demonstrated that VEGF-C induced lymphangiogenesis-mediated tumor spread and the formation of metastatic disease in the lymph nodes[13], while the inhibition of VEGF-C expression reduced lymphangiogenesis, the extent of lymph node metastatic disease, and enhanced survival in mice[14]. The inhibition of VEGF-C expression also dramatically suppresses tumor lymphangiogenesis, tumor growth, and regional lymph node metastasis in mice[18]. Inhibition of VEGFR-3 using small interfering RNA also significantly inhibited tumor growth[19,20]. These *in vitro* and *in vivo* mouse data demonstrate the possible clinical application of lymphangiogenesis as a biomarker and/or as a new target for therapy in CRC in humans.

LYMPHANGIOGENESIS IN HUMAN SAMPLES

The role of intra- and peri-tumoral lymphatics in tumor biology and the initial steps of lymphatic metastatic progression, i.e. the invasion of tumor cells into the lymphatic vessels, are just beginning to be elucidated in human samples[44]. Animal studies have demonstrated that intra-tumoral lymphatic vessels are poorly functional due to high intra-tumoral pressure and may not be required for lymphatic metastatic progression. Conversely, lymphatic vessels in the tumor periphery are functional and can drain colloids from the tumor. In several common human tumors, such as cutaneous melanoma[86,87], head and neck squamous cell carcinoma[38,88], transitional cell carcinoma of the bladder[89,90] and non-small cell lung cancer[91], tumoral lymphangiogenesis detected by lymphatic vessel density (LVD) can be readily appreciated and has been shown to be of prognostic significance. In contrast, in breast[92,93], cervical and prostate carcinoma[94] tumors that metastasize to the lymph nodes, there is little evidence of significant tumoral lymphangiogenesis detected by LVD, with most proliferating vessels lying within the peritumoral tissues[6]. Another important factor to consider is the location of the tumor in relationship to the amount of pre-existing lymphatic vessels, such as in biliary cancer, which is very prone to metastasize *via* the lymphatic system[95,96]. Furthermore, mouse models of cecal cancer metastasis to the liver have demonstrated that both VEGF-C and VEGF-D produced less metastatic disease in the liver compared to primary cecal tumors, suggesting the importance of the tumor microenvironment for the production of these lymphangiogenic factors[97]. Taken together, the pattern of tumoral lymphangiogenesis and metastasis to the lymph node varies between tumor histological type and anatomic location of the tumor, involving both the lymphatic system and the microenvironment. Clearly, further studies are awaited to understand this complex process.

Although several studies have reported the discrepancy between LVD measurements and clinical outcome, it should be noted that there is a great deal of variability in their methodologies and consequently also in their results. In addition to tumor characteristics, the discrepancies in terms of the correlation of LVD with metastasis

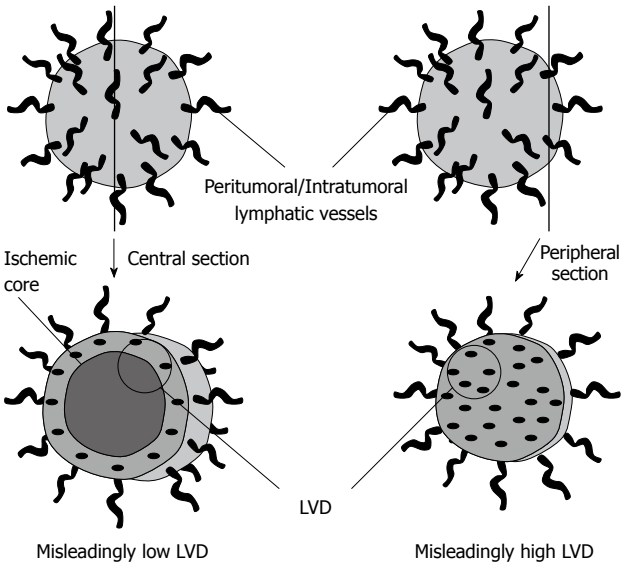

Figure 2 Limitations of lymphatic vessel density estimations. The central section of a tumor with a necrotic central core may estimate a misleadingly low lymphatic vessel density (LVD) (left), while the peripheral section of the same tumor may estimate a misleadingly high LVD (right).

to the lymph nodes and prognosis in these studies can be attributed to the limitations of the methodologies used. The limitations include the different types of tissue preservation, variable immunostaining techniques, different LVD quantification methods employed, and the lack of standardization in the estimation of lymphangiogenesis[98] (Figure 2). To address the limitations of this qualitative analysis methodology, some studies have attempted to quantify the expression of VEGF-C and VEGF-D mRNA or protein in excised primary tumor tissue of patients with various cancers. They frequently report that the levels of these molecules exhibit a strong correlation with parameters associated with poor patient outcome, such as the invasion of lymphatic vessels by tumor cells, the extent of lymph node metastatic disease, and disease-free as well as overall survival[31]. However, even these attempts to quantifiably measure lymphangiogenesis have not been entirely successful. In fact, recent epigenetic studies demonstrated that the analysis of mRNA or protein expression may not reflect actual lymphangiogenesis due to posttranscriptional modifications of proteins. In order to adequately assess the degree of lymphangiogenesis, a better method to accurately quantify the amount of lymphangiogenesis is needed.

LYMPHANGIOGENESIS AS A BIOMARKER FOR CRC PROGRESSION

Although animal models show a strong relationship between lymphangiogenesis and lymph node metastasis and survival, the clinical significance of lymphangiogenesis in CRC remains uncertain, as is the case for other tumors[6]. Parr *et al*[21] showed that the expression of VEGFR-3 receptor, prox-1, 5'-nucleotidase expression, and podoplanin expression in cancer tissue were significantly higher than in the normal background tissue. Jia *et al*[16] showed that the extent of lymph node metastatic disease in VEGF-C-positive patients (81.1%) was significantly higher than that in the negative group (42.1%). Lu *et al*[17] showed that quantitative analysis of podoplanin in CRC specimens correlates with metastasis to regional lymph nodes. Yan *et al*[22] showed that the co-accounting of LVD and microvessel density (MVD) was an independent prognostic factor in CRC. Moehler *et al*[15] showed that the expression of VEGF-D is significantly associated with lymphatic involvement in CRC patients and that cetuximab can block such expression effectively. In addition, the quantification of VEGF-C and VEGF-D in blood samples has the potential to serve as a biomarker to predict the extent of lymph node metastatic disease[23]. Interestingly, Sundlisaeter *et al*[99] showed that LVD was significantly increased in tumor tissue compared with the normal mucosa, but there were no changes in LVD between stage Ⅱ and Ⅲ CRC. This indicates that lymphangiogenesis occurs in CRC, and indeed suggests that it is triggered at an early stage of tumor development. Taken together, these studies suggest that these lymphangiogenesis-related markers indicate an increase in lymphangiogenesis in CRC, and might therefore have prognostic value for CRC patients.

However, other reports failed to find an association between higher LVD, the aggressiveness of tumor behavior and poorer clinicopathological variables. Kazama *et al*[100] revealed that the expression of VEGF-C was significantly correlated with lymphatic involvement, lymph node metastatic disease and tumor size, but not with venous involvement, metastasis to the liver in invasive carcinomas, or overall survival. Miyazaki *et al*[101] showed that an elevated level of plasma VEGF-C correlated with deeper invasion, and more severe venous and lymphatic invasion of the primary tumor, although there was no significant difference in the plasma level between patients with CRC and the healthy controls. Gao *et al*[98] showed that MVD and LVD were higher in the tumor compared with the corresponding normal mucosa, but they were not related to clinicopathological variables and overall survival. However, it should be noted that these studies rely on qualitative analysis methodologies, which are not objectively quantifiable assays. Duff *et al*[102] showed that the balance between the expression of VEGF-C and VEGF-D at the invading tumor edge may enhance lymphatic metastasis by promoting tumor lymphangiogenesis or by activating pre-existing lymphatic vessels. However, no relationship was identified between LVD and clinicopathological variables. Again, it should be noted that these reports rely on qualitative analysis methodologies, which are not objectively quantifiable assays. Taken together, lymphangiogenesis occurs in CRC development, but it has not been clearly linked to CRC patient prognosis. The conflicting reports in the literature regarding the possible correlation of LVD with clinical factors can be attributed to the use of qualitative analysis methodologies. Therefore, the development of a new quantifiable assay that uses standardized metrics is necessary.

In the future, the intra-tumoral expression of specific molecules, e.g. deleted in CRC[103,104] or 18q loss of heterozygosity[104], DNA microsatellite instability[103-105], KRAS mutation[103-105], or thymidylate synthase[103,104] could become biomarkers to predict prognosis or the response to therapy, independently of TNM stage group or histologic grade. It is now clear that there is an interaction between the T and N designations that is likely to rely on the expression of specific molecules within the cancer. In the latest edition of their cancer staging manual, the American Joint Committee on Cancer has stated that they will add molecular profiling information to the TNM classification to enhance the prediction of prognosis and/or even response to therapy[8]. Because lymphatic invasion and metastasis to the lymph nodes have a great impact on patient prognosis, lymphangiogenesis-related molecules are good candidates for the biomarkers that will be included in future editions of the TNM staging system.

LYMPHANGIOGENESIS AS A NEW THERAPEUTIC TARGET FOR CRC

Based upon the importance of angiogenesis and lymphangiogenesis in cancer progression, specific antibodies against angiogenic factors have been developed. The humanized VEGF antibody, known as bevacizumab (Avastin™), has been approved by the United States Food and Drug Administration (FDA) for treating metastatic carcinoma of the colon or rectum, and recurrent or metastatic non-squamous non-small cell lung cancer. Recently, bevacizumab also received accelerated FDA approval for the treatment of metastatic HER2-negative breast cancer[31]. However, the addition of bevacizumab to chemotherapy as adjuvant therapy in CRC did not improve disease-free survival[106]. Bevacizumab is being tested in other clinical settings such as adjuvant therapy, maintenance therapy, and in combination with both cytotoxic chemotherapy and other targeted agents, such as the epidermal growth factor receptor kinase inhibitor, erlotinib[106]. In addition to bevacizumab, other antibody-based therapies targeting the VEGF pathway are being tested. Ramucirumab and IMC-18F1 are monoclonal antibodies that target the VEGF receptors VEGFR-2 and VEGFR-1, respectively.

In addition to anti-angiogenesis therapies, many clinical trials in cancer patients are underway or have been completed with inhibitors that have the potential to suppress tumor-induced lymphangiogenesis. However, analysis of the effects of these treatments on tumor lymphatics is not always explicitly mentioned in the trial descriptions listed by the U.S. National Institutes of Health. There is only one study that has mentioned the role of VEGF-C in tumor progression, with VEGFR-3 being considered a target. This study is a Phase Ⅱ trial of sunitinib for patients with chemo-refractory metastatic gastric cancer[34]. It is hoped that more clinical trials will consistently address the possible effects of novel cancer therapeutics on tumor-induced lymphangiogenesis so that correlative data regarding the possible effects of interfering with tumor lymphatics on patient survival can be generated[34]. It is also important to note that treatment with the VEGFR tyrosine-kinase inhibitors sunitinib and sorafenib is associated with a significant increase in the risk of bleeding[107]. Further assessments need to be performed for treatment with these inhibitors.

Inhibition of metastatic spread may be achieved by restriction of lymphatic vessel growth by using targeted therapeutic strategies against molecules involved in lymphangiogenic signaling, in addition to the inhibition of angiogenesis. Because VEGF-A has been shown to promote tumor lymphangiogenesis, and because VEGF-C and VEGF-D are also able to activate VEGFR-2, the combined inhibition of VEGFR-2 and VEGFR-3, or of VEGF-A and VEGF-C/D, may result in an even more potent blockade of tumor-induced lymphatic vessel growth. Indeed, a combination of both anti-VEGFR-2 and anti-VEGFR-3 blocking antibodies has been shown to be more efficient in reducing experimental lymph node and distant breast cancer metastatic disease than each antibody alone, and it will be of interest to see whether a recently developed biospecific antibody against VEGFR-2 and VEGFR-3 will also show enhanced activity *in vivo*[42]. On the other hand, several recent trials have shown that the addition of anti-EGFR monoclonal antibodies to bevacizumab and chemotherapy resulted in worse outcomes. This was surprising, given that preclinical and early clinical studies had suggested a benefit in combining anti-VEGF and anti-EGFR antibodies. Taken together, further clinical trials are required to reveal the efficacy of the combination of targeted therapies against lymphangiogenesis with other targeted therapies, and/or other anti-cancer therapies.

Which patients will benefit from anti-angiogenesis and anti-lymphangiogenic therapies? Considering that tumors appear to undergo angiogenesis and lymphangiogenesis at an early stage[99], the anti-lymphangiogenic effect may have an even greater impact on the micrometastatic and/or the in-transit metastatic disease of concern after the resection of early stage malignancies. Therefore, these anti-angiogenesis and anti-lymphangiogenesis therapies may be more effective in patients with early stage CRC. However, the role of adjuvant therapy in stage Ⅱ CRC is still controversial[108]. Although there is a cohort of stage Ⅱ CRC patients who will have recurrent disease even after complete resection, there are no markers to identify this cohort. This subgroup appears to be a good candidate for anti-angiogenesis and anti-lymphangiogenesis therapies, if they could be identified with the appropriate biomarkers. Lymphangiogenesis factors have the potential to be used as biomarkers to predict which patients would benefit from adjuvant therapy with anti-lymphangiogenesis therapies to both prevent recurrence and improve overall survival. In the future, tailored treatments consisting of combinations of chemotherapy, other targeted therapies, and anti-angiogenesis and anti-lymphangiogenesis agents will hopefully result in better patient outcomes.

In addition to the development of the ideal combina-

tion therapies, the prevention of CRC is also essential to improve patient outcomes. Cancer chemoprevention is a strategy that uses treatments with natural or synthetic agents to inhibit, delay, or reverse the carcinogenesis process even before the development of invasive cancer[109,110]. The rationale for chemopreventive approaches to prevent CRC comes from epidemiologic and observational studies indicating that the long term ingestion of aspirin may reduce mortality in CRC[111]. Recent clinical trial studies demonstrated that celecoxib, a selective COX-2 inhibitor, is equally effective in reducing colorectal adenomas in animal models and patients with familial adenomatous polyposis (FAP), and it is approved by FDA for the chemoprevention of CRC in patients with FAP[112]. Prostaglandin E2 (PGE2) induced by COX-2 exerts several biological properties that may be advantageous for carcinogenesis, including promoting angiogenesis with increased VEGF, bFGF, and PDGF production[112]. Celecoxib enhances tumor cell apoptosis, thereby inhibiting the growth and angiogenesis of tumors by inhibiting COX-2, PGE2 synthesis, and VEGF expression in tumors in a mouse model of human CRC[113]. Interestingly, VEGF-C and COX-2 are coexpressed and are significantly associated with metastasis to the lymph nodes as well as prognosis in human CRC[114]. Moreover, celecoxib inhibits not only angiogenesis, but also lymphangiogenesis by blocking the VEGF pathway in mouse lung cancer models[115,116]. Taken together, lymphangiogenesis appears to play an important part in carcinogenesis in connection with the COX-2 pathway, and to be one of the important targets in chemoprevention, although the role of lymphangiogenesis in CRC within the adenoma-carcinoma sequence is still unknown. Further investigation will be required in this field.

CONCLUSION

Understanding the molecular pathways that regulate lymphangiogenesis is mandatory to pave the way for the development of new targeted therapies for cancer patients. A new quantifiable assay using standardized metrics is required to measure lymphangiogenesis and evaluate its impact on clinical outcome. In the future, tailored treatments consisting of combinations of chemotherapy, other targeted therapies, and anti-lymphangiogenesis agents will hopefully improve patient outcomes. This progression to the clinic may be guided by new avenues of research such as the identification of biomarkers that predict response to treatment.

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TOPIC HIGHLIGHT

Marco Scarpa, Dr., PhD, *Series Editor*

Health related quality of life after surgery for colonic diverticular disease

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Abstract

Diverticular disease (DD) of the colon is very common in developed countries and is ranked the fifth most important gastrointestinal disease worldwide. The management of acute diverticulitis without perforation and peritonitis is still debated. Health related quality of life (HRQL), subjectively perceived by patients, is becoming a major issue in the evaluation of any therapeutic intervention, mainly in patients with chronic disease. To date only a few published studies can be found on Medline examining HRQL in patients with DD. The aim of this study was to review the impact of surgery for DD on HRQL. All Medline articles regarding HRQL after surgery for colonic DD, particularly those comparing different surgical approaches, were reviewed. DD has a negative impact on HRQL with lower scores in bowel function and systemic symptoms. Both surgery-related complications and disease activity have a significant impact on patients' HRQL. While no significant differences in HRQL between different operations for DD in non-randomized studies were revealed, the only prospective double-blind randomized study that compared laparoscopic and open colectomy found that patients undergoing laparoscopic colectomy had significantly reduced major postoperative complication rates and subsequently had better HRQL scores. Formal assessment of HRQL could be a good instrument in the selection of appropriate patients for elective surgery as well as in the assessment of surgical outcome.

Key words: Colonic diverticular disease; Health related quality of life; Laparoscopy

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INTRODUCTION

Diverticular disease (DD) of the colon is very common in developed countries and is ranked the fifth most important gastrointestinal disease worldwide. It is probably even more common in the Western world with a prevalence of approximately 33% in persons over 60 years[1-3] placing a substantial burden on inpatient and outpatient resources[2]. In

fact, 10%-25% of these patients will suffer an acute attack with a further 30% developing complicated DD[3,4]. The frequency of perforation associated with DD is increasing, indeed it reached a prevalence of almost 4 cases per 100 000 in 2000[4]. Colonic resection is standard practice in cases of perforation and peritonitis in DD and primary anastomosis with defunctioning stoma seems to be the optimal surgical strategy for fit patients, while Hartmann's procedure is recommended in high risk patients[5].

The management of acute diverticulitis without perforation and peritonitis is still debated. In these cases, medical therapy is usually successful[1,6], but up to 25% of these patients may end up requiring an urgent operation[7], and more than half of these procedures involve a colostomy[8-10]. Elective colectomy is, thus, often recommended to avoid the risks and high mortality rate connected with emergency surgery usually associated with recurrent diverticulitis. The timing of elective surgery is, nevertheless, controversial with most advisory bodies recommending surgery after the second episode[2,11]. Many surgeons advise prophylactic colon resection after a single hospitalization in younger patients because the disease is considered more virulent in these subjects[11-15].

The evolution of laparoscopic colorectal surgery in the past decade has brought immediate short-term benefits to patients, including earlier postoperative pain relief and return of bowel function, shorter hospital stay, and better cosmesis. There is some evidence in the recent literature that immediate postoperative health related quality of life (HRQL) is better after certain laparoscopic procedures than after open surgery[16].

In the case of acute complications such as perforation with peritonitis in an emergency setting, the surgical approach can be in one stage or multiple stages and quality of life assessment is increasingly being recognized as an integral factor in surgical decision-making regarding disease management choices[17].

However, the results of one of our previous studies did not show any long-term advantage in terms of quality of life, symptoms recurrence and risk of surgery in submitting patients to colonic resection for DD without perforation and peritonitis[18]. The decision to electively operate on patients after their recovery from an acute episode of diverticulitis is still debated.

Although the use of objective outcome measures after surgical procedures is an important means of defining a patient's degree of health, the patient's subjective perceptions and expectations need to be factored into that objective assessment to determine the patient's actual quality of life, particularly in the treatment of benign disease such as DD. Moreover, HRQL measures have been shown to be useful in predicting health care expenditures. With the development of well-validated reliable and sensitive non-disease specific (generic) questionnaires such as the Short Form survey 36 (SF-36) or disease specific questionnaires like the gastrointestinal quality of life (GIQLI)[19-26], there is a HRQL measuring tool than can be applied to post-operative quality of life research not only to define the long-term outcome in elective operations, but may also be useful in comparing different surgical approaches and techniques. The aim of this study was to review the impact of surgery for DD on HRQL.

A text word literature review was performed using the PubMed and Medline databases. Although this was not a systematic review, the search terms used were as follows: DD, acute diverticulitis, surgery, elective OR emergency resection OR surgery AND HRQL. The reference lists of identified articles were searched for further relevant publications. Aided by a clinical librarian, the databases were consulted from January 1965 to April 2010. Two researchers (Angriman I and Ruffolo C) independently selected the studies, particularly those comparing different surgical approaches. Whenever there was discordance regarding study inclusion the two researchers negotiated an agreement.

DD HAS AN IMPACT ON QUALITY OF LIFE

In the majority of patients, colonic diverticula (diverticulosis) are asymptomatic[27]; nevertheless, an estimated 20% of affected individuals develop symptoms in their lifetime, such as abdominal pain and/or discomfort, bloating and disturbance of bowel habits. This clinical condition is termed DD[28] and may be symptomatic uncomplicated, recurrent symptomatic, or complicated. DD treatment is aimed at relieving symptoms and preventing major complications. HRQL, subjectively perceived by patients, is becoming a major issue in the evaluation of any therapeutic intervention, mainly in patients with chronic disease where the aim of therapy is to keep patients either symptom-free or to reduce the discomfort caused by the disease. To date only a few published studies can be found on Medline examining HRQL in patients with DD.

Bolster *et al*[29] suggested that DD does affect a person's HRQL. In their study they used a disease specific questionnaire which had been validated for patients with inflammatory bowel disease and consisted of 32 questions which assessed four aspects of patients' lives: gastrointestinal symptoms, systemic symptoms, emotional function and social function. Patients with DD had mean scores well below the optimal scores of the questionnaire in all four categories, and compared with a control group of healthy volunteers, patients with DD had statistically significant lower scores in all categories. The authors concluded that DD has a negative impact on HRQL.

Similar results were observed by Comparato *et al*[30] in 58 patients affected by uncomplicated symptomatic DD. They used the SF-36 questionnaire and clinical evaluation was registered by means of global symptomatic score at baseline and after 6 mo. They concluded that DD has a negative impact on HRQL compared with the normal population and medical therapy improves HRQL if symptoms are relieved.

QUALITY OF LIFE AFTER SURGERY FOR DD

Surgical resection for DD, outside of the emergency setting, is primarily intended to obviate future hospitalization and/or emergency surgery for the patient, even though the risk of any single individual suffering a subsequent acute exacerbation is unpredictable. In contrast, symptomatic DD itself causes considerable ongoing disruption in terms of lifestyle and general "well-being". However, little formal weight is currently given to quality of life consideration *per se*. This is probably because it remains unclear whether surgery can restore the missing HRQL, but outcome may instead represent the most compelling reason to offer surgery to an individual.

Several studies observed a significant improvement in quality of life and social function following elective sigmoid resection in the majority of patients.

Most of those studies compared the HRQL before and after surgery. Forgione *et al*[16] performed a prospective analysis of 46 patients undergoing elective, laparoscopic sigmoidectomy for prior acute diverticulitis demonstrated by CT scans over an 18-mo period. Preoperative measures of HRQL were assessed by the GIQLI questionnaire administered at baseline and then again regularly throughout the first postoperative year. Mean preoperative GIQLI score was 99.5 and postoperative scores were significantly higher at each postoperative time point. The mean GIQLI score 12 mo after operation was 111.5 ($P < 0.05$). Postoperative augmentation of GIQLI was correlated most with improvements in the symptoms domain and was inversely correlated with the preoperative score. The authors concluded that the development of a more disease-specific questionnaire for patients being considered for elective surgery after prior diverticulitis may allow even greater discrimination in preoperative selection.

Roblick *et al*[31] observed a significantly higher HRQL after surgery in patients suffering from DD and only slightly below a validated normal population. In this study, a total of 45 patients who underwent surgery for diverticulitis at stage Ⅰ-Ⅱa (Hinchey classification) were included. HRQL was measured using the SF-36 questionnaire and the follow-up period was at least 2 years.

In one of our previous studies[18], to evaluate the impact of colonic resection for DD on the natural history and long-term quality of life in these patients, HRQL of DD patients undergoing surgery was compared to those on medical treatment. The study was particularly focused on the long-term clinical outcome of non-complicated diverticulitis. HRQL was also assessed in 69 healthy subjects [39 males and 30 females with a mean age of 43 (22-85) years] without gastroenteric symptoms enlisted from hospital employees and their relatives.

For HRQL assessment, the Cleveland Global Quality of Life (CGQL) score[32], which consists of three items (current quality of life, current quality of health, and current energy level), each on a scale of 0 to 10 (0, worst; 10, best), was used. The CGQL was created to assess HRQL in patients affected by ulcerative colitis after restorative proctocolectomy and was then used in HRQL analysis of patients with Crohn's disease[33,34]. No significant differences were observed in the rate and in the timing of readmission and surgical procedures for DD in the two groups. The CGQL total score as well as the two items on current quality of life and current energy level responses were similar in the two groups of patients and in the group of healthy subjects. Only the scoring on the current quality of health was significantly worse in patients who had undergone colonic resection. Similarly, in the Hinchey 1 subgroup, no significant differences in CGQL score, current quality of health, current quality of life and current energy level were observed in patients who had been operated on and those who had been treated conservatively. Those results indicated that there are no long-term advantages to colonic resection for DD if the goal of the surgical treatment is to improve HRQL, and these data seemed to be supported by the analysis of the small group of Hinchey stage 1 patients.

QUALITY OF LIFE AFTER OPEN AND LAPAROSCOPIC SIGMOID COLECTOMY

Long-term outcome and HRQL were evaluated in patients undergoing laparoscopic colectomy (LC) *vs* open colectomy (OC) for benign colorectal diseases, based on standardized, validated measures, in a retrospective study[35]. All consecutive patients who underwent elective LC for uncomplicated diverticulitis in an 8-year time period were evaluated and compared to controls treated with conventional OC in the same period. HRQL was assessed by the SF-36 Physical and Mental Component Summary Scale (PCS; MCS) and by the SF-36 Health Survey. None of the 8 SF-36 Health Survey domains or the PCS and the MCS showed significant differences in HRQL between LC patients and OC patients. The occurrence of postoperative incisional hernias and bowel obstructions, which represented the only surgery-associated long-term complications, was comparable in both groups, as was the patients' HRQL. The two limitations of this study were the small patient cohort and the short follow-up (6-9 mo postoperatively with no long-term available). The lack of statistical difference between LC and OC patients in SF-36 scores related to the development of long-term complications may have been due to these limitations. Surgery-related complications were the only events that had a significant impact on the patients' HRQL, reflecting lower SF-36 scores in certain areas. The laparoscopic colorectal surgery itself had no significant influence on the patients' HRQL in the follow-up of these patients. The authors observed that better immediate postoperative HRQL after laparoscopic procedures may have been related to treatment of the disease *per se* and to favourable parameters such as faster convalescence, shorter hospital stay, and better cosmesis. On the other hand, long-term HRQL seemed to be influenced by chronic sequelae of the surgical procedures. Interestingly, more favourable cosmesis

itself had no impact on either the patients' overall HRQL or the emotional and social domains of the SF-36.

Another comparison of long-term HRQL between patients undergoing LC and OC was performed by Seitz *et al*[36] using the GIQLI questionnaire. Fifty-four patients who underwent sigmoid colectomy for recurrent diverticulitis were included. Patients who underwent LC seemed to feel better after surgery compared with those undergoing OC; however, this trend did not reach statistical significance. Patients' satisfaction regarding the cosmetic result was significantly higher in those undergoing LC than patients treated with OC. All patients had a similar GIQLI postoperatively, independent of the type of surgery. Eypasch's GIQLI did not identify clear differences between LC and OC. The limitation of this study was that only the *status quo* was determined and a comparison before and after surgery was not performed. The missing difference between LC and OC may be secondary to the higher rate of persistent symptoms in the LC group compared with the OC group, which was determined by simply asking the patients whether they felt that the disease had recurred. Thus, the authors commented that possible long-term advantages after LC with regard to HRQL may have been lost owing to this difference in "subjective recurrence".

Recently, a prospective, multicentre, double-blind, randomized controlled trial was designed to compare the impact of LC and OC on postoperative complication rates in patients with symptomatic diverticulitis[37]. Quality of life was assessed using the SF-36 questionnaire measured preoperatively and 6 wk after surgery. One hundred and four consecutive patients who underwent elective surgery for symptomatic diverticulitis of the sigmoid colon were randomized in 5 centres. Fifty-two LC patients were compared to 52 OC patients for gender, age, body mass index, American Society of Anesthesiology classification, prevalence of comorbidities, previous abdominal surgery, preoperative workup, and indication for surgery. SF-36 data showed no preoperative intergroup differences. Postoperative SF-36 data were significantly better in LC patients for role limitations due to physical and emotional problems, social functioning, and pain level. The main finding of this randomized controlled trial was that LC patients had significantly reduced major postoperative complication rates as compared with OC patients for symptomatic diverticulitis. Subjectively, patients who underwent LC scored significantly better than OC patients on the Visual Analogue Scale (VAS) for quality of life-pain score and SF-36 questionnaire. Several items in the latter showed improved role limitations due to physical health, role limitations due to emotional problems, social functioning, and pain.

A multicentre study compared HRQL in patients affected by DD submitted to LC *vs* those who underwent OC during long term follow up, using the Padova Inflammatory Bowel Disease Quality of Life (PIBDQL) score, CGQL score, VAS, Body Image Questionnaires (BIQ) and cosmetic score (CS), ad hoc DD symptom score (DDSS) and Bristol Stool Form Scale[38]. Sixty consecutive patients were included: 20 underwent LC, 15 OC and 25 had only medical therapy. The PIBDQL[35,39,40] was validated for use in patients with DD. Preliminary results showed that the PIBDQL scores were significantly worse in all patients with DD than those obtained from healthy subjects and correlated with the symptoms score. The CGQL was similar in patients who had LC compared to healthy subjects. BIQ scores correlated inversely with the presence of a stoma. The intestinal symptoms item was worse in patients who had LC than in those who had OC. On multivariate regression, the DDSS score was the only independent predictor of the PIBDQL score. No significant difference was observed in VAS for quality of life among the three groups of patients and in total CS in the two groups submitted to surgery. Only the scar score item was significantly better in patients who underwent LC compared to that in patients who had OC. Similarly, no significant difference was observed in the BIQ items and total score in the two groups submitted to surgery. The authors concluded that disease activity is the only independent predictor of the disease-specific quality of life.

QUALITY OF LIFE AFTER STAGED RESECTION FOR COMPLICATED DD

Constantinides *et al*[17] assessed long-term differences in HRQL using the SF-36 questionnaire between single and staged resections, in complicated DD. The authors divided the study population into two groups: one group consisting of patients who underwent primary colonic resection and anastomosis and the other group who underwent staged resections followed by restoration of intestinal continuity (HP). Three subgroups were created for each of the single staged and staged resection groups on the basis of when HRQL was assessed (1 group less than 3 years after primary surgery, 1 group 3-6 years after primary surgery and 1 group more than 6 years after primary surgery). One hundred and fifty-eight patients who underwent single staged resections and 30 patients who underwent staged resections with restoration of intestinal continuity were included. Significant differences were observed between the two groups in patients suffering from major comorbidities. No statistically significant differences were found in any of the eight domains between the single and staged resection groups. No significant differences were found between the two surgical methods across any of the eight SF-36 domains, for any of the time periods. The PF and RP domains were both subject to a significant decrease in mean score with advancing age. The BP domain had a progressive, but not statistically significant, decrease in score with advancing age.

Patients with no postoperative complications had significantly higher scores in the PF domain, the RP domain and the BP domain. According to these authors, in the setting of complicated DD, long-term HRQL tends to be similar between surgical interventions but remains significantly lower in selected domains when compared to the general population norms.

The main limitations of this study were that: (1) the two groups were inhomogeneous in terms of patient comorbidities and therefore, the effect of comorbidity on HRQL as a confounder could not be eliminated; and (2) no patient had a residual stoma and as a result, the study did not assess the impact of a colostomy on quality of life. Furthermore, HRQL was not assessed in the preoperative state.

CONCLUSION

The conclusions that can be drawn from these different studies are that DD has a negative impact on HRQL with lower scores in bowel function and systemic symptoms. Currently, the criteria for selection of DD patients for elective surgery remain debatable. Formal assessment of HRQL could be a good instrument in the selection of appropriate patients for elective surgery as well as in the assessment of surgical outcome. Both surgery-related complications and disease activity have a significant impact on patients' HRQL. While no significant differences in HRQL between different operations for DD in the non-randomized studies were revealed, the only prospective double-blind randomized study that compared LC and OC found that patients undergoing LC had significantly reduced major postoperative complication rates and subsequently had better HRQL scores.

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REVIEW

Gene therapy for liver regeneration: Experimental studies and prospects for clinical trials

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Abstract

The liver is an exceptional organ, not only because of its unique anatomical and physiological characteristics, but also because of its unlimited regenerative capacity. Unfolding of the molecular mechanisms that govern liver regeneration has allowed researchers to exploit them to augment liver regeneration. Dramatic progress in the field, however, was made by the introduction of the powerful tool of gene therapy. Transfer of genetic materials, such as hepatocyte growth factor, using both viral and non-viral vectors has proved to be successful in augmenting liver regeneration in various animal models. For future clinical studies, ongoing research aims at eliminating toxicity of viral vectors and increasing transduction efficiency of non-viral vectors, which are the main drawbacks of these systems. Another goal of current research is to develop gene therapy that targets specific liver cells using receptors that are unique to and highly expressed by different liver cell types. The outcome of such investigations will, undoubtedly, pave the way for future successful clinical trials.

Key words: Liver regeneration; Gene therapy; Genetic vectors; Growth factors

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INTRODUCTION

The need to enhance the capacity of liver regeneration has long been recognized but only avidly pursued recently. The clinically successful massive liver resection or small-for-size liver transplant carry the risk of liver failure due to impaired regeneration of the remnant/split liver[1]. Liver regeneration is also an integral component of the recovery processes of liver cirrhosis, fibrosis or liver failure[2]. Major advances in understanding the molecular mechanisms that govern liver regeneration have been made over the past few years[3,4]. Identification and molecular characterization of specific growth factors that promote liver regeneration has allowed the development of recombinant growth factors and their use to promote liver regeneration. The success of this strategy was hampered by the short half-life of these proteins in the circulation and the need for them to be administered continuously[5-9]. To overcome this problem, investigators successfully used gene transfer technology to transfer the genes that encode these growth factors. The intrinsic production of growth factor proteins following the transfer of their encoding genes enhances liver proliferation in various animal models with partial hepatectomy and/or chemical injury. Despite the success of proof of principle studies of gene therapy to enhance liver regeneration, and the potential of translation into clinical settings, no systematic review of published studies has appeared so far. Therefore, an evaluation of the current literature on gene therapy for liver regeneration is required and a look at future perspectives is

warranted. This article briefly summarizes the current key concepts in liver regeneration and gene therapy as they are related to each other, gives an overview of the published studies, and envisions future progress in the field.

LIVER REGENERATION: BASIC CONSIDERATIONS

Following two-thirds partial hepatectomy, the residual liver lobes enlarge within a week to make up for the mass of the removed lobes. Liver regeneration is carried out by proliferation of all adult liver cells including hepatocytes, sinusoidal endothelial cells, biliary epithelial cells, Kupffer cells and hepatic stellate cells (HSCs)[10]. It has been firmly established that mature hepatocytes are not terminally differentiated and that they have an almost unlimited capacity to proliferate, so that the liver can be entirely repopulated by intact hepatocytes that represent 1% of the hepatocyte population[10-15].

The molecular mechanisms of liver regeneration can be divided into two critical steps: the transition of the quiescent G_0 phase hepatocyte into the cell cycle (priming phase), and progression beyond the restriction point in the G_1 phase of the cycle (progression phase). These phases are under separate control; priming by the cytokines tumor necrosis factor (TNF) and interleukin-6 (IL-6), and cell cycle progression by the growth factors hepatocyte growth factor (HGF) and transforming growth factor (TGF)-α[11]. The priming phase does not lead to DNA replication unless the cells can progress through the cell cycle which is accomplished by growth factors. Once hepatocytes pass the G_1 restriction point they are irreversibly committed to replication (Figure 1)[16,17].

The mechanisms that initiate cytokine cascade liver regeneration have not yet been fully identified. It has been proposed that liver injury causes the release of reactive oxygen species and lipopolysaccharide (LPS), which trigger the activation of the complement system. After complement activation, cleavage of C3 or C5 leads to generation of the potent anaphylatoxins C3a and C5a. LPS, C3a and C5a in turn activate the non-parenchymal cells (NPCs) such as Kupffer cells, through the cell surface receptor TLR4 and C3aR and C5aR, which causes activation of the transcription factor nuclear factor (NF)-κB signaling pathway and the production of cytokines such as TNF-α and IL-6[18,19]. Also, the cytokine cascade can be triggered through the binding of TNF to its receptor TNFR1, which leads to activation of the NF-κB in NPCs, with the production of TNF and IL-6. Thus, the released TNF acts on the same NPCs in an autocrine fashion and on hepatocytes by a paracrine mechanism. Released IL-6 binds to its receptor on hepatocytes and leads to activation of the transcription factor STAT3 (signal transduction and activator of transcription), which translocates to the nucleus where it induces transcription of a number of target genes (Figure 2). The precise role played by each cytokines is, however, debatable[3,11]. TNF is not a direct mitogen for hepatocytes. It does, however, enhance the mitogenic effects of direct mitogens such as HGF. For example, it has been shown in stellate cells in culture that TNF and IL-6 activate the transcription factor C/EBPβ (CCAAT/enhancer-binding protein β), which induces HGF mRNA expression[20]. TNF is also involved in the activation of TGF-α[4]. IL-6 has both mitogenic and anti-apoptotic effects on hepatocytes and protects the regenerating liver against ischemic injury[11]. IL-6 has a crucial role in initiating acute phase response in hepatocytes, with the production of many proteins that assist in controlling acute or chronic inflammation[21].

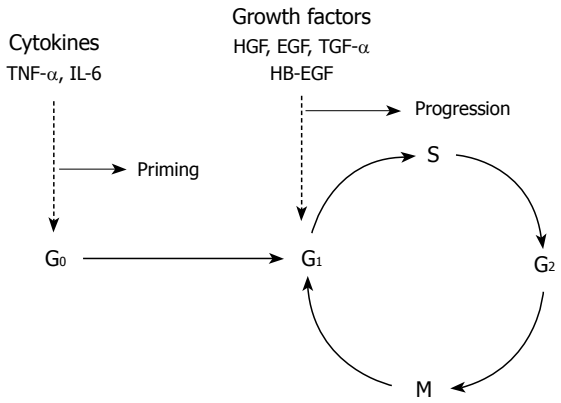

Figure 1 Effect of cytokines and growth factors on hepatocyte cell cycle. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; TGF-α: Transforming growth factor-α; HB-EGF: Heparin-binding EGF-like growth factor.

While cytokines are responsible for the passage of quiescent hepatocytes into the cell cycle (G_0 to G_1), cell cycle progression is then driven by growth factors, which override a restriction point in the late G_1 phase[3]. HGF and ligands of epidermal growth factor receptor (EGFR) are important growth factors that drive cell cycle progression during liver regeneration. Studies have shown that despite the expression of many mitogenic receptors, including receptors for platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF), the only mitogens for hepatocytes are HGF and ligands of EGFR. The family of ligands that bind EGFR, in addition to EGF, includes TGF-α, heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin (AR)[3,22]. Stimulation of the tyrosine kinase receptors for HGF and the EGF ligands activates numerous intracellular signaling pathways that regulate transcription factors involved in liver regeneration[3,4]. It is important to mention, with the possible exception of HGF, that complete elimination of a single growth factor does not entirely abrogate liver regeneration.

HGF is the most extensively investigated growth factor for liver regeneration. It stimulates regeneration in normal and injured liver. It is produced by NPCs and stimulates hepatocytes by a paracrine or endocrine mechanism. Following binding to its receptor, cMet, on hepatocytes, it stimulates DNA synthesis. HGF effects are multiple including mitogenic, motogenic, morphogenic and anti-apoptotic effects[4,11,17,23].

EGFR ligands are direct mitogens for hepatocytes. EGF is continually available to the liver through the portal

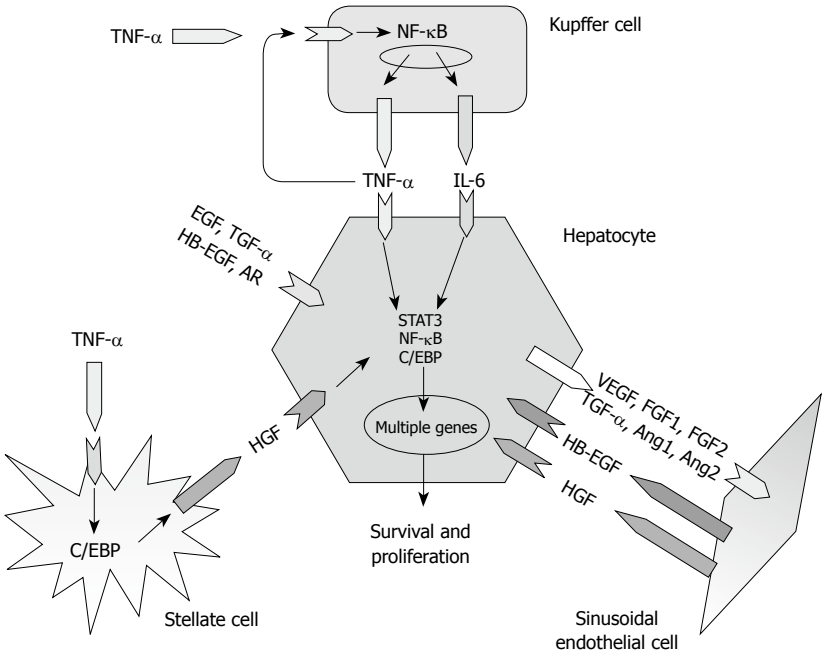

Figure 2 Major cytokine and growth factor signals during liver regeneration. NF-κB: Nuclear factor-κB; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; EGF: Epidermal growth factor; TGF-α: Transforming growth factor α; VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factor; HB-EGF: Heparin-binding EGF-like growth factor; AR: Amphiregulin; C/EBP: CCAAT/enhancer-binding protein; HGF: Hepatocyte growth factor.

vein, and is produced from Brunner's glands of the duodenum. EGF given to intact animals causes hepatocyte proliferation. TGF-α is an autocrine growth factor that is produced by and active on hepatocytes. Transgenic mice that overexpress TGF-α display hepatocyte proliferation and develop tumors[24]. On the other hand, TGF-α knockout mice have no defects in liver regeneration; probably because of the overlap between various ligands of the EGF family. TGF-α is also a mitogen for endothelial cells and bile duct epithelial cells. HB-EGF is produced by endothelial and Kupffer cells and is a key factor for hepatocyte progression through G_1/S transition during liver regeneration[25]. AR also contributes to liver regeneration, because mice deficient in AR have deficient liver regeneration[26]. It is likely that the different growth factors have independent but partially overlapping functions in liver regeneration[3,4].

Cytokine and growth factor pathways interact during different phases of liver regeneration[3,27,28]. For example, TNF activates TGF converting enzyme (TACE) that results in release of TGF-α, activation of EGFR and hepatocyte proliferation[3]. It should be noted at this point that there is significant redundancy between the components of each pathway, such that the lack of a single component generally causes a delay and/or reduction of regeneration. In other words, loss of an individual component gene rarely leads to complete inhibition of liver regeneration[3,23]. In contrast to the large number of hepatocyte growth promoters, very few inhibitors of liver regeneration have been identified. The most potent of these inhibitors is TGF-β[22]. For a more detailed review on molecular mechanisms of liver regeneration, readers should refer to references[1,3,4,23,29-33].

The molecular events involved in liver regeneration are significantly influenced by the extent of resection, as massive (85%-90%) liver resection leads to suppression and delay of liver regeneration, compared to 70% partial hepatectomy (PH), because of suppressed and delayed induction of the regenerative genes TNF-α and IL-6 after 90% PH. Moreover, apoptosis rates are also elevated in 90% PH compared to 70% PH[34]. Several studies have shown that growth factors that promote liver regeneration (HGF and TGF-α) are upregulated in 70% PH, whereas no or only reduced induction occurs after 90% resection. These findings suggest that expression of the factors relevant to the regeneration of liver tissue is influenced by the extent of resection[17,34-36].

A simplified summary of the interactions between cytokines and growth factors and between different cell types during liver regeneration is shown in Figure 2. Hepatocytes are the first to undergo proliferation, based on external stimuli from a variety of sources. HGF is rapidly becoming available to hepatocytes very rapidly through local matrix release and activation induced by urokinase-type plasminogen activator. Stellate and endothelial cells are sources of new HGF, which is synthesized after 3 h following PH. Hepatocytes produce growth factors that are mitogenic for stellate cells (PDGF)[37] and for endothelial cells (VEGF, FGF1, FGF2, stem cell factor, angiopoietins 1 and 2, and TGF-α). Proliferation of endothelial cells aims to restore the network of sinusoids that occurs over a long period of time, from days 3 to 6 after PH. Kupffer cells have not been clearly proven to proliferate during regeneration; however, they do produce TNF and IL-6, which appear to have a contributory role in STAT3 and NF-κB activation during the early stages of liver regeneration. Of note, the original hepatocyte mass is not restored through proliferation of stem cells, but through replication of residual mature hepatocytes. Hepatic stem cells (oval cells) are mobilized and differentiate into hepatocytes, only when proliferation of hepatocytes is totally blocked or when hepatocytes are chronically destroyed[10,23,33,38,39].

Identification and molecular characterization of spe-

Table 1 General characteristics of most commonly used vectors

System	Size of insert (kb)	Infect non-dividing cell	Genomic integration	Duration of expression	Immune response
Adenovirus					
1st generation	5	Yes	No	3-4 wk	High
2nd generation	8	Yes	No	Longer with	High
Gutless	35	Yes	No	Immuno-supression	Less
Adeno-associated virus	< 4.8	Yes	Yes and episomal	Long-term	Low
Herpes simplex virus 1	35	Yes	No	Long	High
Retrovirus	≤ 8	No	Yes, random	Long-term	Low
Lentivirus	≤ 8	Yes	Yes, into active genes	Long-term	Low
Baculovirus	> 20	Yes	No	Transient	Low
Plasmid-naked	Large	Yes	No	Short	Low
Plasmid-polymer	Large	Yes	No	Short	Low
Plasmid-lipid (liposomes)	Large	Yes	No	Short	Low

cific growth factors that promote liver regeneration allow the development of recombinant growth factors and their use to promote liver regeneration[6,40-43]. The success of this strategy is hampered by the short half-life of these proteins in the circulation and the need for them to be administered continuously. To overcome this problem, investigators have successfully used gene transfer technology to transfer the genes that encode these growth factors into liver cells.

GENE THERAPY FOR LIVER REGENERATION: KEY CONCEPTS

The strategy of introducing genetic material into liver cells to enhance proliferation or to inhibit apoptosis has been employed in experimental liver research for more than a decade. The transferred genetic material can be a natural gene[44-46], gene segment[47], chimeric gene[48], oligodeoxynucleotides (ODN)[49,50], or siRNAs. To facilitate transfer (transduction) into cells, the foreign gene (transgene) is packaged into construct named vectors. Gene transfer vectors are classified as either viral or non-viral. Viral vectors provide a powerful means for delivering therapeutic genes to targeted cells due to their high transduction efficiency. They are made replication-defective by deletion of viral genes involved in the replication and pathogenesis of the virus. This allows for the inclusion of non-viral genetic material in the viral genome. The general characteristics of most commonly used vectors are shown in Table 1. The most commonly used viral vectors are retrovirus, adenovirus, adeno-associated virus (AAV), herpes simplex virus, lentivirus and baculovirus. For a gene to be expressed inside a cell, its coding DNA sequence should be linked to an appropriate promoter. These regulatory DNA sequences can be categorized as viral (universal) promoters, which allow transgene expression in most transduced cells, housekeeping promoters, or tissue-specific promoters, which drive gene transcription only in selected cell types[51]. Because of their universal activity, viral promoters were components of many first-generation vectors. However, many of the viral promoters, such as the cytomegalovirus (CMV) promoter, are attenuated or completely shut-off in organs such as the liver. In comparison to viral or housekeeping promoters, tissue- or liver-specific promoters direct higher levels of expression *in vivo*. Successful application of gene therapy depends on the choice of relevant therapeutic genes, appropriate promoters, and effective vectors that allow an adequate level and duration of transgene expression[52-54].

Although retroviral vector transfection results in long-term survival of the gene in the transduced cell, its major disadvantage is the risk of insertional mutagenesis as a result of random integration of the virus into the host chromosome. Moreover, the transduction rate after retroviral gene transfer into hepatocytes *in vivo* is disappointingly low. Efficient retrovirus integration into the host-cell genome requires the active proliferation of target cells with DNA replication and nuclear membrane breakdown during mitosis. Under normal physiological conditions at any given time, only 0.005% of hepatocytes divide. For retrovirus liver transduction, hepatocyte proliferation induced by PH must occur on or about the time of retroviral delivery. To increase gene transfer without hepatectomy, mouse hepatocytes have been transduced *in vivo* with a recombinant adenovirus that transiently expressed urokinase[55], or with recombinant HGF[56]. The induced liver regeneration allowed persistent and efficient retroviral-mediated gene transfer in hepatocytes[55,56].

Adenoviral vectors are the most investigated vectors in animal and human gene therapy studies. Adenoviral vectors exhibit several merits that make them suitable for liver regeneration gene therapy. Adenoviruses are highly hepatotropic and it is relatively easy to produce high titers of recombinant adenoviral particles[57]. Unlike retroviruses, adenoviruses transduce dividing and non-dividing cells and do not integrate into the host chromosomes, thereby eliminating the risk of insertional mutagenesis. These merits make adenoviral vectors suitable for proof of principle experimental studies to verify the effect of overexpression of a specific growth factor gene on liver regeneration. The major limitation of adenoviral vectors is their serious and potentially fatal toxicity as exemplified by the death of an 18-year-old man who received 6×10^{11} viral particles/kg of E1/E4-deleted human adenovirus type 5 vector that contained human ornithine transcarbamylase cDNA[58,59]. Moreover, the severe immune response of the host contributes to the limited survival of the adenovirus

DNA in targeted cells and results in transient expression of the therapeutic gene. Until resolved, adenoviral-vector-induced toxicity will limit its application in clinical gene therapy studies. The transient nature of gene expression with adenoviral vectors may be advantageous because the process of liver regeneration is usually completed in approximately 1 wk. However, liver regeneration is seldom the only goal of therapy. Treating associated liver fibrosis or cirrhosis requires a longer period of gene expression. Furthermore, transduction efficiency of diseased liver is much lower than that of healthy liver. Garcia-Bañuelos *et al*[60] have demonstrated that adenovirus-mediated gene transfer *via* the iliac vein at 3×10^{11} viral particles per rat resulted in approximate 40% transduction in livers made cirrhotic by chronic intoxication with carbon tetrachloride, compared with approximate 80% in control non-cirrhotic livers. In rats made cirrhotic by bile-duct obstruction only, 10% efficiency of transduction was observed. Yu *et al*[61] have shown that NPCs are transduced with greater frequency than hepatocytes at all adenoviral titers tested, both *in vitro* and *in vivo*. After liver injury, adenoviral transduction is reduced for all liver cell types compared with that for cells from normal livers (at all virus titers). Again, transduction efficiency remains greater in NPCs than in hepatocytes after liver injury.

Non-viral vectors can be divided into two categories: physical and chemical. Physical methods involve the introduction of plasmid DNA into cells using electroporation, ultrasound, or hydrodynamic delivery. Chemical methods use lipid or polymer carriers that complex with DNA to deliver the transgene into cells[62,63]. Several non-viral vectors have been used for *in vivo* liver gene therapy including various liposome preparations, protein-DNA conjugates, nanoparticles, and naked or complexed DNA[57,64,65]. Expression is usually both transient and at low level because the DNA is not stable in cells. Despite these limitations, non-viral vectors offer many advantages including being simple to use, ease of production of large quantities, and absence of host immune response.

A major advance in the intravascular delivery of vectors followed the development of the hydrodynamic injection technique. The technique involves rapid tail vein injection of a large volume of the vector (around 10% of the body weight of a mouse or rat) in a short time period (5-7 s in mice and 15-20 s in rats). The hydrodynamic method results in dramatically higher hepatic transfection efficiency compared to conventional injection. Typically, 10%-15% of hepatocytes are transfected in mouse liver following injection of 10 μg plasmid, but levels up to 40% have been reported[66]. Liver enzymes are transiently elevated and liver histology shows minimal damage that resolves within a week, which is similar to the results obtained from intravascular delivery into liver vessels[66,67]. It has been postulated that increased pressure in the inferior vena cava causes retrovenous blood flow from the central to the portal vein, and the resultant increased intrahepatic vascular pressure promotes massive endocytosis that generates intracellular water movement that facilitates gene entry[68,69]. There are multiple lines of evidence that the species differences in the diameter of sinusoidal fenestrae are a critical determinant of transgene expression after adenoviral transfer. The small diameter of fenestrae in humans should be considered in any rational design of gene transfer technology for hepatocyte-directed transfer. Hydrodynamic gene transfer is highly successful in rodents. The significantly lower efficacy in higher species may also partially be due to species differences in liver architecture[70]. Intrinsic factors, in particular compliance (elasticity) of the liver are likely to be crucial in determining the degree of swelling for a given level of intrahepatic vascular pressure. Liver compliance is likely to be the major reason for the low level of hydrodynamic gene delivery in the pig model, and will influence the effectiveness of the approach in humans, both in general and in different disease states[71].

This procedure has great limitations for application to clinical practice, therefore, a clinically relevant method for regional hydrodynamic delivery of vectors has been developed. The method entails the use of an occlusion balloon catheter into the inferior vena cava and retro dynamically injecting towards the liver and through the hepatic vein, 100 mL of the plasmid in saline solution (20 mg/mL), at a rate of 7.5 mL/s. This retrodynamic hepatic vein gene delivery method has been performed in pigs, and was as well tolerated as in mice and led to liver transgene expression, however, the plasma levels of the transgene protein were four orders of magnitude lower than those reached in the murine model[68,72]. A variety of different modifications have been reported recently[73,74].

Recently, retrograde administration of adenoviruses into the common bile duct has been shown to induce efficient transgene expression in the liver without causing severe adverse effects, thus supporting the feasibility of adenovirus-mediated gene transfer into the liver in clinical settings by means of endoscopic retrograde cholangiography[75-77]. Repeat administration of adenoviruses into the common bile duct is successful in re-expressing the transgene in the liver[78]. This contrasts with the failure of re-expression of transgene following intravenous readministration of an adenoviral vector long after the initial administration[79].

OVERVIEW OF PUBLISHED STUDIES

The general features of the reviewed gene therapy studies for enhancing liver regeneration are summarized in Table 2. Gene therapy investigations that fulfilled the following criteria were selected for review: (1) demonstrated, objectively, enhanced liver cell proliferation and or increased survival as compared with controls; (2) animals and/or livers receiving gene therapy were not genetically modified as they do not directly represent human liver diseases (e.g. liver cirrhosis, fibrosis or failure) in which liver regeneration has a critical role in recovery; and (3) gene therapy was administered *in vivo*. The selection of homogeneous cohort studies based on these criteria allows us to delineate the main characteristics of these studies, and more importantly, envision what needs to be done in fu-

Table 2 Main features of reported gene therapy experiments[39,44-50,80-96]

Vector, Ref.	Dose	Transgene (promoter)	Liver model, animals, route	Measured parameters
Adenovirus vector				
Hogaboam *et al*[80], 1999	1×10^8 pfu	r-MIP-2	Acetaminophine injury, mice, IV	↑DNA synthesis, ↑survival
Phaneuf *et al*[46], 2000	1-4 × 10^{11} vp	h-HGF (CMV)	Healthy, mice, IV	↑DNA synthesis, ↓apoptosis and ALT
Shiota *et al*[39], 2000	1×10^9 pfu	r-HGF (CAG)	AAF/70% PH, rats, IV	↑Oval cell proliferation
Nomi *et al*[95], 2000	1×10^9 pfu	r-HGF (CAG)	D-Gal/LPS liver failure, rats, IP	↓Apoptosis, ↑survival
Hecht *et al*[48], 2001	1×10^8 TU	h-HIL-6 (CMV)	D-Gal liver failure, mice, IP	↑Survival, ↑proliferation
Hwang *et al*[81], 2003	1×10^{11} vp	h-HGF (CMV)	TAA liver failure, mice, IV	↑Survival, ↑DNA synthesis, no hepatic necrosis
Iwaki *et al*[49], 2003	2×10^9 pfu	m-MIF antisense	BCG-LPS liver failure, mice, IV	↑Survival
Oe *et al*[45], 2004	7×10^8 pfu	h-VEGF + or r-HGF (CAG)	DMN cirrhosis 70% PH, rats, IV	↑SECs and hepatocytes proliferation
Oe *et al*[82], 2005	7×10^8 pfu	r-HGF, or h-VEGF (CAG)	AAF/70% PH, rats, IV	↑Oval cell proliferation, ↑regeneration
Wullaert *et al*[84], 2005	2.5×10^9 pfu	m-ABIN-1 (CMV)	TNF + Gal-liver injury, mice, IV	↑Survival, ↓apoptosis,
Ichiba *et al*[94], 2005	1×10^9 pfu	r-TPO (CAG)	AAF/70% PH, rats, IV	↑Oval cell proliferation
Khai *et al*[44], 2006	1×10^{11} vp	h-HB-EGF or h-HGF (RSV)	Fas-induced injury, mice, IV	↓Apoptosis and ↑proliferation by both
Ozawa *et al*[47], 2006	5×10^8 pfu each	r-HGF, +/or h-TGFβ2R (CAG)	DMN cirrhosis 10% PH, rats, PV	↑Proliferation, ↑survival, ↓cirrhosis
Tan *et al*[96], 2006	1×10^{11} vp	m-HNF6 (CMV)	70% PH, mice, IV	↑Proliferation
Yuasa *et al*[85], 2007	1×10^9 pfu	r-HGF, (CBA)	85% PH, rats, IV	↓Apoptosis, ↑proliferation, ↑survival
Ueno *et al*[83], 2007	5×10^8 pfu	r-HGF (CAG)	DMN cirrhosis 70% PH, rats, sPV	↑Proliferation, ↑survival, ↓cirrhosis
Atta *et al*[93], 2009	7×10^9 pfu	h-HGF, h-VEGF (CMV)	Healthy, dogs, IV	↑SEC and hepatocytes proliferation
Naked plasmid DNA				
Yang *et al*[90], 2001	10-40 μg/wk × 8	h-HGF (CMV)	Healthy, mice, IV	↑Proliferation
Xue *et al*[89], 2003	50 μg × 3	r-HGF	CCl_4 cirrhosis 70% PH, mice, IM + EP	↑Proliferation
Zhang *et al*[91], 2005	200 μg/kg per 12 h × 4	r-ALR	CCl_4 liver injury, rats, IV, IP	↓ALT and AST, ↑proliferation, ↑survival
Horiguchi *et al*[86], 2009	-	h-HGF	DMN cirrhosis, dogs, IA	↓ALT and AST, ↓fibrosis, ↑survival
HVJ Liposomes				
Ueki *et al*[88], 1999	20 or 40 mg weekly × 4	h-HGF (SRa)	DMN cirrhosis, rats, IM	↓Apoptosis, ↑survival, ↑r-HGF, ↓fibrosis
Ogushi *et al*[92], 2003	50 nmol	NF-κB decoy ODN	*P. acnes*-LPS liver injury, mice, PV	↑Survival, ↑proliferation, ↓apoptosis
Nishino *et al*[87], 2008	20 μg	h-HGF (SRα)	DMN cirrhosis 70% PH, rats, PV	↑Proliferation, ↑survival, ↓apoptosis
Takahashi *et al*[50], 2009	50 nmol	NF-κB decoy ODN	90% PH, mice, PV	↑Survival, ↓apoptosis

AAF: Acetylaminofluorene; ALT: Alanine transaminase; AST: Aspartate transaminase; ABIN-1: A20 binding inhibitor of nuclear factor κB; ALR: Augmenter of liver regeneration; BCG: Bacille Calmette-Guerin; CAG: Chicken β-actin promoter and cytomegalovirus enhancer; CBA: Chicken β-actin; D-Gal: D-galactosamine; TNF: Tumor necrosis factor; HVJ: Hemagglutinating virus of Japan; DMN: Dimethylnitrosamine; EP: Electroporation; Gal: Galactosamine; h: Human; h-HIL-6: Human hyper-interleukin-6 (IL-6) cDNA gene coding the human sIL-6R (amino acid residues 1-323) and human IL-6 (amino acid residues 29-212) fused by a synthetic DNA linker; HNF6: Hepatocyte nuclear factor 6; IA: Intra-arterial injection (hepatic artery); IM: Intramuscular injection; IP: Intraperitoneal injection; IV: Intravenous injection; M: Murine; HGF: Hepatocyte growth factor; MIF: Macrophage migration inhibitory factor; MIP-2: Macrophage inflammatory protein-2; VEGF: Vascular endothelial growth factor; CMV: Cytomegalovirus; ODN: Oligodeoxynucleotides; PH: Partial hepatectomy; LPS: Lipopolysaccharide; SECs: Sinusoidal endothelial cells; *P. acnes*: *Propionibacterium acnes*; PV: Portal vein injection; r: Rat; sPV: Selective portal vein injection; SRα: Simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat; TAA: Thioacetamide; TGFβ2R: Truncated transforming growth factor β type 2 receptor; TPO: Thrombopoietin; TU: Transducing units (1 vp = 25 TU); vp: Viral particles (1 vp = 100 pfu).

ture studies as a preparation for clinical trials. An overview of the different elements of gene therapy for liver regeneration studies are given below.

Vector type

Given the merits of adenoviruses as a powerful vector that has the highest transduction rate for liver cells, it is not surprising that two-thirds of all reviewed studies used it to prove the effect of the therapeutic gene (Table 2). It was the only viral vector used. The non-viral vectors employed in the rest of the reviewed studies are divided between naked DNA and liposomes. Despite the lower transfection rate of the non-viral vectors, their safety makes them suitable candidates for preclinical studies.

Vector dose

The administered adenoviral dose ranged between 1×10^8 pfu and 4×10^9 pfu with a dose of 1×10^9 pfu used in 80% of the studies[39,44-47,80-85,97]. The average vector dose for mice was no different from that for rats despite considerable differences in their body weights. Phaneuf *et al*[46] have examined the effect on liver regeneration of increasing doses (1×10^9 to 4×10^9 pfu) of adenoviral vector encoding for human HGF. They have found that DNA synthesis of hepatocytes and liver weight increased in a dose-dependent fashion, such that the maximal effect was seen after the infusion of 3×10^9 pfu, which resulted at day 5 in a > 130% increase in relative liver mass, with little cytopathic effect. The average single dose of naked DNA was 10-50 μg and that of liposomes was 50 nmol[50,86-91,98].

Therapeutic genes

By far the most studied therapeutic gene was HGF, which is not surprising given the fact that it is the single most important growth factor implicated in liver regeneration. It has been used in about two-thirds of studies, either alone

or in combination with other growth factors (Table 2). Other genetic materials used include growth factors, cytokines or transcription factors involved in direct liver cell proliferation, e.g. VEGF, HB-EGF, C/EBPβ, and IL-6. Two studies have reported the use of antisense ODN to NF-κB (NF-κB decoy ODN) encapsulated in hemagglutinating virus of Japan (HVJ) liposomes to prevent endotoxin- or massive hepatectomy-induced liver failure[50,92]. Antisense ODNs are not natural genes, and they are short (15-20 bases in length) synthetic oligonucleotides that are designed to hybridize to RNA through Watson-Crick base pairing. Upon binding to the target RNA, ODNs prevent expression of the encoded gene product. Although stimulation of the transcription factor NF-κB in Kupffer cells, with production of inflammatory cytokines, has been shown to be involved in liver proliferation, excessive production of cytokines is thought to be responsible for liver failure following excessive hepatectomy[50].

Route of administration

The liver is an attractive target for *in vivo* gene transfer studies because hepatocytes are readily accessible *via* the blood stream. The endothelium of hepatic sinusoids displays fenestrations that are 100 nm wide and that allow macromolecules such as viral particles to cross the endothelium and reach hepatocytes. Moreover, the hepatic blood flow represents one-fifth of the cardiac output. Thus, any particle injected into the blood circulation can quickly reach the liver[54]. For this reason, the vascular route constitutes the most commonly used in 80% of the reviewed studies. The intravenous route is the commonest among the vascular routes not only because it is the easiest route compared with intra-arterial or portal vein administration, but also due to the enhanced transduction rate following the recent modification of the hydrodynamic technique mentioned above.

Duration of transgene expression

Few of the reviewed studies have reported the duration of expression of the transduced gene or its protein[39,45,81,83,85,87,88,93-95]. Those studies that had extended observation periods have shown that the duration of transgene expression does not extend beyond 1 wk following vector administration[39,81,83,87,88,94]. These data agree with the accumulated knowledge that gene therapy using adenoviral vectors or non-viral naked DNA and liposomes confers a limited duration of gene expression. Moreover, it should be noted that the efficiency of gene transduction, which directly affects the duration of gene expression, is lower in cirrhotic liver than in normal liver due to capillarization of sinusoidal endothelial cells as a result of the decreased size or loss of the fenestrae of sinusoidal endothelial cells[99]. Nishino *et al*[87] have demonstrated that only 5%-6% of hepatocytes in cirrhotic rat livers were successfully transfected with human HGF plasmid enveloped in HVJ liposomes.

Non-hepatic gene transfection

There was a tendency towards excluding gene therapy studies for liver regeneration in which gene transduction involved organs other than the liver, e.g. skeletal muscles. Although this could be appropriate for the sake of presenting a homogeneous group of investigations, it was felt however that this would have omitted an important cluster of studies that represented an emerging direction in gene therapy for liver regeneration. In this regard, two studies used liposomes and naked plasmid to transduce skeletal muscles with HGF in animals with liver cirrhosis. They demonstrated expression of the transduced HGF gene and elevation of its plasma levels that exerted proliferative and antifibrotic effects on the liver[88,89].

FUTURE PERSPECTIVES

In 20 years of gene therapy research, there have been few studies that have aimed at enhancing liver regeneration. However, the accumulated knowledge from these studies has allowed the validation of proof of principle gene therapy investigations for promoting liver regeneration in different animal models of liver diseases. Future progress in this field is expected to tackle several points.

First, determination of the combination of gene therapy that works better for a specific disease condition. As mentioned above, enhancing liver regeneration is seldom the only goal of therapy. Treating associated liver fibrosis/cirrhosis or toxic injury requires the combined effects of genetic materials such as growth factor genes and antisense ODN. This should be based on the outcomes drawn from experimental comparative studies of different combinations of therapeutic genes for each defined disease. An example of such comparative studies is that of Ozawa *et al*[47]. In rats with liver cirrhosis, combination gene therapy of HGF, a powerful liver mitogen, and truncated type Ⅱ TGF-β receptor that specifically inhibits TGF-β signaling that is responsible for progression of liver fibrosis[100], resulted in decreased liver fibrosis and improved liver function, compared with monotherapy with either gene alone. These studies provide an opportunity to shed light on how the administered genes influence the pathogenesis of the multifactorial disease process. Also, it could identify synergistic combinations that could enhance regeneration, disease resolution and reduce the amount of transferred genetic material. An example of such studies would make use of HGF and NF-κB decoy ODN, which prevents excessive cytokine production, to prevent hepatocyte apoptosis and enhance regeneration after massive resection or liver injury[50,92].

Secondly, evaluation of the trade-off of risk against the benefits of viral *vs* non-viral gene therapy. Unlike gene therapy for liver genetic diseases that require a high rate of liver transduction to express the therapeutic protein efficiently in the systemic circulation, at a clinically relevant concentration, gene therapy for liver regeneration or resolution of fibrosis aims at locally expressing the desired proteins, which act in an autocrine or paracrine fashion[93]. Thus, despite non-viral systems having a lower transfection rate, they are safer, easy to produce in large quantities, and can be repeatedly administered, which can

aid in gauging the amount and duration of gene expression. Moreover, hydrodynamic injection in murine models and its clinically relevant retrodynamic hepatic vein gene delivery in large animals have dramatically increased transfection efficiency of non-viral systems.

Thirdly, employing the recently developed vectors that target specific liver cell types, and promoters that are capable of liver-specific sustained transgene expression in gene therapy studies to augment liver regeneration and treat associated liver injury. These new developments can be summarized as follows: (1) Cell-specific expression of therapeutic genes of interest is an extremely attractive strategy in gene therapy. Several investigators have developed selective hepatic cell delivery systems using receptors that are unique to and highly expressed by different liver cell types: (A) The asialoglycoprotein receptor (ASGPR) on the hepatocyte membrane is a specific targeting marker for gene and drug delivery. Studies have targeted the hepatocyte ASGPR using its natural ligand, asialoorosomucoid[101,102]. Chiba *et al*[103] recently have developed cationically modified biocompatible phospholipid polymer conjugated with hepatitis B surface antigen for the specific transfer of genes into human hepatocytes; (B) Quiescent HSCs lack specific receptors or motifs on their cell surface, thus, attempts to target HSCs have been a challenging task[104]. (a) The mannose 6-phosphate/insulin-like growth factor Ⅱ (M6P/IGF-Ⅱ) receptor expression is increased on activated HSCs, particularly during fibrosis. The receptor has binding sites for IGF-Ⅱ and M6P-containing ligands[105]. Beljaars *et al* have developed a carrier system that consists of human serum albumin modified with M6P, which binds to the M6P/IGF-Ⅱ receptors on HSCs[104-107]; (b) Vitamin A receptors on HSCs have been used to deliver siRNA against collagen-specific chaperone heat shock protein 47 *via* vitamin A-coupled liposomes[108]; and (c) Liposomes labeled with a cyclic RGD-peptide that recognizes the collagen type Ⅵ receptors[109,110]; (C) Sinusoidal endothelial cells (SECs) possess unique hyaluronan receptors that recognize and internalize hyaluronic acid (HA). SECs have been targeted using HA, the endogenous ligand for the HA receptor for endocytosis[111,112]; and (D) Kupffer cells possess receptors that recognize galactose and N-acetylgalactosamine. Studies have shown that galactosylation can target various DNA preparations including liposomes, low-density lipoprotein and chitosan polymer to Kupffer cells[113-115]; and (2) Liver-specific sustained transgene expression can be obtained at very high levels from optimized promoters[116]. Many experimental gene therapy vectors described in this review express transgenes under the control of non-specific promoters such as CMV, Rous sarcoma virus, simian virus 40 (SV40) and mammalian elongation factor 1α (EF1α) (Table 2). These promoters direct strong gene expression but are shut off rapidly *in vivo*[117,118]. A tissue-specific promoter is a promoter that has activity in only certain cell types. Use of a tissue-specific promoter in the expression cassette can restrict unwanted transgene expression as well as facilitate persistent transgene expression[119]. Ongoing developments are based on two liver-specific promoters, the albumin promoter and the α1 antitrypsin promoter. Wooddell *et al*[116] have demonstrated that when using a plasmid vector that contains albumin promoter combined with an α-fetoprotein (AFP) MERⅡ enhancer, 5' intron from the factor Ⅸ gene, and the 3'UTR from the albumin gene, including intron 14, the reporter gene expression levels remained high for 1 year, at levels comparable to those obtained from the CMV promoter on day 1. Ziegler *et al*[120] have shown that intravenous administration of a recombinant AAV2 vector encoding human α-galactosidase A under the transcriptional control of a liver-restricted enhancer/promoter consisted of human serum albumin promoter (nucleotides -486 to +20), to which were appended two copies of the human prothrombin enhancer (nucleotides -940 to -860). The enhancers were placed 5' of the promoter in the forward orientation. This vector mediated sustained hepatic expression of α-galactosidase A for 12 mo and was associated with a significantly reduced immune response to the expressed enzyme. Several investigators have reported encouraging long expression of transgenes using different modifications of α1 antitrypsin promoter[117,121-123]. Jacobs and his colleagues have compared 22 hepatocyte-specific expression cassettes and have found that a promoter that consists of an 890-bp human α1-antitrypsin promoter and two copies of the 160-bp α1-microglobulin enhancer results in the highest expression levels[124]. Comparisons between different liver-specific promoters have shown that α1-antitrypsin promoters induce higher levels and prolonged expression of transgenes than other liver-specific promoters such as AFP and albumin promoter[125-127]. The most recent investigations have shown the unlimited possibilities for gene therapy modifications. Li *et al*[128] have developed a small DNA fragment (347 bp) from the AAV chromosome 19 integration site that is capable of providing efficient and enhanced liver-specific transcription when used in recombinant AAV vectors. Previously described tissue-specific promoters for gene therapy are typically too big for AAV vectors. Wolff *et al*[129], in an effort to increase long-term expression of transgene products, have designed a plasmid DNA vector under the control of a tissue-specific promoter and have included microRNA target sites in the transcripts, in order to silence expression in antigen-presenting cells.

CONCLUSION

The success of several proof of principle studies of gene therapy for liver regeneration, coupled with the recent extensive search for the mechanisms of selective targeting of specific liver cells, should pave the way towards future clinical trials. As liver regeneration is usually an integral part of the therapeutic goals of many liver diseases, gene therapy to enhance liver regeneration needs to be combined with gene therapy for associated liver disease. Consequently, clinically relevant gene transfer protocols should be developed to address specific goals of such combined gene therapy trials.

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ORIGINAL ARTICLE

Extracellular matrices for gastrointestinal surgery: *Ex vivo* testing and current applications

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Abstract

AIM: To assess the effects of bile and pancreatic juice on structural and mechanical resistance of extracellular matrices (ECMs) *in vitro*.

METHODS: Small-intestinal submucosa (SIS), porcine dermal matrix (PDM), porcine pericardial matrix (PPM) and bovine pericardial matrix (BPM) were incubated in human bile and pancreatic juice *in vitro*. ECMs were examined by macroscopic observation, scanning electron microscopy (SEM) and testing of mechanical resistance.

RESULTS: PDM dissolved within 4 d after exposure to bile or pancreatic juice. SIS, PPM and PDM retained their integrity for > 60 d when incubated in either digestive juice. The effect of bile was found to be far more detrimental to mechanical stability than pancreatic juice in all tested materials. In SIS, the loss of mechanical stability after incubation in either of the digestive secretions was less distinct than in PPM and BPM [mFmax 4.01/14.27 N (SIS) *vs* 2.08/5.23 N (PPM) *vs* 1.48/7.89 N (BPM)]. In SIS, the extent of structural damage revealed by SEM was more evident in bile than in pancreatic juice. In PPM and BPM, structural damage was comparable in both media.

CONCLUSION: PDM is less suitable for support of gastrointestinal healing. Besides SIS, PPM and BPM should also be evaluated experimentally for gastrointestinal indications.

Key words: Extracellular matrix; Intestinal regeneration; *Ex-vivo* testing; Gastrointestinal surgery; Gastrointestinal fistula; Bioscaffolding

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INTRODUCTION

Extracellular matrices (ECMs) have been introduced for clinical therapy of gastrocutaneous, enterocutaneous and anal fistulas and for buttressing of gastrointestinal staple lines. Moreover, they have been evaluated experimentally as bioscaffolds for tissue regeneration in different gastrointestinal hollow organs[1-14] and for reinforcement of gastrointestinal anastomoses[15,16]. In particular, small-intestinal submucosa (SIS) has been tested clinically and experimentally. SIS is a biodegradable, commercially available, acellular, immunologically inert collagen matrix, which is extracted from the submucosal layer of porcine small bowel.

Several clinical studies have been performed to evaluate SIS for plug repair of gastrointestinal fistula. Prospective studies have shown high rates of success in the treatment of anal fistulas[17,18], but enterocutaneous[19,20] and gastrocutaneous[21,22] fistulas have also been treated successfully by implantation of SIS plugs. Furthermore, it has been shown that sealing of intestinal anastomoses promotes the healing of intestinal anastomoses[15,16]. For successful application in luminal gastrointestinal organs, temporary maintenance of structure and stability of ECM against gastrointestinal fluids should be guaranteed. To date, SIS has never been examined because of its resistance against gastrointestinal digestive juices. Other biological collagenous acellular scaffolds, which are promising for the support of gastrointestinal healing, are extracted from porcine dermis, porcine pericardium or bovine pericardium.

The aim of our study was to assess the effect of physiological intraluminal intestinal components human pancreatic juice and bile on 3D surface ultrastructure and mechanical resistance of different natural biological scaffolds *in vitro*.

MATERIALS AND METHODS

Materials

Single-layer SIS was prepared as previously described[23]. Sections of porcine jejunum were obtained from the local slaughterhouse and immediately placed in 0.9% saline solution. Jejunal sections were cut into 10-cm lengths and luminally cleaned with 0.9% saline solution. The mesenteric tissues were removed from the segment of the small intestine, followed by mechanical removal of the tunica serosa and tunica muscularis from its outer surface by gentle abrasion using a scalpel handle and saline-moistened gauze. The segment was inverted and the tunica mucosa was mechanically removed by similar mechanical abrasion and reverted to its original orientation. The remaining 0.1-0.2-mm thick translucent tube actually consisted of the tunica submucosa. The stratum compactum that originally was in contact with the more superficial luminal mucosa was now the luminal surface of the SIS graft. After sterilization of the SIS graft by 2 h incubation in 0.1% perchloric acid, it was rinsed with sterile normal saline and stored in refrigerated 0.05% gentamicin at 4℃. Storage time for the graft materials ranged from 3 to a maximum of 7 d until the material was used for *in vitro* testing.

Four-layer SIS was provided as Surgisis® from Cook Surgical (Lafayette, IN, USA). Porcine dermal matrix (PDM) was provided as Xenoderm® from MBP (Neustadt-Gleve, Germany). Cleansed porcine pericardial matrix (PPM) was provided from aap Biomaterials (Dieburg, Germany). Bovine pericardial matrix (BPM) was provided as Lyoplant® from Braun Dexon (Melsungen, Germany). The materials were divided under sterile conditions into pieces of 1 cm × 1 cm for scanning electron microscopy (SEM) and for measurement of degradation time. For assessment of mechanical properties, samples of 1 cm × 3 cm were used.

Incubation

Human bile had been collected during laparotomy from patients in whom cholecystectomy was performed. Microbiological assessment of bile excluded an infectious biliary syndrome. Pancreatic juice was obtained from patients with pancreatic duct drainage after pancreatic head resection for chronic pancreatitis. Patient consent to use the secretions for the study was given. Both fluids were checked for bacterial contamination in aerobic and anaerobic microbiologic cultures, and only sterile bile and pancreatic juice were used for experiments. ECM specimens of 1 cm × 1 cm and 1 cm × 3 cm were incubated at 37℃ for 1, 7, 14 or 60 d in bile and pancreatic juice. For reference, the material was also incubated in sterile phosphate buffered saline (PBS). The pH of human bile used for incubation was 8.37. Pancreatic enzyme concentrations were measured by routine diagnostic methods at the central laboratory of the University Hospital Freiburg. Initial concentration of amylase was on average 86 040 U/L, concentration of lipase was 210 630 U/L in pancreatic juice, and pH was 8.42. After 24 h of incubation, concentration of amylase was on average 68 440 U/L, and concentration of lipase was 28 670 U/L in pancreatic juice. Due to this degradation of active enzymes during incubation, both media were replaced every 24 h under sterile conditions.

Measurement of degradation time and macroscopic evaluation

All incubated samples were macroscopically inspected daily for signs of degradation for 60 d. After incubation in PBS, human bile and human pancreatic juice, the size of the specimens was measured after 1, 7, 24 and 60 d.

Mechanical testing

Assessment of mechanical resistance using a sero-hydraulic material testing machine with a 200-N force transducer (UTS 20; UTS Systeme GmbH, Germany) was performed after 24 h and 14 d. Test Expert II Software (Zwick GmbH, Ulm, Germany) was used for analysis of force/distension diagrams. All mechanical experiments were carried out in triplicate. ECM strips of 1 cm × 3 cm were removed from incubation medium, rinsed in PBS and fixed in the testing device of the material testing machine. The sample was distended longitudinally at a speed of 12 mm/min until the sample broke. The required force until failure of the SIS strip was measured and reported as Fmax in Newtons.

SEM

After 7 d incubation in PBS, bile or pancreatic juice ECM samples (1 cm × 1 cm) were rinsed in PBS and subsequently fixed with 4% buffered formaldehyde for 48 h at room temperature. The samples were dehydrated in a graded series of acetone, dried in a critical-point dryer, mounted for SEM, and coated with gold in an evaporator unit. The samples were mounted such that one of the sides and the cross-section were visible. Examination was then performed in an LEO 435 VP scanning electron microscope (LEO Electron

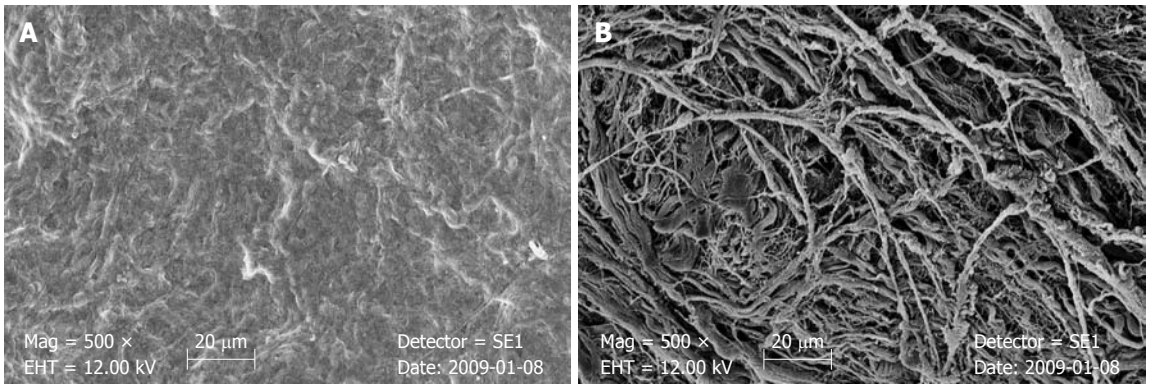

Figure 1 Scanning electron microscopy pictures of the stratum compactum surface (A) and the abluminal surface (B) of small-intestinal submucosa. Magnification × 500.

Table 1 Extracellular matrix degradation ultrastructural grading system

Characteristic	Resistance → Degradation			
	Score 0	Score 1	Score 2	Score 3
Porosity	No change	Light increase	Distinct increase	Strong increase
Surface fibrillar arrangement	No change	Light alteration	Distinct alteration	Native arrangement not recognizable
Erosion of matrix surface	No change	Light surface erosion	Distinct surface erosion	Native surface structure not recognizable

Original materials incubated for 60 min in phosphate buffered saline were used as reference.

Table 2 Degradation time with phosphate buffered saline, bile and pancreatic juice

	PBS	Bile	Pancreatic juice[1]
SIS 4-layer	> 60	> 60	> 60
SIS 1-layer	> 60	> 60	> 60
PDM	40	2	4
PPM	> 60	> 60	> 60
BPM	> 60	> 60	> 60

Degradation time is given in days. Incubation was performed at 37℃ for 60 d. [1]Enzyme concentration: amylase 86040 U/L; lipase 210630 U/L. PBS: Phosphate buffered saline; SIS: Small-intestinal submucosa; PDM: Porcine dermal matrix; PPM: Porcine pericardial matrix; BPM: Bovine pericardial matrix.

Microscopy Ltd., Cambridge, UK). A grading system was developed for comparison of ultrastructural alterations after incubation in bile and pancreatic juice. As reference, original materials incubated for 60 min in PBS were used. Changes in porosity, surface fibrillar arrangement and erosion of the ECM surface were measured and scored (Table 1).

RESULTS

Macroscopic examination and degradation time

SIS one-layer, SIS four-layer, PPM and BPM samples were intact, without macroscopic signs of degradation after 60 d of incubation in bile, pancreatic juice and PBS. Apart from deep green-brown color after incubation in bile, there were no macroscopic differences recognizable after 7, 14 and 60 d of incubation. No shrinkage and no change in size of the patches were macroscopically detectable, regardless of the medium of incubation in SIS, PPM and BPM samples. PDM was dissolved within 40 d in PBS, within 2 d in bile, and within 4 d in pancreatic juice (Table 2).

Mechanical testing

For SIS, PPM and BPM, testing revealed the most distinctive loss of mechanical strength after incubation in bile at 24 h and 14 d. Incubation in pancreatic juice also caused a significant decrease of breaking strength at 14 d. No marked difference could be detected between saline and pancreatic juice incubation for SIS four-layer, PPM and BPM after 24 h. As a result of early structural degradation, PDM could only be tested after 24 h of incubation. No marked differences in mechanical resistance in the three different media were detected for PDM after 24 h. Numerical mean values of mechanical testing are shown in Table 3.

Surface structure of the ECMs

SEM of SIS showed a non-directed fibrous and fine surface structure of abluminal surface of the material. On the opposite side, which represents the stratum compactum of the submucosal layer of the porcine bowel wall, the surface appeared dense without a porous aspect (Figure 1). The single layers of the 4-layer SIS were distinguishable in the cross-sectional view. Arrangement of collagenous fibers in PDM appeared to be more directed. Single fibers looked chubby and showed a scaly surface (Figure 2). The collagenous fibers in PPM appeared fine and were assembled almost straight. Porosity of the material was verified by SEM, although some parts of the heterogeneous surface appeared dense (Figure 2). In BPM, SEM showed

Table 3 Mechanical resistance of the biomaterials after incubation in phosphate buffered saline, bile or pancreatic juice (mean ± SE)

	PBS		Bile		Pancreatic juice	
	24 h	14 d	24 h	14 d	24 h	14 d
SIS 4-layer	31.40 ± 4.3	26.03 ± 3.2	13.70 ± 0.9	4.01 ± 0.3	30.20 ± 1.0	14.27 ± 0.6
SIS 1-layer	4.56 ± 1.2	3.42 ± 0.4	3.60 ± 1.6	1.96 ± 0.2	2.56 ± 0.4	2.59 ± 0.4
PDM	1.05 ± 0.2	0.74 ± 0.1	0.81 ± 0.1	Dissolved	0.97 ± 0.1	Dissolved
PPM	19.03 ± 2.9	13.8 ± 4.9	10.45 ± 1.4	2.08 ± 0.6	21.95 ± 7.2	5.23 ± 1.3
BPM	27.13 ± 7.3	31.07 ± 5.2	10.34 ± 1.3	1.48 ± 0.3	30.07 ± 2.2	7.89 ± 1.8

Experiments were carried out in triplicate. PBS: Phosphate buffered saline; SIS: Small-intestinal submucosa; PDM: Porcine dermal matrix; PPM: Porcine pericardial matrix; BPM: Bovine pericardial matrix.

Table 4 Ultrastructural alteration of extracellular matrices incubated in human bile and human pancreatic juice

	Bile				Pancreatic juice[1]			
	Porosity	Surface fibrillar arrangement	Erosion of matrix surface	Σ	Porosity	Surface fibrillar arrangement	Erosion of matrix surface	Σ
SIS 4-layer	3	2	3	8	2	1	1	4
SIS 1-layer	3	2	3	8	2	1	1	4
PPM	1	3	1	5	1	3	3	7
BPM	0	2	2	4	1	2	1	4

Incubation was performed at 37℃ for 7 d. [1]Enzyme concentration: amylase 86040 U/L; lipase 210630 U/L. SIS: Small-intestinal submucosa; PPM: Porcine pericardial matrix; BPM: Bovine pericardial matrix.

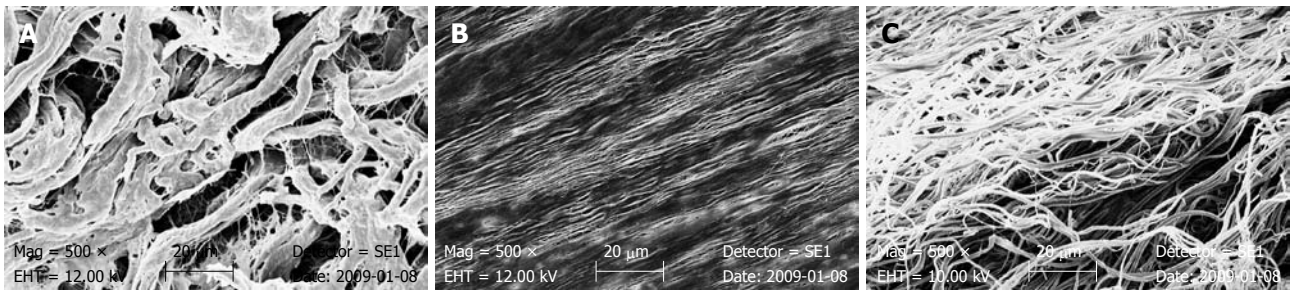

Figure 2 Scanning electron microscopy pictures of the surface structure of the samples with surface characteristics of the different original biomaterials. A: Porcine dermal matrix; B: Porcine pericardial matrix; C: Bovine pericardial matrix. Magnification × 500.

a directed but wormed arrangement of the fibers. Single collagenous fibers appeared fine-structured with a slick surface (Figure 2).

Structural changes after incubation in bile

After 7 d of incubation in bile, the stratum compactum surface of SIS appeared clearly damaged. The dense surface was scarified and a fibrous structure of the deeper parts of the material could be recognized. After biliary incubation in PPM, collagenous fibers were grouped together with a gross scaly surface. The dense surface of the single strands appeared eroded and very fine reticular structures were recognized. In BPM, SEM after 7 d exposure to bile also revealed the phenomenon of collagenous fibrous structures that appeared to be grouped together (Figure 3 and Table 4).

Structural changes after incubation in pancreatic juice

After exposure to pancreatic juice, SEM revealed structural changes in SIS. In the stratum compactum, surface porosity of SIS was increased and the fibrillar structure of the deeper parts was recognizable. Surface structure of the single fibrils and the surface texture of the SIS sheet appeared to be altered. A slight decrease in thickness of the SIS was seen after pancreatic juice incubation for both types of SIS samples in the cross-sectional view. Single layers of four-layer SIS could not be separated in the cross-sectional view. In PPM, the straight arrangement of the collagenous fibers was no longer recognizable after pancreatic juice incubation. The PPM surface appeared dense and partially eroded with a fine undirected fibrillar aspect. In BPM, the fibers of the matrix appeared grouped together with a chubby surface (Figure 3 and Table 4).

DISCUSSION

A prerequisite to the *in vivo* implantation of a biomaterial as a tissue substitute in a gastrointestinal luminal organ is its resistance against gastrointestinal contents, with maintenance of integrity until gastrointestinal healing has progressed and integrity of the host tissue is restored. Physi-

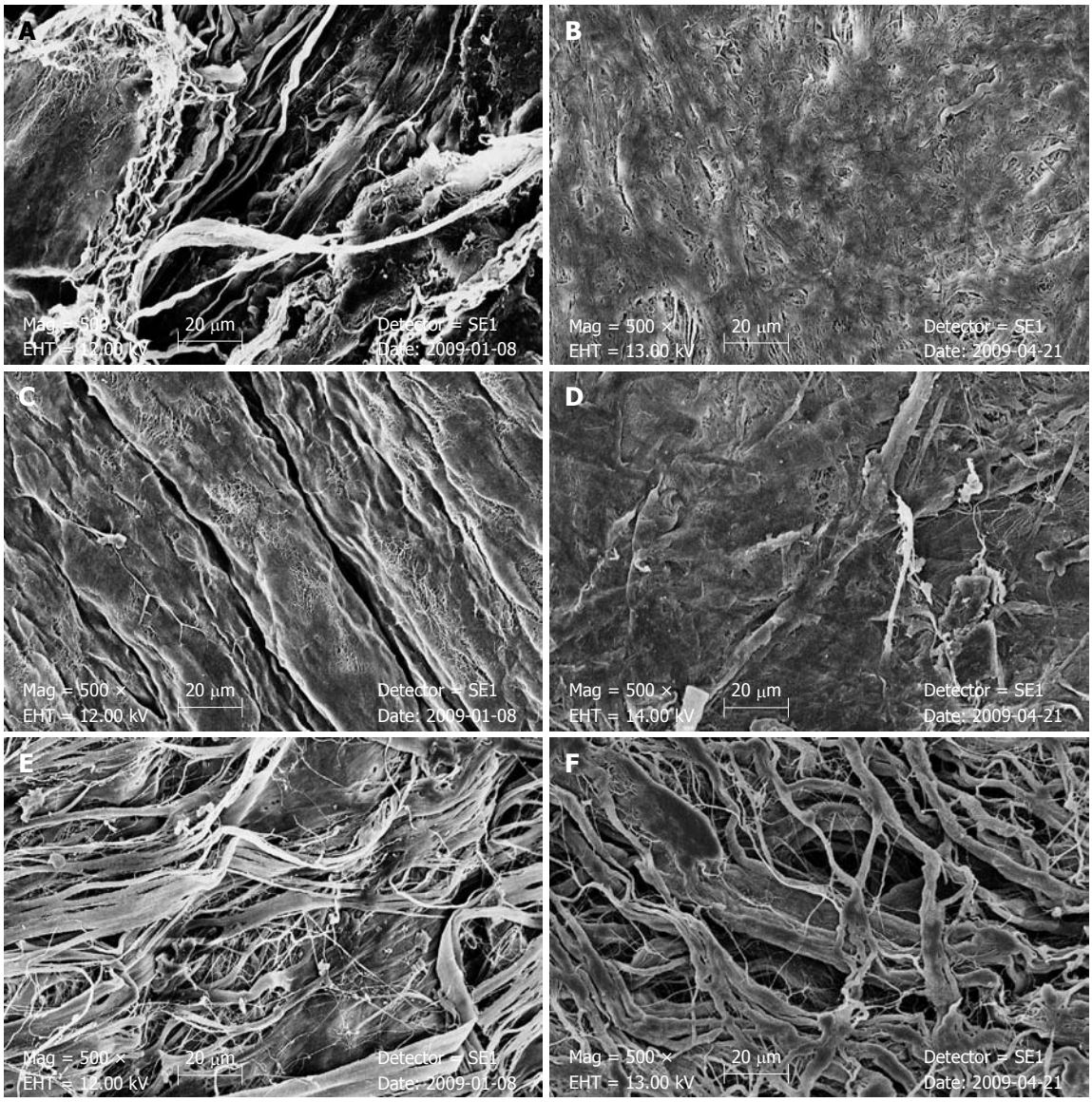

Figure 3 Scanning electron microscopy pictures of small-intestinal submucosa, porcine pericardial matrix and bovine pericardial matrix after 7 d of bile or pancreatic juice incubation. A: Small-intestinal submucosa (SIS) incubated in bile; B: SIS incubated in pancreatic juice; C: Porcine pericardial matrix (PPM) incubated in bile; D: PPM incubated in pancreatic juice; E: Bovine pericardial matrix (BPM) incubated in bile; F: BPM incubated in pancreatic juice.

ological digestive fluids are extremely aggressive substances that are able to destroy most biological tissues. At present, only one experimental study has examined the resistance of ECM against digestive juices[24]. Human placental extracts, human collagen patches, bovine elastin, and bovine collagen matrices were tested for their resistance against bile and pancreatic juice. Only human collagen patches showed sufficient maintenance of integrity, whereas human placental extracts and all bovine materials failed in *in vitro* incubation in bile and pancreatic juice. Recent bioscaffolds like SIS, which have been successfully evaluated for therapy of gastrointestinal fistula, but also have proven potential for gastrointestinal tissue substitution and regeneration at different locations and organs, have so far not been examined systematically for structural and mechanical resistance against digestive contents. This study was designed to examine the effects of biological digestive fluids on the mechanical and structural resistance of current ECM.

It has been shown that ECM, implanted *in vivo* in gastrointestinal luminal organs, is able to induce regenerative responses in the host, and that anatomical tissue structure and tissue function are restored[1-13]. Different expressions of morphological and functional regeneration have been reported in the literature (Table 5). In the past, the majority of experimental work on ECM as a bioscaffold for gastrointestinal regeneration has been performed with SIS[1-13]. Other biological ECMs have only been tested in one single study in an *in vivo* setting in rodents[14]. Nearly complete and anatomical regeneration with SIS as a bioscaffold has been shown for the esophagus and the small bowel. Complete mucosal regeneration and regeneration of muscular layers has been reported within 3-6 mo[2,4,10,11]. After implantation in the rodent stomach, mucosal and muscular

Table 5 Structural regeneration in experimental application of small-intestinal submucosa in the alimentary tract

Autor	Location	Model	Structural regeneration		
			Mucosa	Muscularis	Nerve
Badylak *et al*[4]	Esophagus	Dog	+	+	NR
Lopes *et al*[11]	Esophagus	Rat	+	+	+
de la Fuente *et al*[3]	Stomach	Rat	(+)	-	-
Ueno *et al*[6]	Stomach	Rat	+	+	+
Rosen *et al*[1]	Bile duct	Dog	+	-	NR
De Ugarte *et al*[9]	Duodenum	Rat	(+)	-	NR
Souza Filho *et al*[8]	Duodenum	Dog	(+)	-	NR
Demirbilek *et al*[12]	Jejunum	Rabbit	+	-	NR
Chen *et al*[2]	Small bowel	Dog	+	+	NR
Ansaloni *et al*[10]	Ileum (isolated loop)	Rat	+	+	+
Wang *et al*[13]	Ileum	Rat	+	(+)	NR
Ueno *et al*[5]	Ceacum	Rat	+	+	+
Hoeppner *et al*[7]	Colon	Pig	(+)	(+)	-

+: Complete regeneration; (+): Partial/marginal regeneration; -: Missing regeneration; NR: Not reported.

restoration, along with regeneration of innervation of the stomach wall were evident within 6 mo[6]. In contrast, limited mucosal regeneration and complete absence of muscular regeneration have been reported in the repair of duodenal and lower colonic defects with SIS[7-9]. These differences in SIS-induced gastrointestinal tissue regeneration could be explained by the effects of varying intraluminal chemical and bacterial environments on the integrity and intactness of the collagenous matrix and the 3D structure of the matrix. Biliary and pancreatic enzyme aggression is likely to play a major role in these processes. Toxicity of bile is explained by the detergent capacity of bile salts and alkaline pH of bile. The destructive effects of pancreatic juice on biological tissues and membranes are best explained by its content and the high concentration of enzymes like proteases, glucosidases, elastases and lipases. It is assumed that these enzymes are able to degrade the ECM components that are present in biological and artificial scaffolds[24]. Physiologically, bile and pancreatic juice are slowly secreted and diluted by other intraluminal gastrointestinal contents such as chyme. In our study, ECM was exposed to concentrated and very aggressive media *in vitro*, although they almost never encounter such demanding conditions *in vivo*.

The most pronounced effects of bile and pancreatic juice in our study were seen in PDM. It was dissolved within a few days in both digestive juices. SIS, PPM and BPM maintained their integrity for at least 60 d of incubation in pure bile or pancreatic juice. In ECM of porcine origin, SIS and PPM, the loss of mechanical stability after incubation in the two digestive media was less distinct than in BPM. Our examination revealed bile to be much more efficacious in degrading ECM than pancreatic juice in all tested materials. In SEM, ECM incubated in bile was more eroded than after incubation in pancreatic juice. Moreover, SEM showed obvious changes in the 3D surface structure and arrangement of the collagenous fibers in SIS, PPM and BPM. It is assumed that the 3D structure of the fibrillar collagens and adhesive glycoproteins in the naturally occurring biopolymer SIS are involved in tissue regeneration induced by SIS[25], therefore, it has to be assumed that those changes in 3D structure impair gastrointestinal tissue regeneration. Moreover, destruction of regulatory proteins that are present in SIS, such as fibronectin, heparin sulfate proteoglycan, fibroblast growth factor-2, transforming growth factor-β and vascular endothelial growth factor, by aggressive contents of bile and pancreatic juice could also impair tissue regeneration[25-27]. This is a possible explanation for limited mucosal and missing muscular regeneration in duodenal patch repair by SIS[8,9]. Although nearly complete regeneration of biliary epithelium was reported, no formation of a muscular layer was seen when SIS was used for defect repair of the common bile duct in a canine model[1]. Based on these *in vivo* reports, it can be assumed that in the presence of higher intraluminal concentrations of bile and pancreatic enzymes, in particular, muscular regeneration is impaired if SIS is used as a bioscaffold.

Different clinical studies have reported successful application of SIS as a plug system for the therapy of enterocutaneous fistulas. For this indication, SIS is commercially available as Surgisis AFP Anal Fistula Plug® (Cook Biotech Inc.). For plug repair of anorectal fistulas, Champagne *et al*[17] have reported an overall success rate of 83% in a prospective study in 46 patients with a follow-up of 24 mo. In another prospective examination in 60 patients, Schwandner *et al*[18] have reported effective closure in anorectal fistula systems in 62% of cases, with a follow-up of 12 mo. In both studies, no serious adverse effects like impairment of continence function were reported.

Besides the prospective trials for therapy of anorectal fistulas, only case reports have been published for other enterocutaneous fistulas. Small-bowel-derived fistulas have been reported to be closed successfully in three patients[19,20]. Recently Toussaint *et al*[21] have reported a case series of five patients with gastrocutaneous fistulas after gastric sleeve and gastric bypass with a success rate of 80%. Furthermore, effective closure of persistent gastrocutaneous fistulas after removal of a gastric feeding tube[22], as well as successful therapeutic approaches for rectovaginal and ileal pouch-vaginal fistulas have been reported in case series and reports[28].

Although only experimental work has been published, SIS is commercially available for reinforcement of linear gastrointestinal staple lines (Surgisis Biodesign Staple Line Reinforcement®, Cook Biotech Inc.). It has been proven experimentally that SIS-reinforced staple lines in the porcine small bowel have increased mechanical stability if tested for bursting pressure in small bowel *in vitro*[29] and immediately after application of staple lines *in vivo*[30]. To date, no information is available concerning the effects of SIS in buttressing circular staple lines and its consequences for the intestinal healing process in staple lines. Most experimental and clinical examinations concerning buttressing of staple lines have been carried out on bovine pericardium. Especially in the field of obesity surgery, bovine

pericardium is commercially available and widely used for reinforcement of staple lines. In a prospective randomized trial, Angrisani *et al*[31] have reported significant effects in prevention of staple line bleeding and a reduction of operating time due to dry operating fields compared to non-buttressed staple lines in laparoscopic gastric bypass. However, not only beneficial effects have been reported for bovine pericardium. Ibele *et al*[32] have recently published a retrospective analysis of 500 patients in which buttressing of the circular staple lines with bovine pericardium during laparoscopic Roux-en-Y gastric bypass was associated with an increased staple line leak rate.

Evaluation of ECM for anastomotic reinforcement in terms of sealing of colonic anastomoses by SIS has been experimentally performed in animal models. In rodents, sealing of colonic anastomoses by SIS showed microscopically and mechanically improved intestinal healing in the most vulnerable early phase of anastomotic healing[16]. In the porcine model, although no information about the effects of SIS on early anastomotic healing and long-term effects of SIS on circular stapled colonic anastomoses beyond 30 d was gained, the feasibility and safety of anastomotic sealing by SIS were demonstrated[15].

In summary, most clinical and experimental evaluation of ECM application in gastrointestinal surgery has been performed on SIS. Apart from staple line reinforcement, other biologically derived ECMs have only been rarely examined for gastrointestinal tissues. We therefore compared the mechanical and ultrastructural characteristics of SIS with ECM derived from porcine dermis, porcine pericardium and bovine pericardium in the presence of aggressive gastrointestinal fluids. Compared to SIS and PPM, mechanical resistance of BPM after 2 wk of exposure to bile or pancreatic juice is clearly weaker. These differences, however, could not be reproduced for structural degradation. Therefore, neither ECM of porcine nor bovine origin can be judged as more resistant to one or other of the human digestive juices. Early degradation of PDM in the presence of bile and pancreatic juice could be an explanation for the failure of acellular dermal matrix as a bioscaffold for intestinal regeneration placed in gastrointestinal continuity, whereas after prevention of exposure to bile and pancreatic juice by implantation in a defunctionalized blind jejunal limb, acellular dermal matrix remained sufficient and allowed mucosal regeneration[14]. These findings are important for application of ECM to gastrointestinal luminal organs, because our data suggest that deviation of bile and pancreatic juice upstream from the repair, should be applied in further experimental and clinical testing.

SIS, PPM and BPM retain their integrity in the presence of high concentrations of human bile and pancreatic juice. Ultrastructural degradation with changes in porosity, surface fibrillar arrangement and erosion of matrix surface were detectable in all three ECMs. The extent of ultrastructural alterations in SIS was more pronounced after incubation in bile than in pancreatic juice. In PPM and BPM, these differences were less distinct. Therefore, we conclude that PPM and BPM should also be evaluated as bioscaffolds for intestinal regeneration, as sealing materials for anastomotic reinforcement, and for plug repair of gastrointestinal fistulas in *in vivo* studies. As a result of early dissolution in the presence of digestive juices, PDM is less suitable for application in gastrointestinal luminal organs. To verify the results from *in vitro* testing, the bioscaffolds used should be tested *in vivo* by implantation in different gastrointestinal luminal organs, with and without deviation from bile, pancreatic juice and stool at the site of repair in upcoming experimental studies. Finally, PPM and BPM should also be evaluated experimentally for treatment of gastrointestinal fistula and reinforcement of intestinal anastomoses *in vivo*.

COMMENTS

Background

Extracellular matrices (ECMs) have been introduced for clinical therapy of gastrocutaneous, enterocutaneous and anal fistulas and for buttressing of gastrointestinal staple lines. They have been experimentally evaluated as bioscaffolds for tissue regeneration at different gastrointestinal hollow organs and for reinforcement of gastrointestinal anastomoses.

Research frontiers

ECMs have been tested for different indications and at different locations in the gastrointestinal tract. It is not known if there are any relevant structural and mechanical changes in ECM caused by exposure to digestive juices. *In vivo* models have reported varying degrees of morphological intestinal regeneration after implantation of ECM as a bioscaffold at different gastrointestinal locations. This phenomenon could be explained by the effects of digestive juices on the structural and mechanic traits of the ECM. In this study, the authors investigated the effects of bile and pancreatic juice on different ECMs.

Innovations and breakthroughs

Small-intestinal submucosa (SIS) was found to be mechanically the most resistant tested material. It was demonstrated that porcine dermal matrix (PDM) is not suitable for therapeutic purposes in intestinal tissue regeneration due to its early degradation. However, SIS, porcine pericardial matrix (PPM) and PDM retained their integrity for up to 60 d when exposed to bile and pancreatic juices. Ultrastructural alterations were found to be more important in SIS when exposed to the juices.

Applications

As a result of their proven ultrastructural and mechanical resistance, PPM and bovine pericardial matrix (BPM) should be evaluated for treatment of gastrointestinal fistulas, as bioscaffolds for intestinal regeneration, and for reinforcement of intestinal anastomoses *in vivo*.

Terminology

ECMs are biodegradable, acellular collagen matrices that are derived from biological tissues. SIS is extracted from the submucosal layer of porcine small bowel. PDM is extracted from the porcine dermis. PPM and BPM are extracted from porcine and bovine pericardia.

Peer review

In general, this is an interesting study with new perspectives on various biomatrices that can be used for intestinal regeneration. These analyses could have an impact on the development of therapeutic approaches in the field of bioengineering.

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ORIGINAL ARTICLE

Curcumin suppresses gastric NF-κB activation and macromolecular leakage in *Helicobacter pylori*-infected rats

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Abstract

AIM: To investigate whether curcumin could attenuate nuclear factor (NF)-κB p65 expression and macromolecular leakage in the gastric mucosa of *Helicobacter pylori* (*H. pylori*)-infected rats.

METHODS: Twenty-five male Sprague-Dawley rats were equally divided into five groups: control rats (Control), control rats supplemented with 600 mg/kg curcumin, *H. pylori*-infected rats (*Hp*), *H. pylori*-infected rats supplemented with 200 mg/kg curcumin (*Hp* + cur Ⅰ), and *H. pylori*-infected rats supplemented with 600 mg/kg curcumin (*Hp* + cur Ⅱ). In *H. pylori*-infected groups, rats were inoculated with *H. pylori* suspension twice a day at an interval of 4 h for 3 d. Two weeks later, 200 or 600 mg/kg curcumin was given once daily to curcumin-supplemented groups for 7 d. On the day of the experiment, macromolecular leakage in gastric mucosa was examined by intravital fluorescence microscopy. The stomach tissue was removed to examine NF-κB p65 expression in gastric epithelial cells by immunohistochemistry.

RESULTS: The expression of NF-κB p65 in gastric epithelial cells and the macromolecular leakage from gastric mucosal microcirculation significantly increased in the *Hp* group compared with the Control group. The percentages of NF-κB p65 immunoreactive cells in Control and *Hp* groups were 10.72% ± 2.10% *vs* 16.02% ± 2.98%, $P = 0.004$, respectively. The percentages of macromolecular leakage in Control and *Hp* groups were 10.69% ± 1.43% *vs* 15.41% ± 2.83%, $P = 0.001$, respectively. Curcumin supplementation in *Hp* + cur Ⅰ and *Hp* + cur Ⅱ groups significantly decreased NF-κB p65 immunoreactive cells and macromolecular leakage compared with results in the *Hp* group. The percentages of NF-κB p65 immunoreactive cells in *Hp* + cur Ⅰ and *Hp* + cur Ⅱ groups were 11.79% ± 2.13% ($P = 0.017$) and 11.42% ± 1.68% ($P = 0.010$), respectively. The percentages of macromolecular leakage in *Hp* + cur Ⅰ and *Hp* + cur Ⅱ groups were 12.32% ± 2.13% ($P = 0.025$) and 12.14% ± 1.86% ($P = 0.018$), respectively.

CONCLUSION: *H. pylori*-induced gastric inflammation in rats is associated with increased NF-κB activation and macromolecular leakage which can be reduced by curcumin supplementation.

Key words: Curcumin; *Helicobacter pylori*; Nuclear factor-κB p65; Macromolecular leakage

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral-shaped Gram-negative bacterium. The infection causes chronic gastritis and peptic ulcer diseases in patients. *H. pylori* infection is also related to mucosa-associated lymphoid tissue lymphoma and gastric cancer diseases in patients[1,2]. The pathogenesis of *H. pylori* infection is associated with the bacterial virulence factors. After *H. pylori* bacteria adhere to gastric epithelial cells, they inject their virulence factors into the host cells *via* a type Ⅳ secretory system[3]. The virulence factors can induce the activation of nuclear factor (NF)-κB in gastric epithelial cells[4].

NF-κB is an important regulator of many cellular processes including the control of the immune response and inflammation[5,6]. NF-κB is a dimeric complex composed of the five mammalian Rel proteins, p65, c-Rel, p50/NF-κB1, p52/NF-κB2, and RelB, in almost any combination. In resting cells, the inhibitors of NF-κB (IκB) form complexes with NF-κB. Upon stimulation, specific intracellular signalling pathways are activated, leading to the activation of the IκB kinase complex (IKK complex). The activated IKK complex phosphorylates the IκB at specific amino acids for the poly-ubiquitination of these NF-κB inhibitors. The ubiquitination of IκB and its subsequent degradation by a proteasome are required for NF-κB activation. NF-κB is now free to translocate into the nucleus and regulate NF-κB-dependent gene expression[7]. The target of activated NF-κB includes the genes encoding proinflammatory cytokines and chemokines that are the causes of *H. pylori*-induced gastric inflammation[4,8,9].

In *H. pylori*-associated gastric inflammation, inflammatory mediators could induce vascular damage. A previous study demonstrated that *H. pylori*-infected patients showed erythema, edema, and vasodilation as well as neutrophil infiltration in the mucosa[10]. Our previous study suggested that leukocyte adhesion in postcapillary venules was increased in *H. pylori*-infected rats. Moreover, the degree of leukocyte adhesion was correlated with the level of the proinflammatory cytokine, tumor necrosis factor (TNF)-α[11]. In addition, previous studies have demonstrated that water-soluble extracts of *H. pylori* induced leakage of macromolecules from rat gastric mucosal microcirculation[12-14].

Curcumin (diferuloylmethane) is an active ingredient of *Curcuma longa* (turmeric) and is pharmacologically safe for human and animals. Curcumin has many biological activities, including anti-inflammatory properties[15]. Most of the anti-inflammatory effects can be explained by the efficient inhibition of NF-κB mediated by this substance[16-18]. Recently, a previous study showed that curcumin can inhibit NF-κB activation in *H. pylori*-infected gastric epithelial cells[19]. Curcumin is also a potent antibacterial agent against *H. pylori* as shown in *in vitro* study[20]. In contrast, curcumin did not eradicate *H. pylori* in *H. pylori*-infected patients[21].

However, it is not clear whether curcumin has any *in vivo* effects in *H. pylori*-induced gastric inflammation. Therefore, we examined the anti-inflammatory effect of curcumin, which may reduce mucosal macromolecular leakage through the suppression of gastric epithelial NF-κB p65 expression induced by *H. pylori* infection in rats.

MATERIALS AND METHODS

Experimental design

Male Sprague-Dawley rats (National Laboratory Animal Center, Mahidol University, Nakorn pathom, Thailand) weighing 200-250 g were used. All experiments and procedures carried out on the animals were approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Rats were housed in a controlled temperature room at 25 ± 1℃ under standard conditions (12-h day-night rhythm). Twenty-five rats were divided into five groups (five rats each) as follows.

Control rats (Control): rats were fed normal saline (1 mL/rat) orally *via* intragastric tube twice a day at an interval of 4 h for 3 consecutive days. Two weeks later, 0.1% DMSO (1 mL/rat) was given once daily to the rats by intragastric tube for 7 d.

Control rats supplemented with 600 mg/kg curcumin (Cur): rats were fed normal saline (1 mL/rat) orally *via* intragastric tube twice a day at an interval of 4 h for 3 consecutive days. Two weeks later, 600 mg/kg curcumin (95% purified curcumin, Cayman Chemical, Ann Arbor, MI, USA) dissolved in 0.1% DMSO (1 mL/rat) was given once daily to the rats by intragastric tube for 7 d.

H. pylori-infected rats (*Hp*): rats were inoculated with *H. pylori* suspension according to Thong-Ngam *et al*[22]. Briefly, *H. pylori* suspension (10^{10} CFU/mL; 1 mL/rat) was given to the rats by intragastric tube twice a day at an interval of 4 h for 3 consecutive days. Two weeks later, 0.1% DMSO (1 mL/rat) was given once daily to the rats by intragastric tube for 7 d.

H. pylori-infected rats supplemented with 200 mg/kg curcumin (*Hp* + cur Ⅰ): 2 wk after *H. pylori* inoculation, 200 mg/kg curcumin dissolved in 0.1% DMSO (1 mL/rat) was given once daily to the rats by intragastric tube for 7 d.

H. pylori-infected rats supplemented with 600 mg/kg curcumin (*Hp* + cur Ⅱ): 2 wk after *H. pylori* inoculation, 600 mg/kg curcumin dissolved in 0.1% DMSO (1 mL/rat) was given once daily to the rats by intragastric tube for 7 d.

H. pylori

H. pylori strains used for all experiments were originally

obtained from peptic ulcer patients who visited the King Chulalongkorn Memorial Hospital. The bacteria were grown in Brucella broth (pH 7.0) supplemented with 10% goat serum for 24 h at 37℃ in an automatic CO_2-O_2 incubator (85% N_2, 10% CO_2, and 5% O_2).

Animal preparation

The method of preparing animals for *in vivo* fluorescent microscopy was adapted from a previous study[14]. The animal was anesthetized with thiopental (General Drug House, Thailand; 60 mg/kg, intraperitoneal). A constant level of anesthesia was maintained throughout the experiment by a supplement dose (20% of original dose) every 30-45 min[11]. A tracheotomy was performed. The arterial blood pressure was recorded in the common carotid artery using a pressure transducer (Nihon Kohden, Tokyo, Japan). The abdominal cavity was opened *via* a midline laparotomy. A 1.0 cm incision was made using an electrical microcautery device (Hyfrecator plus®, Conmed, Utica, NY, USA) at the posterior wall, being parallel to the "limiting ridge" of the exteriorized stomach[12]. Next, the stomach was gently extended and placed on a designed board. The incision in the anterior wall was opened using microclamps and covered with Saran wrap to allow visualization of the posterior mucosal surface[12]. The animals were terminated after studying intravital fluorescent videomicroscopy.

Intravital fluorescent videomicroscopy

Observations were made from the glandular portion of the posterior mucosa. Fluorescence tracer [0.3 mL of 0.5% fluorescein isothiocyanate (FITC)-labeled dextran (FITC-dx, MW = 250 000, Sigma-Aldrich, USA)] was injected into the jugular vein[23]. The posterior mucosal microcirculation was visualized under an intravital fluorescence videomicroscope (Nikon Optiphot-2, Nikon, Tokyo, Japan), and examined under × 20 objective lens (Nikon). The selected area included the characteristic honeycomb-like network of mucosal capillaries and at least one postcapillary venule (PCV; diameter 15-30 μm)[24]. Five minutes after FITC-dx administration, a recording was performed as a baseline using a video-recorder (Sony SVT-124p, Sony, Tokyo, Japan). Thirty minutes later, recording was performed again.

Measurement of macromolecular leakage in gastric mucosa

Based on the recorded video images, we measured macromolecular leakage from the PCV in the selected area. Computerized image analysis (GLOBAL LAB® image Ⅱ program, USA) was used to measure fluorescence intensity in the interstitial space and in the PCV at both time points during the experiment. The fluorescence intensities between outside and inside vessels (I_{out}/I_{in}) at baseline and 30-min time points were measured[25]. The molecular leakage in percentage was calculated using the equation: Macromolecular leakage (%) = [(I_{out}/I_{in}) at 30 min - (I_{out}/I_{in}) at baseline]/[(I_{out}/I_{in}) at baseline] × 100.

Assessment of H. pylori infection

The presence of *H. pylori* infection in individual rats was determined by urease test and histological examination by a pathologist. After studying intravital fluorescent microscopy, the rat was terminated by injection of an overdose of thiopental. Then the stomach was removed and longitudinally dissected along the greater curvature. A 2 mm^3 segment of gastric mucosa from the antrum was immediately cut and placed in the urease test tube.

Regarding histological examination, the stomach was fixed with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 at room temperature for 24 h. The tissue was processed, embedded in paraffin, and cut at 5 μm thickness. The sections were stained with hematoxylin and eosin, and microscopically examined for the presence of *H. pylori*. The presence of *H. pylori* was also detected by Warthin-Starry staining in unclear cases. The level of bacterial colonization was recorded by using a grading system as follows, score 0: no bacteria detected; score 1: mild colonization in some gastric crypts; score 2: mild colonization in most gastric crypts; score 3: moderate colonization in all gastric crypts; and score 4: dense colonization in some gastric crypts. The results were presented as the bacterial colonization scores for each group. Moreover, gastric inflammation was classified by using the Sydney system[26]. The infiltrations of mononuclear and polymorphonuclear leukocytes in the gastric mucosa defining the inflammatory scores were recorded. Score 0 to 3 represented normal, mild, moderate, and marked histopathology changes, respectively.

Immunohistochemistry

The stomach sections were deparaffinized with xylene and gradually dehydrated in ethyl alcohol. Next, antigen retrieval was performed by immersing the sections in citric acid buffer (pH 6.0) in a microwave oven for 13 min. Endogenous peroxidase activity and nonspecific binding were blocked with 3% hydrogen peroxide (Merck, Hohenbrunn, Germany) for 5 min and 3% normal horse serum (Gibco, Carlsbad, CA, USA) for 20 min, respectively. After that, the sections were incubated with polyclonal antibody against the p65 subunit of NF-κB (sc109; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100 in a humidified chamber for 1 h at room temperature. Then the sections were incubated with biotinylated anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark) in the humidified chamber for 30 min. The reaction was visualized using the substrate diaminobenzidine (DAKO). The sections were then counterstained with hematoxylin.

Under light microscope, the expression of NF-κB p65 was cytoplasmic with scattered positive nuclear staining[27]. Thus, NF-κB p65 immunoreactive cells were defined as those with dark brown stain in their nuclei. To quantify, one thousand gastric epithelial cells were counted for each rat under × 40 objective lens. All counting was manually performed by an investigator who was blinded to the treatment groups. The data were shown as percentage of immunoreactive cells calculated from this equation: Percentage of immunoreactive cells (%) = (number of immunoreactive cells × 100)/1000.

Table 1 Demonstrated level of *Helicobacter pylori* colonization and gastric inflammation scores

Group	No.	Level of *H. pylori* colonization					Score of gastric inflammation			
		0	1	2	3	4	0	1	2	3
Control	5	5	-	-	-	-	5	-	-	-
Cur	5	5	-	-	-	-	5	-	-	-
Hp	5	-	5	-	-	-	-	3	2	-
Hp + cur Ⅰ	5	-	5	-	-	-	3	2	-	-
Hp + cur Ⅱ	5	-	3	2	-	-	3	2	-	-

Level of *Helicobacter pylori* (*H. pylori*) colonization: Score 0 = no *H. pylori* detected; Score 1 = mild colonization in some gastric crypts; Score 2 = mild colonization in most gastric crypts; Score 3 = moderate colonization in some gastric crypts; Score 4 = dense colonization in some gastric crypts. Score of gastric inflammation: Score 0 = normal gastric tissue; Score 1 = mild inflammation and histopathology changes; Score 2 = moderate inflammation and histopathology changes; Score 3 = marked inflammation and histopathology changes. Control: Control rats; Cur: Control rats supplemented with 600 mg/kg curcumin; *Hp*: *H. pylori*-infected rats; *Hp* + cur Ⅰ: *H. pylori*-infected rats supplemented with 200 mg/kg curcumin; *Hp* + cur Ⅱ: *H. pylori*-infected rats supplemented with 600 mg/kg curcumin.

Statistical analysis

All data were presented as mean ± SD. The means were compared by one-way analysis of variance (one-way ANOVA) followed by LSD post hoc test. Correlation between the percentages of NF-κB p65 immunoreactive cells and macromolecular leakage was analyzed using Pearson's correlation. All the statistical tests were performed using the computer program SPSS, version 13.0, for Windows (SPSS Inc., Chicago, IL, USA). Probability value of less than 0.05 was considered to be statistically significant.

RESULTS

H. pylori colonization and histological changes

H. pylori infection in rats was judged based on a urease test and histological examination. From histological examination, *H. pylori* colonization was observed in the *Hp* group (Figure 1) as well as in the *Hp* + cur Ⅰ and *Hp* + cur Ⅱ groups, whereas colonization was not observed in the Control and Cur groups. Figure 2 demonstrates histological differences between Control and *Hp* groups. The data are shown in Table 1.

Effects of *H. pylori* infection on NF-κB p65 expression and role of curcumin

NF-κB p65 expression in gastric epithelial cells was studied using immunohistochemistry (Figure 3). *H. pylori* infection increased NF-κB p65 expression in gastric epithelial cells. The percentage of immunoreactive cells significantly increased in the *Hp* group (16.02% ± 2.98%) compared with the Control group (10.72% ± 2.1%, $P = 0.004$) (Figure 4A).

The expression of NF-κB p65 in gastric epithelial cells was diminished by curcumin supplementation in both *Hp* + cur Ⅰ and *Hp* + cur Ⅱ groups. The percentage of immunoreactive cells significantly decreased in *Hp* + cur Ⅰ (11.79% ± 2.13%, $P = 0.017$) and *Hp* + cur Ⅱ (11.42% ± 1.68%, $P = 0.010$) compared with the *Hp* group (Figure 4A). However, there was no significant difference between the number of immunoreactive cells in *Hp* + cur Ⅰ and *Hp* + cur Ⅱ. Curcumin administration in the Cur group did not alter the baseline NF-κB p65 expression (Cur group, 9.47% ± 3.46%, $P = 0.447$) in gastric epithelial cells.

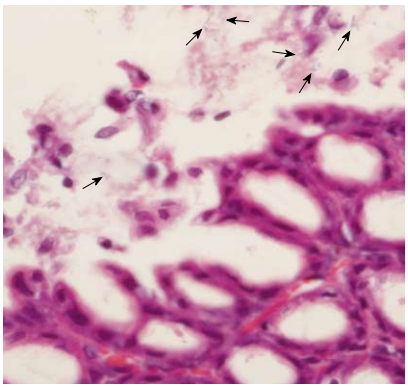

Figure 1 Histological examination (HE stain, × 1000) of *Helicobacter pylori*-infected rats. *Helicobacter pylori* (arrows) in the gastric mucosa identified by the pathologist.

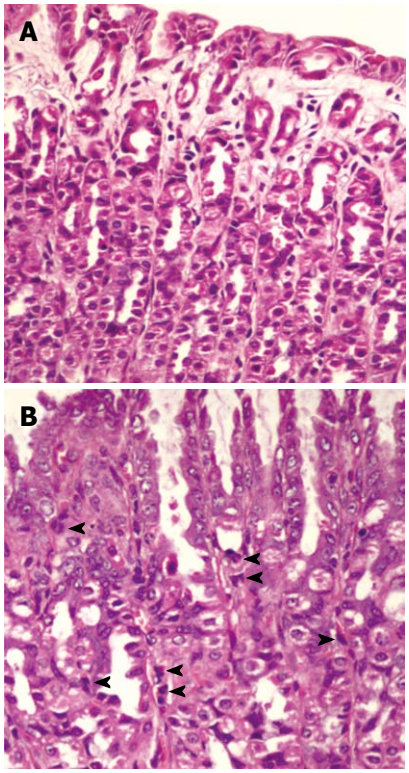

Figure 2 Pathological changes of rat gastric mucosa in control (A) and *Helicobacter pylori*-infected rats (B) (HE stain, × 400). A: Normal gastric mucosa; B: *Helicobacter pylori*-infected gastric mucosa showing inflammatory cell infiltration in the lamina propria (arrowheads).

Effects of *H. pylori* infection on macromolecular leakage and role of curcumin

The macromolecular leakage was studied by intravital fluorescent videomicroscopy. The captured images of

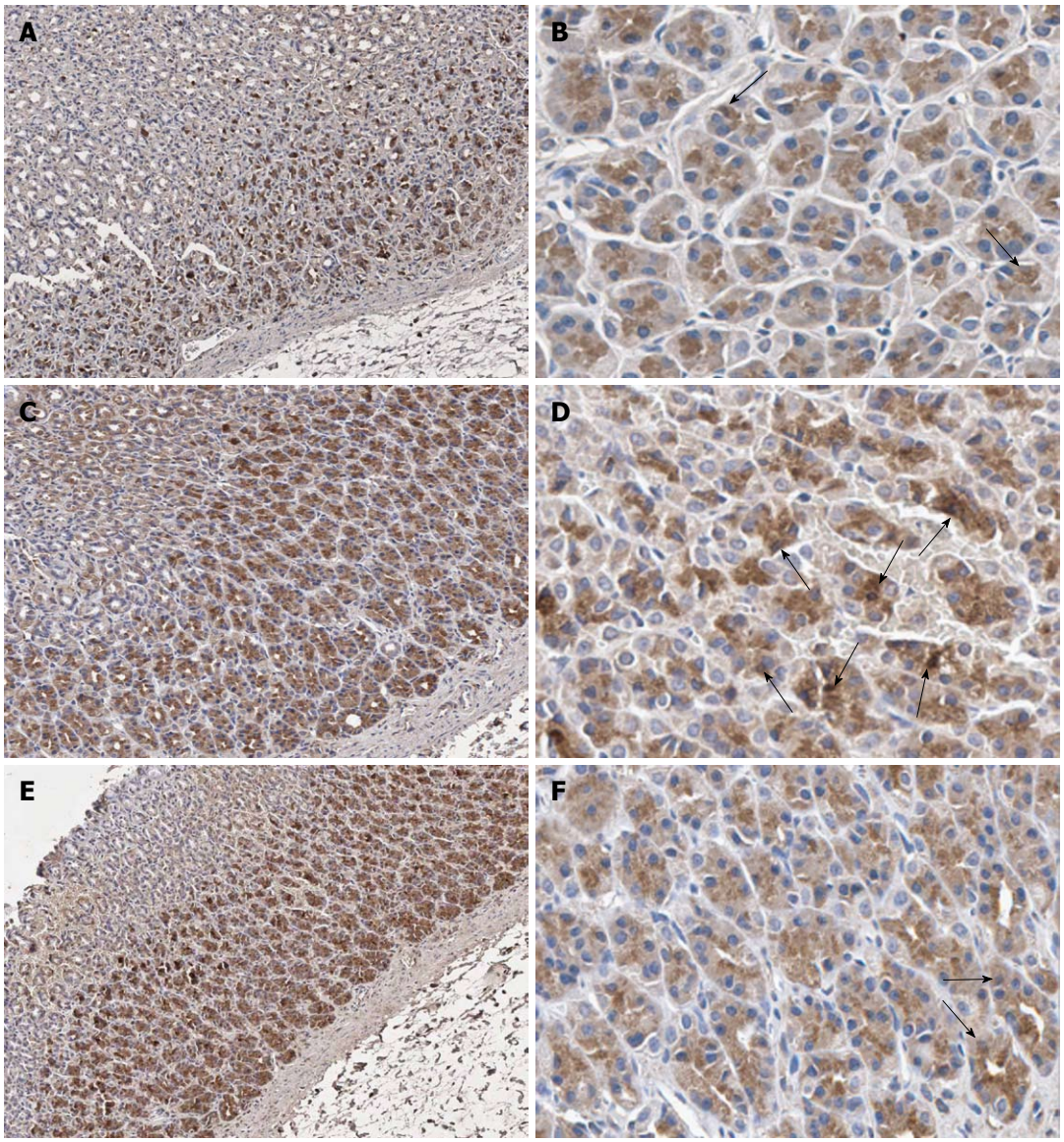

Figure 3 Immunohistochemical staining of nuclear factor-κB p65 antibody in representative tissue specimens. A, B: Control rats; C, D: *Helicobacter pylori* (*H. pylori*)-infected rats; E, F: *H. pylori*-infected rats supplemented with 200 mg/kg curcumin. Nuclear counterstaining was performed with hematoxylin. The examples of immunoreactive cells are those with dark brown stain in their nuclei (arrows). Images were obtained at × 100 (A, C and E) and × 400 (B, D and F).

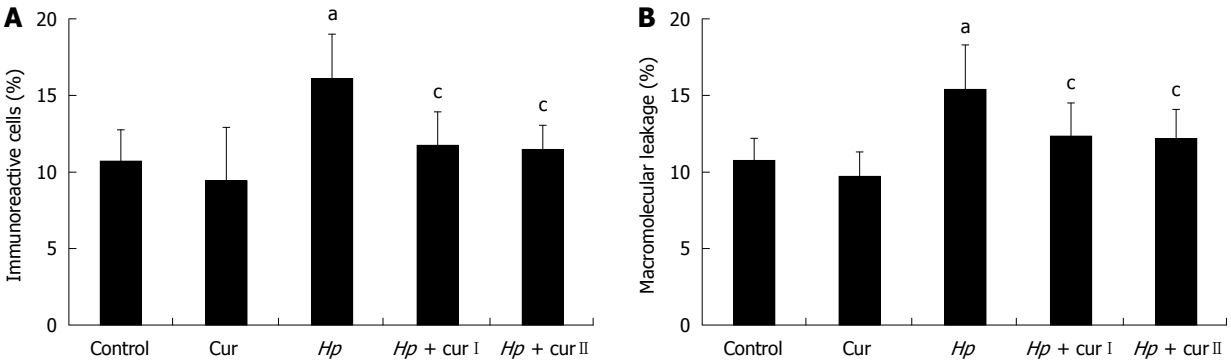

Figure 4 mean ± SD of the percentage of nuclear factor-κB p65 immunoreactive cells (A) and macromolecular leakage (B) in all experimental groups. $^{a}P < 0.05$ *vs* control rats (Control); $^{c}P < 0.05$ *vs Helicobacter pylori* (*H. pylori*)-infected rats (*Hp*). Cur: Control rats supplemented with 600 mg/kg curcumin; *Hp* + cur Ⅰ: *H. pylori*-infected rats supplemented with 200 mg/kg curcumin; *Hp* + cur Ⅱ: *H. pylori*-infected rats supplemented with 600 mg/kg curcumin.

gastric mucosal microcirculation from Control, *Hp*, and *Hp* + cur I groups at the 30-min time point are shown in Figure 5. *H. pylori* infection led to a significant increase of macromolecular leakage in the *Hp* group (15.41% ±

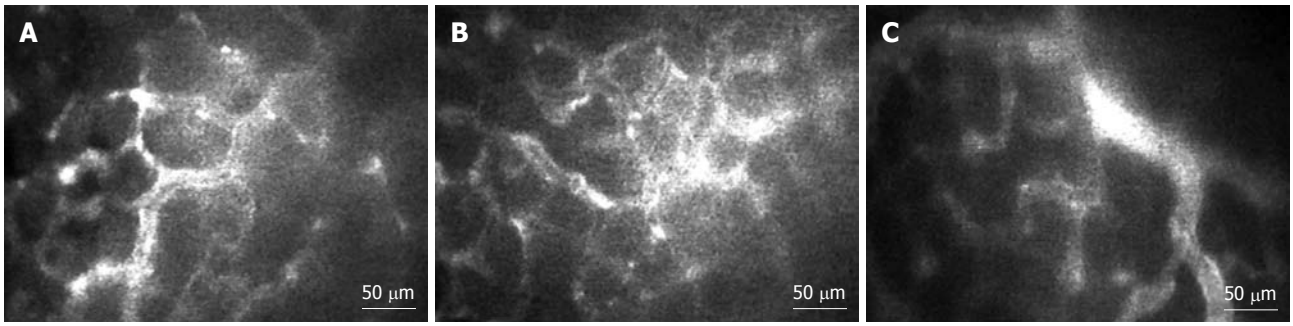

Figure 5 Intravital fluorescent microscopic images (× 200) demonstrate macromolecular leakage from vessels to the interstitial space at 30-min time points. A: Control rats; B: *Helicobacter pylori* (*H. pylori*)-infected rats; C: *H. pylori*-infected rats supplemented with 200 mg/kg curcumin.

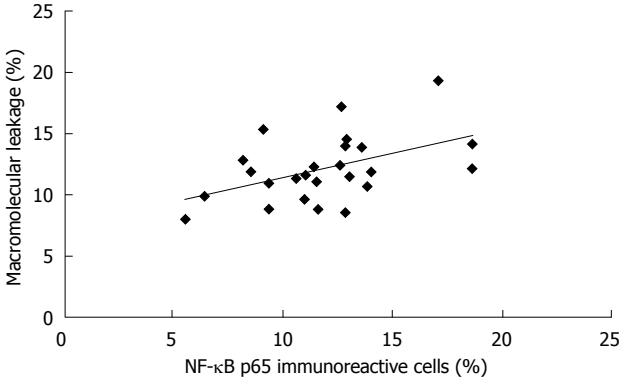

Figure 6 Correlation between the percentage of nuclear factor-κB p65 expression in gastric epithelial cells and the macromolecular leakage (r^2 = 0.2228, P = 0.017). NF-κB: Nuclear factor-κB.

2.83%) compared with the Control group (10.69% ± 1.43%, P = 0.001) (Figure 4B).

Oral treatment with curcumin over 1 wk attenuated *H. pylori* infection-induced macromolecular leakage significantly in both *Hp* + cur I (12.32% ± 2.13%, P = 0.025) and *Hp* + cur II (12.14% ± 1.86%, P = 0.018) groups (Figure 4B). However, there was no significant difference between the extent of macromolecular leakage in *Hp* + cur I and *Hp* + cur II. In addition, macromolecular leakage showed no significant differences between the Control group and the Cur group (Cur group, 9.74% ± 1.5%, P = 0.463).

Relationship between NF-κB p65 expression and macromolecular leakage

The percentages of NF-κB p65 immunoreactive cells and of macromolecular leakage from the same rat in all groups were plotted against each other (Figure 6). Interestingly, the level of NF-κB p65 expression was moderately correlated with the degree of macromolecular leakage (r^2 = 0.2228, P = 0.017).

DISCUSSION

The present study demonstrated that *H. pylori* infection activated NF-κB in gastric epithelial cells. This result corresponds to previous observations studied in both *in vitro* and *in vivo* models[4,19,28]. NF-κB is an important transcription factor which activates many genes involved in inflammatory and immune responses[5,6]. Activated NF-κB induced by *H. pylori* infection upregulates cytokine production and is associated with gastric inflammation[9]. Clinically, NF-κB was also seen to be activated in the stomach of patients with *H. pylori*-induced gastritis[29-31]. Therefore, gastric epithelial NF-κB activation may play an important role in the initiation of *H. pylori*-induced gastric inflammation.

From the results, this study can also demonstrate an increased macromolecular leakage after *H. pylori* infection. This alteration is in good agreement with previous reports[12,14]. Several mechanisms may contribute to the increased macromolecular leakage. A previous study demonstrated that the transmigration of activated neutrophils expressing a specific protein could regulate endothelial permeability, allowing macromolecules to leak[32]. Furthermore, many proinflammatory cytokines such as TNF-α and interleukin (IL)-1β could be directly modulating vascular permeability[33-35]. Neutrophil transmigration and proinflammatory cytokine production were also suggested in a NF-κB-dependent manner in gastric mucosa during *H. pylori* infection[4,8,36-38]. *H. pylori* infection may directly influence the leakage *via* the transportation of *H. pylori* toxin and activation of NF-κB in endothelial cells. Previously, *in vitro* studies indicated that endothelial cells infected with *H. pylori* have changes in protein expression and function[39,40]. Our results substantiate these findings by showing that increased NF-κB p65 expression in gastric epithelial cells is accompanied by increased macromolecular leakage during *H. pylori* infection. Thus, the increased macromolecular leakage may result from inflammatory mediator production and vascular permeability changes through *H. pylori*-induced NF-κB activation.

Our experiments show that curcumin supplementation can suppress *H. pylori*-induced gastric inflammation, as indicated by decreased NF-κB p65 expression in gastric epithelial cells and decreased macromolecular leakage in the gastric microcirculation. The activation of NF-κB is essential for transcription of many genes involved in inflammatory and immune responses influencing gastric inflammation induced by *H. pylori* infection. In the present study, NF-κB p65 expression in the nucleus indicated that curcumin may possibly suppress the translocation of activated NF-κB into transcriptional sites. *H. pylori*-

induced NF-κB activation affects recruitment of neutrophils and vascular permeability that reflect gastric inflammation. Curcumin decreased these parameters, indicating that curcumin could decrease gastric inflammation.

Inhibition of *H. pylori* growth was unlikely to be a mechanism that contributed to the effect of curcumin observed in this study, since positive results regarding *H. pylori* infection were still obtained from both urease test and histological examination after curcumin treatment in *Hp* + cur I and *Hp* + cur II animals. Recently, Di Mario *et al*[21] demonstrated that 7-d treatment with curcumin significantly improved gastric inflammation in *H. pylori*-positive patients despite *H. pylori* persistence. However, eradication by curcumin may be dependent on a high dose of curcumin, the safety of which has to be confirmed in animals and humans[41].

A previous study demonstrated that curcumin at the doses of 200 mg/kg and 600 mg/kg had an anti-inflammatory property[42]. In this study, 200 mg/kg curcumin was a sufficient dose for reducing gastric epithelial NF-κB p65 expression and mucosal macromolecular leakage. The possible mechanism cited was that curcumin inhibited *H. pylori*-induced NF-κB activation. This finding corresponded to an earlier *in vitro* study showing that *H. pylori*-induced NF-κB activation and the subsequent release of IL-8 were inhibited by curcumin[19]. In addition, the correlation between NF-κB p65 expression and macromolecular leakage found in our study suggests that *H. pylori*-induced mucosal macromolecular leakage may be mediated *via* NF-κB activation in gastric epithelial cells. Thus, the decreased macromolecular leakage may be explained by the reduction of inflammatory mediators due to epithelial NF-κB inhibition by curcumin.

In conclusion, the present study showed that *H. pylori* infection induced gastric epithelial NF-κB activation and increased mucosal macromolecular leakage. Curcumin supplementation may exert its anti-inflammatory effect by reducing macromolecular leakage through the suppression of NF-κB p65 expression in gastric epithelial cells. Hence, curcumin might be a novel therapeutic strategy against gastric inflammation induced by *H. pylori* infection.

COMMENTS

Background

The pathogenesis of *Helicobacter pylori* (*H. pylori*) infection is associated with bacterial virulence factors. The virulence factors can induce the activation of nuclear factor (NF)-κB in gastric epithelial cells, causing gastric inflammation and inducing vascular damage. Curcumin has many biological activities, including anti-inflammatory properties resulting from inhibition of NF-κB.

Research frontiers

Curcumin (diferuloylmethane) is an active ingredient of *Curcuma longa* (turmeric) that has many biological activities mediated by the efficient inhibition of NF-κB. *H. pylori* infection induces gastric epithelial NF-κB activation and increases mucosal macromolecular leakage. The hotspots of this study indicate that curcumin supplementation may exert its anti-inflammatory effect by reducing macromolecular leakage through the suppression of NF-κB p65 expression in gastric epithelial cells.

Innovations and breakthroughs

A previous study showed that curcumin is a potent antibacterial agent against *H. pylori* and can inhibit NF-κB activation in *H. pylori*-infected gastric epithelial cells *in vitro*. However, it is not clear whether curcumin has any *in vivo* effects on *H. pylori*-induced gastric inflammation. Therefore, in this study, the authors examined the anti-inflammatory effect of curcumin, which was shown to reduce mucosal macromolecular leakage through the suppression of gastric epithelial NF-κB p65 expression induced by *H. pylori* infection *in vivo* in rats.

Applications

Curcumin might be a novel therapeutic strategy against gastric inflammation induced by *H. pylori* infection.

Peer review

The merits of the manuscript are in showing the conclusion that curcumin can reduce the gastric inflammation reflected in attenuated levels of NF-κB and leakage of dextran.

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ORIGINAL ARTICLE

High expression of ErbB2 contributes to cholangiocarcinoma cell invasion and proliferation through AKT/p70S6K

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Abstract

AIM: To compare the impact of ErbB2 on cell invasion and proliferation in cholangiocarcinoma (CCA) cell lines.

METHODS: Level of endogenous ErbB2 expression in three CCA cell lines, namely HuCCA-1, KKU-100 and KKU-M213, was determined by real-time reverse-transcriptase polymerase chain reaction. Two ErbB2 inhibitory methods, a small molecule ErbB2 kinase inhibitor (AG825) and siRNA, were used to disrupt ErbB2 function in the cell lines. CCA cell invasion, motility and proliferation under ErbB2-disrupted conditions were detected using Transwell and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. In addition, ErbB2 downstream effectors were investigated by Western blotting analysis.

RESULTS: Suppression of ErbB2 activity, using a specific kinase inhibitor (AG825), reduced invasion, motility and proliferation of all three CCA cell lines. The ability of this drug to inhibit neoplastic properties (invasion, motility and proliferation) increased concomitantly with the level of ErbB2 expression. Similarly, knockdown of ErbB2 level by siRNA inhibited cell invasion and proliferation of KKU-M213, a high-ErbB2-expressing cell, better than those of the lower-ErbB2-expressing cells, HuCCA-1 and KKU-100. Thus, both inhibitory methods indicated that there is more ErbB2-dependency for malignancy of the high-ErbB2-expressing cell, KKU-M213, than for that of low-ErbB2-expressing ones. In addition, interrupting ErbB2 activity decreased phosphorylation of AKT and p70S6K, but not extracellular signal-regulated kinase 1/2, in the high-ErbB2-expressing CCA cell line.

CONCLUSION: Our data indicated that high ErbB2 expression enhances CCA invasion, motility and proliferation *via* the AKT/p70S6K pathway, which suggests the possibility of targeting these molecules for CCA therapy.

Key words: AKT; Cholangiocarcinoma; ErbB2; Invasion; p70S6K; Cell proliferation

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INTRODUCTION

Cholangiocarcinoma (CCA) is an incurable and lethal cancer. Its incidence has been increasing worldwide during the past three decades and all cases have almost the same mortality rate[1]. Strikingly, the highest incidence of CCA

has been found in Northeastern Thailand (96 per 100 000 men), followed by China and Japan[2]. As a result of the lack of a specific tumor marker and its silent symptoms, this tumor is difficult to diagnose and the majority of patients present at the late stage (stage Ⅲ or Ⅳ) of disease progression[3]. From 70% to 80% of CCA patients are inoperable and are generally treated by chemotherapy and radiation, which unfortunately have no significant impact on long-term survival[3]. Thus, new diagnostic/prognostic markers and targeted therapies for CCA need to be investigated.

Human epidermal growth factor receptor (EGFR) 2, ErbB2 (also known as HER2/Neu), belongs to subclass I of receptor tyrosine kinases in the ErbB family. Unlike other members of the ErbB family, the conformation of the ErbB2 ectodomain is similar to the ligand-activated state of other ErbBs[4]. Therefore, activation of ErbB2 can occur without ligand binding[5]. Overexpression of ErbB2 results in receptor dimerization (either homodimerization with its own or heterodimerization with other ErbBs). Upon dimerization, cytoplasmic domains of ErbBs become autophosphorylated and act as docking sites for downstream proteins that bear an Src-homology 2 or a phosphotyrosine-binding domain, which recognizes specific phosphorylated tyrosine sites in the receptors, and leads to activation of such downstream pathways as mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/AKT[6]. Furthermore, ErbB2 can block internalization of EGFR for degradation, which results in an elevation in the membrane steady state level[7]. Thus, aberrant ErbB2 expression can lead to an extensive activation of its downstream signals and an aberration from the normal regulation, which promotes cancer development.

ErbB2 overexpression is found in approximately 30% of human breast carcinomas and in many other types of human malignancies, such as prostate, pancreas, colon and ovarian cancers[8]. Cancer patients with high ErbB2 expression tend to have a more aggressive disease, identified by clinical outcomes such as high metastasis and low response to treatment. Typically, ErbB2-positive tumors have high proliferation rates and more extensive invasion, with frequent metastasis[9]. In addition, clinical studies using ErbB2-targeted therapy with trastuzumab (a humanized monoclonal antibody against ErbB2) have shown that the response rates of breast cancer patients with ErbB2 overexpression are higher than those with normal ErbB2 expression[10].

Several lines of evidences have implicated the role of ErbB2 in aggressive forms of CCA. High expression of ErbB2 has been found in a variety of non-cancerous biliary proliferative diseases, such as in hepatolithiasis and primary sclerosing cholangitis, both of which are risk factors associated with CCA[11,12]. Immunohistochemical data have demonstrated that 20%-30% of tumor specimens from CCA patients show moderate to strong immunostaining for ErbB2[13], and most of them confer poor clinical outcomes (high metastasis and low survival rate)[13-16]. Moreover, about 30% of transgenic mice that constitutively express wild-type Neu develop gallbladder cancer and intrahepatic CCA within 8 mo[17]. Although many reports have indicated the involvement of ErbB2 in cholangiocarcinogenesis and CCA progression, the mechanism of aberrant ErbB2 expression in promoting CCA progression remains unclear.

In this study, the role of ErbB2 in governing the malignant phenotype (invasion and proliferation) of CCA was investigated by suppressing ErbB2 function in three human CCA cell lines that expressed different levels of ErbB2. Two strategies, inhibition of its kinase function and siRNA, were used to reduce ErbB2 activity. We demonstrated that downregulation of ErbB2 expression and activity suppressed CCA cell invasion, motility and proliferation, particularly in the high-ErbB2-expressing cells. Downstream signaling pathways of ErbB2 also were investigated.

MATERIALS AND METHODS

Cell culture

HuCCA-1 cell line was a generous gift from Professor Stitaya Sirisinha (Mahidol University, Thailand)[18] and KKU-100[19] and KKU-M213 cell lines were kindly provided by Dr. Banchob Sripa, (Khon Kaen University, Thailand). These three CCA cell lines were developed from Thai patients. The cells were maintained in HAM's F-12 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 2 mmol/L glutamine, 15 mmol/L HEPES and 14 mmol/L sodium bicarbonate, 100 U/mL penicillin G and 100 U/mL streptomycin. All cell cultures were incubated at 37℃, in a 5% CO_2 humidified atmosphere.

Quantitative polymerase chain reaction

Cells (80% confluent) were harvested with 0.1% trypsin/EDTA and RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Two micrograms of RNA were converted into cDNA using the Superscript™ RNase H Reverse Transcriptase kit (Invitrogen), which was amplified by quantitative polymerase chain reaction (PCR) (ABI 7500; Applied Biosystems, Foster City, CA, USA) in a 20-μL reaction volume that contained 0.5 U HotStart *Taq* polymerase (Qiagen), 1 × FastStart Universal SYBR Green Master cocktail (Roche, Germany) and 4 pmol of specific primer pairs (5'-CCAGGACCTGCTGAACTGGT-3' and 5'-TGTACGAGCCGCACATC-3' for ErbB2[20] and 5'-CTCTTCCAGCCTTCCTTCCT-3' and 5'-AGCACTGTGTTGGCGTACAG-3' for β-actin[21], used as internal control). The reactions were started with an initial heat activation step at 95℃ for 15 min and the following thermal cycling conditions: 94℃ for 30 s, 58℃ for 30 s and 72℃ for 1 min. ErbB2 mRNA levels among the test cells were determined using the $2^{-\Delta Ct}$ method[22].

Immunoblot assay

Cells transfected with siRNA (for 72 h) or treated with AG825 (for 6 h) were washed twice with PBS and lysed on ice with freshly prepared lysis buffer that contained 150 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 5 mmol/L EGTA, 5 mmol/L EDTA, 0.1% SDS, 1% so-

dium deoxycholate, 1% Nonidet P-40, 1 × protease inhibitor cocktail (Roche Diagnostics, Germany), 50 mmol/L NaF, 2 mmol/L Na_3VO_4, 40 mmol/L β-glycerophosphate, and 1 mmol/L dithiothreitol. Cells were centrifuged at 12000 × *g* for 15 min. Protein lysate (80 μg) was separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare, Munchen, Germany). After incubating with a blocking solution (5% skimmed milk/TBST), membranes were treated with primary antibodies specific for ErbB2, phospho-ErbB2 Y1248 (Labvision, Fremont, CA, USA), β-actin, AKT, phospho-AKT T308 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERK1/2, phospho-ERK1/2, p70S6K, and phospho-p70S6K T389 (Cell Signaling, Beverly, MA, USA), and then with horseradish-preoxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Signals were detected using enhanced chemiluminescence (ECL plus) (GE Healthcare, Little Chalfont, Bucks, UK) and quantified by Alpha Imager (Alpha Innotech, San Leandro, CA, USA).

siRNA transfection

Two Silencer® validated siRNAs against ErbB2 (Ambion, Austin, TX, USA) were used to target mRNA at different exons. CCA cells were transiently transfected with siRNA using Effectene (Invitrogen) following the manufacturer's protocol. In brief, 3.25 μg of siErbB2 was mixed with Effectene and Enchancer (32.5 and 26.0 μL), incubated for 5 min, and then added to HAM's F-12 medium that contained 10% FBS. The mixture was added to 80% confluent CCA cells in 60-mm dishes that contained 10% FBS medium. After 6 h of incubation, medium was removed, cells were washed with PBS and replenished with fresh medium. Cells transfected with Silencer® Cy™-3 labeled non-targeting siRNA (Ambion) were used as a negative control. Protein expression, cell invasion and motility were determined at 72 h post-transfection and cell proliferation was analyzed during 24-96 h post-transfection.

In vitro invasion and motility assay

Cell invasiveness was determined using a Transwell chamber (6.5-mm diameter polyvinylpyrrolidone-free polycarbonate filter of 8-μm pore size) (Corning, NY, USA) precoated with 30 μg Matrigel (BD Biosciences, San Jose, CA, USA). A 200-μL aliquot of cells (10^5) transfected with siRNA or treated with various concentrations of AG825 in 0.2% FBS medium was added to the upper compartment of the Transwell, and 10% FBS medium was added to the lower chamber. After 6 h of incubation at 37℃ in a humidified CO_2 incubator, non-invaded cells in the upper compartment were removed with a cotton swab, and the invaded cells were fixed and stained with 0.5% crystal violet in 25% methanol for 30 min, followed by washing twice with tap water. Finally, the invaded cells were counted under a microscope with a 10 × objective in five random fields. Cell motility assay was performed as described for the invasion assay but using a Matrigel-free system.

Cell viability assay

Cells (3000/well) transfected with siRNA or resuspended in various concentrations of AG825 were plated onto 96-well plates and incubated for 72 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as follows. Fifty micrograms of MTT were added to each well and the cells were incubated for a further 4 h. The supernatant was removed and insoluble formazan dye produced from MTT by living cells was solubilized by 200 μL DMSO. Percentage cell viability was quantified by measuring absorbance at 540 nm. Cell survival during the invasion assay was determined in the same way, except that 5000 cells/well were incubated for 6 h prior to addition of MTT.

Statistical analysis

Data are presented as the mean ± SE from three independent experiments conducted in triplicate. Comparison of data between groups was analyzed by one-way analysis of variance, followed by Newman-Keuls multiple comparison test (GraphPad Software, La Jolla, CA, USA).

RESULTS

ErbB2 mRNA expression level in human CCA cell lines and inhibition of its phosphorylation by AG825

The role of ErbB2 in CCA was investigated in three cell lines that were established from tumor tissues of Thai CCA patients, namely, HuCCA-1, KKU-100 and KKU-M213. Steady-state level of ErbB2 mRNA was determined by quantitative reverse-transcriptase-PCR, normalized to β-actin mRNA. KKU-M213 cell line expressed the highest level of ErbB2 mRNA, with that of HuCCA-1 and KKU-100 being comparable at 60% (Figure 1A).

Like other tyrosine kinase receptors, ErbB2 is activated by phosphorylation. Total and phosphorylated ErbB2 levels in the three CCA cell lines as analyzed by Western blotting showed correspondence with levels of ErbB2 mRNA, but in this case, the level of phosphorylated ErbB2 in KKU-100 cells was significantly lower than that in HuCCA-1 (Figure 1B). Inhibition of ErbB2 activity by treating for 6 h with its specific kinase inhibitor, AG825 (30 μmol/L), resulted in marked reduction ErbB2 phosphorylation at Y1248 in all three cell lines, without any significant effect on total ErbB2 levels (Figure 1B).

Invasive and proliferative abilities of CCA cell lines

The abilities of the three CCA cell lines to migrate and invade were determined by Transwell *in vitro* assays. Among the three cell lines, KKU-M213 showed the highest invasive and motility abilities, which were related to its level of ErbB2 expression (Figure 2A). However, KKU-100, which contained the lowest phospho-ErbB2 level, still had a somewhat high invasive ability (about four fold higher than HuCCA-1, but still lower than KKU-M213). This suggested that other pathways might be involved in enhancing the invasive ability of KKU-100. However, the proliferative rates of the three CCA cell lines as assessed by MTT assay were comparable (Figure 2B), which suggested that ErbB2 is not the key player in regulating proliferation of these cell lines.

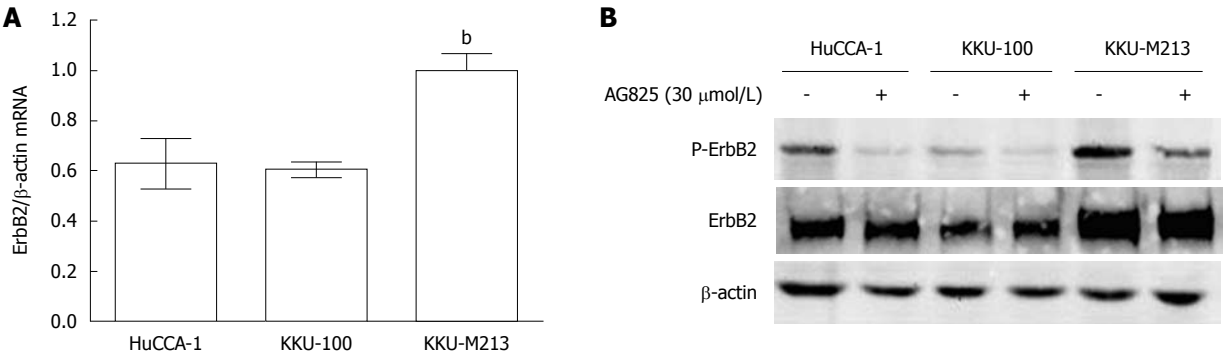

Figure 1 Levels of ErbB2 expression in three cholangiocarcinoma cell lines and inhibition of ErbB2 phosphorylation by AG825. A: Cells cultured in 10% fetal bovine serum (FBS)-containing medium were lysed and the lysates were analyzed for ErbB2 mRNA by real-time reverse-transcriptase polymerase chain reaction. Data are presented as mean ± SE of ErbB2 mRNA level normalized with β-actin mRNA obtained from three independent experiments; B: Cells were incubated without and with 30 μmol/L AG825 in 10% FBS medium for 6 h, then lysed and analyzed for ErbB2, phospho-ErbB2 (Y1248) and β-actin by Western blotting. The results shown are representative of three separate experiments. $^{b}P < 0.01$ *vs* HuCCA-1 and KKU-100.

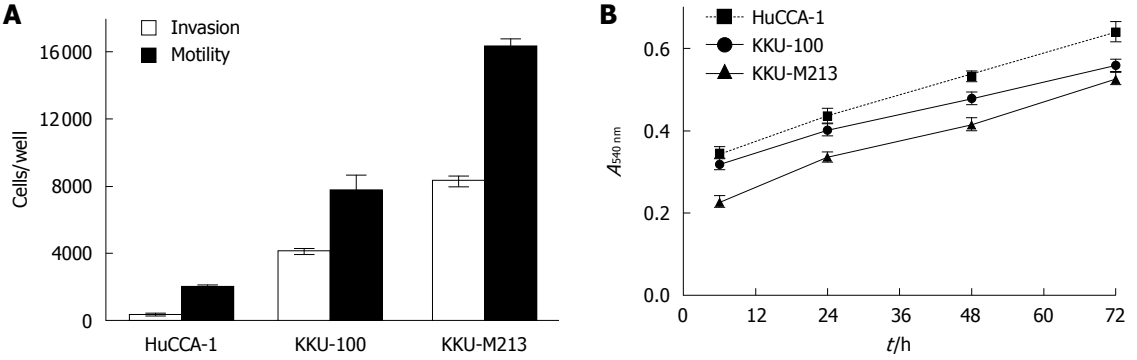

Figure 2 Invasion motility and proliferation of three cholangiocarcinoma cell lines. A: Cells resuspended in 0.2% fetal bovine serum (FBS) medium were plated in the upper compartment of a Transwell chamber coated with and without Matrigel, for invasion and motility assays. The lower compartment was filled with 10% FBS medium. After 6 h incubation, numbers of invading/motile cells were counted; B: Cells were plated on 96-well plates in 10% FBS medium. After incubation for 12-72 h, cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are presented as mean ± SE of the percentage relative to controls obtained from three separate experiments.

Effects of ErbB2 kinase inhibitor on CCA cell invasion and proliferation

As ErbB2 has been detected in CCA and other related cancers, and its expression level has been shown to correlate with highly proliferating diseases of the bile duct and with the degree of CCA local invasion and metastasis[11,23], the role of ErbB2 on invasion and motility of the CCA cell lines was investigated by examining the effects of AG825 on these properties using a Transwell assay. Treatment of AG825 (10-100 μmol/L) for 6 h suppressed cell invasion and motility (Figure 3A-C) in all three CCA cell lines with different IC_{50} values, with KKU-M213 being the most sensitive and KKU-100 the least. During the assay period, the drug marginally affected CCA cell survival (Figure 3A-C), which indicated that inhibition of invasion and motility were not due to drug cytotoxicity. However, incubation with AG825 for 72 h resulted in inhibition of cell proliferation, with KKU-M213 showing the most sensitivity and KKU-100 the least (Figure 3D).

Effects of suppression of ErbB2 expression on CCA cell invasion, motility and proliferation

As ErbB2 kinase inhibitor experiments indicated the role of ErbB2 on malignant phenotypes (invasion, motility and proliferation) of CCA cell lines, we confirmed the specificity of this phenomenon using siRNA targeting of ErbB2 mRNA to suppress *ErbB2* gene expression. In KKU-M213, the CCA cell line that expressed the highest ErbB2 level among the three cell lines, ErbB2 level was reduced in a time-dependent manner (up to 72 h), and was partially restored at 96 h post-transfection (Figure 4A). After 72 h of siRNA transfection, ErbB2 level in all three CCA cell lines was reduced by > 70% (Figure 4B). During this post-transfection period, there was attenuation of invasion and motility of KKU-M213 and HuCCA-1 cells, but not of KKU-100 cells (Figure 5A and B). Although proliferation of KKU-M213 cells was suppressed, that of HuCCA-1 and KKU-100 cells was marginally affected (Figure 5C).

These results on suppression of *ErbB2* gene expression, together with those conducted on inhibition of ErbB2 kinase activity, indicated that a threshold level of ErbB2 is necessary to induce properties associated with malignancy (invasion, motility and proliferation) in CCA cells (viz. KKU-M213).

Involvement of ErbB2 in activation of downstream effectors, AKT, p70S6K and ERK1/2

ERK1/2 and PI3K/AKT are the two major signaling

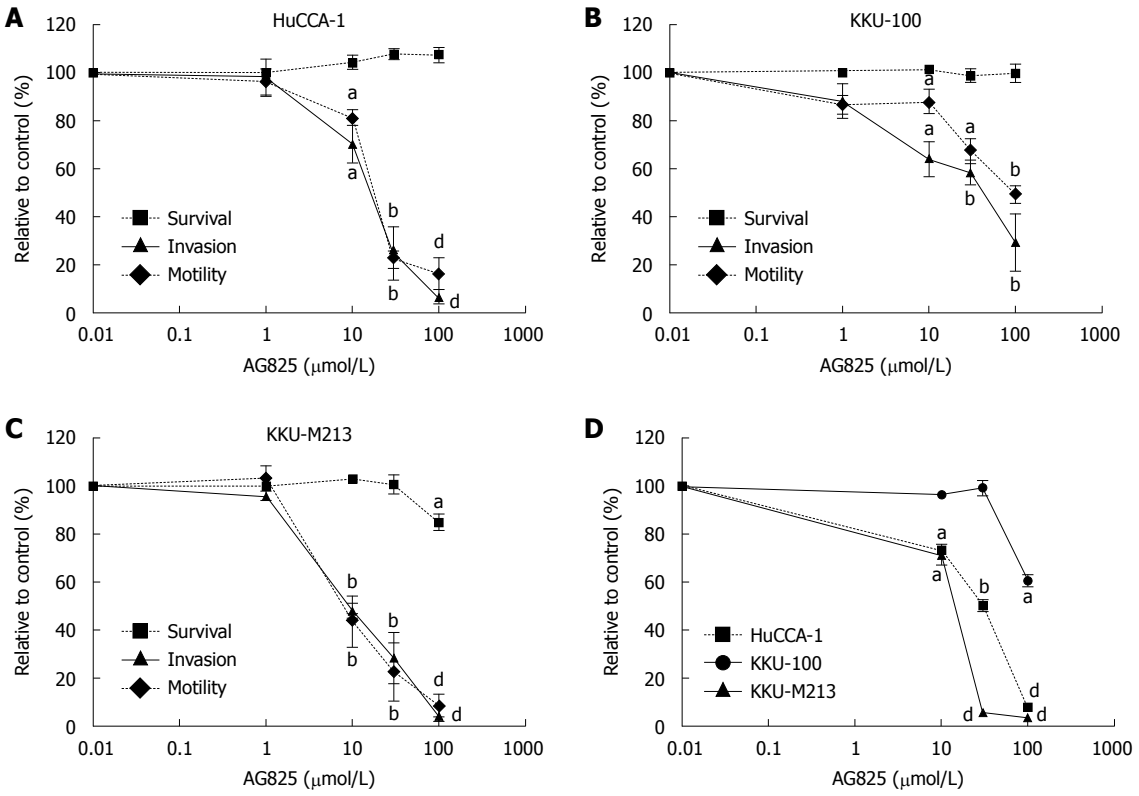

Figure 3 Effects of AG825 on cholangiocarcinoma cell invasion, motility and proliferation. A-C: Cells resuspended in medium that contained 0.2% fetal bovine serum (FBS) and indicated concentrations of AG825 were plated in the upper compartment of a Transwell chamber coated with and without Matrigel, for invasion and motility assays. After 6 h incubation, numbers of invading/motile cells were counted. Cells treated with the same vehicle were used as controls. Cell survival after 6 h of AG825 treatment was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; D: Cells were treated with various concentrations of AG825 in 10% FBS medium for 72 h, and cell survival was determined using the MTT assay. Data are presented as mean ± SE of the percentage relative to the controls obtained from three separate experiments, each done in duplicate. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control.

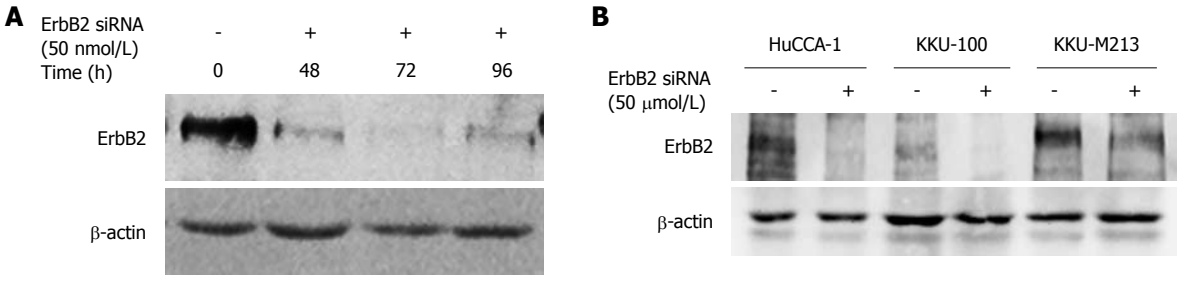

Figure 4 Inhibition of ErbB2 expression by siRNA against ErbB2. A: KKU-M213 cells were transfected with siErbB2 and the levels of ErbB2 at 0-96 h after transfection were determined by Western blotting; B: After 72 h of siRNA transfection, ErbB2 expression in HuCCA-1, KKU-100, and KKU-M213 cells was determined. Scramble siRNA (siNeg) was used as a negative control. The results shown are representatives of two (A) and three (B) separate experiments.

pathways that regulate cell proliferation, motility and invasion in response to a variety of growth factors/receptor tyrosine kinases[24]. To investigate the pathways by which ErbB2 promoted invasion and proliferation of CCA cells, we examined the effects of ErbB2 inhibition (by kinase inhibitor or siRNA) on effectors activated by ErbB2 in these two main signaling pathways. Suppression of *ErbB2* gene expression by siRNA transfection (72 h) reduced phospho-AKT level in all three CCA cell lines, but without affecting total AKT level (Figure 6A). Phosphorylation of its downstream effector, p70S6K, was similarly affected in KKU-M213 and HuCCA-1 cells, but not in KKU-100 cells (Figure 6A). On the other hand, ERK1/2 phosphorylation was marginally, if at all, affected in all three CCA cell lines (Figure 6A). Similarly, treatment of KKU-M213 cells with AG825 (30 μmol/L, 6 h) suppressed AKT and p70S6K phosphorylation but did not affect phospho-ERK1/2 level (Figure 6B). Thus, the PI3K/AKT/p70S6K and not ERK1/2 pathway is responsive to ErbB2 activity related to CCA cell invasion, motility and proliferation.

DISCUSSION

ErbB2 is overexpressed in 20%-30% of epithelial bile duct cancers[13]. Its overexpression is associated with lymph node metastasis in intrahepatic CCA[23]. Unlike

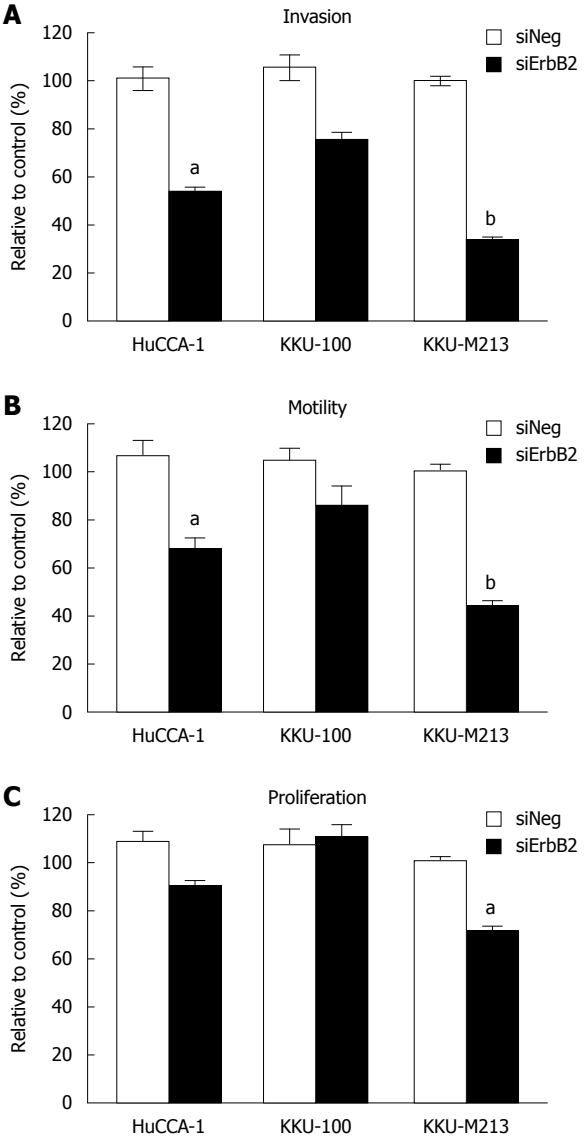

Figure 5 Effects of ErbB2 knockdown on cell invasion motility and proliferation of the three cholangiocarcinoma cell lines. Cells transfected with siRNA (negative control and ErbB2 targeted siRNA) for 72 h were analyzed for *in vitro* invasion (A) and motility (B) by using a Transwell chamber coated with and without Matrigel. After 6 h of incubation, numbers of invading/motile cells were counted. Cell proliferation (C) was analyzed by determining the number of viable cells during 24-96 h post-transfection by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are expressed as mean ± SE of the percentage relative to the controls obtained from three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* controls.

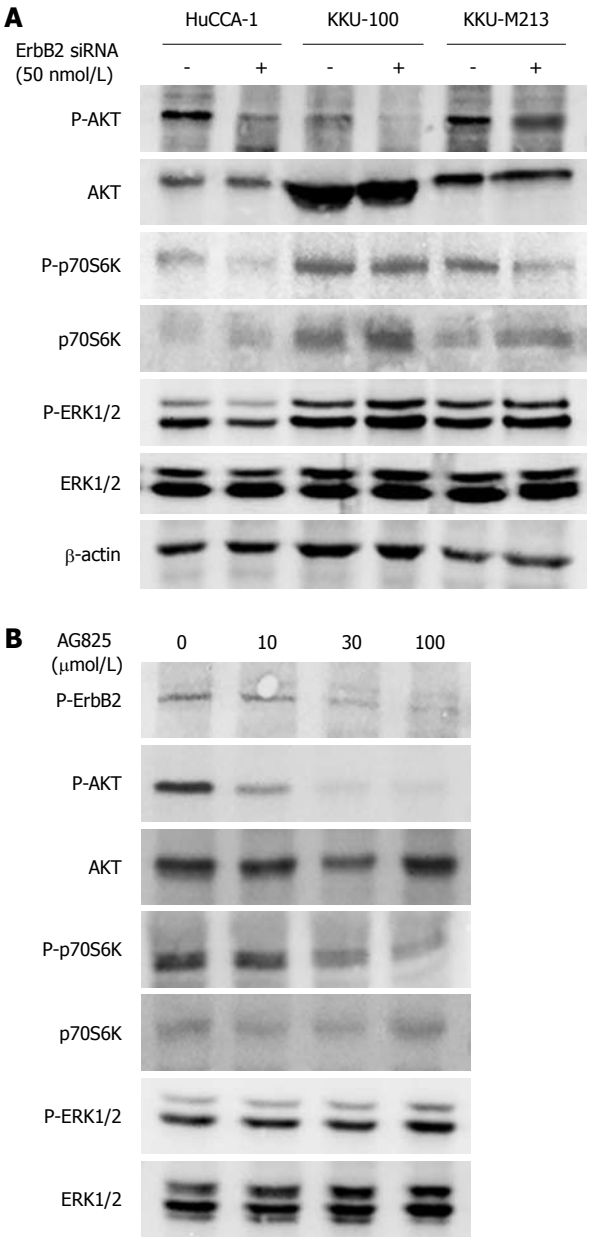

Figure 6 Effects of ErbB2 inhibition (using siRNA and specific kinase inhibitor) on ERK1/2, AKT, and p70S6K phosphorylation in the cholangiocarcinoma cell lines. A: Cholangiocarcinoma cell lines were transfected with siErbB2 and siNeg, as a control, for 72 h and the collected cell lysates were analyzed by Western blotting; B: KKU-M213 cells were treated without and with AG825 in medium that contained 10% fetal bovine serum for 6 h, then lysed and analyzed by Western blotting. The results shown are representatives of three separate experiments.

other ErbBs, ErbB2 is not expressed in normal adult liver (both hepatocytes and cholangiocytes) or in fetal liver[11,25] which makes it an interesting candidate for molecular targeting therapy for liver and bile duct cancers.

The mechanism by which ErbB2 exerts tumorigenicity in CCA is not completely understood. Here, the role of ErbB2 involved in controlling critical characteristics of CCA, namely, invasion, motility and proliferation, was investigated in three human CCA cell lines that expressed different levels of ErbB2, as shown by measurements of mRNA and phosphorylated (P-Y1248) ErbB2 levels. Data showed that ErbB2 was more important for these properties in the highest ErbB2-expressing cell line (KKU-M213), through activation of the AKT/p70S6K pathway.

Upregulation of ErbB2 is implicated in cancer aggressiveness. Studies in transgenic mice have revealed that animals bearing either an activated form of Neu (ErbB2) or overexpressing wild-type Neu frequently develop mammary tumors and lung metastases[26,27]. ErbB2 overexpression plays an important role in cell proliferation and invasion of many cancers, especially those of breast, ovary, stomach and bladder[28,29]. In breast cancer, application of antisense RNA or siRNA against ErbB2 inhibits growth

of high-ErbB2-expressing cells, but has only a little effect on lower-ErbB2-expressing cells[30,31]. In the present study, disruption of ErbB2, by both inhibition of ErbB2 kinase activity using AG825 and knock-down of ErbB2 expression using siRNA, suppressed the neoplastic phenotype of the high-ErbB2-expressing CCA cell line, KKU-M213, to a greater extent than that of the low-ErbB2-expressing cell line, KKU-100. This indicated that the neoplastic phenotype of the high-ErbB2-expressing cell line, KKU-M213, was highly dependent on ErbB2 compared to the low-ErbB2-expressing cells.

There are several possible explanations for the increase in the requirement of ErbB2 in cancer malignancy. Firstly, in cancer cells that have evolved high ErbB2 expression, this pathway has become predominant in regulating cell proliferation and invasion, which results in cells with dependency on this protein. Moreover, Kaelin[32] has suggested that an increase in the requirement of a given protein for cancer cell survival is due either to intrinsic (genetic/epigenetic) or extrinsic (microenvironment) changes. In this case, accumulation of genetic/epigenetic alteration during cancer development might cause a loss of proteins with functional redundancy to ErbB2, or a gain of those with opposing functions. However, cancer cells with ErbB2 overexpression are able to overcome the selective pressures and therefore survive and propagate, thus yielding cells with high dependency on ErbB2. Therefore, the malignant behavior of such ErbB2-dependent cells responds to inhibition of ErbB2 function, whereas those with low or no ErbB2 expression depend on other pathways for their survival, and hence are refractory to such treatment. This has provided the rationale for application of ErbB2-targeted therapy in ErbB2-overexpressing cancer. In fact, trastuzumab, a monoclonal antibody that targets ErbB2, has been used successfully for the treatment of ErbB2-positive metastatic breast cancer as well as for adjuvant therapy of early breast cancer[33]. Although ErbB2-targeted therapy has not been studied in CCA, our data on the inactivation of ErbB2 suggest that this is a crucial oncogene for maintaining the malignant phenotype of CCA, thus becoming a potential target for future therapy.

In the CCA cells, inhibition of ErbB2 by both kinase inhibitor and siRNA suppressed phosphorylation (activation) of AKT and its downstream effector, p70S6K, but not that of ERK1/2. This might be because the regulation of ERK1/2 by other signals overrides the importance of ErbB2 for its activation, or because signaling pathways downstream of ErbB2 are diverse. This diversity depends partly on dimerization partners of ErbB2. For instance, PI3K/AKT is the most important oncogenic signal downstream of ErbB2-ErbB3 heterodimerization[34]. In this case, overexpressed ErbB2 might dimerize with ErbB3, thereby activates PI3K/AKT as a major pathway. The role of AKT in the malignant characteristics of CCA has also been previously reported. Upregulation of AKT pathway has been reported to be related to low survival rate[35]. Moreover, LY294002, a specific inhibitor of PI3K/AKT pathway, attenuates CCA cell proliferation and promotes apoptosis in 10% serum-containing medium[36], and suppresses KKU-M213 and HuCCA-1 cell invasion induced by HGF[37]. In other systems such as ovarian cancer[38,39] and chick embryo fibroblasts[40], both AKT and p70S6K have been reported to regulate cell invasion, motility and proliferation. Therefore, this implies that ErbB2 regulates invasion and proliferation *via* the PI3K/AKT/p70S6K pathway.

In summary, the results presented here demonstrate that ErbB2 is particularly important for malignant properties (invasion, motility and proliferation) of human high-ErbB2-expressing CCA cells. ErbB2 acts through activation of the AKT/p70S6K pathway. These findings lend support for the therapeutic targeting of ErbB2 and/or its effector molecules in CCA with ErbB2 overexpression.

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COMMENTS

Background

Cholangiocarcinoma (CCA), a malignancy of the biliary tract, is an aggressive and currently incurable cancer. The incidence is high in Thailand, China and Japan. Disease progression, especially metastasis, is correlated with ErbB2 overexpression.

Research frontiers

ErbB2 promotes many oncogenic properties, including cell growth, survival, adhesion, motility, invasion, and metastasis. Although many reports have shown the involvement of ErbB2 in CCA, the mechanism that underlies ErbB2-promoted CCA progression is not clearly understood.

Innovations and breakthroughs

Suppression of ErbB2 activity/expression reduced invasion, motility and proliferation in CCA cell lines. This is believed to be the first report to show that, in CCA cell lines with high ErbB2 expression, such neoplastic behavior is more sensitive to ErbB2 inhibition than that in cells with lower ErbB2 expression. Moreover, AKT and p70S6K, but not extracellular signal-regulated kinase 1/2, play important roles as ErbB2 downstream effectors in high-ErbB2-expressing CCA cells.

Applications

As ErbB2 is overexpressed in 20%-30% of CCA, our finding that the malignant phenotypes of CCA cells with high ErbB2 expression are highly dependent on ErbB2 supports the potential use of ErbB2-targeted therapy for the treatment of patients with ErbB2-overexpressing CCA.

Terminology

ErbB2/HER2, human epidermal growth factor receptor 2, is a member of the epidermal growth factor receptor family. Both AKT and p70S6K are downstream effectors of many receptor tyrosine kinases including ErbB2. Their activations induce proliferation and invasion of cancer cells, which leads to metastasis. This study shows that aberrant ErbB2 expression is involved in CCA cell proliferation and invasion *via* AKT/p70S6K.

Peer review

This study provides evidence that high ErbB2 expression in CCA cell lines might be an indicator that blockage of this molecule could be beneficial in cancer therapy. This was a well performed and clearly presented study.

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BRIEF ARTICLE

Relationship between alcohol intake and dietary pattern: Findings from NHANES Ⅲ

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Abstract

AIM: To examine the association between macronutrient dietary patterns and alcohol consumption using the Third National Health and Nutritional Examination Survey Ⅲ.

METHODS: A total of 9877 subjects (5144 males) constituted the study cohort. Dietary interviews were conducted with all examinees by a trained dietary interviewer in a mobile examination center (MEC). Subjects reported all foods and beverages consumed except plain drinking water for the previous 24-h time period. Physical examination and history of alcohol consumption were obtained. Pearson correlation coefficients were used to evaluate the association of the levels of alcohol consumption and the percentage of energy derived from macronutrients. Univariate and multivariate regression analyses were performed accounting for the study sampling weight to further explore the relationships between alcohol consumption and calories derived from each macronutrient.

RESULTS: Subjects who drank were younger than non-drinker controls in both genders ($P < 0.01$). Alcohol intake was inversely associated with body mass index and body weight in women. Of all macronutrients, carbohydrate intake was the first to decrease with increasing alcohol consumption. In the multivariate analyses, the level of alcohol consumption was found to be an independent predictor associated with lower intake of other macronutrients.

CONCLUSION: Our results show that there is an alteration in the daily dietary pattern with increasing alcohol consumption and that energy derived from alcoholic beverages substitutes that from other macronutrients such as carbohydrate, protein, and fat.

Key words: Alcohol; Macronutrients; National Health and Nutritional Examination Survey Ⅲ

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INTRODUCTION

Aside from fat, ethanol is the macronutrient with the highest energy density. Though alcohol can serve as the energy source, how the body processes and utilizes the en-

ergy from alcohol is very complex. Because of additional energy supplementation from alcohol, we might anticipate many drinkers to be obese. In fact, data have shown that drinkers are no more obese than non-drinkers, despite higher caloric intake[1,2]. Moreover, weight loss and malnutrition are common clinical presentations among drinkers. Alcohol intake may be associated with altered patterns of food intake resulting in the replacement of alcohol for other nutrients[1]. We hypothesized that energy derived from alcoholic beverages might substitute energy from other macronutrients such as carbohydrate, protein, and fat. In this study, we examined an association between the macronutrient dietary patterns and alcohol consumption using the Third National Health and Nutritional Examination Survey (NHANES Ⅲ).

MATERIALS AND METHODS

Study population

NHANES Ⅲ was conducted in the United States from 1988 through 1994 by the National Center for Health Statistics of the Centers for Disease Control and Prevention. The NHANES Ⅲ survey used complex, multi-stage, stratified, clustered samples of civilian, non-institutionalized populations of age 2 mo and older to collect information about the health and diet of people residing in the United States. A detailed description of the survey and its sampling procedures is available elsewhere[3]. This study was approved by the CDC Institutional Review Board and all participants provided written informed consent.

During the survey period, 18162 subjects underwent physical examination and laboratory assessment at a mobile examination center (MEC). Exclusion criteria for this study included minors (age < 20 years old), breast feeding or pregnant women, and those with missing values of specific variables (hepatitis B and C serologies, body mass index, aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum creatinine, and drinking history). Additionally, subjects with history of diabetes, congestive heart failure/heart disease, renal insufficiency were also excluded. History of diabetes was defined by self-report and/or taking diabetes medications and/or the subjects having been told by his/her physicians that he/she has diabetes or sugar diabetes. Subjects with history of congestive heart failure/heart disease were defined by self-report of prior myocardial infarction and/or they had been told by their physicians that they have congestive heart failure. For study purposes, we defined subjects with renal insufficiency as those with creatinine ⩾ 2.5 mg/dL. Subjects with diabetes, congestive heart failure and renal insufficiency were excluded from this study because of the possibilities of dietary restrictions due to underlying diseases. Thus, our analytic population included 5144 men and 4733 women.

NHANES Ⅲ dietary data

Dietary interviews were conducted with all examinees by a trained dietary interviewer in the MEC. Subjects reported all foods and beverages consumed except plain drinking water for the previous 24-h time period (midnight to midnight). An automated, microcomputer-based dietary interview and coding system known as the Dietary Data Collection (DDC) System was used to collect all dietary recall data. The detailed method for data collection is described elsewhere[3].

Estimation of alcohol consumption

The amount of alcohol consumed was determined based on the responses to two survey queries that questioned the number of days of drinking over the past 12 mo and the number of drinks per day on a given drinking day. To avoid any arbitrariness in the choice of a cutoff point, we further stratified the extent of alcohol consumption in subjects who reported a history of alcohol use into four groups using quartiles.

Estimation of physical activity

Physical activity assessment was part of the comprehensive interview in NHANES Ⅲ. In brief, subjects were asked to identify specified exercises in which they participated during their free time (jogging or running; riding a bicycle or exercise bicycle; swimming; aerobic dancing; other dancing; calisthenics or floor exercises; gardening or yard work; and weight lifting). They were requested to specify the number of times they participated in an identified activity during the past month. Responses were standardized as "times per week" using the conversion factors 4.3 wk/mo and 30.4 d/mo, then rounded to the nearest whole number. The frequency of performance of other reported exercises, sports or physically active hobbies was also recorded. The physical activity was specified as the sum of intensity rating multiplied by times (of each activity) per month[4].

Laboratory measurements

All venous blood samples were immediately centrifuged and shipped weekly at -20℃ to a central laboratory. The laboratory procedures followed in the NHANES Ⅲ are described in detail elsewhere[3].

Statistical analysis

Descriptive statistics such as means, SD, ranges, and percentages were used to characterize the study patients. Comparisons among groups were made using Analysis of Variance for the continuous and χ^2 test for the categorical variables. Pearson correlation coefficients were used to evaluate the association of the levels of alcohol consumption and the percentage of energy derived from macronutrients. Multivariate regression analyses were performed accounting for the study sampling weight. All statistical analyses and database management were performed using SAS-callable SUDANN software accounting for stratification, sample weight, and clustering. This analysis method also takes into account the different sample weights and the effects of the complex sample design on variance estimation.

Table 1 Clinical characteristics and dietary patterns in male cohorts (n = 5144)

	Levels of alcohol consumption (g/d)					*P* value
	Non-drinkers (n = 3557)	< 16 (n = 372)	16-35 (n = 465)	36-64 (n = 353)	> 64 (n = 397)	
Age (yr)	48.8 ± 19.5	46.4 ± 19.3	45.4 ± 17.8	42.9 ± 16.2	39.5 ± 14.1	< 0.01
Body mass index (kg/m^2)	26.3 ± 4.1	25.5 ± 3.7	25.7 ± 3.6	25.9 ± 3.7	26.0 ± 3.9	0.87
Body weight (kg)	79.3 ± 14.3	76.9 ± 12.7	78.3 ± 12.3	79.1 ± 13.6	79.4 ± 14.0	0.82
Cigarette smoking (%)	24.8	25.5	37	41.9	50.6	< 0.01
Waist to hip ratio	0.96 ± 0.07	0.95 ± 0.07	0.95 ± 0.07	0.94 ± 0.07	0.94 ± 0.07	0.94
AST (U/L)	22.8 ± 12.7	22.6 ± 8.3	24.1 ± 11.9	26.4 ± 18.7	29.7 ± 26.1	< 0.01
ALT (U/L)	19.7 ± 15.2	19.1 ± 12.8	20.1 ± 13.4	21.6 ± 16.5	25.5 ± 23.2	< 0.01
Physical activity	76.7 ± 109.2	91.6 ± 108.4	91.2 ± 113.8	71.6 ± 98.1	71.5 ± 105.5	< 0.01
Total energy from food (kcal/d)	2 213 ± 838	2432 ± 850	2500 ± 848	2606 ± 857	3155 ± 1018	< 0.01
kcal from alcohol (%)	0	3.5 ± 2.1	8.0 ± 3.9	13.6 ± 6.2	24 ± 11.3	< 0.01
kcal from carbohydrate (%)	50.8 ± 10.7	47.7 ± 10.6	44.4 ± 9.8	42.2 ± 9.1	36.6 ± 8.4	< 0.01
kcal from protein (%)	16.1 ± 5.1	15.9 ± 4.6	15.5 ± 4.4	14.6 ± 4.1	12.9 ± 4.1	< 0.01
kcal from fat (%)	34.6 ± 9.0	34.1 ± 9.1	33.2 ± 9.2	30.7 ± 8.3	27.2 ± 9.2	< 0.01
kcal from monosaturated fat (%)	13.1 ± 4.0	13.0 ± 4.1	12.7 ± 4.1	11.7 ± 3.8	10.4 ± 3.8	< 0.01
kcal from polysaturated fat (%)	7.3 ± 3.4	7.0 ± 3.2	7.1 ± 3.5	6.2 ± 2.8	5.7 ± 3.3	< 0.04

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 2 Multivariate linear regression analysis of total daily food energy and other macronutrients adjusting for covariates by level of alcohol intake[1]

	Levels of alcohol consumption (g/d)					*P* value
Male subjects	Non-drinkers (n = 3557)	< 16 (n = 372)	16-35 (n = 465)	36-64 (n = 353)	> 64 (n = 397)	
Total energy from food (kcal/d)	2228.5 ± 818	2419.6 ± 850	2454.5 ± 847	2559.6 ± 857	3052.1 ± 1018	< 0.05
kcal from carbohydrate (%)	50.2 ± 10.6	47.0 ± 10.6	44.1 ± 9.8	41.9 ± 9.0	36.5 ± 8.3	< 0.01
kcal from protein (%)	16.1 ± 5.1	16.0 ± 4.5	15.5 ± 4.4	14.7 ± 4.1	13.1 ± 4.1	< 0.05
kcal from fat (%)	34.8 ± 9.0	34.4 ± 9.1	33.3 ± 9.2	30.7 ± 9.3	27.1 ± 9.2	< 0.05
kcal from monosaturated fat (%)	13.1 ± 4.0	13.1 ± 4.0	12.7 ± 4.1	11.8 ± 3.9	10.4 ± 3.8	< 0.05
kcal from polysaturated fat (%)	7.2 ± 3.3	6.9 ± 3.2	7.1 ± 3.4	6.1 ± 2.7	5.6 ± 3.3	< 0.05
Female subjects	Non-drinkers (n = 4062)	< 13 (n = 161)	13-21 (n = 169)	22-38 (n = 172)	> 38 (n = 169)	
Total energy from food (kcal/d)	1683 ± 668	1794 ± 612	1881 ± 735	1838 ± 651	2256 ± 861	< 0.01
kcal from carbohydrate (%)	51.5 ± 10.8	48.0 ± 10.3	45.4 ± 10.1	43.4 ± 10.1	38.2 ± 8.4	< 0.01
kcal from protein (%)	15.8 ± 5.1	15.7 ± 4.5	15.1 ± 4.4	14.8 ± 4.3	12.4 ± 3.9	< 0.01
kcal from fat (%)	34.1 ± 9.4	33.8 ± 9.1	34.0 ± 10.1	31.5 ± 9.0	27.2 ± 9.0	< 0.01
kcal from monosaturated fat (%)	12.7 ± 4.0	12.7 ± 4.0	13.0 ± 4.5	12.1 ± 4.1	10.1 ± 3.4	< 0.05
kcal from polysaturated fat (%)	7.3 ± 3.7	7.1 ± 3.2	7.0 ± 3.6	6.8 ± 3.6	5.9 ± 3.3	< 0.05

[1]Adjusted for age, body weight, physical activity, and smoking status.

RESULTS

Relationship between dietary pattern and alcohol consumption in males

Among male participants (n = 5144), 69% reported no history of alcohol use (Table 1).

There were no differences in waist-to-hip ratio among groups. In this study cohort, only subjects who drank < 16 g of alcohol/d weighed less than non-drinker controls. The percentage of subjects who smoked increased in accordance with the level of alcohol consumption. As expected, markers of hepatic inflammation, AST and ALT, were increased with the level of alcohol consumption. The total energy consumption per day increased with the level of alcohol consumption. The increment in such energy was mainly due to the calories provided by alcohol. In the univariate analysis (Table 1), we found that the percentage of energy derived from carbohydrate, protein, and fat decreased with increasing alcohol consumption. Carbohydrate intake started to decrease at a daily consumption of ≤ 35 g/d. When the daily levels of alcohol consumption continued to increase (> 35 g/d), subjects consumed less protein and fat. In those who drank alcohol at a level of > 64 g/d, the energy intake which was derived from protein and fat was reduced by 4% and 7%, respectively. In the multivariate linear regression analyses adjusting for covariates (such as age, body weight, smoking status and physical activity), the level of alcohol consumption was found to be an independent predictor associated with lower percent calories derived from macronutrients. The adjusted calories from macronutrients stratified by alcohol consumption are shown in Table 2. In the correlation analyses, the amount of alcohol consumed per day was inversely associated with the percentage of calories derived from each macronutrient (Figure 1A-C).

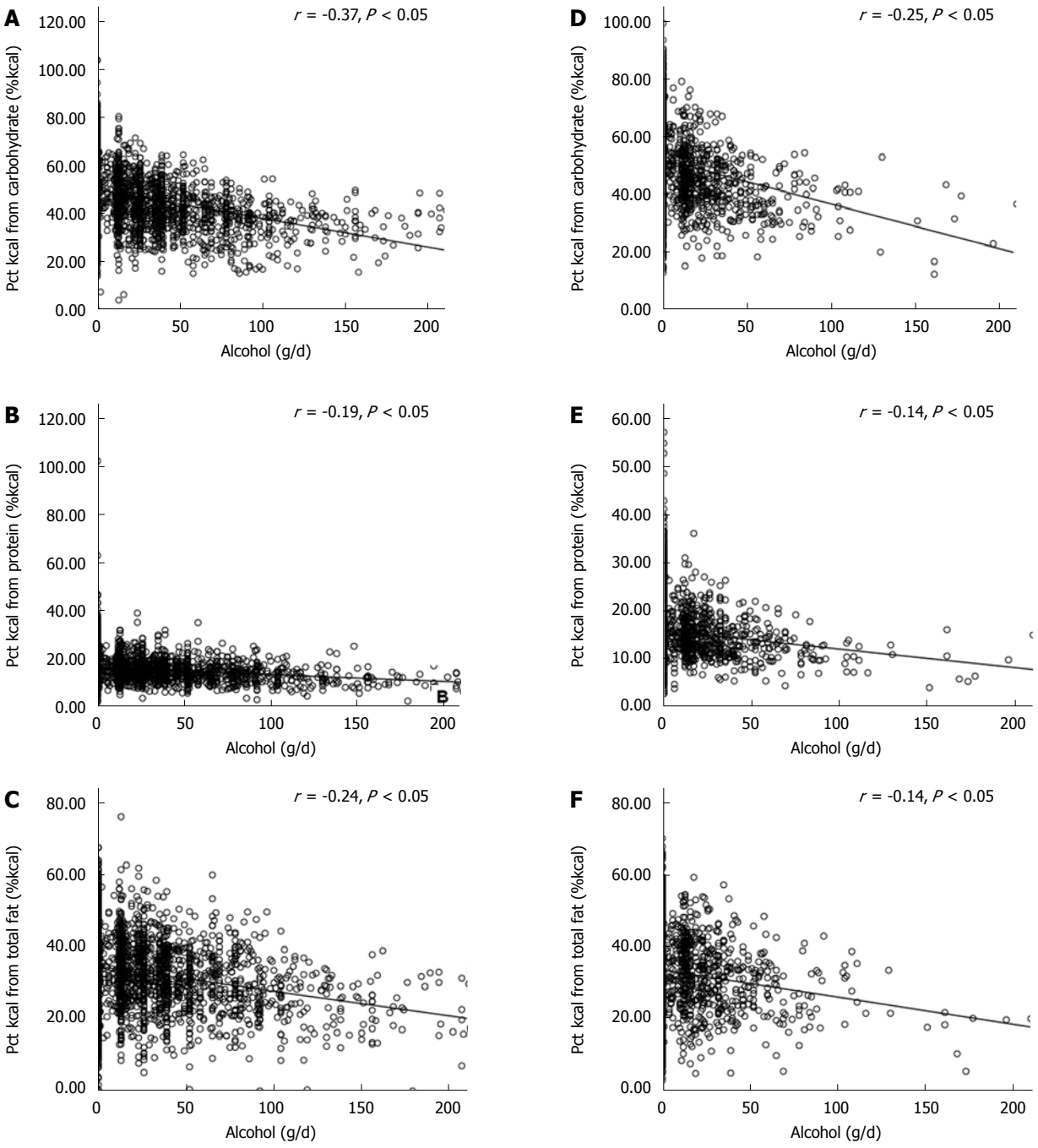

Figure 1 Relationship between the levels of alcohol consumption and the percentage of calories derived from each macronutrient in males (A-C) and females (D-F).

Relationship between dietary pattern and alcohol consumption in females

As observed with male participants, women who drank higher amounts of alcohol were younger than non-drinker controls ($P < 0.05$). Alcohol intake was inversely associated with body mass index and body weight in women. Again, no differences in waist-to-hip ratio were observed among groups (Table 3). As with male subjects, we found a higher prevalence of cigarette smokers in those who drank higher amounts of alcohol. Because of the energy provided from alcohol, the total energy consumed per day was higher in those who drank any amount of alcohol when compared to controls. We again found that female drinkers started to consume less carbohydrate even when they drank < 13 g/d. The calories derived from protein and fat were reduced once the subjects drank > 21 g of alcohol/d. In the multivariate linear regression analyses, the level of alcohol consumption was found to be an independent predictor associated with lower percent calories derived from macronutrients (Table 2). In the correlation analyses, the amount of alcohol consumption per day was inversely associated with the percentage of calories derived from each macronutrient, as observed in male subjects (Figure 1D-F).

Table 3 Clinical characteristics and dietary patterns in female cohorts (n = 4733)

	Levels of alcohol consumption (g/d)					*P* value
	Non-drinkers (n = 4062)	< 13 (n = 161)	13-21 (n = 169)	22-38 (n = 172)	> 38 (n = 169)	
Age (yr)	49.1 ± 19.5	45.6 ± 18.4	45.4 ± 17.6	41.9 ± 15.2	41.3 ± 14.3	< 0.01
Body mass index (kg/m^2)	26.3 ± 5.0	25.1 ± 4.5	24.9 ± 4.9	24.8 ± 4.5	24.8 ± 4.5	< 0.05
Body weight (kg)	67.3 ± 13.9	64.8 ± 11.8	65.7 ± 14.1	66.3 ± 11.9	66.4 ± 13.0	< 0.05
Cigarette smoking (%)	19	25	30.1	35.4	44.9	< 0.01
Waist to hip ratio	0.87 ± 0.08	0.85 ± 0.07	0.86 ± 0.08	0.86 ± 0.11	0.86 ± 0.08	0.94
AST (U/L)	19.8 ± 12.1	18.9 ± 6.7	23.4 ± 30.9	20.9 ± 11.5	20.5 ± 9.4	< 0.01
ALT (U/L)	14.6 ± 13.8	13.8 ± 8.8	15.1 ± 14.2	14.6 ± 10.5	14.6 ± 10.7	< 0.01
Physical activity	56.9 ± 90.9	78.6 ± 105.7	81.6 ± 107.3	70.5 ± 94.8	70.7 ± 104.3	< 0.01
Total energy from food (kcal/d)	1667 ± 667	1816 ± 646	1903 ± 735	1895 ± 651	2316 ± 860	< 0.01
kcal from alcohol (%)	0	3.7 ± 2.2	6.8 ± 3.4	11.6 ± 4.4	23.2 ± 11.4	< 0.01
kcal from carbohydrate (%)	52.4 ± 10.8	48.9 ± 10.5	46.1 ± 10.1	43.8 ± 10.1	38.4 ± 8.7	< 0.01
kcal from protein (%)	15.8 ± 5.0	15.6 ± 4.5	15.0 ± 4.4	14.7 ± 4.2	12.3 ± 3.9	< 0.01
kcal from fat (%)	33.4 ± 9.4	33.2 ± 9.2	33.6 ± 10.1	31.4 ± 9.0	27.3 ± 9.3	< 0.01
kcal from monosaturated fat (%)	12.3 ± 4.0	12.4 ± 4.1	12.8 ± 4.4	11.9 ± 4.1	10.1 ± 3.8	< 0.01
kcal from polysaturated fat (%)	7.3 ± 3.6	7.1 ± 3.3	7.1 ± 3.5	6.9 ± 3.6	6.0 ± 3.4	< 0.04

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

DISCUSSION

In this large population-based study, we found that (1) alcohol consumption was inversely related to body mass index and body weight, primarily in women; (2) energy derived from alcohol replaced that from macronutrients in both genders; and (3) carbohydrate was the foremost macronutrient in which the energy was replaced by that from alcohol. With increasing alcohol consumption, we found a significant reverse relationship between alcohol and all macronutrient intakes.

Although alcohol is an energy source, how the body processes and uses the energy from alcohol is more complex than can be explained by a simple calorie conversion value. Energy derived from alcohol has been consider as "empty calories" because alcohol contains no beneficial nutrients. Additionally, it can also replace the energy derived from other macronutrients. As shown in this study, particularly in female subjects, alcohol provides an average of 23% of the calories when intake is > 38 g/d (Table 2). Despite higher caloric intake from alcohol, females who drank at this level were less obese than non-drinkers. It is postulated that chronic drinking triggers the microsomal ethanol-oxidizing system (MEOS)[1,5,6], an inefficient system of alcohol metabolism. Much of the energy from MEOS-driven alcohol metabolism is lost as heat rather than used to supply the body with energy. The association between gender, body weight, and alcohol intake is debatable. Though our results are consistent with those reported by Colditz *et al*[1], there have been previous reports that men who drank weighed more than non-drinkers[7,8]. The inconsistency in these results is likely due to the study design and data collection.

We observed that the major difference in nutrient intake for both genders was a significantly lower intake of carbohydrates by drinkers (r = -0.37 and -0.25, in male and female subjects, respectively). Our findings are similar to those reported by Thompson *et al*[9], where they observed a decreased absolute intake of carbohydrate, protein, and fat with increasing alcohol intake.

Several limitations in using NHANES datasets deserve discussion. First, the cross-sectional design in NHANES does not enable us to truly address potential temporal associations between significant alcohol consumption and the variables of interest. Second, the accuracy of the alcohol consumption data, as with other retrospective study designs, is unknown. Since the extent of alcohol consumption will be derived from self-report questionnaires, it is vulnerable to a recall bias in each participant.

In summary, our results showed that there is an alteration in the daily dietary pattern with increasing alcohol consumption and that energy derived from alcoholic beverages substitutes that from other macronutrients such as carbohydrate, protein, and fat. Female drinkers were less obese than non-drinkers, suggesting that alcohol calories may be less utilized in female subjects. However, further research is needed to explore the role of gender and body weight in alcoholics.

COMMENTS

Background

Aside from fat, ethanol is the macronutrient with the highest energy density. Though alcohol can serve as the energy source, how the body processes and utilizes the energy from alcohol is very complex. Because of additional energy supplementation from alcohol, the authors might anticipate many drinkers to be obese. In fact, data have shown that drinkers are no more obese than non-drinkers, despite higher caloric intake. Moreover, weight loss and malnutrition are common clinical presentations among drinkers. Alcohol intake may be associated with altered patterns of food intake resulting in the replacement of alcohol for other nutrients. In this study, the authors examined the association between the macronutrient dietary patterns and alcohol consumption using the Third National Health and Nutritional Examination Survey (NHANES Ⅲ).

Research frontiers

In this large population-based study, the authors found alterations in the daily dietary pattern with increasing alcohol consumption and that energy derived from alcoholic beverages substitutes that from other macronutrients such as carbohydrate, protein, and fat.

Innovations and breakthroughs

To the best of the authors' knowledge, this is the first population-based study to address the relationship between alcohol consumption and dietary pattern. The authors found that alcohol consumption was inversely related to body mass index and body weight, primarily in women. With increasing alcohol consumption, they found a significant reverse relationship between alcohol and all macronutrient intakes.

Applications

Energy derived from alcohol has been considered as "empty calories" because alcohol contains no beneficial nutrients. In this study, the authors also found that energy derived from alcohol consumption can replace that from other macronutrients.

Peer review

Dr. Liangpunsakul describes in his manuscript the relationship between alcohol consumption and the macronutrient dietary patterns using data from the NHANES 1988-1994. He found that increasing alcohol intake is associated with an altered daily dietary pattern and that the energy of alcoholic beverages substitutes that from carbohydrates, protein and fat.

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BRIEF ARTICLE

Hepatocellular carcinoma in patients with chronic hepatitis C virus infection without cirrhosis

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Abstract

AIM: To investigate and characterise patients with chronic hepatitis C virus (HCV) infection presenting with hepatocellular carcinoma (HCC) in the absence of cirrhosis.

METHODS: Patients with chronic hepatitis C infection without cirrhosis presenting with HCC over a 2-year period were identified. The clinical case notes, blood test results and histological specimens were reviewed to identify whether additional risk factors for the development of HCC were present.

RESULTS: Six patients (five male, one female) with chronic hepatitis C infection without cirrhosis presented to a single centre with HCC over a 2-year period. Five patients were treated by surgical resection and one patient underwent liver transplantation. Evaluation of generous histological specimens confirmed the presence of HCC and the absence of cirrhosis in all cases. The degree of fibrosis of the background liver was staged as mild (n = 1), moderate (n = 4) or bridging fibrosis (n = 1). Review of the clinical case notes revealed that all cases had an additional risk factor for the development of HCC (four had evidence of past hepatitis B virus infection; two had a history of excessive alcohol consumption; a further patient had prolonged exposure to immune suppression).

CONCLUSION: HCC does occur in patients with non-cirrhotic HCV infection who have other risk factors for hepatocarcinogenesis.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Non-cirrhotic; Screening

Peer reviewer: Dr. Terence C Chua, Department of Surgery, St George Hospital, Sydney, 2217, Australia

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a well-recognised complication of cirrhosis regardless of aetiology; the risk of malignancy differs according to the underlying cause of liver damage. As a consequence, patients with cirrhosis undergo routine interval screening in most liver centres using a combination of serum α-foetoprotein (AFP) and liver ultrasound, although solid evidence to support this

approach post-dates adoption of the strategy[1,2]. Surveillance is restricted to those at higher risk in some centres. Patients with cirrhosis secondary to chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) are at particular risk of HCC[3]. Furthermore, there are numerous reports indicating that chronic infection with HBV is associated with HCC in the absence of cirrhosis.

In contrast, HCC in patients with chronic HCV infection without cirrhosis appears to be very rare[4-7]. We describe six such cases that presented to one tertiary referral centre in the past 2 years (Table 1).

MATERIALS AND METHODS

Patients with chronic HCV infection without cirrhosis presenting with HCC over a 2-year period were identified. The clinical cases were reviewed to identify any additional risk factors for hepatocarcinogenesis. Details obtained included ethnic origin, alcohol consumption, past or present infection with HBV, medical co-morbidity, medication history and family history of liver disease or HCC. Histological specimens were reviewed by an experienced liver pathologist to confirm the presence of HCC and to assess the degree of fibrosis of the background liver tissue. Furthermore, histological specimens were evaluated for the presence of additional hepatic pathology including the presence of iron, steatosis and α-1 antitrypsin globules.

RESULTS

Six cases with non-cirrhotic chronic HCV infection and HCC presented to one centre over a 2-year period. Table 1 summarises the clinical and histological characteristics of the cases including additional risk factors for hepatocarcinogenesis. The first case, a 53-year-old Caucasian male, was referred to the service having been found to be HCV-RNA positive (genotype 1). He had a history of previous injecting drug use and a high alcohol intake exceeding 30 U/wk. Evaluation of hepatitis B serology demonstrated that he was surface antigen (HBsAg) negative, and positive for core antibody (anti-HBc) in keeping with past infection. Screening liver ultrasound demonstrated a focal lesion. Computerised tomography (CT) confirmed a mass with arterial enhancement. Serum AFP was 239 IU/L (normal range < 10 IU/L). Histology of the resected mass revealed a 3.5-cm multi-focal HCC, with moderate to poor differentiation and vascular invasion. Histology of the background liver revealed minimal inflammation but moderate fibrosis. He is recurrence-free 24-mo post resection. HCV-RNA was undetectable at completion of 48-wk therapy with pegylated interferon-α and ribavirin.

The second case, a 57-year-old Caucasian male, presented with fever and abdominal pain. CT of the abdomen revealed a 5 cm × 3 cm, inflammatory mass in the right side of the abdomen in close proximity to bowel and in addition a 2 cm hypodense lesion within the liver. A right hemi-colectomy was performed which revealed extra-colonic fibrosis and abscess formation secondary to a caecal diverticulum. Biopsy of the liver mass demonstrated HCC; the background liver comprised moderate inflammation and mild fibrosis consistent with chronic HCV infection. He was HCV-RNA positive (genotype-3), HBsAg negative, anti-HBc positive with a history of previous injecting drug use. Liver function tests were normal. Serum AFP was 3 IU/L. After hepatic resection, histology revealed a 3.1 cm HCC with moderate differentiation, without vascular invasion; the background liver confirmed mild inflammation and mild fibrosis. He has completed therapy with pegylated interferon-α and ribavirin with sustained virological response. He is recurrence-free 22 mo following surgery.

Case three, a 67-year-old Nigerian lady with chronic HCV infection, had a 4 cm focal lesion detected on screening liver ultrasound. CT imaging confirmed the mass with arterial enhancement. Biopsy of the lesion demonstrated HCC and the background liver revealed moderate inflammation and mild fibrosis consistent with chronic HCV infection. She was HCV-RNA positive (genotype 1), HBsAg negative, anti-HBc positive. Liver function tests were unremarkable. Serum AFP was 206 IU/L. After hepatic resection, histology revealed a 4.5 cm HCC with moderate to poor differentiation and lymphovascular invasion on a background liver with moderate fibrosis. She was unable to tolerate antiviral therapy due to profound anaemia and remains HCV-RNA positive. She is recurrence-free 20 mo after resection.

The fourth case was a 46-year-old Caucasian man known to have chronic HCV (genotype 1) with a history of previous injecting drug use; he was HBsAg negative, anti-HBc positive. Liver biopsy demonstrated moderate activity and moderate fibrosis consistent with chronic HCV infection. He had failed to respond to pegylated interferon and ribavirin. Six years later investigation of a raised AFP (77 IU/L) revealed a 1.5 cm arterially enhancing lesion in the liver. Surgical resection revealed a 1.7 cm moderately - well differentiated HCC without vascular invasion on a background liver with bridging fibrosis but not cirrhosis. He is recurrence-free 16 mo after resection.

Case 5 was a 46-year-old Indian man with chronic HCV infection with a history of renal transplantation 15-year previously, treated with ciclosporin and prednisolone. Liver function tests were unremarkable. Screening ultrasound detected a 3 cm focal lesion in the liver; biopsy of the mass revealed HCC. He was HCV-RNA positive (genotype 1) without evidence of exposure to HBV. Serum AFP was 5 IU/L. Further imaging with CT, magnetic resonance imaging and angiography confirmed the mass and revealed two further smaller lesions. He was deemed to meet Mazzaferro criteria[8] and listed for liver transplantation. The explant revealed a 3 cm HCC with poor differentiation and vascular invasion and several small satellite lesions. The background liver demonstrated moderate fibrosis and moderate inflammation. He developed HCC within the grafted liver after 6 mo leading to death 8 mo following transplantation.

The final case, a 63-year-old Russian doctor, acquired HCV (genotype 1) 9 years previously following a needle stick injury from a patient. He had a long history of excess alcohol consumption (30 U/wk) but was immune

Table 1 Patient characteristics

Age (yr)	Sex	HCC	Background liver histology at time of HCC treatment	AFP (IU/L)	HCV status	HBV status	Alcohol (U/wk)
53	M	3.5 cm Moderate/poor differentiation Vascular invasion	Minimal inflammation Moderate fibrosis	239	RNA positive Genotype 1	HBsAg negative Anti-HBc positive	10-30
57	M	3.1 cm Moderate differentiation No vascular invasion	Mild inflammation Mild fibrosis	3	RNA positive Genotype 3	HBsAg negative Anti-HBc positive	0
67	F	4.5 cm Moderate/poor differentiation Lymphovascular invasion	Moderate inflammation Moderate fibrosis	206	RNA positive Genotype 1	HBsAg negative Anti-HBc positive	0
52	M	1.7 cm Moderate differentiation No vascular invasion	Moderate inflammation Bridging fibrosis	77	RNA positive Genotype 3	HBsAg negative Anti-HBc positive	20
46	M	3 cm Poorly differentiated with satellite lesions	Moderate inflammation Moderate fibrosis	5	RNA positive Genotype 1	Negative	0
62	M	6.5 cm Moderate differentiation Vascular invasion	Moderate inflammation Moderate fibrosis	10	RNA positive Genotype 1	Negative	30

HCC: Hepatocellular carcinoma; AFP: α-foetoprotein; HCV: Hepatitis C virus; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

to HBV and was anti-HBc negative. At presentation, liver biopsy revealed mild fibrosis with moderate inflammation. Nine years later he was found to have a 6.5 cm HCC. Serum AFP was 10 IU/L. He underwent surgical resection; histology revealed a moderately differentiated HCC on a background liver with moderate fibrosis. He is well 5 mo after.

DISCUSSION

Six cases with non-cirrhotic chronic HCV infection and HCC presented to one centre over a 2-year period. All were considered at low risk of HCC and none was in a surveillance programme. One had symptoms but the identification of a liver mass in the remaining five patients was fortuitous. Cirrhosis was excluded confidently in all cases by careful histological review of generous tissue specimens revealing at worst bridging fibrosis. No patient had histological features to suggest any diagnosis other than HCV related injury. All patients had a normal body mass index and none were diabetic. However, all patients had an additional risk factor for liver injury or HCC: four had evidence of past HBV infection; two had a history of excessive alcohol consumption; a further patient had prolonged exposure to immune suppression.

The incidence of HCC is increasing across the developed world[9]. Cirrhosis of any cause is an important precursor for HCC, although liver disorders including chronic HBV or HCV infection, haemochromatosis, non-alcohol-related fatty liver disease (NAFLD) and alcohol-related liver disease carry a particular risk[3]. The risk is also much higher in men and older patients[10,11]. The current increase in the prevalence of chronic liver disease secondary to chronic HCV infection and NAFLD (a consequence of the increasing prevalence of obesity and an ageing population) are the main reasons for the increasing incidence of HCC in the developed world[12-15], although improved screening and diagnosis may also play a part. The underlying mechanisms that lead to malignant transformation of HCV-infected hepatocytes, however, remain uncertain, but as most HCV-related HCC occurs on a background of severe fibrosis or cirrhosis it is thought that the mechanism of carcinogenesis is more likely to be indirect, such that the process of tissue damage, regeneration and repair are important, rather than a direct oncogenic effect of HCV infection or the inflammatory response to the virus.

It is well recognised that chronic HBV infection can lead to HCC in the absence of cirrhosis[16]. HBV is a DNA virus that can integrate into the host cell genome; integration may be mutagenic directly by causing genomic instability, loss of tumour suppressor activity or over-expression of genes involved in regulation of cell cycle proliferation. In addition, HBV encodes HBx protein, which functions as a transcriptional trans-activator of cellular genes that are involved in cell proliferation control such as c-jun, c-fos and c-myc. This may lead to dysregulation of the cell cycle and interference with cellular DNA repair and apoptosis[17].

This series raises the possibility that HCV may be oncogenic. HCV is an RNA virus, which replicates in the cytoplasm and does not integrate into host cellular DNA. However, some HCV proteins, such as HCV core and non-structural proteins NS3, NS4B and NS5A have a regulatory effect on cellular promoters and interact with a number of cellular proteins involved in carcinogenesis under certain conditions[18-21]. In addition, hepatocytes from patients with chronic HCV infection are arrested in G1 and may undergo replicative senescence, which may predispose to malignancy[22]. Direct evidence for a carcinogenic role for HCV *in vivo* is lacking.

HCC has been described very rarely in chronic HCV infection in the absence of cirrhosis[4-7], which suggests that other aetiological factors may be more important. Analyses of case series in Japan have suggested that ageing increases the risk of developing HCC in patients with HCV who do not have cirrhosis, particularly in wom-

en[10,11]. In the series presented here, however, five of six cases were men and the median age at diagnosis of HCC was 55, suggesting that alternative factors are important. None of the cases described had histological evidence of additional injury such as steatohepatitis or iron accumulation that are recognised co-factors in the development of liver injury and HCC[23,24]. However, four of six patients had serological evidence of previous exposure to HBV. It is possible that integration of HBV genes had occurred, increasing the risk of HCC as in HBV infection without cirrhosis. Long-term immune suppression in another may have increased the risk of HCC[25]. Furthermore, it has been reported that renal transplant patients might have an increased susceptibility to HCC even without viral infection purely as a result of immune suppression[26]. In the final patient there was a long history of excess alcohol use but no evidence of alcohol related liver damage on biopsy.

A third of patients with HCV have been exposed to HBV because of common risk factors[27]. In the Cambridge series 35% of 1500 patients with chronic HCV infection were anti-HBc positive/HBsAg negative while 2% were co-infected with HBV. Past HBV infection is associated with an increased risk of progressive liver injury in some series of patients with chronic HCV infection and other liver disorders[28,29] although the presence of anti-HBc was not associated with progressive fibrosis in our own series[30]. HBV genomic material has been identified in liver from patients with HCC who were HBsAg negative but HCV RNA positive[31,32] and the presence of HBV genes in this setting has been linked to HCC[33]. In a prospective observational study, serum anti-HBc was a marker of high risk for HCC among patients with HCV related cirrhosis, but was not a significant risk factor in those without cirrhosis[34]. The presence of HBV genes in HCC tissue of HBsAg negative, HCV negative patients has also been described[35]. Thus, long-term persistence of HBV genes in liver tissue may cause HCC without inflammation, necrosis or regeneration. While abnormal alanine aminotransferase (ALT) fluctuation is associated with carcinogenesis in HCV positive patients[36], the presence of integrated HBV DNA in the liver may promote carcinogenesis independently. Patients with low levels of ALT and minimal histological change may still be at risk of HCC development if they have had previous exposure to HBV.

Surveillance for HCC has been conducted for many years but a survival benefit for screening with 6-monthly ultrasound and AFP monitoring has only been demonstrated recently[1]. However, surveillance is practiced widely and recommended in high-risk groups such as those with cirrhosis due to HBV, HCV, alcohol, or haemochromatosis[2]. In addition, because of the high risk of HCC in non-cirrhotic HBV infection, current guidelines recommend screening in high-risk groups (family history of HCC, Asian males > 40 years, Asian females > 50 years and Africans > 20 years and could be extended to those with high serum HBV DNA levels). In HCV infection with cirrhosis there is a high risk of HCC development (2%-8% per year); surveillance is recommended and cost effective[37]. At the current time it is unclear whether patients with bridging fibrosis should be offered screening and it is not recommended for patients with mild or moderate disease regardless of patient age or length of time of infection.

All HCV-infected patients with cirrhosis in our centre are offered 6-monthly screening with liver ultrasound by a small group of experienced liver radiologists or ultrasonographers; serum AFP is not sought in this particular cohort because of a lack of specificity and sensitivity, as demonstrated in this series. This experience has prompted review of our policy; for example should older men with chronic HCV infection without cirrhosis but with an additional risk factor for HCC, such as anti-HBc or excess alcohol consumption, undergo ultrasound screening? The increased workload for our service would be enormous and it is probable that the number of patients identified with HCC that could be cured by intervention would be too low to justify such an approach. Further data on the incidence of HCC in HCV-infected patients without cirrhosis are required before a change in policy can be recommended as routine practice; a national register of such cases could be helpful.

COMMENTS

Background

Cirrhosis of any cause is associated with a significant risk of developing hepatocellular carcinoma (HCC). Chronic hepatitis B virus (HBV) infection in the absence of cirrhosis is also a recognised risk factor for HCC and screening is recommended in some high risk groups. HCC occurs rarely in non-cirrhotic hepatitis C virus (HCV) infection and there are no recommendations for screening in these patients.

Research frontiers

Due to the observation of an increase in HCC in non-cirrhotic HCV patients, a detailed evaluation of these patients was undertaken.

Innovations and breakthroughs

In each patient with HCC and non-cirrhotic HCV infection, an additional risk factor for hepatocarcinogenesis was identified. These included previous infection with HBV, high alcohol intake and immunosuppression.

Applications

Patients with chronic HCV without cirrhosis may be at risk of developing HCC if there are other risk factors for liver injury and carcinogenesis present. It is possible that these patients should be considered for surveillance programmes although this would result in a dramatic increase in workload for radiological departments and may not be cost effective.

Peer review

This is a well written article.

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BRIEF ARTICLE

Quality of life assessment in patients with chronic pancreatitis receiving antioxidant therapy

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Abstract

AIM: To undertake a baseline study comparing quality of life (QoL) in patients with chronic pancreatitis (CP) on Antox to those with CP, matched for disease duration, who were not on this medication.

METHODS: CP was defined according to the Zurich classification. Sixty eight consecutive patients with CP who were taking Antox (antioxidants) were compared with 69 consecutive control CP patients not on Antox. European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Core questions 30 and Pancreatic Modification (28 questions) were used to assess QoL. Out of a total of 137 patients 28 in each group were matched for disease duration (within 12 mo). Median disease duration was 8 (1-22) years in the Antox group and 7 (1-23) years in the Non-Antox cohort (P = NS, Mann-Whitney U-test). Other parameters (age, gender, etiology, endocrine and exocrine insufficiency) were similar between groups.

RESULTS: Median visual analogue pain score in the Antox group was 3 (0-8) compared with 6 (0-8) in the Non-Antox group ($P < 0.01$). Perceptions of cognitive, emotional, social, physical and role function were impaired in the Non-Antox group compared to Antox patients ($P < 0.0001$, $P = 0.0007$, $P = 0.0032$ and $P < 0.005$ and $P < 0.001$, respectively). Analgesics and opiate usage was significantly lower in the Antox group ($P < 0.01$). Overall physical health and global QoL was better in the Antox group ($P < 0.0001$, 95% CI: 1.5-3).

CONCLUSION: Contemporary quality of life assessments show that after correction for disease duration and cigarette smoking, patients with CP taking antox had better scores than non-antox controls.

Key words: Chronic pancreatitis; Antioxidants; Quality of life; Assessment; Management

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INTRODUCTION

Chronic pancreatitis (CP) is a chronic inflammatory con-

dition of the pancreas characterized histologically by loss of normal pancreatic parenchymal architecture with varying degrees of fibrosis and inflammatory infiltration[1]. Clinically, CP presents a spectrum of disease most often marked by chronic or recurrent abdominal pain together with varying features of pancreatic exocrine deficiency, most typically fat-related, resulting in steatorrhea, and manifestations of pancreatic endocrine deficiency, such as diabetes mellitus[1].

Although there are no national incidence registries, population-based data indicate a frequency of 8.6/100 000 per year[2] with similar incidence being recognized in the United States[3] and other Northern European countries[4,5]. The clinical course of CP can be characterized by variable background abdominal pain with episodic exacerbations. Although the later stages of the illness are marked by pancreatic exocrine and endocrine insufficiency, abdominal pain is the dominant symptom for many sufferers.

Until now there has been no specific therapy for CP. Rose *et al*[6] demonstrated that CP arose as a result of pathological exposure of the pancreatic acinar cells to short-lived oxygen free radicals - a process termed oxidative stress. The peripheral blood samples taken from patients with clinical CP have shown that antioxidants (the term given to inhibitors of the oxidative stress response), their precursors and co-factors in physiologic antioxidant pathways are depleted during the course of this illness[7]. In addition, there is elevation of peripheral blood markers of oxidative injury. Braganza *et al*[8] reasoned that exogenous supplementation with antioxidants or precursors for antioxidant pathways may augment these deficient pathways and help to quench ongoing acinar injury. From a series of exploratory studies they concluded that co-factors of the endogenous glutathione peroxidase pathway were key components for supplementation. Selenium, vitamin C (ascorbic acid) and methionine were proposed as key antioxidants[8-10]. A commercially-available formulation, Antox (Pharma Nord, Morpeth, UK) was developed comprising vitamin C, vitamin E, β-carotene, selenium and methionine.

There is anecdotal evidence from small, underpowered, randomized trials that oral antioxidant therapy reduces the frequency and severity of episodes of pain[11,12]. More recently, a well-conducted randomized trial from India demonstrated that oral antioxidant therapy was associated with a reduction in hospital admission and "pain days"[13].

In contemporary healthcare terms, perhaps the critical issue in the treatment of CP is assessment of the effect of intervention on quality of life (QoL). In this regard, formal, well-validated questionnaire-based QoL scoring systems are now available for assessment of patients with CP[14].

The European Organization for Research and Treatment of Cancer (EORTC) QoL study group has developed a modular approach to the development of QoL instruments designed for use in clinical trials[15]. A 30-item core cancer questionnaire; the EORTC Quality of Life Questionnaire (QLQ) Core questions 30 (C-30) was developed. It was initially developed and validated for use in patients with non small cell lung cancer[16]. The 30-item core questionnaire is intended to be supplemented by additional questionnaire modules to assess disease symptoms and treatment side-effects. The EORTC QLQ Pancreatic Modification (26 questions) (PAN-26) was developed and mainly used for pancreatic cancer[17]. During the development of the QLQ PAN-26, interest was expressed in the feasibility of using this assessment system in patients with CP[14].

Two questions have been added to PAN-26 to produce a questionnaire for use in CP, the QLQ PAN (CP)-28[17].

The aim of this study is to compare QoL, using an appropriately validated, disease-specific questionnaire-based approach in a cohort of patients with CP receiving oral antioxidant therapy to individuals with CP who are not receiving this medication.

MATERIALS AND METHODS

Study design

This is a prospective, single-centre clinical study comparing QoL as assessed using validated, disease-specific EORTC questionnaires[17] in a group of patients with a clinical diagnosis of CP receiving oral antioxidant therapy in the form of Antox (Pharma Nord, Morpeth, UK) to a cohort of patients with CP from the hepatobiliary and gastroenterology services of the same hospital who were not receiving oral antioxidant supplementation.

Patients and treatment algorithm

The terminology advocated by the Zurich International Workshop was used to define alcohol-related CP[18]. All patients had radiological evidence of CP on either computed tomography or magnetic resonance imaging; in addition some patients had supplementary evidence from endoscopic retrograde pancreatography or endoscopic ultrasonography. Routine monitoring of blood glucose was undertaken in the outpatient setting. Pancreatic exocrine function testing was not routinely employed in patients in this study. In general, antioxidant therapy was offered for the treatment of patients with a diagnosis of CP if there was no evidence of a pancreatic lesion potentially requiring surgical intervention, or if there was no marked pancreatic ductal dilatation amenable to endoscopic or surgical drainage. Thus, for the purposes of this study, patients were categorized into those taking Antox and those not taking this medication. Clinical characteristics of these 2 groups are shown in Table 1.

Administration of questionnaire, data registration and analysis

Patients completed the EORTC QLQ C-30 and QLQ PAN-28 questionnaires in the presence of an interviewer as part of a dedicated interview. Pain was assessed using a visual analogue score (VAS), where 0 is no pain and 10 is the maximum (scale 0-10). All interviews were undertaken by the same interviewer (NS). Interviews were conducted

Table 1 Profile of patients with chronic pancreatitis matched for similar disease duration

	CP patients on Antox (n = 28)	CP patients NOT on Antox (n = 28)	P-value
Age (yr), median (range)	53 (24-82)	53 (31-74)	0.30 (Mann-Whitney U-test)
Etiologies	Alcohol: 13 (46%) Idiopathic: 13 (46%) Others: 2 (8%)	Alcohol: 17 (61%) Idiopathic: 11 (39%)	
Gender (male:female)	16:12	18:10	0.79 (Fisher's exact test)
Duration of disease (yr), median (range)	8 (1-22)	7 (1-23)	0.85 (Mann-Whitney U-test)
Current cigarette smoking	8 (27%)	18 (62%)	0.01 (Fisher's exact test)
Alcohol before diagnosis of CP	118 (48-240) g/d per person	160 (28-240) g/d per person	< 0.01 (Mann-Whitney U-test)
Alcohol intake, current mean (range)	25 (0-48) g/d per person	33 (0-96) g/d per person	0.63 (Mann-Whitney U-test)

CP: Chronic pancreatitis.

with patients attending the Hepatobiliary and Gastroenterology clinics in this hospital during the study period February 2007 to February 2009. The interviewer was a clinical research fellow and not involved in the clinical care of any of the patients. Questionnaires were completed prospectively but analyzed retrospectively as a batch after completion of the study. Questionnaire results were not used to inform clinical decision-making. Questionnaire results were transcribed onto an electronic database (Microsoft Excel, Microsoft, Redmond, Washington, USA) for subsequent analysis. The interviews were conducted on a single time point basis: no patients underwent repeat interview. All patients in the Antox group had been receiving therapy for at least 6 mo. No patients had undergone surgery in the 6 mo prior to interview.

Disease duration-matched cohort

Interim analysis of the whole cohort data showed that there were significant differences in the median age and disease duration between patients in the Antox group and those in the Non-Antox cohort. In an effort to correct for at least one of these factors, disease duration matching was undertaken. The disease duration was recorded for each patient from the clinical chart. Patients in the Non-Antox group were then matched with corresponding individuals from the Antox group. A disease duration of the same time period ± 12 mo was selected for matching. No patient was included twice and data were paired by searching chronologically according to date of interview from first interviewee to the last.

Ethics committee approval

This study was approved by regional research ethics committee.

Statistical analysis

Continuous data are presented as median (range). Statistical comparisons were by non-parametric test using the Mann-Whitney U-test for 2 group comparisons and Fisher's exact test for contingency tables. The Wilcoxon signed ranks test (two-sided test) was used for comparison of paired data. Statistical significance was at the $P < 0.05$ level. The StatsDirect software program was used for statistical analyses (StatsDirect version 2.6.5, http://www.statsdirect.com).

RESULTS

Entire cohort comparison (NOT matched for disease duration)

Alcohol was the most common etiologic agent in 84 (61%) of patients. The median age of the group taking Antox was 56 (24-82) years compared to 47 (24-74) years in those not taking Antox. This difference was statistically significant. Disease duration and proportion of patients with diabetes mellitus were also greater in the Antox groups (although the difference in incidence of diabetes mellitus was not significant).

Entire cohort outcome (NOT matched for disease duration)

VAS, overall physical health scores and global QoL were significantly better in patients with CP taking Antox. These results are reflected in the significantly lower number of patients in the Antox group taking analgesics and opiates.

Disease-duration matched cohort outcome

Table 1 shows that the disease duration-matched cohort were also similar in terms of age, etiology of CP and gender ratio. There were more smokers in the Non-Antox group, and alcohol intake prior to diagnosis was also greater in this group.

The outcome data in the disease duration-matched patients show that patients taking Antox had lower pain scores and fewer were taking analgesics (including opiates). There was no difference in the proportions of patients who were diabetic or who were taking pancreatic exocrine supplements (Table 2). A significantly greater number of patients in the Non-Antox group had undergone either surgical or endoscopic intervention.

Detailed global outcome data from the disease duration-matched cohort are shown in Table 3. Answers to questions were ranked on a scale of 1 to 4 [(1) not at all affected; (2) a little affected; (3) quite a bit affected and (4) very much affected]. In addition to lesser pain scores (as above) factors which were significantly better in patients

Table 2 Quality of life, pain scores and analgesic usage in disease duration-matched patients with chronic pancreatitis

	CP patients on Antox (n = 28)	CP patients NOT on Antox (n = 28)	P-value
Median visual analogue pain scores (range 0-10)	3 (0-8)	6 (0-8)	< 0.01 (Mann-Whitney U-test)
Patients taking analgesics	16	26	< 0.01
Patients taking opiate analgesics	11	23	< 0.01
Diabetes	10 (36%)	11 (39%)	0.80
Pancreatic exocrine supplements	14 (50%)	16 (57%)	0.60

CP: Chronic pancreatitis.

Table 3 Detailed European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Core questions 30 and Pancreatic Modification (28 questions) results in disease duration-matched patients with chronic pancreatitis

Scales	Items	CP patients on Antox mean score (n = 28)	CP patients NOT on Antox mean score (n = 28)	P-value	95% CI
Physical Functioning	Q-1-5	1.5	1.97	0.005[1]	-0.8 to 0.1
Role Functioning	Q-6-7	1.84	2.75	0.001[1]	-1.5 to 0.5
Pain	Q-9, 19	2.1	3.1	< 0.0001[1]	-1.5 to 0.7
Fatigue	Q-10, 12, 18	2.05	2.86	0.0001[1]	-1.7 to 0.3
Nausea and vomiting	Q-14, 15	1.5	2.2	0.002[1]	-1.2 to 0.2
Cognitive functioning	Q-20, 25	1.6	2.4	< 0.0001[1]	-1 to 0.5
Emotional functioning	Q-21-24	1.8	2.6	0.0007[1]	-1.2 to 0.5
Social functioning	Q-26, 27	1.8	2.7	0.0032[1]	-1.2 to 0.2
Global quality of life	Q-29, 30	2.6	4.9	< 0.0001[1]	1.5 to 3
Pancreatic Pain	Q-31, 33, 35	1.9	2.9	< 0.0001[1]	-1.5 to -0.7
Digestive function	Q-36, 37	2.14	2.5	0.09[1]	-1 to 0
Jaundice	Q-44, 45	1.1	1.2	0.41[1]	-0.25 to 0
Altered bowel functioning	Q-46, 47	2.1	1.6	0.07[1]	-1 to 0
Body Image	Q-48, 51	1.3	2.5	0.0004[1]	-1.2 to -0.25
Alcohol related guilt	Q-49, 50	1.5	1.4	0.7[1]	-0.25 to 0.5
Satisfaction with health care	Q-55, 56	3.2	3.4	0.44[1]	-0.5 to 0.25
Sexual functioning	Q-57, 58	1.7	2.66	0.0003[1]	-1.5 to -0.5
Dyspnea/shortness of breath	Q-8	1.5	1.6	0.63[1]	-0.5 to 0
Difficulty sleeping	Q-11	1.9	2.9	0.0003[1]	-1.5 to 0.5
Loss of appetite	Q-13	1.8	2.5	0.04[1]	-1.5 to 0
Constipation	Q-16	1.7	1.8	0.86[2]	-1 to 0.5
Diarrhea	Q-17	1.5	1.5	0.03[2]	-1 to 0
Financial problems	Q-28	1.3	1.9	0.02[2]	-1 to 0
Bloated abdomen	Q-32	1.9	2.8	0.002[2]	-1.5 to 0.5
Night pain	Q-34	1.8	3.1	< 0.0001[2]	-2 to -0.5
Taste changes	Q-38	1.2	1.7	0.02[2]	-1 to 0
Indigestion	Q-39	1.7	2.4	0.018[2]	-1 to 0
Flatulence	Q-40	2.0	2.6	0.06[2]	-1 to 0
Weight loss	Q-41	1.2	2.4	< 0.0001[2]	-1.5 to -0.5
Decreased muscle strength	Q-42	1.8	2.3	0.11[2]	-1 to 0
Dry mouth	Q-43	1.6	2.2	0.02[2]	-1 to 0
Treatment side effects	Q-52	1.2	2.0	0.0002[2]	-1 to -0.5
Fear for future health	Q-53	2.4	3.0	0.03[2]	-1 to 0
Ability to plan ahead	Q-54	1.8	2.9	0.0001[2]	-1.5 to 0.5

[1]Wilcoxon signed rank test 95% CI; [2]Wilcoxon t-test. CP: Chronic pancreatitis; Q: Question No. (total C-30 + PAN 28 = 58 questions).

taking Antox were: physical functioning, role functioning and cognitive and emotional functioning. These translated into a significant improvement in global QoL. Digestive function, jaundice and bowel function were not significantly different.

DISCUSSION

This study has examined QoL in patients with CP. Contemporary criteria were used for definition of disease and the Zurich Workshop recommendations were used for assessment of alcohol-related CP[18]. QoL has been evaluated in 2 cohorts of patients: those taking oral antioxidant therapy for CP and those not taking this medication. Specific disease-validated questionnaires were used for assessment of QoL[17].

Antioxidant therapy has been available for the treatment of CP for over 20 years[19]. However, the lack of good-quality randomized trial evidence and the dearth of information about clinical outcome in patients taking antioxidants has meant that this treatment remains on the periphery of practical management.

Thus, the importance of the present study is that it is believed to be the first to utilize specific disease-validated questionnaire methodology to assess QoL in patients with well-defined CP taking oral Antox. Potential sources of bias in these data should be borne in mind when interpreting the results.

Patients were not randomly allocated to Antox or Non-Antox; those receiving Antox were older and had longer disease duration (Table 1). Although disease-duration matching may have corrected for some of these factors, other confounding factors could persist: there were more smokers in the Non-Antox group and a sequential, multiple interview strategy would have yielded a more accurate reflection of QoL. This is accepted but must be balanced against the inconvenience to patients resulting from completing the lengthy questionnaires involved in this study. Also on a practical basis all patients were interviewed at relatively stable points in their disease with no history of recent surgery or change in medication.

Accepting these likely sources of bias, measures of QoL showed a significant benefit in patients on Antox: pain scores, physical health scores and global QoL together with analgesic (including opiate) intake were significantly better in the Antox group.

However, these findings may simply reflect a more mature population in the Antox group who have had more time to adjust to their illness and in some of whom the disease may be "burnt out"[20].

It is accepted that disease duration in a long-term chronic illness such as CP can be unreliably recalled[21] but prospectively recorded duration data were taken from the patients' charts and thus any error should be similar in both groups. The process of matching for disease duration produces a cohort of 28 pairs who are also reasonably well matched in terms of age, gender and etiology (Table 3). Although alcohol consumption in the Non-Antox group was greater prior to diagnosis, there was no difference after diagnosis.

The outcome data from the disease duration-matched cohort show some striking findings. VAS were significantly lower in the Antox group with a corresponding lower use of analgesics including opiate analgesics. There were no differences in the proportion of patients with diabetes or those taking pancreatic exocrine supplements, suggesting that if Antox modified symptoms in CP, there was no effect on the underlying disease course. Examined in detail, using the full EORTC questionnaires, the study showed improvement in global QoL in patients taking Antox. There were no differences in jaundice and digestive function answers, again suggesting that antioxidant therapy may modify symptoms without affecting disease progression.

These data should be considered together with the results of a recent large randomized trial from India of oral multi-compound antioxidants in painful CP. Although the Indian study did not use QoL measurement and had soft principal end-points in the form of reduction in hospital admission and reduction in "pain days" the study showed benefit from treatment with antioxidants.

In summary, this study has used well-validated, disease-specific questionnaires to assess QoL in patients with well-defined CP and compared a cohort of patients taking Antox to a group who were not. When corrected for differences in disease duration and age, patients on Antox had significantly lower VAS, lower analgesic use and better global QoL. Caution in interpretation is required. We would state that these data support a renewal of interest in the role of antioxidant therapy in CP and favor the conduct of a formal, randomized placebo-controlled trial of Antox in painful CP.

COMMENTS

Background

Chronic pancreatitis (CP) is associated with severe, disabling, frequent abdominal pain. It often leads to endocrine (diabetes) and exocrine (diarrhea and weight loss) disorders. In general, patients with CP have very poor quality of life (QoL) and outcome. No treatment has been found to combat long-term pain and cure.

Research frontiers

There has been anecdotal evidence of upregulation of the oxidative stress response and deficiency in antioxidants levels in patients with CP. The cascade of events due to repeated exposure and non correction leads to pancreatic fibrosis. This in turn leads to severe, chronic abdominal pain and poor QoL. In this study we demonstrated improvement in QoL for patients who were on antioxidant therapy.

Innovations and breakthroughs

Antioxidant therapy for CP was suggested in the mid 1990s. There have been a few studies showing the benefit of antioxidant therapy in CP. However, due to the paucity of data, it has not been universally accepted. This is the first comparative study to report QoL assessment in patients with CP on antioxidant therapy and those NOT on antioxidant therapy.

Applications

The study has renewed interest in antioxidant therapy for CP. The data lack randomization. This report supports the progression to a formal randomized double-blind trial of antioxidant therapy assessing QoL and outcome in patients with CP.

Terminology

Antioxidants is the term given to inhibitors of the oxidative stress response. They are typically selenium, vitamin E, methionine and ascorbic acid. European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire Core questions 30 and Pancreatic Modification (28 questions) (PAN-28) (EORTC, QoL core questionnaire and its pancreatic modification, PAN-28) are the QoL assessment tools validated and used for patients suffering from CP. Such a methodology is important and vital in measuring the outcome in chronic diseases.

Peer review

In general, this study by Shah *et al* has high originality and is interesting because they revealed the effectiveness of antioxidant treatment for CP which focused on QoL.

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BRIEF ARTICLE

Diagnostic value of cancer-testis antigen mRNA in peripheral blood from hepatocellular carcinoma patients

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Abstract

AIM: To evaluate the diagnostic value of cancer-testis antigen (CTA) mRNA in peripheral blood samples from hepatocellular carcinoma (HCC) patients.

METHODS: Peripheral blood samples were taken from 90 patients with HCC before operation. Expression of melanoma antigen-1 (MAGE-1), synovial sarcoma X breakpoint-1 (SSX-1), and cancer-testis-associated protein of 11 kDa (CTp11) mRNA in peripheral blood mononuclear cells (PBMC) was tested by nested reverse transcripts-polymerase chain reaction (RT-PCR). Serum α-fetoprotein (AFP) in these patients was also determined.

RESULTS: The positive rate of MAGE-1, SSX-1 and CTp11 transcripts was 37.7%, 34.4%, 31.1% in PBMC samples, and 74.4%, 73.3%, 62.2% in their resected tumor samples, respectively. The positive rate for at least one of the transcripts of three CTA genes was 66.7% in PBMC samples and 91.1% in their resected tumor samples. MAGE-1, SSX-1 and/or CTp11 mRNA were not detected in the PBMC of those patients from whom the resected tumor samples were MAGE-1, SSX-1 and/or CTp11 mRNA negative, nor in the PBMC samples from 20 healthy donors and 10 cirrhotic patients. Among the 90 patients, the serum AFP in 44 patients met the general diagnostic standard (AFP > 400 μg/L) for HCC, and was negative (AFP ⩽ 20 μg/L) or positive with a low concentration (20 μg/L < AFP ⩽ 400 μg/L) in the other patients. The positive rate for at least one of the transcripts of three CTA genes in PBMC samples from the AFP negative or positive patients with a low concentration was 69.2% and 45.0%, respectively. Of the 90 patients, 71 (78.9%) were diagnosed as HCC by nested RT-PCR and serum AFP. Although the positive rate for at least one of the transcripts of three CTA genes in PBMC samples from 53 patients at TNM stage Ⅲ or Ⅳ was obviously higher than that in PBMC samples from 37 patients at stage Ⅰ or Ⅱ (77.9% *vs* 51.4%, $P = 0.010$), the CTA mRNA was detected in 41.7% and 56.0% of PBMC samples from HCC patients at stages Ⅰ and Ⅱ, respectively.

CONCLUSION: Detecting MAGE-1, SSX-1 and CTp11 mRNA in PBMC improves the total diagnostic rate of HCC.

Key words: Hepatocellular carcinoma; α-fetoprotein; Cancer-testis antigen; Diagnosis; Nested reverse transcripts-polymerase chain reaction

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and lethal cancers in the world[1-3]. More than one million cases of HCC occur in the world each year[4]. Although many treatment modalities for HCC are available (including hepatic resection, liver transplantation, radiofrequency ablation, transarterial chemoembolization, *etc.*) at present, the prognosis of HCC patients remains dismal because it is detected at an advanced, non-resectable stage. Early diagnosis of HCC can improve the prognosis of HCC patients. So far, α-fetoprotein (AFP) is the generally accepted serological marker. Serum AFP alone contributes to the diagnosis of HCC if its level is markedly elevated (over 400 μg/L as a threshold value), which occurs in less than 50% of cases at the time of diagnosis[5-8]. Moreover, serum AFP level is negative or slightly elevated in 20%-40% patients, which can significantly reduce the sensitivity of an assay based on over-expression of AFP. The serum AFP level in patients with acute or chronic hepatitis or liver cirrhosis but without malignant disease is often elevated. Since detection of serum AFP level in blood samples appears to be nonspecific[7-10]. Therefore, the diagnostic sensitivity and specificity of AFP are unsatisfactory and questionable. It is thus necessary to select other specific methods for the diagnosis of HCC.

Transcripts of tumor-specific genes can be amplified and detected by reverse transcripts-polymerase chain reaction (RT-PCR), which is a reliable technique to detect circulating tumor cells (CTC). In 1991, Smith *et al*[11] first successfully adopted RT-PCR technique to assess tyrosinase messenger RNA (mRNA) as a tumor marker in detecting circulating melanoma cells. Since then, this technique has been applied to the detection of CTC in solid tumors[12-14]. Melanoma antigen-1 (MAGE-1)[15], synovial sarcoma X breakpoint-1 (SSX-1)[16] and cancer-testis-associated protein of 11 kDa (CTp11)[17] antigens have been designated as cancer-testis antigens (CTA). It has been reported that MAGE-1 and SSX-1 mRNA are expressed with a high percentage and specificity in HCC[16,18-20]. Our group has verified a relatively high and specific expression of CTp11 mRNA in HCC tissues but not in the corresponding adjacent non-HCC and cirrhosis tissues[21]. In this study, we evaluated the diagnostic significance of a highly sensitive nested RT-PCR assay for the MAGE-1, SSX-1 and CTp11mRNA in peripheral blood of HCC patients.

MATERIALS AND METHODS

Cell lines

Human HCC cell lines BEL7405 expressing MAGE-1 mRNA and LM3 expressing both SSX-1 and CTp11 mRNA, purchased from the Cell Bank, Chinese Academy of Sciences, and Liver Cancer Institute, Zhongshan Hospital, Fudan University, respectively, were grown in RPMI1640 medium with 10% fetal calf serum and served as a positive control of the assay used in this study.

Patients and tissue samples

Ninety consecutive patients (79 men and 11 women) with a mean age of 45.6 ± 2.7 years (range 18-79 years) undergoing operation for HCC, including hepatectomy (48 cases) or orthotopic liver transplantation (42 cases), at the 2nd Hospital of Peking University Health Science Centre, were enrolled in this study. Of the 83 patients with virus infection, 79 were infected with hepatitis B virus, 2 with hepatitis C virus, and 2 with both hepatitis B and C viruses. The serum AFP level was negative (≤ 20 μg/L) in 26 patients, positive with a low concentration (20 μg/L < AFP ≤ 400 μg/L) and a high concentration (> 400 μg/L) in 20 and 44 patients, respectively. According to the TNM classification of International Union Against Cancer[22], 12 cases were classified as stage Ⅰ, 25 as stage Ⅱ, 9 as stage Ⅲ, and 44 as stage Ⅳ, respectively. HCC and its adjacent non-cancerous tissue samples (the distance to the edge of HCC tissue > 2 cm) were collected during operation. Control samples collected by surgical biopsy included 20 liver tissue samples from cirrhotic patients and 20 normal liver tissue samples from patients without liver disease. Testis tissue (kindly provided by Urological Department of the 2nd Hospital of Peking University Health Science Centre) was used as a positive control. Clinical diagnosis was confirmed by pathological examination. Each sample was immediately frozen in liquid nitrogen and stored at -80℃ until extraction of total RNA. Informed consent was obtained from each patient before the study. The study protocol was approved by the Ethic Committee of Peking University.

Blood samples

Whole blood samples were taken from the 90 HCC patients on the day before operation. Control blood samples were collected form 20 healthy volunteers and 10 cirrhotic patients. Ten mL of blood from each patient was collected into a heparinzed tube and peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation[15].

Extraction of total RNA and synthesis of cDNA

Total RNA was extracted from frozen tissue specimens (50-100 mg) and freshly isolated PBMC (1×10^7 cells) using TRIZOL reagent (GIBCOL BRL) according to its manufacturer's instructions. Total RNA (2.5 μg) was primed with an Oligo (dT)15 oligonucleotide (Promega, USA) and reverse-transcribed with Superscript Ⅱ (GIBCO BRL, USA) according to their manufacturers' instructions.

PCR amplification of CTAs

PCR amplification reaction (50 μL) contained 5 μL of

Table 1 Primers and conditions used in nested reverse transcripts-polymerase chain reaction

Primers	Primers from 5' to 3'	PCR conditions denaturation annealing extension
MAGE-1 outer primers	Forward primer: 5'-CGGCCGAAGGAACCTGACCCAG-3'	94℃ for 45 s
	Reverse primer: 5'-GCTGGAACCCTCACTGGGTTGCC-3'	65℃ for 45 s
		72℃ for 45 s
MAGE-1 inner primers	Forward primer: 5'-ACAGAGGAGCACCAAGGAGAAG-3'	94℃ for 45 s
	Reverse primer: 5'-AGTTGATGGTAGTGGGAAAGGC-3'	65℃ for 45 s
		72℃ for 45 s
SSX-1 outer primers	Forward primer: 5'-CTAAAGCATCAGAGAAGAGAAGC-3'	94℃ for 60 s
	Reverse primer: 5'-AGATCTCTTATTAATCTTCTCAGAAA-3'	57℃ for 60 s
		72℃ for 60 s
SSX-1 inner primers	Forward primer: 5'-TCAGAGAAGAGAAGCAAGGCCTTT-3'	94℃ for 45 s
	Reverse primer: 5'-TTCTCAGAAATATTTGCTTTTCC-3'	56℃ for 45 s
		72℃ for 45 s
CTp11 outer primers	Forward primer: 5'-CTGCCCCAGACATTGAAGAA-3'	94℃ for 45 s
	Reverse primer: 5'-TCCATGAATTCCTCCTCCTC-3'	57℃ for 60 s
		72℃ for 90 s
CTp11 inner primers	Forward primer: 5'-TGTGAATCCAACGAGGTG-3'	94℃ for 45 s
	Reverse primer: 5'-TTGATTCTGTTCTCTCGGGC-3'	60℃ for 45 s
		72℃ for 45 s

PCR: Polymerase chain reaction; MAGE-1: Melanoma antigen-1; SSX-1: Synovial sarcoma X breakpoint-1; CTp11: Cancer-testis-associated protein of 11 kDa.

cDNA, 1 μL each of 10 μmol/L outer/inner primers, 1 μL of 10 mmol/L dNTP mixture, 2.5 U *Taq* polymerase (GIBCO BRL, USA) in a buffer solution. Thirty-five cycles of PCR amplification of cDNA from liver tissue were performed with a pre-programmed UNO Ⅱ thermocycler (Biometra, German) under the following conditions: an initial denaturation at 94℃ for 5 min, a final extension at 72℃ for 8 min. The PCR products were 421 base pair (bp) (MAGE-1), 422 bp (SSX-1) and 297 bp (CTp11), respectively. Twenty-five cycles of PCR amplification of cDNA from PBMC were performed with its first round conditions identical to those of cDNA from liver tissue. For the second round of PCR, 1 μL of the first-round PCR products was used as a template in combination with 1 μL each of 10 μmol/L inner primers. After heated for 2 min at 94℃, the samples were subjected to 35 cycles of PCR amplification, followed by a final extension at 72℃ for 8 min. The PCR products were 299 bp (MAGE-1), 398 bp (SSX-1) and 188 bp (CTp11), respectively. The PCR conditions and outer/inner primers for MAGE-1[15], SSX-1[16] and CTp11[17] used in this study are shown in Table 1. To verify the integrity of cDNA[18], β2-microglobin (β2-MG) (primers: forward: 5'-CTCGCGCTACTCTCTCTTTCTGG-3' and reverse: 5'-GCTTACATGTCTCGATCCCACTTAA-3', 335 bp) was amplified for 30 cycles (at 94℃, 55℃ and 72℃ for 45 s). For analysis, 8 μL of reaction products was run in 2% agarose gel (Promega, USA), followed by ethidium bromide staining and digital camera photographing (Korda D3.5, USA).

Sensitivity of nested RT-PCR technique

The sensitivity of our nested PCR assay was evaluated by performing the procedure on healthy volunteer blood samples mixed with a certain number of hepatoma cells. Ten-fold serial dilution experiments from 10^4 hepatoma cells were carried out using human hepatoma cell lines BEL7405 expressing MAGE-1 transcript and LM3 expressing both SSX-1 and CTp11 mRNA. Hepatoma cells (1×10^3, 1×10^2, 1×10^1 and 1×10^0) were added to 5×10^6 PBMC from healthy donors, then total RNA was extracted and subjected to RT-PCR amplification with primers for β2-microglobin, MAGE-1, SSX-1 and CTp11 genes.

Sequence analysis of PCR products

PCR amplification-purified cDNA was cloned into the pGEM-T easy vector (Promega) by T4 DNA ligase and amplified in *Escherichia coli*, JM109. Four positive colonies were selected and assessed using EcoR Ⅰ digestion of mini-prepared DNA. Putative MAGE-1, SSX-1 and CTp11 cDNA samples were sequenced with T7 sequencing primers in Sangon Co., Shanghai, China.

Statistical analysis

Statistical analysis was performed by chi-square test and Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Sensitivity of nested RT-PCR technique

After two rounds of PCR amplification, MAGE-1, SSX-1 and CTp11 transcript genes could be detected in the PCR products, indicating that our assay is able to detect a hepatoma cell in 5×10^6 PBMC (Figure 1).

Expression of CTA genes in HCC tissue samples

Expression of MAGE-1, SSX-1 and CTp11 mRNA was detectabled in 74.4%, 73.3% and 62.2% of the 90 HCC tissue samples, respectively. No expression of these genes was detected in the corresponding adjacent non-HCC tissue samples, or in the normal and cirrhotic liver tissue samples. Eighty-two HCC tissue samples (91.1%) were

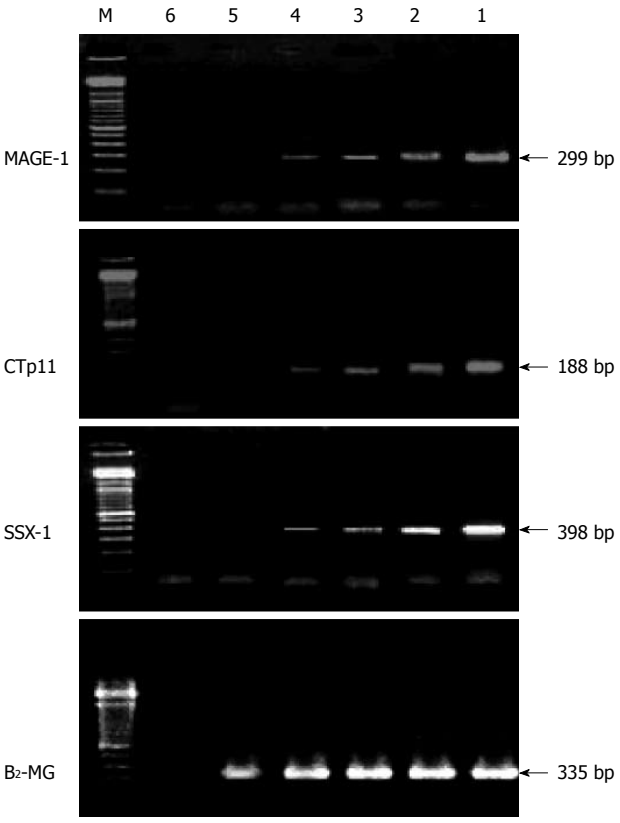

Figure 1 Sensitivity of nested polymerase chain reaction assay to tumor-specific markers in hepatocellular carcinoma cell lines. Lanes 1-4: 1 × 10^3, 1 × 10^2, 1 × 10^1 and 1 × 10^0 hepatoma cells detected by nested reverse transcripts-polymerase chain reaction using melanoma antigen-1 (MAGE-1), synovial sarcoma X breakpoint-1 (SSX-1), cancer-testis-associated protein of 11 kDa (CTp11), and β2-microglobin; Lane 5: Peripheral blood mononuclear cells (PBMC) from healthy donors only; Lane 6: negative control; Lane M: Molecular marker, 100 bp DNA ladder (Gibco).

positive for at least one of the transcripts of three CTA genes.

Nested RT-PCR results in HCC PBMC samples

After two rounds of PCR amplification, MAGE-1, SSX-1 and CTp11 were detected in 37.7%, 34.4% and 31.1% of the PBMC samples, respectively. At least one of the three genes was expressed in 66 PBMC samples (66.7%). Of the 82 MAGEE-1, SSX-1 or CTp11mRNA positive HCC tissue samples, at least one of the transcripts of three genes was detected in PBMC from 60 patients with a positive correlation rate of 73.2%. These gene transcripts could not be detected in PBMC from patients with MAGEE-1, SSX-1 or CTp11mRNA undetectable in their liver tissue samples. MAGE-1, SSX-1 or CTp11 gene was not expressed in the 30 control PBMC samples from 20 healthy volunteers and 10 cirrhotic patients. The typical electrophoresis of nested RT-PCR products amplified from cDNA in PBMC samples from HCC patients is shown in Figure 2.

Sequence analysis of PCR products

Sequence analysis of PCR products verified that the nucleotide sequences of MAGE-1, SSX-1, and CTp11 cDNA fragments were identical to those in GenBank, indicating that the RT-PCR products are MAGE-1, SSX-1, and CTp11 cDNA.

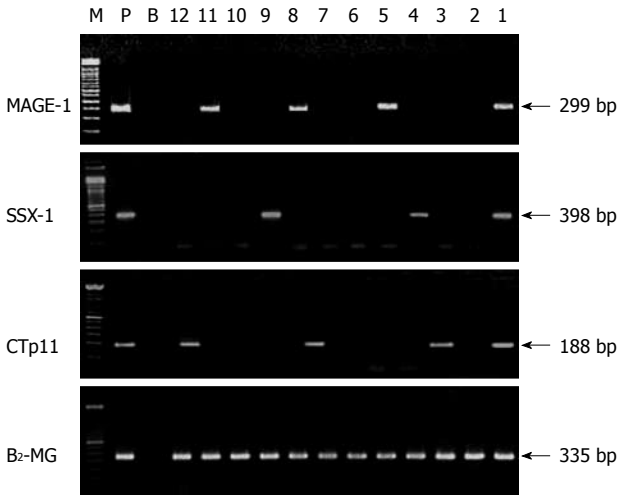

Figure 2 Electrophoresis of second round polymerase chain reaction products amplified from cDNA of peripheral blood mononuclear cells samples. Lane M: Molecular marker, 100 bp DNA ladder (Gibco); Lane P: Positive control; Lane B: Blank control; lane B2-MG (335 bp) as cDNA quality control; Lanes 1, 5, 8, 11: Positive melanoma antigen-1 (MAGE-1) transcript; Lanes 1, 4, 9: Positive synovial sarcoma X breakpoint-1 (SSX-1) transcript; Lanes 1, 3, 7, 12: Positive cancer-testis-associated protein of 11 kDa (CTp11) transcript; Lanes 2, 6 and 10: Negative transcript of all three CTA genes.

CTA transcripts in PBMC and serum AFP level

The overall positive rate of AFP with a high concentration in serum and MAGE-1, SSX-1 and/or CTp11 mRNA in PBMC samples was 48.9% and 66.7%, respectively. No correlation was observed between positive AFP rate and CTA transcripts. However, the serum AFP was negative (≤ 20 μg/L) in 26 HCC patients and positive with a low concentration (20 μg/L < AFP ≤ 400 μg/L) in 20 HCC patients. Of these 46 patients, 27 (18 cases with negative AFP and 9 cases with low concentration AFP) had MAGE-1, SSX-1 and/or CTp11 mRNA transcripts detected in their PBMC samples. By contrast, of the 30 patients with negative CTA transcripts in PBMC samples, 21 had the serum AFP level higher than 400 μg/L. Totally, 78.9% of HCC patients had either the AFP level higher than 400 μg/L in serum or positive CTA transcripts in PBMC. The parameters of AFP in serum and CTA transcripts in PBMC in combination with the results of imaging studies, would enable to make a clear diagnosis of 78.9% of HCC patients, which is much higher than the test with single AFP ($\chi^2 = 17.555$, $P < 0.01$)

CTA transcripts in PBMC from early HCC patients

The frequency of positive MAGE-1, SSX-1 and/or CTp11 transcripts detected in PBMC from patients with HCC was 41.7% at stage Ⅰ, 56.0% at stage Ⅱ, 66.7% at stage Ⅲ, and 79.5%% at stage Ⅳ, respectively. Of note, MAGE-1, SSX-1 and/or CTp11 mRNA was detected in PBMC from 51.4% of HCC patients at stages Ⅰ and Ⅱ and from 77.9% of HCC patients at stages Ⅲ and

Ⅳ, showing that advanced stages of HCC are correlated with the higher expression frequency of MAGE-1, SSX-1 and/or CTp11 gene mRNA (χ^2 = 6.632, P = 0.010). As many as 59.5% of the HCC patients at stages Ⅰ and Ⅱ could be diagnosed when CTA transcripts in PBMC and serum AFP level were combined with imaging findings. However, the serum AFP level was higher than 400 μg/L in 35.1% of the HCC patients. The diagnosis rate made by combined CTAs and AFP was significantly higher than that based on single AFP (χ^2 = 4.391, P = 0.036).

DISCUSSION

The integration of molecular and immunological techniques has led to the identification of a new category of tumor-specific antigens, also known as cancer-testis antigens, such as melanoma antigen (MAGE), synovial sarcoma X breakpoint (SSX), B melanoma antigen (BAGE), G melanoma antigen (GAGE), synaptonemal complex protein-1 (SCP-1), New York esophagus-1 (NY-ESO-1), and CTp11[23]. The CTAs are a distinct and unique class of differentiation antigens. Attributing genes to this gene group is based on their characteristics, including mRNA expression in normal tissues of testis, fetal ovary, and placenta, and mRNA expression in different cancers. Until now, at least 70 families of cancer-testis gene with 140 members have been attributed to this group and their expression has been studied in different types of tumors[23-26]. MAGE-1, SSX-1 and CTp11 belong to the CTA family members. The growing knowledge about CTAs indicates that the expression of CTA often shows a marked specificity for tumor cells[23,27-30]. These markers can be used to target tumor cells for early detection.

In the present study, CTAs (MAGE-1, SSX-1 and CTp11) were expressed with a high percentage and specificity in HCC. The positive rate for at least one of the transcripts of three CTA genes in HCC tissue samples was as high as 91.2%. Conversely, no expression was detected in the adjacent normal and cirrhotic liver tissue samples, or in the PBMC samples from healthy donors and cirrhotic patients. Based on the prevalent invasion of HCC cells to hepatic vessels, it is reasonable to consider that MAGE-1, SSX-1 and CTp11 are the appropriate tumor-specific markers for the detection of circulating HCC cells, which may play a complementary role in HCC diagnosis.

In this study, a sensitive and specific technique was developed, which is capable of detecting circulating HCC cells using MAGE-1, SSX-1 and CTp11 transcripts as tumor-specific markers by nested RT-PCR. Through the ten-fold serial dilution experiments with positive control cell lines, our results verified that exponential amplification of target cDNA converted from mRNA could allow to detect a single malignant cell within millions of normal blood cells and hence, to sensitively detect the metastatic tumor cells in peripheral blood. The sensitivity of this assay is within the range of other published reports[31-34], which is much more sensitive than antibody-based serology[35,36]. The positive rate of nested RT-PCR was as high as 66.7% for at least one of the transcripts of three CTA genes in the PBMC samples from HCC patients. In addition, detecting any of the transcripts of three CTA genes in PBMC samples from HCC patients would directly represent the presence of tumor cells in peripheral blood, suggesting that this method has a higher specificity than serum AFP and is thus able to improve the diagnosis of HCC. However, no expression of CTAs was detected in 8.9% of HCC tissue samples, showing that it is necessary to screen other CTAs or tumor specific antigens in these patients. If we can filter out 1-2 other markers, the diagnostic sensitivity of this method would be further improved.

Both albumin[37,38] and AFP[39-41] mRNA have been widely used as tumor markers for detecting HCC cells in circulation. However, the reliability is challenged, because albumin is abundantly expressed in normal liver cells[37] and AFP is expressed in liver cells infected with hepatitis virus or in cirrhotic liver[34]. In recent years, although an increasing number of genetic markers, such as telomerase reverse transcriptase[42], Des-g-carboxyprothrombin[43], squamous cell carcinoma antigen-immunoglobulin M complexes[44] and human cervical cancer oncogene[45], have been used in the diagnosis of early HCC, they have significant diagnostic limitations in their specific nature. In this study, MAGE-1, SSX-1 or CTp11 was not detected in PBMC from patients with their HCC tissue samples negative for these three CTA genes mRNA or in PBMC from 20 healthy donors and 10 cirrhotic patients, indicating that detecting CTA transcripts in PBMC from HCC patients has a high specificity for HCC.

So far, no methods or biomarkers demonstrate absolute superiority for early detection of HCC. It is difficult to simultaneously solve their sensitivity and specificity. Our assay by nested RT-PCR using MAGE-1, SSX-1 and CTp11 mRNA as tumor-specific makers showed a high sensitivity and specificity, indicating that it can establish the diagnosis of HCC.

Molecular biology technology contributes to the early diagnosis of HCC. However, its disadvantages are also obvious, including its cost and availability. PCR assay, a commonly used molecular biology technology, is more expensive and troublesome than serological tests, and is thus not the first choice in early detection of HCC. However, it plays a supplementary role in the diagnosis of HCC. Hopefully in the not so distant future, this technology will become increasingly popular and automatic with its cost decreased.

At present, the serum AFP level is still the gold standard for diagnosis of liver cancer. The AFP level is normal in 20%-40% of HCC patients at the time of diagnosis and usually remains low even in patients with advanced HCC[7-10]. AFP > 400 μg/L is considered diagnostic for HCC, although fewer than 50% of HCC patients may meet this standard[5-8]. With values of that magnitude, the specificity of AFP is close to 100% at the cost of its sensitivity fallen to less than 45%[6,7]. In this study, 51.1% of HCC patients were negative or positive for AFP with

a low concentration. MAGE-1, SSX-1 and/or CTp11 gene was expressed in 69.2% and 45.0% of HCC patients in the two groups, respectively, suggesting that mRNA, a tumor-specific marker, can be used as an adjuvant diagnostic tool. Detecting the transcripts of three CTA genes combined with serum AFP test in PBMC from HCC patients, can improve the total diagnostic rate of HCC.

In this study, the expression of CTAs in PBMC was significantly correlated with the clinical TNM classification of HCC. Although the positive frequency of CTA mRNA in PBMC was significantly higher in HCC patients at stages Ⅲ and Ⅳ than in those at stages Ⅰ and Ⅱ, MAGE-1, SSX-1 and/or CTp11 mRNA transcripts were detected in 41.7% of the HCC patients at stage Ⅰ and in 56.0% of the HCC patients at stage Ⅱ, showing that these three CTAs are reliable markers for screening hematogenous spread of early HCC cells. The combination of CTA transcripts in PBMC and serum AFP level improves early diagnosis of HCC. The classic TNM staging method[22] does not accurately reflect the actual process of HCC patients. The TNM classification criteria for HCC include tumor size, presence of portal vein invasion, and extrahepatic metastasis, *etc*. According to the TNM criteria, HCC at stage Ⅰ (T1N0M0) or stage Ⅱ (T2N0M0) should have no metastasis of tumor cells except for intra-hepatic metastasis. Assay by nested RT-PCR to detect MAGE-1, SSX-1 and/or CTp11 transcripts, the tumor-specific markers, revealed that 51.4% patients with HCC in early stages (stage Ⅰ and Ⅱ) have already had micrometastasis to the peripheral blood, indicating that blood dissemination of tumor cells has already occurred in the early stage of HCC when distant metastasis cannot be confirmed. Furthermore, it may be the reason why some early HCC patients still suffer from recurrence even after complete removal of the tumor. Detecting CTA transcripts in PBMC of early HCC patients can demonstrate hematogeneous dissemination of tumor cells more specifically than conventional methods, thus playing a supplementary role in the diagnosis of HCC. The traditional TNM staging criteria ignoring the presence of circulating HCC cells need to be perfected.

COMMENTS

Background

The prognosis of hepatocellular carcinoma (HCC) is poor because it is detection at an advanced, non-resectable stage. So far, α-fetoprotein (AFP) is a generally accepted serological marker. Its diagnostic accuracy is unsatisfactory and questionable. Serum AFP alone is helpful when its level is markedly elevated, occuring in less than 50% of cases at the time of diagnosis. Therefore, more sensitive and specific biomarkers are needed.

Research frontiers

The limitations of conventional AFP as a marker has led to a search for more sensitive and specific markers. In recent years, although an increasing number of genetic markers have been used in diagnosis of early HCC, they have significant diagnostic limitations in the specific nature. Cancer-testis antigens (CTAs) are frequently expressed in different types of cancer and have received considerable attention as ideal biomarkers of tumor cells.

Innovations and breakthroughs

The sensitivity and specificity of nested reverse transcripts-polymerase chain reaction assay using melanoma antigen-1, synovial sarcoma X breakpoint-1 and cancer-testis-associated protein of 11 kDa mRNA as tumor-specific multiple-makers are high and can thus be as an adjuvant diagnostic tool. This assay combined with serum AFP level may improve the diagnosis of HCC.

Applications

Detecting transcripts of CTA genes in peripheral blood mononuclear cells from HCC patients, combined with serum AFP test, improves the total diagnostic rate of HCC.

Peer review

The authors investigated the expression of some CTA genes in peripheral blood of HCC patients and showed that the positive rate for at least one of the three CTA genes was 67% in blood samples and 90% in resected tumor samples. They also showed a high specificity and sensitivity of their method for HCC. The study is very interesting and important for early diagnosis of HCC.

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BRIEF ARTICLE

Effects of moxibustion on dynorphin and endomorphin in rats with chronic visceral hyperalgesia

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Abstract

AIM: To observe the analgesic effects of moxibustion in rats with chronic visceral hyperalgesia and its influence on the concentration of dynorphin (Dyn) and endomorphin (EM) in spinal cord.

METHODS: The rat model of chronic visceral hyperalgesia was established by colorectal distention (CRD). In moxibustion (MX) group, moxibustion was applied once daily for 7 d; in sham moxibustion (SM) group, moxibustion was given to the same acupoints but with the non-smoldered end of the moxa stick. Model control (MC) group and normal control group were also studied. The scoring system of abdominal withdrawal reflex was used to evaluate visceral pain for behavioral assessment. Enzyme linked immunosorbent assay was performed to determine the concentrations of Dyn and EM in spinal cord.

RESULTS: Moxibustion significantly decreased visceral pain to CRD in this rat model, and no significant difference was detected between the SM group and the MC group. In MX group, moxibustion also increased the concentrations of Dyn and EM in spinal cord, and no significant difference was found between the SM group and the MC group.

CONCLUSION: Moxibustion therapy can significantly enhance the pain threshold of rats with chronic visceral hyperalgesia, and the effect may be closely related to the increased concentration of Dyn and EM in spinal cord.

Key words: Moxibustion; Analgesia; Hypersensitivity; Dynorphins; Endomorphin

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INTRODUCTION

Acupuncture-Moxibustion is an ancient therapy with a history of 3000 years in China, and it has spread to more than 160 countries for its good effects in management

of pain, nausea induced by radiotherapy/chemotherapy, vomiting, *etc.*[1]. Many studies have proved the analgesic effect of acupuncture from the view point of neurophysiology, neurochemistry, molecular biology, and brain functional imaging[2-6]. As a twin therapy of acupuncture, moxibustion has shown its effects in treatment of irritable bowel syndrome (IBS)[7], ulcerative colitis[8], Crohn's disease[9] and chronic/acute gastritis[10], especially in alleviating visceral pain. Some studies believed that acupuncture could relieve pain by increasing the concentration or expression of dynorphin (Dyn) and endomorphin (EM) in spinal cord[11,12]. It has been reported that visceral sensory nerves are closely associated with the spinal cord fragments[13,14], so that moxibustion might achieve its analgesic effect in treating visceral pain by modulating the concentrations of Dyn and EM in the spinal cord fragments.

Our previous studies have revealed the analgesic effects of moxibustion in reducing abdominal pain in IBS patients[15] and IBS rat models[16,17]. However, the analgesic mechanism of moxibustion has not been clearly elucidated. In this study, a rat model of chronic visceral hyperalgesia was established by colorectal distention (CRD), and abdominal withdrawal reflex (AWR) scoring system was adopted for behavioral assessment in the evaluation of visceral pain after moxibustion intervention. The analgesic effect of moxibustion and increase of the concentration of Dyn and EM in spinal cord were demonstrated, which partially explained the mechanism of the analgesic effect of moxibustion in management of visceral pain.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (5 d old) were obtained from the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (TCM). They were maintained in a plastic cage containing corn chip bedding with controlled temperature (22 ± 2℃), 60% ± 5% humidity and light-dark cycle (12:12 h) with a maximum of five rats per cage. Studies were performed in accordance with the proposals of the Committee for Research and Ethical Issues of the Council for International Organizations of Medical Sciences and approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research, Shanghai University of TCM.

Study design

Neonatal rats were given daily mechanical colon distention beginning 8-21 d after their birth. After the distention was finished, the rats were kept until they reached adulthood (at least 6 wk old), and then experiments were conducted using behavioral test for visceral pain by acute CRD stimulus. Moxibustion (MX) group (n = 10): moxibustion was given to the acupoints of bilateral Tianshu (ST 25) and Shangjuxu (ST 37) using fine moxibustion stick with the smoldered end 2 cm away from the acupoints, once daily, 10 min each time, 7 times in total (Figure 1A). Sham moxibustion (SM) group (n = 10): intervention was given to bilateral Tianshu (ST 25) and Shangjuxu (ST 37) points

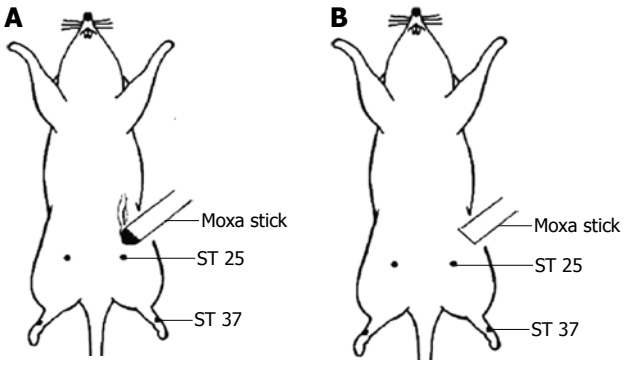

Figure 1 Moxibustion (A) and sham moxibustion (B).

Table 1 Abdominal withdrawal reflex scoring criteria

Score	
0	No behavioral response to colorectal distention
1	Immobile during colorectal distention and occasionally clicked the head at the onset of the stimulus
2	A mild contraction of abdominal muscles, but not lifting the abdomen off the platform
3	A strong contraction of the abdominal muscles and lifting the abdomen off the platform, not lifting the pelvic structure off the platform
4	Arching body and lifting the pelvic structure and scrotum

using fine moxibustion stick with the non-smoldered end 2 cm away from the acupoints, once daily, 10 min each time, 7 times in total (Figure 1B). Normal control (NC) group (n = 10) and model control (MC) group (n = 10): received no treatment except for constraining. After seven treatments, AWR was performed within 90 min, and a segment of spinal cord (L_4-S_1) was harvested and Dyn/EM concentration in spinal cord tissue was detected by enzyme linked immunosorbent assay (ELISA) (Figure 2).

Neonatal CRD irritation

Neonatal rats received CRD daily (the procedure was modified from previous reports[18,19]. Mainly, balloon (constructed from a condom; length: 20.0 mm; diameter: 3.0 mm) was inserted rectally into the descending colon. The balloon was distended with 0.5 mL air for 1 min and then deflated and withdrawn. The distention was repeated twice daily at a 30-min interval.

AWR scores

The AWR was assessed within 90 min after intervention using CRD based on semi-quantitative analysis. Prior to CRD, the rats were gently touched around anus for activating defecation. When the balloon was inserted into the descending colon, CRD was produced by rapidly inflating the balloon at strengths of 20, 40, 60, and 80 mmHg for a period of 20 s. Each score was tested three times, and each rat was tested by two people who were not involved in this research. There was a 3-min intervals between the two tests to allow the rats to adapt. The scoring criteria of AWR were referred to the method of Al-Chaer *et al*[18] (Table 1).

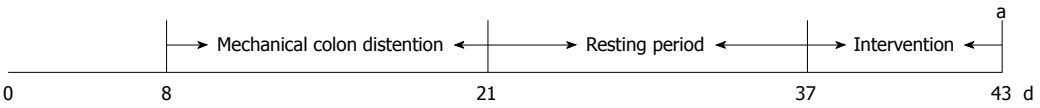

Figure 2 Experimental protocol of the study. [a]Abdominal withdrawal reflex sores after seven treatments.

ELISA for Dyn and EM

The dissected spinal cord tissue (L_4-S_1) was homogenized and weighed (10%), centrifuged for 30 min at 4℃, 4000 r/min. The supernatant was separated for assessment, and 100 μL standards and 100 μL dilution were mixed with 100 μL biotin, respectively. After incubation for 20 min at 20-25℃, 100 μL horse radish peroxidase was added. Followed by another 20 min of incubation at 20-25℃, 100 μL 3,3',5,5'-Tetramethylbenzidine substrate was added. Then 100 μL stop solution was put in after 20 min of incubation at 20-25℃. Calibration curve was drawn with OD value as the Y-coordinate and sample concentration as the X-coordinate. The concentration could be read according to the corresponding OD value. Rat Dyn and EM ELISA kits (THERMO MULTISKAN-MK3) were obtained from Finland.

Dyn in spinal cord (ng/L) = concentration × dilution times of the sample.

EM in spinal cord (ng/L) = concentration × dilution times of the sample.

Statistical analysis

The statistical analysis was done using SPSS 10.0 (SPSS Inc., USA). All data were expressed as mean ± SE for normally distributed continuous variables and as median (QL-QU) for abnormal variables. The differences in the mean values of the AWR score among the four groups (groups NC, MC, MX and SM) at each pressure of CRD were compared using the one-way analysis of variance (ANOVA, $P < 0.05$ as significant in differences). The differences in the median values of the concentration of Dyn and EM among the four groups were compared using the Kruskal-Wallis one-way analysis of variance on ranks. If the Kruskal-Wallis test result was significant ($P < 0.05$), we performed pairwise comparisons using a Wilcoxon rank sum test with a Bonferroni correction at 0.05/4 to correct for multiple comparisons. P value of < 0.05/4 was considered significant in differences.

RESULTS

Analgesic effects of moxibustion on chronic visceral hyperalgesia

At different levels of CRD stimuli (20, 40, 60 and 80 mmHg), the AWR scores in the MC group were significantly higher than in the NC group ($P < 0.01$); the AWR scores of MX group were significantly lower than that of the MC group ($P < 0.01$). There was no significant difference in the AWR scores between the MC group and SM group. This indicated that moxibustion treatment had a beneficial effect in chronic visceral hyperalgesia (Figure 3).

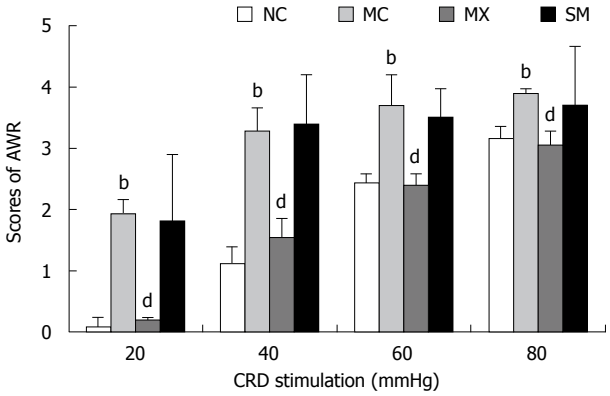

Figure 3 Analgesic effects of moxibustion on chronic visceral hyperalgesia. [b]$P < 0.01$ *vs* normal control (NC); [d]$P < 0.01$ *vs* model control (MC). AWR: Abdominal withdrawal reflex; MX: Moxibustion; SM: Sham moxibustion; CRD: Colorectal distention.

Influence of moxibustion in the Dyn concentration in spinal cord

The statistical analysis for the concentration of Dyn in spinal cord demonstrated a significant difference among the four groups, $F = 25.172$, $P = 0.000$. The concentration of MC group was significantly lower than that of NC group ($P < 0.01$). Compared with the MC group, the concentration of Dyn was significantly higher in the MX group ($P < 0.01$). No significant difference was detected in Dyn concentration between MC group and SM group (Figure 4A).

Influence of moxibustion on the EM concentration in spinal cord

Statistical analysis for the concentration of EM in spinal cord demonstrated a significant difference among the four groups, $F = 43.370$, $P = 0.000$. The concentration of MC group was significantly lower than that of NC group ($P < 0.01$). Compared with the MC group, the concentration of EM was significantly higher in the MX group ($P < 0.01$). No significant difference was detected in the EM concentration between the MC group and SM group (Figure 4B).

DISCUSSION

Visceral pain is commonly encountered by patients with functional intestinal disorders, leading to a miserable life and financial burden of the patients. Mertz *et al*[20] hold that the alterations of rectal sensitivity could be a biological indicator of IBS as IBS is featured by chronic abdominal pain. Alleviating abdominal pain is considered to be the main target in the management of IBS.

Moxibustion has been adopted as an analgesic method

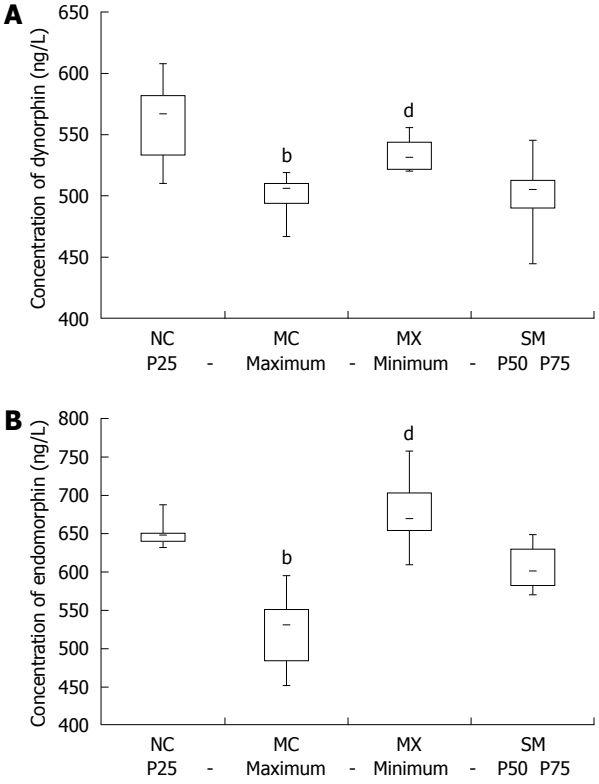

Figure 4 Influence of moxibustion on the concentration of dynorphin (A) and endomorphin (B) in spinal cord. $^{b}P < 0.01$ *vs* normal control (NC); $^{d}P < 0.01$ *vs* model control (MC). MX: Moxibustion; SM: Sham moxibustion.

for thousands of years in China, and is still frequently used in the present clinical practice. Many researches have shown its analgesic effect in treatment of primary dysmenorrhea[21], knee osteoarthritis[22], rheumatoid arthritis[23] and cancer pain[24]. Our previous studies also revealed that moxibustion could alleviate abdominal pain induced by IBS[16,17]. Although moxibustion has been practiced for thousand years, it is still difficult to establish its biological basis.

In the present study, CRD was adopted to establish a rat model of visceral hyperalgesia, and AWR was used for the behavioral assessment. The results showed that the AWR scores in the MC group were significantly higher than in the NC group at various CRD pressure levels (20, 40, 60 and 80 mmHg). Compared with the MC group, a marked reduction in AWR score was detected in the MX group ($P < 0.01$), and no significant difference was found in comparison with the SM group. It indicates that moxibustion has analgesic effects in management of visceral hyperalgesia, which is consistent with the results of our previous studies[16,17]. According to the previous studies adopting the same visceral pain model, herb-partitioned moxibustion could significantly inhibit the increase of AWR score and pain threshold induced by CRD. It has been also found that moxibustion could lower the expression of 5-HT in colon and modulate the expression of 5-HT in spinal cord, indicating a possible relationship between analgesic effect of moxibustion and central nervous system. Rats could keep quiet during the intervention of moxibustion, suggesting that modulating 5-HT was not the only way to reduce visceral hypersensitivity, some endogenous analgesic substances could also play a role in the process.

The analgesic effect of acupuncture has been widely accepted, especially in the study on chronic pain[25]. The endogenous opioid peptides (EOP) have been considered as important fundamental substances in acupuncture analgesia. According to Han JS[6,11,12,26], electro-acupuncture could activate the generation of EOP in spinal cord, such as orphanin, enkephalin, endomorphin, endorphin, and dynorphin.

The present study showed that the concentrations of Dyn and EM in spinal cord of the MC group were significantly lower than that of the NC group ($P < 0.01$). Compared with the MC group, the concentrations were significantly higher in the MX group ($P < 0.01$), and no significant difference was found from the SM group. It suggests that moxibustion could enhance the concentrations of Dyn and EM in spinal cord. Moxibustion may achieve its analgesic effect through multiple pathways and levels. Spinal cord may be the primary integrating center of moxibustion signal, increasing the concentrations of Dyn and EM in spinal cord, inducing a fragmental inhibition (including post-synaptic inhibition and pre-synaptic inhibition), and then blocking the further transmission of pain signal.

It has been shown that midbrain periaqueductal gray descending inhibitory system includes at least three transmitters: EOP, 5-HT and NA. Our findings indicate that moxibustion stimulation accelerates the synthesis and release of central EOP endorphin (dynorphin and endomorphin) and other neurotransmitters (5-HT) in the spinal dorsal horn neurons or nociceptive primary afferents, exerting analgesic effects.

In a word, moxibustion can significantly reduce AWR score and enhance the pain threshold of rats with chronic visceral hyperalgesia, and the analgesic effect may be closely related to the increased concentrations of Dyn and EM in spinal cord.

COMMENTS

Background

Previous studies into the mechanism of acupuncture analgesia have focused on the dynorphin (Dyn) and endomorphin (EM) in spinal cord. Whether analgesic effect of moxibustion is related to Dyn and EM in spinal cord remains unknown. In the previous studies, the authors have demonstrated the analgesic effect of moxibustion in reducing abdominal pain in irritable bowel syndrome (IBS) rats. However, the analgesic mechanism of moxibustion has not been clearly elucidated.

Research frontiers

More and more data have shown that the analgesic effect of moxibustion is closely related to the spinal cord fragments, which has become a hot spot of study.

Innovations and breakthroughs

Moxibustion is found effective against visceral pain. Moxibustion therapy exerts its effect on IBS by increasing the concentration of Dyn and EM in spinal cord

Applications

The experimental data can be used in further studies on moxibustion therapy for visceral pain.

Peer review

This is a good experimental investigation in which authors evaluate the effect of moxibustion, a Traditional Chinese Medicine, and possible involvement of endogenous dynorphin and endomorphin in the spinal cord in rats.

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BRIEF ARTICLE

Serological diagnostic factors for liver metastasis in patients with colorectal cancer

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Abstract

AIM: To investigate the serological diagnostic factors for liver metastasis in patients with colorectal cancer.

METHODS: One hundred and six adult in-patients with colorectal cancer were studied and divided into patients with liver metastasis (n = 56) and patients without liver metastasis (n = 50). Serum levels of tumor and biochemical markers for liver were measured at the time of diagnosis.

RESULTS: The mean survival time was 55.9 mo, 36.8 mo and 68.3 mo for the overall patients, patients with liver metastasis and patients without liver metastasis, respectively. Lactate dehydrogenase (LDH) level was significantly correlated with the survival time of colorectal cancer patients. The levels of alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase (GGT), LDH and carcinoembryonic antigen (CEA) were significantly higher in patients with liver metastasis than in those without liver metastasis. Patients with lymph node metastasis had a higher risk of liver metastasis than those without lymph node metastasis. The cut points of LDH, GGT and CEA for screening liver metastasis were 180 U/L, 30 U/L and 5.0 μg/L, respectively. The sensitivity was 64.3%, 69.6% and 70.4%, and the specificity was 64.0%, 60.0% and 52.4%, respectively. The sensitivity of parallel test was 85.2% for LDH and CEA, and 92.6% for GGT and CEA, respectively. The specificity of serial test was 85.7% for LDH (or GGT) and CEA.

CONCLUSION: Early diagnosis of liver metastasis is of great significance. The sensitivity and specificity of combined tumor and biochemical markers are rather good in screening colorectal liver metastasis.

Key words: Liver metastasis; Colorectal cancer; Lactate dehydrogenase; γ-glutamyltransferase; Carcinoembryonic antigen

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INTRODUCTION

Colorectal cancer is the 3rd most common malignancy worldwide and the second most lethal cancer type in the developed world[1]. Most patients with colorectal cancer succumb to the effects of distant metastatic lesions, especially liver metastasis rather than the primary colorectal cancer itself[2]. The liver is a primary target organ of metastatic lesions and the main cause of death. About 25%

of patients with colorectal cancer have liver metastases at the time of diagnosis and another 25%-30% of them will present with liver metastases in the following 2-3 years[3]. Without treatment, the life expectancy for patients with colorectal metastases is poor and ranges from 5 to 9 mo[2,4]. Thus early diagnosis of liver metastases of colorectal caner leads to timely treatment, which favors a better prognosis.

Laparoscopy has not been advocated as a screening test for colorectal liver metastases due to its invasiveness. Fine needle aspiration cytology also has not been advocated as a screening test, because of its high risk of complications[5]. It has been shown that the incidence of needle tract metastases is 0.4%-5.1% after fine needle aspiration and use of the procedure in abdominal tumors is fatal in 0.006%-0.031% of cases[6,7]. Most deaths are due to hemorrhage of liver tumors[3]. Imaging modalities, such as contrast enhanced computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography CT (PET-CT), may establish the diagnosis of liver metastasis of colorectal cancer[8]. However, it is more difficult to make the clinical diagnosis of early liver metastases of colorectal cancer due to the absence of typical symptoms or signs. Serological examination including tumor and biochemical markers for liver function evaluation is routinely performed, though its accuracy is not high[9]. The level of carcinoembryonic antigen (CEA) is elevated in 63% of patients, while the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) is increased in about 30% of patients with liver metastases of colorectal cancer[10]. To reduce metastases-related mortality, the development of new methods for diagnosis of liver metastases of colorectal cancer is of great significance.

The purpose of the present study was to determine whether CEA and biochemical hepatic tests can be used in assessing liver metastasis in patients with colorectal cancer.

MATERIALS AND METHODS

Patients

One hundred and six in-patients with colorectal cancer admitted to Cancer Institute and Hospital, Tianjin Medical University, from December 1996 to January 2004, were included in this study. Pathological test was performed to confirm their colorectal cancer and contrast enhanced CT, MRI or PET-CT was performed to confirm their liver metastasis. Moreover, liver metastasis was confirmed by operation, biopsy or progression of the disease. The patients who had a history of liver disease and did not undergo contrast enhanced CT and MRI were excluded from this study.

Investigation indexes

Blind tests were performed for total bilirubin (TB), direct bilirubin (DB), ALT, AST, serum total protein (TP), globulin (GLOB), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and CEA. Liver biochemical test was performed within 1 wk after liver metastasis was diagnosed by contrast enhanced CT and MRI in our hospital. The methods to determine biomarkers and upper or lower limits of normality used in our laboratory are shown in Table 1.

Table 1 Methods to determine biomarkers and upper or lower limits of normality used in our laboratory

Diagnostic factors	Methods	Lower and upper limits
Total bilirubin (μmol/L)	Jendrassik-Grof	2-20
Direct bilirubin (μmol/L)	Jendrassik-Grof	0-10
Alanine aminotransferase (U/L)	Rate	0-40
Aspartate aminotransferase (U/L)	Rate	0-42
Serum total protein (g/L)	Biuret	60-80
Globulin (g/L)		27-35
γ-glutamyltransferase (U/L)	Nitrophenol rate	0-50
Alkaline phosphatase (U/L)	P-nitrophenol phosphate rate	45-132
Lactate dehydrogenase (U/L)	Rate	80-240
Carcinoembryonic antigen (μg/L)	Elisa	0-5

Statistical analysis

One-sample Kolmogorov-Smirnov test was used to determine the distribution of ALP, TP, ALB, GLOB, GGT, ALT, AST, TBIL, DBIL, LDH and CEA. Data with the skewed distribution were presented as median (Quartile interval). Two-independent-sample test and χ^2 test were respectively used to determine whether there is any significant difference between patients with and without liver metastasis. Cox regression analysis was performed for GGT, ALP, LDH, TB, DB, ALT, AST, TP, GLB, CEA, lymph node metastasis in order to find the characteristic factors for survival time. Screening test for LDH, GGT and CEA, parallel test and serial test for GGT and LDH, CEA and LDH, CEA and GGT were used to determine the diagnostic factors for liver metastasis in patients with colorectal cancer. Statistical analysis was performed by SPSS (Version: 16.0, Chicago, USA).

The screening tests were evaluated by calculating their sensitivity (SE), specificity (SP), diagnostic index (DI), false positive rate (α), false negative rate (β), crude accuracy (CA), positive predictive value (PV+), negative predictive value (PV-).

SE was defined as the proportion of patients with LM testing positive (A/A + C) where C is the number of false negative cases. SP was defined as the proportion of patients without LM testing negative (D/B + D) where B is the number of false positive cases. DI was defined as the (SE + SP)-1. α was defined as the proportion of negative cases that were erroneously reported as positive while β was defined as the proportion of positive cases that were erroneously reported as negative. CA was defined as the proportion of cases correctly diagnosed by the test (A + D/A + B + C + D) where B + C is the number of cases erroneously diagnosed by the test. PV+ was defined as the proportion of patients testing positive with LM confirmed by pathology (A/A + B) while PV- was defined as the proportion of patients testing negative proved to be free of LM by pathology (D/C + D).

Serial test was defined as positive only if all the re-

Table 2 Characteristics of patients enrolled in our study

	With liver metastasis	Without liver metastasis
Age (yr)	55.69 ± 11.773	55.21 ± 13.225
Sex		
Male	32	36
Female	18	20
Primary tumor (UICC stage)		
T1	1	0
T2	4	0
T3	12	10
T4	22	23
Lymph node metastasis (UICC stage)		
N0	29	12
N1	8	13
N2	2	6

UICC: International Union Against Cancer.

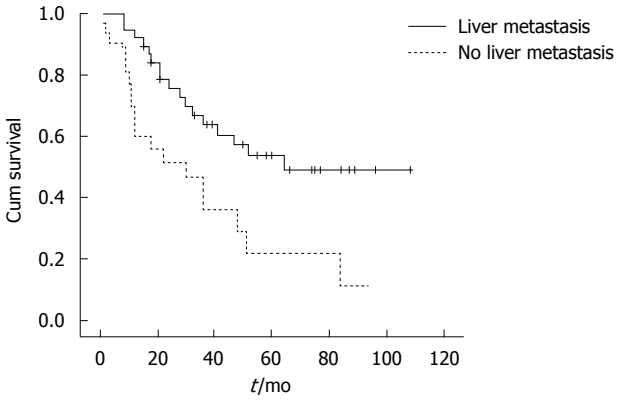

Figure 1 Survival curves for patients with and without liver metastasis of colorectal cancer.

sults were positive when we considered two or more tests together in the single patient. Parallel test was defined as positive if one of the results was positive when two or more tests were considered in the single patient.

RESULTS

Characteristics of patients

The age of our patients ranged 26-80 years with a median of 56 years. Males constituted 68 of patients with a male to female ratio of 1.788 to 1. No significant difference was found in age and sex of the patients with and without liver metastasis. The characteristics of patients with primary tumor and lymph node metastasis are shown in Table 2.

Survival analysis and cox regression

The median survival time was 47, 30 and 64 mo for the overall patients, patients with liver metastasis and patients without liver metastasis, respectively. Their mean survival time was 55.9 ± 5.5 mo, 36.8 ± 6.5 mo and 68.3 ± 7.0 mo, respectively. The survival curves for patients with and without liver metastasis were significantly different (P = 0.005) (Figure 1). Cox regression analysis showed that LDH was significantly correlated with the survival time of colorectal cancer patients with an increased risk of liver metastasis (P = 0.005) (Table 3).

Table 3 Cox regression analysis of patients with colorectal cancer

	χ^2	P
Serum total protein (g/L)	0.093	0.761
Globulin (g/L)	< 0.000	0.994
Alanine aminotransferase (U/L)	1.943	0.163
Aspartate aminotransferase (U/L)	0.143	0.705
Total bilirubin (μmol/L)	0.122	0.726
Direct bilirubin (μmol/L)	0.063	0.801
γ-glutamyltransferase (U/L)	1.126	0.289
Alkaline phosphatase (U/L)	1.006	0.316
Lactate dehydrogenase (U/L)	11.254	0.001
Carcinoembryonic antigen (μg/L)	0.159	0.690
Lymph node metastasis	1.601	0.206

Table 4 Levels of γ-glutamyltransferase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and carcinoembryonic antigen in patients with and without liver metastasis of colorectal cancer

	With liver metastasis	Without liver metastasis	P
GGT (U/L)	43.00 (64.75)	24.00 (35)	0.001
ALT (U/L)	22.00 (22.75)	13.00 (12.50)	< 0.001
AST (U/L)	22.00 (17.50)	16.00 (10.00)	< 0.001
LDH (U/L)	201.5 (169.50)	164.50 (70.75)	0.003
CEA (μg/L)	13.70 (93.8)	4.87 (12.82)	0.039

GGT: γ-glutamyltransferase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactate dehydrogenase; CEA: Carcinoembryonic antigen.

Test of normality and two-independent-sample test

One-sample Kolmogorov-Smirnov test showed that the distribution of ALP, TP, ALB, GLOB and ALB/GLOB was normal, while that of GGT, ALT, AST, TBIL, DBIL, LDH and CEA was skewed. The levels of GGT, ALT, AST, LDH and CEA were significantly higher in patients with liver metastasis than in those without liver metastasis (P < 0.05) (Table 4). Patients with lymph node metastasis had a higher risk of liver metastasis than those without lymph node metastasis (χ^2 = 9.046, P = 0.003). No significant difference was found in ALP, TP, ALB, GLOB, ALB/GLOB, TBIL, and DBIL levels between patients with and without liver metastasis (data not shown).

Screening test

Because the diagnostic indices of LDH, GGT and CEA at 180, 30 and 5.0 for screening liver metastasis were the greatest, the cut off points were selected at 180, 30 and 5.0, respectively (Figure 2). The area under the curves of LDH, GGT and CEA was 0.671, 0. 687 and 0.675, respectively (P = 0.05). The κ of parallel test and serial test for CEA and LDH, CEA and GGT was 0.293, 0.326, and 0.357, 0.284, respectively (P = 0.05). The SE, SP, DI, false positive rate (α), false negative rate (β), CA, adjusted agreement, PV+ and PV- are shown in Table 5.

Table 5 Screening test for liver metastasis in patients with colorectal cancer

	Sen (%)	Spe (%)	DI	CA	AA	PV+	PV-	*P*	α	β
LDH	64.3	64.0	1.283	0.642	0.641	0.667	0.615	0.003	0.360	0.356
GGT	69.6	60.0	1.296	0.651	0.649	0.661	0.638	0.001	0.400	0.304
CEA	70.4	52.4	1.228	0.625	0.623	0.655	0.579	0.039	0.476	0.296
LDH and CEA (serial test)	51.9	85.7	1.376	0.667	0.695	0.823	0.581	0.007	0.143	0.481
LDH and CEA (parallel test)	85.2	42.9	1.281	0.667	0.657	0.657	0.692	0.030	0.571	0.148
GGT and CEA (serial test)	44.4	85.7	1.301	0.625	0.662	0.800	0.545	0.025	0.143	0.556
GGT and CEA (parallel test)	92.6	38.1	1.307	0.687	0.691	0.658	0.800	0.009	0.619	0.074

LDH: Lactate dehydrogenase; GGT: γ-glutamyltransferase; CEA: Carcinoembryonic antigen; Sen: Sensitivity; Spe: Specificity; DI: Diagnostic index; α: False positive rate; β: False negative rate; CA: Crude accuracy; PV+: Positive predictive value; PV-: Negative predictive value.

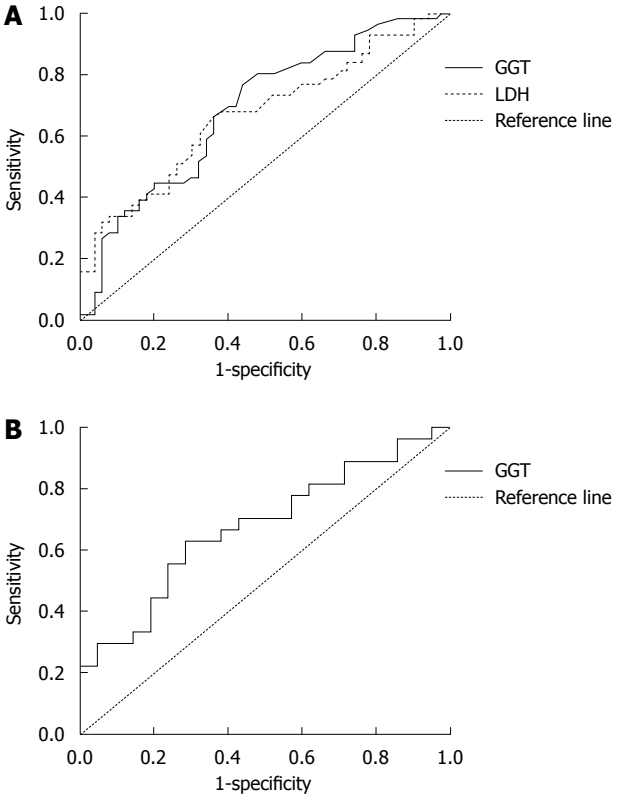

Figure 2 Receiver operator characteristic curves for lactate dehydrogenase and γ-glutamyltransferase (A) and carcinoembryonic antigen (B). LDH: Lactate dehydrogenase; GGT: γ-glutamyltransferase.

DISCUSSION

Colorectal cancer metastasis occurs in various organs, most frequent in lymph nodes and liver[1]. In this study, the patients with lymph node metastasis had a higher risk of liver metastasis than those without lymph node metastasis, indicating that regular imaging modalities, such as contrast enhanced CT and MRI, may be performed every 3 or 6 mo after surgery for patients with colorectal cancer to establish an early diagnosis of liver metastasis.

The overall life expectancy of patients with colorectal cancer is mainly determined by the progression of liver metastasis rather than by the primary carcinoma itself[3]. The median survival time was 47, 30 and 64 mo for the overall patients, patients with liver metastasis and patients without liver metastasis, respectively, with a mean survival time of 55.9, 36.8 and 68.3 mo, respectively. A significant difference was observed in survival curves for patients with and without liver metastasis. The levels of GGT, ALT, AST, LDH and CEA were significantly higher in patients with liver metastasis than in those without liver metastasis. Cox regression analysis showed that LDH was significantly correlated with the survival time of colorectal cancer patients, indicating that LDH may be used to predict the life expectancy of patients with liver metastasis of colorectal cancer.

CEA was demonstrated in fetal gut tissue and gastrointestinal tract tumor four decades ago, and subsequently detected in the circulation of patients and recognized as a serum marker for colorectal cancer. Expression of carbohydrate antigen (CA) 19-9 has been described in colorectal cancer, but its sensitivity is lower than CEA[11]. Lack of sensitivity and specificity precludes the use of any available serum markers, such as CEA, CA 19-9, CA 242, CA 72-4, tissue polypeptide antigen or tissue polypeptide-specific antigen, for the early detection of colorectal cancer[12]. However, a preoperative CEA serum level can predict the prognosis of recurrence and survival time of colorectal cancer patients[11,13]. Moreover, circulating levels of LDH, ALP, and GGT in malignant tissues can directly contribute to liver replacement[14,15]. In patients with metastatic colorectal cancer, CEA, ALP and LDH have been reported as prognostic factors[16-19].

A screening test was performed to show whether LDH, GGT and CEA can be used to screen liver metastasis in patients with colorectal cancer. Because the diagnostic indices of LDH, GGT and CEA at 180 U/L, 30 U/L and 5.0 μg/L for screening liver metastasis were the greatest, the cut off points were selected at 180 U/L, at 30 U/L, and at 5.0 μg/L, respectively. The sensitivity were 64.3%, 69.6% and 70.4%, respectively. The sensitivity were 64.0%, 60.0% and 52.4%, respectively. As a tumor marker, CEA test had a moderate sensitivity and a low specificity for liver metastasis in patients with colorectal cancer. Thus, tumor markers in combination with biochemical markers for liver function may improve the sensitivity and specificity for screening liver metastases in patients with colorectal cancer.

Couples of tests, usually CEA and another, would demonstrate a better accuracy than a single test[20,21], which

is consistent with the findings in our study. In the present study, the sensitivity of parallel test for LDH and CEA, GGT and CEA was 85.2% and 92.6%, respectively. The specificity of serial test for LDH and CEA was 85.7% and the specificity of serial test for GGT and CEA was 85.7% too, indicating that its sensitivity and specificity of tumor marker (CEA) in combination with biochemical markers including LDH and GGT are rather good in patients with colorectal cancer, if LDH > 180 U/L and CEA > 5.0 μg/L, or GGT > 30 U/L and CEA > 5.0 μg/L. Contrast enhanced CT, MRI or PET-CT may be performed immediately to confirm liver metastasis and timely treatment may improve the survival of patients with liver metastasis of colorectal cancer. Thus metastatic liver disease may be diagnosed before symptoms occur and liver metastases of colorectal cancer can be diagnosed more rapidly and accurately.

COMMENTS

Background

The overall life expectancy of patients with colorectal cancer is mainly determined by the progression of liver metastasis rather than by the primary carcinoma. Improved early screening modalities are still needed and molecular beacons may be sufficiently sensitive, specific, and cost-effective for screening of colorectal liver metastases.

Research frontiers

Although various diagnostic modalities, such as ultrasonography, computed tomography scan and magnetic resonance imaging have been used in demonstrating metastases, but their accuracy is low, particularly when the lesions are small. The present study demonstrated the value of carcinoembryonic antigen (CEA) and some biochemical hepatic tests in detection of hepatic metastases in patients with primary colorectal cancer.

Innovations and breakthroughs

Laboratory tests have limits in detecting LM but they can rapidly and accurately evaluate liver metastasis in patients with primary colorectal cancer. This study showed that measurements of plasma biomarkers increase the sensitivity and selectivity of liver metastasis diagnosis.

Applications

The results of this study can improve early screening modalities. Furthermore, combination of markers and even modalities with imaging or endoscopic ultrasound will be needed to achieve a sufficient reliability.

Peer review

It is a very interesting paper describing the diagnosis of liver metastases of colorectal cancer. The authors studied 106 patients with colorectal cancer, showing that the alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase, lactate dehydrogenase and CEA levels are increased in patients with liver metastasis of colorectal cancer.

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BRIEF ARTICLE

Altered expression of MUC2 and MUC5AC in progression of colorectal carcinoma

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Abstract

AIM: To study the expression profiles of MUC2 and MUC5AC in tumorigenesis of colorectal carcinoma and in its different pathologic types.

METHODS: Formalin-fixed, paraffin-embedded human colorectal tissue specimens were immunostained with antibodies against MUC2 and MUC5AC. Six samples of normal mucosa (NM), 12 samples of hyperplastic polyp (HP), 15 samples of tubular adenoma with low-grade dysplasia (LGD), 14 samples of tubular adenoma with high-grade dysplasia (HGD), 26 samples of conventional colorectal adenocarcinoma (CCA), 15 samples of mucinous carcinoma (MC), and 8 samples of signet-ring cell carcinoma (SRCC) were collected.

RESULTS: MUC2 was the most widely expressed protein in each study group, although the number of MUC2-positive cases was less in CCA group than in other groups ($P < 0.05$). The staining score for MUC2 was significantly decreased in the HP-LGD-HGD-CCA sequence ($r = -0.73436$, $P < 0.0001$). Among the neoplasms, MC and SRCC were more frequently associated with the high expression of MUC2 ($P < 0.05$) than with that of CCA. MUC5AC expression was detected in all groups but not in NM group. Furthermore, the staining score for MUC5AC was higher in HP, LGD, HGD, MC and SRCC groups than in NM and CCA groups ($P < 0.05$). The frequency of simultaneous expression of MUC proteins was significantly higher in MC and SRCC groups than in CCA group ($P < 0.05$).

CONCLUSION: Alterations in MUC expression occur during colorectal tumorigenesis. The transformation process in MC and SRCC may be different from that in the traditional adenoma-carcinoma sequence.

Key words: Colorectum; Tumorigenesis; MUC2; MUC5AC; Immunohistochemistry

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INTRODUCTION

Mucins are high-molecular-weight glycoproteins, which are heavily decorated with a large number of O-linked oligo-

saccharides and a few N-glycan chains, linked to a protein backbone[1]. Mucins are known to play a central role in the protection, lubrication and hydration of the external surface of human epithelial tissue layers lining the intricate network of ducts and passageways. Mucins have also been implicated in the pathogenesis of benign and malignant diseases of secretory epithelial cells. The identification of novel transmembrane mucin MUC21[2], means that a total of 20 human mucins have now been recognized. According to their structure and function, mucins can be divided into secreted mucins and transmembrane mucins. Secreted mucins can be gel-forming or non-gel-forming, and include MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9 and MUC19. Transmembrane mucins include MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20 and MUC21, and possess a transmembrane domain for anchoring themselves to the plasma membrane of various cells. These mucin proteins are encoded by various *MUC* genes[1].

The genes for gel-forming mucins MUC2 and MUC5AC are found in a cluster on chromosome 11p15.5, and synthesis of the proteins is regulated by biologically active molecules, including cytokines, bacterial products, and growth factors[3-5]. The *MUC2* gene codes for a typical secretory mucin, which is predominantly found in colorectal goblet cells. The *MUC5AC* gene is mainly expressed in gastric and tracheo-bronchial mucosa. Changes in the expression levels and/or distribution profiles of MUC2 and MUC5AC occur in cancers of the lung, gastrointestinal tract, pancreas, hepatobiliary system and reproductive system. For example, it has been found that MUC5AC is down-regulated in peritumoral epithelium and squamous metaplasia of non-small cell lung carcinoma (NSCLC), and MUC5AC expression is reduced in NSCLC, irrespective of their histologic subtype[6]. The expression of sLe^x is related to MUC5AC protein in NSCLC, and patients with tumors co-expressing both MUC5AC and sLe^x antigen have the poorest survival[7]. MUC2 appears to act as a protective protein and has been shown to be associated with tumors of mucinous type, both in biliary system and in pancreatic system, which carry a more favorable prognosis[8]. MUC5AC expression in intrahepatic cholangiocarcinoma is associated with a higher incidence of lymph node metastasis and has been identified as an independent prognostic factor by multivariate survival analysis[9].

Interestingly, altered expression of MUC in colorectal cancer may be significantly correlated with histologic type, sensitivity to chemotherapeutic drugs, and prognosis of colorectal cancer[10-13]. Colorectal cancer constitutes a suitable model for studying the mechanisms of carcinogenesis and tumor progression in the well-established adenoma-carcinoma sequence. It is possible to observe a dynamic progression from benign adenomatous polyp to adenoma with varying degree of dysplasia, to intramucosal and invasive carcinoma[14]. Moreover, a pathway involving a hyperplastic polyp-adenoma-carcinoma sequence has also been introduced[15]. Alterations in the expression of mucin proteins and genes have been observed in colorectal adenoma and carcinoma, although their significance in neoplastic transformation of the colorectal epithelium is yet to be determined. The present study therefore aimed to study the expression profiles of MUC2 and MUC5AC during tumorigenesis and in different pathologic types of colorectal carcinoma, using immunohistochemical staining.

MATERIALS AND METHODS

Tissue samples

Formalin-fixed, paraffin-embedded human colorectal tissue specimens were obtained from Department of Pathology, Nanjing First Hospital of Nanjing Medical University. Six samples of normal mucosa (NM), 12 samples of hyperplastic polyps (HP), 15 samples of tubular adenoma with low-grade dysplasia (LGD), 14 samples of tubular adenoma with high-grade dysplasia (HGD), 26 samples of conventional colorectal adenocarcinoma (CCA), 15 samples of mucinous carcinoma (MC), and 8 samples of signet-ring cell carcinoma (SRCC) were analyzed in this study. HP was diagnosed when a serrated polyp with no overt cytological atypia showed narrowed crypt bases, predominantly lined with immature cells. Adenoma was further classified as low or high grade based on the degree of glandular intraepithelial neoplasia (dysplasia), according to the World Health Organization classification. Colorectal cancer was defined as mucinous carcinoma if more than 50% of the lesion contained a mucin lake. Cancer where more than 50% of the tumor cells were signet-ring cells was defined as SRCC. Histologically normal mucosa from margins of the specimens served as control tissue. All tissue samples were diagnosed and classified by two pathologists.

Immunohistochemistry

Paraffin-embedded blocks of different tissues were cut into 4-μm thick sections. Slides were deparaffinized in xylene and rehydrated using a graded ethanol series. Antigen was retrieved by boiling the slides in a microwave oven for 15 min in 0.01 mol/L citrate buffer (pH 6.0). Endogenous peroxidase was blocked with a 3% H_2O_2-methanol solution, and the slides were incubated in 10% normal goat serum for 30 min to prevent nonspecific staining. The tissue sections were then incubated overnight at 4℃ with primary antibody (MUC2 or MUC5AC, 1:100; Santa Cruz, CA). The standard biotin-streptavidin-peroxidase method was then used, and the sections were lightly counterstained with hematoxylin. Histologically normal colon mucosa and gastric biopsies were used as positive controls for MUC2 and MUC5AC, respectively. The sections incubated with phosphate-buffered saline (0.01 mol/L, pH 7.4) instead of primary antibody were used as negative controls.

Analysis of immunohistochemical data

Both goblet and non-goblet columnar cells of normal colon and hyperplastic polyps were evaluated. MUC staining was only scored in neoplastic cells of tissues containing either dysplastic epithelium or carcinoma. The range of cytoplasmic staining (0: 0%-5%; 1: 6%-30%; 2: 31%-60%; and 3: 61%-100%) and the intensity of staining (0: no stain; 1: weak staining; 2: intermediate staining; and 3:

strong staining) were assessed in at least 8 high-power fields by two observers, and averages of the grades were taken. The final staining score was defined as the product of scores for the range and intensity of cytoplasmic staining. Staining was designated as negative if the staining score was 0 or 1, intermediate for 2, 3, or 4, and high for 6 or 9. All specimens were scored blindly.

Statistical analysis

Statistical comparison of immunohistochemical staining was performed using SAS software version 9.0 (SAS Institute, Cary, NC). The rank-sum test and Spearman's rank correlation analysis were used to determine differences between the groups and to evaluate correlations, respectively. The T approximation test in Wilcoxon's rank-sum test and Fisher's exact test were used to compare differences between CCA and other groups. $P < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemical localization of MUC2 and MUC5AC

The expression of MUC2 and MUC5AC proteins differed among the study groups, which was prominently characterized by perinuclear and diffuse cytoplasmic staining. The MUC2 was expressed in perinuclear cytoplasm of partial goblet cells in NM group (Figure 1A). The MUC2 labeling was generally increased in cytoplasm of columnar cells and goblet cells in HP group (Figure 1B), and the positive signals were also observed in apical cytoplasm of columnar cells, especially in LGD and HGD groups (Figure 1C and D). MUC2 expression was positive in the cytoplasm of cancerous cells, while the extracellular mucin remained unstained (Figure 1E-G). The staining pattern for MUC5AC was largely similar to that for MUC2 in all groups but not to that in NM group, and positive signals were found in extracellular mucin (Figure 2).

Immunohistochemical analysis of MUC2 and MUC5AC

The frequency of MUC protein expression in different groups was examined with immunohistochemical staining, and the results are summarized in Table 1. MUC2 was the most widely expressed antigen in all groups, but the number of MUC2-positive cases (46.15%) was less in CCA group than in other groups. Both the expression frequency and staining intensity of MUC2 were significantly decreased in the HP-LGD-HGD-CCA sequence ($r = -0.73436$, $P < 0.0001$). The frequency of MUC2 expression was significantly higher in MC and SRCC groups than in CCA group ($P < 0.05$). The MUC5AC expression was detected in all groups but not in NM group. Furthermore, the frequency of MUC5AC expression was dramatically lower in CCA group (30.77%) than in other groups with the exception in NM group. The proportion of high staining scores was significantly higher in MC and SRCC groups than in CCA group ($P < 0.05$), which was similar to that of MUC2 expression. Concordance between MUC2 and MUC5AC expression was also noted in individual specimens from different groups (Table 2). Concordance was defined as positive (intermediate or high) or negative MUC2 and MUC5AC expression. The frequency of simultaneous expression of MUC proteins was significantly higher in MC and SRCC groups than in CCA group ($P < 0.05$).

DISCUSSION

MUC expression has been studied in colorectal carcinoma, but few reports are available on the expression in relation to the hyperplastic polyp-adenoma-carcinoma sequence, or in different pathologic types of colorectal cancer. This study focused on the altered and *de novo* expression profiles of MUC2 and MUC5AC in the tumorigenic sequence, and in different pathologic types of colorectal cancer.

MUC2 is characteristically expressed in goblet cells of native intestinal epithelium and intestinal metastasis of gastric mucosa, but not in normal gastric epithelium. The results of the present study, with immunohistochemical staining of paraffin-embedded human tissue samples, are consistent with those of previous studies showing reduced MUC2 expression in colorectal adenocarcinoma[16-18]. Decreased MUC2 expression in nonmucinous colon cancer can result from methylation of the MUC2 promoter[1]. Gratchev *et al*[19] demonstrated that MUC2 promoter methylation is lower in normal goblet cells than in columnar cells and in specimens of mucinous colorectal carcinoma than in those of nonmucinous adenocarcinoma. Loss of functional p53 is also related to the down-regulation of MUC2 expression in colorectal carcinoma. It has been shown that MUC2 expression is transcriptionally regulated by p53 protein in several cell lines[20]. There are two potential p53-binding sites in the MUC2 promoter, each of which contributes to stimulation of promoter activity. It was reported that MUC2 immunoreactivity is inversely correlated with p53 alteration in mucinous carcinoma, i.e. the level of p53 alteration is lower in regions with a high MUC2 expression level[21]. Decreased *in vivo* expression of MUC2 is related to colon carcinogenesis accompanying increased proliferation, decreased apoptosis, and increased migration of intestinal epithelial cells[22].

MUC5AC is not expressed in normal colonic epithelium, but *de novo* expression occurs in adenoma and colorectal cancer. The number of immunoreactive cells and the intensity of MUC5AC staining are greatest in larger adenoma with moderately villous histology and dysplasia, while immunostaining is lower in highly villous polyps with severe dysplasia[23]. However, in the current study, the score for MUC5AC staining was higher in HGD group than in LGD group, in contrast to the expression of MUC2 in the two groups. This discrepancy in MUC5AC expression in the two studies might be due to the use of a different histologic type of adenoma. Although MUC5AC expression correlates with neural invasion and advanced stage of intrahepatic cholangiocarcinoma[24], the relation between MUC5AC expression and progression of colon cancers may be different. Kocer *et al*[25] found that the expression of MUC5AC in colon cancer is associated with a better

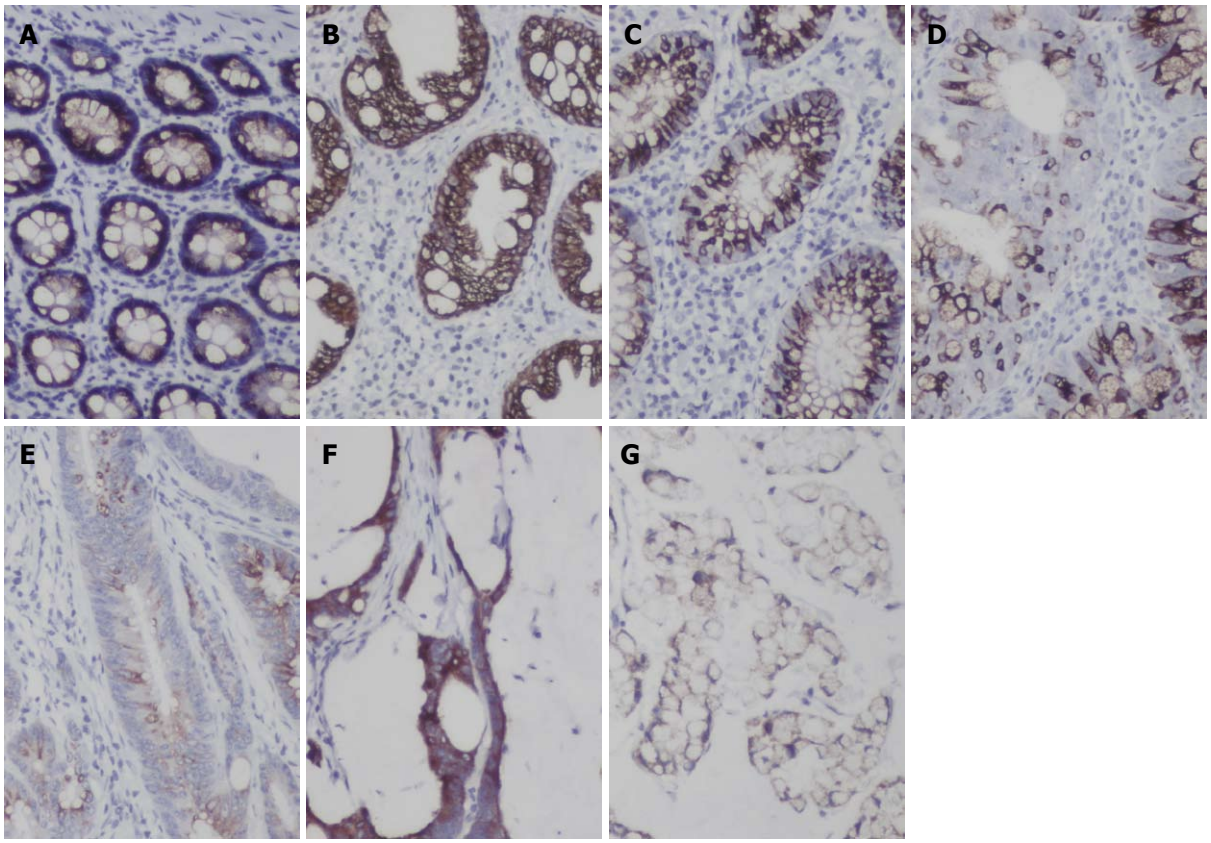

Figure 1 Expression of MUC2 (SP method, × 200). A: Normal mucosa; B: Hyperplastic polyp; C: Tubular adenoma with low-grade dysplasia; D: Tubular adenoma with high-grade dysplasia; E: Conventional colorectal adenocarcinoma; F: Mucinous carcinoma; G: Signet-ring cell carcinoma.

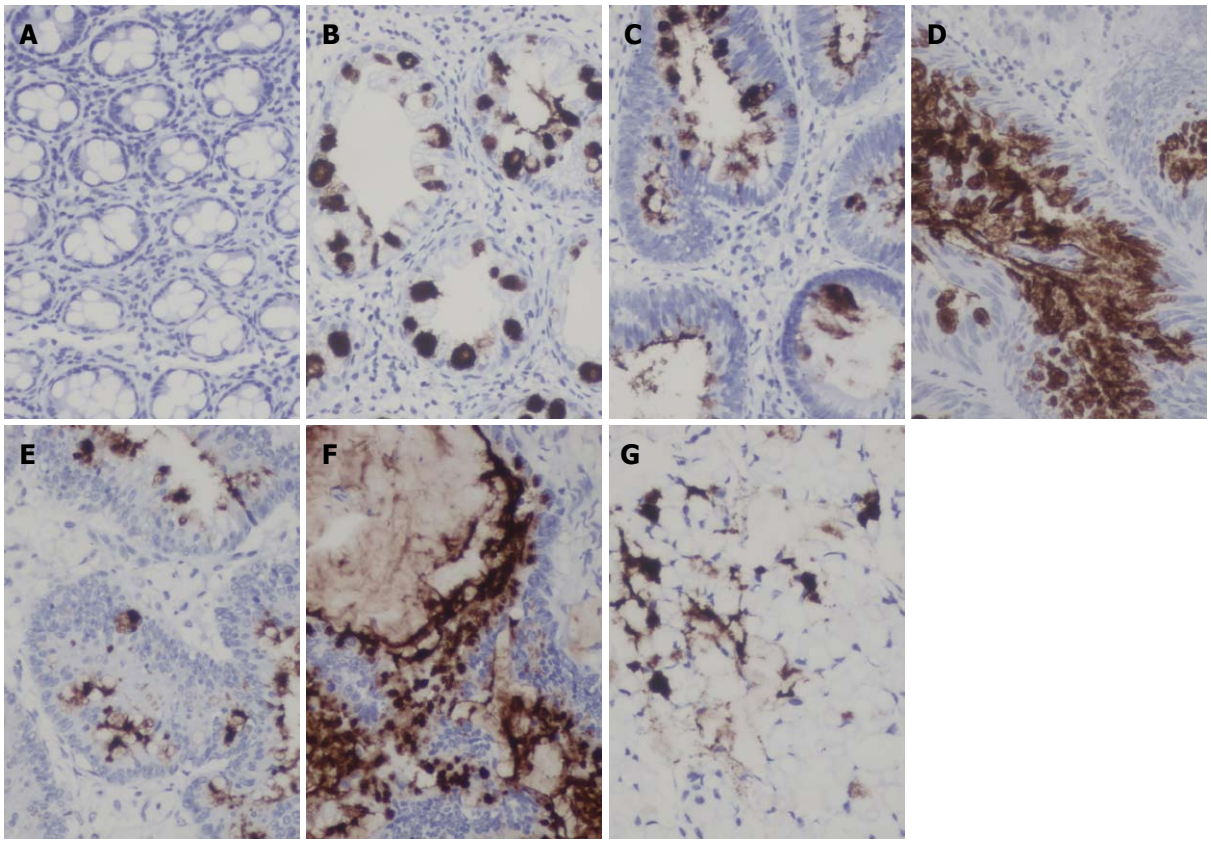

Figure 2 Expression of MUC5AC (SP method, × 200). A: Normal mucosa; B: Hyperplastic polyp; C: Tubular adenoma with low-grade dysplasia; D: Tubular adenoma with high-grade dysplasia; E: Conventional colorectal adenocarcinoma; F: Mucinous carcinoma; G: Signet-ring cell carcinoma.

Table 1 Frequency of MUC2 and MUC5AC protein expression in colorectal tissues *n* (%)

MUC staining score	NM	HP	LGD	HGD	CCA	MC	SRCC
MUC2[1]							
High	6 (100.00)	12 (100.00)	9 (60.00)	5 (35.71)	3 (11.54)	9 (60.00)	4 (50.00)
Intermediate	0 (0.00)	0 (0.00)	6 (40.00)	9 (64.29)	9 (34.62)	6 (40.00)	3 (37.50)
Negative	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	14 (53.85)	0 (0.00)	1 (12.50)
P[2]	0.0013	< 0.0001	0.0003	0.0023	-	0.003	0.0198
MUC5AC							
High	0 (0.00)	5 (41.67)	3 (20.00)	6 (42.86)	3 (11.54)	7 (46.67)	2 (25.00)
Intermediate	0 (0.00)	7 (58.33)	12 (80.00)	8 (57.14)	5 (19.23)	8 (53.33)	6 (75.00)
Negative	6 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	18 (69.23)	0 (0.00)	0 (0.00)
P[3]	0.1444	0.0007	0.0007	0.0003	-	0.0002	0.0047

[1]MUC2 protein expression is significantly decreased in HP-LGD-HGD-CCA sequence (r = -0.73436, P < 0.0001); [2]MUC2 protein expression in CCA *vs* other groups; [3]MUC5AC protein expression in CCA *vs* other groups. NM: Normal mucosa; HP: Hyperplastic polyp; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; CCA: Conventional colorectal adenocarcinoma; MC: Mucinous carcinoma; SRCC: Signet-ring cell carcinoma.

Table 2 Concordance of MUC2 and MUC5AC protein expression in colorectal tissues *n* (%)

Tissue	*n*	Positive cases		Concordance MUC2 *vs* MUC5AC	*P*[1]
		MUC2	MUC5AC		
NM	6	6 (100.00)	0 (0.00)	0 (0.00)	0.5662
HP	12	12 (100.00)	12 (100.00)	12 (100.00)	< 0.0001
LGD	15	15 (100.00)	15 (100.00)	15 (100.00)	< 0.0001
HGD	14	14 (100.00)	14 (100.00)	14 (100.00)	< 0.0001
CCA	26	12 (46.15)	8 (30.77)	4 (15.38)	-
MC	15	15 (100.00)	15 (100.00)	15 (100.00)	< 0.0001
SRCC	8	7 (87.50)	8 (100.00)	7 (87.50)	< 0.0001

[1]Concordance of MUC protein expression in CCA *vs* other groups. NM: Normal mucosa; HP: Hyperplastic polyp; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; CCA: Conventional colorectal adenocarcinoma; MC: Mucinous carcinoma; SRCC: Signet-ring cell carcinoma.

prognosis of its patients. Patients with MUC5AC-negative tumors have a poorer prognosis and a lower survival rate than those with MUC5AC-positive tumors, suggesting that the absence of MUC5AC expression in tumors is a prognostic factor for highly aggressive colorectal carcinoma. The *de novo* expression of MUC5AC in mucinous adenocarcinoma and SRCC was inconsistent in our study. The variability of MUC polypeptide expression in individual adenocarcinomas may reflect tumor cell heterogeneity and aberrant differentiation in invasive tumors, compared with their dysplastic precursors.

SRCC and MC have similar biological behaviors, but their molecular compositions may differ. Signet-ring cells rarely express adhesion molecules, implying disruption of cell-cell adhesion[18], which can explain their aggressive behavior in terms of the invasion and metastasis of tumor cells. Mucinous colorectal carcinoma is associated with microsatellite instability (MSI). It has been hypothesized that MSI may directly influence mucus production in both sporadic and hereditary colon cancer, by alternating the genes involved in mucin synthesis or degradation[26]. Colorectal cancer with a high MSI usually has a higher MUC2- and MUC5AC-positive rate than microsatellite-stable cancer[27]. Boland *et al*[28] recently suggested that colorectal tumor with MSI has more distinctive features, including a poor differentiation, mucinous or signet-ring appearance, as well as a different response to chemotherapeutics, than colorectal tumor without MSI. It is interesting that a similar phenotype (MUC2+/MUC5AC+) has been identified in adenoma and hyperplastic polyp. The classic adenoma-carcinoma pathway usually involves the loss of tumor suppressor genes, although a minority of colorectal cancers may develop in an other pathway associated with mutations in mismatch repair genes or hypermethylation of the hMLH1 gene (in sporadic cancers)[29-31].

In conclusion, alterations in MUC expression occur during colorectal tumorigenesis. The transformation process in MC and SRCC may be different from that in the traditional adenoma-carcinoma sequence. *De novo* expression of MUC5AC can occur in both mucinous and non-mucinous colorectal carcinomas, but its expression is stronger in mucinous colorectal carcinoma than in non-mucinous colorectal carcinoma. Further investigations using molecular biological techniques based on a larger clinical sample size are needed to confirm our findings.

COMMENTS

Background

Although alterations in mucins have been observed in colorectal cancer, little is known about their expression during the development and progression of colorectal tumor. Mucinous and signet-ring cell carcinomas are the two types of colorectal cancer characterized by abundant mucin secretion. However, whether the mechanism underlying the tumorigenesis of mucinous and signet-ring cell carcinomas differs from that of other colorectal cancer remains controversial.

Research frontiers

Mucinous components, such as MUC2 and MUC5AC, are associated with the distinct clinical pathologic features of colorectal cancer and the survival rate of such patients.

Innovations and breakthroughs

The abnormal expression of MUC2 and MUC5AC in mucinous and signet-ring cell carcinomas suggests that a different process may be involved in the tumorigenesis of these types of colorectal cancer.

Terminology

Mucins are high-molecular-weight glycoproteins, which are heavily decorated with a large number of O-linked oligosaccharides and a few N-glycan chains, linked to a protein backbone. Mucins are known to play a central role in the protection, lubrication and hydration of the external surface of human epithelial tissue layers lining the intricate network of ducts and passageways. Mucins have also been implicated in the pathogenesis of benign and malignant diseases of secretory epithelial cells.

Applications

The clinical treatment of mucin-secreting tumor may differ from that of other types of colorectal cancer.

Peer review

This is a good paper that provides new data regarding MUC expression profiles in specific types of colorectal carcinoma.

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BRIEF ARTICLE

Efficacy of telbivudine in HBeAg-positive chronic hepatitis B patients with high baseline ALT levels

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Abstract

AIM: To evaluate the efficacy and safety of telbivudine (LDT) in hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB) patients who have high baseline alanine aminotransferase (ALT) levels between 10 and 20 times the upper limit of normal.

METHODS: Forty HBeAg-positive CHB patients with high baseline ALT levels between 10 and 20 times the upper limit of normal were enrolled and received LDT monotherapy for 52 wk. Another forty patients with baseline ALT levels between 2 and 10 times the upper limit of normal were included as controls. We compared the virological, biochemical, serological and side effect profiles between the two groups at 52 wk.

RESULTS: By week 52, the mean decrease in hepatitis B virus (HBV) DNA level compared with baseline was 7.03 $\log_{10}$ copies/mL in the high baseline ALT group and 6.17 $\log_{10}$ copies/mL in the control group, respectively ($P < 0.05$). The proportion of patients in whom serum HBV DNA levels were undetectable by polymerase chain reaction assay was 72.5% in the high baseline ALT group and 60% in the control group, respectively ($P < 0.05$). In addition, 45.0% of patients in the high baseline ALT group and 27.5% of controls became HBeAg-negative, and 37.5% of those in the high baseline group and 22.5% of controls, respectively, had HBeAg seroconversion ($P < 0.05$) at week 52. Moreover, in the high baseline group, 4 out of 40 patients (10%) became hepatitis B surface antigen (HBsAg)-negative and 3 (7.5%) of them seroconverted (became HBsAg-positive). Only 1 patient in the control group became HBsAg-negative, but had no seroconversion. The ALT normalization rate, viral breakthrough, genotypic resistance to LDT, and elevations in creatine kinase levels were similar in the two groups over the 52 wk.

CONCLUSION: High baseline ALT level is a strong predictor for optimal results during LDT treatment.

Key words: Chronic hepatitis B; Hepatitis B e antigen; Serum alanine aminotransferase level; Telbivudine

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INTRODUCTION

Chronic infection with hepatitis B virus (HBV) affects

approximately 350 million people worldwide and is usually associated with continuing inflammatory activity and progression of liver diseases, which in turn lead to an increased risk of cirrhosis, decompensated liver disease, and hepatocellular carcinoma (HCC)[1,2]. Recently, several prospective follow-up studies of large cohorts of chronic hepatitis B (CHB) patients from Asia found that the presence of hepatitis B e antigen (HBeAg) and high levels of HBV DNA were independent risk factors for the subsequent development of advanced liver diseases[3]. Therefore, suppression of HBV replication is the main therapeutic goal in the treatment of CHB patients.

Up till now, seven drugs have been available for the treatment of CHB: they include conventional interferon α, pegylated interferon α, and nucleoside/nucleotide analogues (NUCs). NUCs for HBV therapy belong to three classes: L-nucleosides [lamivudine, telbivudine (LDT), emtricitabine], deoxyguanosine analogues (entecavir) and acyclic nucleoside phosphonates (adefovir and tenofovir). Lamivudine, adefovir, LDT, entecavir and interferon α have been approved in China for HBV treatment. LDT is an orally bioavailable L-nucleoside with potent and specific anti-HBV activity. It has been proved that LDT has a potent effect and a relatively high seroconversion rate for patients with CHB[4,5].

According to national and international guidelines, the antiviral treatment of patients with CHB is initiated when HBV DNA levels are above 2000 IU/mL and/or the serum alanine aminotransferase (ALT) levels are over 2 times the upper limit of normal (ULN), and liver biopsy shows moderate to severe active necroinflammation and/or fibrosis (e.g. at least A2F2 by METAVIR scoring)[6-8]. Many clinical trials have shown positive results of the antiviral treatments in hepatitis B patients with ALT levels between 2 and 10 times the ULN range. Nevertheless, a proportion of patients have serum ALT level over 10 times the ULN. There are few reports on the issue of whether to treat these patients right away or wait until a decline of ALT level. This paper summarizes the efficacy of LDT treatment in 40 hepatitis B patients with serum ALT level over 10 times the ULN range. We found that these patients obtained a better therapeutic effect when they received LDT treatment immediately.

MATERIALS AND METHODS

Patients and study design

This study was approved by the Ethics Review Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University. All patients provided written informed consent before antiviral therapy was given. The diagnosis of CHB was made according to the diagnostic standard from the National Program for Prevention and Treatment of Viral Hepatitis[9]. All patients were diagnosed as CHB based on hepatitis B surface antigen (HBsAg) positivity for more than 6 mo. Forty HBeAg-positive CHB patients were enrolled in this study. All of them had ALT levels between 10 and 20 times the upper normal level. Another 40 HBeAg-positive CHB patients whose ALT level was between 2 and 10 times the ULN were recruited as controls. All 80 CHB patients had serum HBV DNA level > 10^5 copies/mL and had never received anti-HBV therapy before. Patients were given LDT 600 mg daily as initial antiviral treatment for at least 52 wk. Patients were excluded from this study if they were coinfected with human immunodeficiency virus, hepatitis C virus, hepatitis D virus, had liver cirrhosis or hepatic decompensation, pancreatitis, hepatocellular carcinoma, fatty liver or alcoholic hepatitis.

The present study focused on main therapeutic endpoints at 52 wk for CHB patients with high baseline ALT levels, including proportions of patients with non-detectable serum HBV DNA, serum ALT normalization, HBeAg and HBsAg seroconversion and LDT resistance. Resistance was defined as emergence of treatment-associated resistance mutations, identified by direct sequencing of the amplified HBV DNA at baseline and from sera of all patients with serum HBV DNA > 3 $\log_{10}$ copies/mL at week 52. Viral breakthrough was defined as persistent (two consecutive determinations) on-treatment increase of serum HBV DNA > 1 $\log_{10}$ copies/mL above nadir level[10].

Serum assay

Analyses of liver function, renal function and creatine kinase level were performed at baseline and at week 2, 4, 8, 12, 16, 24, 32, 36, 48 and 52 of LDT therapy using the Automatic Biochemistry analyzer (Hitachi 7600). HBsAg, HBeAg, anti-HBc, anti-HBe and anti-HBs were quantified using radioimmunoassay (Abbott Laboratories Ltd.). HBV DNA was measured using the Amplicor HBV Test (Roche Diagnostics, Basel, Switzerland) with a detection limit of 300 copies/mL. LTD-associated mutations were assessed by direct sequencing.

Statistical analysis

Quantitative data were presented as mean ± SD, categorical data were presented as counts and percentages, and HBV DNA levels were presented as log transformation. Data were analyzed using the SPSS software package version 13.0 (SPSS Inc., Chicago, IL, USA). Pearson chi-square or Fisher exact tests were used for categorical variables. In all cases, *P* values less than 0.05 were considered statistically significant.

RESULTS

Patients

Baseline characteristics for all 80 HBeAg-positive CHB patients are presented in Table 1. In the high baseline ALT CHB patient group, patients consisted of 29 males and 11 females, with ages ranging from 21 to 38 years (28.12 ± 3.71 years). Baseline data are as follows: the median level of serum HBV DNA was 7.78×10^7 copies/mL (range: 4.67×10^5-8.58×10^9 copies/mL), the median ALT level was 658.0 IU/L (range: 513.0-978.0 IU/L).

Virological response

By week 52, the mean decrease in HBV DNA level compared with baseline was 7.03 $\log_{10}$ copies/mL in the high

Table 1 Patient baseline characteristics

Variables	High baseline ALT group	Controls
Patients (*n*)	40	40
Male, *n* (%)	29 (72.5)	28 (70)
Age (yr, mean ± SD)	28.12 ± 3.71	31.12 ± 5.43
ALT (IU/L)	885.6 ± 7.89	128.4 ± 5.33
TBiL (μmol/L)	45.43 ± 6.67	29.12 ± 2.56
HBV DNA (copies/mL)		
Median	7.78×10^8	7.56×10^8
Range	4.67×10^5-8.58×10^9	5.89×10^5-7.34×10^9

ALT: Alanine aminotransferase; TBiL: Total bilirubin; HBV: Hepatitis B virus.

Table 2 Efficacy and safety at week 52 *n* (%)

Variables	High baseline ALT group	Controls	*P* value
Decrease in HBV DNA level (log_{10} copies/mL)	7.03	6.17	< 0.05
HBV DNA negative rate	29/40 (72.5)	24/40 (60)	< 0.05
ALT normalization rate	30/40 (75.0)	31/40 (77.5)	> 0.05
HBeAg negative rate	18/40 (45.0)	11/40 (27.5)	< 0.05
HBeAg seroconversion rate	15/40 (37.5)	9/40 (22.5)	< 0.05
HBsAg negative rate	4/40 (10.0)	1/40 (2.5)	< 0.05
HBsAg seroconversion rate	3/40 (7.5)	0	< 0.05
Viral breakthrough	2/40 (5.7)	3/40 (7.5)	> 0.05
Viral resistance	1/40 (2.9)	2/40 (5)	> 0.05
Increased blood creatine kinase	5/40 (12.5)	4/40 (10)	> 0.05

ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen.

baseline ALT group and 6.17 log_{10} copies/mL in the control group, respectively ($P < 0.05$). The proportion of patients in whom serum HBV DNA levels were undetectable by polymerase chain reaction assay was greater in the high baseline ALT group than in the control group (72.5% *vs* 60%, $P < 0.05$) as indicated in Table 2.

Serological response

At week 52, 45.0% of HBeAg-positive CHB patients in the high baseline ALT group and 27.5% ($P < 0.05$) of controls became HBeAg-negative, and 37.5% of those in the high baseline group and 22.5% of those in the control group had HBeAg seroconversion ($P < 0.05$). Moreover, in the high baseline group, 4 out of 40 patients (10%) became HBsAg-negative and 3 (7.5%) of them seroconverted (became HBsAb-positive). Only 1 patient in the control group became HBsAg-negative, but had no seroconversion (Table 2).

Biochemical response

At week 52, ALT normalization was achieved for 30 of the 40 patients (75.0%) in the high baseline ALT group and 31 of 40 patients (77.5%) in the control group ($P > 0.05$).

Resistance and side effects

As indicated in Table 2, viral breakthrough and genotypic resistance to LDT were similar between patients with high baseline ALT levels and controls. Resistance developed in 2.9% of patients with high baseline ALT levels and in 5% (2/40) of control patients. Consistent with previous reports, M204I was the only mutation associated with LDT resistance in this study. After the emergence of resistance, adefovir dipivoxil was added to treatment. Resistance patients are considered treatment failures in this study.

The frequencies of adverse events through week 52 were similar in both groups treated with LDT. Elevations in creatine kinase level through 52 wk were observed in 12.5% (5/40) of patients in the high baseline ALT group and in 10% (4/10) of controls, respectively. Grade 3 or 4 elevations in creatine kinase level (at least seven times the ULN) were found only in 1 patient in the high baseline ALT group and in 1 patient in the control group, respectively; levels decreased spontaneously during LDT treatment to normal within the next two visits (6 mo). No patients in either group stopped LDT treatment because of creatine kinase elevations in this study (Table 2).

DISCUSSION

The goal of therapy for hepatitis B is to improve quality of life and survival by preventing progression of the disease to cirrhosis, decompensated cirrhosis, end-stage liver disease, HCC and death. This goal can be achieved if HBV replication can be suppressed in a sustained manner, the accompanying reduction in histologic activity of chronic hepatitis lessening the risk of cirrhosis and decreasing the risk of HCC[11]. To date, two types of antiviral drugs can be used in the treatment of CHB: interferon and nucleoside/nucleotide analogs. In China, four types of nucleoside/nucleotide analogs (lamivudine, adefovir dipivoxil, entecavir and LDT) are available. Among them, LDT is potent and induces a relatively high seroconversion rate[12]. LDT has become widely used in anti-HBV therapy in China.

Besides serum HBV DNA levels and histological grade and stage of the liver disease, baseline ALT level of CHB patients is one of the determinants for the initiation of antiviral therapy. The antiviral effect of LDT is associated with the baseline ALT level, as in interferon and lamivudine therapy[13,14]. Taking HBeAg seroconversion as an example, 32% of patients with pretreatment ALT levels between 2 and 5 × ULN and 46% of those with ALT > 5 × ULN achieved HBeAg seroconversion after 2 years of treatment with LDT[5]. Our study focused, we believe for the first time, on the antiviral effect of LDT on HBeAg-positive patients whose baseline ALT level was 10-20 × ULN, showing the HBeAg seroconversion rate was 37.5% at 52 wk, which is the same as reported for peg-interferon therapy at 48 wk[15]. More encouragingly, our results also showed 7.5% (3/40) patients had HBsAg seroconversion at 52 wk after LDT treatment.

The main mechanism of ALT elevation in CHB patients is the activated immune response to eliminate HBV, which theoretically shows the positive association between ALT level and the degree of inmune activation. High baseline ALT level has been shown to be independently associ-

ated with an increased rate of HBeAg response after either interferon or NUC treatment[16,17]. In the present study, our results clearly showed that increased serum baseline ALT levels predict a higher HBeAg seroconversion when patients are treated with LDT.

HBeAg has been recognized as a successful serologic marker in the treatment of HBeAg-positive CHB[18]. Compared with other NUCs, LDT has a relatively high seroconversion rate. Whether this is related to its immune regulation ability needs further exploration. Evans *et al*[19] reported the relatively low expression of programmed death-1 receptor on CD8+ T cells in HBeAg-positive CHB patients who received LDT therapy and had HBeAg seroconversion, compared with those counterparts who did not achieve HBeAg seroconversion.

Entecavir and tenofovir are potent HBV inhibitors and they have a high barrier to resistance. They are widely used as first-line monotherapies in developed countries. However, in China tenofovir is not available yet, and entecavir is expensive for most patients. LDT and lamivudine are still widely used. In order to reduce the incidence of resistance to these drugs, optimal treatment has been used in clinical practice. For example, pretreatment serum HBV DNA $< 10^9$ $\log_{10}$ copies/mL and ALT levels $\geqslant 2 \times$ ULN for HBeAg-positive patients were shown to be associated with a high rate of non-detectable HBV DNA, a high rate of HBeAg seroconversion and lower resistance to LDT treatment after 2 years[5]. Our study also proved that if we select the right patients to treat with LDT, there will be optimal conditions to achieve the desired results. Taken together, if baseline serum HBV DNA $< 10^9$ $\log_{10}$ copies/mL and ALT levels $\geqslant$ 2-20 $\times$ ULN for HBeAg-positive patients, we can consider the administration of LDT treatment in daily clinical practice.

In conclusion, our results indicate relatively higher HBeAg and HBsAg seroconversion in HBeAg-positive CHB patients whose baseline ALT levels were 10-20 $\times$ ULN and who received LDT monotherapy immediately. In addition, there were no significant differences in safety between these patients and their counterparts with lower ALT levels. We suggest that this treatment strategy deserves clinical application.

COMMENTS

Background

There is a proportion of chronic hepatitis B (CHB) patients with serum alanine aminotransferase (ALT) levels over 10 times the upper limit of normal. There are few reports regarding the issue of treatment for these patients, whether to treat them right away or whether to wait until the decline of ALT level.

Research frontiers

In China tenofovir is not available yet, and entecavir is expensive for most patients. Telbivudine (LDT) and lamivudine are still widely used. In order to reduce the incidence of resistance to these drugs, optimal treatment has been used in clinical practice. However, how to select optimally for LDT has not been unequivocally addressed. In this study, the authors demonstrate relatively high hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) seroconversion in HBeAg-positive CHB patients whose baseline ALT levels were 10-20 times the upper limit of normal (× ULN) and who received LDT monotherapy immediately.

Innovations and breakthroughs

Recent reports have highlighted the importance of baseline characteristics, such as serum hepatitis B virus (HBV) DNA level, ALT level and histological grade and stage, in antiviral therapy. This is the first study to report the antiviral effect of LDT on HBeAg-positive patients whose baseline ALT level was 10-20 × ULN, showing the HBeAg seroconversion rate was 37.5% at 52 wk. More encouragingly, our results also showed 7.5% (3/40) patients had HBsAg seroconversion at 52 wk after LDT treatment.

Applications

By understanding that the antiviral results are related to the baseline ALT levels in addition to HBV DNA titer, this study may represent a future strategy for therapeutic intervention in CHB patients with high baseline ALT level.

Terminology

ALT is a common indicator of liver damage and is one of the key predictors of initiation of antiviral therapy. This study suggests that high baseline ALT level is a strong predictor for optimal results during LDT treatment.

Peer review

This study shows favorable results in patients with high baseline ALT values. The authors conclude that in patients with high baseline ALT levels antiviral treatment with LDT should be started immediately.

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BRIEF ARTICLE

Specific shRNA targeting of *FAK* influenced collagen metabolism in rat hepatic stellate cells

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Abstract

AIM: To investigate the effects and mechanism of disruption of focal adhesion kinase (*FAK*) expression on collagen metabolism in rat hepatic stellate cells (HSC).

METHODS: The plasmids expressing *FAK* short hairpin RNA (shRNA) were transfected into HSC-T6 cells, and the level of *FAK* expression was determined by both real-time quantitative polymerase chain reaction (Q-PCR) and Western blotting analysis. The production of type Ⅰ collagen and type Ⅲ collagen in *FAK*-disrupted cells was analyzed by real-time Q-PCR. The level of collagen metabolism proteins, including matrix metalloproteinases-13 (MMP-13) and tissue inhibitors of metalloproteinases-1 (TIMP-1) was also determined by both real-time Q-PCR and Western blotting analysis.

RESULTS: The transfection of *FAK* shRNA plasmids into HSC resulted in disrupted *FAK* expression. Compared with the HK group, the levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in *FAK* shRNA plasmid group were significantly decreased (0.69 ± 0.03 *vs* 1.96 ± 0.15, $P = 0.000$; 0.59 ± 0.07 *vs* 1.62 ± 0.12, $P = 0.020$). The production of TIMP-1 in this cell type was also significantly reduced at both mRNA and protein levels (0.49 ± 0.02 *vs* 1.72 ± 0.10, $P = 0.005$; 0.76 ± 0.08 *vs* 2.31 ± 0.24, $P = 0.000$). However, the expression of MMP-13 mRNA could be significantly up-regulated by the transfection of *FAK* shRNA plasmids into HSC (1.74 ± 0.20 *vs* 1.09 ± 0.09, $P = 0.000$).

CONCLUSION: These data support the hypothesis that shRNA-mediated disruption of *FAK* expression could attenuate extracellular matrix (ECM) synthesis and promote ECM degradation, making *FAK* a potential target for novel anti-fibrosis therapies.

Key words: Focal adhesion kinase; Hepatic stellate cells; Matrix metalloproteinases; RNA interference; Type Ⅰ collagen; Type Ⅲ collagen; Tissue inhibitors of metalloproteinases

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INTRODUCTION

Liver fibrosis results from excessive deposition of extracellular matrix (ECM) components[1]. These components, which are mainly composed of type I collagen and type Ⅲ collagen, are produced by hepatic stellate cells (HSC). The activation, proliferation and migration of HSC play a central role in liver fibrogenesis[2,3]. Activated HSCs are the main producers of collagens and matrix metalloproteinases (MMPs) in the fibrotic liver. The MMP which is able to degrade type I collagen and type Ⅲ collagen is MMP-13. However, this can be specifically inhibited by tissue inhibitors of metalloproteinases-1 (TIMP-1), and its level is found to be high in the fibrotic liver of rats.

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase, whose phosphorylation can promote the proliferation and collagen synthesis of HSC[4-8]. It had previously been shown that when FAK related non-kinase (FRNK) plasmids were transfected into fibronectin (FN)-stimulated HSC using liposome transfection, the over-expression of FRNK significantly decreased the collagen synthesis of HSC *in vitro*[9,10]. This led us to speculate that suppression of FAK expression may provide a new target in the treatment of liver fibrosis.

To date, RNA interference has been the most effective gene silencing technology. It can specifically inhibit the transcription of target genes, and in turn reduce the expression and function of the corresponding proteins[11]. We aim to inhibit FAK expression by transfecting FAK short hairpin RNA (shRNA) plasmids into HSC. To our knowledge, this is the first report that FAK expression is specifically inhibited in HSC cells. This allows us to further analyze the role of FAK in collagen synthesis and degradation in this cell type, and find out how FAK regulates the expression of MMP-13 and TIMP-1.

MATERIALS AND METHODS

Reagents

The shRNA-expressing plasmids, pEGFP-*FAK* shRNA, were purchased from Wuhan Genesil Biotechnology Co. Ltd. (Wuhan, China). One additional plasmid, p-EGFP-HK, was used to express nonsense shRNA and served as the control. Sofast™ Transfection Reagent was purchased from Xiamen Sunma Biological Engineering Co. Ltd. (Xiamen, China).

Cell line and cell culture

The cell line HSC-T6, which is the phenotypically activated HSC, was donated by Professor Xu LM, from Hepatopathy Institute of Shanghai University of Traditional Chinese Medicine. HSCs were cultured in HG-DMEM medium supplemented with 8% FBS, 100 IU/mL penicillin, 100 g/mL streptomycin, 4 mmol/L glutamine and 1 mol/L HEPES. Cells were cultured in a 5% CO_2 humidified incubator at 37℃. All experiments were conducted when cells were at an exponential stage of growth. Cells were seeded into a 25 cm^2 plastic culture flask with a total of 2-3 × 10^5 cells or were seeded in 96-well plates to a density of 3 × 10^4/mL × 200 μL/well. When cells were approximately 70%-80% confluent, shRNA plasmid was transfected into FN-stimulated HSC using a cationic polymer. The cells were divided into five groups: (1) blank control group (control); (2) FN stimulation group (FN); (3) transfection reagent group (Sofast); (4) pEGFP-HK shRNA group (HK); and (5) pEGFP-*FAK* shRNA group (*FAK* shRNA). FN was added to groups 2-5 at a concentration of 10 mg/L.

Efficiency of transfection

At 48 h after transfection, the cells were analyzed by fluorescence microscopy and flow cytometry (FCM) to obtain the efficiency of transfection.

Semiquantitative real-time quantitative polymerase chain reaction

The expressions of the gene *FAK*, *type I collagen* and *type Ⅲ collagen*, *MMP-13* and *TIMP-1* were characterized by semiquantitative real-time quantitative polymerase chain reaction (Q-PCR). Briefly, total RNA was extracted from the cells that had been transfected with the plasmid expressing the *FAK* or HK shRNA and reversely transcripted into cDNA, which was used as the template for PCR. Using the primer design software, Primer Express 2.0, the specific primers for each gene were synthesized by Beijing Saibaisheng Gene Technique Co., Ltd. and the following primers were generated: *FAK*-Forward 5'-ACTTGGACGCTGTATTGGAG-3', *FAK*-Reverse 5'-CTGTTGCCTGTCTTCTGGAT-3' (833 bp amplicon); Collagen type I -Forward 5'-TACAGCACGCTTGTGGATG-3', Collagen type I -Reverse 5'-TTGAGTTTGGGTTGTTGGTC-3' (256 bp amplicon); Collagen type Ⅲ-Forward 5'-ATGGTGGCTTTCAGTTCACC-3', Collagen type Ⅲ-Reverse 5'-TGGGGTTTCAGAGAGTTTGG-3' (425 bp amplicon); MMP-13-Forward 5'-GCGGGAATCCTGAAGAAGTCTAC-3', MMP-13-Reverse 5'-TTGGTCCAGGAGGAAAAGCG-3' (424 bp amplicon); TIMP-1-Forward 5'-TCCCCAGAAATCATCGACAC-3', TIMP-1-Reverse 5'-ATCGCTGAACAGGGAAACAC-3' (329 bp amplicon); *GAPDH*-Forward 5'-GAGGACCAGGTTGTCTCCTG-3', *GAPDH*-Reverse 5'-GGATGGAATTGTGAGGGAGA-3' (298 bp amplicon). Reaction system: 10 μL 2.5 × real master Mix, 1.25 μL 20 × SYBR solution, 0.5 μL upstream primer, 0.5 μL downstream primer and 2 μL DNA template were brought up to 25 μL with purified water. Reaction conditions: 93℃ 5 min, 1 cycle; 93℃ 45 s, 55℃ 1 min, 10 cycles; 93℃ 30 s, 55℃ 45 s, 30 cycles. The PCR reactions were subjected to 93℃ for 5 min, 1 cycle; and then 10 cycles of 93℃ 45 s, 55℃ 1 min, and 30 cycles of 93℃ 30 s, 55℃ 45 s. The size and quantity of amplified prod-

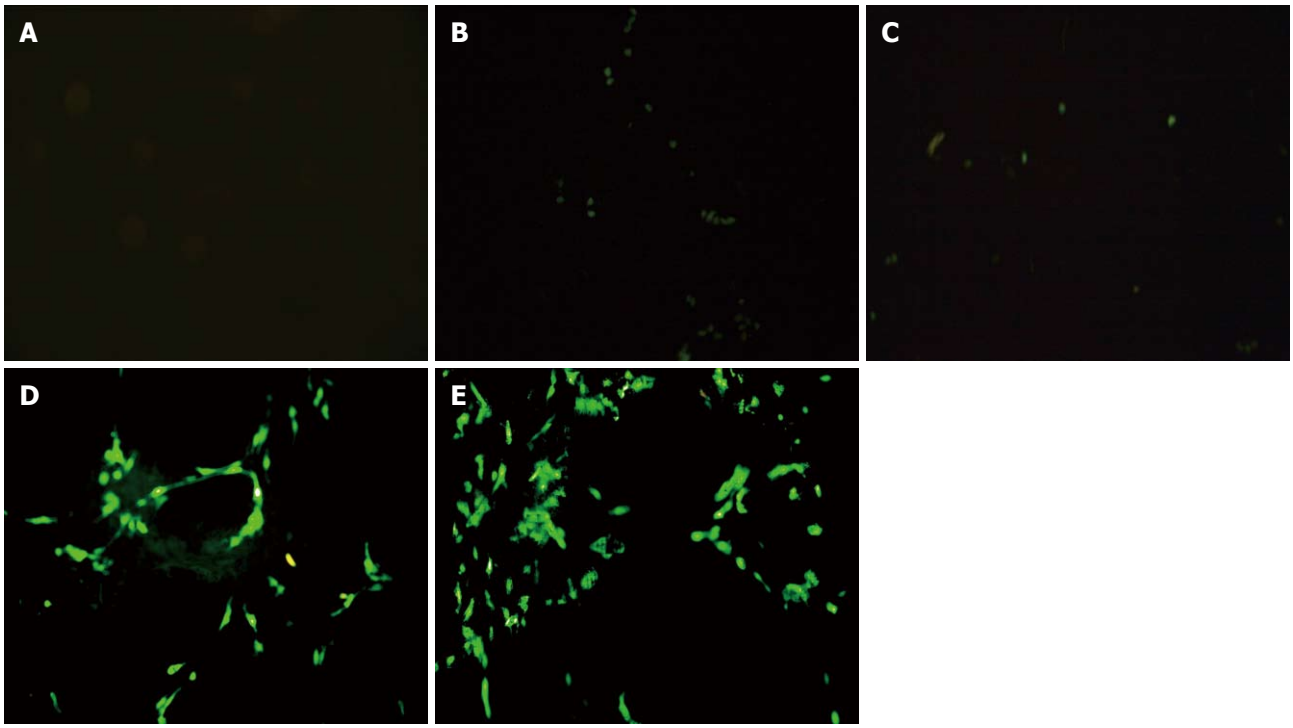

Figure 1 Expression of enhanced green fluorescent protein at 48 h after treatment (fluorescent images, original magnification × 200). A: Control group; B: Fibronectin group; C: Sofast group; D: HK group; E: Focal adhesion kinase (FAK) short hairpin RNA group. *FAK* short hairpin RNA plasmids were successfully transfected into hepatic stellate cells. The results from fluorescence microscopy and flow cytometry showed that the transfection efficiency was 40% at 48 h.

ucts were confirmed by 2% agarose gel electrophoresis. Fluorescent quantitative analysis was performed with the thermal cycler's software package to calculate the ΔCt value. The expression levels of *FAK*, type I collagen and type Ⅲ collagen, *MMP-13* and *TIMP-1* were calculated by the $2^{-\Delta\Delta Ct}$ analysis. The $2^{-\Delta\Delta Ct}$ was presented as the relative expression of the gene expression[12].

Western blotting

At 24 or 48 h after transfection of *FAK* shRNA, HSCs were harvested, washed with phosphate-buffered saline (PBS), and lysed in the improved RIPA buffer (50 mmol/L Tris-HCL, pH 7.5; 100 mmol/L NaCl; 1% NP-40; 0.5% sodium deoxycholate; 2 μg/mL leupeptin; 1% SDS; 2 mmol/L EDTA; 1 mmol/L PMSF; 50 mmol/L HEPES; 1 mmol/L sodium orthovanadate). The supernatant was collected and the protein concentration was determined using comassie brilliant blue assay. Cell extracts containing equal quantities of proteins (100-110 μg) were electrophoresed in 8% or 10% polyacrylamide gel. Subsequently, the separated proteins were transferred to nitrocellulose membrane. The membrane was blocked for non-specific binding for 30 min (5% skimmed milk in PBS), and then incubated overnight at 4℃ with rabbit anti-FAK polyclonal antibody (1:400), rabbit anti-MMP-13 polyclonal antibody (1:200), rabbit anti-TIMP-1 polyclonal antibody (1:200) or mouse anti–GAPDH monoclonal antibody (1:100). The membrane was subsequently incubated at room temperature for 2 h with goat anti-rabbit IgG (1:2000). Blots were developed with enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology Inc.), exposed on Kodak Xdmat blue XB-1 film and quantified by Bandscan 5.0 software using GAPDH as internal control. Densitometry is reported using the integral optical density value (IOD). The results were represented in the form of IOD ratio of the target protein to GAPDH.

Statistical analysis

All the data were expressed by mean ± SD and analyzed with SPSS 13.0 software. The comparison of mean variability among all groups was conducted by one-way ANOVA analysis and two group comparison with LSD test. Student's *t* test was carried out for independent samples. Statistical significance was considered at $P < 0.05$.

RESULTS

Expression of FAK effectively down-regulated by FAK shRNA in HSC

FAK shRNA plasmids were successfully transfected into HSC. The results from fluorescence microscopy and FCM showed that the transfection efficiency was 40% at 48 h (Figure 1). The levels of *FAK* mRNA transcripts and protein expression were determined by real-time Q-PCR and Western blotting analysis. The expression of *FAK* mRNA and FAK protein in the FN group was significantly higher than that of the control group, $P = 0.000$ and $P = 0.024$, respectively. There was no difference between the FN group, Sofast group and HK group. In comparison with the HK group, the expression of *FAK* mRNA and FAK

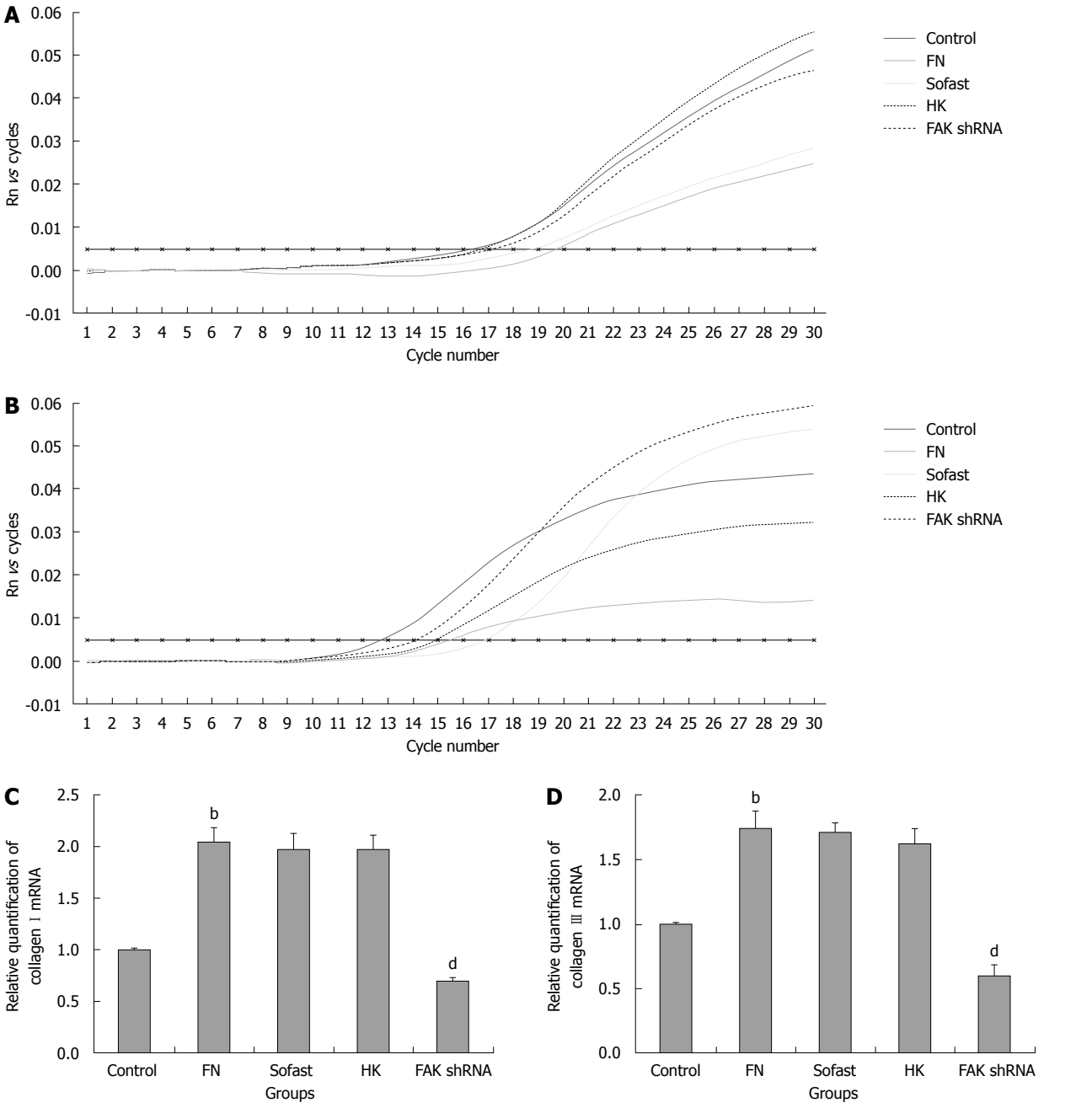

Figure 2 Focal adhesion kinase short hairpin RNA selectively inhibited the expressions of collagen Ⅰ mRNA and collagen Ⅲ mRNA in hepatic stellate cells after focal adhesion kinase short hairpin RNA transfection. A, B: Real-time polymerase chain reaction SYBR Green Ⅰ fluorescence history *vs* cycle number of target gene 1 (collagen Ⅰ, A) and target gene 2 (collagen Ⅲ, B) in sample cDNA. The cycle threshold (Ct) is shown by the darker horizontal line; C, D: The relative quantification of collagen Ⅰ mRNA (C) and collagen Ⅲ mRNA (D) are calculated according to $2^{-\Delta\Delta C(t)}$, [$\Delta\Delta$Ct = ($Ct_{Collagen\ I\ or\ III}$ - Ct_{GAPDH}) experimental group - ($Ct_{Collagen\ I\ or\ III}$ - Ct_{GAPDH}) control group] and shown in the bar graphs (n = 3, ^{b}P < 0.01 *vs* control, ^{d}P < 0.01 *vs* HK). It showed that the levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in fibronectin (FN) group was significantly higher than in the control group. FAK: Focal adhesion kinase; shRNA: Short hairpin RNA.

protein in the *FAK* shRNA plasmid group was significantly decreased (0.37 ± 0.03 *vs* 1.59 ± 0.06, P = 0.000; 0.77 ± 0.03 *vs* 2.24 ± 0.20, P = 0.000), and the rates of down-regulation were 70.51% and 72.53%, respectively.

Effects of FAK by shRNA on the collagen synthesis in HSC

Investigation was carried out in the influence of disruption of FAK expression mediated by *FAK* shRNA on ECM synthesis in HSC. The levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts were determined by real-time Q-PCR. The levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in FN group were significantly higher than that of the control group. The levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in *FAK* shRNA plasmid group were significantly decreased compared with the HK group (0.69 ± 0.03 *vs* 1.96 ± 0.15, P = 0.000; 0.59 ± 0.07 *vs* 1.62 ± 0.12, P = 0.020) and the down-regulated rates were 64.80% and 63.58%, respectively (Figure 2).

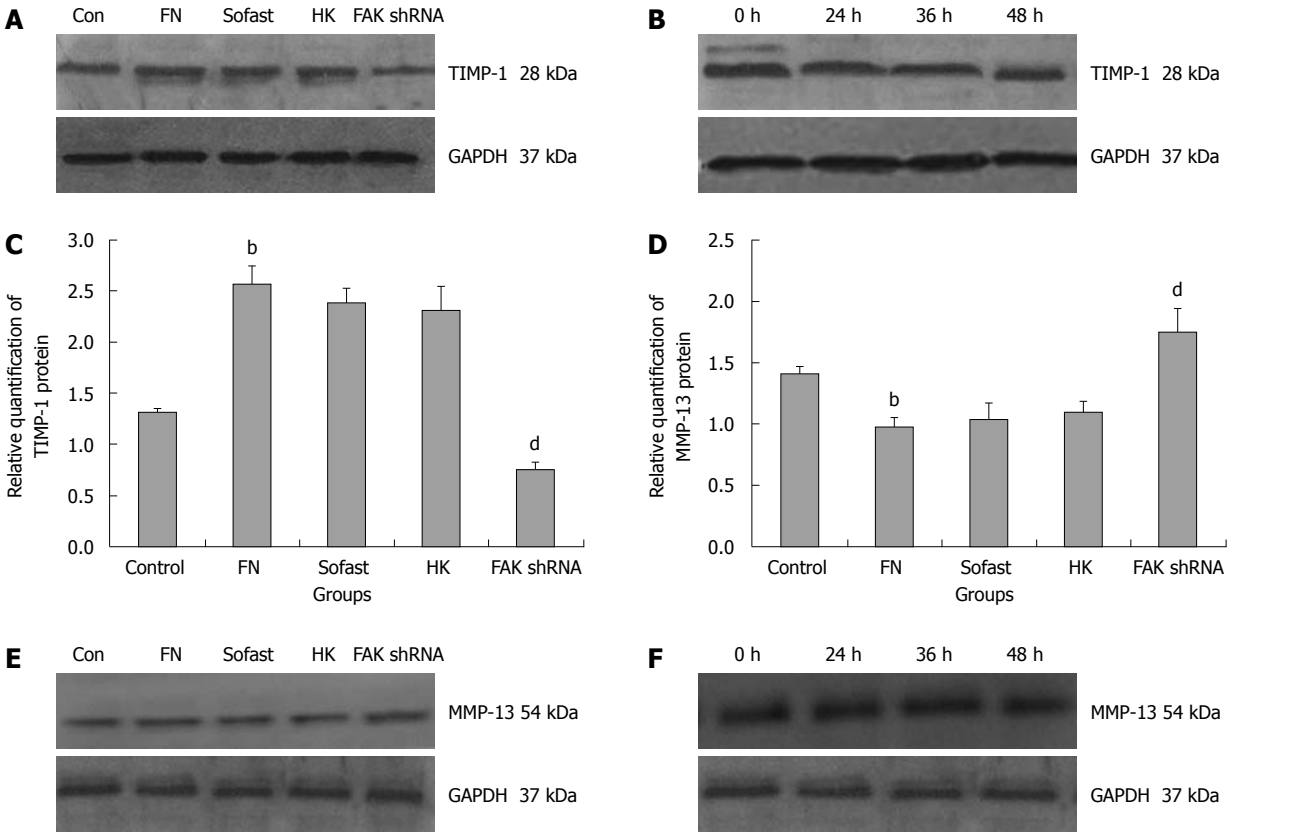

Figure 3 Focal adhesion kinase short hairpin RNA specifically inhibits the expressions of tissue inhibitors of metalloproteinases-1 protein and promotes the expressions of matrix metalloproteinases-13 protein in hepatic stellate cells. A: Cells were harvested, lysed and total protein extracts were separated by SDS-PAGE and analyzed by Western blotting with polyclonal anti-tissue inhibitors of metalloproteinases-1 (TIMP-1) antibody. GAPDH served as a loading control; B: Western blotting analysis was used to detect the expressions of TIMP-1 at different time points; C: TIMP-1 expression levels obtained from scanning densitometry were expressed as a ratio of integral optical density value (IOD) TIMP-1/IOD GAPDH (n = 3, $^{b}P < 0.01$ *vs* Con, $^{d}P < 0.01$ *vs* HK); D: Matrix metalloproteinases-13 (MMP-13) expression levels obtained from scanning densitometry were expressed as a ratio of IOD MMP-13/IOD GAPDH (n = 3, $^{b}P < 0.01$ *vs* Con, $^{d}P < 0.01$ *vs* HK); E, F: Western blotting analysis was carried out at different groups (E) and different time points (F) using polyclonal anti-MMP-13 antibody and monoclonal anti-GAPDH antibody. FN: Fibronectin; FAK: Focal adhesion kinase; shRNA: Short hairpin RNA.

Effects of FAK by shRNA on the collagen degradation in HSC

To further explore the effects of *FAK* shRNA on the ECM degradation in HSC, the levels of MMP-13 and TIMP-1 were determined by real-time Q-PCR and Western blotting analysis. The transfection of HK shRNA did not modulate the levels of MMP-13 and TIMP-1, the cells expressing HK shRNA were similar to that in FN group and Sofast group, $P > 0.05$. However, the knockdown of FAK expression by the *FAK* shRNA significantly reduced the levels of *TIMP-1* mRNA and TIMP-1 protein (0.49 ± 0.02 *vs* 1.72 ± 0.10, $P = 0.005$; 0.76 ± 0.08 *vs* 2.31 ± 0.24, $P = 0.000$), and the down-regulated rates were 69.78% and 67.10%, respectively (Figure 3A-C). The results of real-time Q-PCR and Western blotting analysis showed that the levels of MMP-13 of FN group were significantly down-regulated compared with that of control group. Compared with the HK group, the expression of MMP-13 mRNA was significantly up-regulated by 56.96% at 36 h after transfection of *FAK* shRNA plasmids into HSC (1.24 ± 0.04 *vs* 0.79 ± 0.03, $P = 0.020$), and the expression of MMP-13 protein could be increased by 59.63% at 48 h after transfection (1.74 ± 0.20 *vs* 1.09 ± 0.09, $P = 0.000$) (Figure 3D-F).

DISCUSSION

The current knowledge on the pathophysiology of liver fibrogenesis refers to the increased synthesis and decreased degradation of ECM, mainly type I collagen and type Ⅲ collagen, thereby ECM was overproduced and deposited in the liver. Although several hepatic cell types can synthesize ECM proteins, HSCs are the major source of increased ECM in chronic liver diseases. They can undergo a proliferative and phenotypic change. Excessive deposition of ECM, mainly type I collagen and type Ⅲ collagen, results in liver fibrosis; and the up-regulation of TIMPs blocks activity of MMPs and inhibits the degradation of ECM, thereby aggravating liver fibrosis.

The interaction of HSC and ECM mainly lies between integrins, and FAK plays an integral role in the integrin signal pathway. Activated FAK has been implicated in a diverse array of cellular behaviors, such as cell proliferation[4,5], apoptosis, cell migration[6], collagen metabolism[7,8] and the transfer of tumor cells. It is closely related to numerous fibrotic diseases and it plays a vital role in the occurrence and development of liver fibrosis[13]. This is consistent with our previous studies, which indicated that FAK phosphorylation could promote collagen synthesis

of HSC *in vivo*. Furthermore, using *in vitro* cell culture techniques, we found that the synthesis of total collagen and type I collagen in HSC could be inhibited by the endogenous inhibitor FRNK[9]. We hypothesized that FAK gene silencing may represent a novel method for the treatment and reversal of liver fibrosis. Therefore, in this study, *FAK* shRNA plasmids were transfected into HSC transiently to test our hypothesis, and the expressions of FAK mRNA and FAK protein were significantly decreased, the down-regulation rates being 70.51% and 72.53%, respectively. We have found that *FAK* shRNA can effectively and specially suppress the expression of FAK.

A substantial change in liver fibrosis or liver cirrhosis is the deposition of ECM, which is mainly composed of type I collagen and type Ⅲ collagen, covering approximately 80%-90% of the increased total collagen. The increase of type I collagen and type Ⅲ collagen is an important symbol of liver fibrosis or liver cirrhosis. Therefore, in this study, *FAK* shRNA plasmids were transfected into FN-stimulated HSC transiently and the expression of type I collagen mRNA and type Ⅲ collagen mRNA was significantly down-regulated by 64.80% and 63.58%, respectively. These data show that *FAK* shRNA can effectively suppress the synthesis of collagen and *FAK* gene silencing may, therefore, represent a novel direction for the treatment and reversal of liver fibrosis.

Furthermore, we attempted to assess the role of FAK in the regulation of collagen metabolism in HSC. In the liver, ECM is regulated by MMPs and their specific inhibitors, TIMPs. A principal feature of hepatic fibrosis is a disturbance in the balance between MMPs and TIMPs. Collagenases such as MMP-1 and MMP-13 are able to degrade fibrillar collagens, mainly type Ⅰ, Ⅱ and Ⅲ collagen. These may be responsible for key events in the degradation of ECM. MMP-13 is the interstitial collagenase in rats and its specific inhibitor is TIMP-1. Although the expression of MMP-13 was increased in the liver tissues of CCl_4-induced rat liver fibrosis models, fibrosis still occurred as there was also a corresponding increase in the expression of TIMP-1[14]. This strongly suggests that a disruption in the balance between MMP-13 and TIMP-1 is possibly an important factor in liver fibrogenesis[15]. According to some studies, FAK is closely related to the expression of TIMP-1 and MMP-13[16,17]. In this study, *FAK* shRNA plasmids were transfected into FN-stimulated HSC transiently and the expression of MMP-13 mRNA and MMP-13 protein was significantly up-regulated by 56.96% and 59.63%. Correspondingly, the levels of TIMP-1 mRNA and TIMP-1 protein were significantly down-regulated by 69.78% and 67.10%, respectively. *FAK* shRNA inhibited the ratio of TIMP-1/MMP-13 expression in mRNA and protein levels in HSC after transfection. The data indicate that *FAK* shRNA regulated the collagen metabolism in HSC by disturbing the balance between MMP-13 and TIMP-1.

In summary, we have effectively disrupted the expression of FAK by *FAK* shRNA. The knockdown of FAK expression significantly reduced the synthesis of type I collagen and type Ⅲ collagen, which may be related to the up-regulation of MMP-13 and down-regulation of TIMP-1. These data support the hypothesis that *FAK* disruption by shRNA may be an efficient and specific approach for treatment of liver fibrosis. Future studies will address the signal transduction pathway by which FAK regulates the collagen metabolism in HSC.

COMMENTS

Background

Focal adhesion kinase (FAK) plays an essential role in the activation of hepatic stellate cells (HSCs) which are the major source of collagens and matrix metalloproteinases in the fibrotic liver. Liver fibrosis results from excessive deposition of extracellular matrix components, composed of mainly type Ⅰ collagen produced by HSC.

Research frontiers

The central events in the liver fibrogenesis have been proved to be the activation, proliferation and migration of HSC, and their proliferation and collagen synthesis are promoted by phosphorylation of FAK, a non-receptor protein tyrosine kinase. In the area of knockdown or inhibition of FAK with various molecular biological technologies, an area of intense research is to establish a method to knockdown or inhibit FAK expression thoroughly so as to enhance the collagen metabolism.

Innovations and breakthroughs

Recent reports have highlighted the importance of HSC including activation, proliferation and migration in pathogenesis of liver fibrosis. The collagen metabolism in HSC, particular in activated HSC, is currently an area of intense research. This is the first study to report that shRNA-mediated disruption of *FAK* expression can attenuate extracellular matrix (ECM) synthesis and promote ECM degradation. This represents a potential target for novel anti-fibrosis therapies.

Applications

The results of this study indicated that suppression of *FAK* expression may represent a novel method and direction for the treatment and reversal of hepatic fibrosis.

Peer review

The study focuses on modification of hepatic stellate cell metabolism by shRNA mediated inhibition of FAK, a non-receptor protein tyrosine kinase involved in proliferation and collagen synthesis. The authors demonstrate that FAK inhibition is associated with a decrease in collagen synthesis by HSCs.

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CASE REPORT

Development of fatal acute liver failure in HIV-HBV coinfected patients

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Abstract

Coinfection with hepatitis B virus (HBV) is not uncommon in human immunodeficiency virus (HIV)-infected individuals and patients with HIV-HBV coinfection are at high risk for progression of liver disease. Current guidelines regarding the treatment of HIV infection recommend that patients who are coinfected with HIV and HBV receive highly active antiretroviral therapy (HAART) with activity against hepatitis B. While HIV-HBV coinfected patients often experience liver enzyme elevations after starting antiretroviral therapy, acute liver failure (ALF) is rare and typically occurs with older antiretroviral agents with known potential for hepatotoxicity. We describe two cases of fatal ALF in the setting of HIV-HBV coinfection after initiation of HAART. These cases occurred despite treatment with antiretrovirals that have activity against HBV and highlight the challenges in distinguishing drug hepatotoxicity and HBV immune reconstitution inflammatory syndrome. HIV-HBV coinfected patients should be monitored closely when initiating HAART, even when treatment includes agents that have activity against HBV.

Key words: Hepatitis B virus; Human immunodeficiency virus; Liver failure

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INTRODUCTION

It is estimated that 10% of human immunodeficiency virus (HIV)-infected patients in the United States and Europe are chronically coinfected with hepatitis B virus (HBV)[1,2]. In other regions, rates of HBV coinfection in HIV-infected patients may be even higher[3]. HIV-HBV coinfected patients have higher rates of liver-related morbidity and mortality compared to patients infected with either virus alone[4,5]. Therefore, current guidelines suggest that HIV-infected patients with HBV infection should be treated with a highly active antiretroviral therapy (HAART) regimen that is also active against HBV[6]. However, HIV-HBV coinfected patients are particularly susceptible to certain complications of HAART. The vast majority of antiretroviral medications have been associated with some degree of hepatotoxicity and the presence of HBV infection is an independent risk factor for the development of clinically significant hepatotoxicity[7-9]. Additionally, coin-

fected patients are at risk for HBV immune reconstitution inflammatory syndrome (IRIS), which is characterized by a paradoxical hepatitis flare corresponding to an initial improvement in plasma HIV RNA level and CD4+ T-cell count on HAART[10]. Overall, liver enzyme elevations in HIV-HBV coinfected patients after starting HAART are not uncommon, but acute liver failure (ALF) is rare[11]. Reported cases have typically involved treatment with older thymidine analogue drugs such as stavudine and didanosine[12]. We describe two cases in which fatal ALF occurred in patients with HIV-HBV coinfection after beginning HAART regimens which did not include thymidine analogues but which did have activity against HBV.

CASE REPORT

Patient A

A 42-year-old African-American male with longstanding HIV/HBV coinfection was seen in clinic. He had been diagnosed with HIV infection over 10 years previously but had been on antiretrovirals for only short periods since diagnosis. As a result, his CD4+ T-cell count had reached a nadir of 5 cells/μL (1%) with a plasma HIV RNA level of 51 230 copies/mL. His plasma HBV DNA level was 147 million IU/mL. Both hepatitis B endogenous antigen (HBeAg) and anti-HBe antibody were negative. He was started on a new regimen of ritonavir-boosted atazanavir, lamivudine, and abacavir. At that time, his alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were both slightly elevated at 94 U/L (normal range 17-63 U/L) and 73 U/L (normal range 10-42 U/L), respectively, with a normal bilirubin level. The patient had never undergone a liver biopsy. Eight weeks after starting therapy, he returned to clinic with nausea, vomiting, and jaundice. The ALT level had increased to 1352 U/L and the AST was 1765 U/L. Total bilirubin was 14.1 mg/dL, direct bilirubin was 8.9 mg/dL, and prothrombin time was 18.9 s (INR 1.62). HIV RNA level had decreased to 100 copies/mL. He was admitted to the hospital for further workup. The patient did not drink alcohol and acetaminophen level was undetectable. Hepatitis C virus (HCV) RNA was undetectable, hepatitis D virus (HDV) antibody was negative, and hepatitis A virus (HAV) IgM was negative. HBV DNA had decreased to 4.42 million IU/mL. Antinuclear antibody screen was negative. All medications were held for 48 h. His ALT and AST levels decreased to 992 U/L and 1505 U/L. The appearance of the liver was normal on computed tomography of the abdomen with no suggestion of cirrhosis or portal hypertension. He was then discharged after starting a new regimen of ritonavir-boosted fosamprenavir, emtricitabine, and tenofovir. Ten days later he was re-admitted to the hospital with nausea, vomiting, and abdominal pain. ALT and AST levels had risen to 1214 U/L and 1992 U/L. Total bilirubin was 29.5 mg/dL, direct bilirubin was 18.3 mg/dL, and prothrombin time was 35.4 s (INR 3.19). HBV DNA level had decreased to 83 100 IU/mL. The ritonavir and fosamprenavir were discontinued and he was continued on emtricitabine/tenofovir. A liver biopsy showed marked septal fibrosis with nodule formation that was consistent with cirrhosis (Figure 1A and B). There was a severe mixed inflammatory infiltrate in portal and periportal areas containing lymphocytes, plasma cells and scant eosinophils (Figure 2A). The liver eosinophils were readily identified and no other special stains such as sirius red were used. Peripheral blood eosinophil count was within normal limits. Severe piecemeal necrosis was present diffusely around most of the portal tracts linking some of them together in so-called bridging necrosis (Figure 2B). After 10 d, his ALT and AST levels had come down to 405 U/L and 758 U/L, but total and direct bilirubin remained elevated at 28.8 mg/dL and 13.6 mg/dL. Due to concern for creating HIV resistance, he was switched from emtricitabine/tenofovir to adefovir. After 1 wk, he developed worsening renal failure and was switched from adefovir to emtricitabine and telbivudine. However, his condition continued to deteriorate. After 3 wk in the hospital, he became progressively encephalopathic, thrombocytopenic with a platelet count of 33 000/μL, and coagulopathic with prothrombin time of 37.7 s (INR 3.41). He developed hematemesis and became increasingly unresponsive with an ammonia level of 96 μmol/L (normal range 11-35 μmol/L). The patient was thought to be too unstable to undergo liver transplantation. After discussions with his family, he was placed on comfort care and died.

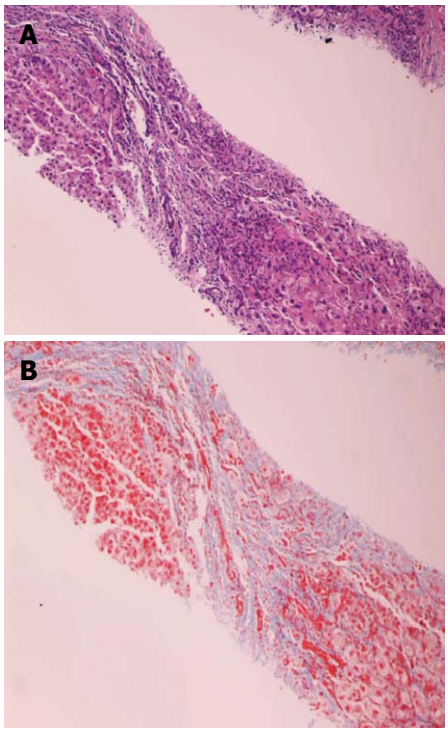

Figure 1 Liver biopsy, low power magnification (100 ×). A: Hematoxylin and eosin stain showing a low power view of two cirrhotic nodule surrounded by fibrosis. The edges of the nodules and the fibrous septi contain intense chronic inflammatory infiltrate; B: Trichrome stain delineating fibrous septa that surround liver nodules in blue color.

Patient B

A 46-year-old African-American male came to clinic with

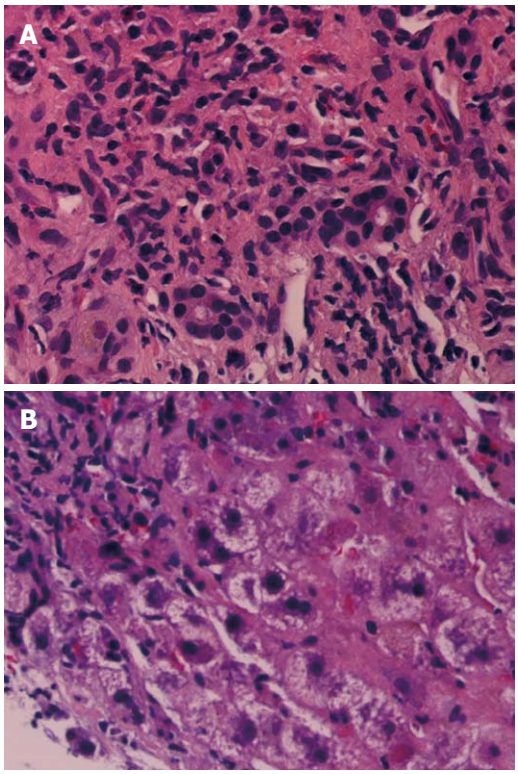

Figure 2 Liver biopsy, high power magnification (hematoxylin and eosin stain, 600 ×). A: Marked portal inflammatory infiltrate composed of plasma cells and a few eosinophils; B: High power view of a cirrhotic nodule composed of markedly swollen hepatocytes, some with granular eosinophilic cytoplasm and intense inflammatory infiltrate at the interface between parenchyma and fibrosis.

a new diagnosis of HIV infection. CD4+ T-cell count was 44 cells/μL (8%) with a plasma HIV RNA level of 9620 copies/mL. He was also diagnosed with hepatitis B infection with HBV DNA level > 500 million IU/mL. Duration of HBV infection was not known but HBV core IgM was negative. HCV antibody was negative, HBeAg was negative, anti-HBe antibody was positive, and HDV antibody was negative. ALT level was normal at 62 U/L and AST was slightly elevated at 53 U/L. He was started on a regimen of once daily ritonavir-boosted darunavir and tenofovir/emtricitabine. Five weeks after starting this regimen, he presented with nausea, vomiting, and jaundice. ALT was 1195 U/L and AST was 1396 U/L. Total bilirubin was 13.5 mg/dL, direct bilirubin was 7.7 mg/dL, and prothrombin time was 27.4 s (INR 2.42). HBV DNA level had decreased to 342000 IU/mL. HIV RNA level was undetectable at < 50 copies/mL and CD4+ T-cell count was 52 cells/μL (10%). HAV IgM was negative and HCV antibody was negative on recheck. Both serum ethanol and acetaminophen levels were undetectable. The patient reported no sexual activity for over 1 year and he never used injectable drugs. Magnetic resonance imaging of the abdomen showed fibrotic changes throughout the liver and portal hypertension evidenced by splenomegaly, a recanalized umbilical vein and minimal perigastric and perisplenic varices. After all medications were held for 72 h, he was restarted on a regimen of ritonavir-boosted fosamprenavir and tenofovir/emtricitabine. Over the next week, his ALT and AST levels trended down to 803 U/L and 773 U/L and his platelets remained within normal limits. However, over that period of time his prothrombin time increased to 42.5 s (INR 3.88) and he became profoundly encephalopathic with an ammonia level of 137 μmol/L. The patient was thought to be too unstable for liver transplantation. After a total of 10 d in the hospital he developed cardiac arrest and died.

DISCUSSION

The development of elevated liver enzymes is not uncommon in HIV-HBV coinfected patients after starting HAART[13]. In the majority of cases, these elevations are mild and do not require modification of treatment[11]. The development of more severe hepatotoxicity (liver enzymes > 10 times the upper limit of normal) is not common and ALF is rare[9]. Among HIV-infected patients in general, those who develop ALF while on HAART have experienced very high rates of mortality[14]. Therefore, an HIV-HBV coinfected patient who develops ALF after starting HAART may be at particularly high risk for mortality, given the presence of underlying liver disease and potentially impaired reserve.

The most appropriate management of such patients is not completely clear, and the optimal management of HIV-HBV coinfected patients who develop liver enzymes > 10 times the upper limit of normal after starting HAART but who do not have evidence of decompensated liver disease may be even more difficult to delineate. One question is whether it is possible to determine if the hepatic injury is from drug toxicity or HBV IRIS and whether this changes management of the patient. Liver biopsy may be helpful in identifying an opportunistic infection, such as mycobacterial disease or cytomegalovirus, that may contribute to liver disease. Liver biopsy may also indicate the presence of cirrhosis. The latter was present in the case of patient A and would have also been found in the case of patient B given radiographic findings. In patient A, liver biopsy showed severe inflammation with lymphocytes, plasma cells, and scant eosinophils. Many cases of HAART-induced ALF in patients without HBV show only mild hepatic inflammatory cell infiltration on biopsy[15]. However, the presence of portal plasma-lymphocytic infiltrate is nonspecific and can be found in chronic viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis and even some medication reactions[16]. The presence of eosinophils may be more specific for a drug reaction[17], though these were scant in this case. Overall, distinguishing between HBV IRIS and drug hepatotoxicity in such cases of ALF may not change management, particularly because it is not clear if anti-inflammatory medications such as corticosteroids are beneficial in HBV IRIS. Corticosteroids have been found to increase HBV replication[18] and this has led some to recommend against the use of corticosteroids in the setting of HBV IRIS[19].

The patients in our series met criteria for ALF according to the American Association for the Study of Liver

Diseases guideline for liver failure[20]. In this guideline, it is recommended that "all non-essential medications" be discontinued in patients with ALF. Due to the fact that the majority of antiretrovirals, including protease inhibitors[8], have been associated with hepatotoxicity, it is advisable to hold HAART at least in the short term. However, HBV flares have been associated with the discontinuation of HAART regimens that contain anti-HBV activity[21]. This theoretically could contribute to ALF. At the same time, continuing single or dual therapy with anti-HBV agents such as lamivudine or tenofovir would create an environment in which HIV resistance might develop. This has led some to recommend stopping HAART and considering treatment of HBV with agents that do not have activity against HIV in cases of significant hepatitis flares on HAART in the setting of hepatitis B cirrhosis[22]. Adefovir is an option, but this drug has less potency against HBV than other agents[23]. Interferon α-based therapies might be considered, but are contraindicated in patients with decompensated cirrhosis. Entecavir was initially thought to have no significant anti-HIV activity, but has subsequently been shown to be associated with the development of HIV resistance mutations when used in the absence of other antiretrovirals[24]. One report suggests that telbivudine may also have activity against HIV[25]. Overall, we believe that the effort to help the patient survive an episode of ALF overrides the preservation of one particular antiretroviral class for future use and that at least one agent with anti-HBV activity should be given in such a scenario. In general, it appears that patients who develop liver failure on HAART, whether coinfected with HBV or not, have a very poor prognosis. These patients should be considered for liver transplantation evaluation, as emerging data show that HIV-HBV coinfected patients have excellent outcomes with liver transplantation[26].

It is prudent to closely monitor the clinical status and liver enzymes of HIV-HBV infected patients after initiation of HAART in order to identify cases of hepatotoxicity early on. Additionally, two new antiretroviral classes have been introduced in recent years. These include the integrase inhibitor class currently represented by raltegravir and the CCR5 antagonist class currently represented by maraviroc. Based on published reports to date, these new agents appear to have minimal hepatotoxicity[27]. While they have only been available since 2007 and toxicities have yet to be fully described, these agents hold promise for HIV-infected patients with chronic liver diseases such as HBV. Perhaps most importantly, efforts should be made to prevent the acquisition of chronic HBV infection, and the HBV vaccine is recommended for all patients with HIV infection[28].

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CASE REPORT

Successful recanalization of acute superior mesenteric artery thrombotic occlusion with primary aspiration thrombectomy

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Abstract

Prompt revascularization of the superior mesenteric artery (SMA) thrombotic occlusion can prevent intestinal infarction and decrease necrosis of the bowel segment. Herein, we describe two cases who underwent successful endovascular recanalization for acute SMA thrombosis using a primary aspiration thrombectomy because of possible consequent laparotomy for survey of bowel viability. The two patients had dramatic pain relief immediately after the procedure and remained symptom-free during the follow-up period.

Key words: Superior mesenteric artery; Thrombosis; Aspiration thrombectomy

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INTRODUCTION

Acute mesenteric ischemia (AMI) is a fatal vascular emergency, representing 1%-2% of acute abdominal emergencies, and develops from a sudden decreased perfusion to the intestines caused by occlusive or non-occlusive impairment of arterial or venous blood flow. The reported overall mortality of AMI is 60%-80%, and its incidence is increasing[1-3]. Superior mesenteric artery (SMA) embolism is the most frequent cause of AMI, which is responsible for approximately 40%-50% of cases[4]. Among the diagnostic tools, conventional angiography shows a complete obstruction of the proximal SMA, 1-2 cm away from the origin of SMA without collateral circulation, which correlates with acute obstruction, and needs relevant treatment[5,6]. We report two cases who underwent successful endovascular thrombolysis of AMI with different methods, despite the presence of early changes in bowel ischemia.

CASE REPORT

Case 1

A 72-year-old man sought evaluation in the emergency room (ER) because of sudden aggravation of abdominal pain and rebound tenderness 3 d ago. He had a 2-year history of diffuse, dull postprandial abdominal pain. The attending physician suspected a surgical abdomen. The patient was on medication for hypertension during the last 16 years. Laboratory data showed no significant abnormalities.An initial abdominal computed tomography (CT) showed a segmental, occlusive acute thromboembolism in the mid-portion of the main stem, and jejunal and colic branches of SMA with circumferential bowel wall

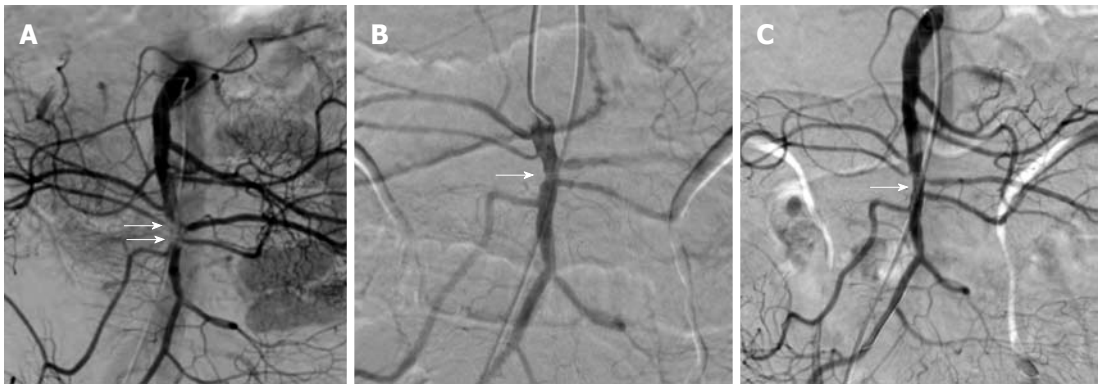

Figure 1 Successful treatment of focal thrombotic occlusion on mid-trunk of superior mesenteric artery with primary aspiration thrombectomy and additional stent implantation. A: Superior mesenteric artery (SMA) angiography showing acute thromboembolism in the mid-trunk of SMA and the origin of jejunal and colic branches (arrows); B: Completely resolved thrombi and focal severe stenosis in the mid-portion of SMA main stem which was considered the leading cause of thromboembolism (arrow) after repetitive primary aspiration thrombectomy using a 6F aspiration catheter; C: A markedly improved stenosis and luminal blood flow of SMA without residual or migrated thrombi after implantation of a 6 mm x 18 mm balloon expandable stent (arrow).

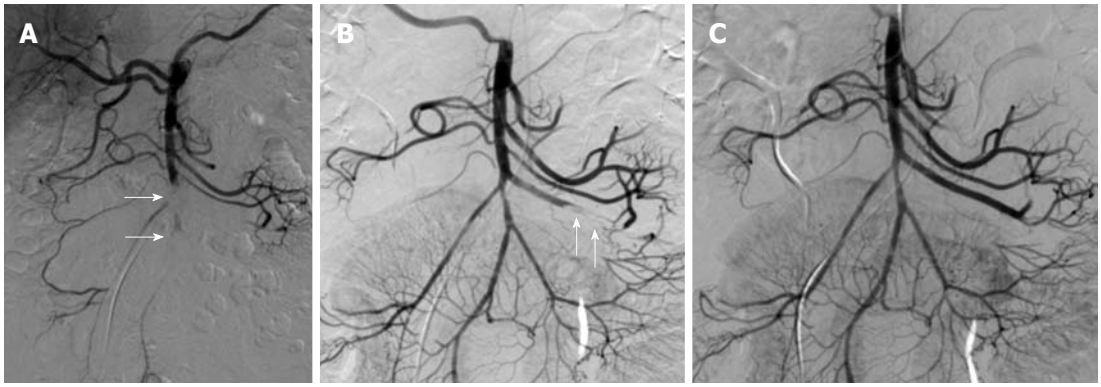

Figure 2 Successful treatment of segmental complete thrombotic occlusion on mid-trunk of superior mesenteric artery with primary aspiration thrombectomy. A: Superior mesenteric artery (SMA) arteriography showing a complete thrombotic occlusion of the mid-portion of the main stem and the origin of ileocolic and right colic branches of SMA due to a large amount of thromboembolism (arrows); B: Complete removal of thromboembolism in the main stem of SMA but an intraluminal filling defect and flow occlusion at the distal portion of jejunal branch of SMA due to migrated thrombi during the procedure (arrows) after multiple courses of aspiration thrombectomy with a 6F aspiration catheter; C: A completely recanalized main stem and jejunal branch of SMA.

thickening and decreased enhancement of mucosal layer in the right colon.

Initial SMA angiography confirmed an acute occlusive thromboembolism in the mid-trunk of SMA and the origin of jejunal and colic branches of SMA (arrows) (Figure 1A). Initially, we performed primary aspiration thrombectomy using a 6F aspiration catheter (Cordis, Johnson and Johnson, San Diego, CA, USA) after IV infusion of 5000 IU heparin. Angiography after repetitive thrombectomy showed nearly complete thrombi removal from the SMA main trunk and its branches and residual focal severe stenosis in the mid-portion of SMA main stem (arrow) (Figure 1B), which was considered the leading cause of thromboembolism. Final angiogram showed completely restored perfusion to the ischemic bowel through the patent main stem, and jejunal and colic branches of SMA after implantation of a 6 mm × 18 mm balloon expandable stent (arrow) (Genesis; Cordis, Johnson and Johnson, San Diego, CA, USA) (Figure 1C). The patient's symptoms improved almost immediately after the operation, and he no longer complained of postprandial abdominal pain. He was instructed to continue taking warfarin after discharge.

Case 2

A 67-year-old man complained of acute abdominal pain 2 d after admission. The patient's symptom was localized at the periumbilical area, and direct tenderness was present at physical examination. The patient had a history of atrial fibrillation and myocardial infarction, which was treated with coronary angioplasty 2 years ago.

Echocardiography showed a 30% ejection fraction and no evidence of thrombi in the left atrium. Laboratory data showed an elevated hs-CRP level of 14.22 mg/dL (normal range, 0-0.3 mg/dL).

Emergent CT angiography showed complete occlusion of the mid-portion of the main stem and origin of the ileocolic and right colic branches of SMA due to thromboembolism, with circumferential bowel wall thickening at the cecum and ascending colon. Surgical laparotomy was later considered because of heart failure.

The initial SMA arteriography showed complete thrombotic occlusion of the mid to distal SMA trunk and the origin of ileocolic and right colic branches of SMA (arrows) (Figure 2A). Initially, we performed primary aspiration thrombectomy using a 6F aspiration catheter (Cordis, John-

son and Johnson, San Diego, CA, USA) after Ⅳ infusion of 5000 IU heparin. After repetitive aspiration thrombectomy, follow-up angiography showed that although most thrombi were removed from the SMA main trunk, small thrombi had a migrated and occluded distal portion of the jejunal branch of SMA during aspiration thrombectomy (arrows) (Figure 2B). Finally, most thrombi in the main stem and jejunal branch of SMA were completely removed immediately after an additional aspiration thrombectomy, and the lumen of SMA was completely recanalized (Figure 2C). After the procedure, the patient's symptoms dramatically improved and the hs-CRP level decreased to 2.2 mg/dL, and he did not have recurrent symptoms during the 12-mo follow-up period.

DISCUSSION

AMI is caused by embolism (40%-50%), SMA thrombosis (20%-25%), non-occlusive mesenteric ischemia (20%), and mesenteric venous thrombosis (5%)[1-4]. Embolic or thrombotic occlusion of SMA frequently occurs on a background of generalized atherosclerotic changes in the involved arteries.

In our two cases, acute mesenteric thrombosis was managed with aspiration thrombectomy without any pharmacologic thrombolysis due to consequent laparoscopic survey to bowel.

Unfortunately, the non-specific symptoms are a frequent cause of delayed diagnosis, and most AMIs and CMIs can be asymptomatic for a long time. Contrast-enhanced CT can shows an arterial occlusion of SMA, frequently accompanied with severe stenosis of the celiac artery, and findings suggestive of bowel ischemia, including pneumatosis intestinalis, bowel wall thickening, ileus, and bowel dilatation[7]. Angiography can define the location and origin of the arterial occlusion and provide the potential for intervention if mesenteric ischemia is diagnosed prior to ischemic bowel necrosis[5,6]. Bloody diarrhea or signs of peritonitis, including abdominal rigidity and rebound tenderness, are signs of advanced bowel ischemia and bowel infarction, requiring urgent surgery. An area of transmural bowel necrosis in AMI can appear within 15 min after onset and after 6 h, and irreversible gangrene of the affected segments may ensue[8]. In our cases, the two patients were diagnosed with AMI by CT examination soon after the onset of symptoms. Although we performed endovascular treatment despite the presence of early ischemic bowel changes, such as bowel wall thickening and engorgement of the vasa recta, on initial or follow-up CT, we obtained good results as reflected by the dramatic improvement in symptoms.

Because of its favorable results, endovascular thrombolysis of acute SMA occlusion has gained popularity and has become one of the most used methods with or without combined open surgery[9]. Pharmacologic thrombolysis with or without mechanical aspiration thrombectomy is a good treatment option in terms of high survival, bowel preservation rates and low complication rate regardless of the nature of thromboembolism, period of occlusion, presence of collateral vessels and severe atherosclerotic disease.

The effect of primary aspiration thrombectomy for acute SMA thrombosis without pharmacologic thrombolysis remains a matter of debate and requires better definition. If consequent laparotomy is considered for assessment of bowel viability and use of anticoagulant is restricted due to a bleeding tendency, aspiration thrombectomy can be performed as the primary procedure. Despite contraindication or strict use of anticoagulant, a small amount of local anticoagulant use is mandatory for prevention of distal migration of small thrombi or recurrent thrombus formation.

Treatment of the underlying stenosis with angioplasty and stent implantation after intra-arterial thrombolysis has been described with at least good short-term results, but the higher restenosis rate of stents during follow-up has been reported in the recent literature[10]. We had a good short-term outcome, despite early ischemic changes, and continued long-term follow-up for recurring symptoms and restenosis of stents with CT angiography.

In conclusion, if the diagnosis of AMI is made early and there are no signs of advanced bowel ischemia, such as peritonitis, endovascular treatment for AMI. Primary aspiration thrombectomy can be performed for rapid and sufficient revascularization of acute thrombotic SMA occlusion in limited cases, consequent laparotomy must be planned to survey for bowel viability or if there is a contraindication to use an anticoagulant.

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January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
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The 7th Biannual International Symposium of Society of Coloproctology

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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

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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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 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/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

Statistical expression

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