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Dependence of fluorodeoxyuridine-induced cytotoxicity and megabase DNA fragment formation on S phase progression in HT29 cells

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Abstract The relationship between cell cycle progression and induction of DNA double-strand breaks and cytotoxicity by exposure to fluorodeoxyuridine (FdUrd) was studied in HT29 human colon cancer cells. Fractionation of drug-treated populations by centrifugal elutriation yielded subpopulations having widely divergent abilities to progress through S phase in the presence of the drug. One of these subpopulations, which appeared to undergo coordinated growth arrest, was resistant to FdUrd cytotoxicity and DNA damage. In contrast, the subpopulation which was able to progress furthest through S phase in the presence of FdUrd underwent unbalanced growth arrest (i.e., increase in size and mass out of proportion to DNA synthesis), and displayed both DNA double-strand break formation (assayed by pulsed field gel electrophoresis) and loss of clonogenicity. When cells were elutriated prior to drug treatment, producing fractions enriched in cells at various cell cycle stages, no significant differences in sensitivity to FdUrd-induced cytotoxicity were detected among elutriation fractions. These findings support the model that, in HT29 cells, progression into and through S phase during drug treatment is an important determinant of FdUrd-induced DNA damage and cytotoxicity, but that the cell cycle position at the start of drug exposure is not a critical factor for these effects.

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Key words Fluorodeoxyuridine · Double-strand break · Cell cycle

Abbreviations *DSB* double-strand break · $F_{released}$ fraction radioactivity released · *FdUrd* fluorodeoxyuridine · *HBSS* Hank's balanced salt solution · *PFGE* pulse field gel electrophoresis · *PBS* phosphate-buffered saline · *SF* surviving fraction · *SSB* single-strand break · *TS* thymidylate synthase

Introduction

Evidence from our [4] and other [7, 13] laboratories supports the hypothesis that, in some cell lines, FdUrd-induced DNA damage and cytotoxicity results from a "futile repair pathway" of uracil misincorporation and misrepair. According to this model, thymidylate synthase inhibition produced by exposure to FdUrd causes an expansion of dUTP pools and excessive incorporation of uracil into DNA. This leads to repeated (futile) cycles of uracil excision and reincorporation. While this model provides a framework for understanding the production of SSBs following FdUrd treatment, it does not specify how SSBs produce DSBs, which appear to be the crucial lesion resulting in cell death.

One mechanism for transformation of SSBs into DSBs is suggested by observations that FdUrd is much more toxic to cells in exponential growth than to plateau-phase cells [3]. This dependence on proliferation is consistent with the hypothesis that, whereas faulty *repair* DNA synthesis causes SSBs, the conversion of SSBs into DSBs may depend upon *replicative* DNA synthesis. More specifically, it is possible that DSBs could be the result of replication fork movement through a region of the genome containing SSBs arising from uracil misincorporation/excision.

In several studies using the HT29 human colon cancer cell line, we have observed a significant degree of

heterogeneity with regard to the ability of cells to progress through S phase in the presence of a highly growth inhibitory concentration of FdUrd. If it is correct that S phase progression is a necessary step in the production of DSBs in FdUrd-treated cells, then it would be expected that cells progressing furthest through S phase in the presence of the drug would show the greatest amount of DNA fragmentation and cytotoxicity. In the present study we have tested this prediction by using centrifugal elutriation to fractionate FdUrd-treated HT29 cells into subpopulations having markedly differing capacities to progress through S phase, and then assaying these subpopulations for DNA DSB formation and for loss of clonogenicity. In accordance with the proposed model, we found that DNA damage and cytotoxicity were correlated with the extent of S phase progression of the subpopulations.

Materials and methods

Cell culture and drug treatment

Human colon carcinoma cells (HT29) were cultured in McCoy's 5a media (Gibco) supplemented with 10% fetal bovine serum, at 37°C, in a 5% CO_2 atmosphere. Cells were tested for mycoplasma every 3 months. FdUrd (Sigma) stocks were made in distilled water and frozen at −20°C; concentrations were verified by UV spectrophotometry. All FdUrd treatments were performed with medium containing 10% dialyzed fetal bovine serum. Media was changed every 24 h for incubations greater than 24 h. A standard clonogenic assay was used to measure cytotoxicity, as described previously [4]. All data were corrected for plating efficiency of untreated asynchronous or synchronized cells, which ranged from 0.60 to 0.85.

Fractionation of cells by centrifugal elutriation

The elutriation system consisted of a Beckman J-B centrifuge and a JE-5.0 rotor with a 5-ml Sanderson separation chamber. Partially assembled components were sterilized by autoclaving or gas sterilization and assembled in a sterile hood. Elutriations were performed at a fixed rotor speed of 2000 rpm at room temperature. Suspensions containing $8–10 \times 10^7$ cells were loaded into the separation chamber at a flow rate of 12 ml/min using a peristaltic pump. Culture medium with 2.5% calf serum was used during loading to maintain high cell viability and minimize mechanical shearing forces. Elutriation fractions were collected at flow rates between 25 and 40 ml/min. To decrease carryover, 50 ml of medium was discarded between fractions. The cells obtained from each fraction were counted and their median cell diameters were assessed with an electronic particle counter (model C1000, Coulter Electronics). For experiments in which elutriation preceded drug treatment, cells were trypsinized and kept suspended in a spinner flask for 16 h prior to elutriation to eliminate the lag phase caused by the trypsinization process. Unsynchronized cell populations examined after this 16-h recovery period were found to have drug responses indistinguishable from exponentially growing monolayer cells (data not shown). Flow cytometry was performed after each elutriation to ensure consistent separations.

Flow cytometry

Cells from each fraction were washed twice with HBSS (Gibco), fixed by dropwise addition of cold 70% ethanol, and resuspended at 1×10^6 cells/ml. Fixed cells were stored at 4°C for up to 2 months before analysis. For staining, cells were washed with HBSS and suspended in 1 ml HBSS containing 16.7 μg/ml of propidium and 40 μg/ml of ribonuclease A for at least 2 h at 4°C. Analyses were performed using a Coulter Epics C flow cytometer. Cell cycle phase distribution was estimated with CytoLogic software, based on a multiple broadened rectangular S-phase model [1].

Analysis of DNA fragmentation by PFGE

Labeling of cellular DNA for quantification of DNA fragmentation was performed by incubating cells with [2-^{14}C]thymidine (0.15 μCi/ml; sp. act. 56 mCi/mmol; Moravek Biochemicals) for 1 day, followed by 8 h chase with fresh medium. Elutriated cells were washed and resuspended in HBSS at a concentration of 2×10^7 cells/ml. Cell blocks were made as described previously [4]. Samples were electrophoresed in a 0.7% agarose gel using a CHEF DR-II apparatus (BioRad) with a 45-min constant switching interval for 45 h at 67 V, in 0.5 × TBE (45 mM TRIS-borate, pH 8.0, 1 mM EDTA) at 14°C. Following electrophoresis, gels were stained with ethidium bromide and photographed. For quantification of DNA fragmentation, each lane was divided into 7- to 8-mm slices which were then melted in 0.1 N HCl and analyzed by scintillation counting. $F_{released}$ was determined as the fraction of cpm which migrated into the lane from its corresponding well.

Results

Centrifugal elutriation and cell cycle analysis

To determine the range of pump speeds to use for fractionating drug-treated HT29 cells, we first established conditions for fractionating unperturbed, exponentially growing HT29 cells. By using pump speeds of 24, 28, 32, and 36 ml/min we were able to obtain subpopulations having mean diameters of 13.9 ± 0.06, 14.5 ± 0.07, 15.3 ± 0.15, and 16.1 ± 0.08 μm, respectively. As shown in Fig. 1, these fractions corresponded to G1, G1/S, S and G2/M subpopulations.

Treatment of exponentially growing HT29 cells with 100 nM FdUrd for 20 h resulted in a population of cells chiefly in the first one-fifth of S phase, as judged by comparison with untreated cells (Fig. 2A). For this reason we initially did not expect to be able to obtain a significant degree of separation by elutriation. Nevertheless, when the same conditions used to fractionate the control cells were applied to the drug-treated population, we were able to reproducibly separate these cells into four fractions, each of which gave a single, sharp, symmetrical peak when analyzed by flow cytometry (Fig. 2B). The use of internal standard particles permitted us to compare the relative DNA contents of the fractions, confirming that there were consistent differences among these groups. Three of the four drug-treated fractions had a peak channel, demonstrating progression into S phase. Based on the positions of the

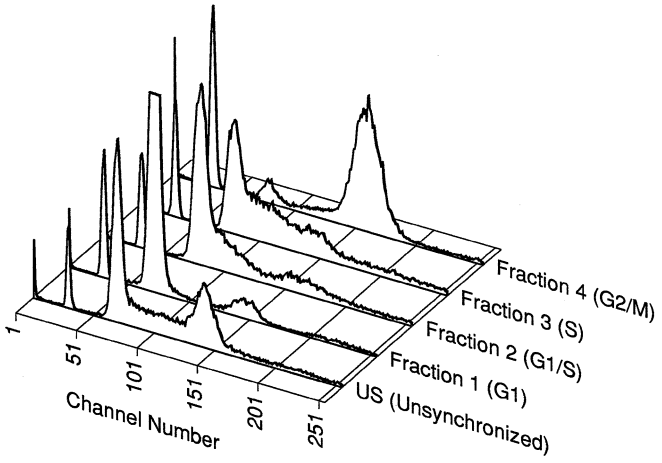

Fig. 1 Single-parameter flow cytograms of elutriated cells. HT29 cells were separated by centrifugal elutriation and stained for DNA content with propidium iodide/RNase. Unsynchronized (*US*) cells and fractions enriched in G1, G1/S, S and G2/M cells are shown. The first peak from the left origin contains trout cells as internal standard particles

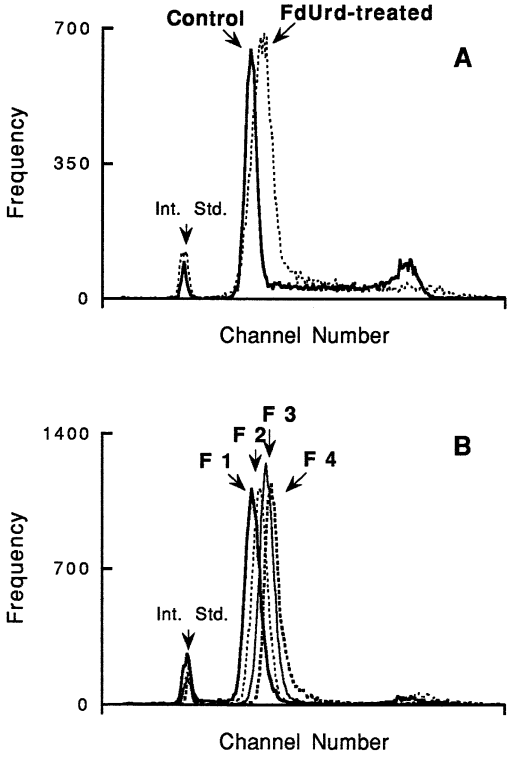

Fig. 2A, B Separation of fluorodeoxyuridine (*FdUrd*)-treated HT29 cells by centrifugal elutriation. **A** Cells were exposed to control medium (*solid line*) or medium containing 100 nM FdUrd (*dashed line*) for 20 h and processed for flow cytometry. **B** After drug treatment as in **A**, four subpopulations (F1–F4) of FdUrd-treated cells were separated by centrifugal elutriation. The peak labeled *Int. Std.* contains normal human lymphocytes as internal standards

G1 and G2 peaks in control cells, the mean extent of progression through S phase in elutriated fractions 1–4 was $0 \pm 2\%$ (i.e., identical to G1 peak in control cells), $3 \pm 1\%$, $6 \pm 1\%$, and $11 \pm 1\%$, respectively.

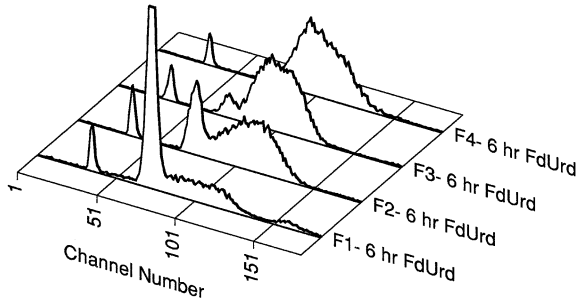

Fig. 3 Cell cycle progression of FdUrd-treated HT29 cells. Four subpopulations (F1–F4) of FdUrd-treated HT29 cells, obtained as shown in Fig. 2B, were incubated with the medium containing 100 nM FdUrd for an additional 6 h and processed for flow cytometry

Before assessing these fractions for FdUrd sensitivity, we wished to evaluate the basis for the heterogeneity of DNA content. One possibility was that replicative DNA synthesis is halted uniformly in all S phase cells within a short time after exposure to FdUrd. In this hypothesis, the observed distribution represents immediately arrested S phase cells plus G2, M and G1 phase cells that arrested shortly upon entering S phase during the period of drug exposure. Alternatively, the heterogeneity in DNA content could be a reflection of a mixture of cell populations which are strongly inhibited from progressing through S phase (and therefore accumulate very early in S phase) with others that are able to move through S phase in the presence of drug. To distinguish between these possibilities, the four fractions shown in Fig. 2B were returned to FdUrd-containing medium after elutriation and incubated for an additional 6 h. If the first explanation was correct, there should be little (if any) movement through S phase in any of the four fractions during the 6-h period. Instead, we found that, whereas cells in fraction 1 progressed little during the 6-h incubation, a substantial portion of cells in each of the other fractions was able to advance through S phase in the presence of FdUrd, and that the percentage of cells able to progress became greater in successive elutriation fractions (Fig. 3).

Relationship between cell cycle progression and FdUrd-induced DNA damage and cytotoxicity

PFGE analysis of DNA damage in the four subpopulations of FdUrd-treated cells revealed that the formation of megabase DNA fragments increased progressively from fraction 1 to fraction 4 (Fig. 4). Correspondingly, the surviving fraction of cells in these four fractions progressively decreased (Fig. 4).

Because the cell populations in the preceding studies were unsynchronized at the start of drug treatment, we considered the possibility that differences in drug

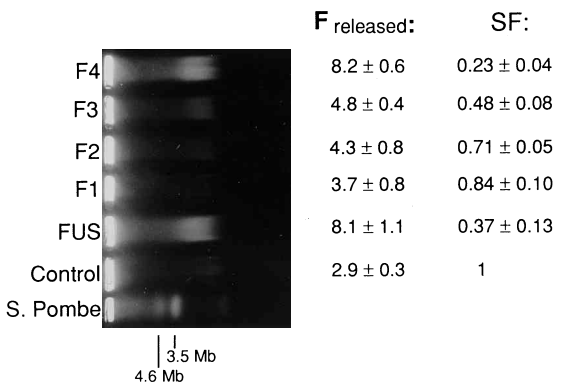

Fig. 4 DNA damage and cytotoxicity in subpopulations of FdUrd-treated HT29 cells. Cells were treated with 100 nM FdUrd for 20 h (*FUS*) and then elutriated to produce the four fractions shown in Fig. 2B. Samples were electrophoresed and prepared for determining DNA fragmentation and cytotoxicity. The results presented here are the mean ± standard error from three independent experiments

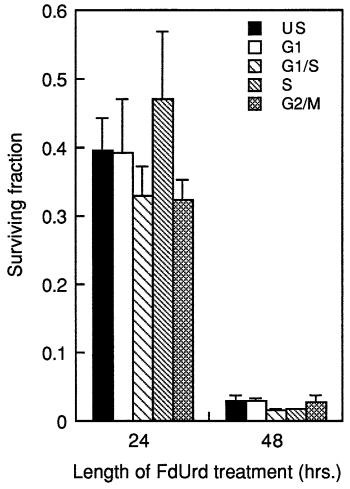

Fig. 5 Relationship of FdUrd-induced cytotoxicity to cell cycle position at the start of drug treatment. Exponentially growing HT29 populations were elutriated to produce fractions enriched in cells at various stages of the cell cycle as in Fig. 1 and then treated with 100 nM FdUrd for 24 or 48 h, after which clonogenic survival was measured. Cytotoxicity to unsynchronized, exponentially growing cells was also determined (*US*). Data are the mean ± standard error with $n \geqslant 3$ for all groups except G2/M, where $n = 2$. No significant differences among the groups were detected at the 95% confidence level, using analysis of variance

response might be related to cell cycle position at the beginning of the drug exposure. We therefore elutriated HT29 cells to produce populations similar to those shown in Fig. 1 and subsequently treated those cells with 100 nM FdUrd. We found that there was no significant difference in sensitivity to FdUrd-induced cytotoxicity among the elutriation fractions enriched in cells at various stages of the cell cycle, nor was there a difference between any of these fractionated cells and unelutriated cells (Fig. 5).

Discussion

We demonstrate here that elutriation of HT29 cells that have been exposed for one doubling time to a high concentration of FdUrd (i.e., > tenfold that required to reduce TS activity below detectable levels [4]) produces subpopulations which are significantly different from each other in several respects. At one extreme is the first elutriation fraction (F1), which has a DNA content indistinguishable from that of G1 cells in an unperturbed population. These cells appear to undergo a coordinated growth arrest, in that their size does not increase out of proportion to their DNA content. In contrast, cells in the last elutriation fraction (F4) have diameters as large as G2/M cells, even though their average increase in DNA content represents progression through less than 20% of S phase. F4 cells also exhibit significantly greater double-stranded DNA damage (as indicated by the appearance of large fragments on a neutral pulsed-field gel) and cytotoxicity, compared to F1 cells, consistent with the model that replication fork movement is necessary to convert FdUrd-induced SSBs into DSBs.

Although it has been shown consistently that fluoropyrimidine cytotoxicity is much greater in proliferating cells than in plateau-phase cells (e.g., [3]), it is less clear if the actions of these drugs are dependent upon the phase of the cell cycle in which drug treatment is begun. Because TS inhibition (and subsequent DNA synthesis inhibition) is such a prominent feature of fluoropyrimidine activity, it is tempting to speculate that cells should be more sensitive to these drugs during S phase. However, whereas some data support this notion [2], other data do not [11]. We have shown previously that the difference in sensitivity to FdUrd of HT29 cells and another human colorectal tumor cell line, SW620, correlates with damage on the parental DNA strand (through uracil misincorporation/misrepair), rather than with inhibition of nascent DNA synthesis [5, 6]. Also, DNA synthesis inhibition caused by aphidicolin is relatively non-toxic in HT29 cells [4]. These data are consistent with the lack of S phase specificity observed in the present study (Fig. 5), and support the model that, in HT29 cells, FdUrd acts as a cycle-specific drug, rather than as an S phase-specific drug.

One factor which complicates interpretation of the literature concerning phase specificity of fluoropyrimidine actions is that studies using fluorouracil may detect either RNA- or DNA-directed toxicity (or both), and it is quite possible that these two types of actions may have different cell cycle phase dependencies. Also, in view of our data showing that there are substantial variations among human tumor cells in the type of DNA damage caused by FdUrd [4], phase specificity

may differ from one cell line to another, depending on whether the process causing the damage is a specific consequence of TS inhibition or a more general response to cell cycle disruption (i.e., a programmed cell death response). In our hands, HT29 cells show neither the morphological characteristics of apoptosis nor the formation of oligonucleosomal ladders during the initial 48-h of treatment with FdUrd [4], indicating that their mode of cell death is primarily non-apoptotic under these conditions. It has been reported recently that oligonucleosomal fragments can be detected in hypoxic HT29 cells, and that a substantial fraction of such cells stain positively in the terminal transferase assay for apoptosis, suggesting that in some cases HT29 cells may die by apoptosis [16]. It should be noted, however, that large DNA fragments can also produce positive staining in the terminal transferase assay, and that this assay may not distinguish between classical apoptosis and cell death involving DNA damage other than nucleosomal laddering. It is therefore still not clear if apoptosis is a major mode of cell death in HT29 cells.

The correlation between the extent of S phase progression and cytotoxicity demonstrated here is consistent with recent findings by Houghton et al. [9], who examined the relationship between cell cycle progression and cytotoxicity due to thymidylate deprivation, using mutant human tumor cell lines lacking TS. Their data suggest that the cytotoxicity of dTTP deprivation may depend upon the integrity of a G1/S checkpoint, in analogy to the ability of wild-type p53 protein to protect cells from radiation-induced DNA damage [10]. HT29 cells have been shown to be homozygous mutant for p53 [12], so it is unlikely that p53 itself is the basis for differences among members of the populations examined here. However, it is possible that the heterogeneity of response to FdUrd seen in the present study could be due to variation among members of the parental HT29 population with regard to factors regulating arrest at the G1/S border that are downstream of p53, such as waf1/cip1 or Rb protein [8]. The existence of stable subpopulations in other human tumor cell lines has been demonstrated previously [14], and cytogenetic data indicate that the parental HT29 population used in the present study does contain a significant cohort of cells which have karyotypic markers that differ from the majority of the population [15]. We are therefore pursuing further studies to determine if there are stable HT29 subpopulations which could account for the heterogeneous response pattern reported here.

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