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ABERRANT CELL CYCLE INHIBITION PATTERN IN HUMAN COLON CARCINOMA CELL LINES AFTER EXPOSURE TO 5-FLUOROURACIL

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Abstract—In this report, we describe the use of two human colon carcinoma cell lines, HCT-8 and HT-29, as potential models to study DNA- and RNA-directed cytotoxicity due to 5-fluorouracil (FUra) exposure by flow microfluorimetric analysis of DNA cell content. The sensitivity of the HT-29 line (EC_{50} = 0.9 μM) to FUra was somewhat greater than that of the HCT-8 line (EC_{50} = 4 μM), but each presented a dramatically different DNA histogram after exposure to FUra. In HCT-8, an unexpected and nearly complete disappearance of cells in S-phase occurred, whereas in HT-29 the expected accumulation of cells at the G_1-S border was observed. The absence of HCT-8 cells in S-phase also occurred as a result of two RNA polymerase inhibitors: actinomycin D and dichloro-D-ribofuranosylbenzimidazole. However, an accumulation of cells in S-phase was observed in the presence of 5-fluorodeoxyuridine. These results suggest that in the HCT-8 cell line, FUra predominantly causes an RNA-related toxicity. By comparison, the rate of formation of 5-fluorodeoxyuridine monophosphate, the increased dUMP pool size, and low thymidylate synthase activity in the HT-29 line are consistent with its greater susceptibility to DNA-directed toxicity. Further evidence was seen in the prevention of FUra cytotoxicity by thymidine in HT-29, but not in HCT-8 cells. Similarly, Leucovorin synergized the action of FUra in HT-29 but not in HCT-8. Enzymatic correlates supporting these observations are seen in the greater activity of uridine kinase than thymidine kinase (20:1) in HCT-8 cells compared with that in HT-29 cells (4:1).

Key words: 5-fluorouracil; cell lines; colon carcinoma; cell cycle; flow cytometry; RNA polymerase

Two major mechanisms are responsible for the cytotoxic activity of FUra†. One mechanism, DNA-directed toxicity, is believed to involve the formation of FdUMP, which in the presence of *N*-5,10-methylenetetrahydrofolate inhibits thymidylate synthase [1–3], or to be consequent to the misincorporation [4, 5] and removal of either FUra or uracil deoxynucleotides from DNA [6, 7]. The other mechanism involves the synthesis of FUra ribonucleotides and their incorporation into various RNA species with consequent effects on their function [8, 9]. The relative ability of thymidine [10] to rescue tumor cell lines *in vitro* from FUra cytotoxicity has provided a means to discern between the different mechanisms, since it should overcome DNA-directed effects with little consequence on RNA.

If it were possible to determine which of these mechanisms predominates in a specific human tumor or class of tumors, modulation therapy could be more rationally employed. In this study, we present data on two human colon tumors *in vitro*, which represent prototypic examples of each mechanism.

In addition to flow cytometric studies, biochemical and enzymatic differences in the activation of FUra are reported and correlated with the response to the effect of biochemical modulators, such as thymidine and Leucovorin.

MATERIALS AND METHODS

FUra, FdUrd, FUrd, uridine, thymidine, Leucovorin, actinomycin D and all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). DRB was a gift from Dr. A. C. Sartorelli. [^{14}C]FUra (55 mCi/mmol), [^{14}C]FUrd (56 mCi/mmol), [^{14}C]FdUrd (56 mCi/mmol), and [^{3}H]-FUra (26 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). Medium, sera and antibiotics for tissue culture were purchased from the Grand Island Biological Co. (Grand Island, NY).

Two human colon carcinoma cell lines, HCT-8 and HT-29, were obtained from the American Type Culture Collection (Rockville, MD) and grown as monolayers. Lines were tested every 3 months and shown to be negative for mycoplasma contamination using a GeneProbe kit (San Diego, CA). Cultures were maintained in RPMI 1640 supplemented with 10% horse serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37° in a 5% CO_2

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† Abbreviations: FUra, 5-fluorouracil; FUrd, 5-fluorouridine; FdUrd, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridine monophosphate; FCM, flow cytometry; DRB, dichloro-D-ribofuranosylbenzimidazole; TCA, trichloroacetic acid; PCA, perchloric acid; and OPRTase, orotate phosphoribosyltransferase.

atmosphere. Under these conditions, the doubling time was 22–24 hr for HCT-8 and 31–34 hr for HT-29.

To measure growth inhibition, monolayers of cells in 25 cm^2 flasks were exposed continuously to various concentrations of FUra. After 72 hr, the medium was removed, and the cell layer was washed twice with PBS and trypsinized. Appropriate dilutions of the resultant cell suspension were counted using a ZB Coulter Counter (Coulter Electronics, Hialeah, FL).

Cytofluorographic analysis was performed on cultures exposed to FUra, washed with PBS, trypsinized, fixed in 70% ethanol, treated with RNase type I-A, and stained with propidium iodide [11]. The fluorescence emission of the stained cells was measured on a FACS IV flow cytometer and sorter (Becton Dickinson, Sunnyvale, CA) interfaced to a Digital PDP 11/73 computer. The laser illumination (Spectra Physics, Mountain View, CA) was at 488 nm (360 mW). Red fluorescence was observed after a 590 nm long pass filter (Ditric Optics, Marlboro, MA). A minimum of 30,000 cells was analyzed for each sample.

Cell extracts were obtained after sonication in 50 mM Tris–HCl, pH 7.4, 3 mM sodium fluoride and 4 μM dithiothreitol, and centrifugation at 100,000 g for 60 min. Uridine and thymidine phosphorylase activities were determined by incubating 10–50 μL of cell extract with 2 mM inorganic phosphate, 20 μM of appropriate ^{14}C substrate, 2 mM ATP and 2 mM $MgCl_2$ in a final volume of 100 μL. Uridine and thymidine kinase activities were measured by incubating the cell extract (5–20 μL) with 2 mM ATP, 2 mM $MgCl_2$ and 20 μM [^{14}C]FUrd or [^{14}C]-FdUrd in a final volume of 100 μL. OPRTase activity was assayed with 200 μM phosphoribosyl phosphate, 2 mM $MgCl_2$ and 20 μM [^{14}C]FUra. Reactions were initiated by the addition of cell extract and incubated at 37° for 30 min; then 10 μL was removed and applied to TLC plates and immediately dried at 80° to stop the reaction. The plates were developed in chloroform : methanol : acetic acid (85 : 15 : 5) [12]. Radioactivity on the plates was determined in 0.5 mL of methanol and scintillation fluid using a Beckman liquid scintillation counter (LS 7000).

Thymidylate synthase activity was determined by the method of Roberts [13]. Briefly, 110 μM [^3H]deoxyuridylate, 1 mM (±)-L-tetrahydrofolate, 0.9 mM formaldehyde, 62.5 mM 2-mercaptoethanol, 50 mM NaF, 130 mM phosphate buffer, pH 7.5, and extract (5–10 μL) were incubated for 60 min at 37° in a final volume of 40 μL. The reaction was stopped by adding 200 μL of a 10% activated charcoal suspension (Norit) in 4% TCA. After centrifugation, 100 μL of supernatant was counted for radioactivity.

Protein concentration was estimated using the method of Bradford [14]. Activity in all enzyme assays is expressed as nanomoles of product formed per milligram of protein per hour.

To assess [^3H]FUra incorporation, cells ($4–6 \times 10^6$) in 150 cm^2 flasks were treated with 10^{-5} M [6-^3H]FUra (1 μCi/mL). At 4, 8 and 24 hr, cells were washed twice with PBS at 0°, trypsinized, collected by centrifugation (1000 g for 5 min) and extracted with 500 μL of 5% PCA at 0°. After centrifugation (2000 g for 5 min), radioactivity in the supernatant was determined directly or after HPLC separation of FUra. The pellet was washed twice with 5% PCA, and radioactivity was determined after digestion with NCS tissue solubilizer (Amersham, Arlington Heights, IL). [6-^3H]Uridine (1.5 μCi/mL) incorporation into cultured cells was also determined after a 1-hr incubation by the above methods.

FUra and uridine metabolites were determined in PCA cell extracts neutralized with KOH, absorbed on a 250 × 4.6 mm i.d. Spherisorb SAX 5 μm column at 20° and eluted with a gradient of sodium phosphate, pH 3.3, from 0.02 to 0.30 M for 40 min at 0.7 mL/min. The effluent was collected in 0.7-mL fractions, and 10 mL of scintillation fluid was added to assay radioactivity.

FdUMP concentrations were assayed in the PCA cell extract after treatment with sodium, periodate and methylamine to eliminate ribonucleotides [15]. The sample was analyzed on a BAX-4 polystyrene anion-exchange column at 50°, eluted with a gradient from 0.4 to 0.6 M ammonium acetate, pH 7.0, at 1.0 mL/min [16]. Samples (1 mL) were collected, and radioactivity was determined after the addition of scintillation liquid.

Intracellular dUMP concentrations were determined in cells exposed to 10^{-5} M FUra and processed by extraction with 1 M acetic acid [17]. The extract was lyophilized to remove acetic acid and resuspended in 50 mM Tris, 10 mM 2-mercaptoethanol and 1 mM sodium EDTA, pH 7.4. dUMP in the extracts was assayed with *Lactobacillus casei* thymidylate synthase (Biopure, Inc., Boston, MA) by measuring the generation of dihydrofolate from *N*-5,10 methylenetetrahydrofolate at 340 nm in an LKB Ultraspec 4050 spectrophotometer.

RESULTS

The human adenocarcinoma cell lines HCT-8 and HT-29 have similar sensitivities to the fluoropyrimidine antimetabolite FUra. HT-29 was more sensitive with an EC_{50} of 0.9 μM, whereas the HCT-8 cell line had an EC_{50} of 4 μM. Determination of the effect of FUra on the cell cycle distribution using an FCM analyzer, however, revealed a unique response in the human HCT-8 line compared with the HT-29 line (Fig. 1A), as well as the T-lymphoblastic leukemia CCRF-CEM. Unlike the other cell lines, a 24-hr exposure to FUra did not cause an accumulation of HCT-8 cells in S-phase. In fact, there was a concentration-dependent ablation of cells in S-phase and an accumulation in the G_1- and G_2 + M-phases. Thymidine up to 1×10^{-4} M could not modify this unusual pattern; however, the presence of 100 μM uridine was able to overcome this effect (data not shown).

In the HT-29 cell line, we observed the typical accumulation in S-phase consequent to an inhibition of DNA synthesis (Fig. 1B), an effect overcome by the addition of thymidine. FUrd (0.1 μM) generated the same disappearance of HCT-8 cells in S-phase (Fig. 2A) compared with the accumulation at the G_1-S border for the HT-29 cell line. When exposed to FdUrd (0.05 μM), however, HCT-8 cells displayed the expected pattern of DNA toxicity with

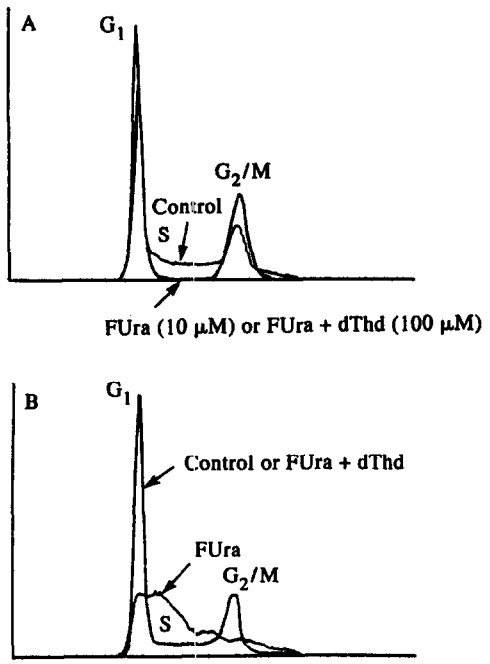


Fig. 1. DNA histograms of HCT-8 (A) and HT-29 (B) cells after a 24-hr exposure to FUra in the presence and absence of dThd. Cells were fixed, stained as described in Materials and Methods, and analyzed by flow cytometry.

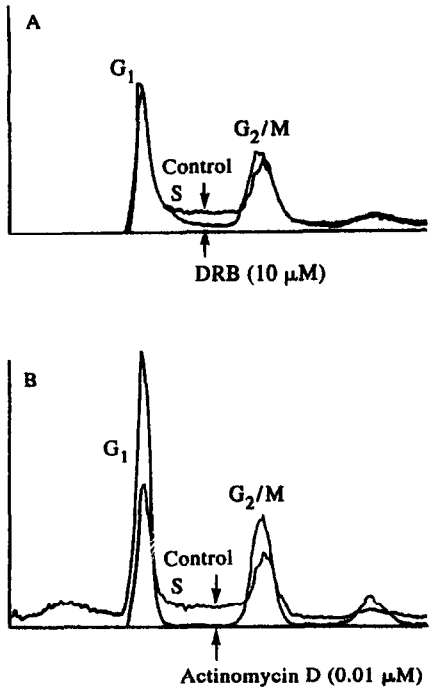
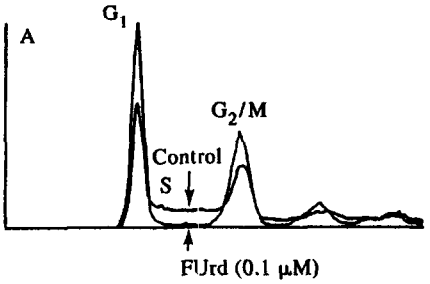


Fig. 3. DNA histograms of HCT-8 cells after incubation for 24 hr in the presence of the RNA polymerases inhibitors DRB (panel A) and actinomycin D (panel B).



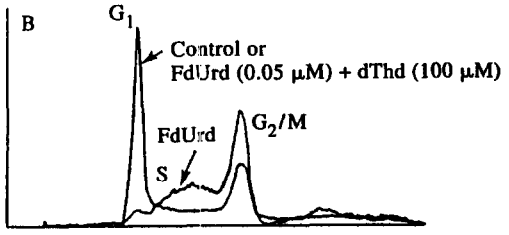


Fig. 2. Effect of a 24-hr exposure to FUrd (A) and FdUrd with or without dTHd (B) on FCM profiles of HCT-8 cells.

Table 1. Activities of enzymes involved in pyrimidine metabolism

	Activity (nmol/hr/mg protein)	
	HCT-8	HT-29
Uridine kinase	89.0 ± 7.7	69.6 ± 8.2
Thymidine kinase	4.7 ± 0.6	17.2 ± 1.8
Uridine phosphorylase	8.8 ± 0.3	24.4 ± 3.0
Thymidine phosphorylase	7.0 ± 2.0	15.0 ± 0.1
OPRTase	2.9 ± 0.4	3.0 ± 0.2
Thymidylate synthase	32.8 ± 2.5	6.0 ± 0.6

Reaction conditions are described in Materials and Methods. Values are means ± SD, N = 3–5.

accumulation of cells in S-phase (Fig. 2B). In this case, thymidine prevented the drug effect on cell cycle distribution.

These cell cycle analyses suggested that FUra toxicity in the HCT-8 cell line was directed more specifically toward an RNA target. To mimic this putative site of inhibition, drugs that primarily inhibit RNA synthesis and function were used [18, 19]. Actinomycin D, a RNA-polymerase I inhibitor, and DRB, a polymerase II inhibitor, caused the same clearance of cells from the S-phase of the cell cycle (Fig. 3).

We examined the activity of enzymes responsible

Table 2. Effect on dUMP pool after exposure to FUra in HCT-8 and HT-29 cells

	dUMP (nmol/10^9 cells)	
	HCT-8	HT-29
Control, 4 hr	5.6	5.3
FUra, 4 hr	5.9	7.4
Control, 8 hr	5.8	5.6
FUra, 8 hr	5.9	16.6

Cells were exposed to 10 μM FUra and then harvested. dUMP was determined on acetic acid extracts, as described in Materials and Methods.

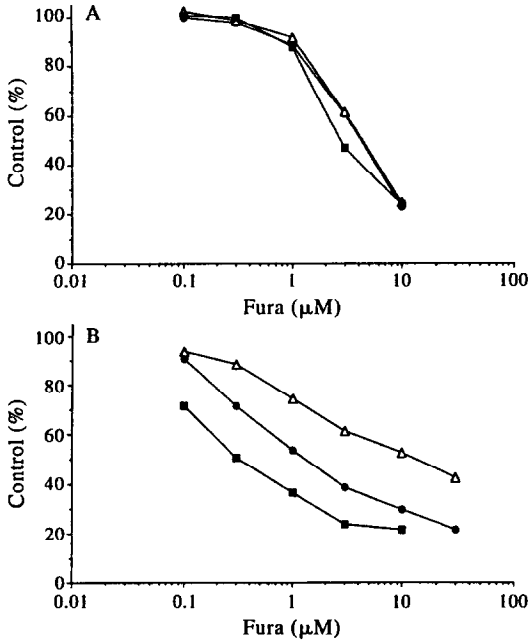


Fig. 4. Effect of FUra on cell growth of HCT-8 (A) and HT-29 (B) in the absence (●) and in the presence of dThd (△) or Leucovorin (■). Monolayers of cells in 25 cm^2 flasks (2–4 × 10^5 cells/flask) were exposed to various concentrations of FUra in the absence or presence of thymidine (50 μM) or Leucovorin (10 μM) for a 72-hr period. The inhibitory effect was measured as described in Materials and Methods.

for FUra metabolism as a possible basis for differences between the two colon cell lines (Table 1). No difference in OPRTase activity was observed between the two cell lines, but both thymidine phosphorylase and uridine phosphorylase activities were 2- to 3-fold greater in the HT-29 cell line compared with the HCT-8 cell line. Thymidine kinase activity was 4-fold higher in HT-29. The ratio of uridine kinase to thymidine kinase activity was 4:1 for the HT-29 cell line versus 20:1 for the HCT-8 cell line. A 5-fold greater activity of thymidylate synthase was observed in HCT-8 than in HT-29 cells.

When the two cell lines were exposed to 10 μM [^{3}H]FUra, the concentration used for the cytofluorometric analysis, the total intracellular accumulation after 24 hr was twice as great in HT-29 as it was in HCT-8 cells. The difference in FdUMP formation after a 24-hr exposure to the same concentration of FUra was even more dramatic: 1.53 pmol/10^6 cells in the HT-29 cell line versus 0.23 pmol/10^6 cells in the HCT-8 cell line. This difference was also reflected in a higher incorporation of FUra into DNA of HT-29 cells during the same period of exposure: 45 pmol/10^6 cells in this cell line compared with 12.5 pmol/10^6 cells in HCT-8 cells.

The accumulation of dUMP consequent to FdUMP inhibition of thymidylate synthase in HT-29 cells was marked at 4 hr and sustained after 8 hr of exposure to 10 μM FUra. This prolonged elevation was not seen in the HCT-8 cell line (Table 2). Overall, these changes are consistent with HCT-8 displaying a greater sensitivity to RNA-directed toxicity.

The biochemical modulation of FUra at the level of its target, thymidylate synthetase, has been utilized clinically to improve the antitumor efficacy of the fluoropyrimidine. The exogenous administration of Leucovorin (5-formyltetrahydrofolate) provides intracellularly the reduced folate cofactor essential for the formation and stabilization of the ternary complex with thymidylate synthetase and FdUMP. This approach, which nowadays represents the standard clinical treatment of colorectal malignancies, has resulted in a substantial improvement in the overall response rate.

Therefore, the exposure of the HT-29 and HCT-8 cell lines to such a regimen containing FUra and Leucovorin should result in increased cytotoxicity, as compared with FUra alone, mostly in the cell line where FUra exerts its antineoplastic activity mainly through the inhibition of thymidylate synthetase. For similar reasons, the same cell line should benefit from the co-administration of thymidine as a rescue agent of the toxic effect of Fura at the level of the same target.

As predicted, in HT-29 cells Leucovorin increased the sensitivity to FUra from an EC_{50} of 0.9 to 0.2 μM, and thymidine decreased the sensitivity almost 10-fold (Fig. 4B). In HCT-8, however, Leucovorin and thymidine were unable to modify the cytotoxic effect of FUra (Fig. 4A). On the contrary, as already demonstrated by FCM analysis, uridine (100 μM) was able to partially reduce the cytotoxic effect of FUra for short term exposures of 6 and 24 hr but did not prevent growth inhibition for a 72-hr continuous exposure (data not shown).

DISCUSSION

Numerous papers have addressed the relative importance of the RNA- versus DNA-directed toxicity of FUra. Maybaum *et al.* [20] proposed that FUra activity can be divided into a dThd-refractory RNA-related effect and a dThd-preventable DNA-directed toxicity. In S49 cells, these authors concluded that the DNA-directed component of toxicity causes immediate growth inhibition and arrest of cells in S-phase, followed by a RNA-

directed toxicity with block in G_1 and cell death after a delay of one doubling time. Washtien [21] has described two human gastrointestinal tumors with different sensitivities to FUra, which respond differently to dThd rescue and deoxyinosine potentiation. A colon adenocarcinoma, rescued by dThd, was suggested to display DNA-directed toxicity, while growth of a duodenum adenocarcinoma cell line was not rescued by dThd and, therefore, assumed to display RNA-directed toxicity.

In this report, we add new elements to this subject using two human colon tumor lines that demonstrate relatively similar sensitivities to FUra but display almost qualitatively different mechanisms of FUra toxicity. The effect on cell cycle distribution, quantitation of relevant enzymes, the fate of FUra, and the modulation of FUra cytotoxicity by Leucovorin and thymidine suggest that these lines may be relatively pure examples of RNA-directed (HCT-8) and DNA-directed (HT-29) cytotoxicity.

In HT-29 accumulation of cells in S-phase, the potentiation by Leucovorin and the protection by dThd are consistent with an anticipated primary effect of dThd on DNA metabolism, cell cycle distribution, and growth. The higher concentrations of FdUMP and dUMP, as well as lower thymidylate synthase activity in HT-29 compared with HCT-8, also favor DNA-directed toxicity.

In HCT-8, however, minimal, if any, evidence for an effect of FUra on DNA synthesis could be seen. Rather than cause an accumulation at different stages of S and at the G_1-S border, FUra caused a remarkable ablation of cells from S-phase, a result similar to that seen with two inhibitors of RNA polymerases: actinomycin D and DRB. These results suggest a RNA or protein effect that prevents entry into S-phase but does not limit the DNA synthetic phase once it is initiated. The incapacity of dThd to prevent FUra toxicity and the failure of Leucovorin to increase FUra toxicity are also consistent with the limited amount of FdUMP formed from FUra and the larger amount of thymidylate synthase activity with which it must compete.

It may be that the failure of Leucovorin to increase the antitumor effect of FUra in some patients treated with this combination reflects an insensitivity of the tumor to inhibition of this pathway, similar to that seen with HCT-8.

Overall, these results suggest that an understanding of the role of activation and metabolism in FUra cytotoxicity and the possibility of using flow cytometry on tumor specimens may help to establish the mechanism of FUra toxicity and, thus, permit a logical choice for biochemical modulation of therapy in specific human tumors.

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