Cancer Chemother Pharmacol (2000) 45: 369–374

ORIGINAL ARTICLE

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The role of p53 in gemcitabine-mediated cytotoxicity and radiosensitization

Received: 4 May 1999 / Accepted: 19 November 1999

Abstract *Purpose*: We compared the cytotoxic and radiosensitizing effects of gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdCyd), a clinically valuable radiosensitizer, in colon cancer RKO cells which differed in their p53 status. The parental RKO cells, RKO-P, contain wild-type p53 protein. In RKO-E6 cells, the p53 function has been disrupted by transfection of the cells with the human papillomavirus type-16 E6 gene. *Results*: We found that the RKO-P cells were significantly more sensitive to dFdCyd-mediated cytotoxicity and apoptosis than RKO-E6 cells (IC_{10} 39.3 ± 5.3 n*M* and 62.0 ± 6.9 n*M*, respectively). The cytotoxic effect of dFdCyd in RKO-P cells was accompanied by induction of the proapoptotic protein Bax at the time when p53 was induced. In contrast, similar treatment of RKO-E6 cells with dFdCyd resulted in only limited expression of Bax, suggesting that the cytotoxic effect of dFdCyd was mediated, in part, by a p53-dependent apoptosis pathway. We also studied the effect of dFdCyd on radiation sensitivity. We found that at minimally cytotoxic concentrations dFdCyd failed to radiosensitize either RKO-P or RKO-E6 cells, whereas at cytotoxic concentrations equal sensitization was produced. Finally, we assessed the influence of dFdCyd on cell cycle distribution. We found that dFdCyd synchronized RKO-P cells, whereas synchrony was not produced in p53-disrupted RKO-E6 cells. *Conclusion*: These results suggest that p53 status may influence dFdCyd-mediated apoptosis, cytotoxicity, and cell cycle progression but do not support an important role for p53 in radiosensitization.

M.C. is a Research fellow supported by National Research Service Award F32 CA69857. This work was supported by CA78554 and Cancer Center Core Grant CA46592

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Key words Apoptosis · Gemcitabine · p53 · Radio-sensitization

Abbreviations *BrdUrd* 5-bromo-2′-deoxyuridine · *dATP* 2′-deoxyadenosine 5′-triphosphate · *dFdCyd* 2′,2′-difluoro-2′-deoxycytidine · *FITC* fluorescein isothiocyanate · *PBS* phosphate-buffered saline · *SDS-PAGE* sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

Gemcitabine (dFdCyd) is an analog of cytosine arabinoside which has produced remarkable radiosensitization in a variety of solid tumors and tumor cell lines [19, 24]. After entering the cell, dFdCyd is phosphorylated sequentially into dFdCMP, dFdCDP and dFdCTP [32]. dFdCTP can block DNA synthesis by incorporating into DNA [10, 33], while dFdCDP can inhibit ribonucleotide reductase [1]. It has been suggested that the effect of dFdCDP is responsible for dFdCyd-mediated radiosensitization [18, 38].

In sensitive cells such as HT29 colon cancer cells and Panc-1 and BxPC-3 pancreatic cancer cells, dFdCyd produces radiosensitization at subtoxic or minimally toxic concentrations (defined as < 10–20% of cell killing by drug alone). Under these conditions, dFdCyd causes significant perturbation of deoxyribonucleotide pools, especially dATP pool depletion [17, 18, 37, 38] and redistribution of cells into early and middle S phase [17, 18, 38]. For example, treating pancreatic BxPC-3 cells with 10 n*M* dFdCyd for 24 h, a condition that produces a radiation enhancement ratio of approximately 1.8, depletes dATP pools to 11% of control values and alters the percentage of S phase cells from 24 ± 2% to 76 ± 3% [17]. These and other findings suggest that dFdCyd-mediated radiosensitization is maximized in cells demonstrating dATP pool depletion and redistribution of cells into S phase under noncytotoxic conditions [17, 38]. However, little is known about the cellular

processes occurring beyond these steps and how these lead to the sensitization of cells to ionizing radiation.

We decided to investigate whether p53 status plays a role in dFdCyd-mediated radiosensitization. p53 is a pivotal transcriptional factor which regulates cellular responses to different types of intracellular stresses, especially those related to DNA damage [14, 15]. In responding to these stresses, increases in p53 can lead to a transient cell cycle arrest which allows damaged DNA to be repaired or can direct cells into a p53-dependent apoptosis pathway (reviewed in references 2, 6 and 28).

To investigate the role of p53 in dFdCyd-mediated radiosensitization, we chose to study RKO human colon cancer cells. The parental RKO cell line, RKO-P, carries wild-type p53. A subline, RKO-E6, was derived by to transfection of RKO cells with human papillomavirus type-16 E6 gene and has disrupted p53 function [13]. We compared the response of RKO-E6 and RKO-P cells to dFdCyd-mediated cytotoxicity and radiosensitization.

Materials and methods

Chemicals and supplies

Gemcitabine, obtained from Eli Lilly and Company (Indianapolis, Ind.), was dissolved in phosphate-buffered saline (PBS) and stored at 4 °C. Other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Fisher Scientific (Pittsburgh, Pa.).

Cell culture, and clonogenic and apoptosis assays

RKO-P and RKO-E6 cells (kindly supplied by Dr. Albert J Fornace, Jr., National Institutes of Health, Bethesda, Md.) were cultured at 37 °C in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum, 2 m*M* glutamine, and the antibiotics penicillin and streptomycin. Cell survival was assessed using a colony formation assay as previously described [38]. After 9 to 10 days cells were fixed, stained with crystal violet, and scored for colonies. The fraction surviving each treatment was normalized to the survival of the control cells. Gemcitabine cell survival curves were fitted using the equation $SF(C_{50})^m/(C_{50})^m + C^m)$ where SF is the surviving fraction, C is the dFdCyd concentration, and C_{50} is the concentration of dFdCyd that produces a 50% cell survival, and *m* is the slope of the sigmoid curve. An analogous calculation was made for the apoptotic fraction. Radiation cell survival curves were fitted using the linear-quadratic equation, and the mean inactivation dose (the area under the cell survival curve) was calculated according to the method of Fertil et al. [5]. The cell survival enhancement ratio was calculated as the ratio of the mean inactivation dose under control conditions divided by the mean inactivation dose after dFdCyd treatment.

We assessed apoptosis by quantifying floating and attached cells as we and others have described previously [3, 29]. We verified that >98% of floating RKO cells demonstrated fragmented DNA (and <3% of adherent cells) using propidium iodide staining (data not shown). Treated or control cells were washed twice with PBS, provided with fresh medium, and cultured for 24–72 h. We found that the full extent of apoptosis was not seen until 72 h after drug exposure (data not shown). Floating and attached cells from each sample were collected and counted. The percent of apoptotic cells in each sample was calculated as: (total number of floating cells/total number of floating plus attached cells) × 100. The results from drug-treated samples are expressed as the percent apoptotic cells relative to untreated control samples. All experiments were performed at least three times.

Irradiation

Cell irradiation was performed with a ^{60}Co unit (Theratron AECL) at 1–2 Gy/min. Dosimetry was carried out using an ionization chamber connected to an electrometer system directly traceable to a National Institute of Standards and Technology calibration. All irradiations were carried out at room temperature.

Antibodies and immunoblot analysis

Cell extracts were prepared using RIPA buffer (50 m*M* NaCl, 50 m*M* Tris, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of protease inhibitors (Boehringer Mannheim, Germany). Total cellular protein from each sample (10 μg) was loaded onto 10% or 12% polyacrylamide gels, separated by SDS-PAGE, and transferred onto Immobilon P membranes (Millipore Corp., Bedford, Mass.). For immunoblotting, the membranes were blocked for nonspecific binding sites with TBST buffer (100 m*M* Tris, pH 7.5, 0.9% NaCl, 0.1% Tween 20) containing 5% dried milk, probed with primary antibody, washed, and incubated with secondary antibody. The primary antibodies used were monoclonal anti-p53 (Oncogene Science, Mineola, N.Y.), monoclonal anti-Bcl-2, polyclonal anti-Bcl-x_L and Bax (Santa Cruz Biotechnology, Santa Cruz, Calif.), and monoclonal anti-β-actin (Sigma Chemical Co., St. Louis, Mo.). Rabbit antimouse IgG and goat antirabbit IgG secondary antibodies were obtained from (Southern Biotechnology Associates, Birmingham, Ala.). Protein bands on membranes were detected using a chemiluminescence detection system as recommended by the manufacturer (Pierce, Rockford, Ill.).

Flow cytometry

Cells were prepared for flow cytometry as described previously [40]. Samples were analyzed on an EPICS C flow cytometer (Coulter Electronics, Hialeah, Fl.). Human leukocytes were used as an internal standard. For two-parameter flow cytometry cells were exposed to 30 μ*M* BrdUrd for 15 min and processed as previously described [9] using a first antibody (mouse anti-BrdUrd (Pharmingen, San Diego, Calif.) for detecting BrdUrd followed by FITC-goat-antimouse IgG (Sigma Chemical Co.). In each experiment a control sample was processed with the second antibody only to determine the background signal. Experiments were performed at least twice.

Results

We began by assessing the effect of dFdCyd on cell survival and induction of apoptosis in RKO-P and RKO-E6 cells treated with dFdCyd for 24 h. We found that RKO-P cells were significantly more sensitive to dFdCyd-mediated cytotoxicity than RKO-E6 cells (Fig. 1A). The IC_{10} values of dFdCyd in RKO-P and RKO-E6 cells were 39.3 ± 5.3 n*M* and 62.0 ± 6.9 n*M*, respectively ($P < 0.04$). We then assessed the effect of a 24-h dFdCyd exposure on the induction of apoptosis in these two cell lines. We found that RKO-P cells were significantly more sensitive to dFdCyd-mediated apoptosis than RKO-E6 cells (Fig. 1B). The IC_{50} of dFdCyd in RKO-P and RKO-E6 cells for apoptotic cell death was 17 ± 3 n*M* and 51 ± 11 n*M*, respectively ($P < 0.05$). In both cell lines, the fraction of cells undergoing apoptosis was similar to the reduction in clonogenic survival, suggesting that dFdCyd-mediated clonogenic cell death occurred largely through an apoptotic pathway. Because RKO-P and RKO-E6 cells are isogenic

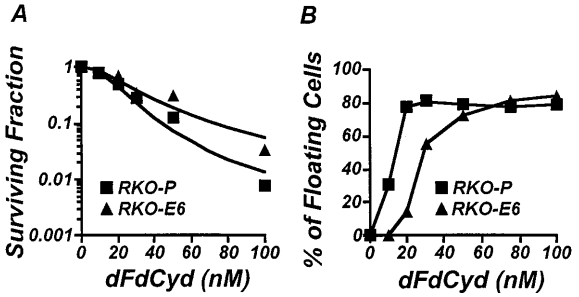

Fig. 1A,B Effect of dFdCyd on cell survival and induction of apoptosis in RKO-P and RKO-E6 cells. RKO-P and RKO-E6 cells were treated with various concentrations of dFdCyd for 24 h, after which drug was removed from the medium. They were then processed for clonogenic survival (**A**) and apoptosis (**B**) as described in Materials and methods. The results of a single representative experiment are shown

except for their p53 function, these findings suggest that a p53-dependent apoptosis pathway may be involved.

We next wished to assess the influence of dFdCyd on p53 and the expression of Bcl-2 family members in RKO-P and RKO-E6 cells under conditions similar to those we had used to assess clonogenic survival and apoptosis. We found that dFdCyd (100 n*M* for 24 h) could induce the expression of both p53 and Bax in RKO-P cells. In contrast, there was no induction of p53 and limited induction of Bax in RKO-E6 cells (Fig. 2). In RKO-P cells, Bax protein was expressed simultaneously with p53, which was between 8 and 16 h after dFdCyd treatment (data not shown). These findings suggest that the induction of Bax by dFdCyd in RKO-P cells was most likely due to the induction of p53. We also sought to determine whether dFdCyd had any effects on the expression of Bcl-2 and Bcl-x_L, two antiapoptotic proteins which have been shown to antagonize Bax-mediated apoptosis [31, 34]. In contrast to the greater levels of Bax in dFdCyd-treated RKO-P cells

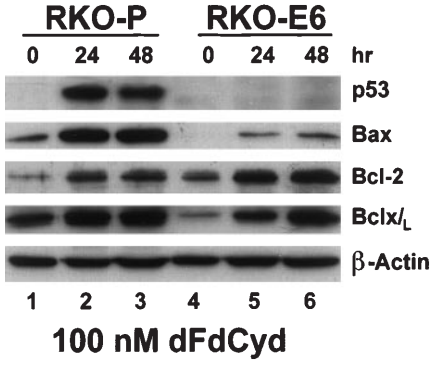

Fig. 2 Effect of dFdCyd on p53 and Bcl-2 family member expression. RKO-P and RKO-E6 cells were incubated under control conditions or treated with 100 n*M* dFdCyd for 24 h, after which drug was removed from the medium. They were analyzed at each indicated time for expression of p53, Bax, Bcl-2 and Bcl-x_L by immunoblotting as described in Materials and methods. The level of β-actin expression was used as loading control. The results of a single representative experiment are shown

compared to RKO-E6 cells, we found that the levels of the antiapoptotic Bcl-2 was greater in RKO-E6 cells, which is consistent with their decreased ability to undergo apoptosis. Both cell lines demonstrated induction of Bcl-x_L after dFdCyd treatment (Fig. 2).

We then wished to study the influence of p53 status on dFdCyd-mediated radiosensitization. We first found that p53 null RKO-E6 cells were more sensitive to radiation than the parental RKO-P cells ($P < 0.01$; Fig. 3). We then sought to determine whether p53 played a role in dFdCyd-mediated radiosensitization. Two types of experiments were performed in which RKO-P and RKO-E6 cells were treated with either moderately toxic (about 50% cell killing by drug alone) or minimally toxic (< 20% cell killing by drug alone) concentrations of dFdCyd for 24 h then subjected to radiation treatment. Under conditions of moderate toxicity (20 n*M* dFdCyd, producing a surviving fraction of 0.55 ± 0.14), dFdCyd caused slight radioenhancement (1.36 ± 0.16) of RKO-P cells (Fig. 4A). In the RKO-E6 cells, 30 n*M* of dFdCyd (surviving fraction 0.48 ± 0.11) produced approximately the same increase in radiation sensitivity, with an enhancement ratio of 1.33 ± 0.17 (Fig. 4B).

In contrast to these similar results under conditions in which dFdCyd produced moderate toxicity, RKO-E6 and RKO-P cells differed in their radiation response under dFdCyd conditions that caused minimal cytotoxicity. Pretreatment of RKO-P cells with minimally cytotoxic concentrations of dFdCyd (10 n*M* dFdCyd, resulting in a surviving fraction of 0.98 ± 0.03) did not produce a significant change compared to control (enhancement ratio 1.03 ± 0.06; Fig. 4A). In contrast, in RKO-E6 cells minimally cytotoxic concentrations of dFdCyd (10 and 20 n*M*) caused radioprotection (Fig. 4B). The enhancement ratios for RKO-E6 cells pretreated with 10 n*M* and 20 n*M* dFdCyd were 0.85 ± 0.04 and 0.74 ± 0.02, respectively.

We hypothesized that the difference between the effect of minimally cytotoxic concentrations of dFdCyd on RKO-E6 cells compared to RKO-P cells could result from different effects of dFdCyd on the cell cycle. As we have previously reported that minimally cytotoxic

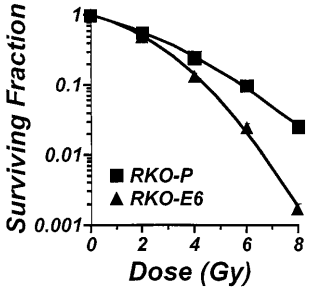

Fig. 3 Clonogenic survival of RKO-P and RKO-E6 cells after radiation. RKO-P and RKO-E6 cells were irradiated and assessed for clonogenic survival as described in Materials and methods. The results of a single representative experiment are shown

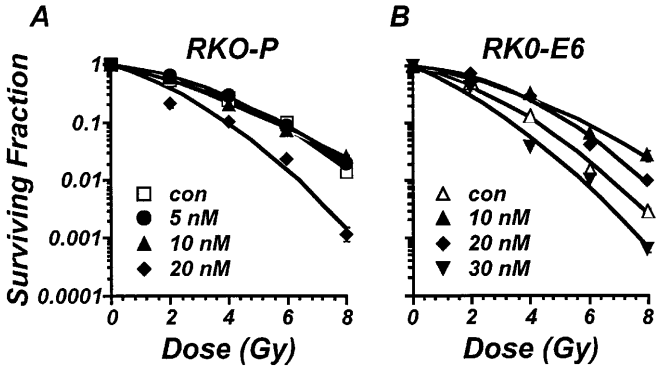

Fig. 4 Effect of dFdCyd on radiation sensitivity. RKO-P or RKO-E6 cells were treated with either moderately or minimally cytotoxic concentrations of dFdCyd (see text for definitions) for 24 h. Cells were then assessed for clonogenic survival as described in Materials and methods. The results of a single representative experiment are shown

concentrations of dFdCyd lead to a significant S phase accumulation [17, 18, 38], we thought it was important to assess cell cycle distribution in RKO-P and RKO-E6 cells. To better distinguish early S phase from late G_1, and late S phase from G_2, we performed two-parameter flow cytometry using BrdUrd (see Methods). Gemcitabine (10 n*M*) produced a similar time-dependent S phase accumulation in both cell types (Fig. 5), with RKO-P and RKO-E6 cells showed similar overall increases in S-phase fraction after a 24-h exposure from 56 ± 3% to 81 ± 4% and 46 ± 4% to 75 ± 2%, respectively. However, RKO-P cells were synchronized by dFdCyd, with progression through S phase (at 16 h), late S to G_2 (at 24 h) followed by G_1 and S (by 32 h). In contrast, RKO-E6 cells showed much less tendency to synchronize (Fig. 5), with cells continuing to cycle throughout the period after drug exposure (note presence of G_1 at all times). Higher dFdCyd concentrations (20 n*M* and 30 n*M*) produced similar changes (data not shown). It is possible that this difference in cell cycle redistribution between RKO-P and RKO-E6 cells contributes to the different effects on radiation sensitivity produced by dFdCyd treatment [39, 41].

Discussion

We studied the influence of p53 status on dFdCyd-mediated cytotoxicity and radiosensitization through the use of RKO-P colon cancer cells (which show normal p53 function) and a subclone in which p53 function is absent secondary to transduction with E6. We found that the RKO-P cells were significantly more sensitive to dFdCyd-mediated cytotoxicity and apoptosis than RKO-E6 cells. Compared to RKO-E6 cells, RKO-P cells showed a substantially greater induction of the proapoptotic protein Bax after drug treatment and lower levels of the antiapoptotic protein Bcl-2. Both cell lines were moderately radiosensitized under conditions which produced moderate cytotoxicity. Neither cell line was radiosensitized by treatment with minimally cytotoxic concentrations of dFdCyd; indeed, RKO-E6 cells were slightly radioprotected. This difference in radiation sensitivity between these two cell types after exposure to subcytotoxic concentrations of dFdCyd is associated with a difference in cell cycle redistribution produced by the drug treatment.

Our findings concerning the effect of p53 status on dFdCyd-mediated apoptosis are consistent with a significant body of literature which suggests that an increase in p53 tends to activate proapoptotic pathways. p53 has been found to be the most important regulator of (proapoptotic) Bax [27, 35]. Increased Bax expression due to p53 induction has been shown to correlate with an increased level of apoptosis, but deficiency of Bax attenuates p53-dependent apoptosis [7, 23]. Conversely, evidence suggests that p53 represses the antiapoptotic protein Bcl-2 [2]. We found that 100 n*M* dFdCyd could effectively induce the expression of p53 and Bax in RKO-P cells but not in RKO-E6 cells. Time-course experiments showed that the expression of p53 and Bax in RKO-P cells occurred simultaneously (data not shown). Although we found that Bcl-2 and Bcl-x_L were induced by dFdCyd treatment, the former was found in greater quantities in RKO-E6 cells. Taken together, these findings suggest that dFdCyd produces a greater shift in Bcl-2 family members toward apoptosis in RKO-P cells compared to RKO-E6 cells. However, we recognize that we have assessed only a limited number of potential mediators of apoptosis, and that other pathways (both p53-dependent and -independent) may be involved. Regardless of the mediators of apoptosis, the finding that RKO-P cells are more sensitive than RKO-E6 cells to dFdCyd when assessed by clonogenic assay suggests that apoptosis plays an important role in overall clonogenic survival of these colon cancer cells. Our findings with solid tumor cell lines are consistent with the result that dFdCyd can cause apoptosis in leukemia cells [10].

The role of p53 in chemo- and radiosensitivity is complex. In the current study, RKO-E6 cells were more resistant to dFdCyd yet more sensitive to ionizing radiation. Such phenomena may be explained by the dual functions of p53 in mediating cell cycle arrest, which increases cell survival by allowing damaged DNA to be repaired, or decreases survival by driving cells into the apoptosis pathways [2, 6, 28]. These dual functions of p53 probably account for the fact that alterations in the cellular p53 status have different impacts on antitumor treatments. In some cases, expression of wild-type p53 is associated with an increase in the sensitivity to anticancer treatment [22, 23, 26], whereas loss of p53 function can also result in increased sensitivity to anticancer treatment [4, 36]. It is possible that the synchrony evidenced in the RKO-P compared to RKO-E6 cells is also a function of p53, which mediates both G_1 and mitotic checkpoints [8, 11, 12, 30].

In this study we found that dFdCyd was not an effective radiosensitizer for either RKO-P or RKO-E6 cells. It produced only moderate radiosensitization even

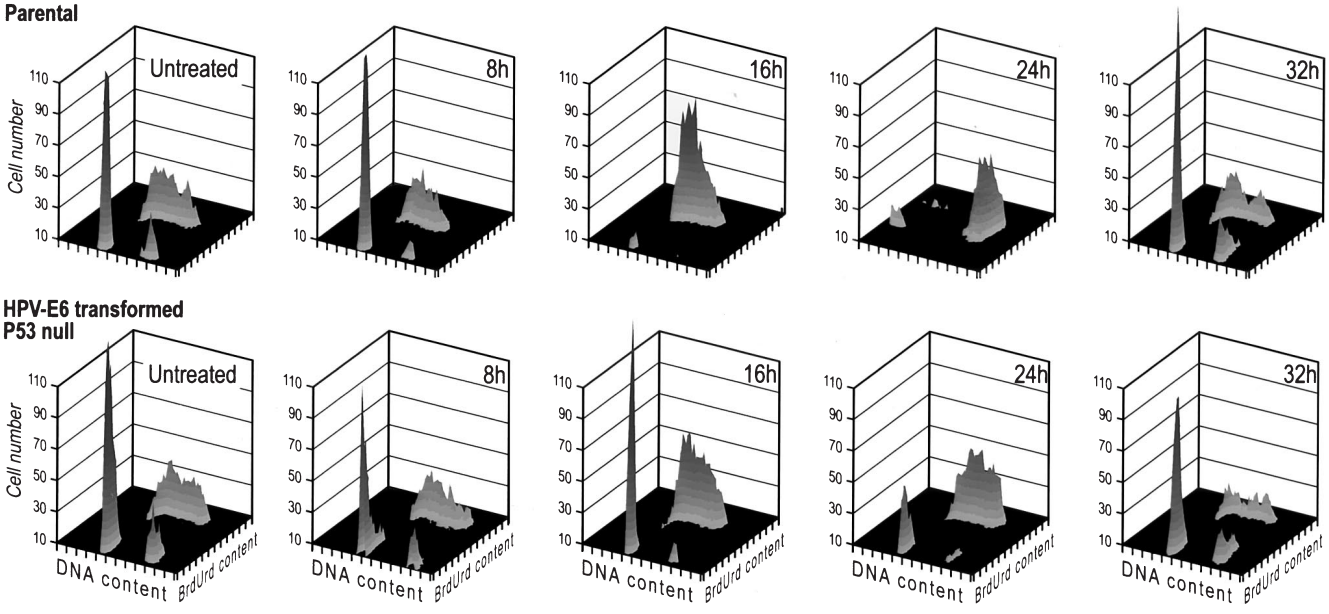

Fig. 5 Influence of dFdCyd on cell cycle distribution. RKO-P or RKO-E6 cells were treated with 10 n*M* dFdCyd for 24 h, after which drug was removed from the medium. At the indicated times, cells were processed for two-parameter flow cytometry as described in Materials and methods. The results of a single representative experiment are shown

under conditions of moderate drug-induced cytotoxicity. Although many cultured human cancer cells are radiosensitized by dFdCyd, including HT29 [38] and SW620 colon cancer cells [21], Panc-1 and BxPC-3 pancreatic cancer cells [17], and MCF-7 breast cancer cells (both wild-type and drug-resistant) [19], and UMSCC-6 squamous cells derived from head and neck cancers (unpublished), A549 lung cancer cells are not radiosensitized under conditions of minimal cytotoxicity (unpublished). The reasons for these differences are not yet known. It appears that dFdCyd-mediated radiosensitization requires simultaneous dATP pool depletion and accumulation of cells in S phase [17, 38], and we have hypothesized that these conditions lower the apoptotic threshold for ionizing radiation [20]. A recent study of V79 cells has suggested that S phase cells show the greatest radiosensitization by dFdCyd [16]. In this study, we found that dFdCyd does produce S phase redistribution but not sensitization, suggesting that cell cycle redistribution alone is not sufficient to produce radiosensitization.

One finding of this study of potential clinical importance is that, under minimally cytotoxic conditions, dFdCyd protected RKO-E6 cells. Radioprotection has also been reported when human cervical carcinoma HeLa cells are exposed to minimally cytotoxic concentrations of dFdCyd [42]. It seems possible that cells which are not sensitized may actually be made more resistant if they are redistributed into a resistant phase of the cell cycle. Radiosensitization (albeit modest) was produced under conditions of moderate cytotoxicity. We have begun attempting to assess this concept through two different phase I clinical strategies using dFdCyd as a radiosensitizer for the treatment of pancreatic cancer. In one of these protocols, dFdCyd is being escalated while radiation is given at its traditional dose (50.4 Gy in 28 fractions) [25]. In the other trial, radiation is being escalated while dFdCyd is given at its traditional dose (1000 mg/m^2 per week) [24]. The findings of the study reported here suggest that the latter strategy of giving dFdCyd at a cytotoxic dose might produce a superior result.

Acknowledgements The authors want to thank Drs. Alnawaz Rehemtulla, Mats Ljungman, and Bruce McKay for valuable discussions, Dr. Mary Davis for help with figure preparation, and Marlene Langley for secretarial assistance.

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