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Advances in Brief

Fluoropyrimidine-mediated Radiosensitization Depends on Cyclin E-dependent Kinase Activation[1]

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Abstract

Fluoropyrimidines radiosensitize human colon cancer cells that progress into S phase in the presence of drug (M. A. Davis, H-Y. Tang, J. Maybaum, and T. S. Lawrence. Int. J. Radiat. Biol. *67:* 509–512, 1995). We hypothesized that progression occurs in cells that generate elevated levels of cyclin E-dependent kinase activity despite the presence of the fluoropyrimidine. To test this hypothesis, we treated HT29 and SW620 human colon cancer cells with fluorodeoxyuridine under conditions that produced nearly complete inhibition of thymidylate synthase but which sensitized only the HT29 cells. We found that, whereas HT29 cells progressed into S phase and demonstrated increased cyclin E-dependent kinase activity, SW620 cells arrested just past the G_1-S boundary and showed no change in kinase activity. Because these cell lines have the same p53 mutation, these findings suggest that there is a p53-independent G_1-S checkpoint that mediates radiosensitization produced by fluorodeoxyuridine.

Introduction

Randomized trials have demonstrated the effectiveness of the fluoropyrimidine radiation sensitizers in the treatment of cancers of the rectum, esophagus, and pancreas. Single-institution trials further suggest that these drugs, in combination with radiation, improve the local control of anal and intrahepatic cancers (for review, see Ref. 1). Despite these encouraging clinical results, many gastrointestinal cancers are either resistant to fluoropyrimidines *de novo* or develop resistance during treatment, giving impetus to studies determining the mechanism of sensitization and resistance.

Using two human colon cancer cell lines, we have found that FdUrd[3]-mediated sensitization correlates with the ability of cells to progress into S phase in the presence of drug. Specifically, HT29 cells, which are markedly radiosensitized by FdUrd, progress approximately 10% of the way into S phase (based on DNA content), whereas SW620 cells, which are minimally sensitized, arrest just past the G_1-S boundary (2). Two lines of evidence suggest that S-phase progression at the time of radiation, rather than simple cell cycle redistribution into a radiosensitive phase of the cell cycle, is critical in producing sensitization. (*a*) FdUrd-treated HT29 cells both from early S phase (obtained by centrifugal elution; Ref. 2) and from mid-S phase (obtained by flow sorting) are radiosensitized to a greater extent than G_1-S FdUrd-treated cells. (*b*) Aphidicolin, which inhibits DNA polymerase α (3) and prevents S phase progression, blocks FdUrd-mediated radiosensitization.[4]

Because S-phase progression seemed to be crucial in producing radiosensitization, we wished to determine the mechanism underlying the difference in responses between these two cell types. The ability of p53 wild-type cells to enter S phase is under control of the G_1-S cyclins, particularly E and D. These cyclins can bind to CDKs, which permits them to become active and carry out the phosphorylation of the retinoblastoma protein, thereby releasing transcription factors such as E2F and facilitating entry into S phase (4–6). DNA damage triggers a sequence of events leading to increases in the levels of GADD45 (7), p53 (8), and p21, the latter of which can inhibit CDK activity (9, 10).

We knew that the difference between SW620 cells and HT29 cells could not depend on p53 status, because both of these cell lines have the same p53 mutation (11). Therefore, we hypothesized that these cells differed in their ability to produce active cyclin–CDK complexes in the presence of FdUrd by a p53-independent mechanism. To test this hypothesis, we assessed cyclin D1 and E levels and CDK activity under identical conditions of FdUrd exposure, which produced equal TS inhibition in both cell types but radiosensitized only HT29 cells. We found that for both SW620 and HT29 cells, FdUrd treatment affected neither cyclin D1 levels nor cyclin D1-dependent kinase activity. In contrast, we found that, whereas both cell types generated significant increases in cyclin E levels in the presence of FdUrd, only HT29 cells showed increased cyclin E-dependent kinase activity. These findings suggest that a p53-independent checkpoint exists at the G_1-S boundary in SW620 cells, but not in HT29 cells, which prevents fluoropyrimidine-mediated radiosensitization.

Materials and Methods

Cell Culture. HT29 and SW620 human colon cancer cells were cultured as described previously (2). FdUrd exposures were carried out in thymidine-free media made with dialyzed serum. Cells were checked for mycoplasma every 3 months.

Flow Cytometry. Cells were trypsinized, washed in PBS, fixed by dropwise addition of 2.5 volume of cold 70% ethanol, and stored at 4°C until the day of analysis. They were then washed with PBS, suspended in propidium iodide, and analyzed on an EPICS C flow cytometer (Coulter Electronics). Human leukocytes or salmon red blood cells were used as internal standards for HT29 and SW620, respectively. Cell cycle phase distribution was estimated with CytoLogic software, based on a multiple broadened rectangular S phase model. For two-parameter flow cytometry, cells were processed for the immunoassay with the first antibody [(mouse anti-BrdUrd (PharMingen) or mouse anti-cyclin E (PharMingen) for detecting BrdUrd and cyclin E, respectively)] followed by FITC-goat anti-mouse IgG (Sigma Chemical Co.; Refs. 12 and 13).

TS Assay. The activity of TS in cell extracts from control or FdUrd-treated cells was determined by measuring the conversion of [^{3}H]dUMP to dTMP with the release of ^3H_2O, using the method of Maley *et al.* (14). Cells were washed with cold PBS, scraped, and sonicated. An aliquot of the extract was then incubated at 37°C for 30 min with [^{3}H]dUMP in the presence of 50 mM Tris, 5 mM $MgCl_2$, 50 mM NaF, 10 μM dUMP, and 1 mM reduced folate. The reaction mixture was then treated with activated charcoal suspended in 4% perchloric acid and centrifuged. The supernatant, containing ^3H_2O produced by TS, was processed for liquid scintillation counting.

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[3] The abbreviations used are: FdUrd, 5-fluoro-2′-deoxyuridine; BrdUrd, 5-bromo-2′-deoxyuridine; TS, thymidylate synthase; CDK, cyclin-dependent kinase.

[4] T. S. Lawrence, M. A. Davis, H-Y. Tang, and J. Maybaum. Fluorodeoxyuridine-mediated cytotoxicity and radiosensitization requires S phase progression. Int. J. Radiat. Biol., in press.

Cyclin Levels. Cyclin content was determined by immunoblotting (15). Logarithmically growing cells were trypsinized, counted, and lysed with detergent buffer containing β-mercaptoethanol. Aliquots of the lysates were electrophoresed using standard SDS-PAGE techniques. Each lane was loaded with an equivalent amount of total protein as determined by the Bradford protein assay. After the gel had been equilibrated with Towbin buffer (Tris/glycine/methanol), it was transferred to Immobilon paper using a Hoeffer TE42 Transphor unit. The blot was then washed in blocking buffer and wash buffer. It was then incubated with the primary antibody, washed, and incubated with anti-IgG2 coupled to horseradish peroxidase, and developed using the ECL chemiluminescence detection system (Amersham). Film densitometry was used to quantify the presence of cyclin.

Cyclin H1 Kinase Assay. Cyclin D1- and E-dependent kinase activity was measured by first immunoprecipitating cell extracts (made by treating the cells with a buffered detergent mixture containing protease inhibitors) with the appropriate cyclin antibody and then binding the precipitate to Protein A Sepharose beads (6). For each sample, equivalent amounts of total protein were mixed with antibody. After washing, the bead-antibody-cyclin E complex was reacted with histone H1, ATP, and [^{32}P]ATP. After incubation, the mixture was boiled in Laemmli buffer and resolved on a 12% SDS-PAGE gel. The gel was then stained with Coomassie blue to assess loading, washed to remove unbound isotope, and dried. The amount of kinase activity was determined by autoradiography, and quantitative analysis was performed by phosphorimaging of the dried gels (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis. All data are presented as the mean ± SE of at least three experiments. The Student's *t* test was used to compare two means. Statistical significance was defined at the level of $P < 0.05$ (two-tailed). In the figures, results of single representative experiments are shown.

Results

Our previous work had characterized the cell cycle distribution after a 20-h exposure to FdUrd (100 nM), which produced modest direct toxicity (2). In that study, we had observed a radiation enhancement ratio of 2.1 ± 0.2 without cytotoxicity after a 14-h exposure. Therefore, we wished to assess the flow cytogram of HT29 and SW620 cells under these latter conditions. Consistent with our previous observations, we found that SW620 cells arrested at the G_1-S boundary, whereas HT29 cells progressed into S phase (Fig. 1). The fact that the majority of cells in both cell lines can incorporate BrdUrd under these conditions (which, as a thymidine analogue, is incorporated by the salvage pathway, thus bypassing the TS block produced by FdUrd) demonstrates that these cells are at or just beyond the G_1-S boundary and not in G_1 or G_0 (Fig. 2).

A simple explanation for the difference between HT29 cells and SW620 cells would be that they have differing degrees of inhibition by FdUrd. Therefore, we assessed the effect of 100 nM FdUrd, the concentration that we have used in the radiosensitization experiments, on TS activity in these two cell types. We found that after varying durations of exposure to FdUrd, the TS activity was inhibited by 94 ± 2% and 93 ± 1% (after 2 h), 78 ± 5% and 80 ± 4% (after 14 h), and 71 ± 6% and 75 ± 3% (after 24 h), in HT29 and SW620 cells, respectively. Therefore, the observed differences between SW620 cells and HT29 cells do not result from differing responses of TS to FdUrd.

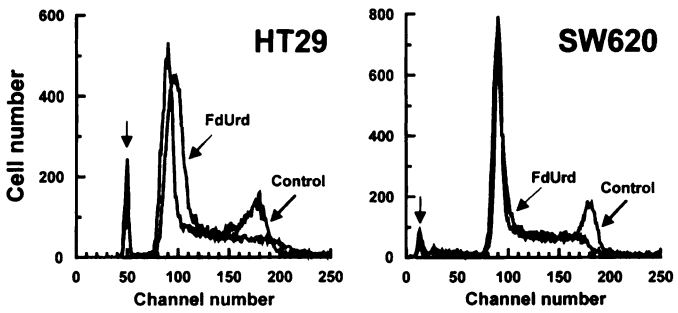

Fig. 1. Effect of FdUrd on cell cycle distribution of HT29 and SW620 cells. Cells were treated with media alone or with 100 nM FdUrd for 14 h. They were then assessed for DNA content by flow cytometry.

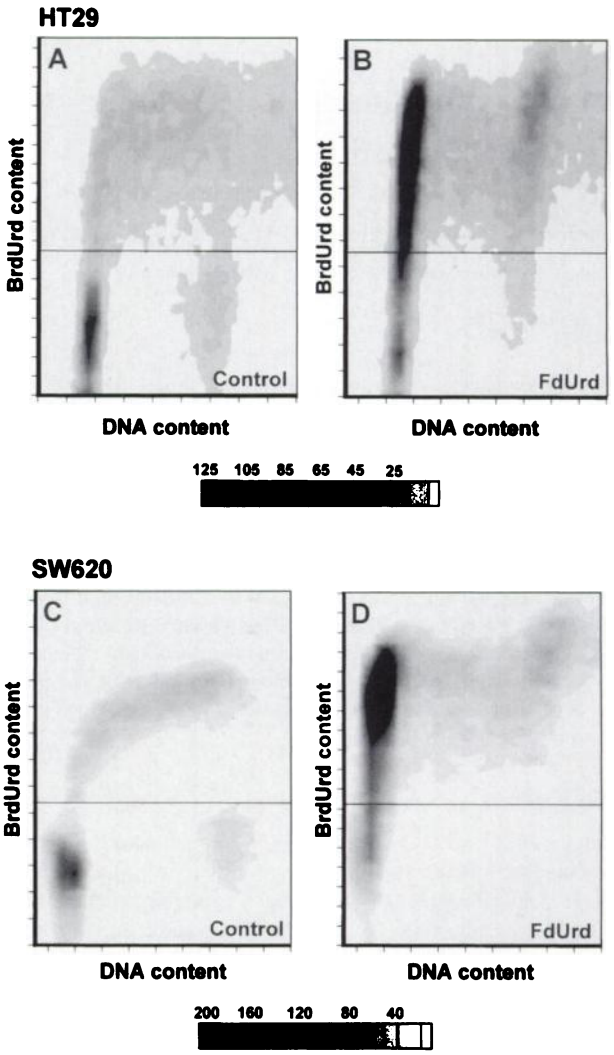

Fig. 2. Influence of FdUrd on entry into S phase of HT29 and SW620 cells. HT29 cells (*A* and *B*) and SW620 cells (*C* and *D*) were treated as described in Fig. 1 but were incubated with 30 μM BrdUrd during the last 15 min of the 14-h FdUrd exposure. They were then processed for two-parameter flow cytometry as described in "Materials and Methods."

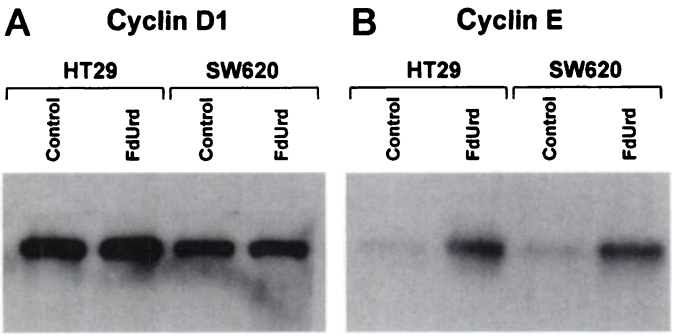

Fig. 3. Influence of FdUrd on cyclin D1 and E levels in HT29 and SW620 cells. Cells were treated with media alone or with 100 nM FdUrd for 14 h. They were then assessed by Western blotting for cyclin D1 levels (*A*) and cyclin E levels (*B*).

To begin to determine whether the differences between these two cell types lay in their cyclin-mediated responses, we evaluated cyclin

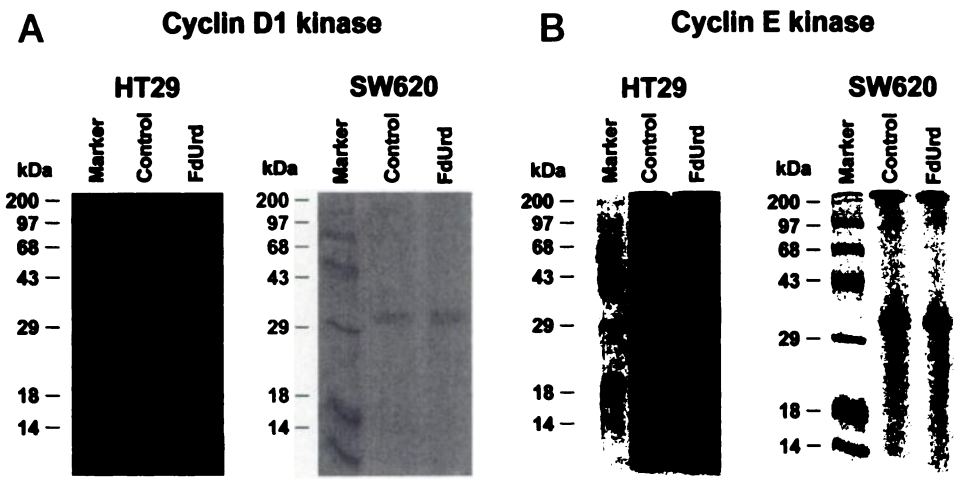

Fig. 4. Effect of FdUrd on cyclin D1- and E-dependent kinase activity in HT29 and SW620 cells. Cells were treated with media alone or with 100 nM FdUrd for 14 h. They were then assessed by the H1 kinase assay for cyclin D1-dependent kinase activity (*A*) and cyclin E-dependent kinase activity (*B*).

D1 and cyclin E levels after exposing cells to FdUrd. We found that cyclin D1 levels were unaffected (Fig. 3*A*). In contrast, cyclin E levels were increased by a factor of 8.9 ± 3.0 and 6.7 ± 1.5 in SW620 cells and HT29 cells, respectively, after FdUrd treatment (Fig. 3*B*). To determine whether this increase occurred in cells at the G_1-S boundary as well as S-phase cells, we evaluated both cell types using two-parameter flow cytometry for DNA content and cyclin E levels. We found that cells throughout S phase demonstrated increased cyclin E levels (not shown).

It was then of interest to determine how these cyclin levels were affecting CDK activity. As would be expected, we found that cyclin D-dependent kinase activity was unaffected by FdUrd exposure (Fig. 4*A*). Furthermore, consistent with the increase in cyclin E levels, HT29 cells showed a significant increase in cyclin E-dependent kinase activity (by a factor of 1.7 ± 0.1; Fig. 4*B*). In contrast, despite the presence of a significant increase in cyclin E levels, SW620 cells showed no change in cyclin E-dependent kinase activity; the ratio of kinase activity in the presence compared to the absence of FdUrd was 1.1 ± 0.2.

Discussion

We have found that SW620 and HT29 human colon cancer cells demonstrate differing cell cycle responses after exposure to FdUrd which may underlie the finding that only HT29 cells are radiosensitized. When treated with FdUrd under noncytotoxic conditions (100 nM for 14 h), HT29 cells progress into S phase, in contrast to SW620 cells, which arrest at the G_1-S boundary. With longer (24 h) exposures to FdUrd, SW620 cells show minimal progression into S phase and significant cytotoxicity (surviving fraction of 0.35 ± 0.1), but they do not become radiosensitized (2). Thus, we feel that the significantly slower progression into S phase of SW620 cells compared to HT29 cells in the presence of FdUrd prevents radiosensitization. Although both cell types exhibit a significant increase in cyclin E levels after FdUrd exposure, only HT29 cells generate increased cyclin E-dependent kinase activity. It seems likely that this greater kinase activity permits HT29 cells to progress into S phase and become radiosensitized, whereas SW620 cells arrest at the G_1-S boundary and show no change in radiation sensitivity.

The fact that both SW620 and HT29 cells show the same p53 mutation suggests that the difference between the cell types and the ability of SW620 cells to arrest at the G_1-S boundary are p53 independent. However, it is possible that p53 plays a role in this arrest in p53 wild-type cells. We have found that exposure of normal skin fibroblasts to FdUrd causes an increase in p53 levels and arrest at the G_1-S boundary, and that these cells are not radiosensitized.[5] This is consistent with previous reports that 5-fluorouracil can elevate p53 levels in cells containing wild-type p53 (16, 17). However, the classic p53-mediated checkpoint (resulting from, *e.g.*, radiation) is a G_1 arrest (18, 19), as opposed to the arrest that we have discussed here, in which cells are crossing the S-phase boundary. Thus, the role of p53 (if any) in the G_1-S arrest observed in p53 wild-type cells exposed to fluoropyrimidines needs to be clarified.

The most important limitation to this study is the use of two different cell lines with different genetic backgrounds. Thus, although we have found a marked difference between the effect of FdUrd on the radiation sensitivity of SW620 and HT29 cells, and we have correlated this difference with cyclin E kinase activity and the ability of cells to progress into S phase, it is possible that some other factor mediates these effects. In addition, we do not yet understand why these two cell types generate different levels of cyclin E-dependent kinase activity. It is possible that p21, which can be activated independently of p53 (20, 21), plays a role in this difference.

Although the fluoropyrimidines remain the most effective chemotherapeutic drugs as well as radiosensitizers in the treatment of gastrointestinal cancers, most patients do not respond to treatment. Therefore, it would be worthwhile to develop predictive assays so that patients who are unlikely to respond can be evaluated for other therapies. An assay for TS level may be one such possibility, in that it has been shown that elevated TS is associated with resistance to fluoropyrimidines in the laboratory (22) and the clinic (23, 24). If the results of the current study can be generalized, it is possible that an assessment of cyclin E-dependent kinase activity may permit the development of a predictive assay for fluoropyrimidine-mediated radiosensitization.

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