Radiosensitization by 5-Fluorocytosine of Human Colorectal Carcinoma Cells in Culture Transduced with Cytosine Deaminase Gene

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ABSTRACT

Recently, use of the suicide gene, cytosine deaminase (CD), has shown a selective antitumor activity of 5-fluorocytosine (5-FC) on human colorectal carcinoma cells grown in vitro and in vivo. We hypothesized that the radiosensitivity of human colorectal carcinoma cells transduced with a retroviral vector encoding the bacterial CD gene would be selectively enhanced by the nontoxic prodrug 5-FC. The radiobiological rationale of using suicide gene therapy is based on the fact that a toxic metabolite of 5-FC, 5-fluorouracil, is a well-known radiation enhancer for the treatment of gastrointestinal and other tumors. 5-FC was found to selectively enhance the radiocytotoxicity of human colorectal carcinoma cells expressing the CD gene. Colorectal carcinoma cells transduced with the CD gene (WiDr-CD) were highly sensitive to irradiation compared with parental cells (WiDr) when exposed to 20 μg/ml 5-FC for 72 h prior to irradiation. The sensitization enhancement ratio was 2.38. This magnitude of radiation enhancement is comparable to that obtained with 5-fluorouracil. These results suggest that the addition of radiation would substantially improve the therapeutic potential of CD gene therapy for the treatment of locally advanced colorectal carcinomas.

INTRODUCTION

One of the major obstacles encountered with chemical modifiers of cancer treatment is the difficulty in identifying biochemical characteristics unique to malignant cells that could be exploitable therapeutically. Recent advances in the molecular biology of gene transduction have permitted significant progress toward the treatment of cancer using gene therapy. As yet, few radiobiological studies have been carried out to explore the new emerging field.

One particularly appealing approach has been to incorporate a drug susceptibility gene, or “suicide gene,” which encodes an enzyme that can activate a prodrug intratumorally, so that the transduced tumor cells can toxify the systemically administered prodrug. The bacterial CD gene encodes an enzyme that catalyzes the deamination of cytosine to uracil (1, 2). Hence, cells that express the CD gene can convert 5-FC to 5-FU, a highly toxic antimetabolite to most mammalian cells (3). Since mammalian cells do not express CD, they cannot deaminate 5-FC (4, 5).

The effectiveness of 5-FC in the killing of carcinoma cells transduced with a vector containing the Escherichia coli CD gene was recently demonstrated by Mullen et al. (6, 7). In vitro, cells expressing the CD gene are killed by 5-FC, while unmodified cells are not. In vivo, CD+ tumors could be eliminated by systemic treatment with 5-FC without significant toxicity to the host. However, this approach was not effective when the solid tumors were grossly large. Similarly, Austin and Huber (8) have shown that a significant antitumor effect is obtained with 5-FC in vivo with human colon carcinoma cells transduced with CD, but no significant antitumor effect was obtained with 5-FU at dose levels not causing systemic toxicities. Furthermore, they enhanced the selectivity of their approach by expressing the CD gene in a tumor-specific manner using 5′ transcriptional regulatory sequences of the tumor-associated marker carcinoembryonic antigen (9).

The rationale of the present radiobiological study is based on the fact that the nontoxic prodrug 5-FC is deaminated into the toxic antimetabolite 5-FU within tumors expressing CD, and 5-FU and its intermediary metabolites enhance the radiosensitivity of tumor cells. The present experiments were carried out with WiDr human colon carcinoma cells transduced with a retroviral vector encoding the E. coli CD gene. The ability of 5-FC to enhance radiation cytotoxicity was investigated.

MATERIALS AND METHODS

Cell Lines. Studies were carried out with WiDr cells, originally derived from human colorectal carcinoma and the same cell line that was transduced with an E. coli CD gene (WiDr-CD). Cells were grown in DMEM with 10% fetal bovine serum. No antifungal agent was used, and a test for Mycoplasma infection was performed routinely.

Gene Transfer. WiDr cells were infected with the CD2 retrovirus produced by PLCD12 cells (6). The retroviral vector CD2 contains the CD gene downstream of the 5′ long terminal repeat sequence and uses the long terminal repeat sequence as its promoter. In addition, CD2 contains the neomycin phosphotransferase gene NeoR, which confers resistance to the neomycin analogue G418. The SV40 early promoter serves as the

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promoter for NeoR. The PLCD12 producer cell line generates a titer of 5.0 × 10^5 colony-forming units/ml. Producer cells were maintained in DMEM with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.1 mM nonessential amino acids. WiDr cells were exposed to the viral supernatant in the presence of protamine (5 μg/ml) for 24 h. To obtain an enriched population of transduced cells, the tumor cells were selected in G418 (1 mg/ml) for 7 days. Surviving colonies were pooled for subsequent radiation and cytotoxic studies of 5-FC and 5-FU.

**Chemicals.** The drugs 5-FU and 5-FC were purchased from Sigma Chemical Co. (St. Louis, MO).

**Irradiation Procedure.** The cells were irradiated using a 5000-Ci ^{137}Cs source (Model Mark I; J. L. Shepherd, Inc., San Fernando, CA) at a dose rate of 1.61 Gy/min.

**Cell Survival Assay.** After drug and/or radiation, cells were plated in drug-free medium. Clonogenic survival was determined by counting colonies at 12–14 days after treatment. Cytotoxicity was assessed using cell survival curves in which the percentage of cells that survive a radiation dose with and without the drug is plotted as a function of radiation dose. Each experiment was repeated at least twice. Cell survival data were analyzed using a nonlinear least-square algorithm developed by Albright (10). Cells were actively dividing and asynchronous at the time of specific treatment.

**RESULTS**

**Sensitivity of WiDr and WiDr-CD Cells to 5-FU and 5-FC.** The cytotoxic effects of 5-FU and 5-FC without radiation were determined as a function of time. The results demonstrate that both WiDr and WiDr-CD cells are sensitive to 5-FU at concentrations ranging from 1.0 to 4.0 μg/ml (Fig. 1A). In contrast, when these cells were exposed to 5-FC, toxicity was seen only with the WiDr-CD cells. For example, at a concentration of 100 μg/ml 5-FC and a 72-h exposure time, the survival of WiDr-CD cells was reduced to 0.06%, while the survival of parental WiDr cells remained unchanged (Fig. 1B). The cell growth rate and plating efficiency of WiDr-CD cells were similar to those of the parental WiDr cells, with a plating efficiency of 25–35% and a doubling time of approximately 36 h.

The sensitivity of WiDr-CD cells to 5-FC varied depending on cell density at the time of drug exposure. Maximum toxicity was obtained when 1 × 10^6 cells were plated 24 h before exposure to drug and/or radiation (data not shown).

**Radiosensitization of WiDr and WiDr-CD Cells by 5-FU.** Prior to combining 5-FC and radiation, experiments were performed to demonstrate the radiosensitizing effects of 5-FU. These results are shown in Fig. 2. Cells were exposed to 1 μg/ml 5-FU for 24 h either before or after irradiation because the drug was not toxic to the cells at this concentration and treatment period (Fig. 1A). As shown in Fig. 2, the radiosensitizing effects of 5-FU were seen with both WiDr and WiDr-CD.
cells only when the cells were exposed to the drug before irradiation.

Effects of 5-FC Concentration on Radiosensitization. Fig. 3 shows the radiation enhancement of WiDr-CD cells as a function of 5-FC concentration. Cells were exposed to 5-FC for 72 h prior to a single-dose irradiation (8 Gy). The survival of irradiated cells decreased gradually as the drug concentration increased from 0 to 30 μg/ml. For example, at 20 μg/ml 5-FC, the drug-alone treatment reduced cell survival by 2-fold, whereas the survival of cells treated with the drug prior to irradiation was reduced by 100-fold.

Effects of 5-FC Treatment Time on Radiosensitization. Fig. 4 shows the radiation enhancement of WiDr-CD cells as a function of drug treatment time. Cells were exposed to 20 μg/ml 5-FC for various periods of time prior to a single radiation dose of 8 Gy. No enhancement of radiation response by 5-FC was seen in the first 24-h exposure to the drug. As treatment time increased to 48 and 72 h, radiation-induced cytotoxicity by 5-FC was clearly observed. The results suggest that cellular conversion of 5-FC to 5-FU by WiDr-CD cells may require at least 24 h to reach toxic concentration.

Selective Radiosensitization of WiDr-CD Cells by 5-FC. Fig. 5 illustrates the survival curves of WiDr and WiDr-CD cells exposed to 5-FC (20 μg/ml) for 72 h before or after irradiation. Toxicity from the drug alone was minimal to moderate during the treatment period as cell survival was reduced approximately to 50% in WiDr-CD cells (Fig. 1B). As expected, the radiosensitizing effect by 5-FC was observed only in WiDr-CD cells exposed to the drug before irradiation (Fig. 5B). The radiosensitivity of parental WiDr cells was not altered by the drug, even at 100 μg/ml (Fig. 5A). As shown in Fig. 5B, WiDr-CD cells exposed to 5-FC (20 μg/ml) prior to irradiation showed a substantial enhancement (sensitization enhancement ratio = 2.38) of radiation-induced cytotoxicity.

DISCUSSION

The present study demonstrates clearly that 5-FC selectively enhances the radiation-induced lethality of human colorectal carcinoma cells expressing the bacterial CD gene (Fig. 5B). The results also indicate that the presence of the CD gene is necessary for the conversion of 5-FC to 5-FU. It appears that conversion of 5-FC to 5-FU by WiDr-CD cells may require at least a 72-h exposure time to the drug for maximum enhancement. Recently, metabolic studies conducted by Huber et al. (11) confirm that tumor cells expressing CD convert 5-FC to 5-FU and 5-FU metabolites, and 5-FU is liberated from WiDr-CD cells in significant quantities. 5-FU can readily diffuse into and out of cells by nonfacilitated diffusion (12).
Radiosensitization by 5-FC

The degree of enhanced cell kill by 5-FC was clearly dependent on drug concentration and exposure time to the drug before irradiation (Figs. 3 and 4). In particular, the potentiating effect was evident only in preirradiated cells. Exposure of cells to the drug after irradiation did not enhance the radiation response, suggesting that 5-FC does not interfere with postirradiation repair process. Cell death following treatment with 5-FU is thought to result from 5-FU anabolism to 5-fluorodeoxyuridine monophosphate, which can inhibit thymidylate synthetase, thereby blocking DNA synthesis, and 5-fluorouridine triphosphate incorporation into RNA, thus disrupting the function of RNA (13, 14).

Despite the clinically favorable results of combined modality treatment regimens using 5-FU and radiation therapy for the treatment of a variety of common human malignancies, laboratory studies on the interaction between radiation and 5-FU are limited and contradictory. Byfield (15) and Byfield et al. (16) showed that treatment of HT-29 and HeLa cells with 5-FU and radiation led to a time-dependent enhancement of cell killing. Only postirradiation incubation with 5-FU demonstrated enhanced cell killing (15, 16). However, data from von der Maase (17) and others (18) showed no effect of 5-FU on radiation sensitivity in C3H/Tif mammary carcinoma and leukemia cells. Smalley et al. (19) reported radiosensitization in both the HT-29 and DU-145 cell lines when 5-FU was present continuously following irradiation. Neither showed significant additional radiosensitization when 5-FU was present overnight (~18 h) prior to irradiation. In contrast, Bruso et al. (20) reported that sensitization occurred when fluorodeoxyuridine, a derivative of 5-FU, was present for at least 8 h before irradiation in HT-29 colon cancer cells. These differences suggest that radiosensitization by 5-FU may be cell-line dependent, which may explain, in part, some of the contradictory results regarding 5-FU radiation interaction.

The approach to potentiate the radiosensitizing effect of 5-FC in colorectal carcinoma cells transduced with CD would be through the use of biochemical modulators. One of the most promising of these agents is leucovorin. The leucovorin metabolite, 5,10-methylene tetrahydrofolate, forms a stable ternary complex with 5-fluorodeoxyuridine monophosphate and thymidylate synthetase leading to more effective enzyme inhibition (21). It has been demonstrated that leucovorin potentiates the cytotoxicity of fluoropyrimidines against colon cancer both in vitro and in clinical trials (22-24).

The present results suggest that combined gene therapy and radiation therapy may have therapeutic potential in the treatment of human colorectal carcinomas. Weichselbaum et al. (25) were the first to introduce gene therapy in an attempt to improve the outcome of radiotherapy. Kim et al. (26) recently reported the first demonstration of a selective enhancement by an antiviral agent of the radiation-induced killing of human glioma cells transduced with the herpes simplex virus-thymidine kinase gene. We are currently investigating the potential of gene therapy, nontoxic prodrug 5-FU administration and radiotherapy in nude mice to assess tumor response and normal tissue toxicity. One of the specific problems with the application of gene therapy in patients would be in vivo delivery of the therapeutic gene to a sufficient number of tumor cells to produce a clinically observable effect. However, several approaches may improve the transduction efficiency in vivo. For example, multiple fractionated implantation of packaging cells into the target tumor tissue may overcome the difficulty of transducing noncycling tumor cells, since fractionated radiation would recruit noncycling cells into the cycling phase. Another approach will be the use of a retroviral vector containing only one promoter. The advantage of such vectors is that they do not exhibit promoter suppression, a phenomenon commonly observed with two promoter vectors (27-29). We have successfully devised and used retroviral vectors that allow for the expression of two genes from a single promoter, which produce over 10 times the CD (and cell killing) activity than double promoter vectors. Finally, adenovirus or aden-associated viral vectors may overcome the limitations of retroviral vectors which are effective only with replicating cells, although this approach would transduce the adjacent critical normal tissue.

![Cell survival curves of WiDr (A) and WiDr-CD (B) cells following the combined treatment of 5-FC and irradiation. Cells were exposed to 5-FC for 72 h before and after single-dose irradiation. ▼, radiation alone; △, 5-FC (100 μg/ml) + irradiation; ○, 5-FC (20 μg/ml) + irradiation. Bars, SEM of six replicates per point.](clincancerres.aacrjournals.org)
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