Adenoviral-mediated Wild-Type p53 Gene Expression Sensitizes Colorectal Cancer Cells to Ionizing Radiation1

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ABSTRACT

Wild-type p53 gene transfer into the SW620 colorectal carcinoma cell line was performed using the replication-defective adenovirus Ad5/CMV/p53 to evaluate the effect of wild-type p53 expression on radiation sensitivity. The results indicated that infection with Ad5/CMV/p53 sensitized the cells. The survival at 2 Gy was reduced from 55 to 23%. Flow cytometric analysis of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay-labeled cells and in situ TUNEL staining of xenograft tumors demonstrated an increase in labeled cells with combination treatment, indicating increased apoptosis in cells treated with Ad5/CMV/p53 before irradiation. A significant enhancement of tumor growth suppression by this combination strategy was observed in a s.c. tumor animal model compared to p53 gene therapy alone. The delay in regrowth to control tumor size of 1000 mm3 was 2 days for 5 Gy, 15 days for Ad5/CMV/p53, and 37 days for Ad5/CMV/p53 + 5 Gy, indicating synergistic interactions. These data indicate that the delivery of wild-type p53 to cells with p53 mutations increases their radiation sensitivity, and this may be accomplished by adenoviral-mediated gene therapy.

INTRODUCTION

Mutations in the p53 tumor suppressor gene are among the most frequently detected genetic abnormalities in human malignancies such as breast, lung, and colon cancer (1). It has been estimated that up to 70% of colorectal cancers have a defect in this gene (2). The function of the p53 gene is not completely understood, but its protein product can function as a transcription factor that is known to have important roles in regulating the cell cycle and apoptosis following exposure of the cell to DNA-damaging agents (3, 4). Cellular p53 protein levels rise quickly in response to treatment with DNA-damaging agents, including ionizing radiation (5). A p53-induced G1 checkpoint may then allow for DNA repair before entry into S phase or, if repair is not possible, the cell may be deleted by apoptosis (6). Recent studies have confirmed that induction of apoptosis by the agents commonly used in cancer treatment, such as chemotherapeutic drugs and ionizing radiation, is highly dependent on normal p53 function (7, 8). Thymocytes from p53 null mice have been demonstrated to be resistant to radiation-induced apoptosis compared to mice with functioning p53 (9, 10). Similar results have been obtained with transformed fibroblasts and cells of hematopoietic origin (11–13).

Several studies have shown that transfection with wild-type p53-expressing plasmids can directly drive cells into apoptosis and/or growth arrest when p53 is overexpressed, suggesting a gene therapy approach for tumor cells lacking normal p53 function (14–16). Recently, three studies using vectors appropriate for gene therapy, such as retrovirus or adenovirus that carry wild-type p53, have illustrated its potential (17–20). However, the limitations of these approaches are that not all cells are infected by the vector, not all infected cells result in adequate transgene expression, and not all cells that express the p53 transgene ultimately die. These and other factors may result in the eventual reestablishment of the tumor if no additional treatment is given. A strategy to overcome this limitation would be to combine p53 gene therapy with a DNA-damaging agent to increase p53-dependent cytotoxicity. This strategy was successful in a previous study in which human lung cancer cells were treated with adenovirus-mediated p53 expression combined with cisplatin (21). In the present study, we sought to test whether wt-p53 gene delivery to cancer cells with p53 mutations can enhance their sensitivity to ionizing radiation. The results indicate that adenoviral-mediated delivery of the wt-p53 gene to SW620 human colorectal adenocarcinoma cells results in an enhancement of their sensitivity to ionizing radiation when treated in vitro and in vivo as xenograft tumors.

MATERIALS AND METHODS

Cell Culture and Xenografts. The SW620 human colorectal carcinoma cell line that has a mutation in p53 (codon 273, Arg→His; Ref. 22) was obtained from the American Type...
Culture Collection. The cells were maintained in RPMI 1640 supplemented with 10% FCS, 10 mm glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Life Technologies, Inc., Grand Island, NY) and incubated at 37°C in a 5% CO₂ incubator.

SW620 xenograft tumors were established by s.c. injection of 5 × 10⁶ viable cells (by trypan blue exclusion), suspended in HBSS into the hind legs of 6–8-week-old athymic nude mice (nu/nu; Charles River, Wilmington, MA). Within 10–14 days, tumors reached a size of ~200 mm³. Tumor growth delay was assessed following treatment. Tumors were measured every other day in two orthogonal dimensions, and volume was estimated assuming an ellipsoid shape. Animals were sacrificed when the tumor diameter exceeded 2 cm or the tumor ulcerated.

Adenovirus Production. The construction and properties of the Ad5/CMV/p53 have been reported elsewhere (21, 23). The Ad5/CMV/β-gal virus was kindly provided by F. Graham (McMaster University, Hamilton, Ontario, Canada). The E1A-deleted vector dl312 (kindly provided by T. Shenk, Princeton, NJ) was used as a control vector. Adenovirus was prepared as described previously (24) and purified by two rounds of cesium chloride ultracentrifugation. Purified virus was mixed with 10% glycerol and dialyzed twice against 1000 ml of a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, and 10% glycerol at 4°C for 6 h. Purified virus was aliquoted and stored at −80°C until used. Viral titer was determined using plaque assay (pfu/ml; Ref. 25). Final viral concentrations for in vitro and in vivo infections were made by dilution of stock virus in PBS. Adenovirus preparations were free of replication-competent adenovirus as determined by techniques described previously (25).

Gene Delivery. In vitro transfection studies for all cell lines were performed by plating 5 × 10⁶ cells in T25 flasks (Falcon Plastics, Lincoln Park, NJ). Forty-eight h after plating, cells were incubated for 2 h with purified virus in 1 ml of RPMI 1640 supplemented with 2% FCS. The MOI was based on cell counts of untreated flasks. A MOI of 50 was used for in vitro experiments. After 2 h, fresh RPMI 1640 supplemented with 10% FCS was added to the flasks.

In vivo experiments were performed on s.c. xenograft tumors induced in nude mice of ~200 mm³. A uniform injection strategy was used. Each injection of purified virus was diluted in a total volume of 200 μl of PBS and was administered in a single pass of a 27-gauge hypodermic needle, using gentle, constant infusion pressure. A total viral dose of 7.5 × 10⁹ pfu was administered in divided doses on 3 consecutive days.

Radiation. For in vitro treatments, cells were irradiated with a high dose-rate ³⁵Cl Cs source (4.5 Gy/min) at room temperature in 100-mm culture dishes. For in vivo treatments, animals bearing SW620 xenograft tumors were irradiated while anesthetized using a ⁶⁰Co teletherapy unit. The mice were positioned in the field such that only the hind leg bearing the tumor was in the irradiation field and the rest of the body was shielded by a lead block.

Clonogenic Assays. The effectiveness of the in vitro treatments was assessed by clonogenic assays. Briefly, monolayers of SW620 cells were treated in 100-mm dishes as described above, and then at various times following treatment, cells were trypsinized and counted. Known numbers were then replated and returned to the incubator to allow macroscopic colony development. Colonies were counted after about 14 days, and the percent plating efficiency and fraction surviving a given treatment were calculated based on the survival of non-irradiated cells treated with either PBS, dl312, or Ad5/CMV/p53.

TUNEL Assay. The TUNEL assay was performed using the procedure described by Gorczyca et al. (26). Briefly, fixed cells were washed in PBS and resuspended in 50 μl of TdT buffer with 5 units of TdT enzyme (Sigma Chemical Co., St. Louis, MO) and 0.5 nmol biotin-16-dUTP (Boehringer Mannheim Co.). Controls were prepared without TdT enzyme. Cells were incubated at 37°C for 1 h, rinsed in PBS, and resuspended in 100 μl of avidin-FITC (2.5 μg/ml; Boehringer Mannheim Co.) in saline-citrate buffer containing 0.1% Triton X-100, 0.1% BSA, 0.5 mM NaCl, and 0.06 mM sodium citrate. Specimens were incubated in the dark for 30 min, washed in PBS with 0.1% Triton X-100, and resuspended in propidium iodine (5 mg/ml) and 0.1% RNAse A. After incubation for 30 min, the specimens were analyzed with the use of an EPICS Profile II flow cytometer (Coulter Corp., Hialeah, FL). An analysis region was set based on the negative controls, and the percentage of labeled cells was calculated from this region.

In Situ TUNEL Assay for Apoptosis. In situ TUNEL assay was performed on paraffin-embedded tissue sections according to the procedure described elsewhere (27). SW620 cells used as positive controls were treated with DNase I (Life Technologies, Inc.) for 1 h at 37°C (50 μg/ml in 10 mM Tris-HCl (pH 7.5), 140 mM sodium cacodylate, 4 mM MgCl₂, and 1 mM DTT).

Statistical Analysis. ANOVA and two-tailed Student's t test were used for statistical analysis of multiple groups and pair-wise comparison, respectively, with P < 0.05 considered significant.
a

Fig. 2  
a. effects of Ad5/CMV/p53 and 5 Gy irradiation on in vitro TUNEL assay labeling of SW620 cells (TUNEL assay): flow cytometric analysis. 
Control, PBS alone; Adp53, Ad5/CMV/p53. 
b. effects of Ad5/CMV/p53 and 5 Gy irradiation on in vitro TUNEL assay labeling of SW620 cells (TUNEL assay). 
Percent Staining, percentage of cells stained via TUNEL assay. Each point is the mean of three independent experiments; bars, SD. 
Cells were irradiated 48 h following infection and harvested 24 h after irradiation or 72 h after infection.
RESULTS

Ads5/CMV/p53 Infection Sensitizes SW620 Cells to Irradiation in Vitro. We performed clonogenic assays on SW620 cells infected with either Ads5/CMV/p53 or dl312 (control vector) 48 h before irradiation in vitro to investigate the role of wt-p53 delivery on cell survival following ionizing radiation. The 48-h time course was chosen based on preliminary results, which demonstrated maximum effects at this time point (data not shown). The MOI used for these cells resulted in 60–80% transduction efficiencies based on an analysis using Ads5/CMV/β-gal, which expresses the reporter gene β-gal (data not shown). The results of the clonogenic assay experiments are presented in Fig. 1. Delivery of the wt-p53 gene resulted in a sensitization to irradiation of the cells that survived infection. A significant reduction in the percent survival was observed at doses of 2 and 4 Gy. These doses were chosen to approximate doses used in the clinical setting. This represents a 50–66% reduction in survival following radiation for the Ads5/CMV/p53-infected cells compared to controls (P < 0.05). The control vector dl312 had no significant effect on survival following irradiation. The values for percent survival in Fig. 1 have been corrected for the cytotoxicity of dl312 or Ads5/CMV/p53 viral infection alone.

Ads5/CMV/p53 Infection Enhances Apoptosis following Irradiation in Vitro. Flow cytometric analysis of TUNEL assays was performed on the SW620 cells 24 h following irradiation with 5 Gy, and the percentage of apoptotic cells was estimated on the basis of TUNEL assay labeling. The TUNEL method is based on detection of DNA strand breaks to identify apoptotic cells, and flow cytometric analysis allows for an estimation of cell populations. The 24-h time point was determined from preliminary data, indicating that apoptotic cells were detectable at this time. As before, infections with either Ads5/CMV/p53 or dl312 were performed 48 h before irradiation. Fig. 2a presents representative flow analysis for each group. The level of fluorescence, which indicates DNA nick end labeling, was relatively low for all groups, except for the cells treated with Ads5/CMV/p53, and greatest with the combination of Ads5/CMV/p53 and 5 Gy. Also, the –2N population of cells, which shows high fluorescence and is consistent with apoptotic cells, is more prominent in the combination treatment group. Fig. 2b graphically presents the cumulative data for the analysis of percentage of cell population labeled via the TUNEL assay. Five Gy alone resulted in only a very slight increase (1.0%) in the proportion of TUNEL assay-labeled cells compared to PBS-treated controls (0.33%). Treatment with the control vector dl312 also had little effect. As expected, Ads5/CMV/p53 infection alone resulted in a modest proportion of TUNEL-labeled cells, 29.5%. However, the 5-Gy treatment of Ads5/CMV/p53-infected cells resulted in a substantial enhancement in the proportion of TUNEL-positive cells (67%) above any of the appropriate controls (P < 0.05). This is consistent with an enhanced apoptotic response due to the combined treatments.

Combined Ads5/CMV/p53 Gene Therapy and Radiotherapy of SW620 Xenograft Tumors. To evaluate whether the in vitro observations described above translated into similar in vivo response, we examined the effects of Ads5/CMV/p53 plus radiation in a s.c. tumor model. SW620 xenograft tumors growing in the hind legs of nude mice were treated with three
consecutive intratumoral injections of Ad5/CMV/p53 (total dose, 7.5 × 10^9 pfu). On day 4, the tumors were treated with a single dose of 5 Gy local irradiation. The dl312 virus was used as a control vector. Histological sections for specimens obtained on day 5, 48 h following final intrascleral injection, are presented in Fig. 3. A central region of cell death was observed in tumors treated with Ad5/CMV/p53 (Fig. 3b) when compared with PBS-treated tumors (Fig. 3a). In situ TUNEL assay staining of the regions of apparent viable tumor cells are presented in Fig. 3, c–h. Consistent with our in vitro data, increased staining was present in tumors that received the combination of Ad5/CMV/p53 and 5 Gy treatment (Fig. 3h) when compared to the other treatment groups (Fig. 3, c–g). The extent of growth delay of the SW620 xenograft tumors achieved with the various treatments is illustrated in Fig. 4. Whereas 5 Gy alone and control virus had minimal effects and Ad5/CMV/p53 virus alone achieved a modest growth delay, a significant growth suppression was observed in tumors treated with Ad5/CMV/p53 plus 5 Gy irradiation (P < 0.01). The delay to regress to 1000 mm^3 compared to the PBS-treated control group was 2 days for treatment with 5 Gy, 15 days for treatment with Ad5/CMV/p53, and 37 days for the combination of Ad5/CMV/p53 and 5 Gy, indicating a greater than additive effect of the combination. The additional treatment of 5 Gy irradiation to dl312-treated tumors resulted in no measurable change in tumor growth (4 days for dl312 treatment alone and 3 days for dl312 treatment plus 5 Gy).

DISCUSSION

In this study, we have demonstrated that in addition to killing a substantial proportion of cells as a single modality, adenoviral-mediated wt-p53 delivery to colorectal carcinoma cells with p53 mutations results in an enhanced sensitivity of the survivors to ionizing radiation. This was demonstrated both in vitro using clonogenic survival assays and in vivo in terms of delayed xenograft tumor growth. Responses of xenograft tumors to intralesional treatment with Ad5/CMV/p53 have varied with cell type and cell line (18, 19, 21). The modest growth delay observed following treatment with Ad5/CMV/p53 of SW620 xenografts is likely related to a relative resistance to p53-dependent cell death. The combination of Ad5/CMV/p53 treatment and 5 Gy irradiation resulted in a significantly greater growth suppression. One potential mechanism for this phenomenon may involve wt-p53-dependent apoptotic pathways. This is supported by our TUNEL data, which indicated increased apoptosis in the cell cultures and xenograft tumors following the combined treatments. Adenoviral-mediated delivery of the wt-p53 gene to the cancer cells in cell culture and s.c. xenograft tumors will result in an induction of apoptosis in a portion of the cell population; however, a population of cells may remain that did not respond by apoptosis. This may be the result of incomplete infection, inadequate p53 transgene expression, and/or failure of the wt-p53 expression to induce cell death. It is this population of cells that we are targeting and that appears to be more sensitive to ionizing radiation.

Studies using thymocytes, transformed fibroblasts, sarcoma cell lines, and hematopoietic cell lines (8–13) indicate that p53 is a mediator of radiation-induced apoptosis. However, some data have led to questions of the role of p53 in radiation sensitivity of epithelioid tumors. Brachman et al. (28) demonstrated no correlation between p53 status and radiosensitivity in head and neck squamous cell carcinoma cell lines. No differences were found in the radiosensitivity of colorectal cell lines with and without p53 mutations (29).

These studies have used a single colorectal carcinoma cell line with a p53 mutation. The differences in response to irradiation of the PBS-treated cells and the Ad5/CMV/p53-infected cells can be attributed to adenoviral-mediated, CMV promoter-driven delivery of wt-p53. The expression of wt-p53 peaks at postinfection day 2–3 (14-fold greater than endogenous expression) and decreases to a low level by day 9 (18). Nonspecific adenoviral infection-related effects are unlikely to be responsible for the effects observed in this study, given the lack of sensitization to radiation of the dl312 control vector-infected cells. This indicates that when studied as an independent factor, transient overexpression of wt-p53 sensitizes colorectal cancer cells to radiation-induced injury.

The role of radiotherapy in the treatment of human malignancies continues to expand. In rectal carcinoma, it is an important adjuvant and improves local control. Our studies have demonstrated that Ad5/CMV/p53 infection induces cell death in colorectal cancer cells and increases the radiation sensitivity of the surviving cell populations. The radiation doses delivered in these studies are in the range of doses used in the treatment of the cancer patient. The s.c. tumor model demonstrates that the radiosensitivity of established colorectal carcinoma cell line tumors can be modulated by in vivo adenoviral-mediated wt-p53 gene transfer. Clinical studies are under way to evaluate adenoviral-mediated wt-p53 gene therapy in other human malignancies. Therapy using the combination of radiation and adenoviral-mediated wt-p53 gene delivery may be a useful multimodality approach to the treatment of colorectal carcinoma and other solid malignancies.

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