Ionizing Radiation Inhibits Chemotherapy-induced Apoptosis in Cultured Glioma Cells: Implications for Combined Modality Therapy

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ABSTRACT

Surgical resection followed by radiation therapy is the mainstay of treatment for glioblastoma multiforme (GBM), the most aggressive of the malignant gliomas. The poor clinical response of GBM and the intrinsic radiation resistance of this tumor type have prompted clinical investigations seeking to define the role of chemotherapy in the treatment of GBM. In this study, we examined the cytotoxic response of GBM-derived cell lines to treatment with both radiation and chemotherapy. We observed that the sensitivity of glioma cells to cisplatin- and FAS-induced apoptosis was diminished by prior treatment with ionizing radiation. Radiation conferred resistance to cisplatin and FAS cytotoxicity in a dose- and time-dependent manner. Radiation diminished the cisplatin-induced cytotoxicity of malignant glioma cells but failed to alter the cisplatin susceptibility of normal primary human astrocytes. Given the role of p53 in the response of cells to irradiation, we evaluated whether p53 function affects the observed radiation-induced resistance to cisplatin. By examining isogenic cell lines differing only in p53 function, we demonstrated that radiation conferred resistance to cisplatin independently of p53. Current clinical strategies in the treatment of astrocytic tumors, which include combined modality therapy, have been empirically derived from limited clinical experience. Further understanding of the molecular determinants of apoptosis associated with combined modality therapy may guide the design of more efficacious multimodality protocols.

INTRODUCTION

Malignant gliomas are the most common primary tumor of the adult central nervous system (1). Radiation therapy is the most important adjuvant treatment for patients with GBM1 (2), the most aggressive of the malignant gliomas (3). Randomized clinical trials have shown that adjuvant treatment with ionizing radiation provides a survival advantage for patients after surgery; however, despite the use of radiation therapy and intensive clinical efforts to improve therapy, the median survival remains less than 1 year (1, 2, 4). The poor clinical response of GBM tumors and their intrinsic radiation resistance (5–7) have prompted clinical investigations seeking to define the role of chemotherapy in the treatment of GBM (8).

In numerous malignancies, combined modality therapy uses chemotherapy in conjunction with radiation. Combined modality therapy serves several purposes such as allowing reduced radiation doses when used in Wilms' tumor (9) and medulloblastoma (10, 11); organ preservation, as exemplified by its use in laryngeal (12) and bladder (13) cancer; increased rates of local control, as evident in anal (14, 15) and rectal cancer (16, 17); and prolonged survival, as observed in nasopharyngeal (18, 19) and pancreatic cancer (20, 21). Several recent promising clinical trials suggest that the sequence of therapies administered in the adjuvant setting is a factor in determining the efficacy of combined modality therapy (22–24); however, the sequencing of chemotherapy and radiation in these trials is only rarely based on a biochemical or biological rationale (25).

In this study, we sought to investigate the effect of sequencing on radiation- and chemotherapy-induced cytotoxicity in cultured cells derived from malignant astrocytic tumors as well as in primary astrocytes, the normal tissue in which these tumors arise. We observed that the sensitivity of glioma cells to cisplatin-induced apoptosis is diminished by prior treatment with ionizing radiation, but this effect is not observed in primary human astrocytes. Furthermore, we observed that these effects on cisplatin-induced apoptosis are not altered by fractionating the administration of radiation, the most clinically relevant mode of radiotherapy delivery. Glioma cells are also susceptible to apoptosis induced by antibodies to FAS, a cytokine receptor belonging to the nerve growth factor/tumor necrosis factor receptor family (26). Activation of this receptor triggers the assembly of a death-inducing signaling complex at the cell membrane that activates a family of cysteine proteases, leading to apoptosis (27). Unexpectedly, we observed that the sensitivity of glioma cells to FAS-induced apoptosis is also diminished by prior treatment with ionizing radiation.

MATERIALS AND METHODS

Cell Culture and Treatment. All cell lines were maintained as exponentially growing monolayer cultures in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.), penicillin, and streptomycin in a humidified incubator at 37°C and 5% CO2. The retroviral vector construction and retrovirus infection procedure used in generating the clonal derivatives of U-87 MG have been described previously (28). Briefly, clonal derivatives expressing either the firefly luciferase (lux) gene (U87-LUX.8 and U87-LUX.6) or a mutant p53 cDNA (U87-175.4 and U87-175.13) under the control of the Moloney murine leukemia virus long terminal repeat were prepared. The retrovirus encoding mutant p53 contained p53 cDNA mutated at codon Arg175→His (29). Retrovirally infected clones were derived by limiting dilution cloning and grown continuously in media containing 400 μg/ml G418 (Geneticin; Life Technologies, Inc.). Normal human astrocytes were isolated from second-trimester elective abortion fetal brain by established methods (30) and confirmed to be astrocytic by uniform staining (data not shown) with an anti-glial fibrillary acidic protein antibody (Boehringer Mannheim). Cells were grown in media prepared from DMEM H-21 with 10 ng/ml epidermal growth factor, 10 ng hydrocortisone, 10 ng/ml insulin, insulin-transferrin-selenium mixture supplement, 50 mg/ml transferrin, 10 ng/ml bixin (all from Sigma), and 1% bovine pituitary extract (Clonetics). This was mixed with DMEM and 10% FCS at a 1:5 ratio. Cells were passaged at confluence by trypsinization and used at population doubling 5–10.

At the start of each experiment, cells were seeded into independent wells at a density of 50–100 cells/mm2. Irradiation of cultures was performed at room temperature in a 150 kV Philips X-ray machine, without a filter, at a dose rate

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5 The abbreviations used are: GBM, glioblastoma multiforme; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PI, propidium iodide; FACS, fluorescence-activated cell-sorting.
RADIATION INHIBITS APOPTOSIS ASSOCIATED WITH CHEMOTHERAPY

Fig. 1. Radiation inhibits cisplatin-induced cell death. A, glioma tumor cell lines were treated with 0 (■) or 8 (▲) Gy of ionizing radiation 24 h before treatment with 20 μM cisplatin. Cell viability was assessed after 2 days of incubation with cisplatin, and the percentage of viability was plotted relative to the number of cells in each treatment group at the beginning of cisplatin treatment. U343 glial tumor cells (■) and T98G glioma tumor cells (▲) were treated with 0 (■) or 8 (▲) Gy of ionizing radiation 24 h before treatment with 20 μM cisplatin and analyzed as described above (A) over a 10-day observation period. Each experimental value is the average of triplicate determinations in a single experiment representative of two comparable experiments. The error bars indicate the SE.

Assessment of Cisplatin- and Anti-FAS Antibody-mediated Cytotoxicity and Apoptosis. Cell proliferation and viability were determined by colorimetric detection of acid phosphatase activity (Abacus Kit; Clontech) and trypan blue dye exclusion. Nonadherent and trypsinized adherent cells were counted together. DNA fragmentation was determined using the TUNEL method (Oncor). Cells collected by trypsinization were pooled with the nonadherent cells, washed with PBS, and fixed for 30 min in 2% paraformaldehyde in PBS. The cells were then washed in PBS and resuspended in 70% ethanol at 4°C for 1 h, and cytopsins were prepared on Superfrost slides (Fisher). Air-dried samples were then microwaved on medium power (approximately 400 W) for 5 min. Apoptotic cells were labeled with digoxigenin-conjugated dUTP using terminal deoxynucleotidyl transferase according to the distributor’s instructions. Quantitative analysis of apoptosis was achieved by flow cytometric analysis of the subdiploid DNA content in fixed cells. After treatment of exponentially growing cell cultures, cells were collected by trypsinization, pooled with the nonadherent cells, washed with PBS, and fixed for 10 min in 70% ethanol. Cells were washed again in PBS, resuspended in PBS containing 50 μg/ml RNase, and stained with PI at a concentration of 5 μg/ml. DNA content, as measured by PI staining, was evaluated using a Becton Dickinson fluorescence-activated cell analyzer with Lysis II software. Of 1.2 Gy/min. For fractionated delivery of radiation, cells received 2 Gy of radiation each day for 4 days, for a total of 8 Gy. During experiments involving radiation treatment followed by cytotoxic drug treatment, cells in all treatment groups were seeded over a range of densities. Data were derived from replicate samples with similar cell densities at the beginning of drug treatment. To examine the cytotoxicity of cisplatin (Sigma), cells were incubated at a concentration of 20 μM for 24–48 h. Fresh media with cisplatin were added every 48 h for longer incubations. Anti-FAS antibody (Kamiya Biomedical Co.) was added to cultures at a concentration of 0.01 ng/ml in combination with 10 μg/ml cycloheximide (Sigma) for 5 h. After this incubation, the cells were rinsed and incubated in fresh medium. For treatment with aphidicolin (Sigma), cells were exposed to 1 μg/ml aphidicolin. Cells were given fresh medium with aphidicolin every 24 h.
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RESULTS

We examined a panel of radioresistant glioma tumor cell lines for their response to treatment with cisplatin alone or after exposure to ionizing radiation (Fig. 1A). All of the cell lines displayed significant cell death after 2 days of incubation with 20 μM cisplatin. All but one of the cell lines exhibited less cell death when the cells were exposed to 8 Gy of radiation before treatment with cisplatin. Cisplatin treatment killed only 22% of the population. Prior irradiation decreased the cisplatin-induced cell death of U-87 MG cells from 75 to 59%. The cytotoxic effect of cisplatin on T98G cells seemed to be unaffected by radiation treatment. Unlike U343 and U-87 MG cells, the T98G cell line harbors a mutation in the P53 gene. To examine a possible role of p53 in mediating this effect, we used transfected derivatives of the U-87 MG cell line to examine whether wild-type p53 function was required for cisplatin-induced cell death or for the radiation-induced resistance to cisplatin observed in the parent cells. The clonal derivative U87–175.4 expresses an exogenous dominant negative mutant p53 that functionally inactivates endogenous wild-type p53 protein, and U87-LUX.8 is a clonal derivative transfected with vector alone (28). We previously verified the effectiveness of dominant negative mutant p53 expression in this cell line by demonstrating that the radiation-induced G1 arrest observed in U-87 MG parent cells and U87-LUX.8 cells was greatly diminished in U87–175.4 cells (28). Radiation treatment administered 24 h before the addition of cisplatin was able to decrease the amount of cisplatin-induced cell death in the clonal derivatives of the U-87 MG cell line despite the differences in the functional status of their p53 (Fig. 1A).

The experimental protocol comparing the cytotoxic action of cisplatin treatment and treatment combining therapeutic radiation and cisplatin was repeated using U343 and T98G cells, and cell viability was assessed every 2 days for 10 days (Fig. 1, B and C). U343 cells treated with 8 Gy of ionizing radiation before incubation with cisplatin sustained greater viability throughout the entire observation period compared with cells that received no prior radiation (Fig. 1B). In contrast, irradiated and unirradiated T98G cells died at approximately the same rate after treatment with cisplatin (Fig. 1C).

We investigated whether reversing the order of administration of radiation and cisplatin would alter the effect of treatment on these
Radiation inhibits apoptosis associated with chemotherapy. A. 10
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B.
X-ray Dose (Gy).

Fig. 4. Resistance to cisplatin-induced apoptosis increases with radiation dose and decreases with time. A, U343 (O) and T98G (■) glioma tumor cells were treated with increasing doses of ionizing radiation 24 h before treatment with 20 μM cisplatin. The percentage of apoptosis was assessed by FACS analysis of cells with a subdiploid DNA content after 24 h of incubation with cisplatin. The percentage of cisplatin-induced apoptosis is plotted relative to that of parallel cultures not exposed to cisplatin. B. U343 glioma cells were treated with 8 Gy of ionizing radiation and incubated for increasing lengths of time as indicated before either sham treatment (Û) or treatment with 20 μM cisplatin (•). The percentage of apoptosis was assessed as described in A. Each experimental point is the average of triplicate determinations in a single experiment representative of two comparable experiments. The error bars indicate the SE.

Cisplatin is known to induce apoptosis in many glioma cell lines (31). We used the TUNEL method to verify that cisplatin-induced cell death of U343 cells was due to apoptosis. DNA fragmentation in individual U343 cells detected by the TUNEL method increased from less than 1% of the population in control cultures to approximately 50% of the population 48 h after treatment with cisplatin (data not shown). We used a FACS-based assay to quantitate apoptosis in U343 cells after treatment with cisplatin (Fig. 3). We detected an increase in the percentage of cells with a subdiploid DNA content beginning 24 h after treatment with cisplatin. The subdiploid population increased from less than 1% in the untreated control sample to 10% in the sample treated with cisplatin for 24 h. Exposure to 8 Gy of ionizing radiation 24 h before cisplatin treatment decreased the amount of cisplatin-induced apoptosis to approximately 3%. An independent experiment quantitating apoptosis 46 h after treatment with cisplatin also revealed an inhibitory effect of radiation on cisplatin-induced apoptosis. The subdiploid population decreased from 55% in cultures treated with cisplatin alone to 45% in cultures exposed to 8 Gy of radiation 24 h before cisplatin treatment (data not shown).

We found that the resistance to cisplatin-induced apoptosis was dependent on the radiation dose (Fig. 4A). U343 cells were exposed to increasing doses of ionizing radiation 24 h before treatment with cisplatin and then analyzed for subdiploid cells 24 h later. As the dose of radiation delivered to U343 cells before treatment with cisplatin increased, the extent of cisplatin-induced apoptosis decreased. Cisplatin-induced apoptosis of T98G cells did not change after exposure to...
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Fig. 6. Aphidicolin-induced G1 cell cycle arrest of glioma cells does not prevent cell death after treatment with cisplatin or anti-FAS antibody. U-87 MG glioma tumor cells were treated with aphidicolin for 2 days to arrest progression of the cell cycle and then either sham-treated (A), treated with cisplatin (O), or treated with anti-FAS antibody and cycloheximide (•). Cell viability was assessed during the incubation with aphidicolin and for 2 more days. The percentage of viable cells is plotted relative to the value obtained at the beginning of the aphidicolin treatment. Each experimental value is the average of eight independent determinations in a single experiment representative of two independent experiments. The error bars indicate the SE.

U-87 MG-derived cell lines that lack functional p53 do not undergo a G1 arrest after irradiation (Ref. 28; data not shown), suggesting that the resistance to apoptosis that we observed was independent of the cells' ability to progress through the cell cycle. We used aphidicolin to further investigate whether arresting the cell cycle in G1 could modulate resistance to cisplatin and anti-FAS antibody treatment. Aphidicolin arrests the cell cycle of eukaryotic cells by inhibiting nuclear DNA replication (32). We treated U-87 MG cells with 1 μg/ml aphidicolin for 2 days before exposure to either cisplatin or ionizing radiation at any of the doses tested. The resistance to cisplatin-induced apoptosis seen in U343 cells after exposure to ionizing radiation depends on time as well (Fig. 4B). U343 cells were exposed to 8 Gy of radiation and then incubated for increasing periods of time before treatment with cisplatin. Cultures treated with radiation alone were analyzed in parallel. As the time between radiation exposure and cisplatin treatment increased, the resistance to cisplatin-induced apoptosis diminished. When cisplatin treatment was administered 4 days after radiation, the amount of apoptosis detected 24 h after treatment with cisplatin returned to the level seen in cultures that had not received radiation (as depicted in Fig. 4A dose 0 Gy).

We assessed the ability of radiation to alter the response of glioma cell lines to another agent known to induce apoptosis. A series of glioma-derived cell lines were treated with anti-FAS antibody and cycloheximide 24 h after exposure to 0 or 8 Gy of ionizing radiation, and cell viability was assessed 24 h later (Fig. 5). FAS-induced cell death was inhibited in irradiated samples of U-87 MG and LN229 cells. The sensitivity of T98G cells to FAS-induced cell death was not changed by exposure to radiation. U343 cells were not susceptible to FAS-induced cell death (data not shown). We used transfected clonal derivatives of the U-87 MG cell line to address the question of whether wild-type p53 function was required for the radiation-induced resistance to FAS-induced cell death. The clonal derivatives U87-175.4 and U87-175.13 express an exogenous dominant negative mutant p53 protein that functionally inactivates endogenous wild-type p53 protein, and U87-LUX.8 and U87-LUX.6 are control clonal derivatives from our laboratory that have been described previously (28). Radiation treatment administered 24 h before the addition of anti-FAS antibody and cycloheximide decreased the amount of cell death in all four of the clonal derivatives of the U-87 MG cell lines to a similar extent, despite differences in the functional status of p53.

Fig. 7. Resistance to cisplatin-induced cell death after radiation treatment is not observed in primary human astrocytes. A, primary human astrocytes were divided into four experimental groups [untreated control cells (•) and samples exposed to 8 Gy of radiation (A), 20 μM cisplatin (B), or the combination of radiation and cisplatin (C)]. The percentage cell viability was determined relative to the average number of live cells in cultures at the beginning of cisplatin treatment (time 0). Samples receiving radiation treatment were exposed to 8 Gy of radiation 24 h before cisplatin treatment. Cell viability was assessed by trypan blue dye exclusion. Each experimental point is the average of three independent determinations in a single experiment. The error bars indicate the SE. B, the viability of primary human astrocytes was assessed by trypan blue dye exclusion for 4 days after treatment with 0 (•) or 8 (○) Gy of radiation 24 h before cisplatin treatment. Each experimental point is the average of three independent determinations in a single experiment. The error bars indicate the SE.
anti-FAS antibody and cycloheximide. Cell viability was assessed during the initial incubation with aphidicolin and for 2 days after cytotoxic treatment (Fig. 6). We determined that aphidicolin treatment effectively arrested these cells, because throughout the entire observation period, they remained viable and did not increase in number (data not shown). Cells exposed to aphidicolin for 2 days before exposure to cisplatin showed a loss of viability due to cisplatin (Fig. 6) that was of similar magnitude to that seen in cultures treated with cisplatin alone (Fig. 1A). Cells exposed to aphidicolin before exposure to anti-FAS antibody and cycloheximide showed a loss of viability similar to that seen in cultures treated with anti-FAS antibody and cycloheximide alone (Fig. 5). Thus, aphidicolin-induced cell cycle arrest did not protect the cells from either of the cytotoxic treatments.

We used primary human astrocytes as a model to determine whether radiation can protect the normal cells in which GBM arises from cisplatin-induced cell death (Fig. 7). Untreated primary astrocytes cultured in defined media proliferated with a doubling time of approximately 48 h. Exposure to 8 Gy of radiation stopped astrocyte proliferation without inducing cell death. In contrast to the data obtained with glioma tumor cell lines, radiation delivered before cisplatin treatment did not make astrocytes resistant to cisplatin-induced cell death in two independent experiments (Fig. 7A). A replication of the experiment using a different preparation of human astrocytes observed for 4 days after cisplatin treatment produced comparable results (Fig. 7B).

Because therapeutic radiation is often delivered as fractionated therapy, we adapted our experimental protocol to examine the effect of fractionated radiation treatment. Radiation was delivered to U343 cells in 2-Gy fractions on 4 consecutive days (cumulative total dose, 8 Gy). One day after the final fraction, the cells were treated with cisplatin, and cell viability was assessed over a 10-day period (Fig. 8). Parallel cultures were treated with cisplatin only. As seen when radiation treatment was delivered in a single 8-Gy dose, the fractionated radiation treatment inhibited the cytotoxic effect of cisplatin treatment, and the effect decreased with time after radiation exposure.

**DISCUSSION**

Malignant gliomas comprise 1% of all adult cancers but are responsible for 2.5% of all cancer-related deaths in the United States (33). The failure of current therapies to significantly prolong survival for patients with GBM has prompted continued clinical interest in combined modality treatment regimens. Sequencing regimens in clinical oncology include three commonly used approaches: (a) neoadjuvant chemotherapy, in which chemotherapy is administered before radiation; (b) adjuvant chemotherapy, in which chemotherapy is given after radiation; and (c) concurrent treatment. A molecular biological rationale for the superiority of a given sequencing regimen has not yet been established. In this study, we used an *in vitro* model to examine the cytotoxic response of GBM-derived cell lines to multimodality treatment. We chose cisplatin as the chemotherapeutic agent to be examined because the activity of cisplatin is not cell cycle dependent. Radiation perturbs cell cycle progression and may alter sensitivity to a cell cycle-specific agent by redistributing the cells within the cell cycle. We sought to exclude such a confounding variable and explore less obvious mechanisms of interaction between radiation and chemotherapy by choosing a cell cycle-independent agent such as cisplatin. Furthermore, in combined modality treatment of numerous human cancers, cisplatin represents a potentially important therapeutic agent, as demonstrated by the survival advantage it has conferred in recent Phase III randomized trials for a variety of tumors (14, 17, 18, 21, 22, 24, 25, 34, 35).

In this study, we demonstrated that radiation impairs the apoptotic response of glioma cells to cisplatin chemotherapy. Given the known function of p53 in modulating apoptosis (36), we examined the effect of p53 status on the observed radiation-induced resistance to cisplatin. By examining isogenic derivatives differing only in p53 function, we demonstrated that radiation-conferred resistance to cisplatin occurred independently of p53. In addition, we observed that radiation conferred such resistance in a dose- and time-dependent manner.

The mechanism by which radiation confers resistance to cisplatin and anti-FAS-induced apoptosis remains unclear. Both anti-FAS and cisplatin execute the apoptotic pathway by activating interleukin 1ß-converting enzyme, a key regulatory enzyme in the cascade of molecular events that mediate apoptotic cell death (31, 37). In addition, both agents share the capacity to induce apoptosis in a p53-independent manner. Radiation may conceivably diminish cisplatin and anti-FAS cytotoxicity by up-regulation of an apoptosis inhibitor such as the transcription factor nuclear factor κB. Pertinent to this hypothesis is the observation that the activation of nuclear factor κB by ionizing radiation was found to protect human fibrosarcoma cells from radiation-induced cell killing (38). Radiation-induced regulation of Bcl-2 gene family members is another potential mechanism of the resistance to apoptotic stimuli observed after radiation (39). In this regard, cyclic GMP-dependent transduction pathways may be important, because radiation-induced resistance to apoptotic stimuli is modulated by this pathway in cisplatin-resistant murine fibrosarcoma cell lines (40).

The clinical relevance of our data is suggested by several of the findings. Radiation decreased the cytotoxicity induced by either cisplatin and anti-FAS antibody, and this effect was seen only when exposure preceded chemotherapy. Resistance was induced when radiation was given 1 day before chemotherapy but not 4 days before chemotherapy treatment. Radiation diminished the cisplatin-induced cytotoxicity of malignant glioma cells but failed to alter the cisplatin susceptibility of normal primary human astrocytes. The lack of a comparable effect in normal tissue raises the possibility that the actual therapeutic index of combined modality therapy would be reduced even more than might be expected by the inhibition of apoptosis in tumor cells. That is, increased doses of cytotoxic therapy would have to be administered to achieve the same level of tumor cell killing, whereas increased toxicity to normal tissue would be predicted. This effect might also be expected in patients receiving fractionated radio-
therapy (41, 42), the most common form of radiation therapy, because such a scheme also inhibited cisplatin-induced cytotoxicity in our experiments.

Combined modality therapy has emerged as a critical approach to the successful treatment of many malignancies. Current clinical strategies for the treatment of astrocytic tumors involve a multimodality approach. Laboratory investigation of the molecular basis for radiation- and chemotherapy-induced cytotoxicity may help improve our understanding of how to optimally use these agents. Further understanding of the molecular determinants of apoptosis induced by combined modality therapy may also guide the design of new clinical multimodality protocols.

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