Fas (APO-1/CD95) Signaling Pathway Is Intact in Radioresistant Human Glioma Cells

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ABSTRACT

Radiation-induced apoptosis can be mediated through pathways initiated by either DNA damage or ceramide-induced Fas signaling. Glioblastoma multiforme is a primary brain tumor that is highly resistant to irradiation, and U-87 MG, SF126, and T98G are glioblastoma-derived cell lines that mimic this characteristic. We found that these radioresistant glioma cells are susceptible to Fas-mediated cell death induced by treatment with either anti-Fas antibody or exogenous ceramide. Fas-mediated cell death in these cell lines is p53-independent. These data demonstrate that apoptosis can be induced by ceramide and mediated through the Fas pathway in glioma cells, although high-dose ionizing radiation fails to trigger this pathway.

INTRODUCTION

Gliomas are the most common primary tumor of the adult human central nervous system (1). Despite current efforts to develop more effective clinical treatment strategies, median survival time for patients with WHO grade IV glioma, glioblastoma multiforme, remains approximately 1 year (1). An important factor contributing to this prognosis is the relative resistance of many gliomas to cytotoxic therapy in general and radiation therapy in particular (2). Nonetheless, radiation therapy is the most widely used modality in the management of gliomas (3). Radioresistance of tumor cells is frequently associated with defects in the pathways leading to apoptosis (4). For example, the product of the p53 tumor suppressor gene has been shown to be responsible for mediating apoptosis after DNA damage in several cell types, and the loss of wild-type p53 function can render cells resistant to radiation (5). Similarly, a relationship between resistance to radiation-induced apoptosis and defective ceramide signaling has been demonstrated in radioresistant tumor cells and in fibroblasts genetically deficient in ceramide synthesis (6, 7). Because activation of Fas signaling by ceramide mediates radiation-induced apoptosis in several cell types (8), we assessed the Fas signaling pathway as to whether it was functional in three highly radioresistant glioma cell lines, U-87 MG (9), SF126 (9), and T98G (10). We found that these cells are susceptible to ceramide-induced and Fas-mediated cell death.

MATERIALS AND METHODS

Cell Culture and Infection. 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 8% CO2. The retroviral vector construction and retrovirus infection procedure used in generating the clonal derivatives of U-87 MG have been described previously (11). Briefly, clonal derivatives expressing either the firefly luciferase (lux) gene, U87-LUX.8 and U87-LUX.6, or a dominant negative mutant p53 cDNA (U87–175.4 and U87–175.13) under control of the Moloney MuLV long terminal repeat were prepared. A retrovirus containing the p53 cDNA mutated at codon 175 (arg to his) was used to infect U-87 MG cells. Retrovirally infected clones were derived by limiting dilution cloning and grown continuously in media containing 400 μg/ml of G418 (Genetec; Life Technologies, Inc.).

TREATMENT OF CELLS. Irradiation of cell cultures was performed as indicated at room temperature in a 150-kV Philips X-ray machine, without a filter, at a dose rate of 1.2 Gy/min. Anti-Fas monoclonal antibody (Kamiya Biochemical Company) was added to cultures as indicated at a concentration of 0.01 ng/ml in combination with 10 μg/ml of cycloheximide (Sigma) for 5 h. After this incubation, the cells were rinsed and incubated in fresh medium. The ICE inhibitor tetrapeptide chloromethylketone Ac-Tyr-Val-Ala-Asp-CMK (Bachem Biosciences) was added to the culture medium (250 ng/ml) at the time of addition of Fas antibody when indicated. C2-ceramide (N-acetyl sphingosine, Calbiochem) stock solutions were prepared in DMSO. Before treatment with ceramide, cells were serum-starved as indicated for 24 h in medium without serum.

Assessment of 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 exclusion. Nonadherent and trypsinized-adherent cells were counted together. For clonogenic survival analysis, cells were trypsinized, counted, and plated at specified concentrations into wells containing an irradiated feeder layer. Cell cultures were then incubated for 21 days, at which time colonies of over 50 cells were counted. The plating efficiency was 30%. In each independent experiment, three different dilutions were made per treatment condition, and each dilution was plated in multiples of three. The presence of apoptotic cells in cultures treated with anti-Fas antibody was confirmed using labeled phosphodiesterase binding protein, Annexin V (TACS Annexin V-FITC; Trevigen). For evaluation of Annexin V binding, adherent cells were trypsinized in the presence of 2% BSA, combined with nonadherent cells and processed according to the manufacturer’s instructions. Cells were analyzed using a multwell fluorescence plate reader (CytoFluor 4000, PerSeptive Biosystems).

Quantitative analysis of apoptosis was performed by flow cytometric analysis of fixed cells to identify cells with a subdiploid DNA content. After ceramide treatment of exponentially growing cell cultures, cells were collected by trypsinization, pooled with 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 propidium iodide at a concentration of 5 μg/ml. DNA content, as measured by propidium iodide staining, was evaluated using a Becton Dickinson fluorescence-activated cell analyzer and Lysis II software.

RESULTS

U-87 MG human glioma tumor cells were treated with a single dose of 0, 8, or 20 Gy ionizing radiation, and their viability was evaluated for approximately 16 days (Fig. 1). Unirradiated cells continued to grow exponentially, doubling about every 2 days. Irradiated cells, however, were growth-arrested. Cell viability and cell density remained unchanged throughout the 16 days of evaluation in cultures
MG cells were incubated for 5 h with anti-Fas antibody in combination with varying doses of cycloheximide in their culture media (data not shown). Cells treated with anti-Fas antibody alone or cycloheximide alone displayed no sign of toxicity 24 h later. In contrast, cells receiving anti-Fas antibody treatment that included 1 μg/ml cycloheximide underwent approximately 43% cell death. Cell death increased as the concentration of cycloheximide was increased. The combination treatment of anti-Fas antibody with 10 μg/ml cycloheximide induced cell death in 90% of cells 24 h after a 5-h incubation. The potentiation of Fas-mediated cell death by cycloheximide has been reported previously and suggests that short-lived cytoprotective proteins synthesized by glioma cells confer resistance to Fas-mediated cell death (12). Combining radiation treatment with cycloheximide treatment did not alter the response of U-87 MG cells to radiation (5 h cycloheximide treatment beginning 2 h before or at the time of irradiation; data not shown).

We evaluated two additional glioma cell lines, SF126 and T98G, for susceptibility to Fas-mediated cell death. Like U-87 MG, these glioma cell lines were previously determined by clonogenic assays to be highly radioresistant (9, 10). These cell lines were incubated with anti-Fas antibody and cycloheximide for 5 h, washed with fresh medium, and 24 h later evaluated for cell viability. Each cell line treated with anti-Fas antibody underwent extensive cell death (Fig. 2). Both SF126 and T98G have been previously characterized and are known to express a mutant p53 protein (13). Although the U-87 MG cell line expresses wild-type p53 protein (13), the response of SF126 and T98G to anti-Fas antibody suggested that wild-type p53 function is not required for Fas-induced cell death in glioma-derived cells. To explore this possibility more critically, we used the U-87 MG clonal derivatives U87–175.4 and U87–175.13, which express an exogenously introduced, dominant negative mutant p53 that functionally inactivates endogenous wild-type p53 protein function (11). U87-LUX.6 and U87-LUX.8 are clonal isolates that express a control vector. We previously verified the effectiveness of dominant negative mutant p53 expression in these cell lines by demonstrating a greatly diminished cell cycle arrest after DNA damage in the U87–175.4 and U87–175.13 cells compared with the U-87 MG parent cells and the U87-LUX.6 and U87-LUX.8 clonal derivatives (11). Parental U-87 MG cells and the clonal derivatives of U-87 MG were examined as described above for anti-Fas antibody-induced cell death. Each of these cell lines underwent significant Fas-mediated cell death with approximately 55–95% of cells dying (Fig. 2). These data indicate that anti-Fas antibody treatment kills radioresistant glioma cells in a p53-independent manner. None of the cell lines examined in Fig. 2 exhibited evidence of cell death when a nonimmune IgG was substituted for anti-FAS antibody (data not shown).

The Fas signaling pathway can trigger apoptotic cell death in susceptible cells (14). In the experiments shown in Fig. 2, we used

![Fig. 1.](image1.png)

**Fig. 1.** U-87 MG glioma cells are susceptible to Fas-mediated cell death. U-87 MG glioma cells were exposed to ionizing radiation, 8 Gy (■) and 20 Gy (▲), or incubated with anti-Fas antibody in combination with cycloheximide (○), and the total number of viable cells was assessed every 4 days for 16 days using the trypan blue exclusion assay. Untreated cells (▲) were assessed only at days 0 and 4. Data points, the mean of triplicate determinations; bars, SE.

treated with either 8 and 20 Gy radiation. Parallel cultures of unirradiated cells were incubated for 5 h with anti-Fas antibody and cycloheximide, washed with fresh medium, and then evaluated at the indicated times for cell viability. Cultures treated with anti-Fas antibody contained no viable cells 4 days after treatment. Analysis of clonogenic survival of these cells following the same experimental treatments yielded similar results (data not shown). In two independent experiments, U-87 MG cells exhibited a relatively radioresistant phenotype (less than 2 log kill after treatment with 8 Gy) as described previously (9), but treatment with anti-Fas antibody and cycloheximide for 5 h completely eliminated the proliferative capacity of these cells (greater than 4 log kill). These data demonstrate that U-87 MG cells are resistant to the cytotoxic effects of high doses of ionizing radiation but are susceptible to Fas-mediated cell death.

To characterize further anti-Fas antibody-induced cell death, U-87

![Fig. 2.](image2.png)

**Fig. 2.** Anti-Fas antibody induces cell death of glioma-derived cell lines. SF126 (p53 mutant), T98G (p53 mutant), U-87 MG (p53 wild-type), and four independent clonal derivatives of U-87 MG were seeded in 96-well plates and treated with anti-Fas antibody and cycloheximide. Cell viability was assessed 24 h later using an acid phosphatase assay. The histogram plots percent cell death relative to control cultures treated only with cycloheximide. Solid bars, the mean of four determinations in a single experiment representative of 2 comparable experiments; bars, SE.
morphological examination and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling analysis to confirm that these cells died of apoptosis (data not shown). To further characterize apoptosis mediating the anti-Fas antibody-induced glioma cell death, we examined U-87 MG cells after antibody treatment for biological changes characteristic of apoptosis. Early in the apoptotic process phosphatidylserine becomes exposed on the cell surface by moving from the inner to the outer surface of the plasma membrane. This alteration in plasma membrane structure can be detected using Annexin V-FITC, which binds to phosphatidylserine and is detectable by fluorescence spectroscopy. We observed a dramatic increase in phosphatidylserine detectable on the surface of U-87 MG cells 5 h after treatment with anti-Fas antibody and cycloheximide relative to cells treated with anti-Fas antibody and cycloheximide relative to cells treated with anti-Fas antibody alone or with ionizing radiation (up to 20 Gy) either in the presence or absence of cycloheximide (Fig. 3).

We sought to further evaluate the involvement of apoptosis in the Fas-mediated death of U-87 MG cells by evaluating cells treated with anti-Fas antibody and cycloheximide in the presence of a tetrapeptide that inhibits caspases, acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-CMK). This synthetic peptide is able to inhibit apoptotic cell death mediated by the ICE in other cell systems. Analysis of cell viability 24 h after treatment revealed that anti-Fas antibody treatment of cycloheximide-treated U-87 MG cells incubated with the ICE inhibitor sustained a level of cell viability comparable to the level observed in cells treated with cycloheximide alone, whereas the viability of cells exposed to anti-Fas antibody without the ICE inhibitor decreased approximately 77% compared with these same control cells (data not shown).

Ionizing radiation rapidly induces the production of ceramide, which in turn can activate the Fas signaling pathway leading to apoptosis (8). We sought to determine whether the addition of exogenous ceramide would induce apoptosis in these glioma cells. We quantitated the induction of apoptosis after the addition of C2-ceramide to U-87 MG cells in the absence of serum. Treatment with ceramide for 24 h induced extensive apoptosis as demonstrated by an increase in the percentage of cells with a subdiploid DNA content (Fig. 4). We found that the subdiploid population increased from approximately 9% in untreated control samples to approximately 45% in samples treated for 24 h with ceramide (100 μM). U-87 MG cells cultured in the presence of serum exhibited a decreased basal level of apoptosis but remained susceptible to ceramide-induced apoptosis. The subdiploid population of U87 MG cells cultured in 10% serum increased from approximately 4% in untreated cultures to nearly 30% in cultures treated with 100 μM ceramide (data not shown). Similarly, when treated with 100 μM ceramide, 20% of SF126 cells and 55% of T98G cells underwent apoptosis (data not shown).

### DISCUSSION

Radiation-induced apoptosis may be initiated by signals originating in the nucleus (15) or in the plasma membrane (16, 17). Ultimately, the molecular pathways that mediate apoptosis lead to the activation of caspases whose substrates direct the terminal events of apoptosis (18). Ionizing radiation initiates the generation of ceramide by hydrolysis of sphingomyelin in the plasma membrane (16), which causes an activation of the Ced-3/ICE-like cysteine protease, CPP32, followed by apoptosis (18). Recently, ceramide was shown to mediate the enhanced expression of the pro-apoptotic molecule, Fas ligand, during radiation- and drug-induced apoptosis of cancer cells (8). Activation of Fas signaling is thought to be the mechanism of ceramide-induced apoptosis (8).
The Fas (APO- l/CD95) receptor-ligand system plays a key role in the regulation of apoptosis. Fas belongs to the nerve growth factor/ tumor necrosis factor receptor superfamily and initiates apoptosis on binding to Fas ligand or anti-Fas antibodies. The Fas receptor is expressed in primary astrocytic brain tumors, with an increasing incidence of expression in higher-grade tumors (19). We investigated whether Fas-mediated apoptosis could be triggered in highly radioreistant human glioma cell lines (9, 10) by assessing the susceptibility of these cells to anti-Fas antibody treatment. We found that U-87 MG, SF126, and T98G cells are killed by treatment with anti-Fas antibody in a p53-independent manner. The ability of cancer cells to undergo apoptosis may be a critical determinant of the outcome of therapy. We found evidence for an intact apoptotic program in radioreistant glioma cells that could be triggered by treatment with either anti-Fas antibody or exogenous ceramide. Thus, the radioreistance of these glioma cell lines cannot be explained by a general inability of these cells to undergo apoptosis as has been suggested for other radioreistant cells (20). The radioreistance of gliomas may result from an inability of these tumor cells to hydrolyze sphingomyelin and to produce ceramide in response to radiation. This possibility is consistent with preliminary observations from our laboratory indicating that ionizing radiation induces ceramide production in some radiosensitive central nervous system tumors but not in any cell lines derived from glioblastoma multiforme that we have examined to date.6 The results of this study suggest that therapeutic approaches that are aimed at enhancing ceramide production or triggering the Fas receptor-ligand system in tumor cells may contribute to the successful treatment of radioreistant glioma tumors.

REFERENCES


6 H. Kuriyama and M. A. Israel, unpublished data.


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