Radiation-Induced Caspase-8 Mediates p53-Independent Apoptosis in Glioma Cells

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Abstract

Malignant gliomas are almost uniformly fatal and display exquisite radiation resistance. Glioma cells lacking wild-type (WT) p53 function are more susceptible to radiation-induced apoptosis than their isogenic counterparts expressing WT p53. We explored the mechanisms of such apoptosis and found that, in the absence of WT p53, radiation increases caspase-8 expression and activity. Inhibition of caspase-8 expression using caspase-8 antisense or small interfering RNA (siRNA) oligonucleotides partially blocks radiation-induced apoptosis. In contrast, inhibition of the mitochondrial death pathway by expression of Bcl-2 has no effect on radiation-induced caspase-8 activity or apoptosis. Our data indicate that, in contrast to commonly accepted models of p53-dependent radiation-induced apoptosis, in our cell system, radiation relies on caspase-8 activity to help mediate p53-independent cell death. In a system of inducible E2F1 activity, E2F1 activated caspase-8 and, accordingly, decreased cellular viability, effects that were abolished by caspase-8 siRNA. In this model, in the absence of WT p53, p21^{Cip1} is not induced, and E2F1 activity is sustained and allows transcription and activation of caspase-8. This model may explain why p53 mutations in adult gliomas paradoxically correlate with improved survival and enhanced response to radiation. (Cancer Res 2006; 66(8): 4223-32)

Introduction

Glioblastoma multiforme is the most common and malignant central nervous system tumor. Patients with glioblastoma multiforme have a median survival time of <1 year (1). Glioblastoma multiformes are treated by surgical resection followed by radiation therapy; however, inevitably, tumors recur usually in the proximity of the original mass (2, 3). Although radiation is the most effective adjuvant treatment, glioblastoma multiformes exhibit radiation resistance that ultimately precludes their cure.

In response to radiation, mammalian cells undergo apoptosis and/or cell cycle arrest. Although p53 mediates both cellular responses, the dominant response in each tumor is influenced by cellular lineage, biological context, and determinants that hitherto remain poorly understood (4). Solid tumors, in which p53 mediates growth arrest after radiation, pose compelling clinical quandaries because more often than not, following a growth arrest, clonogens regrow and lead to tumor recurrence.

Although in many solid tumors the dominant p53-mediated radiation response is a growth arrest, more than half of human malignancies harbor mutations in p53 and are thus deficient in their p53-dependent responses to radiation (5). Specifically, abrogation of wild-type (WT) p53 function in isogenic glioma cell lines renders them more susceptible to radiation-induced apoptosis (6, 7). Therefore, the clinical use of radiation for the treatment of human malignancies must capitalize on p53-independent forms of radiation-induced cell death. p53-independent apoptosis is induced by radiation in several malignancies (8), and previous studies have revealed the importance of p53-independent apoptosis in gliomas (8). We sought to explore the molecular mechanism of p53-independent apoptosis induced in gliomas by ionizing radiation.

Cysteine proteases, known as caspases, constitute key components of the apoptotic pathway (9). Two distinct pathways of apoptosis have been identified as mitochondria initiated and death receptor initiated (9, 10). DNA-damaging agents, such as ionizing radiation, trigger release of cytochrome *c* from mitochondrial intermembrane spaces. Cytochrome *c* forms a complex with an adaptor molecule, Apaf1, which binds and activates caspase-9 (11). Activated caspase-9, in turn, cleaves and activates downstream caspases, effector proteases that execute the cell death program. Release of cytochrome *c* is regulated by the balance between antiapoptotic and proapoptotic members of the Bcl-2 family of proteins.

Whereas mitochondria-initiated apoptosis occurs through caspase-9, the death receptor–mediated pathway requires caspase-8 (12–14). In the death receptor–mediated pathway, binding of a ligand, such as Fas ligand or tumor necrosis factor-α (TNF-α), induces conformational changes of oligomerized death receptors (15, 16). The death domain–containing cytoplasmic region of the receptor recruits an adaptor molecule known as Fas-associated death domain (FADD) either by direct binding to the death domain of FADD or through yet another death domain–containing protein named TRADD. The death effector domain of FADD binds with procaspase-8. Binding to FADD triggers proteolytic processing of procaspase-8 to the active caspase-8 form, which in turn activates downstream caspases (9, 10).

Ionizing radiation increases protein levels of the tumor suppressor p53, which in turn regulates many cellular responses to DNA damage. Caspase-9 has been shown to execute p53-dependent radiation-induced apoptosis (11, 17). In contrast, radiation-induced apoptotic pathways executed in the absence of

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WT p53 have not been well defined. The same key molecules frequently coordinate apoptosis and cell cycle progression. By extension, following irradiation, molecules that mediate cellular responses to DNA damage are also key cell cycle regulators. Some such molecules, including pRb and E2F1, control proliferation and apoptosis partly in p53-dependent manners. In the presence of WT p53, radiation induces $p21^{Cip1}$ expression, which in turn inhibits pRb phosphorylation. Hypophosphorylated pRb binds E2F1, preventing expression of genes required for entry into S phase and arresting cell cycle progression. Disruptions of the p53 and/or pRb pathways free E2F1 and promote proliferation, although E2F1 activity induces not only proliferation but also apoptosis. Overexpression of E2F1 in glioma cells has been shown to induce apoptosis independent of p53 function, a response that is further enhanced by ionizing radiation (18).

An improved understanding of the mechanism of p53-independent apoptosis will facilitate the exploitation of this cell death pathway in the treatment of the majority of human cancers that lack WT p53. In this study, we identify a novel mechanism of p53-independent apoptosis that is distinct from the traditional p53-dependent radiation-induced apoptosis pathways.

Materials and Methods

Cell culture, transfection, and irradiation. U87 MG, U251 MG, and SF188 glioma cell lines and primary human astrocytes (19) were grown in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) and 100 units/mL penicillin-100 μg/mL streptomycin (Life Technologies) in a humidified incubator at 37°C and 8% CO_2. U87-pBabe and U87-E6 cells were described previously (20) and grown similarly.

Plasmid transfections were done using Fugene 6 transfection reagent (Roche). Cells were trypsinized and aliquoted into five T-75 flasks 12 to 16 hours after transfection. Following cell adherence, each flask was either treated with TNF-α (20 ng/mL) and cycloheximide (30 μg/mL), irradiated (J.L. Shepherd and Associates Mark I Cesium irradiator; dose rate, 2.3 Gy/min), or left untreated as a control. Staurosporine was purchased from Cell Signaling and used at 100 nmol/L.

For transfection with antisense oligonucleotides, 20-mer oligonucleotides were used, containing a central contiguous stretch of eight oligodeoxy residues flanked by 2′-methoxyethyl nucleotides with a phosphorothioate backbone (21). Oligonucleotides containing 8-bp mismatches were used as controls. For transfection of oligonucleotides, 60 μL Lipofectin (Life Technologies) were mixed with 12-mL serum-free medium for 30 minutes at room temperature. Oligonucleotide solution (18 μL/200 μmol/L) was used for transfection of each T-150 flask. The transfection, carried out according to the manufacturer's instructions, was repeated once 24 hours after the first transfection followed by treatment with radiation or TNF-α/cycloheximide as described above.

For small interfering RNA (siRNA) experiments, caspase-8-targeting siRNA [sense r(GGAGCUGCUCUUCCGAAUU)d(TT) and antisense r(AAUUCGGAAGAGCAGCUCC)d(TT)] or nonsilencing siRNA [sense r(UUCUCCGAACGUGUCACGU)d(TT) and antisense r(ACGUGACACGUUCGGAGAA)d(TT); Qiagen-Xeragon] was transfected using 160 nmol/L siRNA with 4 μL/mL Oligofectamine (Invitrogen, Carlsbad, CA) in Opti-MEM (Life Technologies) according to the manufacturer's instructions. Transfections with antisense oligonucleotides and siRNA were carried out twice in each experiment and spaced by 24 hours.

Clonogenic and 3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assays. Cells were transfected with caspase-8 siRNA or nonsilencing siRNA and irradiated 72 hours after the first transfection. Clonogenic survival assay was done as described previously (22). Cell survival measurements were fitted to a linear quadratic mathematical model using the FIT 2.5 program (23, 24). Within each of at least two independent experiments, two to four different dilutions were made per radiation dose, and each dilution was plated in multiples of six.

For 3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assays, siRNA-treated cells were plated in 96-well plates at concentrations of 1,500 (for U87 MG) or 2,000 (for SF188) per well 24 hours after transfection as described above. Eight hours after TNF-α/cycloheximide treatment or 72 hours after irradiation, MTS (Promega, Madison, WI) assays were done according to the manufacturer's instructions.

cDNA array analysis. Primary human astrocytes and SF188 glioma cells were treated with 20 Gy ionizing radiation or left untreated as controls (19). Total RNA was isolated 8 hours after irradiation by guanidinium isothiocyanate extraction method (Atlas Pure RNA isolation kit, Clontech, Palo Alto, CA), with 20 mg/mL glycogen added as a carrier to aid precipitation. RNA was reverse transcribed into ^{32}P-labeled cDNA and purified on a Sephadex G-25 Quick Spin Column (Boehringer Mannheim). The purified sample was denatured, neutralized, and hybridized with cDNA array membranes (Atlas 1.2 arrays, Clontech) overnight at 68°C. After a series of washes, the membranes were exposed to phosphorimaging screens for quantification using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RNA isolation and Northern blot analysis. Total RNA was isolated using RNeasy Midi kit (Qiagen, Inc., Chatsworth, CA). Total RNA (10 μg) was electrophoresed on 1% agarose/formaldehyde gels. RNA was transferred to nylon membranes (Hybond-N, Amersham) and UV cross-linked. Caspase-8 probe was prepared by reverse transcription (Pro-STAR first-strand reverse transcription-PCR kit, Stratagene, La Jolla, CA) of total RNA from U87 MG cells using oligo(dT) primer and amplified by PCR using AmpliTaq polymerase (Roche) and 5′-CCATGAATCAGAAGGGAAGAC-3′ and 5′-AAGATGGACTTCAGCAGAAATC-3′ caspase-8-specific primers. The resulting 1.5-kb cDNA fragment was radiolabeled by random priming (Rediprime, Amersham Pharmacia) and purified using Sephadex G-50 Quick Spin Columns (Boehringer Mannheim). β-Actin probe was obtained from Clontech. Northern blots were prehybridized and hybridized with Rapid-hyb (Amersham Pharmacia) at 65°C and washed several times in 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, and 0.7× SSC/0.1% SDS at 65°C. The blots were exposed to Kodak BioMax film. Northern blot data were quantitated after normalization to the β-actin loading controls.

Immunoblot analysis. Cells were lysed in cell lysis buffer (Clontech), and equal amounts of protein were electrophoresed by standard SDS-PAGE. Proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), and the membrane was blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 (TBST). The membrane was incubated with primary antibody in 5% nonfat dry milk in TBST followed by incubation with secondary antibody conjugated to horseradish peroxidase diluted in 5% nonfat dry milk in TBST. Proteins were detected with Enhanced Chemiluminescence Plus (Amersham) and exposed to Hyperfilm MP (Amersham). The primary antibodies used were directed against caspase-8 (Ab-3, Oncogene), caspase-9 (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Sigma), pRb (PharMingen, San Diego, CA), $p21^{Cip1}$, cleaved poly(ADP-ribose) polymerase (PARP), Bid, E2F1 (Cell Signaling), and Bcl-2 (Transduction Laboratories, Franklin Lakes, NJ).

Caspase activity assay. Caspase-8 activity was determined by measuring the fluorescence emission of 7-amino-4-trifluoromethyl coumarin (AFC) on its cleavage from the IETD-AFC substrate (ApoAlert Caspase Assay kit, Clontech). For each sample, three independent measurements were made using 25 μg total lysate.

Generation of E2F1-inducible cell lines. Amphotropic retrovirus, either pBabe-puro-HA-ER-E2F1 or control retrovirus pBabe-puro, was generated as described previously (25). The virus yield from one dish of amphotropic Phoenix cells, supplemented with 8 μg/mL polybrene (Aldrich), was used for transduction of 7.5×10^6 cells in a 10-cm dish. Cells were incubated in retrovirus-containing medium for 7 hours and then allowed to recover overnight in full medium. The following day, cells were harvested by trypsinization and seeded at a 1:4 dilution in medium containing 5 μg/mL puromycin (Sigma) to select for transduced cells. Expression of the transgene was confirmed by immunoblot analysis. In pooled derivatives expressing the pBabe-puro-HA-ER-E2F1 construct, E2F1 activity can be conditionally induced with 100 nmol/L 4-hydroxytamoxifen (4OHT; ref. 26).

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E2F1 luciferase assay. Cells were transiently cotransfected with pE2F-LUC and pRL-TR (Clontech) and irradiated with 0 or 8 Gy 24 hours later. Cells were irradiated 24 hours after transfection because initial experiments showed that transient transfections resulted in 60% to 80% transfection efficiency in U251 MG and U87 MG cells with sustained exogenous protein expression for 96 hours. We sought to assess luciferase reporter activity for 72 hours after irradiation and therefore irradiated cells 24 hours after transient transfections. The dose of 8 Gy, rather than 20 Gy, was chosen to avoid effects of early cell death. To document that 20 Gy resulted in extensive cell death, specifically apoptosis, in the mutant p53-expressing cell line U251 MG but not in the WT p53-expressing cell line U87 MG, we used the flow cytometry–based Annexin V-FITC/propidium iodide (PI) apoptosis detection method. Annexin V–positive cells were quantitated 72 hours following irradiation with either 8 or 20 Gy. In U251 MG, 7.3 $\pm$ 0.1%, 23.7 $\pm$ 0.2%, and 37.8 $\pm$ 0.3% cells were apoptotic after 0, 8, and 20 Gy, respectively. In U87 MG, 2.1 $\pm$ 2.5%, 2.7 $\pm$ 0.1%, and 0.5 $\pm$ 0.02% cells were apoptotic 72 hours after 0, 8, and 20 Gy, respectively.

For experiments addressing the effects of $p21^{Cip1}$ on E2F1 activity, cells were transiently cotransfected with pE2F-LUC, pRL-TR, and either cytomegalovirus-Bam-NeoP21 or pcDNA control vector. Luciferase activity was measured at indicated time points after irradiation using Dual-Luciferase Reporter Assay System (Promega).

Cell death analysis. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (*In situ* Cell Death Detection kit, TMR Red, Boehringer Mannheim) according to the manufacturer's instructions. The cell nuclei were visualized by Hoechst staining (Molecular Probes, Eugene, OR). Fluorescence microscopy was used to visualize and quantitate the apoptotic response. For each condition, a minimum of 500 cells were counted, and the data represent the mean and SD of at least three independent experiments. Flow cytometry–based apoptosis assays used double staining with Annexin V-FITC/PI using Annexin V apoptosis detection kit (EMD Biosciences, Inc., CA).

Results

Caspase-8 transcription is induced by irradiation of glioma cells. p53-independent apoptosis of glioma cells in response to ionizing radiation was shown previously to require new protein synthesis (19). We therefore conducted a pilot experiment using cDNA expression arrays to identify candidate genes whose expression was induced by radiation in radiosensitive glioma cell lines but not in normal human astrocytes. cDNA was prepared from the radiosensitive glioma cell line SF188 as well as from primary human astrocytes 8 hours after irradiation with 20 Gy. cDNA from irradiated cells and unirradiated control cells were each hybridized to cDNA expression arrays for semiquantitative comparison of expression levels, and results of this analysis have been reported previously (19). The caspase-8 gene stood out as having the most robustly induced transcript levels following irradiation of SF188 cells ($\sim$3- to 4-fold), whereas levels remained unchanged in irradiated normal human astrocytes (data not shown). The potential for false-positive signals using expression arrays prompted us to corroborate the induction of caspase-8 transcription in additional cell lines using another method of gene expression analysis (i.e., Northern blot analysis).

Induction of caspase-8 transcription by irradiation was confirmed by Northern blot analysis. Time courses of caspase-8 mRNA levels were done after irradiation of radiosensitive SF188 cells, the markedly radioresistant glioma cell line U87 MG, and U251 MG cells, whose radiosensitivity lies between SF188 and U87 MG cells. In radiosensitive SF188 cells treated with 20 Gy, caspase-8 transcript levels increased after irradiation as seen in Fig. 1*A*. In addition to mRNA levels, protein levels of procaspase-8 increased following irradiation as shown in Fig. 1*D*. U251 MG cells, second only to SF188 cells in radiosensitivity, also showed an increase in caspase-8 transcript levels after 20 Gy irradiation (Fig. 1*A*), whereas, in U87 MG cells, radiation induced no significant changes in caspase-8 transcript levels (data not shown).

Radiation activates caspase-8 in glioma cells. We sought to extend our studies of caspase-8 transcript levels to analyses of caspase-8 activity following irradiation. Activity was determined by a well-established assay, in which caspase-8 cleaves a AFC-conjugated substrate, and release of the fluorescent AFC is measured by a fluorometer. Whereas caspase-8 activity progressively increased after irradiation of SF188 and U251 MG cells, normal human astrocytes and U87 MG cells exhibited no change in caspase-8 activity following radiation (Fig. 1*B* and *C*). As a positive control, we treated the glioma cell lines with TNF-α and cycloheximide to trigger death receptor pathways known to be intact in these cell lines (Fig. 1*B*). Addition of the pharmacologic caspase-8 inhibitor IETD-fmk reduced caspase-8 activity to near or below the levels measured in unirradiated control samples.

Caspase-8 activation mediates p53-independent apoptosis of glioma cells. Although glioma cell lines exhibit marked resistance to ionizing radiation, within this radioresistant spectrum, SF188 and U251 MG, both expressing mutant p53, are two of the most radiosensitive cell lines we have characterized (27). Accordingly, radiation induces caspase-8 transcription and activation in these two cell lines. In contrast, WT p53-containing U87 MG cells and normal astrocytes, in which radiation does not induce caspase-8 activity, undergo no cell death after radiation. We confirmed that this cell death is apoptotic using the flow cytometry–based Annexin V-FITC/PI apoptosis detection method (Supplementary Fig. S1). To confirm that caspase-8 plays a role specifically in p53-independent apoptosis, we used U87-E6-transfected derivatives that stably express human papillomavirus-16 (HPV-16) oncoprotein E6 that binds to p53 and targets it for degradation. We first documented that, indeed, U87-E6 cells are significantly more susceptible to radiation-induced cell death as shown microscopically and biochemically through PARP cleavage (Fig. 2*A* and *B*, respectively). Although radiation did not induce caspase-8 activity in U87-pBabe cells, consistent with its expression of WT p53, inhibition of p53 function by HPV-16-E6 now allowed induction of caspase-8 activity by radiation in U87-E6 cells (Fig. 2*C*).

Beyond these correlative observations, we sought to establish that radiation-induced activation of caspase-8 plays a functional role in p53-independent apoptosis. To this end, we used siRNA and antisense oligonucleotides directed against caspase-8. Antisense oligonucleotides directed against caspase-8 efficiently blocked procaspase-8 expression (Fig. 1*D*) and inhibited radiation-induced apoptosis. Caspase-8 antisense oligonucleotides decreased apoptosis of SF188 cells from 47 $\pm$ 6% to 34 $\pm$ 2% (P = 0.03) when assayed 72 hours after exposure to 20 Gy. To solidify the key role of caspase-8 in radiation-induced apoptosis of gliomas lacking WT p53, we used siRNA oligonucleotides directed against caspase-8. Transfection of caspase-8 siRNA efficiently inhibited procaspase-8 expression (Fig. 3*A*) and blocked TNF-α-induced cell death as reflected by cleavage of both PARP and caspase-8 (Fig. 3*A*) as well as by MTS assays (Fig. 3*B*).

Caspase-8 siRNA effectively inhibited receptor-mediated cell death in both SF188 and U87 MG cells, and we proceeded to ask whether radiation responses would be similarly blocked. Following irradiation, p53-independent apoptosis of SF188 cells and

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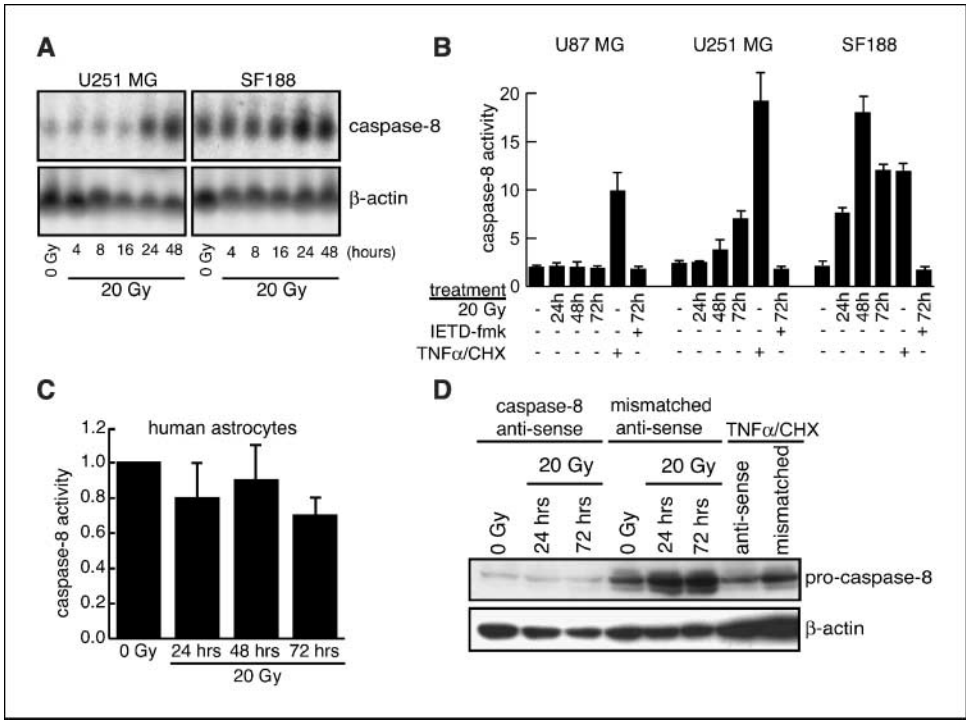

Figure 1. Irradiation induces caspase-8 expression and activity. *A,* Northern blot analysis of SF188 and U251 MG cells following irradiation showing increased expression. Caspase-8 activity is increased after irradiation of (*B*) U251 MG and SF188 cells but not U87 MG cells or (*C*) normal human astrocytes. For caspase-8 activity assays, whole-cell lysates were prepared at the specified times following exposure to 20 Gy or treatment with TNF-α/cycloheximide (*CHX*). Treatment of the lysates with the pharmacologic caspase-8 inhibitor IETD-fmk indicates that the documented increase in fluorescence emission is due to caspase-8 activity. *Columns,* mean of three independent experiments; *bars,* SD. *D,* irradiation increases procaspase-8 protein levels, a response blocked by caspase-8 antisense oligonucleotides. Immunoblot analysis of SF188 cells shows that both the basal caspase-8 protein levels and the radiation-induced increase in caspase-8 levels are blunted by antisense oligonucleotides designed to target caspase-8.

p53-dependent growth arrest of U87 MG cells both result in reduced MTS values. We hypothesized that caspase-8 siRNA would block radiation-induced reductions in MTS values in SF188 cells, in which a p53-independent pathway is engaged but not in U87 MG cells, which expresses WT p53. Indeed, caspase-8 siRNA expression in SF188 cells inhibited radiation-induced decreases in MTS activity (Fig. 3*C*) but did not alter the radiation-induced reduction in MTS values seen in U87 MG cells (Fig. 3*D*). To further confirm that reductions in MTS activity in irradiated SF188 cells reflected apoptosis, we used the flow cytometry–based Annexin V-FITC/PI apoptosis detection method. Irradiation with 20 Gy resulted in accumulation of Annexin V–positive cells reflecting radiation-induced apoptosis (Supplementary Fig. S1). As the gold standard of radiation sensitivity is the clonogenic survival assay, we sought to show that the effects of caspase-8 on apoptosis would translate into effects on capacity for cell renewal. Indeed, inhibiting caspase-8 expression using siRNA resulted in a significant enhancement in cell survival as measured by the clonogenic assay (Fig. 3*E*).

Ectopic expression of Bcl-2 does not affect radiation-induced apoptosis of gliomas. Radiation-induced apoptosis is reported to occur through activation of caspase-9 rather than caspase-8 (28, 29). Because caspase-9 and caspase-8 are known to reciprocally activate each other through Bid cleavage, the radiation-induced caspase-8 activation we observed might have been a secondary event that resulted from caspase-9 activation. We therefore sought to show that radiation-induced caspase-8 activity is a primary event that is not mediated through the mitochondrial machinery or caspase-9.

To test this hypothesis, we overexpressed Bcl-2, an antiapoptotic protein that blocks mitochondria-dependent apoptosis by inhibiting cytochrome *c* release and caspase-9 activation. SF188 cells were transiently transfected with either pBabe-Bcl-2 or pBabe-puro empty vector as a control and irradiated with 20 Gy after 7 days of puromycin selection. Bcl-2 is well known to inhibit death induced by staurosporine through a mechanism dependent on Bid cleavage (30). We therefore examined the susceptibility of pBabe-Bcl-2 or pBabe-puro to staurosporine-induced cell death to document the activity of Bcl-2 in the transfected derivatives. Transfected Bcl-2 was clearly functional as shown in Fig. 4*A*. Bcl-2 inhibited staurosporine-induced apoptosis but had no effect on radiation-induced apoptosis as quantitated by Annexin V and PI staining. Consistent with the failure of Bcl-2 to affect radiation-induced cell death, Bcl-2 expression did not affect caspase-8 activation by radiation as shown by the persistent cleavage of procaspase-8 (Fig. 4*B*) and release of the fluorescent AFC from an AFC-conjugated caspase-8 substrate (Fig. 4*C*). Similar results were found in U251 MG cells, in which Bcl-2 also failed to inhibit apoptosis following irradiation (data not shown), showing that radiation induces caspase-8 activity through a mechanism independent of caspase-9 and the mitochondrial machinery. To further confirm that radiation-induced apoptosis is a direct result of caspase-8 activity, independent of the mitochondrial death machinery, we examined Bid cleavage, known to occur following staurosporine treatment. Consistent with our model, immunoblots showed Bid cleavage only after staurosporine treatment but not after irradiation (Fig. 4*D*).

Radiation-induced caspase-8 expression and activation is induced by E2F1 and inhibited by $p21^{Cip1}$. The cyclin-dependent kinase (Cdk) inhibitor $p21^{Cip1}$ is a key mediator of the p53-dependent cell cycle arrest induced by DNA damage. In cells expressing WT p53, radiation leads to increased $p21^{Cip1}$ protein levels, which in turn inhibit Cdk2/cyclin E and prevent phosphorylation of pRb (31–33). Hypophosphorylated pRb binds and sequesters E2F1, thus preventing transcription of S-phase genes that are required for progression through the cell cycle. In addition to arresting cell cycle progression, $p21^{Cip1}$ inhibits apoptosis induced by many cellular insults, including ionizing radiation (34–36).

E2F1 antagonizes both growth-inhibitory and antiapoptotic functions of $p21^{Cip1}$. E2F1 promotes apoptosis specifically by

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promoting accumulation of caspase proenzymes through a direct transcriptional mechanism (18, 37). These observations led us to propose a model by which irradiation causes apoptosis only in gliomas lacking WT p53 because, in the absence of WT p53, $p21^{Cip1}$ is not induced and E2F1 activity is sustained and allows persistent transcription and activation of caspase-8. To test this model, we first verified that U87 MG cells and primary astrocytes, which express functional WT p53, display increased $p21^{Cip1}$ levels after irradiation. In contrast, the mutant p53-containing U251 MG and SF188 cell lines and U87-E6 cells showed little or no $p21^{Cip1}$ induction after irradiation (Figs. 2*B* and 5*A*). We next confirmed that irradiation reduced E2F1 transcriptional activity in cells expressing WT p53 but not in cells expressing mutant p53 using E2F1 luciferase reporter assays (Supplementary Fig. S2).

To directly test the hypothesis that $p21^{Cip1}$ induction blocks radiation-induced apoptosis through inhibition of caspase-8, we first transiently transfected $p21^{Cip1}$ into U251 MG cells followed by irradiation 24 hours later. Enforced expression of $p21^{Cip1}$ decreased E2F1 activity as reflected by luciferase reporter assays (Fig. 5*B*) and blocked caspase-8 activation by radiation (data not shown). $p21^{Cip1}$ blunted radiation-induced apoptosis quantitated by TUNEL assays in gliomas expressing mutant p53, decreasing cell death from $32 \pm 6.3\%$ to $20 \pm 4.4\%$ 72 hours after irradiation of U251 MG cells ($P < 0.01$), with similar effects in SF188 cells.

We next generated a system of inducible E2F1 activity. U251-ER-E2F1 and U87-ER-E2F1 cells express an inducible form of E2F1, the activity of which is fully dependent on the presence of the modified estrogen receptor ligand 4OHT (Fig. 6*A*; refs. 26, 38). Activation of E2F1 on addition of 4OHT activated caspase-8 (Fig. 6*B*) and, accordingly, decreased MTS activity (Fig. 6*C*). However, inhibition of caspase-8 expression by siRNA transfection inhibited E2F1-induced reduction in MTS activity (Fig. 6*D*). To confirm that apoptosis underlay the reductions in MTS activity in 4OHT-treated U87-ER-E2F1 cells, we used the flow cytometry–based

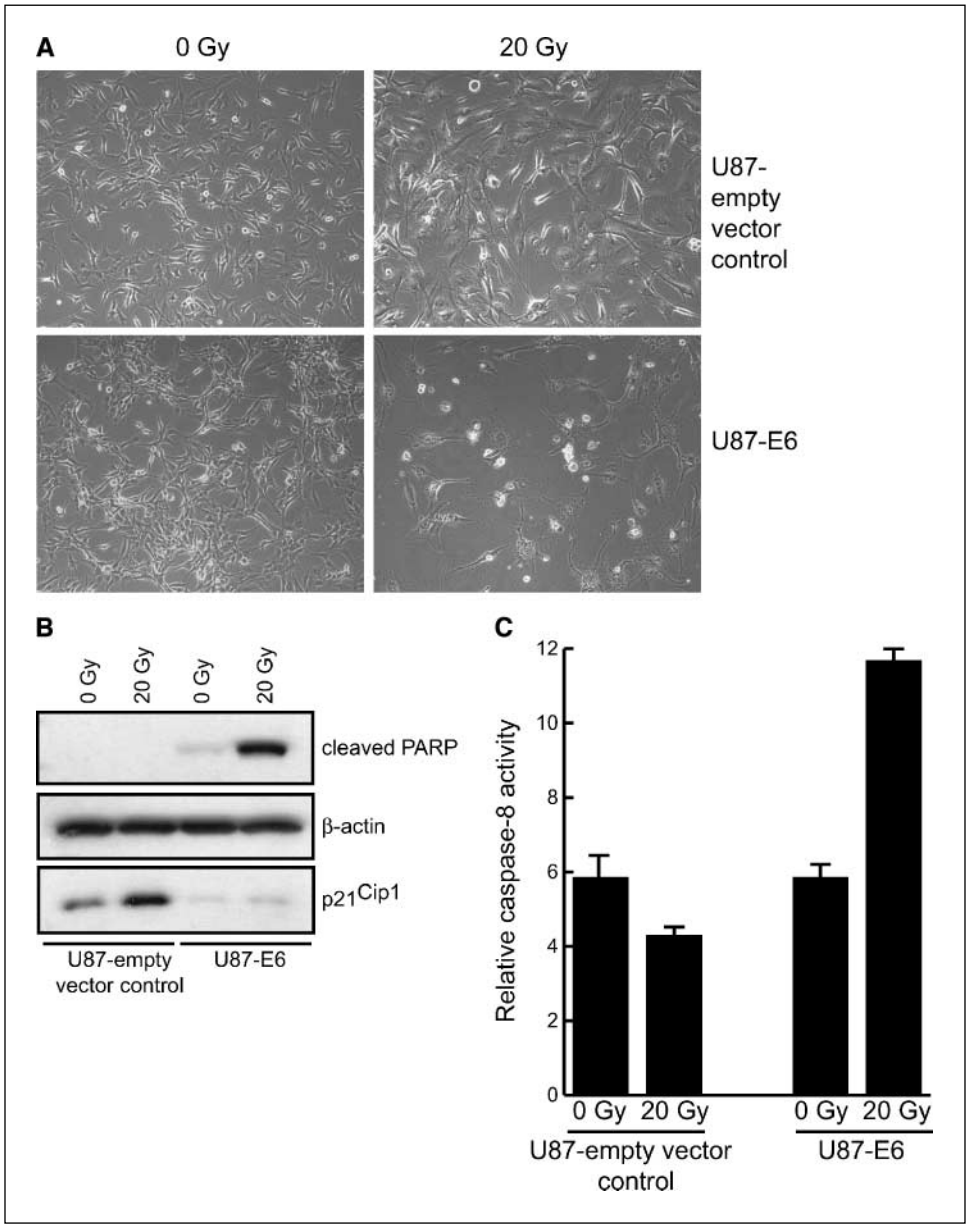

Figure 2. Inhibition of p53 function allows radiation to induce caspase-8 activity and triggers cell death. Radiation induces apoptosis in U87 MG cells stably expressing HPV-16 oncoprotein E6 (U87-E6) but not in parental cells expressing WT p53 as shown (*A*) microscopically and (*B*) through PARP cleavage (72 hours after radiation). As expected, up-regulation of $p21^{Cip1}$ after irradiation is blunted by inactivation of WT p53. *C,* degradation of p53 allows radiation-induced activation of caspase-8 (72 hours after radiation).

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Annexin V-FITC/PI apoptosis detection method. Treatment with 4OHT resulted in accumulation of Annexin V–positive cells reflecting apoptosis (Supplementary Fig. S3). Our results show that, in U87 MG cells expressing WT p53, radiation fails to induce E2F1 activity, caspase-8 expression, caspase-8 activity, and cell death. However, activation of E2F1 using an inducible system decreased U87 MG cell viability following irradiation, an effect that was blocked by caspase-8 siRNA. Taken together, these data

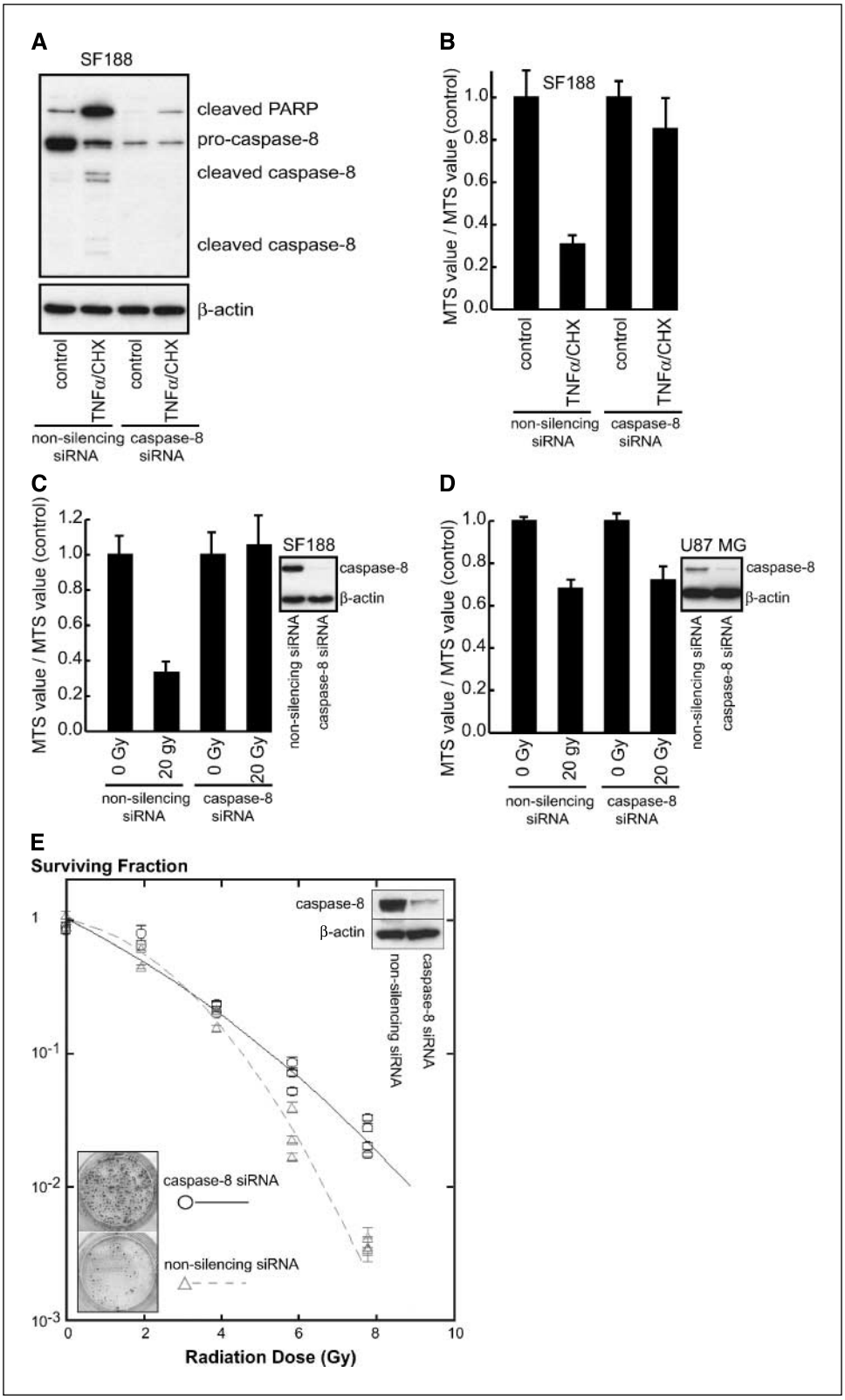

Figure 3. Caspase-8 siRNA blunts cytotoxicity induced by TNF-α/cycloheximide and inhibits radiation-induced MTS reduction in SF188 cells (reflecting cell death) but not in U87 MG cells (reflecting cell cycle arrest). Cells were transfected with either nonsilencing siRNA or caspase-8 siRNA. *A,* SF188 cells were treated with TNF-α/cycloheximide and harvested 8 hours later for immunoblot assays. *B,* diminished MTS values in this system reflect apoptosis given the PARP cleavage that is documented using an antibody that recognizes only the cleaved form of PARP. SF188 (*C*) or U87 MG (*D*) cells were irradiated and evaluated by MTS assays 72 hours later. Immunoblots for both cell lines document markedly reduced caspase-8 expression after siRNA transfection and before irradiation. *E,* inhibition of caspase-8 expression by siRNA enhances clonogenic survival of irradiated U251 MG cells.

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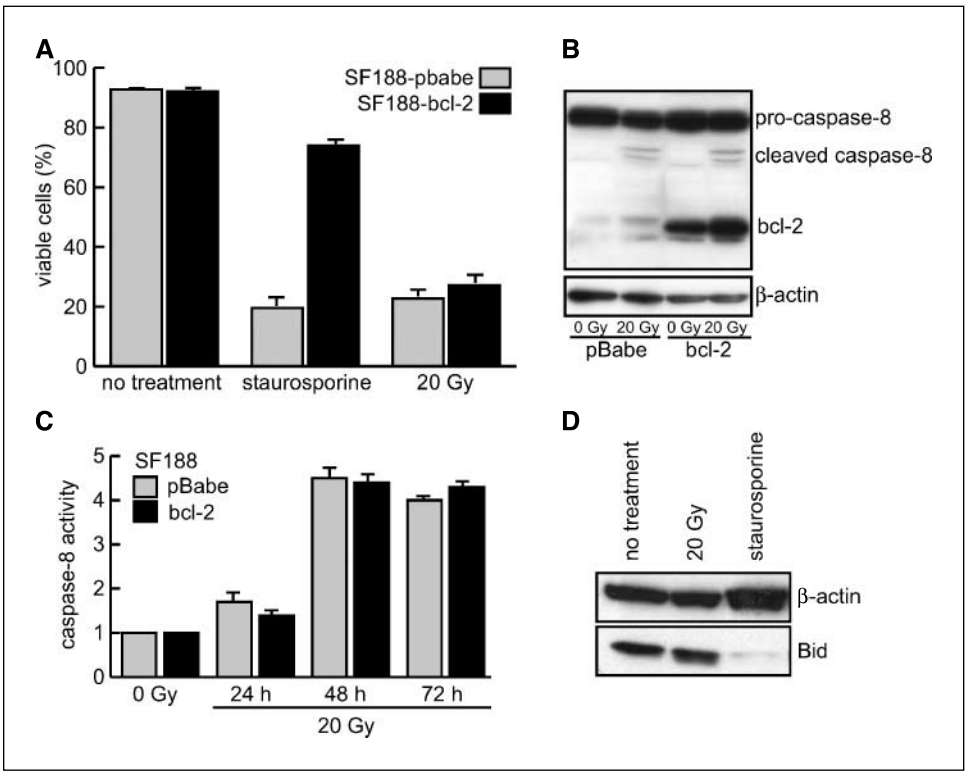

Figure 4. Bcl-2 fails to protect gliomas from caspase-8-mediated cell death after irradiation. *A,* Bcl-2 blocks staurosporine-induced but not radiation-induced apoptosis (both 72 hours after treatment). *B,* immunoblot analysis 48 hours after irradiation of SF188 cells shows that caspase-8 is cleaved regardless of Bcl-2 expression. *C,* caspase-8 activity is unaffected by ectopic expression of Bcl-2. *D,* immunoblot showing Bid cleavage after staurosporine treatment but not after irradiation (both 72 hours after treatment).

suggest that persistently low caspase-8 activity and enhanced cell survival in irradiated U87 MG cells may be causally related and due in part to dampened E2F1 activity following radiation. The data further suggest that sustained, elevated E2F1 activity after radiation of gliomas lacking WT p53 enhances caspase-8 expression and activity and ultimately plays an important role in p53-independent cell death.

Discussion

Concerted efforts and advances in surgical and radiation technologies have failed to cure patients with gliomas. However, of all adjuvant treatments, radiation is the most effective in prolonging patient survival (39, 40). In glioma cells, radiation induces two distinct responses: p53-dependent growth arrest and p53-independent apoptosis (18, 41). Clinical investigations indicate that these distinct responses to radiation influence the prognosis of human malignancies in general and gliomas in particular. Several human tumors exhibit a higher sensitivity to DNA-damaging agents when p53 is mutated (42–44). In primary gliomas, expression of mutant p53 confers an improved prognosis in adult glioma patients specifically because of better responses to radiation therapy (45). The improved responses to radiation therapy seen in patients with p53 mutations may very well reflect a shift to p53-independent apoptosis when the growth arrest response of p53 is compromised (46). This is consistent with experiments reported herein and published previously *in vitro* studies, in which glioma cells lacking WT p53 function are more susceptible to radiation-induced apoptosis than their isogenic counterparts expressing WT p53 (6, 7).

We sought to explore the mechanism of p53-independent apoptosis of gliomas in response to radiation. We found that radiation increased caspase-8 expression level and activity in radiosensitive glioma cell lines, with minimal effect in radio-resistant glioma cells and primary astrocytes. The functional relevance of caspase-8 activation by radiation is indicated by the decrease in radiation-induced apoptosis and enhanced clonogenic survival seen with agents that block caspase-8 expression.

Although radiation did not induce caspase-8 activity or apoptosis in U87 MG cells, this was not due to a deficit in the

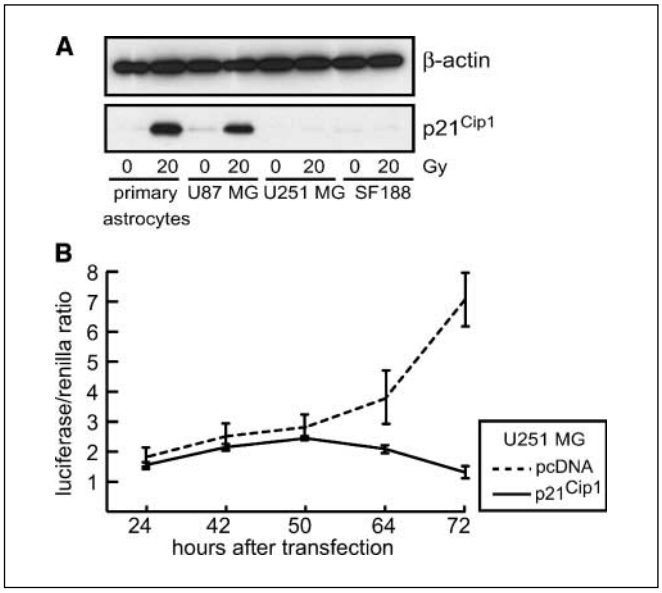

Figure 5. *A,* radiation induces $p21^{Cip1}$ protein levels in primary astrocytes and U87 MG cells but not in U251 MG and SF188 cells. *B,* ectopically expressed $p21^{Cip1}$ inhibits E2F1 activity. U251 MG cells were transiently cotransfected with an E2F1 luciferase reporter construct and either $p21^{Cip1}$ or control vector (pcDNA), and luciferase activity was measured at the indicated time points.

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death receptor–mediated apoptotic pathway, as treatment of U87 MG cells with TNF-α/cycloheximide resulted in caspase-8 activation and cell death similar to those seen in SF188 and U251 MG cells. U87 MG cells transfected with caspase-8 siRNA were rescued from death triggered by TNF-α/cycloheximide treatment. Moreover, previous studies have shown that U87 MG cells are susceptible to Fas receptor–mediated cell death (47). These results indicate that the death receptor pathway mediated by caspase-8 activity is functional but is not induced by irradiation in these radioresistant cells.

The finding that caspase-8 helps mediate radiation-induced apoptosis of glioma cells is novel in light of extensive literature that indicates a role for caspase-9 in radiation-induced apoptosis (48–51). Caspase-9 is an apical caspase that is activated through the mitochondria-mediated apoptotic pathway (52). Caspase-9 is also activated indirectly by caspase-8 through Bid cleavage, thus establishing cross-talk between receptor-mediated and cytochrome *c*–mediated pathways of apoptosis. Release of cytochrome *c* from the intermitochondrial membrane space in response to stress stimuli, such as radiation, is a critical step in the activation of caspase-9. However, in our experiments, overexpression of Bcl-2, which inhibits release of cytochrome *c*, blocked neither caspase-8 activation nor radiation-induced apoptosis of gliomas. Bcl-2 successfully inhibited Bid-mediated cell death induced by staurosporine, indicating that it was expressed and functional in these transfected cells. Further confirmation of the role of caspase-8 in directly inducing cell death after irradiation is the finding that, although staurosporine successfully cleaved Bid, no such Bid cleavage was observed after irradiation.

Thus, in contrast to commonly accepted models of p53-dependent radiation-induced apoptosis, radiation relies on caspase-8 activity to help mediate p53-independent cell death. The distinct mechanism mediating p53-independent apoptosis is highlighted by our finding that, whereas irradiation of WT p53-expressing gliomas fails to stimulate caspase-8 activity, inhibition of WT p53 function with HPV-16-E6 now allows induction of caspase-8 activity and resultant apoptosis.

Our study is the first to show a direct role for transcriptional activation of caspase-8 in radiation-induced apoptosis. As raised in a previous report by Nahle et al., increased levels of procaspase-8 alone may not be sufficient to induce apoptosis but rather sensitize cells to apoptosis in the context of other effects of ionizing radiation (37).

Depending on the cellular context, p53 mediates apoptosis and/or cell cycle arrest. The growth-inhibitory functions of p53 and its effector $p21^{Cip1}$ are known to block radiation-induced apoptosis (35, 53). Our model of p53-independent apoptosis predicts that enforced expression of $p21^{Cip1}$ in cells lacking WT p53 would block E2F1 activity, prevent sustained caspase-8 expression and activation by radiation, and thus inhibit apoptosis. In accordance with these predictions, ectopic expression of $p21^{Cip1}$ in glioma cells inhibited E2F1 transcriptional activity and prevented radiation-induced caspase-8 activation and apoptosis.

Previous work suggests a mechanism through which the induction of $p21^{Cip1}$ may prevent caspase-8 activation. Expression of $p21^{Cip1}$ inhibits the transcription factor E2F1 (31, 54, 55). E2F1, in turn, promotes both cell cycle progression and apoptosis (37, 56). It has been shown that the caspase-8 promoter contains an E2F1-responsive element (37), and it is therefore not surprising that E2F1 up-regulates caspase-8 proenzyme levels through a direct transcriptional mechanism. Moreover, analyses of gene expression patterns in mice that carry homozygous deletions of E2F1

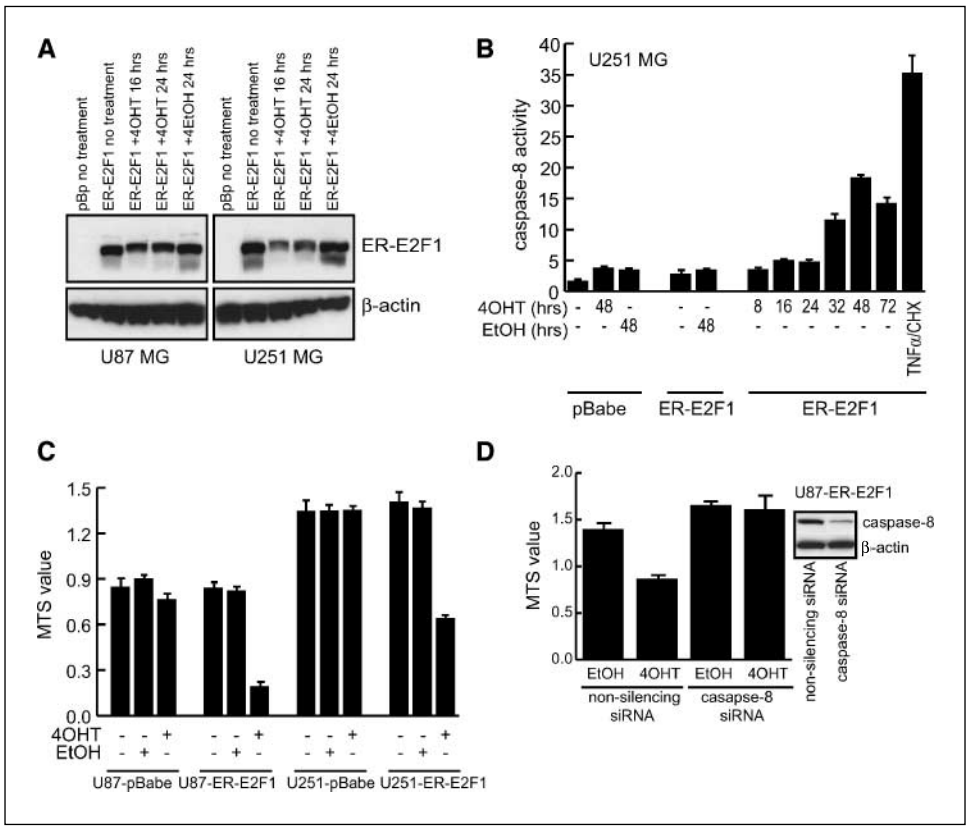

Figure 6. E2F1 activates caspase-8 and induces cytotoxicity that is inhibited by caspase-8 siRNA. Pools expressing either control vector (pBabe) or a conditionally active form of E2F1 (ER-E2F1) were generated. E2F1 was fused to a mutant form of the murine estrogen receptor that is responsive to the synthetic steroid 4OHT. *A,* immunoblot shows expression of the ER-E2F1 fusion protein. *B,* E2F1 activity was induced by addition of 4OHT followed by measurements of caspase-8 activity at the specified times. Treatment with TNF-α/cycloheximide was used as a positive control for caspase-8 activation. *C,* derivatives expressing ER-E2F1 were treated with 4OHT to induce E2F1 activity followed by assessment of cell viability 72 hours later. *D,* following transfection with either nonsilencing siRNA or caspase-8 siRNA, E2F1 activity was induced in U87 MG derivatives by incubation with 4OHT, and cell viability was quantitated by MTS assays. Immunoblot analysis documents reduced caspase-8 protein levels after siRNA transfection.

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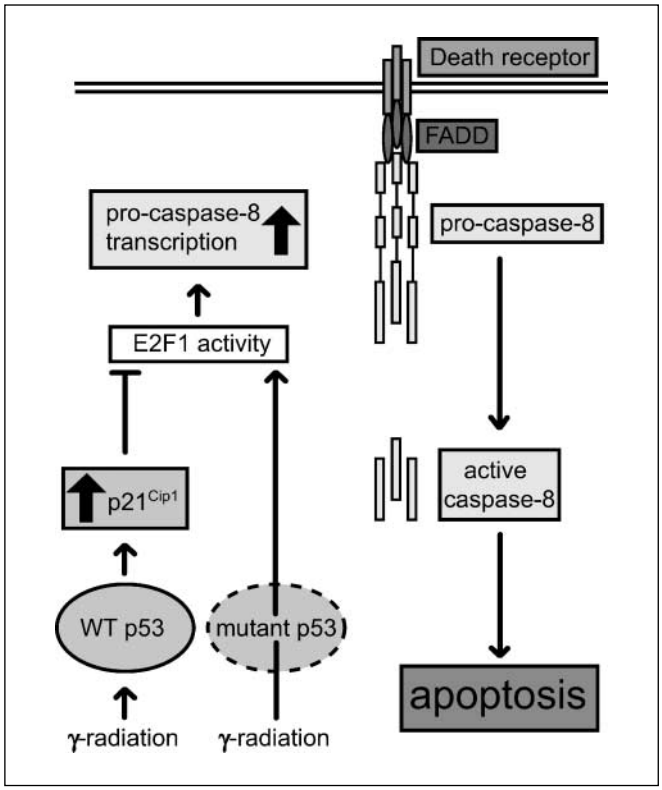

Figure 7. Model of p53-independent apoptosis induced by radiation. In this model, $p21^{Cip1}$-induced cell cycle arrest leads to down-regulation of E2F1 after irradiation and thus prevents caspase-8 expression and its subsequent activation. In cells that lack WT p53, radiation does not increase $p21^{Cip1}$ levels, a cell cycle arrest does not ensue, and enhanced, persistent E2F1 activity allows the transcriptional activation of caspase-8 and resultant apoptosis. This model may explain the observation that p53 mutations in adult gliomas correlate with improved response to irradiation and prolonged patient survival (46).

identified caspase-8 as one of the genes whose expression level is induced by E2F1 (57). These observations led to our model (Fig. 7) by which a $p21^{Cip1}$-induced cell cycle arrest, for instance, through activation of WT p53, leads to down-regulation of E2F1 after irradiation and thus prevents caspase-8 expression and its subsequent activation. In cells that lack WT p53, radiation does not increase $p21^{Cip1}$ levels, a cell cycle arrest does not ensue, and enhanced, persistent E2F1 activity allows the transcriptional activation of caspase-8 and resultant apoptosis.

Our proposed model applies to cell types, such as fibroblasts and astrocytes, which have a propensity to undergo p53-dependent cell cycle arrest in response to ionizing radiation (58). In these cell types, caspase-8 mediates radiation-induced apoptosis that occurs only in the absence of functional p53. In contradistinction, other cell types, such as hematopoietic and neuronal cells, display a propensity to undergo p53-dependent apoptosis in response to radiation (59, 60).

The molecular pathways that radiation induces in p53-dependent apoptosis clearly differ from the pathways invoked in p53-independent apoptosis. It is therefore not surprising that the majority of the studies describing the role of mitochondria and caspase-9 in radiation-induced apoptosis have been done in cell types that display a propensity to undergo p53-dependent apoptosis rather than p53-dependent cell cycle arrest. Because half of all human malignancies, including gliomas, harbor p53 mutations, antineoplastic therapy must capitalize on p53-independent pathways of cell death to broadly affect cure rates of human cancers. The distinct responses to radiation exhibited by gliomas expressing or lacking WT p53 function are all the more poignant given the heterogeneity in p53 expression commonly described in gliomas *in vivo*. Our data suggest that radiation therapy may more readily kill mutant p53-expressing glioma cells within the tumor mass, and multimodality treatments will be required to effectively treat cells with other genetic features within the same tumor.

Little progress in glioma treatment has been accomplished by clinical trials that use agents, such as traditional chemotherapy, which rely on caspase-9-mediated cell death. Such approaches are unlikely to prove effective even in the face of higher doses or altered dosing schedules. Rather, our study indicates that clinical progress for gliomas is most likely to derive from innovative agents that engage caspase-8-mediated mechanisms of cell death.

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