Overexpression of E2F1 in glioma-derived cell lines induces a p53-independent apoptosis that is further enhanced by ionizing radiation[1]

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Glioma cell lines show variable responses to radiation in a manner influenced by their p53 status. Irradiation of glioma cell lines does not generally induce apoptosis. When wild-type p53 is present, these cells undergo a G_1 arrest that is closely associated with increased radiosensitivity as measured by clonogenic survival. Previously, others have shown that dysregulated overexpression of E2F1 induces apoptosis in cell lines with either functional or inactivated p53. We found that regardless of p53 status, apoptosis induced by overexpression of E2F1 in glioma cell lines was further enhanced by treatment with ionizing radiation. *BAX* induction did not follow E2F1 overexpression or irradiation in the glioma cell lines tested. Thus, the apoptotic response of glioma-derived cells to irradiation can be enhanced by E2F1 by a mechanism that does not involve the induction of *BAX*. *Neuro-Oncology 2, 16–21, 2000 (Posted to Neuro-Oncology [serial online], Doc. 99-36, December 13, 1999. URL <neuro-oncology.mc.duke.edu>)*

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[3]Abbreviations used are as follows: GBM, glioblastoma multiforme.

Introduction

Radiation therapy is a primary modality of treatment for many brain tumors including those of glial origin. Despite its use in the treatment of gliomas, local recurrence of tumors occurs frequently. Survival of patients diagnosed with high-grade gliomas is especially poor, with few five-year survivors. Although the clinical resistance of gliomas to irradiation does not generally correlate with standard measures of radiation resistance in vitro (Taghian et al., 1993), virtually all glioma-derived cell lines exhibit resistance to radiation and do not undergo radiation-induced primary apoptosis (Haas-Kogan et al., 1996a, 1996b). The contribution of apoptosis to radiation-induced cell death depends on the cell type. Apoptotic death significantly modifies clonogenic survival in some cases (Rupnow et al., 1998), but relatively little in others (Palayoor et al., 1997; Voehringer et al., 1997). The lack of an apoptotic response to radiation may contribute significantly to the poor local control of gliomas achieved by cytotoxic therapies, including irradiation.

E2F1 belongs to a family of transcription factors that activates transcription of *S*-phase–specific genes. Its transcriptional activity is regulated through direct interactions with the product of the retinoblastoma gene (for review see Dyson, 1998). In addition to its activity in normal cell cycle control, E2F1 expression in vitro can induce apoptosis in a number of cell types, and in most cell types, its activity has been shown to be p53-dependent (for review see Dyson, 1998). Although initial in vitro studies implicated E2F1 as a potential transforming protein (Johnson et al., 1994; Singh et al., 1994), more recently mice with homozygous deletion of the E2F1 locus have been observed to develop tumors, suggesting a role of E2F1 as a

tumor suppressor (Field et al., 1996; Yamasaki et al., 1996). The mechanism by which E2F1 acts as a tumor suppressor may reflect its ability to induce apoptosis, lending support for the importance of this molecule in the normal molecular pathways that regulate apoptosis (Field et al., 1996; Yamasaki et al., 1996). Fueyo et al. have recently demonstrated that glioma cell lines are also sensitive to apoptosis induced by overexpression of E2F1 (Fueyo et al., 1998). Because glioma cell lines are notoriously resistant to radiation-induced, p53-dependent apoptosis (Haas-Kogan et al., 1996a, 1996b), we sought to further characterize E2F-mediated apoptosis in these tumors and its potential to modify the therapeutic effects of radiation.

We found that overexpression of E2F1 in multiple glioma cell lines led to apoptosis that was further enhanced by exposure to ionizing radiation irrespective of their p53 status. Consistent with this observation and the findings of others (Fueyo et al., 1998), E2F1 overexpression resulted in significant levels of apoptosis that were p53-independent. *BAX* was not induced in glioma cell lines found to undergo apoptosis after expressing exogenous E2F1. Therefore, although E2F1 cannot complement the defect in p53-dependent apoptosis in glioma cell lines, it is still capable of inducing significant levels of apoptosis in a cell type that is very resistant to apoptosis induced by cytotoxic DNA damage and further increases the apoptotic response of these cells in combination with irradiation.

Material and Methods

Cell Culture, Adenoviral Infection, and Treatment with Ionizing Radiation

The GBM[3] cell lines U-87 MG, U-87 MG derivatives (lux.6, 175.4 and 175.13), U-343 MG, and SF210 were all cultured as previously described (Shu et al., 1998). U-87 MG clonal derivatives containing either the luciferase gene or a dominant-negative mutant of the *TP53* gene introduced via a retroviral vector (lux.6 or 175.4 and 175.13) were previously described (Haas-Kogan et al., 1996b; Yount et al., 1996). A defective adenoviral vector containing no exogenous gene (AdCMV) or the *E2F1* gene (AdE2F) has also been previously described (kindly provided by Joseph Nevins) (Kowalik et al., 1995; Schwarz et al., 1995). All infections were carried out at a multiplicity of infection of 100 utilizing standard procedures (Kowalik et al., 1995; Schwarz et al., 1995). Irradiation experiments involved the exposure of $4–5 \times 10^6$ GBM cells in approximately 10 ml of media in a 10-cm petri dish that were treated with 250 KVp X-rays at a dose rate of 250 cGy/min on a Philips RT-250 X-ray machine. When exposure to ionizing radiation was combined with adenoviral infection, cells were first infected with either AdCMV or AdE2F for a minimum of 30 min and then irradiated within 1 h after removal of the infectious supernatant.

Apoptosis Assays

Determination of the subdiploid apoptotic fraction with propidium iodide staining has been previously described (Shu et al., 1998). In some cases, the induction of apoptosis was qualitatively confirmed using a terminal deoxynucleotidyl transferase assay to detect DNA ends (Oncor, Gaithersburg, Md.). For inhibition of caspases, the inhibitory peptide zVAD-FMK (Alexis, San Diego, Calif.) (stock solution of 2 mM in 100% methanol) was used. AdE2F-infected U-87 MG and U-343 MG cells were cultured with complete media containing a 1:500 dilution of either the zVAD-FMK stock solution or an equal volume of 100% methanol. Fresh media containing a 1:250 dilution of either the zVAD-FMK stock solution or 100% methanol was added at a 1:1 ratio to each petri dish 1–2 days postinfection. Cells were harvested 3 days postinfection, and apoptosis was assayed as described above.

Western and Northern Blotting

For Western blotting, total cellular protein was prepared as previously described (Shu et al., 1998). Protein concentration was quantitated by a Bradford protein assay (Bio-Rad, Hercules, Calif.); 100 μg of total cellular protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred to Immobilon-P (Millipore, Bedford, Mass.), and examined by immunoblotting by standard techniques. Anti-$p21^{WAF1}$ antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, Calif.) and anti-E2F1 mixed monoclonal antibody (KH20/KH95, Upstate Biotechnology, Lake Placid, N.Y.) were used at a concentration of 0.1 μg/ml in blocking solution. Goat antirabbit IgG antibody conjugated to horseradish peroxidase (Gibco-BRL, Gaithersburg, Md.) was used as the secondary antibody according to the manufacturer's recommendation. Detection was performed by enhanced chemiluminescence according to the manufacturer's recommendation using Hyperfilm ECL (Amersham, Arlington Heights, Ill.).

Total RNA isolation and Northern blotting were performed as previously described (Shu et al., 1998). Serial probing of the membranes with cDNAs for human $p21^{BAX}$, E2F1, and GAPDH labeled with ^{32}P-dCTP using Rediprime (Amersham), a random primer labeling kit, was performed according to manufacturer's recommendations. Membranes were washed at high stringency prior to exposure on either Hyperfilm MP (Amersham) or Reflection autoradiography film (New England Nuclear, Boston, Mass.).

For quantitation of either protein or RNA expression, densitometry was performed on the appropriate exposed film. Films were scanned using a standard flatbed scanner, and the resulting digital images were analyzed using NIH Image 1.61 software (National Institutes of Health, Bethesda, Md.). For quantitation of protein bands, equal loading of lanes with protein was confirmed by staining the Immobilon-P membrane with Ponceau S according to standard protocol prior to immunoblotting. For quantitation of RNA, equal loading of lanes with total RNA was performed, but further normalization using GAPDH expression was also performed to assure maximal accuracy of measurement of RNA expression despite small variations in lane loading.

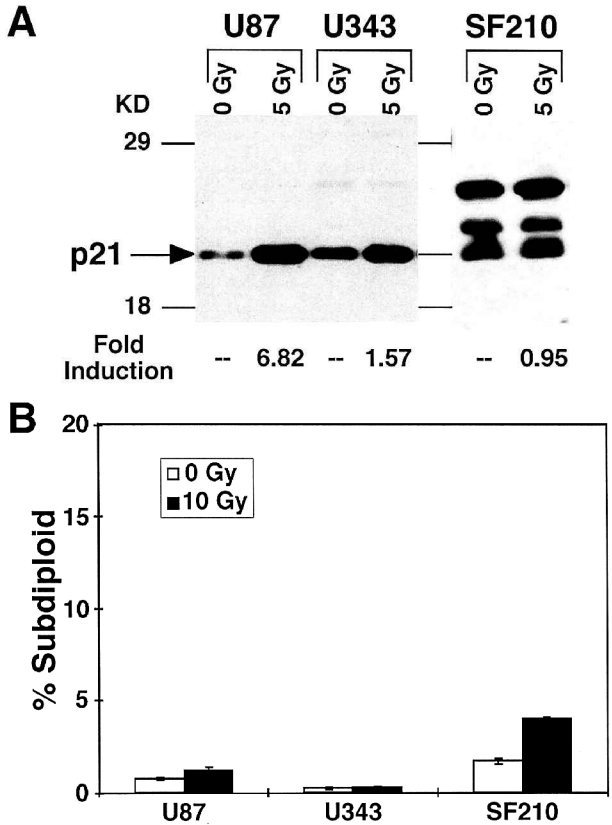

Fig. 1. $p21^{WAF1}$ induction and apoptotic response after irradiation of glioma-derived cell lines. (A) Glioma-derived cell lines U-87 MG, U-343 MG, and SF210 were treated with either 0 Gy or 5 Gy of ionizing radiation, and lysates of total cellular protein were prepared 24 h posttreatment. Total cellular protein was resolved on a 12.5% gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred to a polyvinylidene difluoride membrane, and examined by immunoblotting for $p21^{WAF1}$ expression. Quantitative densitometric analysis determining the fold induction of $p21^{WAF1}$ expression on exposed film is shown below the appropriate lanes of the Western blot. (B) The bar graph summarizes the results of experiments to determine the subdiploid, apoptotic fraction of U-87 MG, U-343 MG, and SF210 three days after treatment with 0 Gy or 10 Gy of ionizing radiation. Each bar represents the mean of the results of three independent experiments. The error bar is ± SD.

Results

Resistance of Glioma Cell Lines to Radiation-Induced Apoptosis

We previously determined that in the glioma-derived cell lines U-87 MG and U-343 MG, p53 is wild type, whereas it is mutant in SF210 (Chen et al., 1995; H.-K.G.S. and M.A.I., unpublished data, 1996). The cyclin-dependent kinase inhibitor $p21^{WAF1}$ has previously been shown to be induced by ionizing radiation after stabilization of wild-type but not mutant p53 (el-Deiry et al., 1993). Thus $p21^{WAF1}$ levels after radiation exposure can serve to assess the functional status of p53 transcriptional activation in U-87 MG, U-343 MG, and SF210 glioma cell lines. Twenty-four hours after treatment with 5 Gy ionizing radiation, $p21^{WAF1}$ protein levels were shown to increase in the U-87 MG and U-343 MG cell lines but remained unchanged in the SF210 cell line (Fig. 1A). Thus U-87 MG and U-343 MG contain a functional, wild-type p53, while SF210 has a mutant p53 phenotype. We used a fluorescence-activated cell sorting assay to quantitate the level of apoptosis after exposure to ionizing radiation by measuring the percentage of treated cells that had subdiploid DNA content. When U-87 MG, U-343 MG, and SF210 glioma cells were treated with 10 Gy of ionizing radiation, <5% of cells underwent apoptosis by 3 days posttreatment (Fig. 1B) (Haas-Kogan et al., 1996a, 1996b; Shu et al., 1998).

E2F1 Overexpression Induces Apoptosis in Glioma Cell Lines Irrespective of p53 Status

We used a recombinant adenoviral vector encoding E2F1 to transiently express this protein in U-87 MG, U-343 MG, and SF210 glioma cell lines. Each glioma cell line was infected with either the control adenoviral vector (AdCMV) or the recombinant adenoviral vector containing the *E2F1* transgene (AdE2F) at a multiplicity of infection of 100 (Kowalik et al., 1995; Schwarz et al., 1995). Twenty-four hours postinfection, E2F1 protein was detected in each of the AdE2F-infected cultures, but at lower levels in AdCMV-infected cells (Fig. 2A). Apoptosis was assayed after this *E2F1* transgene was expressed in each of the glioma cell lines. We quantitated apoptosis by determining the percentage of cells with subdiploid DNA content 3 days postinfection. Apoptosis was induced after overexpression of E2F1 in the U-87 MG and U-343 MG glioma cell lines that contained wild-type p53 and in the SF210 glioma cell line that contained a mutant p53 (Fig. 2B). Additionally, each of the cell lines was treated with 10 Gy of ionizing radiation immediately after they were infected with adenovirus. Treatment with 10 Gy ionizing radiation further increased the levels of apoptosis in cells expressing E2F1 (Fig. 2C), although it had little effect on uninfected cells (Fig. 1B) or AdCMV-infected cells (data not shown). Induction of apoptosis by E2F1 in the U-87 MG and U-343 MG glioma cell lines was confirmed by a deoxynucleotidyl transferase assay (data not shown).

We found high levels of apoptosis induced by E2F1 overexpression in cell lines resistant to apoptosis after DNA damage. E2F-induced apoptosis has been found to be p53-dependent in some cell types and p53-independent in others (Dyson, 1998; Fueyo et al., 1998; Hunt et al., 1997; Nip et al., 1997). Because E2F1 overexpression induced apoptosis in SF210, a glioma cell line with mutant p53, it appeared likely that E2F1 could induce a p53-independent apoptosis in glioma-derived cell lines. To confirm this observation, previously characterized U-87 MG clonal derivatives expressing a dominant negative p53 mutant were used (Haas-Kogan et al., 1996b; Yount et al., 1996). This included two U-87 MG clones expressing mutant p53 (175.4 and 175.13) and a corresponding U-87 MG clone expressing the luciferase gene (lux.6). The 175.4 and 175.13 U-87 MG clones have been shown

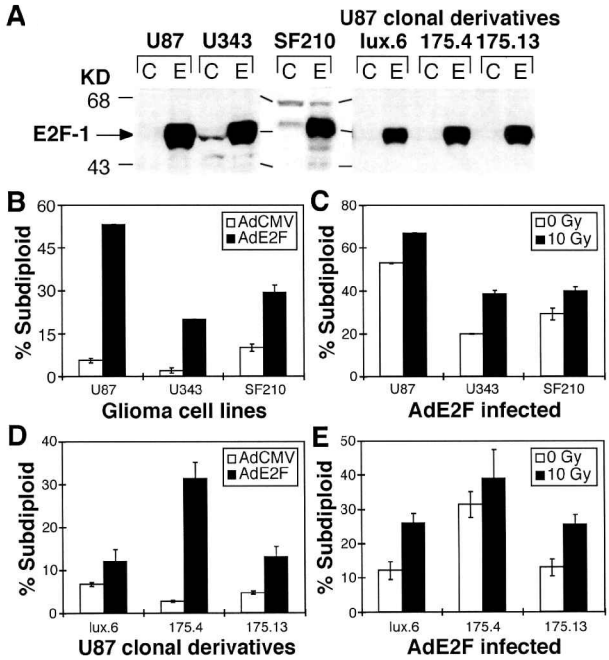

Fig. 2. Apoptotic response after introduction of E2F1 ± irradiation in glioma-derived cell lines. (A) Glioma-derived cell lines U-87 MG, U-343 MG, and SF210 and the U-87 MG clonal derivatives lux.6, 175.4, and 175.13 were infected with either a control recombinant adenovirus AdCMV (C) or one expressing E2F1 AdE2F (E), and lysates of total cellular protein were prepared 24 h postinfection. Total cellular protein was resolved on a 10% gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred to a polyvinylidene difluoride membrane, and examined by immunoblotting for E2F1 expression. Bar graphs summarize the results of experiments to determine the subdiploid, apoptotic fraction of (B) U-87 MG, U-343 MG, and SF210 three days after infection with AdCMV or AdE2F; (C) U-87 MG, U-343 MG, and SF210 three days after infection with AdE2F ± exposure to 10 Gy ionizing radiation; (D) U-87 MG derivatives lux.6, 175.4, and 175.13 three days after infection with AdCMV or AdE2F; and (E) U-87 MG derivatives lux.6, 175.4, and 175.13 three days after infection with AdE2F ± exposure to 10 Gy of ionizing radiation. Each bar represents the mean of the results of three independent experiments. The error bar is ± SD.

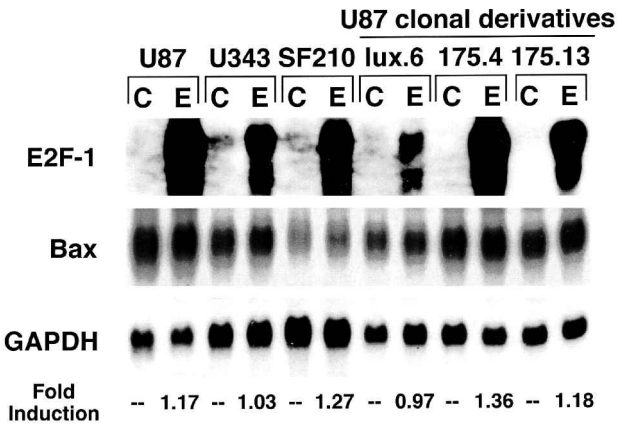

Fig. 3. Effect of E2F1 overexpression on *BAX* expression in glioma-derived cell lines. Representative Northern blot analysis is shown. Total RNA was prepared from U-87 MG, U-343 MG, SF210, and the U-87 MG derivatives lux.6, 175.4, and 175.13 24 h after infection with either AdCMV (C) or AdE2F (E). The blot was serially probed for expression of E2F1, BAX, and GAPDH mRNA. Quantitative densitometric analysis determining the fold induction of BAX mRNA normalized for GAPDH expression with E2F1 overexpression is shown below the appropriate lanes in the Northern blots.

not only to express a mutant p53 protein but also to no longer arrest in G_1 in response to ionizing radiation, while the lux.6 U-87 MG clone behaves similarly to the parental U-87 MG with wild-type p53 (Haas-Kogan et al., 1996b; Yount et al., 1996).

To examine E2F1-mediated apoptosis, each of these U-87 MG derivatives was infected with either AdCMV or AdE2F. Expression of E2F1 was assessed 24 h after infection. Each of these infected U-87 MG clones expressed high levels of E2F1 after being infected with AdE2F (Fig. 2A). Next, as detailed previously, we assayed for the induction of apoptosis by determining the subdiploid fraction 3 days after infection with either AdCMV or AdE2F. In each case, significant levels of apoptosis were induced after AdE2F infection (Fig. 2D), and this induction was further augmented by treatment with 10-Gy ionizing radiation (Fig. 2E), while again, no increase in apoptosis was noted in the AdCMV-infected U-87 MG clonal derivatives treated with radiation (data not shown).

E2F1-Mediated Apoptosis in Glioma Cell Lines Does not Depend on Transcriptional Activation of BAX but Results in Activation of Caspases

While E2F1-mediated apoptosis in glioma cell lines results from a p53-independent mechanism, a p53-dependent process could still contribute to E2F1-induced apoptosis in U-87 MG and U-343 MG glioma cell lines. The pro-apoptotic *BCL2* family member *BAX* has previously been shown to be a transcriptional target of p53, implicating it as the p53-dependent apoptotic signal in some cell types (Miyashita and Reed, 1995). To test whether *BAX* expression levels increased after E2F1 overexpression, all the glioma cell lines tested previously were infected with either AdCMV or AdE2F and assessed for E2F1 mRNA levels 24 h postinfection. Northern blots analyzed serially for E2F1, BAX, and GAPDH mRNA expression revealed unchanged BAX levels in all of the cell lines despite high levels of E2F1 expression in AdE2F-infected cells (Fig. 3). The hybridization pattern of RNA encoding GAPDH confirmed that equivalent amounts of RNA were added to each lane (Fig. 3). These results confirm that E2F1-mediated apoptosis in gliomas does not have a p53-dependent component mediated through *BAX* induction (Gomez-Manzano et al., 1999).

Because we and others have shown that E2F1 induces a p53-independent apoptosis in gliomas (Fueyo et al., 1998), we sought to examine downstream mediators of

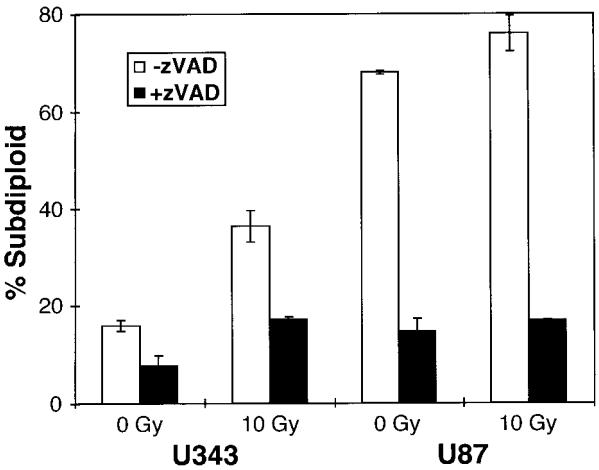

Fig. 4. Effect of caspase inhibition on E2F1-mediated apoptosis in glioma-derived cell lines. The bar graph summarizes the results of experiments to determine the subdiploid, apoptotic fraction of U-343 MG and U-87 MG glioma cell cultures three days after infection with AdE2F and treatment with 0-Gy or 10-Gy ionizing radiation ± zVAD-FMK, a tripeptide caspase inhibitor. Each bar represents the mean of the results of three independent experiments. The error bar is ± SD.

apoptosis in this system. We evaluated whether caspase activation occurred during induction of apoptosis by E2F1. This was assessed by determining the subdiploid fraction in the U-87 MG and U-343 MG cell lines infected with AdE2F in the presence or absence of 10 μM zVAD-FMK, a small peptide that is a broad spectrum inhibitor of caspases. At three days postinfection, cells in the absence of zVAD-FMK had levels of apoptosis comparable to those seen previously (Figs. 2B–C and 4). In the presence of zVAD-FMK, however, E2F1-mediated apoptosis was inhibited greatly (Fig. 4). These results implicate caspase activation as a critical step in the induction of apoptosis by E2F1 and confirm that apoptosis is in fact responsible for the DNA fragmentation seen in our cells.

Discussion

The prognosis of patients with GBM remains poor despite treatment with radiation therapy. Local recurrence of these tumors occurs frequently, indicating the resistance of these tumors to primary therapy. The failure to eradicate these tumors after treatment may be related to their intrinsic radioresistance (Haas-Kogan et al., 1996a, 1996b). We and others have noted that glioblastoma cell lines generally undergo very little primary apoptosis in response to ionizing radiation (Gomez-Manzano et al., 1996; Haas-Kogan et al., 1996a, 1996b). This may contribute to the poor response seen in GBM patients. We were interested in exploring whether activation of divergent cellular pathways could be used to increase the apoptotic response in glioma cell lines. We overexpressed E2F1 in glioma cell lines, and like Fueyo et al. found that apoptosis could be induced at high levels (Fueyo et al., 1998). Importantly, this apoptotic response could be further enhanced by treatment of the glioma cell lines with ionizing radiation in combination with E2F1 expression. E2F1 was able to induce apoptosis in glioma cell lines containing either a wild-type or mutant p53. Inhibition of caspase activity by the peptide inhibitor zVAD-FMK was sufficient to inhibit apoptosis in response to E2F1 overexpression, indicating that one or more member(s) of the caspase family act as downstream effector(s) of E2F1-mediated apoptosis in glioma-derived cell lines. As recently reported by others, we found that *BAX* induction was not found to be associated with the overexpression of E2F1 in these settings (Gomez-Manzano et al., 1999).

As we previously demonstrated, glioma cells that contain a wild-type p53 protein appear to have an intrinsic defect that prevents them from undergoing apoptosis in response to stabilization of p53 (Shu et al., 1998). Instead, they undergo a G_1 cell cycle arrest. The molecular basis for this defect in p53-dependent apoptosis may involve the selective inability of p53 to transcriptionally activate the *BAX* gene product (Shu et al., 1998). However, when we examined the status of *BAX* expression in glioma cell lines overexpressing E2F1, we found it was not induced in response to E2F1 overexpression. Thus, E2F1 does not appear to be complementing the defect in p53-dependent apoptosis that we observed, but instead is activating a p53-independent pathway leading to the induction of apoptosis.

In each of the glioma cell lines examined, a potentiation of the induction of apoptosis was observed after treatment with 10 Gy of ionizing radiation. This is particularly striking, because radiation-induced apoptosis does not occur to any great extent in the absence of E2F1 overexpression (see Fig. 1B) (Haas-Kogan et al., 1996a, 1996b; Shu et al., 1998). While the potentiation of E2F1-mediated apoptosis by ionizing radiation appears to be a small effect, this increase in cell kill may be magnified greatly in the setting of fractionated radiotherapy. Theoretically, even very small increases in cell kill will increase exponentially over the course of fractionated radiotherapy with a typical course for GBM consisting of more than 30 fractions. Utilizing *E2F1* in gene therapy concurrently with radiotherapy may have other theoretical advantages. E2F1 has been shown to drive cells that have undergone a radiation-induced G_1 arrest into *S*-phase (DeGregori et al., 1995) and may also promote the redistribution of cells into more sensitive portions of the cell cycle (for example, late G_2 and M) thereby increasing mitotic-linked cell death.

Treating gliomas, especially GBMs, remains a clinical challenge. Because GBM is typically infiltrative in nature, surgical resection may leave behind microscopic or gross disease. Radiation therapy, as currently practiced, will generally only delay local progression. Therefore, novel approaches to improve therapy in the treatment of GBMs are needed to make a significant impact in the survival of patients with this disease. Others have suggested the feasibility of using gene transfer of *E2F1* as a gene therapy strategy for the treatment of brain tumors (Fueyo et al.,

1998). Our finding suggests that new modalities of treatment that activate E2F-induced apoptosis may also be effective in increasing the sensitivity of these brain tumors to irradiation by enhancing tumor cell death.

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