Introduction of mutant *p53* into a wild-type *p53*-expressing glioma cell line confers sensitivity to Ad-*p53*-induced apoptosis¹

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Transient expression of the tumor suppressor gene *p53* via adenoviral-mediated gene transfer induces apoptosis in glioma cells expressing mutant *p53*, while causing cell cycle arrest in cells with wild-type *p53*. To determine whether a change in *p53* status of a wild-type *p53*-expressing cell line such as U-87 MG would alter its apoptotic resistant phenotype in response to Ad-*p53* infection, we generated cell lines U-87-175.4 and U-87-175.13 via retroviral-mediated gene transfer of the *p53* (175H) mutant into the U-87 MG parental line. Control cell lines U-87-Lux.6 and U-87-Lux.8 were also generated and express the reporter gene luciferase. Both U-87-175.4 and U-87-175.13, but not control cell lines, exhibited morphology characteristic of apoptosis after Ad-*p53* infection. Furthermore, expression of other *p53* mutants (248W, 273H) in U-87 MG also sensitized cells to Ad-*p53*-induced apoptosis. Apoptosis was confirmed by TUNEL and cell cycle analysis. Several *p53* response genes were examined in cells infected with Ad-*p53*, and among these, *BCL2*, *p21*^{WAF1/CIP1}, *CPP32/caspase 3*, and *PARP* showed differences in expression between U87-175 and U87-Lux cell lines. Taken together, our data demonstrate that the introduction of *p53* mutants in U-87 MG

promotes an apoptotic response in association with adenoviral-mediated wild-type *p53* gene transfer. These results underscore the importance of glioma *p53* genotype for predicting tumor response to *p53*-based gene therapy. *Neuro-Oncology* 3, 113–122, 2001 (Posted to *Neuro-Oncology* [serial online], Doc. 00-043, February 20, 2001. URL <neuro-oncology.mc.duke.edu>)

The *p53* tumor suppressor gene is a nuclear phosphoprotein that transcriptionally regulates the expression of a number of target genes including *p21*^{WAF1/CIP1} (el-Deiry et al., 1993), *GADD45* (Hollander et al., 1993), *BAX* (Miyashita and Reed, 1995), and *FAS/APO1* (Owen-Schaub et al., 1995). In normal cells, the *p53* protein is expressed at low levels and has a short half-life due to a rapid turnover rate mediated by ubiquitination and proteolysis. Upon exposure to a number of stressful stimuli, including DNA-damaging agents and hypoxia (Carmeliet et al., 1998), the *p53* protein is stabilized and, in turn, regulates biological functions including cell cycle arrest (el-Deiry et al., 1994), apoptosis (Liu et al., 1995), senescence (Sugrue et al., 1997), differentiation, and antiangiogenesis (Dameron et al., 1994). However, *p53* function is often deregulated or impaired after the occurrence of mutations or deletions resulting from neoplastic transformation.

Astrocytomas and glioblastomas are the most frequently occurring CNS tumors of neuro-epithelial origin and the most challenging brain tumors to treat. In more than 30% of astrocytomas, mutations exist in the *p53* tumor suppressor gene whereby inactivation occurs through missense mutation or loss of heterozygosity (Van Meir et al., 1994). The *p53* gene (located on chromosome 17) is frequently associated with the loss of 1 allele in malignant gliomas, although a large number of malignant gliomas have no *p53* mutations (Fults et al., 1992). Some of the latter tumors have an amplified *MDM2*

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³Abbreviations used are as follows: AdΔE1, replication defective adenovirus; Ad-*p53*, wild-type *p53* adenovirus; MOI, multiplicity of infection; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

(murine double minute 2) gene (He et al., 1994) that suppresses *p53* gene activity. Both the loss of normal *p53* function and the acquisition of oncogenic functions by mutant *p53* proteins may contribute to tumorigenesis.

Consistent with the definition of a tumor suppressor gene, the reintroduction of wild-type *p53* into many tumor cell lines, including gliomas, results in the activation of cell cycle arrest and/or apoptosis (Gomez-Manzano et al., 1996). The general conclusion from these studies is that the introduction of wild-type *p53* into neoplastic cells in vitro is lethal in most cases, so long as the cells are *p53^{mut}* or *p53^{null}*. However, the reintroduction of wild-type *p53* into *p53^{wt}* tumor cells results solely in cell cycle arrest (Nielsen and Maneval, 1998). The molecular mechanisms underlying the differential response of these cell lines to exogenous *p53* remains unclear.

The current phase I clinical trial using Ad-p53³ to treat glioma is based on laboratory studies in which Ad-p53 efficiently suppresses the growth of tumor cells bearing a mutant *p53* both in vitro and in vivo (Gomez-Manzano et al., 1996). However, given the fact that most tumors are heterogeneous in nature, and the ability of exogenous *p53* to affect only tumor cells harboring mutant *p53*, it is conceivable that the sole use of Ad-p53 for the treatment for cancer patients would not yield a satisfactory outcome. Under this notion, it is imperative to investigate the mechanism whereby cells with different *p53* gene status respond differently to Ad-p53.

Introduction of mutant *p53* (175H) into the wild-type *p53* glioblastoma cell line U-87 MG confers a dominant negative phenotype (Yount et al., 1996). When synchronized cells were irradiated in G₁, the control cell line (U87-Lux.8) expressing functional *p53* was twice as sensitive to radiation as the isogenic dominant negative mutant derivative (U87-175.4), in which *p53* was inactivated. Here we have investigated the apoptotic response of the U87-175 cell lines as well as other isogenic cell lines expressing different mutant *p53* genes (248W or 273H) after adenoviral-mediated gene transfer of wild-type *p53*. Our findings demonstrate that the presence of mutant *p53* is a major determinant in whether cells are responsive to Ad-p53 induced apoptosis.

Materials and Methods

Cell Lines

Both U87-Lux.8 and U87-175.4 were derived from the human glioblastoma cell line U-87 MG after recombinant retroviral infection and clonal selection in 400 µg/ml of G418 selection media. Both U87-Lux.8 and U87-175.4 cell lines were generated by infection of U-87 MG with recombinant retrovirus containing a mutant *p53* cDNA in codon 175H or a luciferase reporter gene, respectively (Hsiao et al., 1994). Isogenic cell lines of U-87 MG containing other *p53* mutants (248W or 273H) (Zhang et al., 1993) were generated by stable transfection with either pRc/CMV-248W or pRc/CMV-273H expression vector. After G418 selection, colonies were isolated and the expression of *p53* was assessed by Western blot analysis. Control cell lines were generated by transfection of only the expression vector pRc/CMV into

U-87 MG and similarly selected. Cells were cultured in Dulbecco's modified Eagle's/F12 medium (1:1, vol/vol) containing 10% fetal bovine serum supplemented with penicillin and streptomycin. Cells were maintained in a humidified atmosphere containing 5.0% CO₂ at 37°C.

Generation of Recombinant Adenovirus and Conditions of Infection

The recombinant *p53* adenovirus (Ad-p53) and Ad-βgal contain the cytomegalovirus promoter, wild-type *p53* cDNA or β-galactosidase, and SV40 polyadenylation signal in a mini-gene cassette inserted into the E1-deleted region of modified adenovirus type 5 (Ad5) (Liu et al., 1995). Replication-defective adenovirus AdΔE1 (provided by Dr. Bingliang Fang (Department of Thoracic and Cardiovascular Surgery, M.D. Anderson Cancer Center, Houston, Tex.)) was generated by cotransfecting the pXCJL-1 and pJM 17 plasmids Ad-p53 and AdΔE1 (Liu et al., 1999). The cell lines were infected as previously reported (Gomez-Manzano et al., 1996), and culture medium was used for mock infection. Infection was performed at 100 MOI for 1 h of incubation at 37°C with constant shaking.

Western Blot Analysis and Antibodies

Total cell lysates were prepared at various time points from mock-infected cells and cells infected with Ad-p53 or AdΔE1 virus. Cell pellets were washed in PBS and resuspended with Lysis buffer (150 mM NaCl, 1% Triton X-100, 1.0% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 20 mM EDTA, and 50 mM Tris, pH 7.4) for 30 min on ice. Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Richmond, Calif.). Fifty micrograms of protein were subjected to gel electrophoresis and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After separation, proteins were transferred to a nitrocellulose membrane and blotted with either anti-p53 DO-I, Ab-3 (pAb240; Oncogene Science, Mineola, N.Y.), anti-p21^{WAF1/CIP1} (Santa Cruz Biotechnology, Santa Cruz, Calif.), anti-BAX, anti-BCL-X_L, anti-MDM2, anti-BCL2, or anti-pro-CPP32 antibodies (Santa Cruz Biotechnology). For PARP detection, cells were sonicated on ice for 20 s and stored in -20°C. Lysates were incubated at 65°C for 15 min prior to gel loading. One hundred micrograms of protein was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel analysis and was probed with an anti-PARP C2-10 monoclonal antibody at a dilution of 1:2000. Equal loading was determined by using an antiactin antibody (Amersham Corp., Arlington Heights, Ill.).

Cell Viability Assay

Cell cultures were harvested with 0.05% trypsin and seeded in triplicate at 1×10^5 in 6-well culture plates for 24 h prior to viral infection. Cells were infected with Ad-p53 or AdΔE1 at 100 MOI. Culture medium was used for mock infection. Cell populations were counted at various time points after infection. Cell viability was assessed by trypan blue (0.1%) exclusion.

Caspase Activity Assay

Cells ($1.0\text{--}2.0 \times 10^6$) were scraped, washed in PBS, and lysed using 25–50 μl of cell lysis buffer (RandD Systems, Minneapolis, Minn.) to obtain cell lysates. Each enzymatic reaction was performed using 100 μg total protein and was carried out in a flat-bottom 96-well plate. Five microliters of the caspase fluorogenic substrate was added to each reaction and samples were incubated at 37°C for 1 to 2 h. Fluorescence was measured on a microplate reader using a filter that allowed a light excitation at 400 nm and light collection at 505 nm (Perkin Elmer, Norwalk, Conn.). Substrate for each caspase is as follows: caspase-1, WEHD-AFC; caspase-2, VDAD-AFC; and caspase-3, DEVD-AFC.

Flow Cytometry

Cells were infected with 100 MOI of Ad-p53 or replication-defective virus Ad Δ E1. They were harvested at 48 h and 96 h postinfection, and pellets were washed 3 times in PBS and fixed with 70% ethanol for 24 h at –20°C. The cells were then stained with a solution of PBS containing 1.0% propidium iodide for 20 min at 37°C to measure DNA content. Flow cytometry of cell cycle profiles was performed on an EPICS (I) Profile Analyzer (Coulter Corp., Hialeah, Fla.).

TUNEL Assay

The assay was performed according to the manufacturer's procedure (Phoenix Flow Systems, San Diego, Calif.). Briefly, after fixation and washing, cells were resuspended in 50 μl of TdT buffer containing 0.2 M sodium cacodylate (pH 7.0), 2.5 mM Tris-HCl, 2.5 mM CoCl₂ (Sigma Chemical Company, St. Louis, Mo.), 0.1 mM DTT (Sigma), 0.25 mg/ml bovine serum albumin (Sigma), 5 units of terminal transferase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and 0.5 nmol biotin-16-dUTP along with dATP, dGTP, and dCTP at a concentration of 20 μM . Controls were prepared by incubating a separate aliquot of each test sample without dUTP. The cells were incubated in the solution at 37°C for 30 min, rinsed in PBS, and resuspended in 100 μl fluorescein isothiocyanate staining solution containing 4X saline-sodium citrate, 0.1% Triton X-100, and 2.5 $\mu\text{g}/\text{ml}$ fluoresceinated avidin (Vector, Burlingame, Calif.). Tubes were incubated for 30 min in the dark at room temperature. Cells were rinsed in PBS with 0.1% Triton X-100 and resuspended in 0.5 ml PBS containing propidium iodide (5 $\mu\text{g}/\text{ml}$) and 70 μl (1 mg/ml) RNase. Tubes were incubated in the dark on ice for 30 min prior to flow cytometric analysis.

Results

In Vitro Growth Suppression of Isogenic Mutants as a Result of Transient p53 Expression

To investigate the biological effect of wild-type p53 in glioma cells that differ in their p53 status, we infected isogenic cell lines U87-175.4 and U87-Lux.8 with Ad-p53 virus. Comparable transduction efficiency was

observed in these cell lines after Ad- β gal reporter virus infection (data not shown). After Ad-p53 infection, U87-Lux.8 displayed morphology characteristically observed in senescence including flat, multinucleated cells. This behavior was similar to that which has been reported for U87 MG cells infected with Ad-p53 (Gomez-Manzano et al., 1996). In contrast, a significant percentage of rounded, dead cells and cell membrane blebbing indicative of apoptosis was observed in U87-175.4 cultures after Ad-p53 infection (Fig. 1). Moreover, elevated levels of p53 in U87-175.4 cells led to a significant ($P < 0.001$) reduction in cell viability (Fig. 2). No features of apoptosis were evident in mock-infected U87-Lux.8 or U87-175.4 cells nor in cells infected with Ad Δ E1, a replication-defective virus void of E1 and E3 genes. Although trypan blue exclusion results indicated similar degrees of cell viability in Ad Δ E1 infection control for both cell lines, some cytotoxicity was noted in Ad Δ E1-infected cultures compared with mock control. The apparent decrease in cell number can be attributed to growth suppression and not to cell death, as later determined by flow cytometry.

Growth Suppression as a Consequence of Apoptosis

Cell cycle analysis was performed using flow cytometry, and the sub-diploid (sub-G₁) population was measured as an indication of apoptosis. A time-dependent increase in the sub-G₁ population of Ad-p53-infected U87-175.4 cells was observed, as it escalated to 60.9% by 96 h postinfection. In contrast, no significant increase was evident in U87-Lux.8 cells infected with Ad-p53 (Table 1 and Fig. 3). These results correlate with cell viability measurements after Ad-p53 infection. Interestingly, a high percentage of cells in G₁ was detected in both cell lines before Ad-p53 infection. This can be attributed to slow cell growth rates previously recorded by Cell Titer 96AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, Wis.) (data not shown). Cell cycle analysis of U87-Lux.8 cells after Ad-p53 infection resembled that of infected U87 MG (Gomez-Manzano et al., 1997). Both cell lines undergo a G₁ arrest postinfection; however, a small population of U87-Lux.8 cells resumes cycling at 96 h (Table 1). In contrast, U87-175.4 cells infected with Ad-p53 exhibited no build-up in G₂/M before undergoing apoptosis. The cell cycle distribution of U87-175.4 cells is unlike that of the control cell line U87-Lux.8. Cells infected with Ad Δ E1 virus experienced a background level of cytotoxicity often associated with adenoviral infections. However, flow cytometry and morphologic evidence portray healthy growing cells and indicate that minimal cell death present in control infections was not a result of apoptosis.

To examine whether acute levels of p53 have a generalized effect on other U87-175 transfectants, we performed TUNEL assays on cell lines U87-Lux.6, U87-Lux.8, U87-175.4, and U87-175.13. Results shown in Table 2 demonstrated that a higher percentage of TUNEL-positive cells was detected in both isogenic mutant cell lines than in the control cell lines after Ad-p53 infection for different time intervals. Some toxicity was noted in U87-Lux.6 cells infected with Ad Δ E1.

Fig. 1. Morphological analysis. Phase-contrast photomicrographs (original magnification $\times 100$) were taken of the U87-Lux.8 and U87-175.4 cells infected with either Ad Δ E1 or Ad-p53 for 48 and 96 h by using a diaphotomicroscope. A. Mock infection of U87-Lux.8 cells for 48 h. B. Ad Δ E1 infection of U87-Lux.8 cells for 48 h. C. Ad-p53 infection of U87-Lux.8 for 48 h. D. Mock infection of U87-175.4 cells for 48 h. E. Ad Δ E1 infection of U87-175.4 cells for 48 h. F. Ad-p53 infection of U87-175.4 for 48 h. G. Mock infection of U87-Lux.8 cells for 96 h. H. Ad Δ E1 infection of U87-Lux.8 cells for 96 h. I. Ad-p53 infection of U87-Lux.8 for 96 h. J. Mock infection of U87-175.4 cells for 96 h. K. Ad Δ E1 infection of U87-175.4 cells for 96 h. L. Ad-p53 infection of U87-175.4 for 96 h. Note there are fewer cells in panel L and cells with blebbed membranes, which is indicative of an apoptotic event occurring in these cells.

Taken together, these results suggested that the introduction of mutant *p53* (175H) into a cell line containing a wild type *p53* resulted in a generalized apoptotic-sensitive phenotype in response to Ad-p53 infection. It is noteworthy that both U87-175.4 and U87-175.13 cell lines exhibited a high rate of spontaneous apoptosis of 8.6% and 7.1%, respectively, as detected in 72 h post-Ad-p53 infection.

To test the ability of other *p53* mutants in sensitizing U-87 MG to Ad-p53-induced apoptosis, 2 additional isogenic *p53*-mutant cell lines overexpressing a mutant *p53* (248W or 273H) were generated. Twenty different clones from each mutant construct were isolated and

analyzed for *p53* expression by Western blot analysis (data not shown). Based on Western blot analysis, 2 clonal cell lines expressing highest levels of *p53*, U87-248-1, and U87-273-D, as well as a cell line U87C expressing an empty vector, were infected with 100 MOI of Ad-p53. DNA content and cell cycle profiles were determined by propidium iodide staining and fluorescence-activated cell sorting analysis. Both cell lines exhibited an increase in the percentage of sub-G₁ population after Ad-p53 infection (Table 3). These results suggest that at least 3 different *p53* mutants can sensitize wild-type *p53*-expressing cells to Ad-p53-induced apoptosis to varying degrees.

Fig. 2. Viability assay: Effects of exogenous p53 on the growth of U87-Lux.8 and U87-175.4 cells. The cells were plated at low density and were infected with AdΔE1 or Ad-p53 for 96 h before being assessed for their viability by using trypan blue exclusion dye. The number of viable cells displayed includes the mean $\pm$ SD of triplicate experiments, and *P* values were determined by *t*-test analyses between AdΔE1 and Ad-p53 data. SDs of U87-175.4 experiments were too small to appear in the graph. The assay was repeated a minimum of 3 times.

Expression of p53 Target Genes After Ad-p53 Infection

Immunoblots for p53 expression revealed up to a 4-fold increase in protein levels in both U87-175.4 and U87-Lux.8 infected with Ad-p53 (Fig. 4A). U87-175.4 exhibited higher endogenous levels of p53 than did U87-Lux.8, presumably due to the expression of mutant p53 (175H). Western blot analyses were performed to examine differential expression of known p53 target genes in response to Ad-p53 infection.

Overexpression of MDM2, a negative regulator of p53, has been shown to inactivate wild-type p53 and prevent apoptosis (Chen et al., 1996). We sought to determine if irregular expression of MDM2 was responsible for resistance to p53-mediated apoptosis in our system. Western blot analysis indicated that similar levels of MDM2 were detected in both U87-Lux.8 and U87-175.4 up to 96 h after infection, whereas undetectable levels of MDM2 were noted in the mock and AdΔE1-infected control cells (Fig. 4B). These levels were consistent with those recorded after overexpression of p53 through DNA damage or apoptotic insult (An et al., 1998). Although allelic exclusion or mutations in MDM2 have

been associated with abnormalities in p53 in numerous cell lines (Cheng et al., 1995), it appears that differential MDM2 expression was not responsible for the differences in apoptotic response demonstrated by U87-Lux.8 and U87-175.4.

Overexpression of BCL2 has been shown to inhibit p53-mediated apoptosis after various apoptotic stimuli. We examined whether there were alterations in BCL2 protein levels involved in Ad-p53-induced apoptosis in U87-175.4. Endogenously, U87-Lux.8 and U87-175.4 express comparable levels of BCL2 as shown in mock- and AdΔE1-infected samples (Fig. 5). However, BCL2 expression in U87-Lux.8 cells decreased at 48 h post-Ad-p53 infection and were sustained at such a level until 96 h (Fig. 5). In contrast, after Ad-p53 infection, BCL2 levels in U87-175.4 cells appeared to increase modestly at 48 h and 72 h with respect to the 24 h level, yet were not significantly greater than mock or AdΔE1 samples. These findings, however, did not fully account for cell viability or TUNEL results and suggested that the involvement of other BCL2 family members may contribute to inhibiting apoptosis in the wild-type p53 glioma (McDonnell et al., 1996). Therefore, Bcl-X_L protein expression was examined under the same conditions. No differences in expression were detected between uninfected and Ad-p53-infected cells (data not shown). Similarly, there was no indication that differences in BAX protein levels measured after Ad-p53 infection were responsible for the observed differences in apoptotic response displayed by U87-Lux.8 and U87-175.4. The role of other BCL2 family members in this model system remains to be determined and is currently under investigation.

p21^{WAF1/CIP1} is a key cell cycle regulator that mediates growth arrest by inhibiting the action of G₁ cyclin-dependent kinases (Waga et al., 1994). As a downstream target of p53, p21^{WAF1/CIP1} levels can be induced after Ad-p53 infection in glioma cell lines regardless of their p53 status (Gomez-Manzano et al., 1997). We sought to determine whether Ad-p53 infection induces p21^{WAF1/CIP1} expression and promotes cell cycle arrest in U87-Lux.8 and U87-175.4 cells. Western blot analysis showed higher levels of p21^{WAF1/CIP1} expression in U87-Lux.8 cells than in U87-175.4 cells during 24–72 h postinfection (Fig. 5). Protein levels appeared to decrease in both cell lines at 96 h postinfection. Decreases in p21^{WAF1/CIP1} expression correlated well with the concomitant reduction of G₁ cell population observed in cell cycle analysis (Fig. 5 and Table 1).

Table 1. Cell cycle analysis of U87-Lux.8 and U87-175.4 cells treated for 48 h and 96 h with AdΔE1 or Ad-p53

Cell phase	U87-Lux.8				U87-175.4			
	48 h		96 h		48 h		96 h	
	ΔE1	p53	ΔE1	p53	ΔE1	p53	ΔE1	p53
G ₁ (% cells)	73.9	82.9	84.1	76.0	81.4	81.7	83.1	66.0
S (% cells)	10.8	7.0	11.2	17.9	11.5	12.2	11.2	25.2
G ₂ /M (% cells)	10.3	10.0	4.7	6.1	7.0	6.1	5.7	8.8
Sub-G ₁ (% cells)	1.2	1.7	8.4	5.0	2.0	19.9	2.5	60.9

Cells ($1-2 \times 10^6$) were infected with 100 MOI of AdΔE1 or Ad-p53 for 48 h or 96 h. After infection, total cells, including the floating cells, were harvested and subjected to flow cytometric analysis.

Fig. 3. Cell cycle analysis. U87-Lux.8 and U87-175.4 cells were infected with AdΔE1 or Ad-p53 virus for 48 h and 96 h. Cells were then harvested for cell cycle analysis. A. U87-Lux.8 cells infected with AdΔE1 (A for 48 h and C for 96 h) or with Ad-p53 (left panel; B for 48 h and D for 96 h); and U87-175.4 cells infected with AdΔE1 (right panel; E for 48 h and G for 96 h) or with Ad-p53 (F for 48 h and H for 96 h). The sub-G₁ population of cells is as indicated. B. Percentage of sub-G₁ cell population. The sub-G₁ percentage represents the cellular fraction containing less than 2n DNA indicative of apoptosis.

PARP Cleavage Was Evident in U87-175.4 Cells upon p53-Induced Apoptosis

Involvement of caspases in cell death is a hallmark for apoptosis. We examined whether overexpression of p53 in both U87-Lux.8 and U87-175.4 cells would lead to differential expression of a central caspase, CPP32 (caspase-3), and subsequently apoptosis in U87-175.4 cells. Western blot analysis was used to measure the levels of the proform of CPP32 as an indicator of caspase activation. In the U87-Lux.8 cell line, CPP32 levels increased at 48 h post Ad-p53 infection compared with controls (Fig. 6A). The significance of the accumulation of CPP32 proform is unclear. However, U87-Lux.8 cells do not undergo apoptosis when assayed longer than 96 h post-Ad-p53 infection (data not shown). Interestingly, no changes in the proform of CPP32 were detected in Ad-p53-infected U87-175.4 cells (Fig. 6A).

Characteristic with the onset of apoptosis is the cleavage of enzymes that are responsible for DNA fragmentation. PARP functions as a DNA repair enzyme and is cleaved by caspases during apoptosis. PARP is a downstream target of multiple caspases, including caspase-2,

CPP32, caspase-7, and caspase-9 (King et al., 1998). We examined whether the apoptotic event detected in U87-175.4 cells involved PARP cleavage. Western blot analysis for the identification of PARP cleavage was performed using C2-10 anti-PARP antibody. The 85-kDa cleaved product can be detected as early as 48 h after Ad-p53 infection and is visible for up to 96 h after infection (Fig. 6B). However, in the mock-infected and AdΔE1-infected controls, a cleaved product of PARP was also detected, yet to a lesser degree. A similar analysis was performed for U87-Lux.8 cells. Western blot analysis indicates that no cleavage product corresponding to the molecular mass of 85 kDa was evident in U87-Lux.8 cells infected with Ad-p53 virus (Fig. 6B). Furthermore, in contrast to what was observed in U87-175.4 cells, no basal level of the 85-kDa protein was detected in the mock- and AdΔE1-infected controls for U87-Lux.8 cells.

Enzymatic Activity of Caspases After Ad-p53 Infection

To further confirm the apoptotic induction by p53 in U87-175.4, we performed a more sensitive assay by measuring the activity of several caspases. Both U87-Lux.8

Table 2. TUNEL analysis of U-87 MG derivatives after infection with Ad-p53, AdΔE1, or culture medium (mock)

	U87-Lux.8		U87-175.4		U87-Lux.6		U87-175.13	
Vector	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
Mock	0.7	0.8	6.2	8.6	3.5	3.8	2.4	7.1
AdΔE1	0.8	3.7	5.0	6.0	5.3	26.3	2.6	6.3
Ad-p53	0.8	4.6	8.2	39.4	7.9	23.9	3.3	29.3

Cells (2×10^5) were infected with 100 MOI of AdΔE1 or Ad-p53 for 24 h or 72 h. Mock infection with medium was used as a control. After infection, cells were harvested and 1×10^6 cells were subjected to TUNEL analysis. The numbers shown are the percentage of TUNEL-positive cells indicating apoptosis.

Table 3. Cell cycle analysis of U87-248-1 and U87-273-D cells after treatment with Ad Δ E1 or Ad-p53 for 48 h or 96 h

Cell phase	U87-248-1				U87-273-D				U87 control			
	48 h		96 h		48 h		96 h		48 h		96 h	
	Δ E1	p53	Δ E1	p53	Δ E1	p53	Δ E1	p53	Δ E1	p53	Δ E1	p53
% cells G ₁	65.6	81.1	72.6	81.4	59.8	81.9	70.7	80.6	68.8	80.9	73.3	75.1
% cells S	17.6	6.5	8.7	8.9	18.5	7.0	8.5	8.7	14.3	6.3	6.9	8.3
% cells G ₂ /M	16.8	14.5	18.7	9.8	21.7	11.1	20.7	10.7	16.9	12.8	19.7	16.6
% cells Sub-G ₁	3.8	4.8	4.8	15.4	2.0	1.9	4.8	13.3	2.8	1.0	3.3	3.7

Cells (2×10^5) were infected with 100 MOI of Ad Δ E1 or Ad-p53 for 48 h or 96 h. After infection, total cells, including the floating cells, were harvested and subjected to flow cytometric analysis.

and U87-175.4 cell lines were mock-infected, Ad Δ E1 infected, or Ad-p53 infected for 96 h, and cell extracts were assayed for caspase-1, -2, and -3 activity using a commercial kit (R&D Systems, Minneapolis, Minn.). There was no induction of caspase-1 activity in both cell lines after Ad-p53 infection. However, U87-175.4 cells exhibited approximately a 2-fold induction of caspase-2 and -3 enzymatic activity after Ad-p53 infection compared with Ad Δ E1-infected extracts (Fig. 7). A minimal induction of both caspase-2 and caspase-3 activities was observed in U87-Lux.8 cells after Ad-p53 infection. Furthermore, induction of caspase-2 and caspase-3 enzymatic activity in U87-175.4 correlated with TUNEL and cell viability results.

Discussion

Current ideology aims to use gene therapy (Fujiwara et al., 1996) or synthetic peptides (Hupp et al., 1995) to restore wild-type *p53* function in cells harboring a mutant *p53* protein or those that have defective *p53* pathways. Although some success with this approach has been achieved, cell types remain that are nonresponsive to these forms of therapy. Despite the ongoing gene therapy trial using Ad-p53 for glioma patients, the underlying mechanism of differential response to apoptotic induction after Ad-p53 infection is unclear. In this study, we sought to investigate the importance of *p53* status in response to Ad-p53-induced apoptosis. We hypothesized that the introduction of a mutant *p53* into a wild-type *p53*-containing cell line would sensitize the cells to Ad-p53-induced apoptosis. Our results demonstrated that *p53* status in glioma cells is integral to determining cellular fate after Ad-p53 insult.

Isogenic mutant cell lines of U87-Lux.8 and U87-175.4 have been used to demonstrate that the overexpression of a mutant *p53* in a wild-type *p53*-expressing cell line alters its radiosensitive phenotype (Yount et al., 1996). Using these cell lines, we demonstrated that the reintroduction of a functional wild-type *p53* via adenoviral-mediated gene transfer into U87-175.4 cells results in an apoptotic-sensitive phenotype similar to cell lines with endogenous *p53* mutations. Interestingly, other *p53* mutants such as 248W and 273H also exert similar effects as 175H, albeit less effectively. Several studies support the active role of multiple mutant *p53* genes in transcription and protein-protein interactions

(Friedlander et al., 1996; Selivanova et al., 1997). Evidence by Zhang et al. (1993) indicates that both 273 and 175 mutants can retain their DNA binding and transcriptional activity. Alternatively, Hachiya et al. (1994) have demonstrated that both the 175 and 248 mutants, but not 273, can suppress wild-type *p53* transcriptional activity when co-expressed in cells. Because the extent of apoptotic induction elicited by these *p53* mutants varied, the effect on apoptotic response by these mutants may reflect their differential binding activity to the *p53*-responsive element.

A

U87-Lux.8

U87-175.4

B

Fig. 4. A. Time-dependent expression of p53 protein in U87-Lux.8 and U87-175.4 cells after Ad-p53 infection. Western blot analysis was performed on the cell lysates by using an anti-p53 antibody (DO-1). Expression of p53 protein increased in a time-dependent fashion. Mutant p53 protein was also detected only in the mock and Ad Δ E1 controls of U87-175.4 cells compared with the same controls of U87-Lux.8 cells. B. Expression of MDM2 protein in U87-Lux.8 and U87-175.4 cells after Ad-p53 infection. Western blot analysis was performed on cell lysates extracted from cells 96 h after Ad-p53 infection. In both cell lines, MDM2 expression was induced only in Ad-p53 infected cells compared with controls, indicating that the exogenous p53 retained its normal function.

Fig. 5. Expression of *p53*-responsive genes in U87-Lux.8 and U87-175.4 cells after Ad-*p53* infection. Western blot analyses were performed to examine *BCL2* and *p21*^{WAF1/CIP1} expression at indicated time intervals in response to Ad-*p53* infection in both cell lines.

The most outstanding difference between the two cell lines examined in response to Ad-*p53* infection was the degree of apoptosis exhibited by U87-175.4 cells. The solitary difference between U87-Lux.8 and U87-175.4 cells is the presence of mutant *p53* in U87-175.4 cells. Although expression of mutant *p53* in U87-175.4 cells altered its phenotype in response to Ad-*p53*, its degree of sensitivity to *p53*-mediated apoptosis is not identical to that of the natural *p53* mutant cell such as U-251 MG. One possible explanation for the observed difference lies in the frequency of multiple mutations in glioblastoma genesis. Alterations in genes such as *RB*, *MMAC/PTEN*, and *p16/p14^{arf}* result

in a multitude of destabilized pathways and may contribute to the sensitivity to Ad-*p53*. In addition, the formation of wild-type *p53* and mutant *p53* heterodimers may affect transcriptional regulation of key apoptotic genes and account for the phenotype of the isogenic cell lines. These heterotetramers may preferentially activate genes or interfere with regulators that induce apoptosis.

We determined that the expression of *p53* was elevated in both U87-Lux.8 and U87-175.4 cells after Ad-*p53* infection. Therefore, the apoptosis observed in U87-175.4 cells was not a consequence of differential *p53* expression, rather the difference in apoptotic response

Fig. 6. Western blot analyses of the activation of caspase-3 (CPP32) on U87-Lux.8 and U87-175.4 cells after *p53* transfer. A. Expression of CPP32 protein was detected with a polyclonal antibody. In the U87-Lux.8 cell line, CPP32 levels increased at 48 h post-Ad-*p53* infection as compared with controls, whereas the levels of CPP32 in U87-175.4 remained constant. B. Cleavage of PARP was detected in U87-175.4 cells.

Fig. 7. Enzymatic activity of caspases. Both U87-Lux.8 and U87-175.4 cell lines were mock AdΔE1- or Ad-p53-infected for 96 h. Cellular extracts were assayed for enzymatic activity of caspase-1, caspase-2, and caspase-3 by using commercial kits. Fold induction was calculated by dividing the activity of each experiment with that of the mock control. Experiments were repeated 3 times with similar results.

may reflect differences in the transcription of *p53* target genes. With regard to this possibility, both cell lines expressed elevated levels of $p21^{\text{WAF1/CIP1}}$ after Ad-p53 infection, suggesting that the $p53/p21^{\text{WAF1/CIP1}}$ pathway was not compromised in either cell line. Additionally, the levels of MDM2, a negative regulator of p53 function, were unaltered in these 2 cell lines after Ad-p53 infection. The potential involvement of both *BCL2* and *BAX* was also examined. After Ad-p53 infection, *BCL2* levels appeared to decrease in U87-Lux.8 cells over time, in contrast with those that remained unchanged in U87-175.4-infected cells. No noticeable changes of *BAX* expression in either U87-Lux.8 or U87-175.4 were evident. Results of *BCL2* and *BAX* expression in the isogenic mutant cell lines contradict the results from cell viability and TUNEL assays showing that U87-Lux.8 cells are resistant to *p53*-induced apoptosis.

One of the most important downstream mediators of the caspase cascade is CPP32. Differences in the endogenous levels of CPP32 were evident in the 2 cell lines studied whereby U87-175.4 cells display higher levels of the pro-CPP32 protein than did U87-Lux.8 cells. Interestingly, levels of the proform of the enzyme increased after Ad-p53 infection in U87-Lux.8 but not in U87-175.4 cells. It is likely that this increase is a result of the inability of cellular machinery to activate CPP32 and contributes to its apoptotic insensitive phenotype. In contrast, the levels of pro-CPP32 in Ad-p53-infected U87-175.4 cells remain constant and may be due to continual processing of the procaspase. A more sensitive enzymatic assay for caspase activity revealed that several caspases, including caspase-2 and caspase-3, were activated after Ad-p53 infection on U87-175.4 cells. These results point toward the involvement of complex proteolytic systems whereby a combination of proteases, cofactors,

inhibitors, feedback loops, and thresholds act in concert to execute an apoptotic signal (Thornberry and Lazebnik, 1998). Finally, the DNA repair enzyme PARP was cleaved in U87-175.4 cells but not in U87-Lux.8 cells after Ad-p53 infection. These data confirm that PARP cleavage is indeed an active part of *p53*-mediated apoptosis in glioma and includes the activation of multiple caspases. In this study we examined the effect of Ad-p53 on the expression of only a few key target genes. It is likely that other genes may contribute to the sensitization of U87-175.4 cells to *p53*-induced apoptosis. Efforts to employ techniques such as the DNA array, for the profiling of multiple gene expression in response to Ad-p53, are under way.

Taken together, our results demonstrate the importance of *p53* status in governing cellular fate after Ad-p53 treatment. The ability to alter an apoptotic-resistant phenotype indicates that intrinsic apoptotic pathways are functional in U-87 MG cells and can be reinitiated upon the change in *p53* status. Understanding *p53* pathways and the mediators of the apoptotic response in glioma will significantly facilitate the design of novel antitumor strategies. Gaining insight into the intricate mechanisms of resistance in glioma will enable us to pinpoint the genes or pathways to target for radiation or gene therapy.

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