

# Restoration of p53 function for selective Fas-mediated apoptosis in human and rat glioma cells *in vitro* and *in vivo* by a p53 COOH-terminal peptide

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## Abstract

We have shown that a COOH-terminal peptide of p53 (amino acids 361–382, p53p), linked to the truncated homeobox domain of Antennapedia (Ant) as a carrier for transduction, induced rapid apoptosis in human premalignant and malignant cell lines. Here, we report that human and rat glioma lines containing endogenous mutant p53 or wild-type (WT) p53 were induced into apoptosis by exposure to this peptide called p53p-Ant. The peptide was comparatively nontoxic to proliferating nonmalignant human and rat glial cell lines containing WT p53 and proliferating normal human peripheral marrow blood stem cells. Degree of sensitivity to the peptide correlated directly with the level of endogenous p53 expression and mutant p53 conformation. Apoptosis induction by p53p-Ant was quantitated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay and Annexin V staining in human glioma cells *in vitro* and in a syngeneic orthotopic 9L glioma rat model using

convection-enhanced delivery *in vivo*. The mechanism of cell death by this peptide was solely through the Fas extrinsic apoptotic pathway. p53p-Ant induced a 3-fold increase in extracellular membrane Fas expression in glioma cells but no significant increase in nonmalignant glial cells. These data suggest that p53 function for inducing Fas-mediated apoptosis in gliomas, which express sufficient quantities of endogenous mutant or WT p53, may be restored or activated, respectively, by a cell-permeable peptide derived from the p53 COOH-terminal regulatory domain (p53p-Ant). p53p-Ant may serve as a prototypic model for the development of new anticancer agents with unique selectivity for glioma cancer cells and it can be successfully delivered *in vivo* into a brain tumor by a convection-enhanced delivery system, which circumvents the blood-brain barrier. [Mol Cancer Ther 2006;5(1):20–8]

## Introduction

p53, known as the “guardian of the genome,” is a sequence-specific transcription factor that binds DNA and transactivates cellular proteins involved in growth and cell cycle regulation (1–6). p53 is the most frequently mutated gene in human neoplasms (7). Major transcriptional targets of functional p53 are p21WAF/Cip1, which mediates cell cycle arrest; GADD45, which mediates growth arrest and DNA repair; and the proteins Bax, Bak, Bcl-2, and Fas/Apo-1, which mediate programmed cell death (4, 8–11). In addition, functional p53 can induce activation of Fas/Apo-1-mediated apoptosis without transcriptional/translational changes, although this is not completely understood.

The p53 molecule contains three major domains: NH<sub>2</sub>-terminal transactivation domain, central sequence-specific DNA-binding domain, and a COOH-terminal negative regulatory domain involved in modulating p53 function (1, 12, 13). Fifty percent of all human malignancies and up to 65% of human gliomas have missense or nonsense mutations of p53. The great majority of these missense mutations have been mapped to the central DNA-binding region of the protein. The sequence-specific DNA-binding activity of p53 is negatively regulated by its 30–amino acid COOH-terminal sequence (amino acids 363–393) in concert with its NH<sub>2</sub>-terminal proline-rich region located between amino acids 80 and 93. Synthetic peptides derived from the COOH-terminal sequence bind directly to wild-type (WT) and mutant p53 *in vitro*, and binding activity was dependent on the presence of both the proline-rich NH<sub>2</sub>-terminal amino acids 80 to 93 sequence and the COOH-terminal amino acids 363 to 393 sequence in the p53 protein (13). We have calculated computational docking models of low-energy conformations of COOH-terminal

Received 6/7/05; revised 9/19/05; accepted 10/14/05.

**Grant support:** NIH grant RO1 CA 82528, Columbia University Cancer Center Translational Award, and Vinzinni Brain Cancer Award (R.L. Fine), NIH grant RO1 CA 89395 (J.N. Bruce), NIH grant RO1 OH 07590 (P.W. Brandt-Rauf), Catherine O'Rourke/American Brain Tumor Association Fellowship (P.B. Senatus), Reza Khatib Fellowship (C. Mandigo), American Brain Tumor Association Medical Student Fellowship (G. Moise), and George Becker Fellowship (M.D. Brown).

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**Note:** P.B. Senatus and Y. Li contributed equally to the work. J.N. Bruce and R.L. Fine are senior authors.

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doi:10.1158/1535-7163.MCT-05-0181

and NH<sub>2</sub>-terminal p53-derived sequences that predict a unique low-energy, favorable, and stable complex between the two domains (14). Addition of exogenous COOH-terminal p53 peptide restored sequence-specific DNA-binding function to many mutant p53 molecules, including p53-273 (Arg-to-His) *in vitro*. This mutation is the second most common p53 mutation in human cancers. Moreover, introduction of COOH-terminal p53 peptide, via micro-injection or Antennapedia (Ant) transduction protein, has restored transactivation of a p53-responsive reporter construct and induced apoptosis in SW480 colon carcinoma cells with mutant p53-273 (Arg-to-His; refs. 15, 16). COOH-terminal p53 peptide alone cannot traverse the cell membrane because of its multiple basic charged amino acids unless it is linked to a carrier peptide, such as Ant.

We reported that p53 COOH-terminal peptide (p53p-Ant) induced rapid apoptosis in breast cancer cell lines carrying either endogenous p53 mutations or overexpressed WT p53. The peptide was not toxic to nonmalignant breast or colon cells and null p53 human breast or colon cancer cells (14, 17, 18). The mechanism of apoptosis by p53p-Ant in breast cancer cells was through the Fas/Apo-1 pathway involving an increased surface expression of Fas/Apo-1 and Fas ligand due to increased "flipping" of the Fas intracellular membrane receptor to the extracellular membrane milieu without transcriptional or translational changes (14, 18). We studied the three-dimensional structure of p53p-Ant by proton nuclear magnetic resonance spectroscopy and found that it did not have any rigid secondary or tertiary structure; rather, it was flexible and able to conform to multiple mutant p53 conformations (19). Because various p53 mutations are prevalent in human gliomas, restoration of p53 function in malignant glioma may present a novel approach for therapy. In addition, the prolonged half-life of mutant p53 compared with WT p53, due to decreased ubiquitination-proteasomic degradation, leads to mutant p53 accumulation, which increases the target and selectivity of the COOH-terminal p53 peptide. This also occurs with WT p53 that is overexpressed but inactive because of accumulation in the cytoplasm instead of the nucleus, such as in neuroblastoma and some breast cancers. Thus, cells with either mutant p53 or overexpressed WT p53 present more p53 target protein for the peptide and explain why these cells are selectively more sensitive to the peptide. Conversely, normal cells have very low or nondetectable levels of WT p53 in Western blots and are relatively insensitive to the peptide (14, 17, 18, 20). Thus, our hypothesis for selective apoptosis induced by the p53 peptide is that it is regulated mainly by the status (mutant > WT) and the level of p53 expression: the more p53, the more potential for inducing cell death, especially if it is mutant p53. This unique characteristic for the peptide hypothetically allows it to be more selective for mutant and overexpressed WT p53 tumors (18, 20). We tested this hypothesis in this article with human and rat glioma and nonmalignant glial cell lines with different levels of p53 expression and p53 status (mutant and WT).

## Materials and Methods

### Cell Lines and Tissue Culture

Human glioma cancer cell lines U138 (mutant p53) and U87 (WT p53) and rat glioma cancer cell lines 9L (mutant p53), D74 (mutant p53), and F98 (mutant p53) were purchased from American Type Culture Collection (Rockville, MD) and maintained according to their cell culture guidelines. The nonmalignant human glial cell line RAV (WT p53) was derived from normal human brain tissue of an anterior temporal lobe resection for intractable seizures. The normal rat glial cell line NL (WT p53) was harvested from normal brain cortices of Fischer rats. All of the malignant glioma and the nonmalignant glial cell lines were cultured in DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, and 100 µg/mL penicillin/streptomycin. Assessment of cell proliferation in glial and glioma cell lines was done by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

### Peptides

The COOH-terminal p53 peptide (amino acids 361–382, p53p), Ant, and p53p-Ant peptide were chemically synthesized by Research Genetics (Huntsville, AL) as described previously (14). All peptides were high-performance liquid chromatography purified to >95%. Peptide stocks (4 mmol/L) were prepared in sterile distilled water and stored in aliquots at –80°C.

p53p: N-GSRAHSSHLKSKKGQSTSRHKK-C (22 amino acids, 361–382 of p53),

Ant: KKWKMRNRFVVKVQRG (17 amino acids), and

p53p-Ant: N-GSRAHSSHLKSKKGQSTSRHKKWKMRNRFVVKVQRG-C (37 amino acids).

The chimeric peptide has 37 amino acids instead of 39 amino acids, because KK was eliminated from the NH<sub>2</sub> terminus of the Ant sequence because p53p has a KK at the COOH terminus.

### Antibodies

Anti-human p53 monoclonal antibody DO-1 (epitope: amino acids 11–25) and anti-human and anti-rat p53 polyclonal antibody PAb-240 (epitope: amino acids 212–217) were obtained from Santa Cruz Biotechnology (San Diego, CA). FITC-conjugated mouse anti-human Fas/CD95 monoclonal antibody (clone DX2) was obtained from PharMingen (San Diego, CA). Specific caspase-8 inhibitor (Z-IETD-AFC) and caspase-9 inhibitor (Z-LEHD-FMK) were obtained from MBL International Corp. (Watertown, MA). Anti-α-tubulin monoclonal antibody was obtained from Sigma (St. Louis, MO).

### DNA Sequencing of p53 in F98 and D74 Cell Lines

Total RNA was extracted from rat glioma D74 and F98 cells by using RNeasy Mini kit (Qiagen, Valencia, CA) and its cDNA was reverse transcribed with SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). The PCR product was amplified by using 5' primer 5'-ATGGAGGATTCACAGTCGGATATGAGCAT-3 and 3' primer 5'-TCAGTCTGAGTCAGGCCCCACTTTCTT-3 with Platinum Pfx DNA Polymerase. After running the gel, the

PCR product with the corresponding band was purified and sequenced with the primers as above at the Columbia University DNA Sequencing Core Laboratory. Sequencing for all of the *p53* exons (1–11) were done for *p53* mutations.

#### MTT Assay

Cells (3,000) were seeded per well in 96-well plates overnight for 18 hours and then treated with various concentrations of peptide for 12 hours. The assay was done with a Cell Proliferation Kit I (MTT; Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

#### Western Blot Analysis

Cell lysates were prepared in 1 mL lysis buffer [20 mmol/L Tris-HCl (pH 7.6), 1 mmol/L EDTA (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100, 10 µg/mL aprotinin, 5 mmol/L benzimidazole, 50 µg/mL leupeptin, 10 µg/mL pepstatin A, and 1 mmol/L phenylmethylsulfonyl fluoride] for 15 minutes on ice and centrifuged at  $10,000 \times g$  for 30 minutes. Equal amounts of lysates (40 µg) were determined by the Bradford method and then boiled in SDS sample buffer and loaded on SDS-PAGE. After transferring, immunoreactive products were detected by the enhanced chemiluminescence system (Amersham, Piscataway, NJ).  $\alpha$ -Tubulin staining was used for a second loading control in the gels. *p53* expression was quantitated by densitometry and the cell line with the lowest expression band for *p53* was given the arbitrary number of 1 (i.e., NL glial line). Cell lines expressing more *p53* by densitometry than the arbitrary number of 1 were given numbers that equal the  $x$ -fold increase over the NL glial line, which has a densitometric *p53* expression value of 1.

#### Terminal Deoxynucleotidyl Transferase – Mediated dUTP Nick End Labeling Assay

Cells were treated with 0, 30, or 50 µmol/L *p53p*-Ant for 6 hours. After washing with PBS, cells were fixed in 4% paraformaldehyde for 1 hour. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was done with a MEBSTAIN Apoptosis Kit Direct (MBL, Nagoya, Japan) according to the manufacturer's instructions. TUNEL-positive cells were measured from the fluorescence intensity of 5,000 cells using FACSCalibur flow cytometry.

#### Annexin V Assay

Cells were treated with 30 µmol/L Ant, *p53p*, or *p53p*-Ant for 2 hours. Annexin V assays were done according to the manufacturer's protocol (PharMingen). Annexin V-positive cells were measured from the fluorescence intensity of 10,000 cells using FACSCalibur flow cytometry.

#### Membrane-Bound FAS Expression

Human U138 glioma and RAV glial cells were treated with either saline or 30 µmol/L Ant alone, *p53p* alone, or *p53p*-Ant for 6 hours. For cell surface Fas analysis, the FITC-conjugated mouse anti-human monoclonal Fas/CD95 antibody (clone DX2) was used by measuring their fluorescent intensity in FACSCalibur flow cytometry according to the manufacturer's protocol.

#### Collection of Stem Cells and Colony-Forming Units for Granulocyte, Erythroid, Monocyte, and Macrophage Cells Assay

Human peripheral blood stem cells from normal volunteers were collected after informed consent by standard apheresis in acid-citrate-dextrose formula A solution. Permission for use of these stem cells in our experiments was obtained from each volunteer. This protocol followed Health Insurance Portability and Accountability Act regulations and was institutional review board approved. Aliquots were separated by Ficoll-Hypaque and washed once in PBS and centrifuged  $1,000 \times g$  for 5 minutes. Cells were resuspended in PBS and added to the methylcellulose medium MethoCult GF H4434 (StemCell Technologies, Vancouver, British Columbia, Canada) containing growth factors for colony-forming units for granulocyte, erythroid, monocyte, and macrophage cells (CFU-GEMM; CD34<sup>+</sup> stem cells). For the CFU-GEMM assay, the cells were seeded in four-well plates (100,000 per well), treated with either 30 µmol/L Ant, *p53p*, or *p53p*-Ant, and incubated at 37°C with 5% CO<sub>2</sub> for 2 weeks. Cell colonies containing >50 cells per colony were counted as positive and scored after a 14-day incubation by an inverted microscope. CFU-GEMM assays were done three separate times each in triplicate from three separate normal donors.

#### Convection-Enhanced Delivery

Male Fischer 344 rats (ages 12–14 weeks, 250 g) were anesthetized and a 20-gauge plastic guide cannula was cemented into their skulls. After allowing for a 48-hour recovery, a catheter was stereotactically inserted into the right caudate nucleus and  $10^5$  syngeneic 9L glioma cells in a volume of 5 µL were injected. The catheter was then withdrawn and the guide cannula was sealed with a dummy stylet. The tumor cells were allowed to grow for 10 days to an average size of  $2 \times 2$  mm. *In vivo* *p53* peptide doses for convection-enhanced delivery were determined previously in the laboratory (J.N.B.) in *in vitro* dose escalation toxicity trials (21, 22). Either 100 µmol/L Ant, *p53p*, *p53p*-Ant, or saline (30 µL) was constantly infused by convection-enhanced delivery over a 6-hour period and repeated once after a 12-hour hiatus between infusions. Animals were monitored for adverse symptoms, such as ataxia, lethargy, or seizure, and sacrificed 12 hours after the second infusion of peptide. Thus, the total exposure time to peptide was over a 36-hour period for the experiment. Brains were immediately fixed, then sectioned, and stained by the TUNEL method for apoptotic cells.

## Results

#### Growth Rates and Transduction of *p53* COOH-Terminal Peptide into Normal Glial and Malignant Glioma Cells

We initially assessed whether growth rates and peptide transduction into the cell lines were similar to ensure that the proliferation status and the ability of the peptides to enter the various cell lines were not different (human glioma U138 and rat glioma F98 versus human glial RAV



and rat glial NL). These control experiments were necessary to ensure that any differential toxicities from the peptide in nonmalignant glial cells versus malignant glioma cells were not due to differences in growth rates or differential transduction of peptide. Growth rates, as measured by MTT assays between the cell lines, showed a mean  $\pm$  SD doubling rate in the glioma cell lines of  $30 \pm 6$  hours, and for glial cell lines, it was  $36 \pm 7$  hours ( $n = 3$ ). Because the mean  $\pm$  SD of doubling times overlapped, we considered the lines to have no major significant differences in growth rates. To test for differences in peptide transduction across cell membranes, we used p53-Ant linked to rhodamine B (30  $\mu$ mol/L p53p-Ant-RhoB) in the human glioma U138 and human glial RAV cell lines as assessed by fluorescence microscopy. p53-Ant-RhoB quickly entered both cell lines by 1 minute and maximized at 10 minutes after peptide exposure. The peptide entered cytoplasmic and nuclear compartments equally in both cell lines (data not shown). Our previous work with p53-Ant-RhoB in normal and malignant colon and breast cell lines also showed equal peptide transduction (14, 18).

#### **Inhibition of Cell Proliferation by p53 COOH-Terminal Peptide**

MTT assays were employed to test the hypothesis that activation and restoration of latent WT and mutant p53 activity, respectively, will selectively decrease the growth rates and viability of glioma tumor cells. The half-life of functional p53p-Ant was determined by peptide incubation at 37°C in DMEM with 10% FCS and found to be  $\sim 36$  to 48 hours (data not shown). The two human glioma cell lines (U138 and U87) and the two rat glioma cell lines (9L and D74) were exposed to p53p-Ant for 12 hours at concentrations from 10 to 100  $\mu$ mol/L. Cell viability after exposure to p53p-Ant was compared with background and controls consisting of either Ant alone, p53p alone, or saline after 72 hours of growth. The  $IC_{50}$  was determined by graphing the MTT results from titrations of p53p-Ant from 10 to 100  $\mu$ mol/L added to the cell lines. Human glioma line U138 with mutant p53 showed dose-dependent inhibition of growth from p53p-Ant with an  $IC_{50}$  of 30  $\mu$ mol/L and the human glioma line U87 with WT p53 showed dose-dependent inhibition of growth from p53p-Ant with an  $IC_{50}$  of 50  $\mu$ mol/L (Fig. 1A). Rat glioma lines 9L and D74, both with mutant p53, showed dose-dependent sensitivity to p53p-Ant with  $IC_{50}$ s of 30 and 50  $\mu$ mol/L, respectively (Fig. 1B). Similar results were also obtained in the rat glioma cell line F98 with mutant p53 (Table 1). All human and rat glioma lines were completely insensitive to unlinked control peptides, including Ant and p53p alone at concentrations tested up to 100  $\mu$ mol/L (Fig. 1A and B). In comparison with the toxicity of p53p-Ant to human and rat glioma cell lines, p53p-Ant was much less toxic to the human and rat nonmalignant glial cell lines, RAV and NL, respectively, both with WT p53 (Table 1). These glial cell lines showed zero cytotoxicity from p53p-Ant up to 100  $\mu$ mol/L and the  $IC_{50}$ s were  $\sim 200$   $\mu$ mol/L for both lines. These results indicated that p53p-Ant was selectively

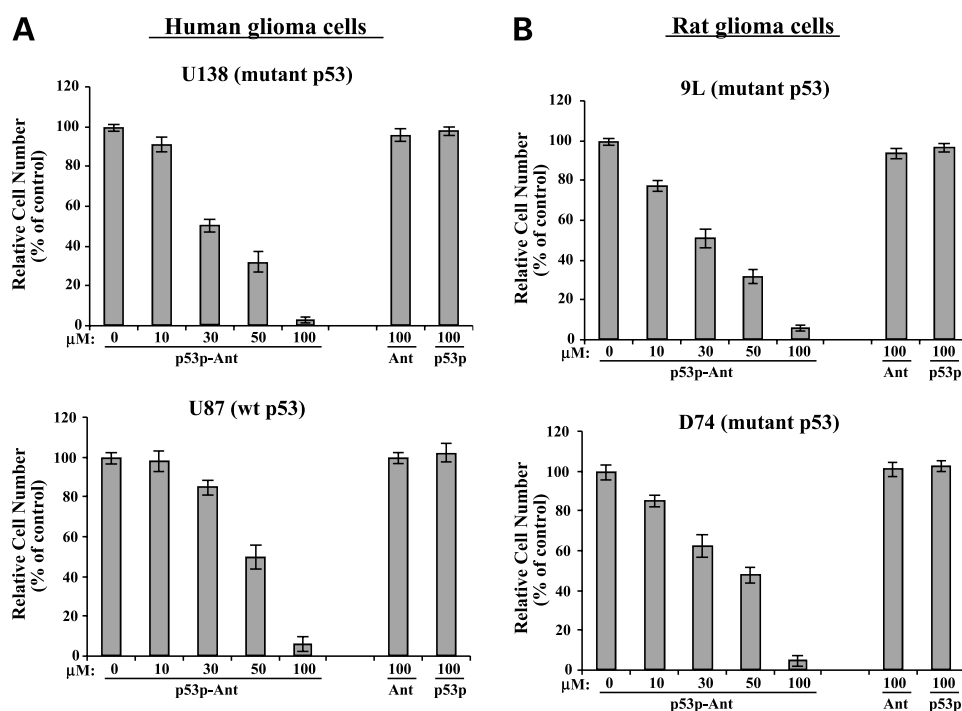
toxic to human and rat glioma cells and much less toxic to proliferating human/rat nonmalignant glial cells. In addition, this preferential toxicity of p53p-Ant in glioma cell lines compared with normal glial cell lines, both rat and human, was not due to major differences in growth rates in MTT assays or due to differences in peptide transduction into the nonmalignant glial or malignant glioma cell lines.

#### **Correlation of p53 Levels and Status with Sensitivity to p53p-Ant**

The p53 status (mutant versus WT) for all cell lines used in this study has been either published or available from American Type Culture Collection, except for the D74 and F98 rat glioma cell lines. To test our hypothesis, we determined the correlation of p53 levels and status with sensitivity to p53p-Ant. First, we sequenced the entire p53 gene in the rat glioma cell lines D74 and F98 by using total cellular cDNA. The sequence results showed a mutation at amino acid 174 (Trp-to-Cys) in the D74 line and a mutation at amino acid 54 (Gly-to-Ala) in the F98 line. The anti-p53 monoclonal antibody DO-1 and anti-p53 polyclonal antibody PAb-240 were both used for human and rat lines to quantitate the p53 levels in Western blots. We found the expression level of p53 protein was much higher in two of four mutant p53 lines (U138 and 9L) than in all WT p53 lines from both human and rat glioma cells (Table 1). The p53 levels in Western blots were quantitated by densitometry and the p53 status (mutant versus WT) was then compared with the sensitivity of each line to p53p-Ant. As predicted by our hypothesis, these results showed that sensitivity to p53p-Ant generally correlated with p53 expression levels and its status (Table 1). Interestingly, the peptide was more toxic to malignant glioma lines than nonmalignant glial cell lines with approximately equal p53 expression in Western blots (WT p53 human glioma U87 versus WT p53 human glial RAV and mutant p53 rat glioma F98 versus WT p53 rat glial NL) as shown in Table 1. In addition, nonmalignant glial cells with low WT p53 expression had  $IC_{50}$ s to p53p-Ant at  $\sim 200$   $\mu$ mol/L, suggesting that p53p-Ant had preferential toxicity to human or rat glioma cells with low WT or low mutant p53 expression than to glial lines with low WT p53 expression. However, among malignant glioma lines, the peptide was clearly more toxic to the lines with high mutant p53 expression, human U138 and rat 9L.

#### **Mechanism of p53p-Ant-Induced Death**

To determine whether apoptosis was the mechanism of cell death from the peptide, TUNEL assays were done on the mutant p53 human glioma line U138 and the nonmalignant WT p53 human glial line RAV. In the presence of 30 or 50  $\mu$ mol/L p53p-Ant for 6 hours, TUNEL-positive cells increased rapidly from 4% to 42% in a dose-dependent manner in the mutant p53 human glioma cell line U138. However, TUNEL-positive cells only increased from 4% to 11% in the human nonmalignant glial cell line RAV (Fig. 2). Control peptides Ant or p53p alone showed no significant induction of TUNEL positivity



**Figure 1.** p53p-Ant induces inhibition of growth in human and rat glioma cells. Cells (3,000) in MTT plate wells were treated with different concentrations of p53p-Ant (0 – 100  $\mu\text{mol/L}$ ) or Ant alone (100  $\mu\text{mol/L}$ ) or p53p alone (100  $\mu\text{mol/L}$ ) for 12 h and cell viability was detected by MTT assay in human glioma cell lines U138 and U87 (**A**) and rat glioma cell lines 9L and D74 (**B**). Columns, mean of three experiments done in triplicate; bars, SD.

(<5%) in all cell lines when tested at 100  $\mu\text{mol/L}$  (data not shown). These results suggested that the mechanism of death induced by p53p-Ant was apoptotic and occurred mainly in glioma cells and much less in nonmalignant glial cells.

One of the most sensitive and specific tests for apoptosis compared with necrosis is the early increase in Annexin V binding. The Annexin V assay detects flipping of intramembrane phosphatidylserine to the outer membrane, which is indicative of early apoptosis. The results of Annexin V staining in the human mutant p53 glioma U138 cells are shown in Fig. 3. U138 glioma cells exposed to p53p-Ant for 2 hours had a 55% increase in positivity for Annexin V, whereas cells exposed to control peptides exhibited only 10% (Ant alone) and 3% (p53p alone)

increase over the basal level of staining. These data suggested that p53p-Ant induced an apoptotic cell death preferentially in human glioma cells ~48% higher than the average of the controls.

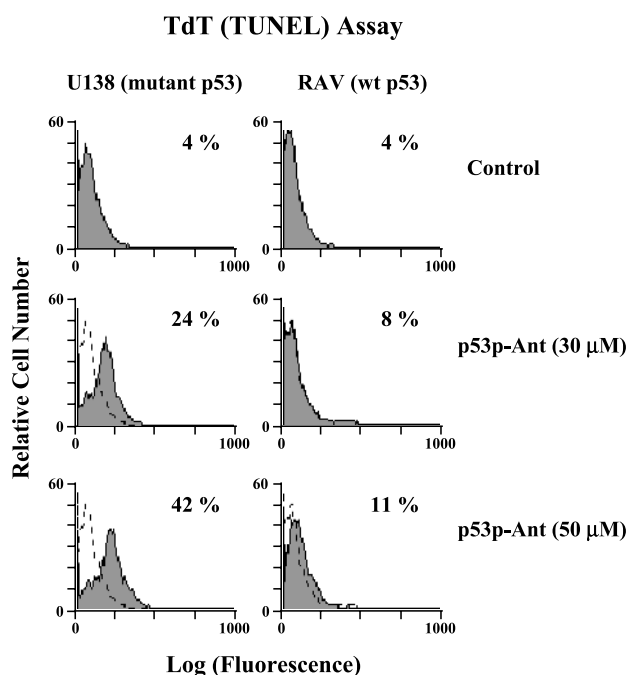
#### Effect of p53 COOH-Terminal Peptide on the Intrinsic and Extrinsic Pathways of Apoptosis

Our previous studies in human cancer lines showed that the mechanism by which p53p-Ant induced apoptosis was through a nontranscriptional/nontranslational activation of the Fas/FADD complex, which led to death signaling through the extrinsic pathway for apoptosis. This could be blocked by dominant-negative FADD overexpression (18, 20). There was a downstream activation of the initiator caspase-8 and effector caspase-3 without changes in the intrinsic pathway proteins (caspase-9, Bcl-2, Bax, Bcl-xL,

**Table 1.** Inhibition of cell proliferation by p53p-Ant correlates with p53 status and expression

Cell lines	Cell type	p53 status	p53 expression	IC <sub>50</sub> ( $\mu\text{mol/L}$ ) $\pm$ SE
U138	Human glioma	Mutant	13	30 $\pm$ 2.8
U87	Human glioma	WT	2	50 $\pm$ 3.2
RAV	Human normal glial	WT	2	210 $\pm$ 10.1
9L	Rat glioma	Mutant	8	30 $\pm$ 4.0
D74	Rat glioma	Mutant	2	50 $\pm$ 4.2
F98	Rat glioma	Mutant	1	50 $\pm$ 3.4
NL	Rat normal glial	WT	1	194 $\pm$ 9.0

NOTE: Cells were treated with p53p-Ant for 12 hours and cell viability was assessed by MTT assay. IC<sub>50</sub>s  $\pm$  SE were determined from the MTT assay results. The p53 band values were determined by densitometric analysis of the Western blots and standardized to the lowest band density, which was given the arbitrary number of 1 (NL glial line = 1). The p53 band expression numbers represent the fold increase of each respective line for their p53 level over the NL glial line, which equals 1. The number 13 was the highest x-fold increase (i.e., the U138 glioma line was 13-fold over the arbitrary number 1 value).



**Figure 2.** Effect of p53p-Ant on TUNEL staining in human glioma and nonmalignant glial cells. U138 glioma and RAV glial cells were treated with 0, 30, or 50  $\mu\text{mol/L}$  p53p-Ant for 6 h. Apoptotic cells were determined by TUNEL assay and quantitated by flow cytometry from 5,000 cells according to the manufacturer's instructions. Representative of three separate experiments showing relative apoptotic cell numbers.

Bak, and Bid; refs. 14, 18). In the mutant p53 human glioma cell line U138, exposure to p53p-Ant did not alter caspase-9 activity, indicating that the intrinsic pathway (Bcl-2, Bax, Bcl-xL, etc.) was not involved in the mechanism of apoptosis by peptide in this glioma cell line. In addition, exposure to p53p-Ant in the U138 glioma line increased caspase-8 activity over 3-fold, indicating an important role for the extrinsic pathway (Fas) in glioma cells for peptide-induced cell death. In addition, flow cytometry experiments with FITC anti-Fas labeling antibody showed increased outer membrane Fas expression in U138 glioma cells exposed to 30  $\mu\text{mol/L}$  peptide as shown in Fig. 4. By cell sorting flow cytometry, we isolated the human mutant p53 U138 cells exposed for 2 hours to 30  $\mu\text{mol/L}$  p53p-Ant, which expressed increased Annexin V staining, and labeled these cells with FITC anti-Fas antibody. There was a 300% increase in Fas expression in these cells over the controls p53p or Ant alone (30  $\mu\text{mol/L}$ ) or saline. In comparison, the RAV human glial cell line exposed to p53p-Ant at 30  $\mu\text{mol/L}$  increased outer membrane Fas expression only 20%. The controls, p53p, and Ant alone minimally increased Fas expression (<10%) in the U138 and RAV cell lines. These results suggested that the mechanism of selective apoptosis in the glioma U138 line was via the extrinsic Fas apoptotic pathway. Thus, the mechanism of apoptosis in the glioma cell line U138 appeared similar to the former results we obtained in multiple nonglioma cancer cell lines (prostate,

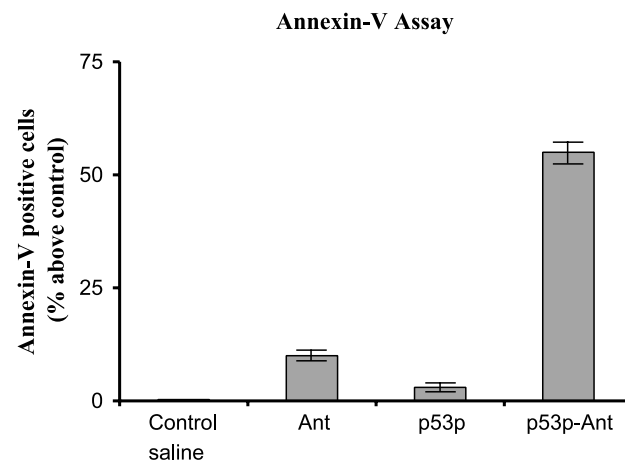
breast, and lung) where the mechanism for apoptosis was also through the Fas pathway and it was toxic to the glioma cell line U138 and nontoxic to the human glial line RAV.

#### Effect of p53 COOH-Terminal Peptides on Normal Human Peripheral Blood Stem Cells

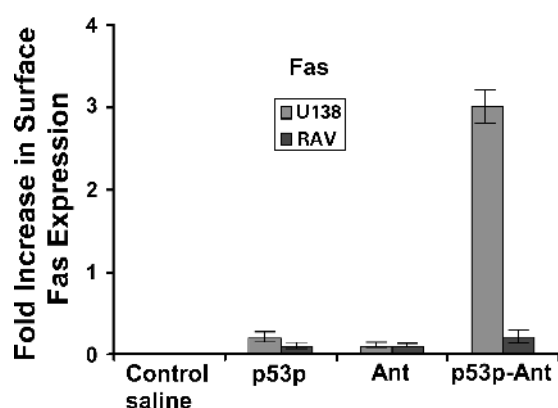
We used the CFU-GEMM assay to determine whether p53p-Ant was toxic to rapidly proliferating, normal peripheral blood stem cells. The *in vitro* CFU-GEMM assay is highly predictive for clinical toxicity to blood or marrow-derived stem cells *in vivo* and assesses toxicity to precursors of granulocyte, erythroid, monocyte, and macrophage cell lineages (CFU-GEMM) also known as the CD34<sup>+</sup> stem cell population. There was only a minor toxic effect on these blood stem cells when exposed to p53p-Ant for 14 days as shown in Table 2. These results suggested that p53 COOH-terminal peptide was minimally toxic to proliferating normal human peripheral blood stem cells for CFU-GEMM, which normally have low levels of WT p53 and thus less target for the peptide.

#### *In vivo* Effects of p53p-Ant

To determine the efficacy of p53-Ant *in vivo*, a syngeneic, orthotopic 9L rat glioma convection-enhanced delivery model was used as described in Materials and Methods. This rat glioma model has been described previously in our reports (21, 22). Intracerebral cannulas were implanted stereotactically into the brain striata of Fischer rats. During and after the peptide infusions, no toxicity was observed in the animals (i.e., seizures, lethargy, and ataxia). The protocol consisted of a 6-hour infusion of 30  $\mu\text{L}$  of 100  $\mu\text{mol/L}$  peptide or controls followed by a 12-hour hiatus and repeated once. After another 12-hour hiatus, the animals were sacrificed and brains were assessed for TUNEL positivity (total 36 hours; Fig. 5A). p53p-Ant induced 52% TUNEL-positive cells in the



**Figure 3.** Annexin V assay in human glioma U138 cells exposed to p53p-Ant. Cells were treated with 30  $\mu\text{mol/L}$  Ant, p53p, or p53p-Ant for 2 h. Apoptotic cells were detected by Annexin V assay and analyzed with a FACSCalibur flow cytometer for 10,000 cells. Columns, mean of three experiments done in triplicate; bars, SD.



**Figure 4.** Fas involvement in p53p-Ant-induced apoptosis of glioma cells. Human U138 glioma and human RAV glial cell lines were untreated or treated for 2 h with 30  $\mu\text{mol/L}$  p53p-Ant and controls (p53p alone and Ant alone). Results are expressed as fold change in fluorescent intensity above controls in flow cytometry as determined for 5,000 cells that bound FITC-labeled anti-Fas antibody. Columns, mean of three experiments done in triplicate; bars, SD.

*in vivo* 9L tumor, whereas control Ant alone, p53p alone, and vehicle alone produced only 2% to 4% TUNEL positivity (Fig. 5B). The normal brain tissue surrounding the tumor did not display any TUNEL-positive cells above 2%. In addition, increased inflammatory cells were not detected in the glioma or surrounding normal brain tissue. Thus, collectively p53p-Ant was relatively nontoxic to nonmalignant glial cell lines *in vitro* and normal brain tissue *in vivo*. The results showed that p53p-Ant induced preferential apoptotic cell death *in vivo* in the glioma tumor model at a percentage (49%) similar to the increases detected in the *in vitro* Annexin V experiments (48%) and the *in vitro* TUNEL assay in 9L and U138 glioma cell lines (42%).

## Discussion

This study investigated whether p53 function for induction of apoptosis in glioma cell lines could be restored or increased by a peptide derived from the COOH-terminal domain of human p53. Three murine malignant glioma cell lines, three human malignant glioma cell lines, and two nonmalignant glial cell lines from normal murine and human brain tissue were used. MTT assays (Fig. 1; Table 1) showed that, among both human and murine cell lines, malignant cells displayed a much greater sensitivity for induction of apoptosis than nonmalignant cells from exposure to p53p-Ant. The  $\text{IC}_{50}$  of malignant glioma cell lines was between 30 and 50  $\mu\text{mol/L}$  dependent on the level of p53 expression and phenotype, whereas the  $\text{IC}_{50}$  for nonmalignant glial cell lines was  $\sim 200$   $\mu\text{mol/L}$ . The controls, Ant, and p53p alone had no significant toxicity to either glioma or glial cell lines. Our previous studies showed that the control p53p alone without Ant did not enter malignant or normal cells (14). We also found that p53p-Ant mediated selective apoptosis in multiple cell

types with mutant p53 status, including premalignant colon and breast and malignant colon, prostate, breast, lung, and mesothelioma cell lines, in a p53-dependent manner (17, 18, 20).

More interestingly, the differences in sensitivity to p53p-Ant were not totally explained by the levels of p53 expression alone. The human U87 glioma and human RAV glial cell lines have approximately equal p53 expression as do the rat F98 glioma and rat NL glial cell lines (Table 1). Yet, there is  $\sim 4$ - to 7-fold increased sensitivity to peptide in the malignant glioma cells compared with the nonmalignant glial cell lines, although the lines have equally low expression of p53. Preliminary *in vitro* experiments with purified p53p-Ant and whole mutant or WT p53 in the Biacore surface plasmon resonance assay suggest that the peptide binds more tightly to the mutant p53 conformation than WT p53 conformation. We found dissociation constants ( $K_d$ ) in the picomolar range ( $10^{-12}$  mol/L) for p53p-Ant binding to mutant p53 and  $K_d$  of  $10^{-10}$  mol/L for peptide binding to WT p53 conformation (19). This 2-log increase in binding of peptide to mutant versus WT p53 could explain why p53p-Ant was more toxic to the mutant p53 F98 glioma cells than the WT p53 NL glial cells, although their p53 levels were similar in Western blots (Table 1). As to why the peptide was more toxic to WT p53 U87 glioma cells compared with WT p53 RAV glial cells, both with equal WT p53 levels, is unknown, but it suggests other factors within malignant cells that make them more sensitive to peptide compared with nonmalignant cells other than WT p53 expression levels.

In this study, human peripheral blood pluripotent stem cells for CFU-GEMM were much less sensitive to p53p-Ant, again showing the relative lack of toxicity of the p53 peptide to nonmalignant, proliferating cells with low WT p53 expression. These data as well as the lack of significant toxicity to glial cell lines suggest that cell kinetics was not a major determinant for sensitivity to the induction of apoptosis by the peptide (Table 2).

The relative insensitivity of human RAV and rat NL glial cells as well as human peripheral blood stem cells to p53p-Ant may be related to the instability and short half-life of WT p53 under normal cellular conditions or to a subthreshold level of activated WT p53 insufficient

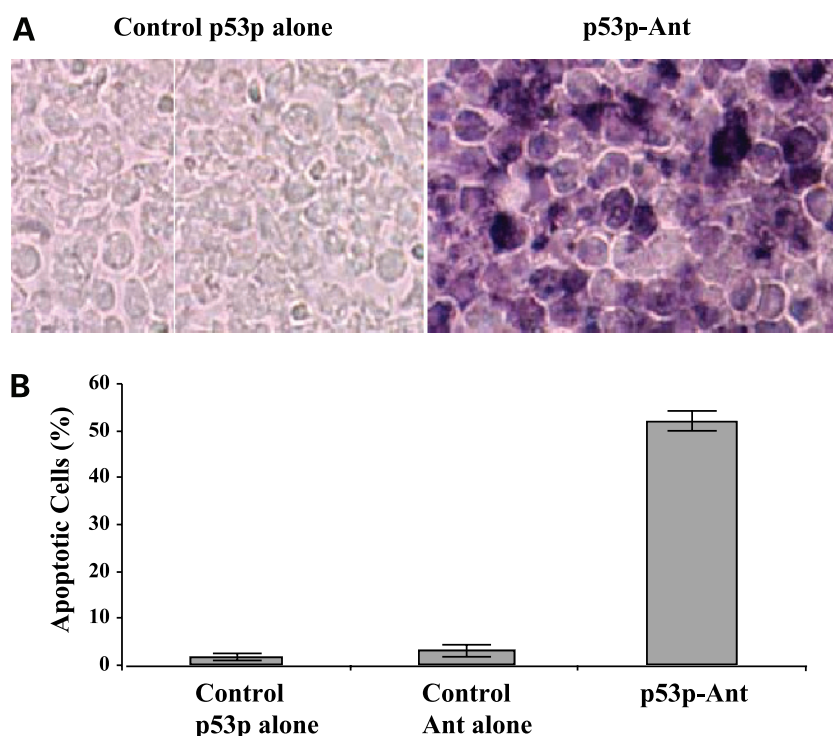
**Table 2.** Effects of p53p-Ant on normal human peripheral blood stem cells for CFU-GEMM

Treatment	% of Control (mean $\pm$ SE)
Vehicle	100 $\pm$ 4.7
Ant	98 $\pm$ 3.5
p53p	90 $\pm$ 5.3
p53p-Ant	83 $\pm$ 11.3

NOTE: Three separate CFU-GEMM experiments from three separate normal donors. Mean  $\pm$  SE from a total of three experiments in triplicate. The concentration of Ant, p53p, and p53p-Ant was 30  $\mu\text{mol/L}$ .



**Figure 5.** *In vivo* effects of p53p-Ant given by convection-enhanced delivery. Male Fischer rats (ages 12–14 wks) had a 20-gauge cannula cemented to their cranium. Either saline, 100  $\mu$ mol/L Ant, p53p, or p53p-Ant (30  $\mu$ L) was infused over a 6-h period by a convection-enhanced delivery system and then repeated once, separated by a hiatus of 12 h. There were 4 animals per group, total of 16 animals for each experiment, and a total of three experiments. Animals were monitored for adverse symptoms, such as ataxia, lethargy, or seizure, and sacrificed 12 h after the second infusion of peptide (total 36 h). Brains were immediately fixed, sectioned, and stained for presence of apoptosis using the TUNEL method. **A**, morphologic pictures. *Left*, a representative brain slice from an animal that received control p53p without Ant. Animals that received the controls Ant alone or saline were identical to the control p53p alone (data not shown). *Right*, a representative brain slice from an animal that received p53p-Ant, showing a marked increase in TUNEL-positive cells. **B**, percentage of apoptotic cells. Numbers of apoptotic cells staining positive for TUNEL were scored in at least 400 cells in each sample by fluorescence microscopy. The controls are p53p without Ant and Ant without p53p that showed 2% to 4% TUNEL staining. The p53p-Ant group showed 52% TUNEL staining. *Columns*, mean of three experiments; *bars*, SD.



to trigger apoptosis. This has been described in human null p53 Saos-2 osteosarcoma cells stably transfected with a tetracycline-inducible WT p53 gene. These cells underwent either apoptosis or growth arrest depending on the intracellular level of p53 expression. Low p53 levels induced p21WAF/Cip1, which induced transient growth arrest without apoptosis, and high p53 levels induced Bax, which induced apoptosis without cell cycle arrest (8). Our hypothesis is that high intrinsic levels of mutant p53, secondary to its long half-life from defective ubiquitination through the proteasome, can be converted to "functional" p53 by p53p-Ant, thus inducing a Fas/FADD-mediated apoptosis without the need for other apoptotic inducers, such as chemotherapy agents. In support of this concept of restoring partial WT p53 function to mutant p53 by p53p-Ant, which restores Fas-mediated apoptosis only occurred with the expression of WT p53 and not with the mutant p53 form in null p53 H1299 lung cancer cells expressing a stably transfected temperature-sensitive p53 gene (19, 20). However, Fas-mediated apoptosis was restored to the mutant temperature-sensitive p53 H1299 cells if these cells were exposed to p53p-Ant. Thus, Fas-mediated apoptosis occurred only in cells with normal WT or "partially functional" p53, the latter as in our mutant p53 glioma cells exposed to p53p-Ant.

Preliminary data from our laboratory using human breast and prostate cancer lines resistant to either topotecan, CPT-11 (irinotecan), docetaxel, or paclitaxel show equal preservation of sensitivity to the apoptotic

effects of p53p-Ant compared with their respective parental cell lines.<sup>4</sup> This could potentially be important for the treatment of glioma patients who have failed these agents due to drug resistance mechanisms. Thus, this peptide may possibly have equal cytotoxic effects to tumor cells irrespective of different mechanisms of drug resistance or growth rates, presumably because it acts through a novel mechanism dependent on the level and status of endogenous p53. The therapeutic window for this peptide may be significant because "normal" cells, including nonmalignant glial, breast and colon cells and human marrow peripheral stem cell progenitors, have intrinsically low levels of WT p53, which reduces the target for the peptide and thereby allow the "normal" cells to be less sensitive to the apoptotic effects of p53p-Ant.

Flow cytometry analyses for TUNEL and Annexin V showed much higher staining in the glioma cell line U138 after exposure to p53p-Ant than in the glial cell line RAV (Figs. 2 and 3). Significantly increased Annexin V binding by 2 hours in the U138 cell line, in response to 30  $\mu$ mol/L p53p-Ant compared with the controls, strongly supports that the mechanism of cell death was apoptotic. Thus, p53p-Ant could be restoring partial WT p53 function to mutant p53 phenotypes for their ability to activate the Fas/FADD pathway for apoptosis. As shown in Fig. 4, after exposure to p53p-Ant, the Annexin V-positive,

<sup>4</sup> Y. Li and R.L. Fine, unpublished data.



mutant p53 U138 malignant glioma cells had increased surface Fas expression above control (300%). In comparison, Fas expression did not increase significantly above control in the RAV human glial cell line (20%) on exposure to the same concentration of p53p-Ant. As in our previous breast and lung cell line studies, increased outer membrane Fas expression (extrinsic pathway) was the only death pathway induced in glioma cells without changes in the intrinsic caspase-9 pathway for apoptosis (23). Thus, in glioma cell lines, in accordance with our hypothesis, the extrinsic pathway (Fas) seems to be the major mechanism for peptide-induced apoptosis and the ability of the peptide to cause apoptosis seems, in general, related to the endogenous levels of p53, mutant more so than WT.

Induction of apoptosis in the syngeneic, orthotopic rat 9L glioma model by convection-enhanced delivery of p53p-Ant suggested that *in vitro* results could be translated to the *in vivo* model. *In vivo*, p53p-Ant induced 52% apoptosis in the 9L glioma by TUNEL staining, whereas the controls p53p and Ant alone induced only 2% to 4% apoptosis. Histochemical analysis of the normal rat brain surrounding the 9L glioma did not display TUNEL positivity or alteration in the number of inflammatory cells. This was of concern because Ant is derived from *Drosophila*. However, the time window for the convection-enhanced delivery experiments was probably too short to examine this fully (36 hours). A preliminary survival study with controls versus p53p-Ant in the above *in vivo* 9L glioma model has shown an increase in survival from a median of 21 to 37 days, respectively, translating to a 76% increase in median survival from p53p-Ant.<sup>5</sup> This increase by Kaplan-Meier survival analysis was statistically significant ( $P < 0.001$ ). However, further studies are needed to corroborate this preliminary experiment. Long-term survival studies in this syngeneic, orthotopic rat glioma model are in progress.

Investigation into synthetic small molecules, which partially restore p53 function to mutant p53 tumor cells, is also an active area of research by many laboratories. We have shown recently that the small-molecule PRIMA-1, which is cell permeable without a carrier, induced apoptosis selectively in premalignant and malignant mutant p53 colon and breast cells through the c-Jun NH<sub>2</sub>-terminal kinase pathway without affecting the intrinsic or extrinsic pathways of apoptosis (24).

In total, this work supports the potential clinical utility of p53p-Ant as a selective novel therapeutic agent for gliomas and the clinical advantages of convection-enhanced delivery as a system to circumvent the blood-brain barrier for delivery of large and/or charged therapeutic moieties, such as p53 peptides.

<sup>5</sup> J.N. Bruce and R.L. Fine, unpublished data.

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*Mol Cancer Ther* 2006;5:20-28.

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