Intracerebral Adenovirus-mediated p53 Tumor Suppressor Gene Therapy for Experimental Human Glioma

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ABSTRACT

Malignant gliomas of astrocytic origin are good candidates for gene therapy because they have proven incurable with conventional treatments. Although mutation or inactivation of the p53 tumor suppressor gene occurs at early stages in gliomas and is associated with tumor progression, many tumors including high-grade glioblastoma multiforme carry a functionally intact p53 gene. To evaluate the effectiveness of p53-based therapy in glioma cells that contain endogenous wild-type p53, a clinically relevant model of malignant human glioma was established in athymic nude mice. Intracerebral, rapidly growing tumors were produced by stereotactic injection of the human U87 MG glioma cell line that had been genetically modified for tracking purposes to express the Escherichia coli lacZ gene encoding β-galactosidase. Overexpression of the p53 gene by adenovirus-mediated delivery into the tumor mass resulted in rapid cell death with the eradication of β-galactosidase-expressing glioma cells through apoptosis. In long-term experiments, the survival of mice treated with the p53 adenoviral recombinant was significantly longer than that of mice that had received control adenoviral recombinant. During the observation period of 1 year, a complete cure was achieved in 27% of animals after a single injection of p53 adenoviral recombinant, and 38% of the animals were tumor free in the group receiving multiple injections of p53 adenoviral recombinant into a larger tumor mass. These experiments demonstrate that overexpression of p53 in gliomas, even in the presence of endogenous functional wild-type p53, leads to efficient elimination of tumor cells. These results point to the potential therapeutic usefulness of this approach for all astrocytic brain tumors.

INTRODUCTION

Malignant astrocytic gliomas, although highly invasive, are almost always confined to the central nervous system. Hence, any therapeutic approach that would lead to complete eradication of malignant cells would be expected to provide a cure for the original tumor. Many genetic alterations have been documented during the transformation of glial cells and the subsequent tumor progression into the most malignant form, glioblastoma multiforme [summarized by Kleihues et al. (1)]. Mutation or inactivation of the p53 tumor suppressor gene is one of the early genetic alterations (2, 3) that may confer a proliferative advantage to these cells and is associated with tumor progression (4). Because p53 mutations are common in astrocytic gliomas, therapies aimed at restoring wild-type p53 function (5) or specifically targeted at killing cells with mutant p53 (6) might be of benefit. However, even high-grade glioblastoma cells may have intact p53 genes, and there may be molecular heterogeneity within the same tumor bed (7). In in vitro studies, gene transfer of wild-type p53 into glioma cell lines expressing mutant p53 leads to cell death by apoptosis (8–10), whereas in glioma cells with an intact p53 gene, overexpression of p53 has been associated with growth arrest (8, 9) or apoptosis (10).

From a therapeutic point of view, there are profound implications with regard to whether malignant cells stop proliferating and undergo growth arrest or are eliminated completely. Wild-type p53 is a potent activator of apoptosis in cell lines carrying mutant p53 [reviewed by Elledge and Lee (11)]. On the other hand, recent studies suggest that in cells expressing wild-type p53, the cellular levels of p53 may direct the cell response toward either growth arrest at lower levels of expression or apoptosis when higher levels of p53 are achieved (12). Our previous in vitro experiments have shown that AV4-mediated gene transfer and overexpression of the wild-type p53 tumor suppressor protein leads to cell death by apoptosis in the U87 MG human glioma cell line (10), which carries a functionally active wild-type p53 gene (10, 13). Here we report the effects of AV-mediated gene transfer of wild-type p53 (AVp53) in an experimental model of human malignant glioma produced by intracerebral inoculation of human U87 MG cells into athymic nude mice.
nu/nu (nude) mice. Stereotactic injection of AVp53 into the preestablished tumor resulted in potent tumoricidal activity and highly significant increases in survival of the treated mice. Furthermore, complete eradication of the tumor could be achieved by overexpression of p53 in this experimental human glioma.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. The human glioma cell line U87 MG was obtained from the American Type Culture Collection (Rockville, MD). Cell lines were maintained at 37°C in 5% CO₂ in DMEM supplemented with 10% FCS and 20 μg/ml gentamicin. U87 MG cells were cotransfected with plasmid pUT535 containing the Escherichia coli lacZ gene encoding β-galactosidase and plasmid pCRSV carrying the neomycin phosphotransferase gene conferring resistance to G418 using Lipofectin (Life Technologies, Inc., Mississauga, Ontario, Canada). Stable transfectants (U87/lacZ) were established after selection in G418 (600 μg/ml) for 14 days and histochemical staining for β-galactosidase activity.

Recombinant Adenoviruses. The recombinant adenovirus AVp53 is a replication-deficient AV in which the wild-type p53 cDNA driven by the CMV promoter/enhancer has been inserted into the E1 region of E1–E3-deleted human adenovirus type 5 (14). The control adenoviral recombinants expressed lacZ (AVlacZ; Ref. 15) or GFP (AVGFP; a gift of Dr. Bernard Massie, Biotechnology Research Institute, Montreal, Quebec, Canada) under the control of CMV promoter/enhancer. The production and screening of adenoviral recombinants as well as the conditions for the infection of cells in culture were described previously (10, 16).

Flow Cytometry Analysis. Approximately 1 × 10⁵ U87/lacZ cells/well were seeded in 6-well plates, and cells were infected 16–18 h later with AVp53 or AVLacZ at a multiplicity of infection of 100. At 24, 48, and 72 h after infection, both attached cells and floating cells were harvested, washed in PBS, and fixed with 70% ethanol. After RNase A treatment, cells were stained with 100 μg/ml propidium iodide in PBS. Flow cytometry was performed on an argon laser-equipped Becton Dickinson (Sunnyvale, CA) FACScan instrument with excitation set at 488 nm and emission at 525 nm; the data were analyzed by LYSYSII software.

Animal Experimentation. Stereotactic injections were based on the work of Martuza et al. (17). CD1 nu/nu athymic nude mice (6 weeks old; Charles River Canada) were anesthetized by i.p. injection of sodium pentobarbital (25 mg/kg) and placed in a stereotactic apparatus (Kopf). A burr hole was drilled 1 mm anterior and 2 mm lateral to the bregma. The U87/lacZ cell suspension (1 × 10⁵ cells in 3 μl of HBSS) was injected stereotactically over a 10-min period using a Hamilton syringe at a depth of 3.5 mm. After 10 or 20 days, animals were anesthetized again for intratumoral administration of adenoviral recombinants. The same coordinates were used for the injection of virus, except that the needle was placed 1 mm deeper than with the injection of tumor cells, and the virus was injected at six points at 0.5-mm intervals; 2 × 10¹⁴ particles/ml AVp53 or AVGFP (control) were injected in a volume of 0.5 μl at each of the six levels over a 15-min period. The needle was withdrawn within a 20-min period. All animal experimentation was carried out according to the guidelines of the Canadian Council on Animal Care.
Western Blot Analysis. The mass of the tumor was dissected out and homogenized in an appropriate volume of SDS-loading buffer [2% SDS, 10% glycerol, 0.125 M Tris-HCl (pH 6.8), and 3% mercaptoethanol]. The concentration of total protein was determined by the BCA Protein Assay (Pierce, Rockford, IL). Protein samples (50 μg/lane) were electrophoresed on a 10% or 12% polyacrylamide-SDS gel and then transferred to nitrocellulose (Schleicher & Schuell, Keene, NH), and blots were reacted with the antihuman p53 antibody DO-1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-b-actin antibody (1:5000; Sigma, Oakville, Ontario, Canada), or anti-GFP antibody (1:1000; Boehringer Mannheim, Laval, Quebec, Canada). Detection was accomplished by the enhanced chemiluminescence system (Amersham, Pharmacia Biotech, Baie d’Urfé, Quebec, Canada) according to the manufacturer’s instructions.

Brain Tissue Analysis. After euthanasia, brains were removed and quickly frozen in isopentane chilled with liquid nitrogen. Coronal sections (10 μm) were prepared and stained histochemically for β-galactosidase activity as described previously (15) before counterstaining with H&E. In situ DNA cleavage was assessed by the TUNEL technique as described previously (18). Incorporation of biotinylated dUTP was visualized with the ABC kit (Vector Laboratories, Burlingame, CA).

Reconstruction of Tumors. Three-dimensional reconstruction was performed on 10-μm-thick sections that were mounted on glass slides and stained for β-galactosidase activity. Coronal sections 100 μm apart were scanned at 400 dpi, and the images were used to produce a three-dimensional surface using custom software. Calculated surfaces were rendered using POV-Ray software. Tumor volumes were calculated using the formula \( a \times b^2 \times 0.4 \) in which \( a \) represents the longest axis, and \( b \) represents the width perpendicular to this axis.

RESULTS AND DISCUSSION

To monitor glioma cell growth in vivo, the U87 MG cell line was genetically marked by stable transfection with the E. coli lacZ gene that encodes β-galactosidase. The U87/lacZ cell line maintained strong β-galactosidase expression during at least 30 passages in vitro (data not shown). Furthermore, U87/lacZ cells that were injected intracerebrally were capable of forming tumors that constitutively expressed β-galactosidase as revealed by histochemical staining of tumor tissue sections in the presence of the substrate X-gal (see Figs. 2A and 3). During subsequent experiments, the identification of single blue-stained glioma cells allowed a rapid and efficient evaluation of tumor growth and spread in brain tissue.

To confirm that the U87/lacZ cell line behaves as the parental cell line in response to p53 overexpression, U87/lacZ

Fig. 3 Expression of p53 and GFP 2 days after adenovirus-mediated gene transfer by intratumoral injection of AV. A, Western blot of homogenates prepared from two tumors injected with AVGFP (Lanes 1 and 2) or two tumors injected with AVp53 (Lanes 3 and 4) were reacted with the anti-p53 antibody DO-1. The blot was stripped and subsequently reacted with an anti-b-actin antibody. B, the same samples were electrophoresed on a 12% SDS-polyacrylamide gel and reacted with an anti-GFP antibody.
cells were infected in vitro with an adenoviral recombinant expressing p53 under the control of the CMV promoter/enhancer (AVp53), and the total population of cells was collected at various times after infection for flow cytometric analysis of DNA content. As shown in Fig. 1A, little effect was observed 24 h after infection as compared to cells infected with the control adenoviral recombinant AVlacZ; on the other hand, at 72 h after infection with AVp53, there was a highly significant increase in the number of cells with sub-G1 DNA content (from 11% to 85%). Only U87/lacZ cells that were infected with AVp53 were positive for in situ DNA fragmentation with the TUNEL assay (data not shown). These results indicate that stable transfection of U87 MG with the lacZ gene did not alter the response of the cell line to the overexpression of p53.

In view of the rapid apoptotic death of AVp53-transduced U87/lacZ cells in vitro, we tested whether direct intratumoral transduction with p53 would cause tumor regression. Tumors were established bilaterally by stereotactic injection into the caudate region of immunodeficient nude mice. Ten days after tumor cell injection, when the tumors were approximately 0.83 ± 0.19 mm³ in volume (as seen in Fig. 5A), 6 × 10⁸ particles of AVp53 (or AVGFP as a control) were injected into tumor beds using previously established coordinates. An examination of the brain coronal sections of mice euthanized 25 days after the injection showed that the tumor in the left hemisphere (injected with AVp53) had disappeared and had been replaced by areas of necrosis/gliosis (Fig. 2A). In contrast, the implanted tumor on the right side injected with the control adenoviral recombinant AVGFP had continued to grow (Fig. 2A).

To study in more detail the tumoricidal effect of AVp53, single tumors were established for 10 days, followed by injection of AVp53 or AVGFP. Animals were euthanized at 2, 3, 4, 5, or 25 days after viral injection (n = 4 for each time point) to examine brains for the presence of tumor cells. In tumors harvested shortly after viral injection, overexpression of either p53 or GFP (depending on the adenoviral recombinant used) could be demonstrated by Western blot analysis (Fig. 3). As early as 3 days after viral infection, approximately 30% of the glioma cells revealed a positive nuclear reaction by TUNEL labeling (18) in the AVp53-treated group (Fig. 1B), suggesting that tumor cells were undergoing apoptosis. In contrast, no positive nuclei were seen in tissue sections from the control AVGFP-treated animals (Fig. 1B).

In accordance with these results, all of the animals in the AVGFP-treated group had intact cerebral tumors (Fig. 4, B, D, and F) that were characterized by blue-stained cytoplasm indicating the

Fig. 4 Rapid destruction of implanted U87/lacZ tumors after infection with AVp53 (A, C, and E) as compared to infection with AVGFP (B, D, and F). Animals were euthanized at day 2 (A and B), 5 (C and D), and 25 (E and F) after treatment with AV. Sections were stained for β-galactosidase activity (blue), followed by H&E staining.
presence of β-galactosidase activity. Little necrosis was observed in these tumors, but there was a mild inflammatory response as well as some microhemorrhages. In the case of tumors treated with AVp53, focal necrosis and moderate inflammatory response were detectable as early as 2 days after virus injection (Fig. 4A); extensive necrosis/hemorrhages with fewer remaining tumor cells were apparent by 5 days (Fig. 4C). After 25 days, only a small number of residual cells surrounded the injection tract with a large area of necrosis/gliosis (Fig. 4E). Immunocytochemical analysis revealed that CD45⁺ lymphocytes and Mac-1⁺ macrophages were present within all of the tumors and in the immediately adjacent parenchyma, with the inflammatory reaction being most pronounced in the AVp53-treated animals (data not shown). However, by 25 days after viral injection, the extent of cellular infiltration had declined significantly. At this time period, microscopic examination of semi-thin sections of Epon-embedded tissue showed that CD45⁺ lymphocytes and Mac-1⁺ macrophages were present within all of the tumors and in the immediately adjacent parenchyma, with the inflammatory reaction being most pronounced in the AVp53-treated animals (data not shown). However, by 25 days after viral injection, the extent of cellular infiltration had declined significantly. At this time period, microscopic examination of semi-thin sections of Epon-embedded tissue showed that there was little evidence of damage to the adjacent neuropil from AVp53 expression (data not shown).

Long-term experiments were then undertaken in nude mice implanted with U87/lacZ cells that were allowed to proliferate for either 10 days or 20 days to form tumors of different mass (Fig. 5, A and C). The different groups of animals received either AVp53, AVGFp, or saline by stereotactic injection as outlined in Table 1. Mice injected with either saline or AVGFp were all dead by 65 days after glioma cell inoculation (Fig. 5, A and C). In contrast, mice injected on a single occasion with AVp53 10 days after tumor cell inoculation had a median survival time of 193 days (P < 0.0001). Nine months after inoculation, one of the three remaining survivors died of unrelated causes. Histological analysis of brain sections from this animal showed no tumor cells, but there was evidence of a small area of gial scar tissue in the original tumor bed (Fig. 2B) and along the needle tract (data not shown).

Two additional mice euthanized 1 year after intracerebral tumor implantation were also free of tumor cells (data not shown).

The observed therapeutic effect of AVp53 injection was dependent on the mass of the original tumor. Although animals in which tumor cells had proliferated for 20 days before a single adenoviral injection survived significantly longer than controls (median survival time of 124 days; P < 0.0001), they were all dead by 156 days (Fig. 5D; Table 1). However, in this experi-

**Table 1** Survival times (in days) of the different experimental groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10 days*</th>
<th>20 days*</th>
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<tbody>
<tr>
<td></td>
<td>Single injection</td>
<td>Single injection</td>
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<tr>
<td>Saline</td>
<td>n = 7</td>
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<tr>
<td>Range</td>
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<td>45–65</td>
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<tr>
<td>Median</td>
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<td>58</td>
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<tr>
<td>AVGFp</td>
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<td>n = 10</td>
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<tr>
<td>Range</td>
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<tr>
<td>Median</td>
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<td>59</td>
</tr>
<tr>
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<td>n = 12</td>
</tr>
<tr>
<td>Range⁵</td>
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<td>88–156</td>
</tr>
<tr>
<td>Median</td>
<td>193⁵</td>
<td>124⁵</td>
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<tr>
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<td>250⁵</td>
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* The number of days refers to the time after initial glioma cell inoculation.

⁵ Incomplete range indicates animals did not die from tumor.

⁶ Statistically significant (P < 0.0001) in relation to the saline and AVGFp groups by log-rank analysis of the Kaplan-Meier survival curve (Bonferroni correction).

⁷ Statistically significant (P < 0.0001) in relation to the single injection group by log-rank analysis of the Kaplan-Meier survival curve (Bonferroni correction).
mental group, survival was improved significantly by performing multiple injections (n = 4) of AVp53 at monthly intervals using the same initial coordinates of the implanted tumor (Fig. 5D). At 240 days, 61% of the mice were still alive; one mouse that died at 264 days of unrelated causes had only a glial scar remaining at the site of the original tumor, similar to what was observed in Fig. 2B. After a 1-year period, 38% of the animals were free of tumor cells upon pathological examination at euthanasia (Fig. 5D; Table 1).

Our study demonstrates that even a single injection of an adenoviral recombinant expressing wild-type p53 leads to near complete eradication of an implanted intracerebral malignant glioma. However, it is evident in Fig. 4E that a few tumor cells expressing β-galactosidase remain at 25 days after infection. Because these cells may form the nidus for recurrence of the tumor, multiple sequential injections with AVp53 are likely to be required to eradicate the implanted tumors (Fig. 5D).

These in vivo results are in agreement with our previous data on the effect of gene transfer and overexpression of wild-type p53 in U87 MG, a human glioma cell line in which the endogenous p53 gene is not mutated (10). Rapid cell death occurred only in the p53-transduced cells and was characterized by nuclear condensation, the formation of nucleosomal DNA ladders, and positive in situ end-labeling of DNA, which, taken together, suggested that apoptosis had been induced (10). As shown in Fig. 2A, overexpression of p53 did not merely lead to the growth arrest of the cells forming the mass of the implanted tumor, thereby inhibiting tumor growth, but caused rapid cell death, probably through the induction of apoptosis. Because the p53 gene is not consistently mutated in all malignant astrocytic gliomas (7), it is significant that its overexpression through gene transfer can nevertheless cause apoptosis in cells harboring an endogenous wild-type p53 gene.

The cell death within the implanted tumors was accompanied by a marked inflammatory response consisting mainly of macrophage infiltration, which abated with time. No edema developed, and the surrounding neuropil was largely unaffected, although there was evidence of generalized astrocytic and microglial activation. Because these experiments were carried out in immunodeficient animals, it is expected that the nature of the inflammatory response will be very different in immunocompetent animals in which the adenoviral particles themselves may elicit an immune reaction (19, 20). This in itself may contribute to additional tumoricidal activity. Taken together, these experiments demonstrate that the AV is very efficient in transducing these tumors and that adenovirus-mediated overexpression of p53 may have therapeutic value in the treatment of astrocytic gliomas, irrespective of their molecular characteristics.

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