Adenovirus-mediated Transfer of the p53 Gene Produces Rapid and Generalized Death of Human Glioma Cells via Apoptosis


Abstract

Wild-type p53 is involved in several aspects of cell cycle control and suppression of transformation, inducing either apoptosis or G1 block in cell cycle progression. Using a recombinant adenovirus containing the wild-type p53 cDNA, the biological effects of the newly expressed wild-type p53 protein were examined in six human glioma cell lines. Three cell lines (U-251 MG, U-373 MG, and A-172) expressed endogenous mutant p53, and the other three (U-87 MG, EFC-2, and D54 MG) expressed wild-type p53. The restoration of normal p53-encoded protein in the mutant cell lines induced apoptosis as assessed by morphological studies using nuclear staining, electron microscopy, and flow cytometric assays. In wild-type p53 cell lines, however, the overexpression of wild-type p53 did not result in apoptosis but inhibited cellular proliferation rather drastically and modified the neoplastic phenotype. Differential effects suggest two pathways for glioma oncogenesis and a possible therapeutic strategy.

Introduction

The necessity of developing new approaches for cancer treatment is particularly urgent in brain tumors. The current results of conventional therapy for malignant glioma, the most common primary brain tumor, are disappointing. Despite intensive therapy using surgery, radiation, and chemotherapy, the median survival time of patients with glioblastoma multiforme is still only 12 months. Furthermore, current therapies have many and often serious secondary effects. Based on the tenet that cancer is a disease generated through a process of genetic alterations, new approaches to cancer therapy are being developed. One of these includes the use of gene replacement as a therapeutic tool. Thus, wild-type p53 transfer has been shown to suppress cell transformation and to induce apoptosis in several tumors. In gliomas, p53 mutations are among the most frequently observed genetic findings (1), and inactivation of p53 is an early event in these tumors, suggesting that p53 is involved in glial tumorigenesis. Interestingly, the incidence of p53 mutations in cell lines is similar to that reported in primary brain tumors (2–4), indicating that in vitro culture of human glioma cells is not usually associated with a selection of mutations of the p53 gene. Therefore, glioma cell lines may constitute a suitable system for investigating the antineoplastic role of wild-type p53 gene in glial tumors.

Materials and Methods

Human Glioma Cell Lines and Culture Conditions. The cell lines U-251 MG, T-98 G, U-87 MG, U-373 MG, U-138 MG, and A-172 were obtained from the American Type Culture Collection (Rockville, MD). The human glioma cell lines LG and EFC-2 were established from glioblastoma multiformes at the Department of Neuro-Oncology, University of Texas M. D. Anderson Cancer Center. D54 MG was provided generously by Dr. Darell Bigner (Duke University, Durham, NC). Cells were maintained in DMEM:F12 medium [1:1 (v/v)] supplemented with 10% FCS (Hyclone Laboratories, Inc., Logan, UT) in a humidified atmosphere containing 5% CO2 at 37°C.

RT-PCR. The RT-PCR conditions and the primer set used have been described previously (6). The sequencing reactions were carried out using the termination mixture of a Sequenase kit (United States Biochemical, Cleveland, OH) and analyzed on a 6% polyacrylamide gel containing 7 M urea for 2 h at 40 W, and the dried gel was exposed to Kodak XAR5 film (Eastman Kodak Co., Rochester, NY) overnight. The procedure for automated sequencing has been reported already (6). The experiments were repeated three times using three different PCR reactions to confirm every mutation.

Restriction Enzyme Analysis. A portion of the PCR-amplified product (1.5 μl) from the U-138, LG, and A-172 cell lines was subjected to AulNI (GIBCO-BRL, Grand Island, NY) restriction enzyme digestion at 37°C for 4 h. All samples were analyzed by 3% NuSieve agarose (BioRad, Rockland, ME) gel electrophoresis. The enzyme did not cut the PCR product if the transversion from G:C to T:A at codon 242 was present, rendering two different possible patterns: (a) homozygous for the mutation, only one fragment of 563 nucleotides; and (b) heterozygous for the mutation, two fragments of 346 and 217 nucleotides each.

Recombinant Adenoviruses and Infection Conditions. The construction and generation of Ad5CMV-p53 was described previously (5). Infection of the cell lines was carried out by diluting viral stock to certain concentrations, adding viral solutions to cell monolayers (0.5 ml/60-mm dish), and incubating at 37°C for 30 min with brief agitation every 5 min. This was followed by the addition of culture medium and the return of the infected cells to the 37°C incubator.

Cell Growth Rate Determination. Glioma cells were seeded in six-well culture plates for 20 h before viral infection. The cells were infected with...
Ad5CMV-p53 or Ad5CMV at a MOI of 100 plaque-forming units/cell. Culture medium was used for mock infection. Triplicate dishes of each treatment were counted at regular intervals until the eighth day after infection. The viability of the cells was assessed using trypan blue staining.

**Western Blotting Analysis.** Total cell lysates were prepared by incubating the cells 0, 4, 8, 12, 24, 48, and 72 h and 5 and 7 days after the infection in radioimmunoprecipitation assay-buffer [150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM EDTA, and 50 mM Tris (pH 7.4)] for 1 h at 4°C. Proteins (20 µg/sample), assessed by BCA protein assay; Pierce Chemical Co., Rockford IL) were subjected to 14% SDS-Tris glycine gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). The membrane was blocked with Blotto-Tween [3% nonfat milk, 0.05% Tween 20, 0.9% NaCl, and 50 mM Tris (pH 7.5)] and incubated with the secondary antibody: horseradish peroxidase-conjugated, goat anti-mouse lgG (Amersham). The membranes were developed according to the manufacturer’s instructions.

**Electron Microscopy.** Pellets from U-251 MG and U-373 MG cells after infection with either a mock virus control or Ad5CMV-p53 were fixed in 2% glutaraldehyde and then postfixed for 1 h in 1% osmium tetroxide. Specimens were counterstained with uranyl acetate and lead citrate before being examined by transmission electron microscopy.

**Flow Cytometry.** Flow cytometric analyses were performed using an Epics Profile II flow cytometer (Coulter Corp., Hialeah, FL) equipped with an air-cooled argon ion laser emitting 488 nm at 15 mW with the standard optical configuration. Forward-angle and side-scatter light signals were collected for gating purposes. A minimum of 10,000 events/sample was analyzed. DNA cell cycle analysis was performed using 70% ethanol-fixed cells, which were stained subsequently with propidium iodide (5 µg/ml) in the presence of RNase (1 mg/ml) and determined using ModFit (Verity Software House Inc., Topsham, ME). Terminal deoxynucleotidyltransferase analysis was performed as described previously (7).

**Results and Discussion**

To evaluate the effect of the exogenous wild-type p53 expression on glioma cell proliferation, we selected nine human glioma cell lines. We examined the p53 status of five cell lines, and it was already known (8–10) in the other four (Table 1). D54 MG and EFC-2 cell lines exhibited the wild-type p53 sequence within exons 5–8. Interestingly, the other three cell lines (U-138 MG, A-172, and LG) displayed a similar mutation at the hot-spot codon 242. We demonstrated that the three cell lines were either homozygous or hemizygous for the 242 mutation by RT-PCR amplification and restriction enzyme digestion. Mutations at the 242 codon have been described previously in an anaplastic astrocytoma (11) and in the germ line of a patient with ependymoma (12). In addition, we detected another mutation in the U-138 MG glioma cell line at the codon 232 consisting of a transition from T:A to G:C that also has been found in prostate cancer (13).

To induce expression of the exogenous p53, we used the Ad5CMV-p53 adenovirus, which comprised a wild-type p53-cDNA expression cassette carried in an E1 substitution adenovirus 5 vector (5). Its direct tumoricidal effect has been reported previously in the 9L rat glioma cell line and other human tumors (7, 14–16). This study represents the first time that an adenovirus has been used to transfer p53 into human glioma cells. To assess the ability of Ad5CMV-p53 to inhibit human glioma cells, we performed a β-galactosidase transduction assay using a recombinant adenovirus, Ad5CMV carrying the Escherichia coli β-galactosidase.

**Table 1 Analysis of p53 status and gene transfer efficiency of the Ad5CMV-p53 virus in human glioma cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53 statusa</th>
<th>MOI</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-87 MG</td>
<td>wt (exons 2–11)</td>
<td>Homozygous</td>
</tr>
<tr>
<td>U-251 MG</td>
<td>mut (c. 273: CGT/CAT; Arg/His)</td>
<td>Inability to trans-activate a test gene</td>
</tr>
<tr>
<td>D-54 MG</td>
<td>wt (exons 5–8)</td>
<td>Homozygous</td>
</tr>
<tr>
<td>EFC-2</td>
<td>wt (exons 5–8)</td>
<td>Homozygous</td>
</tr>
<tr>
<td>U-373 MG</td>
<td>mut (c. 273: CGT/CAT; Arg/His)</td>
<td>Inability to trans-activate a test gene</td>
</tr>
<tr>
<td>A-172</td>
<td>mut (c. 242: TGC/TTG; Cys/Phe)</td>
<td>Homozygous</td>
</tr>
<tr>
<td>T-98 G</td>
<td>mut (c. 237: ATG/ATA; Met/Ile)</td>
<td>Homozygous</td>
</tr>
<tr>
<td>U-138 MG</td>
<td>mut (c. 232: ATC/ACC; Ile/Thr)</td>
<td>Homozygous</td>
</tr>
<tr>
<td></td>
<td>(c. 242: TGC/TTG; Cys/Phe)</td>
<td>homzygous</td>
</tr>
</tbody>
</table>

a The p53 status in the cell lines U-87 MG, U-251 MG, U-373 MG, and T-98 G was reported previously (8–10). wt, wild type; mut, mutant; c, codon; ND, not done.

b The gene transfer efficiency of the recombinant adenovirus was evaluated by X-galactosidase staining, after infection with Ad5CMV-LacZ. In the first six glioma cell lines, 80% to 100% of the cells were blue at the indicated MOI. However, in the cell lines T-98 G, U-138 MG and LG, the percentage of blue cells was equal to or less than 40% using 1000 MOI.

c Determined by AlwNI restriction analysis.
Ad5-p53 PRODUCES GENERALIZED APOPTOSIS IN GLIOMAS

Days after infection

Fig. 2. Growth curves of U-251 MG, U-373 MG, A-172, U-87 MG, EFC-2, and D-54 MG cell lines. The effect of exogenous p53 expression was very different depending on the endogenous p53 status. In the p53 mutant cell lines U-251 MG (A), U-373 MG (B), and A-172 (C), exogenous p53 expression was followed by progressive cell death. In p53 wild-type cell lines U-87 MG (D), EFC-2 (E), and D-54 MG (F), there was a significant inhibition of the growth after Ad5CMV-p53 infection.
Ad5-p53 PRODUCES GENERALIZED APOPTOSIS IN GLIOMAS

Fig. 3. Phase-contrast photomicrographs (× 100) of the Ad5CMV (A) and Ad5CMV-p53 (B) U-251 MG-infected cells 36 h after treatment: acridine orange staining of U-251 MG glioma cells. The glioma cells were seeded in two-well chamber slides (Nunc, Inc., Naperville, IL) and infected with either Ad5CMV (C) or Ad5CMV-p53 (D). After 36–48 h, glioma cells were fixed in 70% ethanol and stained with acridine orange. Fluorescence microscopy; × 600. Electron microscopy study of U-251 MG cells 30–40 h after the infection with Ad5CMV-p53. E, glioma cell displaying electrondense chromatin. F, apoptotic body phagocyted by a glioma cell that did not present any change of apoptosis. Bar, 1 μm. G, morphological changes of U-87 MG glioma cells. Five days after the infection, the treated cells were polygonal, stellate, and rounded. The diameters of the cells were bigger than those of the cells infected with the control virus (H). × 100.
Ad5-p53 produces generalized apoptosis in gliomas

The expression level of the newly expressed p53 protein was more than 10 times higher than the expression of the endogenous wild-type protein in the same cell line (U-87 MG). The protein was detected as early as 8 h after the treatment, peaked at postinfection day 3, and was detectable in the three wild-type p53 cell lines for at least 7 days. At day 7, the expression of the p53 protein was still greater than 50% of the level observed at 72 h (Fig. 1B). The decrease in expression observed after the third day probably was caused by the episomal localization of the adenovirus vector, the degradation of the viral DNA, or the outgrowth of noninfected cells (14). In glioma cells, the expression level of the exogenous p53 lasted longer than in lung cancer cells. Thus, in lung cancer cells, the expression level at the fifth day after infection was 29% and at the ninth day was only 2.3% of the maximum expression (14). The differences between these and our results indicate that the expression of the exogenous p53 is influenced not only by the promoter and the virus enhancer, but also by the specific characteristics of the different host cells.

The high expression of the newly transduced protein had different effects on the growth rate of the infected cell lines depending on the p53 status of the cells. The treated mutant p53 cells U-251 MG and U-373 MG showed a drastic decrease in their viability, and practically 90% of cells died within 3 days after the Ad5CMV-p53 infection (Fig. 2, A and B). To examine the decreased viability, U-251 MG and U-373 MG cells were stained with acridine orange, and practically 90% of cells died within 3 days after the Ad5CMV-p53 infection to study the level and chronicity of the inhibition of the growth observed in wild-type p53 cells when containing the Rb gene (17). Furthermore, p53-mediated growth arrest or differentiation in glioma cell lines has been reported previously (18–20).

In four cell lines, the efficiency of the adenovirus infection was not very high (Table 1). Our results showed that the number of infection-refractory cells influenced the final outcome of the gene treatment. Thus, with a low number of infected cells, the effect of the p53 protein on the growth curve was negligible. Moreover, in these cell lines, signs of growth arrest or apoptosis were not detectable.

Wild-type, p53-mediated, negative cell growth regulation, including both the inhibition of cell proliferation and the induction of cell death, is critically important as an anticancer mechanism. Restoration of this negative regulation can contribute to the modulation of the transformed phenotype or to the induction of apoptosis. Our results indicate it is possible to express wild-type p53 in glioma cells using a recombinant adenovirus vector. The expression of p53 in wild-type p53 cells produced the modification of the neoplastic phenotype, suggesting that even wild-type p53 tumor cells may be a target for this therapy. However, the critical contribution of the exogenous wild-type p53 to cancer cell growth was more evident in cells in which p53 was mutated. Following the expression of the exogenous wild-type p53 protein, these cells underwent rapid cell death via apoptosis. These results demonstrate that overexpression of the wild-type p53 protein in glioma cells using an adenoviral vector has antineoplastic effects and should propel the development of clinical therapeutic strategies based on the transfer of the wild-type p53 gene into brain tumors.

Acknowledgments

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References


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Candelaria Gomez-Manzano, Juan Fueyo, Athanassios P. Kyritsis, et al.


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