Restoration of Wild-Type p16 Down-Regulates Vascular Endothelial Growth Factor Expression and Inhibits Angiogenesis in Human Gliomas

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ABSTRACT

Recent studies have indicated that the loss of p16 is a frequent event in the progression of malignant gliomas. The loss of p16 promotes the acquisition of malignant characteristics in gliomas, which are among the most angiogenic of all human tumors. High-grade gliomas are distinguished from low-grade gliomas by intense angiogenesis in addition to their frequent loss of p16. New therapeutic strategies aimed at inhibiting tumor angiogenesis on the basis of molecular mechanisms are theoretically attractive. Here we evaluate the effect of p16 gene replacement on the angiogenesis of gliomas. Infection with a recombinant replication-defective adenovirus vector containing the cDNA of wild-type p16 significantly reduced the expression of vascular endothelial growth factor, which is thought to be a pivotal mediator of tumor angiogenesis, in p16-deleted glioma cells. Restoring wild-type p16 expression into p16-deleted glioma cells markedly inhibited angiogenesis induced by tumor cells in vivo. Furthermore, wild-type p16 inhibited neovascularization more potently than did wild-type p53 transfer. These findings indicate that the p16 gene plays an important role in the regulation of glioma angiogenesis, suggesting a novel function of the p16 gene.

INTRODUCTION

Angiogenesis is an essential prerequisite for aggressive tumor proliferation and spreading (1). Prominent neovascularization and vascular endothelial proliferation are features frequently encountered in high-grade gliomas (2); thus, the interaction between glioma cells and the tumor vasculature has received considerable attention. The onset of tumor angiogenesis seems to occur at various steps induced by tumor-producing angiogenic-related molecules during the progression of malignant transformation (2–4). Among these angiogenic factors, VEGF plays a pivotal role in tumor angiogenesis. VEGF is a dimeric glycoprotein secreted by normal and transformed cells, and it acts as both a selective endothelial cell mitogen and a potent inducer of microvessel permeability (5, 6). Correspondingly, two VEGF receptors (Flt-1 and KDR/Fk-1) are preferentially expressed in invading and proliferating endothelial cells (4). The expression of VEGF and its receptors, which markedly contribute to tumor-associated neovascularization, seems to be correlated with the malignant transformation of gliomas and the patient prognosis (2).

The link between tumor suppressor genes and angiogenesis remains obscure. However, alteration of such genes may contribute to transformation into an angiogenic phenotype following changes in the production of cytokines, which act as angiogenic or antiangiogenic factors (7, 8). Recent experiments have shown that induction of wild-type p53 down-regulates VEGF expression and inhibits angiogenesis in several tumor cells (9, 10). Clinical investigation has also revealed that mutant-type p53 expression correlates well with VEGF expression and tumor vascularity in several cancers (11, 12).

A multistep cascade proceeding from a nonneoplastic cell to a low grade and finally to a high grade of malignancy has been molecularly characterized in gliomas (13). Mutations of p53 are frequently observed and represent consistent molecular genetic alterations in low-grade gliomas. This suggests that loss of p53 function is involved in the early phases of glioma development. In contrast, much evidence has been obtained with regard to the genetic loci and genes affected during the malignant progression of gliomas. In particular, a high frequency (greater than 50%) of homozygous p16 gene deletion has been demonstrated in gliomas and is significantly more common in high-grade gliomas (14–16). Angiogenic capacity correlates with the degree of malignancy, and loss of p16 activity frequently occurs in high-grade gliomas. However, there are no reports demonstrating that inactivation of the p16 gene promotes angiogenesis in gliomas. To explore the possibility that the p16 gene regulates glioma angiogenesis, we examined the regulatory role of the tumor suppressor gene p16 in the expression of VEGF in vitro and in the process of neovascularization in vivo. Wild-type p16 gene was introduced using the recombinant adenoviral vector Ad5CMV-p16 in human glioma cells.

MATERIALS AND METHODS

Recombinant Adenovirus and Cell Lines. Ad5CMV-p16, a recombinant adenoviral vector, was constructed by following a previously published procedure (17). Ad5CMV-p16 contains the cytomegalovirus promoter, wild-type p16 CDNA, obtained from Dr. D. Beach (Institute of Child Health, London, United Kingdom; Ref. 18) as well as a SV40 polyadenylation signal in a minigene cassette, which was inserted into the E1-deleted region of the modified adenovirus 5. The adenoviruses Ad5CMV-p53 and Ad5CMV-p21, which contain the p53 and p21 gene, respectively, were kindly provided by Dr. Jack A. Roth (The University of Texas M. D. Anderson Cancer Center, Houston, TX).

The human glioma cell lines U-87 MG and SW 1783 were obtained from the American Type Culture Collection (Manassas, VA), and U-251 MG was generously provided by Dr. N. Arita (The University of Osaka, Osaka, Japan). U-251 MG and U-87 MG cells have an endogenously deleted p16 gene (19), whereas SW 1783 cells contain the wild-type p16 gene (20). The p53 gene is mutated in U-251 MG [codon 273, CGT→CAT; Ref. 21] and SW 1783 cells [codon 273, CGT→CAT; confirmed by direct sequencing using a ABI PRISM 377 DNA sequencer (Perkin-Elmer, Foster City, CA)]. However, p53 is intact in U-87 MG cells (21). U-87 MG and SW 1783 cells were cultured in Eagle’s minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, and nonessential amino acids. U-251 MG cells were maintained in DMEM containing 10% heat-inactivated fetal bovine serum. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. Cell infection was carried out according to a previously described technique (22). Culture medium was used for mock infection.

Western Blot Analysis. Cells were infected with medium alone, Ad5CMV-LacZ viral control, Ad5CMV-p21, Ad5CMV-p53, and Ad5CMV-p16. Twenty-four h after the infection, total cell lysates were prepared by lysing cell monolayers in plates with SDS-PAGE sample buffer [125 mM...
Tris-HCl, 2% sodium dodecyl sulfate, 5% mercaptoethanol, and 10% glycerine (pH 6.8) for 1 h at 4°C after rinsing the cells with PBS. Cell lysate protein (10 μg) from each sample was measured by the BCA protein assay (Pierce, Rockford, IL) and subjected to 13.5% SDS-Tris-glycerine gel electrophoresis; it was then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 1% dry milk and 0.1% Tween 20 in Tris-buffered saline and incubated with the primary antibodies, mouse anti-human p21/WAF-1 monoclonal antibody (Oncogene Research Products, Cambridge, MA), mouse anti-human p53 monoclonal antibody (DAKO, Carpinteria, CA), and mouse anti-human p16 monoclonal antibody (PharMingen, San Diego, CA); membranes were also incubated with a secondary antibody, horseradish peroxidase-conjugated sheep antimouse IgG (Amersham, Arlington Heights, IL). The membranes were developed according to the Amersham enhanced chemiluminescence protocol.

Flow Cytometric Assay. To analyze the DNA histogram, cell suspensions were prepared by trypsinization at 36 and 72 h after infection with Ad5CMV-LacZ viral control, Ad5CMV-p21, Ad5CMV-p53, and Ad5CMV-p16. Total RNA was isolated from cultured cells using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) 24 h after infection. Total RNA sediment (400 ng) was reverse-transcribed in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM MgCl2, 0.5 mM deoxynucleotide triphosphates, and 0.2 μg of oligo(dT)12–18 primer (Life Technologies, Inc.) with 200 units of M-MLV reverse transcriptase (Life Technologies, Inc.) at 37°C for 60 min. The PCR was performed with human VEGF- and GAPDH (as an internal standard)-specific primers. The sequence of the VEGF primer 1 was 5′-GGATGTCTATCAGCGCAGCTAC-3′, and the sequence of VEGF primer 2 was 5′-TCACGCGCTCGGTGTGCACT-3′ (23). This primer set can detect mRNA encoding all four molecular species of VEGF giving rise to 322-, 454-, 526-, and 577-bp bands in the case of VEGF121, VEGF165, VEGF189, and VEGF206, respectively. The sequence of GAPDH primer 1 was 5′-CCAAATGTGTTAAGGCTAGACC-3′, and the sequence of GAPDH primer 2 was 5′-CCATGGAAGAAGCCTGGG-3′, which was expressed as a 195-bp band (24). Reverse transcription products (3 μl) were amplifiable by Taq DNA polymerase (Promega, Madison, WI) in 25 μl of a reaction mixture containing 5 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 μM EDTA, 0.1 mM DTT, 1.5 mM MgCl2, 0.15 mM deoxynucleotide triphosphates, and 0.2 μM VEGF or GAPDH primers. The PCR consisted of 30 cycles of 1 min at 94°C, 2 min at 61°C, and 2 min at 72°C. Products were then electrophoresed on a 1% agarose gel. The intensity of the ethidium bromide luminescence was measured using a Fluor Imager SI (Amersham). The amplified 322- and 454-bp fragments of VEGF cDNA were used as a VEGF control. The probe recognizes a VEGF transcript of approximately 4.0 kb. The VEGF DNA probe was purified by 1% agarose gel electrophoresis, recovered with GENECLEAN III (BIO 101, La Jolla, CA), and radiolabeled with [γ-32P]ATP (Amersham) using a DNA labeling kit (Amersham).

**RESULTS**

Gene Transduction Efficiency and p21, p53, and p16 Expression in Human Glioma Cells. β-Galactosidase transduction was assessed to determine adenoviral transduction efficiency as described previously (22). All cell lines infected with Ad5CMV-LacZ over a m.o.i. of 100 exhibited 100% blue cells. Infection with Ad5CMV-p21 and Ad5CMV-p16 at a m.o.i. of 100 and infection with Ad5CMV-p53 at a m.o.i. of 50 had no effect on cell growth until 36 h after infection and maintained a 100% viability that was determined by trypan blue exclusion in the examined cells (data not shown). With these results, Ad5CMV-p21 and Ad5CMV-p16 at a m.o.i. of 100 and Ad5CMV-p53 at a m.o.i. of 50 were used in this study to exclude the possibility that the change in VEGF expression is attributed to growth inhibition induced by gene transduction.
Western blot analysis was performed to confirm p21, p53, and p16 expression in virally transduced cells. Endogenous p21 was hardly detectable in U-251 MG and SW 1783 cells but was detected in U-87 MG cells. U-251 MG and SW 1783 cells exhibited high levels of mutant-type p53 protein. Endogenous p16 was absent in U-251 MG and U-87 MG cells but was detected in SW 1783 cells. In the gene transfection study, exogenous p21, p53, and p16 were clearly demonstrated 24 h after infection with Ad5CMV-p21, Ad5CMV-p53, and Ad5CMVK-p16, respectively (Fig. 1).

**Cell Cycle Analysis on Exogenous p21, p53, and p16 Protein Expression.** We performed flow cytometric analysis of the cell cycle to evaluate the effect of exogenous wild-type p21, p53, and p16 expression on the cell growth of U-251 MG, U-87 MG, and SW 1783 glioma cells (Table 1). U-251 MG cells accumulated significantly in the G0-G1 phase at 36 and 72 h after infection with Ad5CMV-p21, Ad5CMV-p53, and Ad5CMVK-p16, as compared with infection with Ad5CMV-LacZ. G0-G1 accumulation occurred at the same levels among cells infected with Ad5CMV-p21, Ad5CMV-p53, and Ad5CMVK-p16. In contrast, U-87 MG cells did not arrest in the G0-G1 phase of the cell cycle 36 h after infection with Ad5CMV-p21, Ad5CMV-p53, and Ad5CMVK-p16 but significantly accumulated in the G0-G1 phase 72 h after infection with Ad5CMVK-p16, as compared with infection with Ad5CMV-LacZ, Ad5CMV-p21, and Ad5CMV-p53. SW 1783 cells did not accumulate in the G0-G1 phase at 36 and 72 h after infection with Ad5CMV-p21, Ad5CMV-p53, and Ad5CMVK-p16.

We confirmed that no significant change in the G0-G1 phase was found among Ad5CMV-p21-, Ad5CMV-p53-, and Ad5CMVK-p16-treated U-251 MG, U-87 MG, and SW 1783 cells at 36 h after infection. Therefore, VEGF expression was assessed within 36 h after infection.

**Reduction of VEGF RNA by Introduction of Wild-Type p16.** RT-PCR was performed to detect VEGF RNA expression in virally transduced cells 24 h after infection (Fig. 2). Two bands corresponding to VEGF165 and VEGF121, confirmed by sequencing, were observed in all cells analyzed. In U-251 MG cells, VEGF165 and VEGF121 RNA expression in Ad5CMV-p53-treated cells decreased as compared with that in Ad5CMV-p21-treated cells (P < 0.01 and P < 0.05, respectively). Ad5CMVK-p16 reduced VEGF165 and VEGF121 RNA expression more potently than Ad5CMV-p53 (P < 0.01). In U-87 MG cells, Ad5CMV-p53 did not affect VEGF RNA expression, but Ad5CMVK-p16 significantly reduced VEGF165 and VEGF121 RNA expression as compared with Ad5CMV-p21 (P < 0.05) and Ad5CMV-p53 (P < 0.05). In SW 1783 cells, Ad5CMV-p53 reduced VEGF165 and VEGF121 RNA expression as compared with Ad5CMV-p21 (P < 0.05) and Ad5CMVK-p16 (P < 0.05), but Ad5CMVK-p16 had no effect on the expression of VEGF RNA.

Furthermore, VEGF RNA expression in U-251 MG cells was estimated by Northern blot analysis (Fig. 3). Twenty-four h after infection with Ad5CMV-p16, VEGF RNA expression decreased markedly as compared with Ad5CMV-LacZ, Ad5CMV-p21, and Ad5CMV-p53 infection (P < 0.01). This result was compatible with that of RT-PCR.

**Suppression of VEGF Secretion Attributed to Wild-Type p16 Expression.** To measure the VEGF protein levels in a medium of cells transduced with adenoviral vectors, cell supernatants were collected 36 h after infection with Ad5CMV-LacZ, Ad5CMV-p21, Ad5CMV-p53, and Ad5CMVK-p16. VEGF expression did not change in any of the cell lines after infection with Ad5CMV-LacZ and Ad5CMV-p21. VEGF secretion in Ad5CMV-p53-treated U-251 MG cells was significantly suppressed by 23.0% as compared with that of Ad5CMV-p21-treated cells (P < 0.05). Furthermore, Ad5CMVK-p16 reduced VEGF secretion in U-251 MG cells by 78.4% and 72.0% as compared with Ad5CMV-p21 (P < 0.0001) and Ad5CMV-p53 (P < 0.001), respectively. In U-87 MG cells, Ad5CMV-p53 had no effect on VEGF expression, but Ad5CMVK-p16 reduced the expression of VEGF by 82.5% as compared with Ad5CMV-p21 (P < 0.001). In SW 1783 cells, no significant reduction of VEGF protein levels was found between the mock-infected and virally infected cells (Fig. 4). Adenovirus-mediated overexpression of wild-type p53 significantly decreased VEGF protein expression in U-251 MG cells. In p16-deleted cell lines (U-251 MG and U-87 MG cells), overexpression of wild-type p16 suppressed VEGF production more potently than wild-type p53.

**Inhibition of Tumor Cell-induced Angiogenesis in Vivo by Wild-Type p53 and p16 Gene Transfer.** We examined the development of a capillary network on the air sac fascia of nude mice (four mice/group). The photographs shown in Fig. 5 display air sac fascia in contact with a chamber containing U-87 MG cells or PBS on day 2 after implantation into the nude mice. Mock-, Ad5CMV-LacZ-, and Ad5CMV-p21-infected cells were found to be involved in the development of more extensive capillary networks than PBS, whereas Ad5CMVK-p16-infected tumor cells induced only a few newly formed vessels. In U-251 MG cells, the mean area of newly formed vessels was reduced by 34.6% in mice implanted with Ad5CMV-p53-treated cells as compared with those implanted with Ad5CMV-p21-treated cells (P < 0.05; Fig. 6). Furthermore, Ad5CMVK-p16 diminished newly formed vessels more potently than Ad5CMV-p53 (P < 0.05). Similarly, in U-87 MG cells, infection with Ad5CMV-p53 and Ad5CMVK-p16 reduced newly formed vessels by 36.1% and 66.9%, respectively, as compared with Ad5CMV-p21 (P < 0.05 and P < 0.001, respectively). Ad5CMVK-p16 decreased neovascularization more potently than Ad5CMV-p53 (P < 0.05). In SW 1783 cells, Ad5CMV-p53 inhibited angiogenesis induced by tumor cells more potently than Ad5CMV-LacZ (P < 0.05), but no significant change in neovas-
cularization was found among treatments with Ad5CMV-p21, Ad5CMV-p53, and Ad5CMVK-p16. Wild-type p16 transfer inhibited angiogenesis more markedly than wild-type p53 in p16-deleted cell lines.

**DISCUSSION**

In the present study, Ad5CMVK-p16 significantly decreased VEGF RNA and protein expression in p16-deleted cells (U-251 MG and U-87 MG cells) but did not change expression in SW 1783 cells with wild-type p16. On the other hand, Ad5CMV-p21 did not suppress VEGF expression in any of the cells. The degree of growth inhibition and cell cycle arrest did not change between p21- and p16-treated cells 36 h after infection. Therefore, we speculate that the suppression of VEGF RNA and protein expression in Ad5CMVK-p16-infected cells might not be related to cell growth or cell cycle regulatory ability. These findings indicate that the p16 gene plays an important regulatory role in VEGF synthesis in a cell cycle-independent manner. To further ascertain the effect of tumor suppressor gene transfer on angiogenesis, we used a dorsal air sac assay in nude mice. Ad5CMV-p21 had no effect on angiogenesis induced by tumor cells in vivo. Ad5CMVK-p16 significantly reduced tumor neovascularization in p16-deleted cells but not in SW 1783 cells. Furthermore, restoration of wild-type p16 markedly

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**Table 1: Effect of Ad5CMV-p21, Ad5CMV-p53, and Ad5CMVK-p16 on cell cycle regulation**

<table>
<thead>
<tr>
<th></th>
<th>U-251 MG</th>
<th>U-87 MG</th>
<th>SW 1783</th>
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<tbody>
<tr>
<td>% in G0-G1</td>
<td></td>
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<tr>
<td>Ad5CMV-LacZ</td>
<td>53.8 ± 4.9</td>
<td>64.1 ± 4.0</td>
<td>60.6 ± 6.6</td>
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<tr>
<td>Ad5CMV-p21</td>
<td>79.2 ± 5.0³</td>
<td>87.6 ± 0.9³</td>
<td>66.7 ± 2.7</td>
</tr>
<tr>
<td>Ad5CMV-p53</td>
<td>82.1 ± 2.6³</td>
<td>90.7 ± 3.8³</td>
<td>61.2 ± 4.8</td>
</tr>
<tr>
<td>Ad5CMVK-p16</td>
<td>80.3 ± 4.7³</td>
<td>87.2 ± 9.6³</td>
<td>68.7 ± 3.1</td>
</tr>
</tbody>
</table>

³ Each cell line was infected with Ad5CMV-LacZ, Ad5CMV-p21, Ad5CMV-p53, and Ad5CMVK-p16. Cells were analyzed for DNA content using flow cytometry 36 and 72 h after infection. Data are expressed as the mean ± SD of the percentage of G0-G1 (n = 3).

P < 0.05 versus Ad5CMV-LacZ.

P < 0.05 versus Ad5CMV-LacZ, Ad5CMV-p21, and Ad5CMV-p53.

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**Fig. 2. Effect of Ad5CMV-p21, Ad5CMV-p53, and Ad5CMVK-p16 on VEGF RNA expression in U-251 MG, U-87 MG, and SW 1783 cells.** Cells were infected with medium alone (mock), Ad5CMV-LacZ (lacZ), Ad5CMV-p21 (p21), Ad5CMV-p53 (p53), and Ad5CMVK-p16 (p16). cDNA preparations of 454 and 322 bp corresponding to VEGF165 and VEGF121, respectively, were analyzed by RT-PCR in each cell. GAPDH transcripts were used as an internal control. A, PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining. M, marker (λ-HindIII digest). B, the amount of VEGF165 (□) and VEGF121 (■) RNA was estimated as described in “Materials and Methods.” Values are expressed as a percentage of the mock control group from four experiments. Bars, SD. a, P < 0.05 versus lacZ and p21. b, P < 0.05 versus lacZ, p21, and p53. c, P < 0.05 versus lacZ, p21, and p16.
and p21.

...and incubated for another 12 h. The supernatants were assayed for VEGF using an ELISA. Values are the means... 

...cancer effect of p16 may be modulated by different factors, such as... 

...and the resulting inhibition of tumor neovascularization would... 

...lates VEGF is unknown, it is possible that p16 down-regulation of... 

...cell invasion by an inhibition of matrix metalloproteinase 2 in glioma... 

...recent study demonstrated that p16 overexpression suppressed tumor... 

...effective induction of apoptosis, which could not be achieved by... 

...transduction. In glioma cells, wild-type p53 probably induces the release of an unidentified inhibitor of angiogenesis. Van Meir et al. (8) demonstrated that p53 regulates the expression of glioma-derived angiogenesis inhibitory factor. Recently, a candidate glioma-derived angiogenesis inhibitory factor was identified as a p53-inducible gene product containing five thrombospondin 1 repeats specifically expressed in the brain (brain-specific angiogenesis inhibitor 1; Ref. 32). This study indicates that angiogenesis inhibitors might be up-regulated by wild-type p53 transduction. 

...Because conventional adjuvant therapy using surgery, radiotherapy, and chemotherapy against malignant gliomas has limitations in improving the prognosis (33), new rational and effective strategies are required to achieve a therapeutic breakthrough. Malignant gliomas are the most common primary brain tumors, which are characterized by increased mitotic activity, a highly invasive...
nature, and prominently neovascularized neoplasm (34). In this study, wild-type p16 transfer inhibited tumor neovascularization markedly more potently than did wild-type p53, which has been noted to inhibit tumor angiogenesis. Our results indicate that the vector-mediated transduction of p16 may have a potential role in the treatment of malignant gliomas. Recent studies (35, 36) have identified effective antiangiogenic agents such as angiostatin and endostatin that inhibit tumor growth. Combined delivery of p16 and other genes that code antiangiogenic substances may promote further tumor regression. The present study and more recent data disclose that p16 performs multiple functions as a tumor suppressor in fundamental processes during malignant transformation; such processes include cell cycle progression, tumor invasion, and angiogenesis. Although additional studies will be necessary to...
assess the suppression of tumor growth in vivo, wild-type p16 gene replacement may be an effective strategy in the treatment of malignant gliomas.

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REFERENCES

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