Antiangiogenesis Treatment for Gliomas: Transfer of Antisense-Vascular Endothelial Growth Factor Inhibits Tumor Growth in Vivo

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ABSTRACT

Presently, there is no effective treatment for glioblastoma, the most malignant and common brain tumor. Angiogenic factors are potentially optimal targets for therapeutic strategies because they are essential for tumor growth and progression. In this study, we sought a strategy for efficiently delivering an antisense cDNA molecule of the vascular endothelial growth factor (VEGF) to glioma cells. The recombinant adenoviral vector Ad5CMV-αVEGF carried the coding sequence of wild-type VEGF165 cDNA in an antisense orientation. Infection of U-87 MG malignant glioma cells with the Ad5CMV-αVEGF resulted in reduction of the level of the endogenous VEGF mRNA and drastically decreased the production of the targeted secretory form of the VEGF protein. Treatment of s.c. human glioma tumors established in nude mice with intraleSIONal injection of Ad5CMV-αVEGF inhibited tumor growth. Taken together, these findings indicate that the efficient down-regulation of the VEGF produced by tumoral cells using antisense strategies has an anti-tumor effect in vivo. This is the first time that an adenoviral vector is used to transfer antisense VEGF sequence into glioma cells in an animal model, and our results suggest that this system may have clinical and therapeutic utility.

INTRODUCTION

New strategies are needed for the treatment of glioblastoma, the most common type of brain tumor. Among targets suitable for new therapies are regulators of angiogenesis. These molecules are especially important in gliomas because hypervascularization is a major feature of these tumors. Indeed, the progression of a astrocytoma from a low-grade to high-grade malignancy is characterized by increased neovascularization. Angiogenesis modulators are extraordinarily important in tumor growth, as shown by the fact that neovascularization should occur for solid tumors to grow beyond a diameter of 2–3 mm (1). One of these molecules, VEGF, was discovered and cloned in the conditioned medium of bovine pituitary folliculostellate cells (2). VEGF is up-regulated in several tumors, including glioma (3). The VEGF protein is a M, 34,000–42,000, heparin-binding, dimeric, disulfide-bound glycoprotein that exists as four isoforms of 121, 165, 189, and 206 amino acids. Analysis of various cDNA libraries derived from cultured cells and tissue by the PCR technique revealed that the transcript encoding the 165-amino acid species is the most abundant product of the VEGF gene. VEGF is a pivotal molecule in tumoral angiogenesis that promotes endothelial cell growth and is efficiently secreted by cells (4). In addition, several lines of evidence suggest that VEGF plays a major role in the neovascularization and growth of gliomas. For example, in situ hybridization studies demonstrate expression of VEGF mRNA at high level in the hypervascularized glioblastoma multiforme (5). In addition, the transfection of VEGF165 cDNA to rat glioma cells results in hypervascularized tumors with abnormally large vessels, and the abrupt withdrawal of VEGF results in the regression of preformed tumor vessels (6). Moreover, the transfection of antisense VEGF cDNA results in the suppressed ability of glioma cells to form tumors in mice (7, 8).

Increasing knowledge of molecular concepts has yielded a battery of potential therapies for cancer, but the clinical application of these methods has not been completely successful. This is in part because in vitro models used for screening often do not duplicate in vivo conditions. In this regard, proof of the role of VEGF in tumor angiogenesis requires the demonstration that inhibition of VEGF action prevents tumor growth in vivo. In the study we describe here, an adenovirus (Ad5CMV-αVEGF) was used to transfer antisense VEGF165 cDNA into a human malignant glioma cell line, with the result that the exogenous sequence was efficiently transferred and highly expressed. After the treatment of glioma cells, production of the endogenous VEGF165 mRNA and production of the secretory VEGF protein levels were both decreased. The availability of an adenovirally mediated system capable of blocking VEGF message in vitro allowed us to test the hypothesis directly in vivo. Treatment of human glioma tumors implanted s.c. in nude mice with the Ad5CMV-αVEGF resulted in consistent suppression of growth. To our knowledge, this is the first time that a recombinant adenovirus has been used to down-regulate VEGF in human gliomas. In addition, our in vivo results suggest that a brain tumor treatment based on antisense VEGF strategies may be feasible and effective.

MATERIALS AND METHODS

VEGF cDNA. VEGF cDNA has been amplified by reverse transcription-PCR using the following primers 5′-AACCATGAACTTTCTGCT-3′ (forward) and 5′-TTGCAGATGTGACAAGCC-3′ (backward). Subsequently, the 574-bp VEGF cDNA was cloned using the pCRII vector (Invitrogen, Carlsbad, CA) and sequenced using the T7 promoter and M13-reverse primers. The VEGF165 cDNA has been extracted from the pCRII vector with HindIII (Boehringer Mannheim Co., Indianapolis, IN) and NotI (Boehringer Mannheim Co.) restriction enzymes and then inserted in an antisense orientation into the E1-deleted expression plasmid pXCL-CMV shuttle vector (generously given by Dr. W. W. Zhang, Urogen Corp., San Diego, CA) between the CMV promoter and SV40 polyadenylation signal site (pXCL-CMV-αVEGF).

Construction and Generation of the Adenoviral Vectors. To down-regulate endogenous VEGF expression and enhance the in vivo applicability of the antisense VEGF strategy, we constructed a replication-deficient recombinant adenoviral vector containing the cDNA for VEGF165 in an antisense orientation (Fig. 1A) following a procedure described previously (9). The expression plasmid pXCL-CMV-αVEGF was cotransfected with the plasmid pJM17 (10) into the transformed human embryonic kidney cell line 293 cells (American Type Culture Collection, Rockville, MD) by calcium phosphate method. Homologous recombination of the expression plasmid and pJM17 in

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4 The abbreviations used are: VEGF, vascular endothelial growth factor; CMV, cytomegalovirus; Ad5CMV-αVEGF, recombinant adenovirus 5 containing the coding sequence of the wild-type VEGF165 in an antisense orientation; MOL, multiplicity of infection; Ad5dl312, E1-deleted, replication-defective adenovirus type 5; bFGF, basic fibroblast growth factor; PPU, plaque forming unit.
293 cells replaced the E1 region with the expression cassette from the expression plasmid. After cotransfection, individual viral plaques were isolated. Plaques containing the human VEGF165 cDNA were identified by the PCR and restriction enzyme digestion. Then, these plaques were amplified in 293 cells. Ad5(dl312), an E1-deleted adenovirus type 5 carrying no exogenous gene, was used as an adenoviral control vector (11). Ad5CMVαVEGF, Ad5(dl312), and Ad5CMV-LucZ were propagated in 293 cells and then underwent CsCl density purification. Subsequently, the preparations were dialyzed and stored in the dialysis buffer (10 mmol/l Tris-HCl and 1 mmol/l MgCl2, pH 7.4) with 10% glycerol at −80°C. The titer of each viral stock was determined in 293 cells by plaque assays (12).

Infection Conditions. The cell lines were infected as described previously (13). Briefly, the cell lines were infected by diluting the viral stock to certain concentrations, adding viral solutions to cell monolayers (0.5 ml/60-mm dish), and incubating the cells at 37°C for 30 min with a brief agitation every 5 min. After this, 10% serum containing culture medium was added, and the infected cells were returned to the 37°C incubator. Control cells were infected with the Ad5(dl312) and the mock (culture medium). Ad5CMV-LucZ, which is an adenovirus type 5-based vector lacking E1 and containing the CMV promoter driving the Escherichia coli LacZ gene coding for the β-galactosidase protein, was used for determining the efficiency of infections.

Human Glioma Cell Line and Tissue Culture Conditions. The human glioma cell line U-87 MG was obtained from the American Type Culture Collection. U-87 MG cells were maintained in DMEM/Ham’s F-12 medium (1:1, v/v) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO2 at 37°C. The 293 cells were maintained in high-glucose DMEM with 10% heat-inactivated fetal bovine serum.

Determination of Transduction Efficiency. Ad5CMV-LacZ was used to infect the U-87 MG cells (105 cells/well) in six-well plates at different MOIs, further defined as the ratio of the number of infectious virions to the number of susceptible cells, ranging from 5 to 100. Forty-eight h later, cells were fixed with 4% paraformaldehyde in a phosphate-buffered NaCl solution at 4°C for 5 min, washed with the phosphate-buffered NaCl solution, and then stained with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl2 in phosphate-buffered NaCl solution at 37°C overnight. The percentage of β-galactosidase-positive cells, or the infection efficiency, was determined by scoring the number of blue cells among 500 cells each on replicate dishes.

Northern Blot Analyses. U-87 MG cells at a density of 2 × 105 were seeded onto a 15-cm plate and allowed to adhere overnight. The next day, the cells were infected with Ad5CMVαVEGF at 50 MOI. The total cellular RNA was isolated, 1, 2, 3, 4, and 5 days after infection by the acid-guanidinium thiocyanate method, as described previously (14). Dose/effector experiments were performed as follows. U-87 MG cells at a density of 2 × 105 were seeded onto a 15-cm plate and allowed to adhere overnight. The next day, the cells were infected with either Ad5CMVαVEGF or Ad5(dl312) at five different MOIs (25, 50, 100, 200, and 400). Three days after infection, the total cellular RNA was isolated. For the Northern analysis, 15 µg of total cellular RNA prepared from each sample was subjected to electrophoresis on a 1% agarose gel containing 2% formaldehyde, stained with ethidium bromide, photographed, transferred to a nylon membrane (Zetaprobe; Bio-Rad Laboratories, Hercules, CA), and hybridized to an [α-32P]dCTP-labeled VEGF165 cDNA probe. Random priming was performed using the Prime It II kit (Stratagene, La Jolla, CA), after which the membrane was washed in high-stringency conditions and autoradiographed for 5 h.

ELISA. We performed human VEGF ELISA analyses to quantitate the secretory VEGF165 in conditioned medium according to the manufacturer’s protocol (R & D Systems, Minneapolis, MN). To prepare the conditioned medium, 105 cells were seeded overnight on six-well plates with medium containing 10% serum. Then, cells were infected with either Ad5CMVαVEGF or Ad5(dl312) at different MOIs. Culture medium was used for the mock infection. Triplicate dishes of cells subjected to each treatment were used. Conditioned media were processed 3, 6, and 9 days after infection. Thirty h before collecting conditioned media, the cells were washed three times with 2 ml of serum-free medium/well. After which the cells were preconditioned by incubation in 2 ml of serum-free medium/well for 6 h. After the preconditioned medium was aspirated, the cells were washed again with 2 ml of serum-free medium/well and incubated for 24 h in 1 ml of medium containing 2% serum. The medium was collected in a tube containing 1 µl of 100 mM phenylmethylsulfonyl fluoride.

The bFGF ELISA was performed according to the manufacturer’s protocol (R & D System) using total cellular lysate and conditioned medium. Total cell lysates for bFGF ELISA were prepared by incubating the cells at 3, 6, and 9 days after infection in radiomunomoprecipitation 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. Conditioned medium for bFGF ELISA was prepared following the procedures described above for VEGF ELISA.

Determination of the Cell Growth Rate in Vitro. U-87 MG glioma cells were seeded at a density of 105 cells/well in six-well culture plates and allowed to adhere overnight. The next day, the cells were infected with either Ad5CMVαVEGF or Ad5(dl312) at an MOI of 100 or plain medium. Triplicate dishes of each treatment were counted at regular intervals until the 12th day after infection. The population doubling level was calculated using the following formula: Nh/Ni = 2Nh (where Nh = cell harvest number, Ni = cell inoculum number, and X = number of population doubling).

Ad5CMVαVEGF Treatment in Vivo. The animal study was carried out at the animal facility of M. D. Anderson Cancer Center in accordance with institutional guidelines. In this study, athymic female nu/nu mice, 4–6 weeks of age, were acclimated and caged in groups of five or fewer. All mice were fed a diet of animal chow and water ad libitum. The animals were anesthetized with methoxyflurane before all procedures and were observed until fully recovered. U-87 MG cells (105) in 100 µl of serum-free medium were injected into the right flank of each animal. The animals were observed daily for evidence of tumor growth. Upon the observation of tumors, the mice were randomized to treatment groups. The mice were monitored daily for evidence of tumor growth, and those mice that were judged to be tumor-free died were censored.
s.c. into the flank of the nude mouse. By 4 days, visible and palpable s.c. nodules had developed at all injection sites. Intratumoral treatment with serum-free medium or 10^7 PFUs of either Ad5(dl312) or Ad5CMV-αVEGF (eight mice/group) was started and repeated every other day for a total of four times. At the end of the measurement period, the mice were sacrificed by a lethal dose of CO₂, and the tumors were excised and fixed in neutral-buffered formalin for routine histological examination and H&E staining. To measure tumor volume, the largest (a) and smallest (b) diameters of each tumor were measured, and the volume was calculated by the formula: \( a \times b^2 \times 0.4 \) (15).

The tumor volumes for the different treatment groups were compared using the SPSS software package (SPSS, Inc., Chicago, IL).

RESULTS

Adenovirus-mediated Gene Transfer in U-87 MG Cell Line. We constructed a replication-deficient recombinant adenoviral vector Ad5CMV-αVEGF, containing the cDNA for VEGF_{165} in an antisense orientation (Fig. 1A). The extent of adenovirus gene transfer in a glioblastoma cell line was determined by measuring reporter gene expression 48 h after infection with Ad5CMV-LacZ at different MOIs. The transduction efficiency was shown by the percentage of blue cells seen after 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. The transduction efficiencies of U-87 MG cells at 20 MOI were 78%, and the efficiency increased with higher MOIs, reaching >99% at 100 MOI (Fig. 1B).

Ad5CMV-αVEGF Down-Regulated Endogenous mRNA VEGF_{165}. We decided to perform this study using an antisense VEGF_{165} cDNA for several reasons: (a) the transcript encoding the 165- amino acid species is the most abundant product of the VEGF gene; (b) VEGF_{165} and VEGF_{121} promote endothelial cell growth and are efficiently secreted by cells; and (c) VEGF_{165} cDNA contains the full sequence for the VEGF_{121} cDNA. In addition, the two larger forms, VEGF_{189} and VEGF_{206}, are mostly cell associated, and mRNA conditioned by stable cell lines expressing VEGF_{189} and VEGF_{206} have a very low level of mitogenic activity for endothelial cells. The human glioblastoma cell line, U-87 MG, was chosen for this study because this cell line expresses high levels of VEGF mRNA, secretes the VEGF protein, and expresses the four VEGF splice variants, including VEGF_{165} (7). In addition, these malignant glioma cells are able to form tumors in mice.

Northern blot analyses were performed to determine the level of endogenous VEGF mRNA expression. In this series of experiments, cells were treated with Ad5CMV-αVEGF at 50 MOI, and then the total cellular RNA was isolated at different times (1–5 days) after infection. Maximal down-regulation of the endogenous VEGF mRNA was observed between 3 and 5 days after infection (Fig. 2A). To determine the influence of different MOIs, U-87 MG cells were infected with 25–400 MOIs, and the total cellular RNA was isolated 3 days after infection. The levels of the 4.3-kb, endogenous VEGF mRNA detected in the samples isolated from Ad5CMV-αVEGF-infected cells were down-regulated 20% at 25 MOI (Fig. 2B, Lane 2), and the degree of down-regulation increased with increasing MOIs (Fig. 2B, Lanes 3–6). In particular, at 100 MOI, the endogenous VEGF mRNA was down-regulated by >50%, and at 200 MOI, the endogenous VEGF mRNA was almost undetectable. The levels of endogenous VEGF mRNA detected in the samples isolated from mock-infected cells (Fig. 2B, Lane 1) and from Ad5(dl312)-infected cells (Fig. 2B, Lanes 7–9) were similar. In addition, exogenous, 0.8-kb antisense VEGF mRNA was present in the cells infected with Ad5CMV-αVEGF (Fig. 2B, Lanes 2–6), indicating that the exogenous VEGF_{165} antisense cDNA was successfully transduced into these cells and then efficiently transcribed. The decreased levels of endogenous VEGF mRNA in the Ad5CMV-αVEGF-infected cells are likely due to their antisense RNA-targeted degradation.

The Ad5CMV-αVEGF Treatment Induces a Reduction of the Expression Level of VEGF_{165} Protein. We performed ELISA analyses to determine the amounts of secretory VEGF proteins in conditioned media collected 3, 6, and 9 days after infection with either Ad5(dl312) or Ad5CMV-αVEGF. The mock-infected and Ad5(dl312)-infected U-87 MG cells secreted VEGF protein at a concentration of 1451.7 ± 178.5 and 1468 ± 328.5 pg/ml/10^5 cells/24 h, 6 days after infection, respectively; Ad5CMV-αVEGF-infected cells produced 888.7 ± 153.8 pg/ml/10^5 cells/24 h at 100 MOI (Fig.

![Fig. 2. A, expression of VEGF mRNA at different times after treatment with 50 MOI of Ad5CMV-αVEGF. Maximal down-regulation was observed between 3 and 5 days after infection. B, expression of VEGF mRNA in different times. C, expression of VEGF protein in different times. D, expression of VEGF protein in different times.](http://cancerres.aacrjournals.org/issue)
The effect of antisense VEGF on VEGF protein secretion was maximal at 9 days after infection. Secreted VEGF protein levels in Ad5CMV-αVEGF-infected cells dropped to 654.5 ± 18.2 pg/ml/10⁵ cells/24 h at 100 MOI, 9 days after infection (Fig. 2D).

To determine whether the Ad5CMV-αVEGF is specific to VEGF, the expression of bFGF was studied by ELISA. ELISA of the cellular and conditioned media proteins were performed on days 3, 6, and 9 after adenoviral infection. Down-regulation of the bFGF was not observed, indicating that the Ad5CMV-αVEGF effect is probably specific to VEGF (Fig. 3A).

The Ad5CMV-αVEGF-infected U-87 MG Cells Did Not Show Any Modification in the Growth Rate in Vitro. Although endogenous VEGF mRNA expression and VEGF protein secretion are decreased in Ad5CMV-αVEGF-infected U-87 MG cells as shown in Fig. 2, we did not observe any reduction in the growth rate of Ad5CMV-αVEGF-infected U-87 MG cells compared with that of mock- and Ad5(dl312)-infected U-87 MG cells in vitro, as shown by the fact that the population doubling levels of mock-, Ad5 (dl312)-, and Ad5CMV-αVEGF-infected U-87 MG cells were 2.55, 2.62, and 2.42, respectively (Fig. 3B).

The Ad5CMV-αVEGF Treatment Suppresses Tumor Growth in Vivo. The U-87 MG (10⁵) cells were injected s.c. into the flank of the nude mouse. By 4 days, visible and palpable s.c. nodules (mean size, 54.6 mm³) had developed at all injection sites. To determine the potential effectiveness of Ad5CMV-αVEGF therapy, intratumoral treatment with serum-free medium or 10⁸ PFU of either Ad5(dl312) or Ad5CMV-αVEGF (eight mice/group) was started and repeated every other day for a total of four times. Four weeks later, tumor sizes were measured, and the mean tumor size was 977.2 ± 234.89 mm³ in the serum-free medium treatment group and 1475.6 ± 722.66 mm³ in the Ad5(dl312) treatment group. In contrast, tumor size in the Ad5CMV-αVEGF treatment group was 236.3 ± 223.12 mm³. The difference between sizes of tumor in the control and test groups was significant (P = 0.004; Fig. 4), providing direct evidence that Ad5CMV-αVEGF has an antitumoral effect in vivo. No gross adverse effect of this inoculation on the animals health and behavior was observed during experimental periods. Data from these animal experiments demonstrate that adenovirus-mediated antisense-VEGF gene transfer can suppress the growth of tumors derived from the human glioblastoma U-87 MG cells.

DISCUSSION

In this study, we demonstrate that treatment of gliomas may be achieved by using an adenovirally mediated antisense-VEGF transfer. It is now evident that a number of specific growth factors play a significant role in the growth, invasion, and progression of gliomas. Thus, although a therapy that interrupts paracrine and/or autocrine growth factor pathways that impinge on the tumor cells themselves might be a successful anticancer approach, a drawback to such treatment is that it would not be widely applicable because it would be difficult to find a pathway that is universally present in all tumors and because there is wide variation in the extent to which growth factor-mediated pathways are important to the growth of each tumor. Progressive growth of solid tumors depends on neovascularization. Tumor cells influence this process by producing angiogenic stimulators and inhibitors. Major physiological stimulators include VEGF, bFGF, angiogenin, interleukin 8, and platelet-derived endothelial cell growth factor, which are produced by the tumor cell itself, as well as by macrophages recruited by the tumor, and proliferating endothelial cells. Several angiogenic inhibitors have been identified; these include thrombospondin, platelet factor 4, IFN-α-2A, and angiostatin (16). The rationale for antiangiogenic therapy is that progressive tumor growth is angiogenesis dependent. This concept has recently been the subject of renewed interest in the development of new therapeutic strategies.

The present study describes the in vitro and in vivo effect of Ad5CMV-αVEGF, a replication-deficient recombinant adenoviral vector that carries the cDNA for the human form of VEGF₁₆₅ in an antisense orientation. Our data demonstrated that the transfer of an antisense cDNA in vitro efficiently down-regulates VEGF mRNA and secretory VEGF protein. In addition, the transfer of antisense VEGF cDNA did not result in the down-regulation of bFGF, another angiogenic factor unrelated to VEGF, which showed that the antisense effect is specific to VEGF and does not result from a nonspecific suppression of angiogenic factors. In addition, Ad5CMV-αVEGF did not seem to have a direct effect on the tumor cells themselves, as demonstrated by the growth studies which showed that the adenoviral construct was unable to slow the growth of glioma cells in vitro. Our results also demonstrated that Ad5CMV-αVEGF can be efficiently delivered in vivo and significantly suppresses the growth of preestablished tumor xenografts with reduction in the levels of VEGF, a finding consistent with the anticancer effect reported recently for
the VEGF protein, a directly acting endothelial cell mitogen and proliferating endothelial cells (1). One of the angiogenic stimulators is angiogenic stimulators and inhibitors produced by tumor cells and vascularization appears to result from a homeostatic balance between the proliferation. This is indicated by the fact that tumor-induced neovascularization is characteristic of the cancer cells. It is such genetic abnormalities specific to the tumor may be necessary to improve the delivery of the antisense VEGF. The presence of the blood-brain barrier limited the use of systemic therapies for brain tumors. In this study, we showed that local administration of antisense-VEGF is enough to suppress tumor growth. This is of significance because angiogenesis, besides being a characteristic of malignancy in most solid tumors, is also a physiological process in some organs and constitutes a normal response to injury (4, 24–26). Our local approach to brain tumor treatment may therefore not only be effective but also free of the potential adverse effects of systemic antiangiogenesis. The downside of any intratumoral approach using adenovirus is the inability of current vectors to deliver the exogenous message to every tumor cell in vivo. The incomplete delivery of the antisense VEGF cDNA might be responsible for the lack of a complete anticancer effect. Thus, although we showed tumor inhibition, we did not observe tumor regression. One explanation for this fact is that noninfected cells can still release VEGF and induce angiogenesis. Future vectors able to infect the majority of the cells in vivo and multiple injections of the vector into the tumor may be necessary to improve the delivery of the antisense message and either induce tumor regression or inhibit tumor growth for a long period of time.

In summary, the present study shows the in vitro and in vivo effect of Ad5CMV-αVEGF, a replication-deficient recombinant adenoviral vector that carries the cDNA for the human form of VEGF in an antisense orientation. Taken together, our data demonstrate that the cDNA encoding a secreted form of the VEGF can be efficiently delivered in vivo and significantly inhibits the growth of preestablished experimental tumors. Although several angiogenic mediators are involved in tumor angiogenesis, our finding that decreased levels of endogenous VEGF leads to significant tumor inhibition underscores the pivotal role of the VEGF system in tumor angiogenesis. Importantly, our data suggest that the Ad5CMV-αVEGF may be a useful tool for successful vascular targeting to achieve directed and effective therapy for gliomas.

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