rAAV-mediated Long-term Liver-generated Expression of an Angiogenesis Inhibitor Can Restrict Renal Tumor Growth in Mice

Andrew M. Davidoff, Amit C. Nathwani, William W. Spurbeck, Catherine Y. C. Ng, Junfang Zhou, and Elio F. Vanin

ABSTRACT

It is now well established that tumor growth is angiogenesis dependent. Inhibition of angiogenesis, therefore, is likely to be an effective anticancer approach. A gene therapy-mediated approach to the delivery of antiangiogenic agents using adeno-associated virus (AAV) vectors has a number of advantages, including the potential for sustained expression. We have constructed a rAAV vector in which the expression of a soluble, truncated form of the vascular endothelial growth factor receptor-2 (Flk-1), a known inhibitor of endothelial cell activation, is driven by a composite β-actin-based promoter. After intraportal injection of this vector, high-level, stable transgene expression was generated in mice. This established a systemic state of angiogenesis inhibition; sera from these mice inhibited endothelial cell activation in vitro and Matrigel plug neovascularization in vivo. Significant antitumor efficacy was observed in two murine models of pediatric kidney tumors. Tumor development was prevented in 10 of 15 (67%) mice, with significant growth restriction of tumors in the remaining mice. For the first time, long-term, in vivo expression of a functional angiogenesis inhibitor has been established using rAAV, with resultant anticancer efficacy in a relevant, orthotopic tumor model. These findings establish the feasibility of using rAAV vectors in antiangiogenic gene therapy.

INTRODUCTION

Inhibition of tumor-associated angiogenesis is an anticancer strategy in which the neovascularature supporting the growth of a progressing tumor is targeted. Although an increasing number of studies have demonstrated the efficacy of recombinant antiangiogenic proteins in murine tumor models (1, 2), a gene therapy-mediated approach to the long-term delivery of these therapeutic proteins has a number of potential advantages (3–6): (a) angiogenesis inhibition is likely to be tumoristic therapy; therefore, long-term administration or expression of these agents may be necessary. Gene therapy may make this feasible after even just a single administration of a vector, making this approach a potentially cost-effective, clinically convenient method for chronic delivery of these proteins; (b) difficulties with protein production and maintenance of function especially when “scaling up” for clinical trials may be avoided by in situ expression in host tissues; (c) continuous, low-level delivery of these proteins, as would be generated from gene-modified cells, may be the optimal schedule (7); and (d) there is the potential to target several angiogenic pathways with a single vector through the delivery of multiple genes.

Gene therapy strategies for tumor angiogenesis have, in fact, already been tested in a number of different murine tumor models, with some success. However, most of these studies have used either retroviral vector producers, naked DNA, or adenoviral vectors (for review, see Ref. 8). Unfortunately, retroviral vector producers may be impractical for human use; the transfer of naked DNA is typically an inefficient process, and adenoviral-mediated gene transfer is complicated by a host immune response to transduced target cells. In addition, only transient transgene expression is achieved by these approaches in which, after an initial delay, tumor growth often resumes. This likely occurs, at least in part, because antiangiogenic therapy is only cytostatic; tumor growth can resume once the restrictions of angiogenesis inhibition are removed. Therefore, long-term expression of angiogenesis inhibitors is likely to be required for sustained anticancer efficacy. Thus, alternative gene therapy approaches are needed to ensure long-term expression of these proteins.

AAV is a nonpathogenic, helper-dependent member of the parvovirus family. It has been shown to direct long-term transgene expression from largely differentiated tissues in vivo without host toxicity or eliciting a cellular immune response to the transduced cells (for review, see Ref. 9). It is, therefore, a logical choice for vector-mediated delivery of antiangiogenic factors (10). In addition to recent studies that have shown significant progress in the application of AAV-based vectors in gene therapy (9), the technology of high-titer, contamination-free rAAV production has made this a more practical endeavor (11, 12). We have chosen to target the liver in this study because of the high levels of sustained, AAV-mediated transgene expression that can be achieved after in situ liver transduction (13–15).

The target for inhibition in this study is the VEGF signaling pathway. VEGF is one of the primary tumor-expressed endothelial cell mitogens, and it is expressed at high levels in various types of tumors (16, 17). Numerous studies have shown that overexpression of VEGF is strongly associated with tumor invasion and metastasis (for review, see Ref. 18). The activity of this ligand can be inhibited by a soluble, truncated form of one of its receptors, Flk-1/KDR (VEGFR-2; Refs. 19 and 20). We have shown previously that autocrine expression of tsFlk-1 from either ex vivo or in situ gene-modified tumor cells results in significant inhibition of local angiogenesis and tumor growth in vivo (21, 22). The purpose of this study was to determine whether the approach of in situ liver transduction with AAV vectors encoding tsFlk-1 could, through long-term expression and systemic delivery of this angiogenesis inhibitor, provide the same antiangiogenic and antitumor efficacy.

MATERIALS AND METHODS

Cell Lines. SK-NP-1 (human Wilms’ tumor cell line, American Type Culture Collection, Manassas, VA), RTK-1 (J. Dome and S. Skapek, Memphis, TN), and 293T cells (human embryonic kidney cells expressing SV40 large T antigen; Ref. 23) were maintained in DMEM (Mediatech, Inc., Herndon, VA), supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Washington, PA). 

RTK-15 and SK-NP-1 were selected for their high expression of Flk-1/KDR and Flt-1 (VEGFR-1), respectively. RTK-1 cells were maintained in high-glucose DMEM (11) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Washington, PA). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Worth, CO), 100 units/ml penicillin to 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY), and 2 mM L-glutamine (Life Technologies, Inc.). HUVECs (Clonetics, Walkersville, MD) were maintained in endothelial growth medium (Clonetics).

**RNA Extraction and RT-PCR.** Total cellular RNA was isolated from HUVEC, SK-NEP-1, and RTK-1 cell pellets using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). cDNA was generated by reverse transcription of 1 μg of DNase-treated (Fisher Scientific, Pittsburgh, PA) RNA using SuperScript II reverse transcription (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s protocol. The cDNA (100 ng) was then subjected to PCR amplification with 2 units of Taq DNA polymerase, 0.2 μM each primer, and 200 μM deoxyribonucleotide triphosphates (Life Technologies, Inc.). The conditions and primers for cDNA amplification of VEGF (24), VEGFR-1, and VEGFR-2 (25) have been reported previously. The primers for VEGF are able to detect two of four different isoforms produced by alternative splicing of mRNA-VEGF165 and VEGF121 (24). The annealing temperature used in our study for VEGFR-1 and VEGFR-2 was 54°C. Primers for β-actin were used as an internal control (14). All PCR reactions were for 35 cycles, performed in a PTC-200 Programmable Thermal Cycler (MJ Research, Waltham, MA). The PCR products (20 μl) were electrophoresed on a 1.5% agarose gel.

**rAAV Vector Construction and Purification.** To construct the rAAV vector plasmid pAV CAGG tsFlk-1, a cloning intermediate (pAV CAGG) was first created by the ligation of a 2.2-kb HindIII-BamHI (blunt) fragment from the plasmid pCAGGS (26), encompassing the cytomegalovirus-IE enhancer, β-actin promoter, a chicken β-actin/rabbit β globin composite intron, and a rabbit β-globin polycladenylation signal (CAGG), into the SnaBI-Stul sites of pSUB201 (27). This created a plasmid in which the CAGG expression cassette was flanked by the AAV internal terminal repeats. cDNA for the truncated, soluble, ligand-binding extracellular domain of the VEGFR-2 (Flk-1) was provided by Dr. P. Lin (Durham, NC). The tsFlk-1 cDNA was contained within an AdExFlk-6His plasmid that has been described previously (20). A 2.3-kb HindIII-BamHI fragment containing the tsFlk-1 transgene was excised from this vector and ligated into pSP72 (Promega, Madison, WI) cut with HindIII and BamHI to make p72-tsFlk-1. pAV CAGG tsFlk-1 was then generated by ligating the 2.3-kb EcoRI-PstI fragment from p72-tsFlk-1 with the 5.3-kb EcoRI-Ecl136II fragment from pAV CAGG. To construct pAV CAGG GFP, the enhanced GFP gene (Clonetech, Palo Alto, CA) cDNA was first cloned into the EcoRI-XbaI site of pSP72 (Promega) and then into the KpnI-Xhol site of pCAGGS. A 3.9-kb SnaBl-Nhel fragment was recovered from this plasmid and cloned into the SnaBl-Stul sites of pSUB201 to generate pAV CAGG GFP.

AAV particles were made by the transient transfection method described previously (28). In brief, subconfluent 293T cells were cotransfected with either the pAV CAGG tsFlk-1 or pAV CAGG GFP plasmid vector, a helper plasmid encoding the adenoviral helper genes necessary for AAV production (80-XX6; Ref. 28) and a packaging plasmid (XX2; Ref. 11) using the calcium phosphate precipitation method. Cells were harvested between 65 and 70 h post-transfection and lysed by incubation with 0.5% deoxycholate (Fisher Scientific) in the presence of 50 units/ml Benzonase (Sigma, St. Louis, MO) for 30 min at 37°C. After centrifugation at 6000 × g, the rAAV particles were isolated by affinity column chromatography (12).

**In Situ Liver Transduction via Portal Vein Injection.** B6.CB17-PkdcszJ (Jackson Laboratory, Bar Harbor ME) and C.B-17 SCID (Charles River Laboratory, Wilmington, MA) male mice were used. AAV CAGG tsFlk-1 or AAV CAGG GFP (either 2.5 × 1011 or 1 × 1012 genomes) in a maximum volume of 400 μl was injected into the portal vein of these mice using a technique described previously (14). Identification and quantitation of tsFlk-1 expression in mouse sera were performed by Western blot analysis (21) and ELISA assay (23) as described previously.

**Inhibition of In Vitro Migration and Proliferation.** Endothelial cell migration assays were performed as described previously (21). Proliferation assays were performed by initially plating 1 × 105 cells (HUVEC, SK-NEP-1, or RTK-1) in 24-well plates. The next day, the medium was replaced with DMEM supplemented with 0.1% fetal bovine serum, 100 units/ml penicillin to 100 μg/ml streptomycin, and 2 mM L-glutamine. After 48 h, 5% mouse serum pooled from either tsFlk-1- or GFP-expressing mice was added to each well. Certain wells also received 10 ng/ml VEGF (R&D Systems, Inc., Minneapolis, MN). The SK-NEP-1 and RTK-1 cells were harvested and counted 48 h later; the HUVECs were harvested and counted 72 h later. Each condition in both assays was performed in triplicate.

**Inhibition of bFGF/Heparin-stimulated Matrigel Plug Neovascularization.** Matrigel plugs consisting of 500 μl of phenol-free Matrigel (BD Biosciences, Bedford, MA), 250 ng/ml bFGF (R&D Systems, Inc.), and 40 units of heparin (Elkins-Sinn, Inc., Cherry Hill, NJ) were established in the s.c. space along the ventral midline of tsFlk-1- or GFP-expressing mice. The plugs were harvested 6 days after injection, photographed, and then fixed in 10% formalin for histological evaluation.

**Murine Tumor Models.** Heterotopic anaplastic Wilms’ tumors were established in C.B-17 SCID mice by s.c. injection of 2 × 106 SK-NEP-1 cells in 200 μl of PBS. Tumor measurements were performed in two dimensions with calipers twice weekly, and volumes were calculated as width2 × length × 0.5. Mice were sacrificed after 42 days, at which time their tumors were excised and fixed in formalin. Orthotopic renal tumors were established in both C.B-17 SCID and B6.CB17-PkdcszJ mice by direct subcapsular injection of 5 × 104 tumor cells in 100 μl of PBS after exposure of the kidney via a flank incision. All mice had previously undergone liver transduction via portal vein injection of either AAV CAGG tsFlk-1 or AAV CAGG GFP. Mice receiving intrarenal tumor injections were sacrificed 30 (C.B-17 SCID) or 60 (B6.CB17-PkdcszJ) days after tumor cell injection. Both the tumor-bearing kidney and uninvolved contralateral kidney from each mouse were harvested and weighed to determine net tumor weight. These experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of St. Jude Children’s Research Hospital.

**Immunohistochemistry.** Immunohistochemical analysis for CD34, GFP, and vimentin was performed by indirect immunoperoxidase staining. Sections (5 μm) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene. Heat-induced epitope retrieval in citrate buffer (CD34, GFP; Zymed, South San Francisco, CA) or Target Retrieval buffer (vimentin; DAKO, Carpinteria, CA), pH 6.0, was performed at >95°C for 15 min. Endogenous peroxidase activity was blocked by incubation with 3% H2O2 (Humco, Texarkana, TX), and the slides were then incubated with rat anti-mouse CD34 (553, Pharmingen, San Diego, CA), rabbit anti-GFP (Living Colors Peptide Antibody, Palo Alto, CA), or mouse antiwesine vimentin (V9; DAKO) for 10 min followed by a biotinylated secondary antibody, either rabbit antirabbit, goat antirabbit (both from Vector, Burlingame, CA), or goat antiebant (DAKO). After incubation with streptavidin conjugated to horseradish peroxidase (DAKO), a substrate containing the chromogen 3,3’ diaminobenzidine tetrahydrochloride was added. Control concentration-matched, species-appropriate immunoglobulins (DAKO) were also used. Slides were counterstained with hematoxylin. Endothelial cell density in section hot spots was determined on sections stained with the CD34 antibody using a method described previously (23).

**Statistical Analyses.** Results are reported as means ± SEM. Student’s t test was used to analyze statistical differences between final tumor volumes (s.c.) and weights (intrarenal) and endothelial cell densities. A P < 0.05 was considered to be statistically significant.

**RESULTS**

**Long-term Transgene Expression after in Situ Liver Transduction.** One of the main theoretic advantages of using rAAV in a gene therapy approach to antiangiogenesis is the ability of the vector to mediate long-term transgene expression. We chose to use a construct in which transgene expression was directed by the CAGG composite promoter because this has been shown previously to generate high levels of transgene expression from the liver without being silenced (14). We have also found that the portal vein route of administration gives the highest systemic levels of transgene expression, with minimal vector DNA and transgene expression detectable in organs other than the liver (14). The time course of tsFlk-1 expression in different strains of mice receiving different doses of rAAV vector is shown in Fig. 1A. Peak levels of expression were attained after ~8 weeks and were sustained for the duration of the study (nearly 6 months). C.B-17 mice receiving 2.5 × 1011 particles expressed a mean of 6.4 ± 0.6 μg/ml tsFlk-1 after 8 weeks, whereas those receiving 1 × 1012...
AAV CAGG tsFlk-1. The ability of this serum to inhibit endothelial cell proliferation and migration, hallmarks of endothelial cell activation, was then tested in vitro. Fig. 2A shows that serum from these mice was able to inhibit endothelial cell migration in vitro in a dose-dependent manner. Serum from control mice had no effect on endothelial cell migration. Similarly, serum from tsFlk-1-expressing mice showed marked inhibition of endothelial cell proliferation in vitro (>75% inhibition), both in the presence and absence of supplemental VEGF (Fig. 2B). Interestingly, serum containing tsFlk-1 also had a marked effect on RTK-1 in vitro proliferation, an effect that was nearly completely abrogated by the addition of VEGF. Serum containing tsFlk-1 had no effect, however, on the in vitro proliferation of the SK-NEP-1 tumor cells.

Further confirmation that a systemic state of angiogenesis inhibition had been established was demonstrated in vivo by the Matrigel plug assay. Heparin plus bFGF were used to stimulate angiogenesis, as this combination has been shown to be among the most potent in this assay. In control mice, bFGF and heparin-impregnated Matrigel plugs showed gross and histological evidence of neovascularization.

Fig. 1. Systemic expression of tsFlk-1. A, measurement by ELISA of tsFlk-1 expression in the serum of CB-17 mice receiving 2.5 × 10^11 vector particles by intraportal injection (n = 10; ○ ). CB-17 mice receiving 1 × 10^12 vector particles by intraportal injection (n = 5; ● ). B, Western blot analysis of serum from three GFP- and two tsFlk-1-expressing mice. Purified, recombinant tsFlk-1 (100 ng) was run in the first lane. C, representative anti-GFP staining of a liver transduced with 2.5 × 10^11 AAV-GFP vector particles delivered by intraportal injection (×100).

particles expressed a mean of 31 ± 4.3 μg/ml. Interestingly, greater levels of expression were detected in B6.CB17-PrkdcSzJ mice that expressed a mean of 73.2 ± 6 μg/ml after receiving only 2.5 × 10^11 particles. This higher level of expression appears to be both promoter and transgene independent, as we, and others, have observed this phenomenon previously with different AAV vectors (14, 15). Western blot analysis confirmed that the tsFlk-1 protein being detected by ELISA was of the correct molecular weight (Mₐ, 105,000). Fig. 1B shows abundant tsFlk-1 present in sera from mice that 3 months before had received intraportal administration of AAV CAGG tsFlk-1. No tsFlk-1 was detectable in sera from control mice. Liver transduction efficiency was assessed by anti-GFP immunohistochemical staining of livers harvested 3 months after intraportal administration of 2.5 × 10^11 AAV-GFP vector particles. Approximately 2–5% of hepatocytes were found to express GFP (Fig. 1C), consistent with a previous report by Maio et al. (29).

Serum-mediated Inhibition of Endothelial Cell Activation in Vitro. We have shown previously that the cDNA encoding this truncated, soluble form of Flk-1 generates a protein which, when expressed by tumor cells after retrovirial vector-mediated transduction, could inhibit endothelial cell activation in situ (21). In the current experiments, we wanted to establish a systemic state of angiogenesis inhibition based on rAAV-mediated in situ transduction of the liver with the same tsFlk-1 cDNA. To confirm that this had been achieved, serum was collected from mice whose livers had been transduced with

Fig. 2. Inhibition of endothelial cell activation. Sera were pooled for testing from 4 mice expressing either tsFlk-1 or GFP. A, effect of sera from mice transduced with AAV CAGG tsFlk-1 on VEGF-stimulated HUVEC migration. Also shown are the effects of diluting sera from the tsFlk-1 mice with sera from GFP mice at ratios of 1:2 and 2:1 and the effect of mouse serum with and without VEGF. B, effect of sera (5%) from mice transduced with AAV CAGG tsFlk-1 on HUVEC, SK-NEP-1, and RTK-1 proliferation, as compared with sera (5%) from control mice transduced with AAV CAGG GFP, with and without VEGF (10 ng/ml). C, expression of VEGF and its receptors by HUVEC, SK-NEP-1, and RTK-1 cells. RT-PCR products obtained with primers specific for VEGF (165, 121), VEGFR-1, VEGFR-2, and β-actin transcripts. Sizes of the PCR products are also shown.
However, in those mice with high levels of tsFlk-1 expression, there was marked inhibition of angiogenesis, as there was evidence for only minimal neovascularization of the Matrigel plugs (Fig. 3).

**VEGF, VEGFR-1, and VEGFR-2 Expression.** To determine whether the primary components of the VEGF signaling pathway were present in HUVECs and the two kidney tumor cell lines, expression of the VEGF ligand, as well as two of its receptors, VEGFR-1 and VEGFR-2, was assessed. RT-PCR analysis of total RNA recovered from HUVEC, SK-NEP-1, and RTK-1 cells revealed that each of these lines expressed VEGF (165, 121; Fig. 2C). Both HUVECs and RTK-1 cells also expressed the VEGFR-2 receptor; no expression of this receptor was detected in the SK-NEP-1 cells. Only the HUVECs were found to express the VEGFR-1.

**Inhibition of Heterotopic Tumor Growth.** The effect that this systemic state of antiangiogenesis would have on in vivo tumor growth was first tested using a heterotopic xenograft model of anaplastic Wilms’ tumor. The growth of SK-NEP-1 tumor cells implanted in the s.c. space of C.B-17 SCID mice was significantly restricted in mice that, 10 weeks before tumor cell injection, had received portal vein injection of 2.5 × 10¹¹ particles of the AAV CAGG tsFlk-1 vector. Mean tumor volume 6 weeks later was 146.6 ± 84.5 mm³ when compared with 1770.9 ± 704.1 mm³ in the AAV CAGG GFP mice (P < 0.04), a difference of >90% (Fig. 4A).

To confirm that this restriction of tumor growth was attributable, at least in part, to inhibition of angiogenesis, endothelial cell densities in the tumors were compared. Immunohistochemical analysis with an anti-CD34 antibody revealed that tumors in the control mice had a mean endothelial density of 13.8 ± 2.4, whereas those in the tsFlk-1-expressing mice had a mean density of 7.2 ± 1.1 (P < 0.02), a difference of 48%.

**Inhibition of Orthotopic Tumor Growth.** Because it is likely that angiogenic factors responsible for tumor growth may be influenced by the site of tumor growth, it was important to test our antitumor strategy in an orthotopic tumor model. In an initial set of experiments, SK-NEP-1 cells were injected into the kidneys of C.B-17 SCID mice 10 weeks after intraportal vein injection of 2.5 × 10¹¹ vector particles of AAV CAGG tsFlk-1 or AAV CAGG GFP. Tumor weight was measured at necropsy 30 days later. The average tumor weight in the tsFlk-1-expressing mice was 83% less than that of the tumors in the control mice, 0.9 ± 0.3 grams as compared with 5.2 ± 0.7 grams (P < 0.01; Fig. 4B).

In a second set of experiments, a higher dose of vector (1 × 1₀¹² particles) was administered to C.B-17 mice. This resulted in a higher level of tsFlk-1 expression (Fig. 1A). When these mice were necropsied 30 days after intrarenal injection of SK-NEP-1 cells, the average tumor weight in the high tsFlk-1-expressing mice was 0.3 ± 0.2 grams as compared with 4.8 ± 1.4 grams in the control mice (P < 0.01; Fig. 4C). In fact, in three of five high tsFlk-1-expressing mice, no gross tumor was visible. The improved antitumor effect seen

---

**Fig. 3.** Inhibition of Matrigel plug neovascularization. A, photographs of Matrigel plugs, containing bFGF (250 ng/ml) and heparin (40 units), excised from GFP- and tsFlk-1-expressing mice after 6 days. B and C, H&E staining of formalin-fixed sections of the Matrigel plugs. D and E, immunohistochemical analysis of formalin-fixed sections of the Matrigel plugs stained with an anti-CD34 antibody (×40, original magnification).
fig. 4. inhibition of tumor growth. a, s.c. growth of sk-nep-1 cells in c.b-17 mice expressing gfp (∗••• or tsflk-1 (□□□□□), n = 7 for each group. in each of the subsequent panels, mean orthotopic renal tumor weight in 5 mice transduced with aav cagg gfp (left) and 5 mice transduced with aav cagg tsflk-1 (right) is shown. b, sk-nep-1 tumors in c.b-17 mice transduced with 2.5 × 1011 vector particles. c, sk-nep-1 tumors in c.b-17 mice transduced with 1 × 1012 vector particles. d, sk-nep-1 tumors in b6.cB17-PkdcSzJ mice transduced with 2.5 × 1011 vector particles. e, rtk-1 tumors in c.b-17 mice transduced with 2.5 × 1011 vector particles.

Discussion

These experiments confirm the potential of recombinant aav vectors for antiangiogenic gene therapy. for the first time, long-term, in vivo expression of a functional inhibitor of endothelial cell activation, with anticancer efficacy in a relevant, orthotopic tumor model, has been demonstrated using rAAV. Serum from mice with rAAV-mediated expression of tsFlk-1 was able to inhibit endothelial cell migration and proliferation in vitro and bFGF/heparin-stimulated neovascularization of Matrigel plugs in vivo, demonstrating that a systemic state of angiogenesis inhibition had been established. the fact that rAAV-mediated expression of the tsFlk-1 transgene was long lasting was confirmed by ELISA in which stable peak levels of expression were detected >1 year after vector administration, even in immunocompetent mice (data not shown). we chose to perform these studies in immunodeficient mice to avoid any potential immune-mediated antitumor effects to more clearly define antitumor efficacy of angiogenesis inhibition. finally, the systemic state of angiogenesis inhibition achieved by rAAV-mediated expression of tsFlk-1 resulted in significant tumor inhibition at sites remote from the liver.

the decision to study models of anaplastic Wilms’ tumor and malignant rtk was made for several reasons: (a) both tumor types have very poor prognoses with conventional chemotherapy. overall, 5-year survival for patients with anaplastic Wilms’ tumor is 40% (31) and even worse, at 20%, for those with malignant rtk (32). clearly, alternative treatment modalities are needed; and (b) clinical studies have suggested that VEGF plays a significant role in driving angiogenesis in these tumors (33, 34), and preclinical studies have demonstrated that experimental anaplastic Wilms’ tumor is highly susceptible to anti-VEGF therapy (35). rowe et al. (35), e.g., showed that anti-VEGF antibodies can suppress primary tumor growth of anaplastic Wilms’ tumor xenografts without treatment-related morbidity. these investigators also found, however, that when the anti-VEGF therapy was terminated, there was “rebound” tumor growth in the treatment group. although the mechanism of this rebound effect is unknown, this observation is of concern when considering anti-VEGF therapy for the treatment of Wilms’ tumor. it provides a strong rationale for a gene therapy approach in which long-term targeting of the VEGF-mediated angiogenic pathway can occur.

in addition to expressing high levels of VEGF, the renal tumor cells used in this study also express other prongangiogenic factors, including bFGF and platelet-derived growth factor-α (data not shown). it was, therefore, somewhat surprising that a single angiogenesis inhibitor delivered as monomodality therapy would be so efficacious in restricting tumor growth. our anti-VEGF strategy prevented tumor development (both anaplastic Wilms’ or malignant rtk) in 10 of 15 (67%) mice expressing high levels of tsFlk-1. this finding may be explained in part by several studies that have highlighted the critical role of VEGF in angiogenesis (36, 37) and the subsequent development and progression of a wide variety of malignancies, including Wilms’ tumor (33, 34). it may be that different angiogenic pathways have requirements that overlap in their dependence on certain critical factors, such as VEGF. we and others have shown, for example, that blocking VEGFR function can inhibit bFGF-stimulated neovascularization, even though it has been suggested that bFGF and VEGF angiogenesis signaling pathways are separate and distinct (38). it is unclear whether “resistance” to our antiangiogenic strategy developed, thereby permitting delayed renal tumor development in the remaining mice (33%) expressing high levels of tsFlk-1.

Additional antitumor efficacy may have been achieved, at least in the RTK-1-derived tumors, by interfering with autocrine stimulation of the tumor cells by VEGF. RT-PCR analysis demonstrated that the RTK-1 cells expressed both VEGF and VEGFR-2, and the in vitro
proliferation assay confirmed that these cells were susceptible to inhibition by tsFlk-1 expressed in sera from experimental mice. Tumor cell stimulation through VEGF-mediated autocrine loops has been observed in some solid neoplasms (39). Thus, inhibition of VEGF signaling may have impaired not only the neovascularization necessary for tumor growth but may also have interrupted autocrine stimulation through VEGFRs of the tumor cells themselves.

Finally, despite the generally held belief that antiangiogenic therapy is cytostatic, and that chronic angiogenesis inhibition would, therefore, leave a small, dormant focus of tumor cells, evaluation of the kidneys in those mice expressing high levels of tsFlk-1 that did not develop tumors (the majority of the mice) did not reveal even microscopic residual disease by immunohistochemistry. The absence of detectable tumor cells suggests that a cytotoxic effect may have been effected. This observation has important implications for planning anticancer strategies with an antiangiogenic agent, such as a VEGFR-2 inhibitor. Efficacy may be greater when treating minimal residual disease rather than when treating larger tumors with established neovascularulature, as this approach may prevent new blood vessel development but does not destroy existing vessels.

In conclusion, this study is the first to demonstrate that sustained expression of a functional angiogenesis inhibitor can be directed through rAAV-mediated in situ transduction of the liver after a single administration of vector via the portal vein. This established a systemic state of angiogenesis inhibition within the host and effected significant, dose-related inhibition of tumor growth in all mice. Although it is often stated that one of the benefits of a gene therapy approach to angiogenesis inhibition is the ability to establish sitespecific expression to avoid systemic side effects, successful treatment of widely metastatic disease that has spread to occult sites may require a systemic state of angiogenesis inhibition. The ability to treat established tumors with this approach remains to be tested, perhaps in very slow growing, spontaneously arising tumors (e.g., transgenic mice with a tissue-specific promoter driving an oncogene). In addition to continuing to evaluate the feasibility of this approach as an anti-cancer strategy, however, the effects of chronic angiogenesis inhibition on physiological, angiogenesis-dependent processes, such as wound healing and fertility, are being critically evaluated. As well, the use of an inducible promoter, whereby transgene expression could be carefully regulated, may be beneficial.

ACKNOWLEDGMENTS

We thank Dorothy Bush, Bonnie Greer, and Adriana Nance for their assistance with immunohistochemistry. We also thank Drs. Brian Sorrentino, Jeffrey Dome, and Stephen Shochat for their critical review of this manuscript.

REFERENCES


rAAV-mediated Long-term Liver-generated Expression of an Angiogenesis Inhibitor Can Restrict Renal Tumor Growth in Mice


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/11/3077

Cited articles
This article cites 39 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/11/3077.full.html#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
/content/62/11/3077.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.