

Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2

(cancer/adenovirus/soluble receptor/tumor angiogenesis)

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Edited by Webster K. Cavenee, University of California, La Jolla, CA, and approved May 12, 1998 (received for review December 18, 1997)

ABSTRACT Angiogenesis is required for tumor growth and metastasis, and inhibition of angiogenesis is a promising approach for anticancer therapy. Tie2 (a.k.a. Tek) is an endothelium-specific receptor tyrosine kinase known to play a role in tumor angiogenesis. To explore the therapeutic potential of blocking the Tie2 pathway, an adenoviral vector was constructed to deliver a recombinant, soluble Tie2 receptor (AdExTek) capable of blocking Tie2 activation. Two days after i.v. injection of AdExTek, the plasma concentration of ExTek exceeded 1 mg/ml and was maintained for about 8 days. Administration of AdExTek to mice with two different well established primary tumors, a murine mammary carcinoma (4T1) or a murine melanoma (B16F10.9), significantly inhibited the growth rate of both tumors (64% and 47%, respectively). To study the effect of ExTek on tumor metastasis, both tumor cell lines were coinjected i.v. with either AdExTek or a control virus. Mice coinjected with control virus developed numerous large, well vascularized lung metastases. In contrast, mice coinjected with AdExTek virus developed few, if any, grossly apparent metastases, and histologic examination revealed only small avascular clusters of tumor cells. Administration of AdExTek also inhibited tumor metastasis when delivered at the time of surgical excision of primary tumors in a clinically relevant model of tumor metastasis. This study demonstrates the potential utility of gene therapy for systemic delivery of an antiangiogenic agent targeting an endothelium-specific receptor, Tie2.

One of the most remarkable advances in our understanding of cancer pathogenesis is the notion that the progression of solid tumors depends on tumor angiogenesis. This fundamental principle states that tumor growth beyond a few mm³ in size strictly depends on tumor angiogenesis (1). By extrapolation, the same is true for tumor metastasis (2, 3). Consistent with this notion, recent studies indicate that tumors with a luxuriant vasculature have a higher fraction of dividing cells and lower necrosis rates than tumors with a poorly developed vasculature (4, 5). Moreover, clinical studies have shown a direct correlation between the density of tumor vessels and an adverse prognosis in patients with a variety of solid tumors, including breast, colon, lung, kidney, bladder, and head and neck tumors (6–14). Taken together, these studies suggest that the ability of a tumor to induce neovascularization determines its rate of growth and its likelihood of metastasis. Considering the importance of vascular growth in tumor progression, therapeutic

approaches targeting the tumor endothelium may provide long-term, effective control of the disease.

Angiogenic polypeptide growth factors, such as fibroblast growth factor and vascular endothelial growth factor (VEGF), are produced by tumor cell lines *in vitro* and by tumors *in vivo* and are likely to be key regulators of tumor angiogenesis (15). VEGF is currently the leading candidate for an endogenous mediator of tumor angiogenesis, because blocking the VEGF pathway inhibits the growth of several murine tumors and human tumor xenografts (16–19). However, a recent study has demonstrated that although many tumors are inhibited by blockade of the VEGF/VEGF receptor pathway, others are unaffected, which suggests that alternative pathways for vascular growth can drive tumor angiogenesis (17).

Tie2 (a.k.a. Tek) is a newly cloned endothelium-specific receptor tyrosine kinase that has crucial roles during the development of the embryonic vasculature (20–23). Disruption of Tie2 function in transgenic mice results in embryonic lethality because of defects in vascular development characterized by a reduction in endothelial cell number and a defect in the morphogenesis of microvessels (24, 25). Disrupting the function of the Tie2 ligand, angiopoietin (Ang) 1 and overproduction of Ang2, an inhibitory ligand, yielded a phenotype similar to the Tie2 knockout, confirming the importance of the Tie2/Ang1 pathway during embryonic vascular development (26, 27). To determine whether Tie2 played a role in pathologic angiogenesis in adult tissues, we have demonstrated that blocking Tie2 activation by local administration of a recombinant, soluble Tie2 receptor (ExTek) could inhibit tumor angiogenesis and tumor growth (28). These findings demonstrated a role for the Tie2 pathway in the formation of the tumor vasculature and suggested that targeting the Tie2 pathway might yield useful anticancer therapy.

Although our previous findings demonstrated a role for the Tie2 pathway in tumor angiogenesis, local application is not likely to be a clinically useful means of delivering a Tie2 inhibitor because many primary tumors will be inaccessible and metastases may be too numerous. In addition, using a recombinant protein is likely to be problematic because of the expense and inconvenience involved with frequent dosing, especially if delivered systemically. Gene therapy using viral vectors offers promise as an approach to the long-term delivery of therapeutic proteins (29). Adenoviruses are common and relatively benign human pathogens that have not been associated with persistent infections or neoplasias in humans (30).

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: VEGF, vascular endothelial growth factor; Ang, angiopoietin; pfu, plaque-forming units; TBST, 10 mM Tris-Cl, pH 8.0/150 mM NaCl/0.1% Tween20.

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Recombinant adenoviruses can be produced in high titer [up to 10^{11} plaque-forming units (pfu)/ml], and they can efficiently infect a variety of replicating or nonreplicating cells to yield high-level expression of exogenous proteins (30). In the present study, a replication-deficient adenoviral vector was used for systemic delivery of soluble Tie2 in several models of primary and metastatic cancer.

METHODS

Cell Lines, Antibodies, and Recombinant Soluble Tie2 (ExTek.6His). Recombinant adenovirus was generated and propagated in monolayer cultured 293 cells (American Type Culture Collection) maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (GIBCO/BRL) at 37°C with 5% CO₂. The murine mammary carcinoma cell line 4T1 (31) and murine melanoma cell line B16F10.9 (32) were maintained in DMEM plus 10% FBS at 37°C with 5% CO₂. The human endothelial cell line, EAHy926, was maintained in DMEM plus 10% FBS in the presence of hypoxanthine/aminopterin/thymidine at 37°C. Soluble, recombinant murine Tie2 fused at the C terminus to a 6His tag (ExTek.6His) was produced in insect cells and purified by Ni²⁺-nitrilotriacetic acid agarose chromatography as previously described (28). A mouse monoclonal anti-Tie2 antibody (Ab33) was generated by using human ExTek.6His as an antigen (33, 34).

Ang Binding Competition Assay. Purified rat Tie2-Fc fusion protein (25 µg/ml in 10 mM sodium acetate) was immobilized on a CM5 BIAcore chip activated with *N*-hydroxysuccinimide/EDC to produce a surface of 5,500 resonance unit. After coupling, the chip surface was deactivated with 1.0 M ethanolamine, pH 8.5. The running buffer for BIAcore was 10 mM Hepes, pH 7.4 containing 150 mM NaCl and 0.005% nonionic detergent P20. Ang1* and Ang2 were diluted to 800 ng/ml in a running buffer that was supplemented with 0.066 mg/ml of dextran. Ang1* is a slightly modified version of Ang1 that is easier to express and purify (26). Soluble receptors were used at concentrations ranging from 1 to 50 µg/ml. Tie1-Fc at 50 µg/ml was used to verify specificity of inhibition of ligand binding to the immobilized Tie2-Fc. Samples (40 µl) were injected at 5 µl/min, and the response was measured 30 s after the end of injection. The Tie2-Fc surface was completely regenerated between samples with two injections (13 and 8 µl) of 100 mM glycine buffer, pH 10.7. All samples were run in duplicate. The binding activity of Tie2-Fc surface remained unchanged throughout the experiment.

Ligand Stimulation and Immunoprecipitation. Endothelial cells (EAHy926) grown in 10-cm dishes were starved for 16 hr in DMEM alone. The cells were pretreated with 1 mM sodium vanadate for 10 min before incubation with Ang1* at 150 ng/ml plus various amounts of ExTek.6His protein or control protein ExFms.6His for 8 min. Cells were washed three times with ice-cold PBS plus vanadate at 1 mM and lysed in RIPA (50 mM Tris·Cl, pH 7.4/150 mM NaCl/1 mM EGTA/1% Nonidet P-40/0.25% Na deoxycholate) plus 1 mM of phenylmethylsulfonyl fluoride, 1 mg/ml of pepstatin, 1 mg/ml of leupeptin, and 1 mM vanadate. The cell lysate was centrifuged for 10 min to remove the cell debris and then incubated with Ab33 at 4°C overnight. After the incubation with protein G agarose beads for 1 hr, the beads were washed three times with TBST (10 mM Tris·Cl, pH 8.0/150 mM NaCl/0.1% Tween20). Proteins were eluted with sample buffer, separated by SDS/PAGE, transferred to nitrocellulose filter, and blotted with an antiphosphotyrosine antibody (PY-99, Santa Cruz Biotechnology). The same filter was stripped by using TBST pH 2.4 overnight at room temperature and reblotted with Ab33.

Tie2 Inhibition Assay. NIH-3T3 fibroblasts were stably transfected with an expression plasmid encoding a chimeric receptor consisting of the ectodomain and transmembrane

portion of Tie2 fused to the cytoplasmic kinase domain of TrkC. These cells are able to survive and proliferate under low-serum conditions in response to added Ang1*. To assess the ability of a soluble Tie2 to neutralize Ang1 activity, the Tie2/TrkC cells were incubated in serum-free medium that contained increasing concentrations of ExTek.6His and a constant amount (400 ng/ml) of Ang1*. The vital dye MTT was added to the cultures after 2 days, whereupon the cells were incubated further and then solubilized. The amount of MTT-derived blue product in the cell lysates, indicative of the number of live cells present at 2 days of culture, was determined spectrophotometrically. All values were corrected by subtracting the value of a culture with no added factors, and represent the mean ± SD of three replicate wells.

Construction of Recombinant AdExTek. A novel adenoviral vector system was used to construct AdPacβ-gal as previously described (35). AdPacβ-gal served as a control virus and was used to generate AdExTek by replacing the β-galactosidase gene with the murine ExTek cDNA. Briefly, the ExTek cDNA consisting of the mouse Tie2 extracellular domain fused to a "strep-tag" at its C terminus was subcloned into the *Bam*HI-*Nhe*I site of the transfer plasmid pGEM cytomegalovirus (CMV)/bovine growth hormone (BGH) poly(A) (35). The resulting plasmid, pGEM CMV ExTek/BGH poly(A), then was digested with *Xba*I and *Bst*BI, and a 3.6-kb plasmid fragment containing the CMV early promoter and enhancer, ExTek and BGH-poly(A) signal was directly ligated to the *Xba*I site of predigested Ad.Pacβ-gal viral DNA. The ligation mixture was purified by phenol-chloroform extraction and ethanol precipitation, and then transfected into 293 cells by using the calcium phosphate method. After observation of a cytopathic effect (7–10 days), the cells were lysed by multiple freeze/thaw cycles, and the recombinant virus (Ad-ExTek) was isolated by one round of plaque purification on 293 cells.

Propagation, Purification of Recombinant Adenoviruses, and Detection of ExTek Expression. Plaque-purified AdExTek or Ad.Pacβ-gal virus was used to generate high-titer virus stock by infecting 40 150-mm plates of confluent 293 cells at a multiplicity of infection (MOI) of 2 in DMEM plus 2% fetal bovine serum. The viruses were purified from the infected cell lysates as described previously (35). The virus was stored in virus storage buffer (20 mM Tris, pH 7.4/150 mM NaCl/5 mM KCl/1 mM MgCl₂) plus 1 mg/ml of mouse albumin (Sigma) and 10% glycerol and immediately frozen in aliquots at -80°C. Titters of viral stocks were determined by plaque assay on 293 cells by using standard techniques (35). ExTek expression was tested in 293 cells infected with AdExTek at MOI = 1. Twenty-five microliters of culture medium was harvested 3 days after viral infection, analyzed in SDS/PAGE, transferred to nitrocellulose membrane, and blotted with Ab33 followed by incubation with anti-mouse IgG-horseradish peroxidase conjugate, and the signal was detected by enhanced chemiluminescence (Amersham). Purified ExTek.6His protein expressed by baculovirus was used as a control (28).

Determination of ExTek Plasma Concentration. ExTek plasma concentration was determined by an ELISA assay. Briefly, AdExTek adenovirus (5×10^8 pfu) was administered i.v. into five BALB/c mice via the retro-orbital sinus. For plasma ExTek levels, a small amount of blood was collected from the tail vein into a heparinized micro capillary tube at days 0, 2, 4, 6, 8, and 11. Plasma was recovered after brief centrifugation to remove cells. Serial dilutions of the plasma in PBS were incubated in microtiter wells overnight at 4°C. The coated wells were blocked with 5% milk in TBST for 30 min. With three washes of TBST in between each step, a biotinylated Ab33 diluted in TBST (0.5 µg/ml) was incubated for 1 hr followed by incubation with 1:2,000 diluted streptavidin alkaline phosphatase conjugate (GIBCO/BRL) in TBST for 30 min. The phosphatase activity was determined by addition of

Sigma @104 phosphatase substrate, and absorbency at 405 nm was measured in V_{max} Kinetic Microplate Reader (Molecular Devices). The ExTek concentration in blood was calibrated by using purified recombinant ExTek in the ELISA assay.

Primary Tumor Growth. All animal protocols were approved by the Duke Institutional Animal Care and Use Committee. To determine the inhibitory effect of AdExTek on well established primary tumor growth, a murine mammary tumor cell line 4T1 or a murine melanoma cell line B16F10.9 was implanted into the left flank of female BALB/c or C57/BL mice, respectively (5×10^5 cells/mouse in 50 μ l of PBS). After the development of an easily palpable tumor (7–10 days, approximately 5 mm in diameter), either AdExTek virus or a control Ad.Pac β -gal virus (5×10^8 pfu/mouse in 100 μ l of PBS) was administered i.v. Tumor size then was measured by using a caliper every other day for the next 12 days. Tumor volume was calculated by using the following formula: tumor volume = $1/2(\text{width})^2 \times \text{length}$.

Tumor Metastasis. Cells (5×10^5) of either tumor cell line, 4T1 or B16F10.9, were mixed with either AdExTek virus (5×10^8 pfu) or an equal amount of Ad.Pac β -gal virus, and the mixture was administered i.v. into female BALB/c or C57/BL mice, respectively. Twenty to 25 days after injection, the animals were sacrificed, and the lungs were removed, weighed, and fixed in Bouin's solution. Lung weight increase was calculated by using the formula: lung weight increase (%) = (tumor lung weight – normal lung weight)/normal lung weight \times 100. Surface metastases then were counted by using a dissecting microscope by a technician who was blinded as to the experimental groups involved. The lungs then were decolorized in 70% ethanol several times before being embedded in paraffin. Serial sections of eight microns were cut and stained with hematoxylin/eosin for histologic examination.

Footpad Tumor Metastasis Model. 4T1 tumor cells (5×10^5) in 20 μ l of PBS were injected into the foot pad of female BALB/c mice. The primary tumors were removed by amputating the leg 3 weeks after tumor implantation when tumors had reached approximately 5 mm diameter. Immediately after surgery, 5×10^8 pfu of AdPac β -gal or AdExTek virus was administered i.v. The mice were sacrificed 25 days after amputation. The lungs were removed, weighed, and fixed in Bouin's solution. Surface metastases then were counted by using a dissecting microscope by a technician who was blinded as to the experimental groups involved.

Statistics. Results are reported as means \pm SE for each group. A two-tailed Student's *t* test was used to analyze statistical differences between control and AdExTek-treated groups. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Soluble Tie2 (ExTek) Blocks Ang1* and Ang2 Binding, Ang1-Mediated Tie2 Autophosphorylation, and Ang1-Mediated Cellular Responses. To explore the mechanism of soluble Tie2 inhibition of tumor angiogenesis, the ability of ExTek to block Ang1* and Ang2 binding to an immobilized rat Tie2-Fc fusion protein was tested by using a BIAcore device. As anticipated from previous studies (26, 27), ExTek blocked binding of both Tie2 ligands to the immobilized Tie2-Fc (Fig. 1 *A* and *B*). Although these analyses did not permit a precise determination of the binding affinity of ExTek for the Tie2 ligands, the IC_{50} for ExTek inhibition of Ang1 and Ang2 binding was determined to be 110 nM and 159 nM, respectively.

Consistent with its ability to block binding to endogenous Tie2, ExTek was able to block Ang1*-mediated Tie2 phosphorylation in cultured endothelial cells (Fig. 1*C*). Tie2 phosphorylation levels increased after stimulation with Ang1* at 150 ng/ml for 8 min. This increase in Tie2 phosphorylation was inhibited by addition of excess of ExTek protein, decreasing to

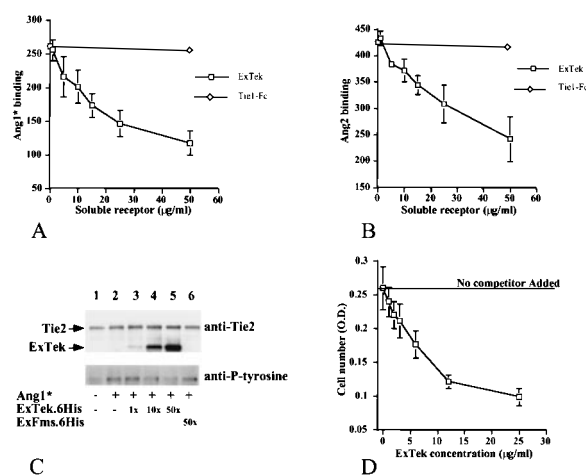


Fig. 1. ExTek.6His blocks Ang binding and Ang-stimulated cellular responses. ExTek.6His blocks binding of Ang1* (*A*) and Ang2 (*B*) to immobilized Tie2-Fc. Binding of Ang1* and Ang2 (800 ng/ml) to immobilized Tie2-Fc was done in the presence of increasing ExTek.6His concentrations ranging from 0 to 50 μ g/ml. The closely related Tie1-Fc (50 μ g/ml) was used to verify specificity of inhibition of ligand binding to the immobilized Tie2-Fc. Data are expressed as the means \pm SD of three replicate experiments. ExTek.6His blocks Tie2 phosphorylation induced by Ang1* in endothelial cells (*C*). EAhy926 cells were serum-starved overnight followed by stimulation with Ang1* (150 ng/ml) plus the indicated amount of ExTek.6His or control protein, ExFms.6His, for 8 min. Tie2 and Tie2 phosphorylation levels then were detected by immunoprecipitation/Western blot (anti-Tie2 and anti-P-tyrosine, respectively). ExTek.6His blocks Ang1*-mediated cell survival/proliferation (*D*). Tie2/TrkC expressing cells were incubated in serum-free medium that contained increasing concentrations of ExTek.6His and a constant amount of Ang1* (400 ng/ml). The vital dye MTT was added to the cultures after 2 days of treatment, and the amount of MTT-derived blue product in the cell lysates was determined spectrophotometrically and used as an index of live cells present. All values were corrected by subtracting the value of a culture with no added factors and represent the means \pm SD of three replicate wells.

baseline levels when a 50-fold molar excess of ExTek was used. No decrease in Tie2 phosphorylation was found when a 50-fold molar excess of a control protein ExFms.6His was used.

In addition to blocking Tie2 autophosphorylation, ExTek was able to block Ang1*-mediated cell survival/proliferation signals in NIH 3T3 fibroblast expressing a Tie2/TrkC chimeric receptor (Fig. 1*D*). Interestingly, the IC_{50} of ExTek for inhibition of Ang1-mediated cell growth and survival was somewhat lower (56 nM) than the IC_{50} for Ang1 binding in spite of the high level of Tie2/TrkC chimeric receptor expression in these cells and the higher concentrations of Ang1* used in the cell-based assay. Taken together, these data suggested that ExTek was a potent inhibitor of Tie2 activation and may be an effective therapeutic agent for cancer.

Development of a Recombinant Adenovirus for Gene Transfer of ExTek (AdExTek). Because therapeutic use of recombinant ExTek protein may be difficult and expensive, the efficacy of systemic ExTek delivery by gene transfer using a recombinant adenovirus, AdExTek, was tested. AdExTek was constructed as described above (see *Methods*) and used to infect 293 cells. Three days postinfection, Western blotting with a Tie2 mAb (Ab33) demonstrated easily detectable levels of ExTek protein in the culture media of the AdExTek-infected cells (Fig. 2*A*). Purified ExTek.6His protein expressed in baculovirus was used as a positive control. The gel mobility difference between baculovirus-expressed ExTek and adenovirus-expressed ExTek is likely caused by a different epitope tag (6His tag vs. Streptag, respectively) and/or differences in glycosylation.

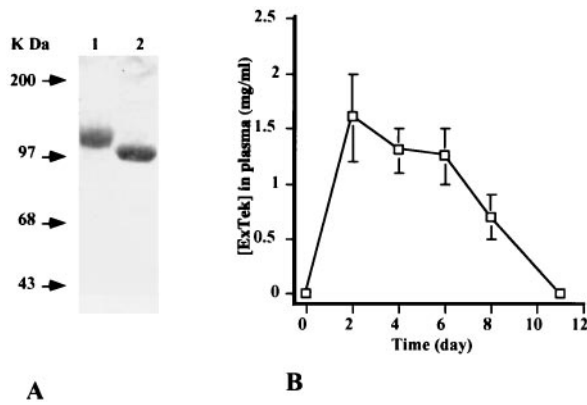


FIG. 2. AdExTek delivers ExTek serum levels in excess of levels required to block Ang binding and Ang-stimulated cellular responses. (A) ExTek expression from cultured 293 cells infected with AdExTek virus. Three days after AdExTek infection, 25 μ l of culture media was separated by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with anti-Tie2 Ab33 (lane 1). Purified ExTek.6His protein (100 ng) was used as a control (lane 2). (B) Plasma ExTek concentrations after systemic administration of AdExTek. AdExTek (5×10^8 pfu) was administered to mice i.v. ExTek concentration in plasma was determined by a simple ELISA as described in *Methods*. Data are plotted as the mean \pm SE of five mice. For some points error bars are smaller than the symbols.

The AdExTek virus (5×10^8 pfu) next was administered to BALB/c mice by i.v. injection, and plasma ExTek levels were measured on days 2, 4, 6, 8, and 11. The peak ExTek concentration was reached by 2 days after viral injection, was maintained for days 6–8, and thereafter fell rapidly to baseline by day 11 (Fig. 2B). Importantly, the peak concentrations of ExTek were in excess of 1 mg/ml, which was well above the concentrations required to inhibit Ang1* or Ang2 binding, Tie2 autophosphorylation, and Ang1*-mediated cellular responses.

AdExTek Inhibits the Growth of Two Well Established Primary Murine Tumors. To determine whether administration of AdExTek could inhibit the growth rate of well established primary tumors, two murine tumor cell lines, a mammary adenocarcinoma (4T1) and a melanoma (B16F10.9), were used. To produce primary tumors, tumor cells (5×10^5 in 50 ml of PBS) were implanted into the left flank of female BALB/c (4T1 cells) or female C57/BL6 mice (B16F10.9 cells). Tumors produced in this manner from either cell line expressed Tie2 by Western blot (data not shown). Mice with easily palpable primary tumors (approximately 5 mm in diameter) underwent i.v. administration of either AdExTek or Ad.Pac β -gal virus (5×10^8 pfu). Tumor size then was measured every other day for 12 days. In this experiment, administration of AdExTek significantly inhibited the growth rate of both tumors, compared with Ad.Pac β -gal virus-injected animals (Fig. 3). At day 12 after viral injection, AdExTek-treated mammary tumors were 64% smaller, and the melanoma tumors were 47% smaller than tumors in Ad.Pac β -gal virus-treated animals.

AdExTek Suppresses the Growth of Tumor Metastases. To determine whether blocking Tie2 action could suppress the growth of tumor metastases, 4T1 and B16F10.9 cells (5×10^5) were mixed with either AdExTek or Ad.Pac β -gal (5×10^8 pfu) and then coadministered by i.v. injection. By 25 days after injection, three of 15 mice injected with Ad.Pac β -gal and 4T1 tumor cells died from massive lung metastases, but all of the mice that received AdExTek were alive. The remaining animals were sacrificed, and the lungs were removed, weighed, and fixed in Bouin's solution. Gross examination revealed numerous lung surface metastases in Ad.Pac β -gal-treated mice coinjected with either 4T1 or B16F10.9 cells (Fig. 4, *Left*).

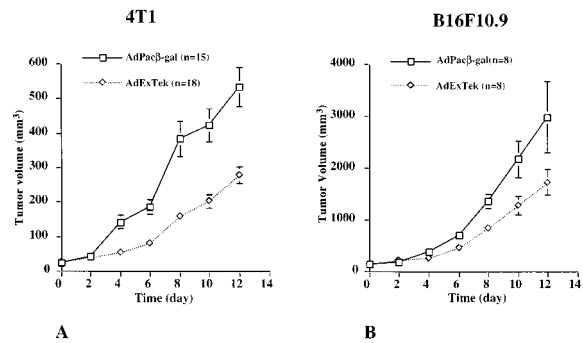


FIG. 3. AdExTek inhibits the growth of well established 4T1 (A) and B16F10.9 (B) primary tumors. A murine mammary tumor (4T1) and a murine melanoma (B16F10.9) were implanted into the right flank of BALB/c and C57/BL6 mice, respectively (5×10^5 cells/mouse). After a palpable tumor was achieved (day 0), the mice were randomly divided into two groups for each tumor type. One group of mice received i.v. Ad.Pac β -gal, and the other group mice received AdExTek. Tumor size then was measured every 2 days for 12 days. Data were plotted as the mean \pm SE. For some points error bars are smaller than symbols.

In contrast, there were few, if any, visible lung surface metastases in AdExTek-treated mice coinjected with either 4T1 or B16F10.9 cells (Fig. 4, *Left*). Comparison of tumor metastasis by counting the lung surface metastases (Fig. 5B and D) or by measuring increases in lung weight (Fig. 5A and C) confirmed that mice treated with Ad.Pac β -gal carried a significantly greater metastatic burden than mice treated with AdExTek.

Histologic examination of hematoxylin/eosin-stained lung sections revealed numerous large, well vascularized pulmonary metastases in lungs from Ad.Pac β -gal-treated mice harboring either 4T1 or B16F10.9 tumors (Fig. 4, *Right*). In contrast, only small, avascular metastases could be seen in lungs from AdExTek-treated mice harboring either 4T1 or B16F10.9 tumors. No difference in inflammatory infiltrates was noted in tumors from AdExTek- vs. Ad.Pac β -gal-treated animals. Furthermore, no difference in viability or proliferation was detected when cultured tumor cells were infected with AdExTek vs. Ad.Pac β -gal (data not shown).

To demonstrate that AdExTek also could inhibit the growth of spontaneous metastases, primary tumors were grown in the foot pad and surgically removed 3 weeks after implantation of 4T1 tumor cells. At the time of surgery, mice were divided into two groups to receive either Ad.Pac β -gal or AdExTek by i.v. injection ($n = 10$ for AdExTek group, $n = 7$ for Ad.Pac β -gal group). The lungs were harvested 25 days after amputation and weighed, and lung surface metastases were counted. A significant inhibition ($P < 0.05$) of tumor metastasis was achieved when animals were treated with AdExTek vs. Ad.Pac β -gal as assessed either by measuring lung weight or by counting lung surface metastases (Fig. 6). Two mice from each group did not develop any visible lung surface metastases. When sectioned, these mice had no lung metastases, suggesting failure of tumor metastasis in these animals.

DISCUSSION

Angiogenesis is required for the growth and metastasis of solid tumors. Establishment of the "angiogenesis dependence" of solid tumor progression suggested that inhibiting tumor angiogenesis should provide a practical approach to long-term control of the disease. Here, we demonstrated that a gene therapy strategy to deliver a specific antiangiogenic agent, ExTek, inhibited both the growth of well established primary tumors and vascularization and growth of tumor metastases.

Other soluble receptors such as soluble VEGF receptor (36, 37) and soluble platelet-derived growth factor receptor (38)

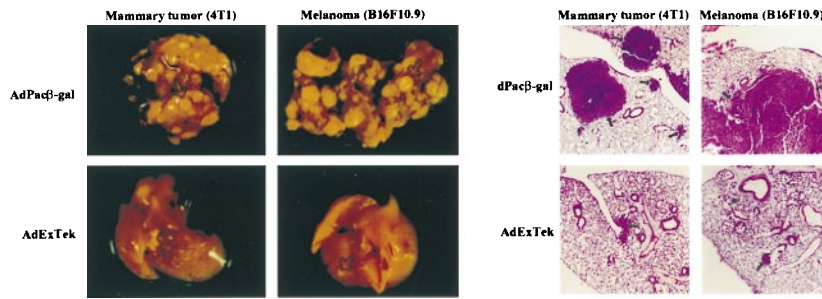


FIG. 4. AdExTek inhibits the growth of lung metastases after coadministration with tumor cells. (Left) AdExTek inhibits the appearance of grossly visible lung metastasis. Murine mammary tumor cells (4T1) or murine melanoma cells (B16F10.9) were mixed with AdExTek or Ad.Pacβ-gal and coadministered i.v. into BALB/c or C57/BL mice, respectively. Twenty to 25 days later, the mice were sacrificed, and the lungs were harvested, fixed in Bouin's solution, and photographed. Arrows indicate tumor metastases. (Right) Histologic comparison of metastases from mice treated with AdExTek or AdPacβ-gal. Hematoxylin/eosin-stained lung sections from mice with 4T1 tumors or B16F10.9 tumors. Magnification: ×40.

bind their cognate ligands with high affinity and function as competitive inhibitors, preventing receptor activation by competing with endogenous receptors for ligand binding. Recently, we have demonstrated that a soluble VEGFR-2 (ExFlk.6His) also functions as a “dominant-negative” inhibitor, preventing VEGF signaling by forming nonproductive heterodimers with endogenous cell surface VEGF receptors (39). A dominant negative mechanism of action for ExTek inhibition of Tie2 signaling is suggested by the coimmunoprecipitation of ExTek with the endogenous receptor (Fig. 1C). The ability of ExTek.6His to function as a dominant negative inhibitor also may explain why inhibition of Ang1*-mediated cell survival/proliferation required lower ExTek concentrations compared with inhibition of Ang1* binding.

Despite our promising results, there are limitations to the approaches used in this study. First, the adenoviral vector used in this study yielded only short-term ExTek expression, which limits its application for antiangiogenic therapy. However, there are new adenoviral vectors under development that promise to prolong protein expression for a few weeks to months (40, 41). In addition, other viral vectors such as lentivirus and adeno-associated virus also hold the promise of long-term expression (29, 42, 43). Another limitation of this study is that only partial tumor growth inhibition was achieved. This result indicates that blocking Tie2 activation may not be sufficient to completely block tumor angiogenesis. Because the VEGF pathway also has been shown to be critical for tumor angiogenesis, future work is directed toward testing the effi-

cacy of combining Tie2 pathway inhibitors with VEGF pathway inhibitors.

Although the present study suggests that Tie2 signaling is important for tumor angiogenesis and tumor growth, the precise step at which ExTek inhibits assembly of the tumor vasculature is not known. When the function of Tie2 was disrupted in transgenic mice, although the primitive patterning of the vasculature appeared normal, homozygous mice died *in utero*, secondary to profound abnormalities of vascular morphology characterized by a reduction in endothelial cell number and an apparent defect in the recruitment of perivascular pericytes and smooth muscle cells (24–27). These results suggested a role for Tie2 downstream of angiogenesis initiators such as fibroblast growth factor and VEGF in the stabilization, maturation, and subsequent maintenance of the neovasculature.

The recent discovery of an agonist and antagonist ligand for Tie2 (Ang1 and Ang2, respectively) further suggested that careful titration/regulation of Tie2 activation is required during vascular assembly (26, 27). Ang2 expression is highest during the early stages of angiogenesis, perhaps curtailing Tie2 activity to allow the established vasculature to respond to angiogenic stimuli. Subsequently, Ang2 expression is decreased and superseded by Ang1 expression, perhaps activating Tie2 and resulting in the stabilization and maturation of the neovessel (26). Failure of the switch from Ang2 to Ang1 expression, as occurs in atretic ovarian follicles, may trigger vascular regression secondary to the absence of a maintenance signal provided by Tie2 activation. Based on this concept of Tie2 action, it may be that ExTek mimics the action of Ang2,

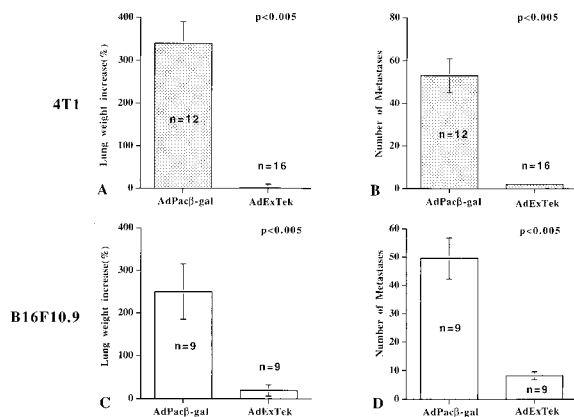


FIG. 5. AdExTek suppresses the growth of lung metastases. Mice were coinjected with adenovirus (AdExTek or AdPacβ-gal) and tumor cells (4T1 or B16F10.9) as described in the legend to Fig. 4. Growth of lung metastasis then was analyzed by measuring the lung weight for calculating lung weight increase as described in *Methods* (A and C) and by counting lung surface metastases (B and D). Data are plotted as mean ± SE.

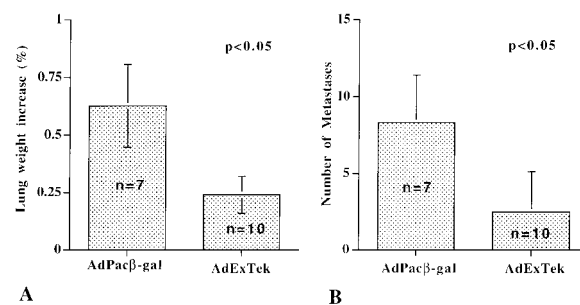


FIG. 6. AdExTek inhibits the growth of spontaneous lung metastases. Primary tumors (4T1) grown in the footpad were surgically removed after reaching 5 mm in diameter. At the same time, the animals were divided into two groups: one group was injected i.v. with control virus AdPacβ-gal ($n = 7$), and the other group was injected i.v. with AdExTek virus ($n = 10$). Animals were sacrificed 25 days after removal of the primary tumor. The lungs were weighed and fixed in Bouin's solution, and lung surface metastases were counted (B). Lung weight increase (A) was calculated as described in *Methods*. Data are plotted as mean ± SE.

preventing the activation of Tie2 and resulting in regression of nascent tumor vessels, thereby inhibiting tumor growth.

If the above scenario is true, then one might predict that blocking the Tie2 pathway would have dire consequences for the maintenance of the mature vasculature, especially in light of recent studies demonstrating the expression and activation of Tie2 in the endothelium of the quiescent adult vasculature (33). However, despite high levels of ExTek expression, animals treated with AdExTek appeared well, and histologic examination of the vasculature in normal tissues revealed no gross abnormalities (P.L. and K.G.P., unpublished data). These findings suggest three possibilities: (i) The growing tumor vasculature may be more susceptible to disruption of the Tie2 maturation/maintenance signals than mature, preformed vessels. (ii) Delivery of ExTek, a relatively large molecular weight inhibitor, may be augmented in the relatively leaky tumor vasculature compared with the normal vasculature. (iii) ExTek could block an as-yet-unidentified Ang2-mediated Tie2 signaling event that is required for the growth and assembly of neovessels but not for the maintenance of existing vessels. Clearly, realizing the full potential of therapeutic modulation of the Tie2/Ang pathway will require a greater understanding of the biology of this important pathway in both the normal and pathologic adult vasculature.

We thank Drs. Wolfgang Joklik, J. Dirk Iglehart, Jack Keene, and Gary Stiles at Duke University Medical Center for their comments on the manuscript. We also thank Dr. Donald Berry, Dr. Mark Olah, Jenifer Beaty, and Conrad Ireland for technical support and Katie Hunter for editorial assistance. This work was supported by the National Cancer Institute Special Program of Research Excellence (SPORE) in Breast Cancer at Duke University (CA-66228), an R01 from the National Heart, Lung, and Blood Institute (HL-54479), and a scholarship from the James McDonnell Foundation.

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