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Vascular Endothelial Growth Factor Chimeric Toxin Is Highly Active against Endothelial Cells

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ABSTRACT

Angiogenesis is a critical step in a benign tumor's evolution toward malignancy and metastasis. Tumor cells acquire such a phenotype by their ability to secrete angiogenic factors such as vascular endothelial growth factor (VEGF). VEGF receptors (VEGFRs) flt-1/VEGFR-1 and Flk-1/KDR/VEGFR-2 are restricted to activated endothelial cells, with the highest expression being in the tumor vasculature. The present study was undertaken to target the VEGFRs. Targeted toxins were developed by recombinant methods by fusing $VEGF_{165}$ or $VEGF_{121}$ to the diphtheria toxin (DT) translocation and enzymatic domain (DT_{390}-$VEGF_{165}$ or DT_{390}-$VEGF_{121}$). Both fusion proteins were found to be highly toxic to proliferating endothelial cells but not to vascular smooth muscle cells. The fusion protein is also active in Kaposi's sarcoma, a tumor type that expresses high levels of VEGFRs. These fusion proteins completely inhibit the basic fibroblast growth factor-induced growth of new blood vessels in the chick chorioallantoic membrane assay. Furthermore, the fusion toxin substantially retards the growth of Kaposi's sarcoma tumors in mice. Because nearly all tumors induce local angiogenesis with high VEGFR expression, VEGF-derived toxins may have wide application in cancer therapy.

INTRODUCTION

Tumor growth beyond a few millimeters and metastasis are dependent on the induction of angiogenesis mediated by the release of angiogenic factors secreted by the tumor cells (1). Angiogenesis is a complex process in which endothelial cells undergo activation, proliferation, and migration through the extracellular matrix by dissolution and remodeling, followed by tube formation (1, 2). Through concerted regulation, vascular smooth muscle cells then encase the newly formed blood vessels (3). VEGF[3] is among the major factors mediating tumor angiogenesis (4). Antibodies against VEGF are able to suppress tumor growth in nude mice (5). VEGF mRNA by alternate splicing results in proteins of 208, 189, 165, or 121 amino acids in length (6). $VEGF_{208}$ and $VEGF_{189}$ are secreted but remain bound to the extracellular matrix (7). $VEGF_{165}$ and $VEGF_{121}$ are secreted as soluble factors (7). $VEGF_{165}$ is the most abundantly expressed splice variant, and its binding to the cell surface receptor is induced by heparin sulfates, whereas $VEGF_{121}$ lacks a heparin binding domain (8, 9).

VEGF homodimers function by binding to two distinct cell surface receptor tyrosine kinases, flt-1/VEGFR-1 and KDR/flk-1/VEGFR-2 (referred to hereafter as VEGFR-1 and VEGFR-2), with the exception of $VEGF_{121}$, which binds selectively to VEGFR-2 (9–12). VEGF mitogenic activity appears to occur exclusively through VEGFR-2 (13). VEGFRs are expressed most abundantly in the tumor vasculature and less abundantly in the endothelium of resting blood vessels (10, 11). High levels of VEGFR expression in the tumor vasculature thus provide a unique opportunity for tumor targeting with agents that kill cells (4). Cytokines and antibodies conjugated with translocation and enzymatic domains of bacterial toxins have been studied to target various cell types (14). For example, IL-2 fusion toxins target certain T-cell neoplasms (15). Such toxins thus have a potential use in specific tumor types only. To direct therapy to a wide range of cancers, VEGF fused with the translocation and enzymatic domains of bacterial toxins may cause selective toxicity to the tumor vasculature.

We have chosen DT for fusion with VEGF. DT is secreted as a mature protein of 535 residues with a M_r of 58,342 (16). It is cleaved into two fragments, DTA fragment (residues 1–193) and DTB fragment (residues 194–535), by the action of proteolytic enzymes within a disulfide loop. The DTB fragment is responsible for binding to the cell surface and translocation of the DTA fragment into the cytosol. The DTA fragment ADP ribosylates a unique amino acid, diphthamide, present in elongation factor 2 to inhibit new protein synthesis in mammalian cells (17, 18). DT truncated at 389 lacks the eukaryotic cell binding domain and is nontoxic to human cells. Substitution of the binding domain with ligands to specific cell surface receptors can direct the toxin to the desired cells. For example, fusion with the IL-6 coding region directs toxicity to cells expressing IL-6 receptors (19, 20).

The present study was undertaken to determine the potential use of DT-VEGF fusion protein against activated endothelial cells in tumor angiogenesis. We used an *in vitro* and *in vivo* murine tumor model of KS, a tumor that is commonly seen in patients with HIV-1 infection. We and others have shown that KS cells express functional VEGFRs (21). This is the only tumor cell type shown to use VEGF as an autocrine growth factor. VEGF toxin was found to be highly active in KS cells (*in vitro* and *in vivo*) and is a potential therapeutic agent for a wide range of tumors.

MATERIALS AND METHODS

Reagents and General Procedures. Restriction endonucleases and DNA-modifying enzymes were purchased from Life Technologies, Inc. (Gaithersburg, MD), Boehringer Mannheim (Indianapolis, IN), Pharmacia Biotech (Piscataway, NJ), or New England Biolabs (Beverly, MA). *Escherichia coli* DH5α competent cells were purchased from Life Technologies, Inc. Low-melting point agarose (Sea Plaque) was obtained from FMC Corp. (Philadelphia, PA). Plasmid pGEX-KG was purchased from the American Type Culture Collection (Manassas, VA). Glutathione-Sepharose 4B was purchased from Pharmacia Biotech. MTT and thrombin were purchased from Sigma (St. Louis, MO). Oligonucleotides were synthesized on a PCR mate (Applied Biosystems, Foster City, CA). DNA fragments were amplified using Amplitaq DNA polymerase from Perkin Elmer Cetus (Norwalk, CT) on a thermal cycler with deoxynucleotides from Boehringer Mannheim. The PCR amplification involves melting the DNA strand at 94°C for 1 min, annealing at 55°C for 2.50 min, and amplification at 72°C for 3 min. After 30 cycles, a final amplification reaction was done at 72°C for 10 min.

Plasmid Construction. $VEGF_{165}$ or $VEGF_{121}$ fusion proteins containing 390 amino acids of DT with the enzymatic and translocation domains were produced as tripartite fusion proteins with GST in vector pGEX-KG (22). This

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; KS, Kaposi's sarcoma; bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; IL, interleukin; DT, diphtheria toxin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GST, glutathione *S*-transferase; HUVEC, human umbilical vein endothelial cell; AoSM, aortic smooth muscle.

vector contains a *tac* promoter for high-level expression followed by a GST gene, a sequence encoding a thrombin cleavage site, a linker to facilitate cleavage, and a multiple cloning site. The vector also contains the *LacI*^{q} gene encoding the *lac* repressor. Fusion proteins expressed after induction by isopropyl-1-thio-β-D-galactopyranoside were conveniently purified by adsorption to the glutathione affinity column.

DT_{390} sequences were amplified by PCR using primers R1 and R2 (Table 1) at residues 1 and 390 of DT that added an *Xba*I and a *Mlu*I site at the 5′ and 3′ ends, respectively. The DT template used was DTM1-E6-sFv-PE40 (23), which was kindly provided by Peter Nicholls (Food and Drug Administration, Bethesda, MD). Another PCR amplification using cDNA from KS cells was done with R5 and R6 primers that added *Mlu*I and *Xho*I sites at the 5′ and 3′ ends of the product for $VEGF_{165}$, respectively. A small fragment of $VEGF_{121}$ was amplified with the R5 and R13 primers using cDNA from KS cells as the template. The amplification product was gel-purified and used to amplify full-length $VEGF_{121}$ with primers R5 and R12 that added *Mlu I* and *Xho*I sites at the 5′ and 3′ ends of the product, respectively. This strategy adds Thr-Arg at the junction of the two domains for both of the fusion proteins. The amplified DNA fragments and pGEX-KG expression vector were digested with appropriate restriction enzymes. Vector DNA was dephosphorylated with bacterial alkaline phosphatase for 30 min. All three DNA fragments and vector pGEX-KG were purified by electrophoresis by running a low-melting point agarose gel. The three fragments were ligated overnight at 16°C, transformed into *E. coli* DH5α cells, and plated on Luria-Bertani agar containing 100 μg/ml ampicillin. Recombinant clones were verified by restriction enzyme digestion.

Expression and Purification of Fusion Protein. Fusion proteins DT_{390}-$VEGF_{165}$ and DT_{390}-$VEGF_{121}$ were expressed from pGEX-KG vector (23) in *E. coli* SG12036 (which was kindly provided by Susan Gottesman, National Cancer Institute, Bethesda, MD) that has mutations in the *gal, lon* and *sulA* genes (24). Cultures were grown separately in super broth (32 g of tryptone, 20 g of yeast extract, 5 g of NaCl, and 5 ml of 1 M NaOH/liter) to an A_{600} of 0.6–0.8; isopropyl-1-thio-β-D-galactopyranoside was added to the supernatant to a final concentration of 1 mM. The cultures were incubated for an additional 4 h, and cells were harvested by centrifugation. Bacterial cells were resuspended in 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl with 1% Triton X-100, 2 mM EDTA, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml AEBSF, and 750 μg/ml benzamidine hydrochloride and disrupted by sonication in a heat systems sonicator operated at 50% of maximum power. Extracts were centrifuged at 20,000 $\times$ *g* for 30 min at 4°C to remove unbroken cells and debris. The supernatants were loaded on glutathione-Sepharose 4B columns, equilibrated with 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl with 1% Triton X-100. After the supernatant was loaded, the column was extensively washed with 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl. The fusion proteins were eluted with 10 mM glutathione in the same buffer and concentrated on Centriprep-30 (Amicon, Danvers, MA). The fusion proteins were cleaved with thrombin (4 μg/ml) for 2 h in buffer containing 3 mM $CaCl_2$. The samples were passed again on the glutathione column to remove the GST domain, and purified proteins were analyzed on SDS gels (23). Western blot analysis was also performed for the recombinant proteins to show the reactivity with specific antibodies.

Cell Culture Methods. KS Y-1 and AoSM cells and HUVECs were grown in their respective media and plated at a density of 10,000 cells/ml in 24-well gelatin-coated plates on day 0. The cells were treated with various concentrations of DT_{390}-$VEGF_{165}$ and DT_{390}-$VEGF_{121}$ fusion proteins in fresh medium. After 72 h of incubation, cells were either counted in a Coulter counter or treated with MTT at a final concentration of 0.5 mg/ml. The cells treated with MTT were dissolved in solution containing 90% isopropanol, 0.5% SDS, and 40 mM HCl, and the color developed was read in an ELISA reader at 490 nm (Ref. 21; Molecular Devices Corp., Sunnyvale, CA).

Table 1 *Primers for amplifying DT and VEGF fragments*

Primer	Restriction enzyme	Sequence of the primer (5′ to 3′)
R1	*Xba*I	T CTA GAT CTA GAC GGC GCT GAT GAT GTT GTT GAT TCT TC
R2	*Mlu*I	ACG CGT ACG CGT AAG AAA TGG TTG CGT TTT ATG CCC
R5	*Mlu*I	ACG CGT ACG CGT ATG AAC TTT CTG CTG TCT TGG GTG
R6	*Xho*I	CTC GAG CTC GAG TCA CCG CCT CGG CTT GTC ACA TTT
R12	*Xho*I	CTC GAG CTC GAG TCA CCG CCT CGG CTT GTC ACA ATT TTG TTG TCT TGC TCT ATC TTT CTT
R13		ATT TTC TTG TCT TGC TCT ATC TTT

Chick CAM Assay. The CAM assay has been extensively used to study angiogenesis (25). On 10-day-old embryos, 0.01, 0.05, 0.1, or 1 μg of either DT_{390}-$VEGF_{165}$ or DT_{390}-$VEGF_{121}$ was introduced on a filter disc with 200 ng of bFGF. For control, the vehicle alone was added on the filter disc, and for positive control, 200 ng of bFGF were used. After 72 h, CAMs under the filter paper were harvested, washed with PBS, and evaluated by three independent observers for evidence of angiogenesis. In addition, branching blood vessels were counted under the stereomicroscope (Olympus SZH10).

Cytotoxicity Assay: Inhibition of Protein Synthesis. KS Y-1 cells (5×10^3 cells/well) were plated on gelatin-coated 48-well plates. Fibroblasts (T1) and B lymphoma cells (23-2) were seeded at a density of 5×10^3 cells/well in 48-well plates. DT_{390}-$VEGF_{165}$ was diluted to various concentrations ranging from 0.1 to 100 ng/ml with the appropriate culture medium. After a 20-h incubation at 37°C in a 5% CO_2 atmosphere, the medium was replaced with 0.5 ml of leucine-free medium containing 1.0 μCi/ml [^{14}C]leucine (325 mCi/mmol; DuPont New England Nuclear, Boston, MA) and 2 mM glutamine and incubated for 2 h. The medium was removed, and the cells were washed and lysed by the addition of 75 μl of 4 M KOH over a 10-min period. The proteins were precipitated by the addition of 10% trichloroacetic acid, and insoluble material was collected on glass fibers using a cell harvester (Micromate 196; Packard Instruments, Downers Grove, IL). Filters were washed with 5% trichloroacetic acid, dried, and counted in a Beckman (Fullerton, CA) liquid scintillation counter. All assays were performed in quadruplicate.

***In Vivo* Studies in Immunodeficient Mice.** Mice were divided into three different groups of four mice each. KS SLK cells (4×10^6 cells/100 μl) were injected s.c. into the lower back of 5-week-old BALB/c Nu^+/nu^+ athymic mice (21). After 7 days of tumor development, control mice were injected with PBS. Other groups of mice were injected with either 20 μg/kg or 200 μg/kg DT_{390}-$VEGF_{121}$ i.p. on the 8th and 10th day of the experiment. The tumor growth in mice was measured three times/week. Mice were sacrificed after the 19th day of tumor measurement.

RESULTS

Plasmid Construction. The VEGF sequence required for binding to the receptor was genetically fused to the DTA chain and the part of the DTB chain that lacks receptor binding domain. The fusion gene was constructed in the pGEX-KG vector, resulting in a three-fragment chimeric protein. The addition of linker and the alteration of a base after the *Xba*I site before the 5′ end of the $DT1_{390}$ gene fragment resulted in a modified 14-residue spacer (GSPGISGGGGGILE) between GST and the required protein (Fig. 1). The addition of a *Mlu*I site to the 3′ end of DT and the 5′ end of VEGF added two additional residues, TR, between the DT and VEGF domains.

Expression and Purification of Fusion Protein. The recombinant proteins DT_{390}-$VEGF_{165}$ and DT_{390}-$VEGF_{121}$ were expressed as GST fusions purified by affinity chromatography, and the GST domain was cleaved by thrombin. One liter of the culture typically yielded 400 μg of DT_{390}-$VEGF_{165}$ and 1 mg of DT_{390}-$VEGF_{121}$ purified proteins. Electrophoresis on SDS gels showed their respective M_rs of 63,000 and 55,000. Specificity of the cleaved fusion proteins was confirmed with rabbit antiserum to DT in Western blots (Fig. 2).

Inhibition of Cell Proliferation by the DT-VEGF Fusion Protein. The toxicity of DT_{390}-$VEGF_{165}$ and DT_{390}-$VEGF_{121}$ was measured in HUVECs, KS cell line (KS Y-1), and AoSM cells. HUVECs and KS Y-1 cells express both VEGFR-2 and VEGFR-1 on their surface, and AoSM cells have either no or very low level receptor expression. DT_{390}-$VEGF_{165}$ was toxic to both HUVECs and KS Y-1 cells (Fig. 3*A*). The EC_{50} was 0.6 (10 pM) and 1.4 ng (22 pM) for HUVECs and KS Y-1 cells, respectively. The lower toxicity to KS cell lines may be due in part to the engagement of VEGFRs by endogenously produced VEGF (21). In a similar manner, DT_{390}-$VEGF_{121}$ was toxic to the same cell types, *i.e.*, HUVECs and KS Y-1

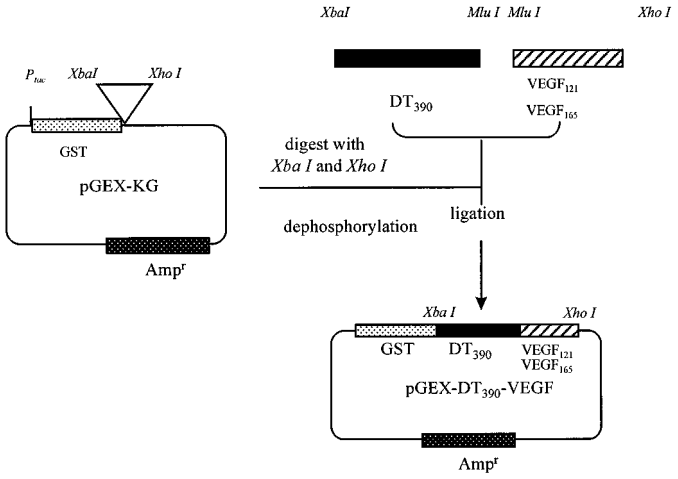

Fig. 1. Schematic construction of the plasmid for the expression of DT_{390}-$VEGF_{165}$ or DT_{390}-$VEGF_{121}$ fusion protein in *E. coli*. *GST*, a M_r 26,000 GST domain from the pGEX-KG vector; *DT_{390}*, the coding sequence of residues 1–390 of mature DT; *$VEGF_{165}$* or *$VEGF_{121}$*, the coding sequence of VEGF. Both fusion proteins contain two non-native amino acids, Thr-Arg (TR), between the DT and VEGF domains. The recombinant fusion proteins made from the pGEX-KG vector contain 14 amino acids (GSPGISGGGGGILE) added at the amino terminus of the fusion protein that are obtained from the expanded linker.

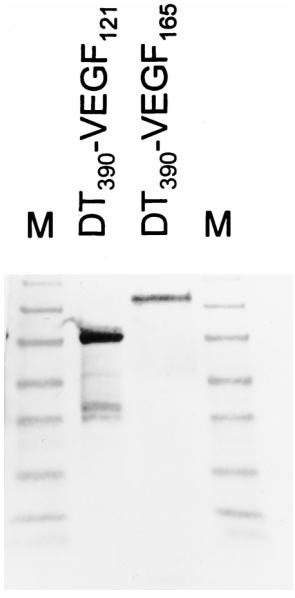

Fig. 2. Western blot analysis of the recombinant protein against DT antibody raised in goats. Horseradish peroxidase-conjugated antigoat antibody was used as a second antibody. *Lane M*, molecular weight markers (Novex, San Diego, CA).

cells but not AoSM cells (Fig. 3*B*). The EC_{50} for DT_{390}-$VEGF_{121}$ was 2 (36 pM) and 5 ng (91 pM) for HUVECs and KS Y-1 cells, respectively. The low toxicity of DT_{390}-$VEGF_{121}$ may be because $VEGF_{121}$ selectively binds to VEGFR-2 (9). Lack of fusion protein toxicity to vascular smooth muscle cells is also consistent with little or no VEGFR expression.

Inhibition of Protein Synthesis by DT-VEGF Fusion Protein. *De novo* protein synthesis in the presence of increasing amounts of DT_{390}-$VEGF_{165}$ was assayed by the incorporation of [^{14}C]leucine. Protein synthesis was inhibited in KS Y-1 cells, which express VEGFR-2 and VEGFR-1, but not in fibroblast (T1) or B-cell lymphoma (23-2), which both lack these receptors (Fig. 3*C*).

DT-VEGF Fusion Protein Inhibits Angiogenesis. To determine whether DT_{390}-$VEGF_{165}$ or DT_{390}-$VEGF_{121}$ inhibits the formation of new blood vessels, CAM assays were done. The data shows that bFGF induced vascular sprouting in the CAMs (Table 2). This increase in vascularization was completely inhibited by both fusion proteins at a dose level of 0.1 μg and above. The lower concentration of 0.05 μg/disc only blocked new blood vessel formation for DT_{390}-$VEGF_{165}$ fusion protein without any toxicity to the existing vessels (Fig. 4).

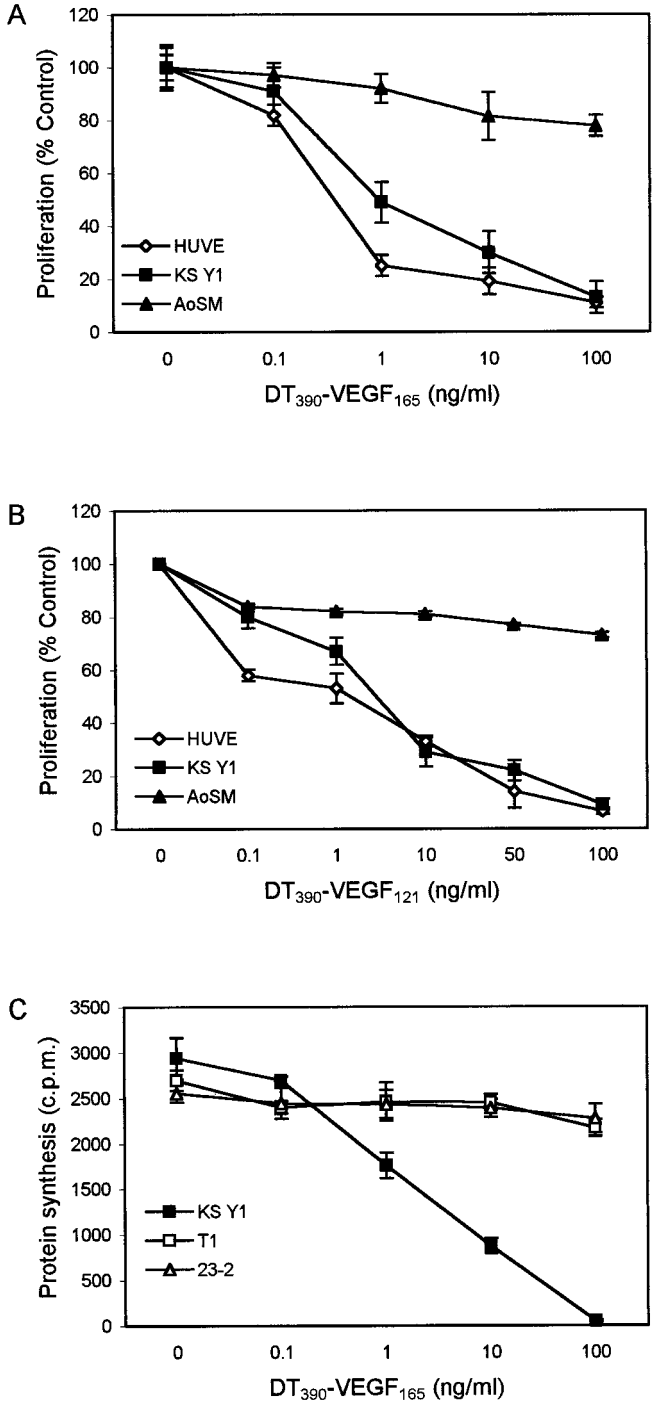

Fig. 3. Inhibition of cell proliferation by (*A*) DT_{390}-$VEGF_{165}$ or (*B*) DT_{390}-$VEGF_{121}$. HUVECs, KS Y-1 cells, or AoSM cells were plated in 24-well tissue culture plates. The next day, fusion toxin was added in different doses. After 72 h, the cells were incubated with 0.5 mg/ml MTT for 2 h. The MTT-stained cells were lysed in isopropanol solution and read in an ELISA reader. *C*, effect of DT_{390}-$VEGF_{165}$ on protein synthesis in KS Y-1 cells, fibroblasts (T1 cells), and B-cell lymphoma (23-2 cells). Cells were plated and treated as described in *A*. The amount of [^{14}C]leucine incorporated into cells in a 2-h period after a 20-h incubation with fusion toxin is shown. Results are the mean ± SD of two experiments performed in quadruplicate.

Table 2 *Angiogenic effect of fusion protein on CAMs*

Sample	bFGF (0.2 μg)	Dose (μg)	Blood vessels (mean ± SE)
Control	−	PBS	72 ± 20
Positive	+	0.0	292 ± 32
DT_{390}-$VEGF_{165}$	+	0.01	166 ± 23
DT_{390}-$VEGF_{165}$	+	0.05	78 ± 15
DT_{390}-$VEGF_{165}$	+	0.10	50 ± 18
DT_{390}-$VEGF_{165}$	+	1.00	48 ± 15
DT_{390}-$VEGF_{121}$	+	0.01	286 ± 24
DT_{390}-$VEGF_{121}$	+	0.05	200 ± 30
DT_{390}-$VEGF_{121}$	+	0.10	90 ± 21
DT_{390}-$VEGF_{121}$	+	1.00	60 ± 12

DT-VEGF Fusion Toxicity Is VEGFR Dependent. Specificity of the mode of action of DT_{390}-$VEGF_{165}$ was demonstrated by antibodies to VEGF and its mitogenic signal-transducing receptor, VEGFR-2. Preincubation of the DT-VEGF fusion protein with either VEGF polyclonal antibody (R & D Systems, Minneapolis, MN) or VEGF monoclonal antibody (Sigma) almost completely inhibited the activity of the fusion protein (Fig. 5*A*). This verifies that the VEGF moiety of the fusion protein is necessary for toxicity. As a control, preincubation with a nonspecific isotype antibody (mouse IgG2b) had no effect on the DT_{390}-$VEGF_{165}$ activity (Fig. 5*A*). To further confirm the specificity of targeting of the DT-VEGF fusion toxin, we preincubated KS Y-1 cells with antibodies to VEGFR-2. VEGFR-2 antibody blocked toxin-mediated toxicity (Fig. 5*B*), whereas nonspecific isotype antibody had no effect (Fig. 5*A*). These two results indicate that DT-VEGF fusion proteins act through a VEGFR-2-mediated mechanism, which requires the VEGF moiety of the fusion protein.

Inhibition of Tumor Growth *in Vivo* by Recombinant Fusion Protein. To study the effect of the fusion protein *in vivo*, KS SLK cells were implanted in immunodeficient mice, and tumors were allowed to grow for 1 week. DT_{390}-$VEGF_{121}$ was given i.p. on days 8 and 10 at a dosage of 20 or 200 μg/kg body weight. Tumor growth was inhibited in mice receiving either of the fusion toxin doses when compared to control mice treated with vehicle alone (Fig. 6). Higher doses of the toxin induced tumor response with a marked reduction in the tumor volume. In all cases, there was no detectable tumor at the dose of 200 μg/kg body weight. Thus, the toxin specifically targets tumor growth after systemic administration.

DISCUSSION

The presence of restricted cell surface receptor tyrosine kinases in the tumor vasculature/activated endothelium at high levels provides a unique opportunity to target the delivery of desired molecules (2, 15, 25). VEGFR-1 and VEGFR-2 represent such surface molecules. We have developed toxin conjugates to target activated endothelial cells and endothelial-derived neoplastic cells. To accomplish this goal, we used the VEGF coding regions for either the 165- or 121-amino acid forms to direct the delivery of DT, which lacks receptor binding domain. This truncated toxin is nontoxic to the cells but retains the functions of translocation and protein synthesis inhibition when delivered with targeting molecules (19).

The resulting chimeric toxins DT_{390}-$VEGF_{165}$ and DT_{390}-$VEGF_{121}$ were highly toxic to the cells expressing VEGFRs. DT_{390}-$VEGF_{165}$ was 3–4-fold more toxic to endothelial cells and KS cells compared to DT_{390}-$VEGF_{121}$. The specificity of the fusion proteins was also demonstrable by the lack of toxicity to human AoSM cells, fibroblasts (T1), and B-cell lymphoma (23-2), which do not express VEGFRs. By individually blocking the VEGF moiety and VEGFR with their respective antibodies, we showed that the DT-VEGF fusion proteins are dependent on both the ligand moiety and the receptor. DTA fragment (residues 1–193) inhibits protein synthesis by ADP ribosylation of diphthamide, an amino acid present on elongation factor 2 (17, 18). We showed that the *de novo* synthesis of protein was inhibited in cells with VEGFR-2 (KS Y-1), but cells lacking this receptor showed no decrease in protein synthesis in the presence of DT_{390}-$VEGF_{121}$. The two other possibilities for DT-VEGF binding VEGFR-2 are not consistent with these results. It is possible that DT-VEGF could bind VEGFR-2 and act as either a receptor agonist or an antagonist. In the first case, protein synthesis would increase as the mitogenic signal stimulated cell growth. In the second case, the VEGF autocrine loop, which functions in these cells, would be interrupted, leading to apoptosis. This would also result in a decrease in protein synthesis, but after a prolonged exposure.

The *in vivo* activity of these fusion toxins was also demonstrated in CAM assays and in the murine model of KS. CAM assays demonstrated that both of these fusion toxins inhibit bFGF-induced neovas-

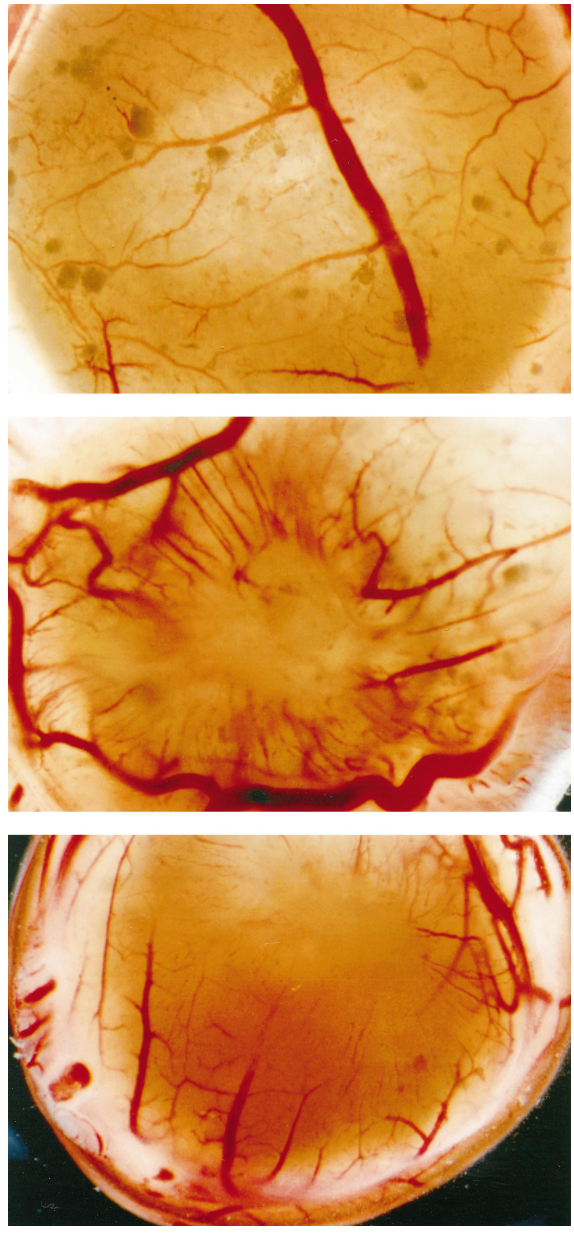

Fig. 4. Chicken CAM assay of angiogenesis. Representative CAMs from 10-day-old chick embryos treated for 48 h as described below are depicted. *Top*, control (PBS). *Middle*, the induction of new branching with 200 ng of bFGF. *Bottom*, DT_{390}-$VEGF_{121}$ fusion protein (100 ng/disc) abrogates the angiogenic effect of bFGF.

cularization *in ovo*. In the mouse, the fusion toxin dose of 200 μg/kg injected i.p. beginning a week after tumor implant and given twice was highly effective in inducing KS tumor response and inhibiting tumor progression. DT_{390}-$VEGF_{165}$ is more active *in vitro*; thus, it is likely to be more effective.

Ramakrishnan *et al.* (26) chemically conjugated $VEGF_{165}$ to DT 385 residues with activity shown in murine hemangioma cell lines representing another endothelial cell tumor (26). The activity was significantly lower, with an EC_{50} of 25 nM compared to an EC_{50} of 10–40 pM with our recombinant fusion toxins. This may be because chemically conjugated toxin competes poorly with native VEGF. Alternatively, the chemical conjugation may reduce the function of the DT. Most of the conjugates constructed with DT use the amino-terminal 389 residues (15), but Ramakrishnan *et al.* (26) have used 385 residues, which may also alter the function of DT. Furthermore, they show that VEGF-conjugated DT385 reduces tumor neovascularization in mice when a 10-μg dose is given for 22 consecutive days (27). In contrast, the present study shows that two doses of the fusion toxin at 20–200 μg/kg body weight (*i.e.*, two doses of 5 μg each) were sufficient to result in *in vivo* activity in a mouse model.

Whereas these studies show a potential use of fusion protein in a variety of disorders in which angiogenesis plays a role, certain limitations are inherent to the use of bacterial proteins due to the generation of antibodies (28). Thus, the repeated use of these fusion toxins may be limited (29). Monitoring the development of neutralizing antibodies would be required during the clinical development of these fusion toxins.

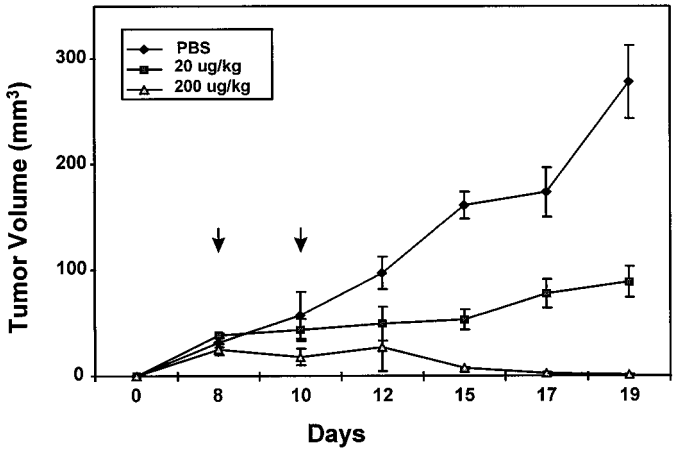

Fig. 6. *In vivo* studies in tumor-deficient mice. Mice were injected with 4×10^6 cells s.c. After 1 week of tumor growth, mice were injected with either PBS or DT_{390}-$VEGF_{121}$ (20 or 200 μg/kg body weight) fusion toxin i.p. on the 8th and 10th days of the experiment, as indicated by the *arrow*. Tumor growth was measured three times/week.

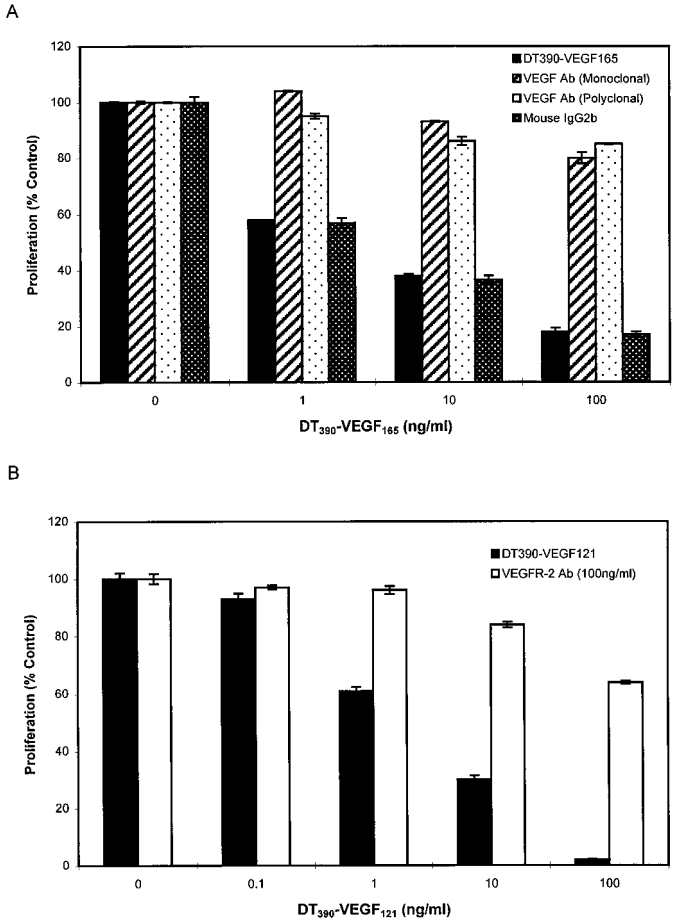

Fig. 5. DT-VEGF fusion toxin acts specifically through the VEGFR. *A*, the effect of the preincubation of increasing concentrations of DT_{390}-$VEGF_{165}$ with polyclonal (R & D Systems) or monoclonal antibody (Sigma) against VEGF. Mouse IgG2b is an isotype-nonspecific antibody and serves as a negative control. All three antibodies were used at 1 μg/ml. *B*, the effect of incubating cells with 100 ng/ml VEGFR-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) before the addition of DT_{390}-$VEGF_{121}$ fusion toxin in increasing amounts. KS Y-1 cell proliferation was assayed as described in the Fig. 3 legend. Results are the mean ± SD of four experiments.

In conclusion, we have developed fusion toxins that target VEGFRs with therapeutic potential in tumors, because angiogenesis is a critical component of tumor growth and metastasis. Strategies to reduce the antigenic potential of these toxins with the concurrent use of modulators of humoral response may be advantageous.

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