Inhibition of Tumor Growth by Targeting Tumor Endothelium Using a Soluble Vascular Endothelial Growth Factor Receptor

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Abstract

Vascular endothelial growth factor (VEGF) is a leading candidate for an endogenous mediator of tumor angiogenesis. Recently, two endothelial cell surface receptors, flk-1 and fli-1, have been shown to mediate the angiogenic activities of VEGF. In this study, we have evaluated whether a soluble receptor could suppress tumor angiogenesis and thereby inhibit tumor growth. A soluble VEGF receptor was constructed by fusing the entire extracellular domain of murine flk-1 to a six-histidine tag at the COOH terminus (ExFlk.6His). In vitro, recombinant ExFlk.6His protein bound VEGF with high affinity (Kₐ = 16 nM) and blocked receptor activation in a dose-dependent manner and inhibited VEGF-induced endothelial cell proliferation and migration. ExFlk.6His bound to endothelial cells only in the presence of VEGF, and cell surface cross-linking yielded a high molecular weight complex consistent with the VEGF-mediated formation of a heterodimer between ExFlk.6His and the endogenous VEGF receptor. In vivo, ExFlk.6His potently inhibited corneal neovascularization induced by conditioned media from a rat mammary carcinoma cell line (R3230AC). Moreover, when ExFlk.6His protein was administered into a cutaneous tumor window chamber concomitantly with R3230AC carcinoma transplants, tumor growth was inhibited by 75% (P < 0.005) and vascular density was reduced by 50% (P < 0.002) compared with control-treated tumors. These results demonstrate the potential of ExFlk.6His to inhibit VEGF action by a potent "dominant-negative" mechanism and suggest that targeting VEGF action using a soluble receptor may be an effective antiangiogenic therapy for cancer and other "angiogenic" diseases.

Introduction

Over the past several decades, a variety of findings have suggested a link between tumor angiogenesis and tumor progression (1–5). Increased vascularization may allow for both an increase in tumor growth and a greater likelihood of hematogenous tumor embolization. Thus, it has been hypothesized that inhibition of tumor angiogenesis will block tumor growth and decrease the potential for tumor metastasis. This hypothesis has led to an intensive effort to identify the molecular mechanisms of tumor angiogenesis to serve as the basis for novel anticancer therapy.

VEGF³ is a leading candidate for an endogenous mediator of angiogenesis (6, 7). VEGF induces vascular growth in vivo and promotes endothelial cell responses thought to be important for angiogenesis such as proliferation, migration, and protease production (6–8). The angiogenic actions of VEGF are mediated via two closely related endothelium-specific receptor tyrosine kinases, flk-1 and fit-1 (9–11). Disruption of either flk-1 or fit-1 in transgenic mice resulted in early embryonic lethality due to profound abnormalities of the embryonic vasculature (12, 13). Taken together, these data establish a role for VEGF in normal vascular growth and development.

Other findings indicate that VEGF and its receptors also play important roles in pathological vascular growth such as tumor angiogenesis. Recently, both VEGF and its receptors have been found to be expressed in a variety of tumors (14–20). Moreover, forced expression of VEGF in a human breast carcinoma cell line dramatically increased its tumorigenicity, apparently by increasing the tumor’s vascularity (21). Conversely, disrupting VEGF action in experimental models of tumorigenesis potently inhibits tumor growth (22, 23). These studies indicate that therapeutic agents designed to block VEGF receptor activation should provide effective cancer therapy. Here, we used a recombinant, soluble form of flk-1 (ExFlk.6His) to explore the mechanism of action of soluble VEGF receptors in vitro and to test the ability of soluble VEGF receptors to block tumor angiogenesis in vivo.

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; NTA, nitrilo-tri-acetic acid; HUVEC, human umbilical vein endothelial cell; EBM, endothelial cell basal medium.
**Results**

**Design and Production of A Recombinant Soluble VEGF Receptor (ExFlk.6His).** To generate a soluble VEGF receptor, a baculovirus vector (BvExFlk.6His) was generated directed the expression of a fusion protein consisting of the entire extracellular domain of the murine VEGF receptor, flk-1, fused to a six histidine-tag at the COOH terminus (Fig. 1A). The six-histidine tag was chosen because it is relatively small, it has little effect on the conformation of proteins to which it is fused, it is relatively nonimmunogenic, and it allows one-step purification under non-denaturing conditions (24–26). ExFlk.6His protein was purified from the supernatant of SF9 cells infected with BvExFlk.6His virus by one-step Ni2+ NTA resin chromatography (see "Materials and Methods"). As controls, ExFms.6His was purified from the supernatant of SF9 cells infected with a baculovirus vector directing the expression of a fusion between the extracellular domain of the CSF-1 receptor, c-fms, fused to a six-histidine tag. A mock control was purified from uninfected SF9 cells using the same protocol. By this approach, ExFlk.6His and ExFms.6His proteins were purified to near homogeneity, yielding a single major band with the expected molecular masses of approximately 105 and 70 kDa, respectively (Fig. 1B).

**ExFlk.6His Protein Binds VEGF, Blocks the Activation of Endothelial VEGF Receptor, and Neutralizes VEGF-stimulated Endothelial Proliferation and Migration in Vitro.** To determine the binding affinity of ExFlk.6His with VEGF, binding assays were done by adding 125I-labeled ExFlk.6His to microtiter wells precoated with human recombinant VEGF as described (see "Materials and Methods"). Bound 125I-labeled ExFlk.6His was then detected by counting the washed wells in a gamma counter. Under the conditions of this assay, ExFlk.6His demonstrated high affinity ($K_d$, 16 nM) saturable VEGF binding (Fig. 2A). This binding was specifically competed by the addition of cold ExFlk.6His, and no competition of binding was observed with excess cold ExFms.6His (data not shown).

The ability of ExFlk.6His to block activation of VEGF receptors on cultured endothelial cells was tested. Stimulation of endothelial cells (ECRF) with VEGF produced easily detectable autophosphorylation of endogenous Flk-1 (Fig. 2B). This high-level phosphorylation of Flk-1 was significantly blocked by the addition of ExFlk.6His in a concentration-dependent manner. Even in the presence of a saturating concentration of VEGF, a 3-fold molar excess of ExFlk.6His resulted in substantial inhibition of Flk-1 activation. With the addition of a 12-fold molar excess of ExFlk.6His, the receptor phosphorylation level was reduced almost to baseline. This result clearly demonstrated the ability of ExFlk.6His to block activation of endogenous VEGF receptors.

Next, the ability of ExFlk.6His to block VEGF-stimulated mitogenesis and migration of cultured HUVECs was tested. Stimulation of HUVECs with VEGF (10 ng/ml) produced a characteristic 2–3-fold increase in [3H]thymidine incorporation 24 h after stimulation ($P < 0.005$) compared with unstimulated cells (Fig. 2C). This VEGF-stimulated mitogenic activity was completely blocked by simultaneous addition of ExFlk.6His protein (2.5 μg/ml) but was not blocked by ExFms.6His at the same concentration ($P < 0.0005$). Similarly, HUVEC migration rate in a modified Boyden chamber assay increased approximately 3-fold by the addition of...
VEGF at 10 ng/ml ($P < 0.0005$), and this increase in migration was reduced to background levels after preincubation of VEGF with ExFlk.6His (2.5 μg/ml) but not with ExFms.6His at the same concentration ($P < 0.0005$; Fig. 2D). These data demonstrate that ExFlk.6His could specifically bind VEGF and inhibit VEGF-mediated endothelial cell responses.

**ExFlk.6His Forms a VEGF-dependent Heterodimer with Endogenous VEGF Receptors on the Surface of Cultured Endothelial Cells.** To gain insight into the mechanism of ExFlk.6His inhibition of VEGF-mediated endothelial responses, $^{125}$I-labeled ExFlk.6His was mixed with VEGF to form a $^{125}$I-labeled ExFlk.6His/VEGF complex. This preformed complex was then tested for its ability to bind to VEGF receptors on the surface of cultured endothelial cells (Fig. 3A). In these experiments, the binding of $^{125}$I-labeled ExFlk.6His to the endothelial cell surface could only be detected in the presence of VEGF; no $^{125}$I-labeled ExFlk.6His binding was detected in the absence of VEGF or in the presence of FGF. Excess unlabeled ExFlk.6His (30×) totally competed off this binding. When $^{125}$I-labeled ExFlk.6His was
covalently cross-linked to endothelial cells in the presence of VEGF, a large molecular mass complex was revealed (Fig. 3B, Lane 2). No complex formation was detected in the absence of VEGF (Fig. 3B, Lane 1) or in the presence of FGF (Fig. 3B, Lane 4), and excess, unlabeled ExFlk.6His competitively blocked complex formation (Fig. 3B, Lane 3). These data are consistent with the formation of a VEGF-mediated heterodimer between 125I-labeled ExFlk.6His and endogenous VEGF receptors. To further confirm that the complex indeed contained the cell surface receptor, the cross-linked complex was immunoprecipitated with an antibody against the c-tail of Flk-1 (antibody 1158; Santa Cruz Biotechnology) and analyzed by SDS-PAGE and autoradiography (Fig. 3C). Again, consistent with the formation of VEGF-mediated receptor heterodimer, a single high molecular complex containing both the endogenous cell surface receptor and the radiolabeled soluble receptor was immunoprecipitated only in the presence of VEGF. The formation of such a heterodimeric complex between ExFlk.6His and cell surface VEGF receptors indicates that ExFlk.6His could function as a "dominant-negative" inhibitor of VEGF receptor activation and should be a potent inhibitor of angiogenesis in vivo.

**ExFlk.6His Inhibits Corneal Angiogenesis in Vivo.** To determine whether ExFlk.6His could inhibit angiogenesis in vivo, a rat corneal micropocket assay was used (27). When pellets containing R3230AC tumor cell-conditioned media were implanted into rat corneas, a strong angiogenic response was seen as early as 5 days after implantation in comparison with Hydron pellets containing fresh media when no angiogenic response was seen (Table 1). With the addition of ExFlk.6His (100 ng) to the pellets containing tumor cell-conditioned media, the angiogenic response induced by tumor-conditioned media was totally blocked in five corneas (72%), and a weak growth was seen in two corneas (28%).

**Table 1** Inhibitory effect of ExFlk.6His on angiogenic response induced by tumor-conditioned media

<table>
<thead>
<tr>
<th>Samples</th>
<th>Proportion of corneal angiogenic responses (%)</th>
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<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Fresh media</td>
<td>3/3 (100%)</td>
</tr>
<tr>
<td>Conditioned medium</td>
<td>2/2 (100%)</td>
</tr>
<tr>
<td>ExFlk.6His</td>
<td></td>
</tr>
<tr>
<td>ExFms.6His</td>
<td></td>
</tr>
<tr>
<td>CM + ExFlk.6His</td>
<td>5/7 (72%)</td>
</tr>
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<td>CM + ExFms.6His</td>
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* Percentage of rats with the indicated response.
The addition of a control protein, ExFms.6His, to pellets containing tumor cell-conditioned media did not block vessel formation. These results demonstrated that ExFlk.6His could inhibit angiogenesis induced by tumor-conditioned media in vivo and suggested its potential application as an antiangiogenic agent for cancer therapy.

**ExFlk.6His Inhibits Tumor Growth in Cutaneous “Window Chambers.”** To evaluate whether the VEGF-neutralizing protein ExFlk.6His could reduce tumor growth and tumor angiogenesis in vivo, a rat cutaneous window chamber model bearing an R3230AC mammary tumor was used (28). Previous work has demonstrated that small fragments of tumor (0.1 mm³) placed in a window chamber become vascularized and undergo rapid growth within 10–14 days (28). Typically, vascularization of tumors in the window chamber is first detected at about 5 days after implantation and is followed by a rapid growth phase of the tumor.

In this model, a single dose of purified ExFlk.6His protein (100 μg) or mock control solution was administered directly into the window chamber at the time of tumor implantation. After 10 days, gross inspection of live tumors in the tumor window and examination of histological sections demonstrated that tumors (n = 10 pairs) treated with ExFlk.6His protein were thinner, smaller, contained more connective tissue, and had a less well-developed vasculature compared with tumors in the control group (Fig. 4). Comparison of tumor volumes measured from the histological sections confirmed that the ExFlk.6His-treated tumors were, on average, 75% smaller than the control tumors (n = 10 pairs, P < 0.005; Fig. 5A). These results showed that blockade of the VEGF pathway by ExFlk.6His could inhibit tumor growth and suggested that ExFlk.6His blocked tumor growth by inhibiting tumor angiogenesis.

**ExFlk.6His Inhibits Tumor Vascularization.** To determine whether inhibition of tumor growth by ExFlk.6His was secondary to inhibition of tumor angiogenesis, tumor vascular length density was measured from photomicrographs of live tumor window chambers bearing ExFlk.6His-treated or control-treated tumors 10 days after implantation. Consist-

ent with the sparse appearance of the vasculature of ExFlk.6His-treated tumors, there was an approximately 50% reduction in tumor vascular length density in ExFlk.6His-treated tumors versus control-treated tumors (n = 10 pairs, P < 0.005; Fig. 5B). This finding is in accordance with the ability of ExFlk.6His protein to neutralize VEGF-mediated endothelial responses in vitro and to block tumor cell-conditioned media-stimulated angiogenesis in the rabbit cornea. It is also consistent with the notion that the primary action of the ExFlk.6His protein is to inhibit tumor neoangiogenesis.

**ExFlk.6His Does Not Directly Affect Tumor Cell Proliferation or Viability.** To further confirm that the primary effect of ExFlk.6His protein was on the tumor vasculature, the cytotoxicity of the ExFlk.6His protein on cultured R3230AC cells was assayed. When cells were cultured in the presence of 3 μM ExFlk.6His protein, which was roughly equivalent to the protein concentration used in tumor window chambers, no significant difference in tumor cell proliferation or viability was observed compared with control (Fig. 6). Thus, inhibition of tumor growth by ExFlk.6His protein was not mediated by direct tumor toxicity, further supporting the notion that the primary action of ExFlk.6His is to inhibit tumor angiogenesis.

**Discussion**

Antiangiogenesis is a promising new therapeutic approach for the treatment of cancer and other “angiogenic” diseases (29). Solid tumor growth is dependent on tumor neovascularization. The expression of VEGF and its receptors have been closely linked to tumor vascularity, metastasis, and progression (22, 23, 30, 31). This study is the first to demonstrate that a recombinant, soluble VEGF receptor, ExFlk.6His, capable of neutralizing VEGF action in vitro could also block tumor angiogenesis in vivo. ExFlk.6His inhibited corneal angiogenesis mediated by tumor cell-conditioned media and also inhibited the growth and vascularization of tumors in a cutaneous window chamber after a single administration. Moreover, binding and cross-linking experiments demonstrated, for the first time, that ExFlk.6His could
function as a dominant-negative inhibitor of VEGF receptor activation, perhaps explaining the efficacy of ExFlk.6His in vivo.

The first approach used to block VEGF action in tumors was to coinject various tumor cell lines with a retroviral packaging cell line producing a retrovirus directing the expression of a membrane-bound, kinase-deficient VEGF receptor (23). This approach resulted in significant inhibition of the growth and vascularization of a variety of tumors. The efficacy of this approach is likely related to the ability of the kinase-deficient Flk-1 to inhibit activation and autophosphorylation of endogenous VEGF receptors by a potent dominant-negative mechanism (23). However, regardless of its potency, the clinical utility of this approach is limited by the necessity of gene transfer techniques to deliver the inhibitor to the tumor endothelium.

Because of the likely importance of VEGF in tumor angiogenesis, a variety of other approaches to inhibit VEGF receptors are under investigation. In murine models, neutralizing antibodies against VEGF were potent inhibitors of tumor growth (22). Although results with inhibitory antibodies were encouraging, their therapeutic use in clinic, even with highly "humanized" versions, may be limited by the development of human anti-monoclonal antibodies (32, 33). Other approaches, such as small molecule inhibitors of the Flk-1 kinase and of VEGF binding, have been developed but have not yet been tested in animal models of tumor angiogenesis (34).

Recently, several studies have shown that recombinant, soluble extracellular domains of VEGF receptors can block VEGF-mediated responses in vitro (35, 36) and block hypoxia-induced angiogenesis in the neonatal mouse retina (36). These results suggest that soluble VEGF receptors might be effective therapeutic agents for the inhibition of pathological angiogenesis in tumors and other angiogenic diseases. In the present study, we have demonstrated for the first time that a soluble receptor of VEGF could block tumor angiogenesis and then inhibit tumor growth.

Soluble receptors such as soluble fms-like tyrosine kinase (Flt-1) receptor (35, 37) and soluble platelet-derived growth factor receptor (38) bind their cognate ligands with high affinity and could function as competitive inhibitors, preventing receptor activation by competing with the endogenous receptors for ligand binding (Fig. 7B). Alternatively, soluble receptors might function as dominant-negative inhibitors, preventing receptor activation by forming nonproductive heterodimers with endogenous cell surface receptors (Fig. 7C). The present study is the first to directly demonstrate that a ligand-bound soluble receptor (ExFlk.6His) can form a heterodimer with an endogenous cell surface receptor and function as dominant-negative
inhibitor (Figs. 3 and 7C). The ability of ExFik.6His to function as a dominant-negative inhibitor may explain the substantial inhibition of Fik-1 receptor activation with a 3-fold molar excess of ExFik.6His, even in the presence of saturating concentrations of VEGF. Thus, this alternative mechanism is also consistent with the potent antiangiogenic activity of ExFik.6His in vivo.

Besides functioning as a dominant-negative inhibitor to block receptor activation and function, using a soluble receptor offers several practical advantages: (a) the delivery of a recombinant, soluble receptor does not require genetic modification of endothelial cells as do membrane-anchored dominant-negative inhibitors (23); (b) because soluble receptors are composed of the ligand-binding domain of endogenous receptors, they are likely to have low immunogenicity and thus avoid problems of immune clearance that plague antibody inhibitors; and (c) recombinant, soluble receptors can be easily made and tested and could be used directly in patients as demonstrated by other recombinant proteins, particularly those with intravascular targets (39).

Materials and Methods

Cell Lines and Antibodies. Recombinant baculovirus was generated and propagated in monolayer cultured SF9 cells maintained in Grace's Insect Medium Supplement (Life Technologies, Inc.) at 28°C. Protein expression was carried out in suspension-cultured SF9 cells in Protein-Free Insect Medium (Life Technologies, Inc.). The R3230Ac rat adenocarcinoma cell line was maintained in DMEM plus 10% fetal bovine serum (Life Technologies, Inc.) at 37°C with 5% CO₂. HUVECs were purchased from Clonetics, Inc. ECRF24, an immortalized HUVEC line, was provided by Dr. Hans Pannekoek (40). Endothelial cells were maintained at 37°C, 5% CO₂ in complete endothelial cell growth medium (Clonetics, Inc.), and grown on 2% gelatin (Sigma Chemical Co.)-coated plates. Endothelial cells were serum starved in EBM (Clonetics, Inc.). Anti-Fik antibodies (C-20 and 1158) and protein A-agarose were purchased from Santa Cruz Biotechnology. Anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology. Human recombinant VEGF165 was purchased from R&D Systems.

Construction of the ExFik.6His and ExFms.6His Baculovirus Vectors and Production of Recombinant Viruses. The entire extracellular domain of murine fik-1 was generated by PCR from a plasmid containing a cDNA encoding the extracellular and transmembrane domains of fik-1. The forward primer consisted of nucleotides 208–223 with a BamHI site introduced upstream of the start codon; the reverse primer consisted of nucleotides 2493–2478 in the reverse orientation with a ClaI site introduced at the 5' end. The resulting PCR product was digested with BamHI/ClaI and ligated to the same sites of an intermediate vector (BSK fik-1/6His) to generate a CDNA encoding a fusion protein consisting of the entire extracellular domain of fik-1 with a six-histidine tag at the COOH terminus (BSK/ExFik.6His). A 2.3 kb EcoRI/Wnt fragment from BSK/ExFik.6His was subcloned into the same sites of pVL 1939, a baculoviral expression transfer vector (PharMingen). pVL 1393/ExFik.6His and Baculogold baculoviral DNA (PharMingen) were cotransfected into SF9 cells for production of the recombinant baculovirus (BvExFik.6His) according to the manufacturer's instructions. Second-passage virus was used to infect serum-free SF9 cells for ExFik.6His protein production. The same approach was used to generate a recombinant baculovirus (BvExFms.6His) expressing the entire extracellular domain of the human c-fms receptor fused to a six-histidine tag at the COOH terminus.

Purification of ExFik.6His and ExFms.6His Proteins. Suspension-cultured SF9 serum-free insect cells (1 liter) were infected with approximately 1 plaque-forming unit/cell of second-passage BvExFik.6His or BvExFms.6His for 5 h at 28°C. Cells were removed by centrifugation at 3000 rpm (Sorvall) for 20 min at 4°C. The supernatant was dialyzed against 8 liters of PBS (pH 8.0) for 48 h with one change of buffer. The dialyzed supernatant was then incubated with 4 ml of Ni²⁺-NTA resin (Qiagen). After 1 h at room temperature, the resin-bound ExFik.6His protein or ExFms.6His protein was loaded onto a 10-ml column. The column was then washed with 200 ml of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole pH 8.0), and the protein was eluted with elution buffer (wash buffer plus 250 mM imidazole), followed by a buffer change to PBS (pH 7.2) by ultra filtration (Centricon 10; Amicon Co.). Mock control material used in the tumor study was generated from the supernatant of uninfected SF9 cells following the same purification procedure. Aliquots of purified ExFik.6His and ExFms.6His proteins were analyzed by SDS-PAGE on a 7.5% gel.

Binding of ExFik.6His to VEGF. Purified ExFik.6His was radiolabeled with Na¹²⁵I (Amersham) using a modified iodogen procedure (41) to a specific activity of 2866 cpm/ng. Labeled ExFik.6His was separated from free ¹²⁵I by gel filtration on a Sephadex G-25 column. There was >95% precipitability with trichloroacetic acid.

Binding of ExFik.6His to VEGF was done basically as described by Duan et al. (38). Human recombinant VEGF was diluted [25 μM Hepes (pH 7.4), 75 mM NaCl, and 20 mM NaHCO₃] and 10 ng/well of VEGF in 100 μl was coated onto 96-well plates (Dynatech plates with detachable wells) overnight at 4°C. The wells were washed once with blocking buffer [25 μM Hepes (pH 7.4), 100 mM NaCl, 0.5% gelatin, and 20 μg/ml BSA] and then blocked with the same buffer for 2 h at room temperature. Increasing amounts of ¹²⁵I-labeled ExFik.6His (0.03–300 ng) in binding buffer [25 μM Hepes (pH 7.4), 100 mM NaCl, 20 μg/ml BSA, and 0.5 μg/ml heparin] were added to each well and incubated for 1 h at room temperature. Nonspecific binding was determined by incubation of ¹²⁵I-labeled...
ExFlk.6His in the presence of 60-fold excess of unlabeled ExFlk.6His. The wells were washed three times with binding buffer and counted in a gamma counter. Scatchard analysis of binding was performed with the aid of a Scatchard program.

Flik-1 Activation Assay. ECRF cells, a transformed HUVEC line expressing Flik-1, were grown in endothelial cell growth medium in a 60-mm dish (40). Upon reaching confluence, the cells were serum starved in EBM overnight followed by stimulation with recombinant VEGF at 20 ng/ml plus different amounts of ExFlk.6His at 37°C for 5 min. The cells were then washed three times with ice-cold PBS and lysed with lysis buffer [20 mM Tris (pH 8.0), 137 mM NaCl, 1% glycerol, 1% Triton X-100, and 2 mM EDTA] supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, and 1 mM sodium vanadate. Flik was immunoprecipitated with anti-Flik antibody (antibody 1158, Santa Cruz Biotechnology) for 4 h at 4°C, and immunocomplexes were collected by the addition of protein A-agarose beads. After thorough washing with TBST [20 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Tween 20], the bound proteins were eluted with SDS sample buffer, subjected to SDS-PAGE, and transferred to nitrocellulose filter. The filter was blocked with 5% milk in TBST and incubated with anti-phosphotyrosine antibody (4G10) in TBST buffer for 1 h at room temperature. With TBST washes between each step, the filter was incubated with a horseradish peroxidase secondary antibody conjugated and detected with ECL working solution (Amerham Corp.) for 1 min and exposed to X-ray film. The same filter was stripped with TBST (pH 2.4) and rebotted with anti-Flik antibody (antibody c-20; Santa Cruz Biotechnology).

Endothelial Cell Proliferation Assay. HUVECs were plated in 24-well plates at a density of 25,000 cells/well. After 24 h, the medium was replaced by EBM as quiescence media (Clenetics, Inc.) and incubated for an additional 24 h. The EBM medium was then replaced with either fresh quiescence medium alone as a control or quiescence medium plus human recombinant VEGF at 10 ng/ml, or quiescence media plus 10 ng/ml VEGF and purified ExFlk.6His at 2.5 μg/ml, or quiescence medium plus 10 ng/ml VEGF and purified control ExFms.6His at 2.5 μg/ml. The cells were incubated for 24 h, followed by a 3-h pulse-labeling with 2 μCi/ml [3H]thymidine (Amersham Corp.). The reaction was stopped by aspirating the medium and washing the cells with HBSS (Life Technologies, Inc.). The DNA was precipitated by treating the cells with cold 10% trichloroacetic acid at 4°C for 30 min, followed by an absolute ethanol wash. The precipitated material was resuspended in 0.5 ml of 0.5 mM NaOH, and [3H]thymidine incorporation was determined from a 400-μl aliquot using a Beckman LS6000SC scintillation counter.

Endothelial Cell Migration Assay. The rate of migration of HUVECs was determined by using a modified Boyden chamber assay as described by Clyman et al. (42). Briefly, polycarbonate filter wells (Costar Transwell with an 8-μm pore size) were coated with 2% gelatin in PBS for 30 min at room temperature and subsequently incubated at 37°C for 1 h with DMEM containing 0.1% BSA (DMEM/BSA). Confluent HUVECs were trypsinized, pelleted by centrifugation, washed with DMEM/BSA to remove residual serum, and resuspended in fresh DMEM/BSA to a final concentration of 2 × 10⁵ cells/ml. Aliquots of cells (1 × 10⁵) were applied to the upper chamber of the filter wells. The filter inserts with cells were placed in wells of a 24-well culture plate containing either 600 μl of DMEM/BSA alone as control, or DMEM/BSA plus human recombinant VEGF at 10 ng/ml, or DMEM/BSA plus VEGF at 10 ng/ml preincubated with ExFlk.6His or pre-incubated with ExFms.6His at 2.5 μg/ml for 30 min at room temperature. After a 4-h incubation at 37°C, the cells that have migrated to the lower surface of the filter inserts were fixed with 10% formalin (Fisher) and stained with Harris’ hematoxylin (Fisher). Six randomly selected high power (>400) fields were counted on each filter.

Experiments. Six hundred ng of radiolabeled 125I-labeled ExFlk.6His (2666 cpm/ng) generated as described above was incubated at room temperature for 30 min with an equimolar amount of VEGF in a final volume of 500 μl of binding buffer (DMEM plus 0.5 μg/ml of heparin). The binding mixture was then added to 90% confluent ECRF24 cells (60-mm dish) and incubated for 4 h at 4°C. The cells were then washed three times with ice-cold PBS, followed by cell lysis in either 0.5 ml of 1 N NaOH for direct counting with 1.5 ml BS3 (Pierce) in PBS for cross-linking. After a 30-min incubation at 4°C, the cross-linking reaction was stopped by the addition of 1 ml Tris-HCl (pH 7.4), followed by three washes with cold PBS. The cells were then lysed at 4°C for 10 min in lysis buffer containing 50 μg Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.25% sodium deoxycholate, and protease inhibitors. The lysate was recovered after centrifugation to remove cell nuclei and either directly analyzed by 5% SDS-PAGE, followed by autoradiography, or immunoprecipitated with an anti-Flik antibody raised against a c-tail peptide of Flik (antibody c-20; Santa Cruz Biotechnology) before the SDS-PAGE analysis.

Rat Corneal Micropocket Assays. The In vivo angiogenic activity of ExFlk.6His was tested in the avascular cornea of F344 female rat (Harlan Laboratories, Madison, WI) as described (27). Briefly, each sample was coimplanted with sterile hydrogel casting solution (IPN Sciences, New Brunswick, NJ), and the solution was pipetted onto the surface of 1.5-mm diameter Teflon rods (Dupont Co., Wilmington, DE). The pellets were air-dried in a laminar flow hood for 1 h and refrigerated overnight. The following day, pellets were rehydrated with a drop of PBS and then placed in a surgically created pocket within the corneal stroma, 1.5 mm from the limbus. Corneas were observed every other day until day 5 or 7, when the animals were anesthetized and perfused with lactated Ringer’s solution, followed by colloidal carbon solution to enumerate the vessels. Responses were scored as positive when vigorous and sustained directional ingrowth of capillary sprouts and hairpin loops toward the implant were detected. Negative responses were recorded when no growth was detected or when there was only an occasional sprout or hairpin loop with no evidence of sustained growth. Negative controls consisted of Hydron pellets containing media alone. Medium was incorporated into pellets at a concentration of 1 μg of total protein/pellet. Soluble receptor proteins were incorporated into pellets at 100 ng/pellet. Histological examination of representative corneas revealed that nonspecific inflammation was not a contributing factor in any of the corneal responses except for occasional neutrophils found in the limbus of both control and test corneas (data not shown).

Tumor Window Chamber Model. R3230AC tumors were grown in cutaneous window chambers in Fisher 344 rats (ages 12–14 weeks; weight, 140–160 g; purchased from Charles River Labs, Raleigh, NC) as described previously (43). Briefly, two 1-cm diameter holes were dissected in opposing epithelial surfaces of the dorsal skin flap as it was retracted away from the posterior surface of the back. The underlying tissue was dissected away until two fascial planes with associated vasculature remained. One hundred μl of ExFlk.6His protein (0.5 μg/μl) or 100 μl of mock-purified control solution was injected between the fascial planes. A 0.1-mm³ piece of tumor from a donor rat was then placed onto the fascial plane, and an additional 100 μl of protein or control solution was added, and the chambers were sealed with glass covesrips. A pair of tumor window chambers were done at each time, one treated with ExFlk.6His and the other with control solution. The tumor implants in each pair were taken from the same region of grossly viable donor tumor tissue. Ten days after implantation, tumors in window chambers (200 μm thick) were photographed using transillumination and a dissecting microscope (Zeiss, Stemi SV5) for vascular length density measurement and were subsequently harvested for H&E staining.

Measurement of Tumor Volume and Tumor Vascular Length Density. To obtain an estimate of tumor volume, H&E-stained sections representing the largest cross-sectional area of each tumor were photographed, and the thickness (t) and the diameter (d) of tumors were measured from the photographs. Tumor volumes, which were assumed to approximate a flat cylinder in shape, were calculated using the formula:

\[ \text{Tumor volume} = 3.14 \left( \frac{d}{2} \right)^2 \times t \]

Tumor vascular length density as an indicator of tumor vascularity was measured from photographs of 10-day-old tumors within the window chamber using a method described previously (28). Three to five areas inside the tumor were randomly selected for measurement. The vascular length density in mm/mm² was calculated using the formula:

\[ \text{Length density} = \frac{N}{(d/p)d} \]

where \( N \) is the average number of intersections between vessels and grid per sheet; \( g \) is number of blocks in grid (54); \( d \) is the length of one grid square (0.25 mm); \( s \) is the diameter of surface binding with a calibration of new microns per millimeter; \( r \) is the measured depth of field through which microvessels could be discerned (0.2 mm).

Tumor Cell Toxicity Assay. R3230AC tumor cells were seeded at 2 × 10⁴/well into 24-well plates and maintained in the presence of purified light.
ExFlk.6His protein (β μm) or control solution. Cell morphology was monitored daily by light microscopy. Cells were trypsinized and suspended in PBS containing 0.02% trypan blue (Life Technologies, Inc.), and live cells were counted with a hemacytometer on each following day for 3 days.

Statistics. Results are reported as means ± SE for tumor volume and tumor vascular length density for each group. A two-tailed Student’s t test was used to analyze statistical differences between control and ExFlk.6His-treated groups. Differences were considered statistically significant at P < 0.05.

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