The Antiangiogenic Agent Neovastat (Æ-941) Inhibits Vascular Endothelial Growth Factor-mediated Biological Effects[1]

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ABSTRACT

Purpose: **Vascular endothelial growth factor (VEGF) is a potent regulator of angiogenesis, which exerts direct effects on vascular endothelial cells, including endothelial cell proliferation and survival, tubulogenesis, and vascular permeability. In this study, we examined whether Neovastat, a naturally occurring multifunctional antiangiogenic drug, could inhibit the endothelial cell response to VEGF stimulation.**

Results: **We demonstrated that Neovastat was able to block the VEGF-dependent microvessel sprouting from Matrigel-embedded rat aortic rings, and it also blocked the VEGF-induced endothelial cell tubulogenesis *in vitro*. *In vivo* studies showed that Neovastat was able to specifically inhibit VEGF-induced plasma extravasation in numerous tissues, including pancreas and skin. The mechanism of action of Neovastat on VEGF-mediated effects was also evaluated at the molecular level. Neovastat was shown to compete against the binding of VEGF to its receptor in endothelial cells and significantly inhibited the VEGF-dependent tyrosine phosphorylation of VEGF receptor-2, whereas it had no significant effect on VEGF receptor-1 activity. Moreover, the inhibition of receptor phosphorylation was correlated with a marked decrease in the ability of VEGF to induce pERK activation. Neovastat does not compete against the binding of basic fibroblast growth factor, indicating a preferential inhibitory effect on the VEGF receptor.**

Conclusions: **Because Neovastat was shown previously to inhibit metalloproteinase activities, these results suggest that Neovastat is able to target multiple steps in tumor neovascularization, further emphasizing its use as a pleiotropic, multifunctional antiangiogenic drug.**

INTRODUCTION

Angiogenesis, the formation of new capillary blood vessels from preexisting vessels, is a tightly regulated phenomenon that plays essential roles in reproductive functions, wound healing, and embryonic development (1). However, angiogenesis may become pathological when capillary growth is uncontrolled, and the resulting excessive neovascularization may then sustain the development of numerous pathologies including retinopathies, hemangiomas, rheumatoid arthritis, psoriasis, and tumor growth (1). In this latter case, it is now clearly established that the aggressive growth of tumors and of their metastases is strictly dependent on angiogenesis (2), thus suggesting that the inhibition of angiogenesis may represent an effective approach for blocking tumor progression. This has led to the development of a new class of products having antiangiogenic activity with almost 300 inhibitors discovered to date, from which >50 are under clinical investigation. This new class of drug targets mechanisms that play a critical role during angiogenesis, such as cell adhesion and degradation of the extracellular matrix, as well as stimulation of endothelial cells by angiogenic cytokines (3).

The first inhibitors of angiogenesis were discovered >25 years ago by Brem and Folkman (4), who demonstrated that fractions or liquid extracts obtained from cartilage were able to inhibit the proliferation of capillaries induced by tumor cells. The antiangiogenic property of cartilage was observed in several species including shark, bovine, and human (Ref. 5 and references therein), and there is an increasing number of inhibitors of angiogenesis found in cartilage. The purification of some cartilage factors were mainly correlated with the presence of at least two distinct type I collagenase inhibitors that share extensive homologies with tissue inhibitors of matrix metalloproteinases 1 and 2 (6, 7). This has led to the suggestion that these MMP[3]

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[3] The abbreviations used are: MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; bFGF, basic fibroblast growth factor; VVO, vesiculo-vacuolar organelle; BAEC, bovine aortic endothelial cell; HUVEC, human umbilical vein

inhibitors may account for many of the antiangiogenic properties contained in cartilage and may thus represent an attractive source of therapeutic anticancer agents. Since then, other cartilage-associated proteins with antiangiogenic properties were identified, including troponin-1, U-995, stem cell factor-2, thrombospondins, and metastatin (8–12), further emphasizing the potential use of cartilage as an abundant source of natural inhibitors of neovascularization. However, the mechanisms of action of most of these products were not fully characterized.

Neovastat is a naturally occurring inhibitor of angiogenesis derived from marine cartilage (dogfish) that contains a high concentration of biologically relevant molecules. Two Phase III (double-blind, randomized, placebo-controlled) clinical trials and one Phase II pivotal trial are currently ongoing to determine the efficacy of Neovastat in patients with advanced unresectable non-small cell lung cancer (stages IIIA and IIIB; sponsored by the US National Cancer Institute), metastatic renal cell carcinoma, and refractory multiple myeloma, respectively (13). Both preclinical and clinical studies demonstrate the nontoxic oral bioavailability of Neovastat. A recent analysis of a Phase II trial using two doses of Neovastat administered to patients with metastatic renal cell cancer demonstrated a dose-survival relationship (14).

The inhibitory effect of Neovastat on angiogenesis has been characterized recently. It was first demonstrated that Neovastat inhibits chick embryo vascularization and Matrigel-induced angiogenesis *in vivo* (15). It also inhibits tumor growth of the DA3 breast adenocarcinoma (16) and the HGD human glioblastoma (17) in mice. Moreover, it inhibits bone and lung metastasis using the MDA-231 human breast carcinoma and Lewis lung carcinoma models in mice, respectively (14, 18). At the molecular level, the antiangiogenic activity of Neovastat was correlated with the inhibition of metalloproteinases (MMP-2, MMP-9, and MMP-12) and serine elastases (19). Neovastat also inhibited the proliferation of endothelial cells *in vitro*, thus supporting the hypothesis that Neovastat could antagonize the action of angiogenic growth factors, such as VEGF.

VEGF and bFGF are mitogens that are usually associated with the activation of tumor-induced angiogenesis. VEGF was first identified by its ability to induce vascular leakage (20, 21) and subsequently shown to induce plasma protein extravasation through the VVOs found in vascular endothelium cells and in the microvasculature that accompanies tumors (22, 23). VEGF was also found be a specific mitogen for endothelial cells (24). Moreover, it functions together with angiopoietin-1 during vascular development, with VEGF acting early during vessel formation, and angiopoietin-1 acting later during vessel remodeling, maturation, and stabilization (Ref. 25 and references therein). The central role of VEGF in embryonic angiogenesis was exemplified in recent studies showing that heterozygote knock-out mice suffer fatal deficiencies in vascularization (26). In tumors, VEGF secretion has been reported to be induced by hypoxia (27) and by oncogenes such as *src* and *ras* (28). VEGF seems highly specific for the endothelial cell surface, where it binds to receptors of the tyrosine kinase family, the fms-like tyrosine kinase (Flt; VEGFR-1) and fetal liver kinase-1 (Flk-1/KDR; VEGFR-2; Refs. 29, 30). The binding of VEGF to VEGFR-2 triggers the tyrosine phosphorylation of a number of key signaling intermediates (30–37) that appears to account for most of the biological activities mediated by VEGF including mitogenesis (30), chemotaxis (35), antiapoptosis (36), and vascular hyperpermeability (37).

Here we report that Neovastat is able to inhibit several functions of endothelial cells that are mediated by VEGF, including capillary sprouting, tubulogenesis, and hyperpermeability. Moreover, the inhibition of the VEGF-mediated signaling pathway by Neovastat emphasizes its unique inhibitory potential on the overall angiogenesis process because it blocks two of the main events involved during neovascularization, inhibition of specific MMPs and of VEGF action on endothelial cells.

MATERIALS AND METHODS

Chemicals. EB dye, formamide, bradykinin, and histamine were purchased from Sigma Chemical Co. (St. Louis, MO). VEGF was from R&D Systems (Minneapolis, MN), and Neovastat, a naturally occurring antiangiogenic drug (38), was supplied by Æterna Laboratories. Anti-VEGFR-1 and VEGFR-2 polyclonal antibodies as well as an anti-phosphotyrosine monoclonal antibody (PY99) were from Santa Cruz Biotechnology (Santa Cruz, CA). Matrigel was purchased from Collaborative Biomedical (Bedford, MA).

Cells

HUVECs. HUVECs (Clonetics Corp., San Diego, CA) were cultured in EBM bicarbonate-buffered medium supplemented with 10 ng/ml human EGF, 20 μg/ml endothelial cell growth supplement, 10% FBS, gentamicin (10 μg/ml), and amphotericin-B (0.25 μg/ml).

BAECs. BAECs were isolated from fresh aortas in our laboratory (39). Their identification was established by their cobblestone monolayer morphology, factor VIII immunohistochemistry, and negative α-actin staining of the smooth muscle cells. The BAECs were cultured in DMEM supplemented with 5% FBS and penicillin-streptomycin (1%).

Rat Aortic Ring Culture

Rat aortic sections were prepared as described by Bauer *et al.* (40). Briefly, thoracic aortas excised from juvenile male Sprague Dawley rats were dissected into 1-mm-long cross-sections, placed into a 12-well plate coated with Matrigel, and then covered with another layer of Matrigel. Aortic rings were cultured overnight with EGM-2 medium (Clonetics). Afterward, the medium was replaced with either EBM-2 medium supplemented with SingleQuot (Clonetics), 2% FBS, heparin, hydrocortisone, ascorbic acid, and gentamicin (FBS-EBM-2) or EBM-2 medium supplemented with SingleQuot (Clonetics), VEGF, heparin, hydrocortisone, ascorbic acid, and gentamicin (VEGF-EBM-2). Each aortic ring was exposed to either vehicle or Neovastat. Additional control aortic rings were exposed to 12.0 μg/ml CAI (40). Aorta preparations were cultured for 6 days, with microvessel growth assessed daily. The presence of

endothelial cell; EBM, endothelial cell basal medium; CAI, carboxyamido-triazole; FBS, fetal bovine serum; EB, Evans blue; ERK, extracellular signal-regulated kinase.

endothelial cells in new microvessel growths was confirmed by staining the aorta section for factor VIII and CD34. Pixel density was determined by image analysis using NIH Image Software (version 1.62).

Tubulogenesis

HUVECs were isolated according to the method of Jaffé *et al.* (41) and routinely cultured on fibronectin in M199 supplemented with 20% human serum (obtained from the blood bank center of Grenoble), antibiotics, and 2% glutamine. Human fibrinogen was purified in our own laboratory, according to the method of Keckwick *et al.* (42). For the tubulogenesis assay, 2×10^5 HUVECs (from passage 2 or 3) were seeded on a 1-ml fibrin gel (0.5 mg/ml) in a 33-mm diameter tissue culture dish in M199 supplemented with 2% human serum, in the presence or in the absence of VEGF (40 ng/ml) and Neovastat (31–62 μg protein/ml) that were added at the time of cell seeding. The effects of Neovastat and VEGF on the formation of tubules were evaluated after 12 and 24 h of culture according to a method developed in our laboratory (43–45). Vascular index was quantified as the ratio existing between cell-free areas delimited by the network and the total surface of cell culture on fibrin gel. At the onset of cell reorganization, cells were fixed (4% paraformaldehyde) and stained. Pictures were taken on the entire tissue culture dish using a binocular microscope (LEICA CLS 150E) coupled to a camera (SONY 3CCD) and the Vision Explorer software (ETCC3000; Grafteck). Image analysis were performed with the Visiolab system software (Biocom).

Vascular Permeability Assay

Unanesthetized male CD-1 mice weighing 20–25 g (Charles River, Wilmington, MA) were maintained in a retention cage and were given a bolus injection in the caudal vein of EB dye (20 mg/kg) in a final volume of 100 μl, together with saline for the basal level group, or with VEGF (0.1 nmol/kg), histamine (45 nmol/kg), or bradykinin (235 nmol/kg) for the stimulated groups. These permeabilizing agents and the EB dye were injected at the same time. Mice were gavaged with 0.5 ml of Neovastat (200 μg of protein) or saline once a day for 3 days and received the last dose 30 min before the permeability test. The dye was allowed to circulate 10 min before sacrifice of the mice. Free intravascular dye was removed with PBS perfusion in the left ventricle (15 ml at 10 ml/min).

The pancreas and skin were harvested, dissected, and weighed, and a portion of each was immersed in formamide (4 ml/g wet weight at 24°C for 24 h). The remaining portions were dried at 60°C for 24 h. The concentration of EB dye extracted in formamide from these tissues was determined by spectrophotometry at 620 nm and expressed as μg of EB per g of dry tissues after comparison to a standard curve.

Radioreceptor Binding Assay with ^{125}I-labeled VEGF or ^{125}I-labeled bFGF

Binding studies were performed on HUVECs and BAECs grown to confluence in 12-well tissue culture plates. The binding buffer was M199 medium supplemented with 5 mM HEPES and 0.1% gelatin at pH 7.3. Saturation of VEGF and bFGF receptors was determined by adding 0.5 ml of binding buffer containing increasing concentrations of ^{125}I-labeled VEGF or ^{125}I-labeled bFGF (0.05–10 ng/ml) to the endothelial cells in culture for a 2-h period at 4°C. Nonspecific binding was determined at each concentration by adding a 100-fold excess of unlabeled VEGF or bFGF. At the end of incubation, cells were washed twice with ice-cold M199 containing 1 mg/ml of BSA. The cells were collected after trypsin incubation, and the radioactivity was evaluated on a gamma counter. Binding site competition studies for ^{125}I-labeled VEGF or ^{125}I-labeled bFGF (8 ng/ml; a concentration close to the saturation) were evaluated in the presence of increasing concentrations of unlabeled VEGF, bFGF, or Neovastat. Each determination was performed in triplicate.

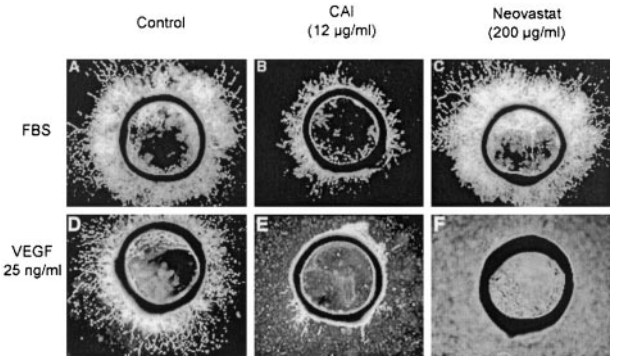

Fig. 1 Neovastat inhibits VEGF-induced rat aortic ring microvessel sprouting. Neovastat was tested in the rat aortic ring assay using FBS or VEGF as a stimulus. *A*, control FBS medium. *B*, CAI (12 μg/ml) in FBS medium. *C*, Neovastat (200 μg/ml) in FBS medium. *D*, control VEGF medium. *E*, CAI (12 μg/ml) in VEGF medium. *F*, Neovastat (200 μg/ml) in VEGF medium. Photographs are representative pictures from a triplicate assay.

VEGFR Phosphorylation

VEGF-dependent tyrosine phosphorylation of VEGFR-1 and VEGFR-2 was measured essentially as described (34). Briefly, BAECs were treated for 18 h in a serum-free medium in the absence or in the presence of various concentrations of Neovastat, followed by a 1-min incubation with VEGF (1 nM). Equal amounts of proteins from the resulting cell lysates were subjected to immunoprecipitation using polyclonal antibodies specific for either the type 1 or type 2 receptors. The extent of phosphorylation of the two receptors was determined by immunoblotting with an anti-phosphotyrosine monoclonal antibody (PY99). The activation of the ERK signaling cascade by VEGF was monitored using phosphospecific antibodies raised against pERK (New England Biolabs).

RESULTS

Neovastat Inhibits VEGF-dependent Endothelial Sprouting. The effect of Neovastat on microvessel sprouting from vascular tissues was tested using an *ex vivo* rat aortic ring angiogenesis assay. In this model, microvessel sprouting is stimulated by both specific (VEGF) or nonspecific (FBS) stimuli (Fig. 1, *A* and *D*), both processes being sensitive to the presence of CAI, an inhibitor of the calcium-mediated nitric

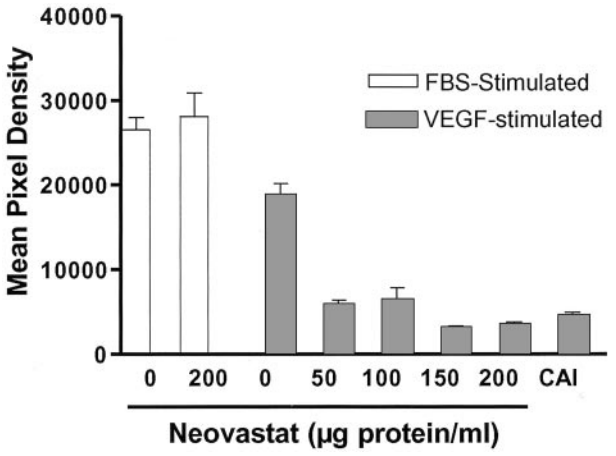

Fig. 2 Dose-dependent inhibition of VEGF-induced rat aortic ring microvessel sprouting by Neovastat. Microvessel density was analyzed for FBS-induced and VEGF-induced rat aortic rings treated with Neovastat. *Bars*, SD of a triplicate assay.

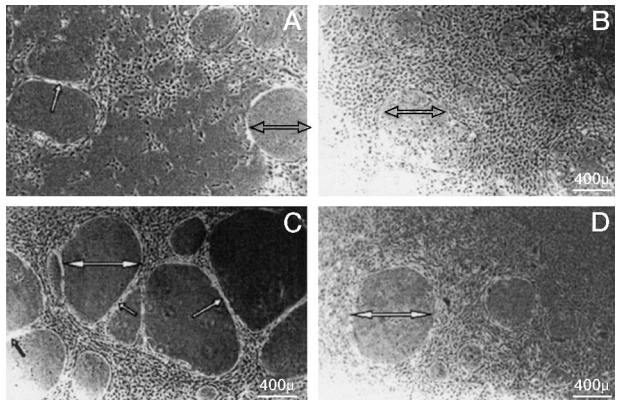

Fig. 3 Effect of Neovastat on endothelial cell tubulogenesis *in vitro*. *A*, HUVECs seeded on fibrin formed tubes (*arrow*) and areas depleted of cells (*double arrows*). *B*, Neovastat (31- and 62.5 µg of protein/ml) inhibited the formation of tubules and of areas depleted of cells. *C*, VEGF (40 ng/ml) augmented the formation of tubules, a process that is inhibited by Neovastat (*D*).

oxide synthase-VEGF pathway used as negative control (Ref. 40; Fig. 1*B*). Addition of Neovastat to the VEGF-dependent sprouting assay showed a potent inhibition of microvessel sprouting in a dose-dependent manner (Fig. 1*F* and Fig. 2). Under these conditions, the inhibitory effect of Neovastat appears slightly greater than CAI (84 *versus* 76% of inhibition; Fig. 1*E* and Fig. 2). By contrast, no inhibitory activity of Neovastat could be detected in the nonspecific, FBS-dependent assay (Figs. 1*C* and 2).

Neovastat Inhibits the Formation of Capillary-like Structures *in Vitro*. To further determine whether Neovastat could modulate endothelial cell migration and vessel formation, two other processes positively regulated by VEGF, we tested the effect of Neovastat on the VEGF-dependent promotion of capillary-like structures on HUVECs seeded on fibrin gel. HUVECs plated on fibrin gel formed capillary-like structures as we described previously (44, 45). This cell reorganization, named tubulogenesis, occurred in three major steps. In the first step, which lasts ~2 h, HUVECs adhered onto the fibrin matrix and were homogeneously distributed. During the second step, 8–10 h later, areas free of cells and of fibrin started to be seen (lumen, see *double-sided arrows* in Fig. 3*A*). Finally, between 12 and 24 h after cell seeding, the cell-free areas increased in size and number, and then thin tubules were formed at the junction of the cell-free areas (Fig. 3*A*, *arrow*). In our assay, VEGF (40 ng/ml) increased the rate of formation of tubule and lumen as compared with the control untreated cells (Fig. 3, *A versus C*). This effect was reverted by the addition of Neovastat in the culture medium (Fig. 3*D*). Tubulogenesis was also inhibited by Neovastat in the untreated cells (Fig. 3, *A versus B*). Quantitative analysis of tubulogenesis indicated that VEGF induced a 2-fold induction of the area occupied by the tubules in the culture dish as compared with control (60% of the area *versus* 30%; Fig. 4). Neovastat decreases the effect of VEGF on tubule formation in a concentration-dependent fashion, a maximal inhibition of 66% being observed in the VEGF-treated cells (decrease of the area occupied by tubules from 60% to 20%). A similar effect of

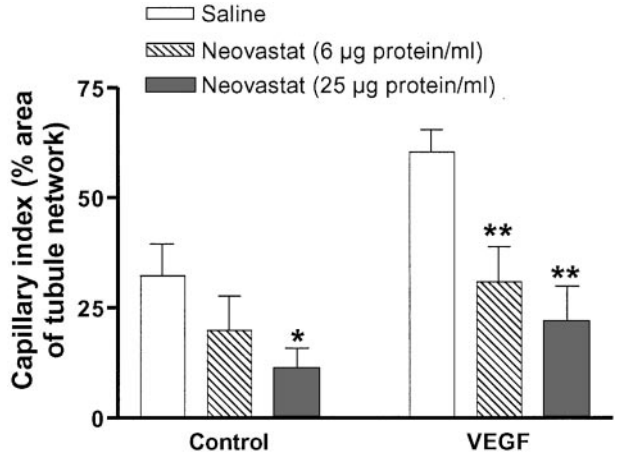

Fig. 4 Quantification of the effect of Neovastat on tubulogenesis. Effect of Neovastat on the area occupied by tubules in cells cultured in the presence or absence of VEGF (40 ng/ml). The capillary index was performed as described in "Materials and Methods." Each histogram represented the mean of three separate experiments done in duplicate; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$.

Neovastat was observed in the cells cultured in the absence of VEGF (30% for control *versus* 10% for Neovastat-treated cells). These results strengthen the hypothesis that Neovastat could interfere with the action of VEGF on endothelial cells.

Neovastat Inhibits VEGF-dependent Vascular Permeability. In addition to its angiogenic effects, VEGF enhances vascular permeability (46, 47). We thus tested whether oral administration of Neovastat could inhibit dye extravasation induced by VEGF in adult mice previously injected with EB dye. The animals were treated p.o. with Neovastat (0.5 ml/day; 200 µg protein/day) or saline for 3 days before i.v. administration of VEGF (0.1 nmol/kg) and EB (20 mg/kg). The amount of EB present in selected tissues was evaluated 10 min after the administration of VEGF. Pancreas and skin were used because

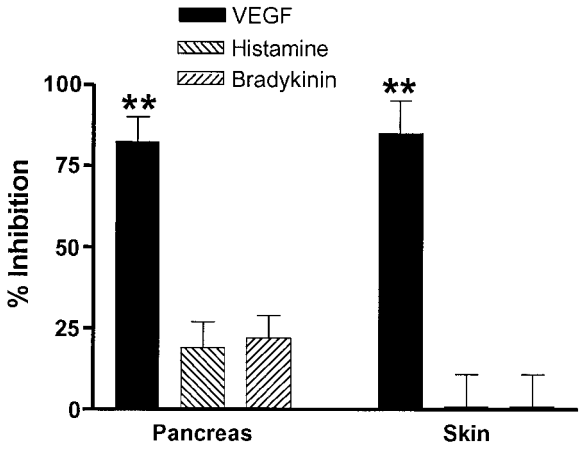

Fig. 5 Effect of Neovastat on VEGF-, histamine-, and bradykinin-induced vascular permeability in pancreas and skin. Plasma extravasation was determined by the tissue concentration of EB (μg of EB/g of dry tissue), 10 min after an i.v. injection (20 mg/kg). Vascular permeability of mouse pancreas and skin was evaluated after administration of VEGF (0.1 nmol/kg), histamine (45 nmol/kg), or bradykinin (235 nmol/kg). EB levels in mice gavaged with 500 μl of Neovastat, daily for 3 days, and 20 min before EB and agonist injection are shown. Values are means of 10, 8, and 4 experiments for VEGF, histamine, and bradykinin, respectively; *bars*, SE. **, $P < 0.01$ between the stimulated group and the group treated with Neovastat.

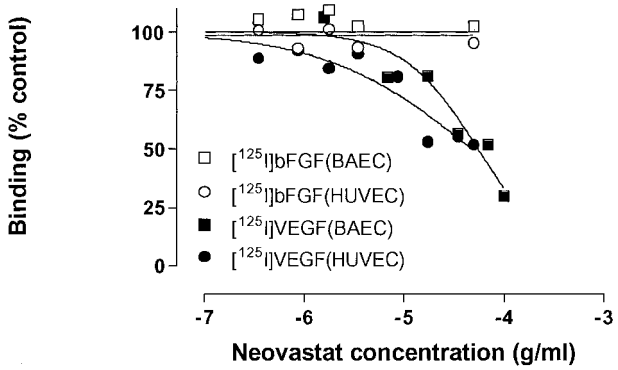

Fig. 6 Effect of Neovastat on the binding of VEGF and bFGF to the endothelial cell surface. A competitive binding study was performed against ^{125}I-labeled VEGF (8 ng/ml) and ^{125}I-labeled bFGF (8 ng/ml) on BAECs and HUVECs. VEGF and bFGF binding reached a saturation plateau at around 20 ng/ml (0.52 and 1.25 nM, respectively) in both endothelial cell types. The *Bmax* values for VEGF in BAECs and HUVECs were 2.6 and 2.7 fmol/10^5 cells, respectively, whereas the *Bmax* values for bFGF in BAECs and HUVECs were 4.5 and 30.1 fmol/10^5 cells, respectively (data not shown). A competitive binding study indicated that VEGF IC_{50}s on BAECs and HUVECs were 17 and 1.84 ng/ml and the bFGF IC_{50}s on BAECs and HUVECs were 1.07 and 27.7 ng/ml.

these tissues showed a significant response to VEGF (43% increase, $P < 0.05$; 59% increase, $P < 0.01$). Neovastat significantly blocked the vascular permeability induced by VEGF, with >80% inhibition ($P < 0.01$) of the increase in dye extravasation observed in the pancreas and skin (Fig. 5). In contrast, the induction of vascular permeability by histamine and bradykinin was not altered by Neovastat (Fig. 5). These results indicate that oral administration of Neovastat in mice specifically reduces the induction of vascular permeability by VEGF and support its oral systemic effect.

Neovastat Competes for the Binding of VEGF to the Endothelial Cell Surface. In light of its inhibitory effect on VEGF-dependent endothelial cell migration and vascular hyperpermeability, we next determined whether Neovastat could compete with the binding of ^{125}I-labeled VEGF to its receptor in two endothelial cell lines (HUVECs and BAECs). Under our conditions, Neovastat has shown an important competition against the binding of ^{125}I-labeled VEGF (8 ng/ml) to its receptors on BAECs and HUVECs with corresponding IC_{50}s of 13.6 and 24 μg protein/ml (Fig. 6). By contrast, Neovastat did not significantly alter the binding of ^{125}I-labeled bFGF (8 ng/ml) to BAECs and HUVECs (Fig. 6).

Neovastat Inhibits VEGF-dependent VEGFR-2 Tyrosine Phosphorylation. We next attempted to determine the role of the two endothelial cell-specific VEGFRs, Flt-1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2), in mediating the antiproliferative and antipermeability activities of Neovastat. Because these two receptors possess intrinsic tyrosine kinase activities, we tested the effect of Neovastat on the extent of their VEGF-dependent phosphorylation. BAECs were incubated for 18 h in serum-free medium in the absence or in the presence of Neovastat and both VEGFRs (VEGFR-1 and VEGFR-2) were immunoprecipitated from the resulting cell lysates using specific antibodies against these proteins. As illustrated in Fig. 7*A*, Neovastat preferentially blocks the VEGF-dependent phosphorylation of VEGFR-2 (Flk-1), reaching almost complete inhibition of receptor autophosphorylation at 100 μg of protein/ml. By contrast, Neovastat had no effect on VEGFR-1 phosphorylation. Interestingly, the inhibition of receptor phosphorylation by Neovastat was correlated with a marked decrease in the ability of VEGF to induce pERK activation, a well-characterized consequence of VEGFR-2 activation (Ref. 48; Fig. 7*B*). These results suggest that VEGF receptor blockade by Neovastat interferes with the signaling pathways triggered by VEGF.

DISCUSSION

Neovastat is a naturally occurring antiangiogenic drug that has reached Phase III clinical trial evaluation. In contrast with other inhibitors of angiogenesis derived from natural sources, there is significant information available about the mechanism of action of Neovastat. We have reported recently that it contains inhibitors of MMPs as well as of serine elastases (19). We have also reported that Neovastat inhibits endothelial cell proliferation, whereas it had no significant effect on the proliferation of fibroblast and muscle cells or on several tumor cell lines (15), thus suggesting that Neovastat acts directly at the endothelial cell level. This behavior is also consistent with its cytostatic rather than cytotoxic properties, as observed with many endogenous antiangiogenic molecules described to date including angiostatin (49, 50), endostatin (51, 52), and cleaved antithrombin (53).

VEGF is the most potent angiogenic growth factor, showing a strong effect on the proliferation, survival, and differentiation of endothelial cells. Therefore, much research was recently focused on the development of synthetic and natural drugs that

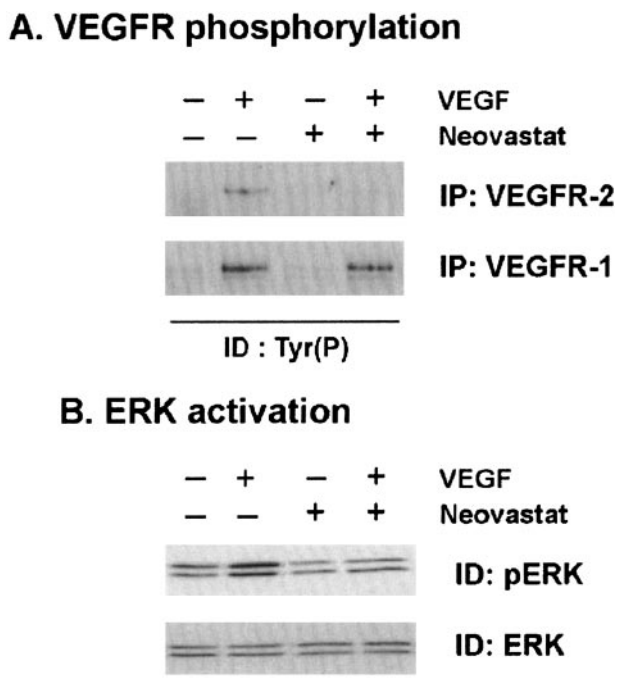

Fig. 7 Effect of Neovastat on VEGF-dependent VEGFR tyrosine phosphorylation. *A*, BAECs were incubated in the absence or presence of Neovastat, and both VEGFRs R1 and R2 (VEGFR-1 and VEGFR-2) were immunoprecipitated from the resulting cell lysates using specific antibodies against these proteins. The extent of tyrosine phosphorylation of the receptors was monitored using a monoclonal phosphotyrosine antibody (PY99). *B*, lysates from control and Neovastat-treated cells were separated by SDS-PAGE and immunoblotted with anti-phospho-specific ERK antibodies (*upper panel*) or with anti-ERK antibodies (*lower panel*).

could block its activity, which has led to the development of products that target VEGF, VEGFR-1 and VEGFR-2 (54–58). In this work, we thus investigated whether Neovastat could exert its antiangiogenic effects by interfering with VEGF-mediated biological effects.

This is supported by two different lines of evidence:

(*a*) We observed that Neovastat significantly competed against the binding of VEGF to the endothelial cell surface. Under these conditions, Neovastat preferentially inhibited VEGF binding, because the binding of bFGF to its receptor was unaffected under these conditions.

(*b*) The incubation of endothelial cells with Neovastat markedly reduced the extent of VEGF-dependent tyrosine phosphorylation of the VEGF receptor VEGFR-2, whereas it had no effect on that of VEGFR-1.

We also demonstrate that Neovastat has potent inhibitory activity against a number of VEGF-dependent biological events. We observed that Neovastat inhibited VEGF-mediated microvessel sprouting *ex vivo* using the rat aortic ring angiogenesis assay. This assay has been used previously to study the effects of a variety of angiogenesis inhibitors, including thalidomide, endostatin, CAI, and endothelial monocyte activating polypeptide II (40, 59–61). In our study, the assay conditions were modified to induce the system with VEGF only, allowing the comparison of VEGF-stimulated microvessel formation *versus* nonspecific sprouting induced by 2% FBS, as described previously (40). Neovastat showed no inhibitory activity when FBS was used as the stimulus but demonstrated potent inhibition in a VEGF-driven system. These results suggest a selectivity of the activity of Neovastat against VEGF-induced microvessel formation, supporting the hypothesis that Neovastat affects VEGF signaling in this model.

We also observed that Neovastat was able to disrupt tubulogenesis *in vitro*. In our assay (44, 45), the formation of tubules was associated with fibrin degradation and remodeling of the nearby tubules. This remodeling allowed the establishment of contacts between endothelial cells and the resulting formation of tubules. We reported that α_2-antiplasmin totally inhibits fibrin degradation and cell reorganization, thus suggesting that the plasminogen/plasmin system plays a key role under these experimental conditions (44, 45). Because some MMPs are also able to degrade fibrin (62), the inhibitory effect observed with Neovastat could be attributable to the presence of MMP inhibitors in Neovastat (19). This could explain the inhibitory effect of Neovastat observed on tubule formation in the cells cultured in the absence of VEGF. Importantly, we also show that VEGF is able to stimulate the formation of tubules, a process that is significantly inhibited by incubation of the cells with Neovastat. Because binding of VEGF to VEGFR-2 stimulates endothelial cell migration and tubulogenesis, these data indicate that Neovastat may inhibit tubulogenesis through its interference with the VEGF signaling pathway.

It is well established that in addition to its angiogenic effects, VEGF enhances vascular permeability (63), which could be associated with pinocytotic vesicular transport (64), increase of VVO function (23), or with fenestrations in the endothelium (65, 66). Moreover, the striking leakiness of microvessels that supply tumors is largely attributed to VEGF (67). $VEGF_{165}$ was shown recently to induce vascular permeability by its specific binding to VEGFR-2 (68, 69), whereas histamine and bradykinin exert their effect through their binding to distinct receptors (70–72). VEGFR-2 as well as bound VEGF were localized to VVOs in an animal model (23). Taken together with the inhibitory effect of Neovastat on VEGF-dependent capillary sprouting and tubulogenesis, the observation that Neovastat inhibits VEGF-dependent vascular permeability, whereas it has no effect on that induced by histamine or bradykinin, thus strongly suggesting that Neovastat acts at the VEGFR level.

Our results indicate that Neovastat blocks the action of VEGF. VEGFR-2 has been shown to be responsible for most of VEGF-mediated biological actions, including proliferation, migration, cell survival, and vascular permeability. For this reason, targeting of VEGF and/or its receptor has been a strategy that has received considerable attention recently. Neutralizing antibodies, a small peptide derived from the immunoglobulin-like domain of the receptor, or compounds that interfere with the kinase activity of the receptor have been developed and have shown efficacy in animal tumor models (73–75).

The presence within Neovastat of specific inhibitor(s) of VEGF-mediated signal transduction may thus significantly contribute to the antiangiogenic activity of the compound. Given the recent observations of antimetalloproteinase and antielastase activities in Neovastat (19), it thus appears that it may act as a multitarget drug with inhibitory efficacy against several crucial

steps of the angiogenic cascade closely linked to tumor progression. Identification of the molecular entities responsible for the antiangiogenic activities of Neovastat is currently under way and should be of considerable importance in our understanding of the antitumor effects of this drug.

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