Angiostatin Binds to Smooth Muscle Cells in the Coronary Artery and Inhibits Smooth Muscle Cell Proliferation and Migration In Vitro

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Abstract—Angiostatin is an inhibitor of angiogenesis that is known to reduce endothelial cell proliferation and consequently prevent the progression of tumor metastases. However, the modest effect of angiostatin on endothelial cell proliferation raises the possibility that angiostatin might exert its effects on other cells. To determine the cellular distribution of angiostatin binding in tissues with neovasculature (atherosclerotic coronary arteries), we developed a fusion protein consisting of placental alkaline phosphatase and the first 3 kringles of plasminogen. Angiostatin binding colocalized with smooth muscle cells and could be inhibited by a 50-fold molar excess of plasminogen and 10 mmol/L e-amino-n-caproic acid. The fusion protein also bound to smooth muscle cells in culture. Angiostatin inhibited hepatocyte growth factor–induced proliferation and migration of smooth muscle cells, suggesting that they are a target for the antiangiogenic effect of angiostatin. (Arterioscler Thromb Vasc Biol. 1999;19:2041-2048.)

Key Words: angiostatin ■ angiogenesis ■ plasminogen ■ hepatocyte growth factor ■ smooth muscle cells

Angiogenesis, the elaboration of blood vessels by endothelial cell outgrowth from preexisting vessels,1 plays a vital role in embryogenesis and physiological processes such as menses formation and wound healing. The pathogenesis of a variety of diseases, including tumor growth and metastasis, diabetic retinopathy, arthritis, psoriasis, and atherosclerosis, is also influenced by either excessive or deficient angiogenesis.1,2

A variety of stimulators and inhibitors of angiogenesis have been described. One of the most novel antiangiogenesis compounds is angiostatin, a 38-kDa protein containing the first 4 of the 5 plasminogen kringles.3 Although angiostatin is derived from plasminogen, reportedly through the activity of either a serine proteinase4 or metalloelastase,5 the antiangiogenic activity of angiostatin is unique to this derivative. Angiostatin has been shown to dramatically suppress the growth and metastases of experimental tumors in animal models.6 This effect is thought to be mediated via the inhibition of endothelial cell proliferation, resulting in suppressed tumor angiogenesis. Although endothelial cell growth is a vital component of the process of angiogenesis,1,2 the near-total inhibition of tumor metastasis is paralleled by a much more modest effect on endothelial cell proliferation.4,5 This apparent discrepancy raises the possibility that angiostatin might exert some of its effects by an alternate mechanism, or that other cells, in addition to endothelial cells, could be its target. Localizing the distribution of angiostatin binding could potentially lead to a further understanding of its mechanism of action. Because the atherosclerotic coronary artery is a site of intense neovascularization,7 we examined the cellular distribution of angiostatin binding to coronary artery sections. In this study, we report that the smooth muscle cell (SMC) is the principal binding site in the coronary artery for binding angiostatin and further demonstrate that angiostatin inhibits SMC proliferation and migration. Because there is evidence that SMCs participate in angiogenesis,8 these effects could contribute to the antiangiogenic effect of angiostatin.

Methods

Materials

The full-length plasminogen cDNA and the APtag-4 vector were generous gifts from L.O. Heden9 (Department of Microbiology, University of Lund, Lund, Sweden) and J. Flanagan,10 (Department of Cell Biology, Harvard Medical School, Boston, Mass), respectively. Competent Escherichia coli cells (MAX efficiency DH10B/P3) were purchased from Gibco/BRL. The restriction enzyme BglII was purchased from Promega. Ampicillin and tetracycline were purchased from Sigma Chemical Co. COS-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Va). Opti-MEMI and LipofectAMINE were purchased from Gibco/BRL. All cell culture reagents, including Dulbecco’s modified Eagle’s medium (DMEM), FCS, the serum-free medium AIMV, l-glutamine, penicillin/streptomycin (pen/strep), and trypsin/EDTA were purchased from Gibco/BRL. Placental alkaline phosphatase (PAP) activity was detected by using either the soluble substrate p-nitrophenyl phosphate with diethanolamine buffer from Bio-Rad or, for in situ analyses, the ProtoBlot NBT (nitro blue tetrazolium) or BCIP (5-bromo-4-chloro-3-indolyl phosphate) color development system (Promega). 1-Homourguinine, an inhibitor of cellular phosphatases but not of PAP, was purchased from Sigma.
Centricron-30 concentrators were purchased from Amicon. The polyclonal antibody to plasminogen was purchased from Calbiochem, and the monoclonal antibody to PAP was purchased from Genzyme Diagnostics. A monoclonal antibody to smooth muscle actin (HHP-35) was purchased from R&D Systems, Inc. A monoclonal antibody to the endothelial cell antigen CD31 was purchased from Dako Corp. The enhanced chemiluminescence kit, which contained peroxidase-conjugated anti-rabbit IgG and peroxidase-conjugated anti-mouse IgG, was purchased from Amersham. e-Aminocaproic acid (EACA), heparanase growth factor (HGF), and BSA were purchased from Sigma. Rabbit aortic SMCs) were a gift from M. Nachtigal (Department of Pathology, University of South Carolina School of Medicine, Columbia), and human aortic SMCs (CRL 1999) were obtained from ATCC. Purified angiostatin, used in the proliferation and migration assays, was purchased from Angiogenesis Research Industries. The Transwell apparatus was purchased from Corning Costar Corp, and staining was performed with Diff-Quick from Dade International. Oligodeoxynucleotide primers were synthesized on an ABI 394 DNA/RNA synthesizer by a standard cyanoethyl phosphoramide procedure in the DNA Synthesis Core Laboratory of the Comprehensive Cancer Center of Wake Forest University.

**Development of Fusion Protein Containing PAP and Plasminogen Kringle 1 through 3 (PAP/K1-3)**

The fusion vector was constructed by amplifying the human plasminogen cDNA using the polymerase chain reaction (PCR) primers listed as follows: sense, 5'-GA AGA TCT (BgII) GCC (Gly) GCC (Gly) GTG TAT CTC TCA GAG TGC-3'; antisense, 3'-GG CTC TTT GAC CGA ACT (stop) TCT AGA (BgII) AG-5'. These primers create 5' and 3' BgII sites, Gly-gly linkers between PAP and kringle 3, and amplify the first 3 kringles of plasminogen (residues Lys78 to Ala344). The 3-kringle form of angiostatin has been found to be more potent than the 4-kringle derivative form.12 PCR amplification was performed using a denaturing temperature of 94°C for 1 minute, annealing temperature of 60°C for 1 minute, and extending temperature of 72°C for 2 minutes.

**Expression of PAP/K1-3 in COS-1 Cells**

Transient expression of PAP/K1-3, as well as the wild-type vector APtag-4, was achieved by transfecting COS-1 cells that were grown in DMEM supplemented with 10% FCS and pen/strep. After 24 hours, the medium was changed to DMEM/F12K supplemented with 2 mmol/L glucose, 10 mmol/L HEPES, 10 mmol/L TES, 50 µg/mL ascorbic acid, 10 µg/mL insulin, 10 µg/mL transferrin, 10 ng/mL Na2SeO3 with 10% FCS. The cells were cultured in Ham’s F12K supplemented with 2 mmol/L glutamine, 10 mmol/L HEPES, 10 mmol/L TES, 50 µg/mL ascorbic acid, 10 µg/mL insulin, 10 µg/mL transferrin, and 10 ng/mL Na2SeO3 with 10% FCS. The cells were cultured in 75-cm² plastic tissue-culture flasks and incubated at 37°C in an atmosphere of 5% CO2 in air. The cells were subcultured at a 1:4 split ratio at confluence with the use of 0.02% trypsin/EDTA.

**Conditions for SMCs in Culture**

Rabbit aortic (Rb-1) SMCs were grown in DMEM supplemented with l-glutamine and 10% FCS. Human aortic SMCs (CRL 1999) were cultured in Ham’s F12K supplemented with 2 mmol/L glutamine, 10 mmol/L HEPES, 10 mmol/L TES, 50 µg/mL ascorbic acid, 10 µg/mL insulin, 10 µg/mL transferrin, and 10 ng/mL Na2SeO3 with 10% FCS. The cells were cultured in 75-cm² plastic tissue-culture flasks and incubated at 37°C in an humidified atmosphere of 5% CO2 in air. The cells were subcultured at a 1:4 split ratio at confluence with the use of 0.02% trypsin/EDTA.

**Binding of PAP/K1-3 to Human Aortic SMCs**

Human aortic SMCs were grown to confluence in 96-well plates. The 10×-concentrated medium from the transfection of PAP/K1-3 or APtag-4 was incubated at the concentrations shown for 90 minutes at room temperature. Unbound material was removed with HBHA buffer and fixed for 30 seconds with 60% acetone, 3% formaldehyde, and 20 mmol/L HEPES, pH 7.5. The slides were then washed 150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.5) twice for 5 minutes each and floated on a 65°C water bath for 60 minutes to inactivate cellular phosphatases. The slides were then rinsed with 100 mmol/L Tris-HCl, pH 9.5, 100 mmol/L NaCl, and 5 mmol/L MgCl2. To stain the slides, the ProtoBlot NBT and BCIP color development system containing 0.17 mg/mL BCIP and 0.33 mg/mL NBT in 100 mmol/L Tris-HCl, pH 9.5; 100 mmol/L NaCl, and 5 mmol/L MgCl2 was added to each slide for up to 72 hours.

**Binding of PAP/K1-3 to Human Aortic SMCs**

Rabbit aortic (Rb-1) SMCs were grown in DMEM supplemented with 10% FCS and pen/strept. The cells were transfected with Opti-MEMI at 80% confluence with 2 µg of PAP/K1-3 or APtag-4 and 8 µL of LipofectAMINE. After 1 hour, fresh Opti-MEMI plus 10% FCS was added for 4 hours, followed by fresh DMEM, FCS, and pen/strept. After 24 hours, the medium was changed to AIM-V serum-free medium with BSA. The medium from transfected COS-1 cells was harvested at 4 days and assayed for PAP activity.

**PAP Assay**

A sample of the harvested medium (10 µL) was heated to 65°C for 10 minutes to eliminate background alkaline phosphatase activity (PAP is resistant to heat inactivation). The OD405 was measured after incubating the heated sample for 1 hour at room temperature in 1 mol/L diethanolamine (pH 9.8), 0.5 mmol/L MgCl2, 10 mmol/L L-homoarginine (a cellular phosphatase inhibitor that does not inhibit PAP), 0.5 mg/mL BSA, and 12 mmol/L p-nitrophenyl phosphate.

**Analysis of Secreted Protein**

The medium from the transfections was concentrated 10-fold by using a Centricron-30 concentrator. A 10% SDS–polyacrylamide gel electrophoresis (PAGE) of the concentrated media was performed, followed by transfer of the proteins to nitrocellulose and blocking with 3% nonfat milk in 1× Tris-buffered saline, pH 7.4, and 0.1% Tween-20. A polyclonal antibody to plasminogen (1:500) was used, followed by a peroxidase-conjugated anti-rabbit IgG (1:5000). For detecting PAP, a monoclonal antibody to human PAP (1:2000) was used, followed by a peroxidase-conjugated anti-mouse IgG (1:5000). Chemiluminescence detection was performed with an enhanced chemiluminescence kit.

**In Situ Binding Assays**

The medium from the transfections was concentrated so that it had equal alkaline phosphatase activity (OD405 APtag-4=1.37, OD405 PAP/K1-3=1.32). The unfused-PAP medium was used as a control for nonspecific binding and for background alkaline phosphatase activity that was not destroyed by heating.

The in situ binding analysis of the fusion protein to the frozen coronary arteries was performed by following the method of Flanagan and Leder.10 OCT-embedded frozen sections were thawed briefly at room temperature and then immediately washed with HBHA buffer (Hanks’ balanced salt solution with 0.5 mg/mL BSA, 0.1% NaN3, and 20 mmol/L HEPES, pH 7.0). The slides were then incubated with the 10×-concentrated PAP/K1-3 protein or unfused PAP, with or without a 50-fold molar excess of plasminogen or 10 mmol/L EACA, for 90 minutes. The slides were rinsed 7 times with HBHA buffer and fixed for 30 seconds with 60% acetone, 3% formaldehyde, and 20 mmol/L HEPES, pH 7.5. The slides were then washed in 150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.5) twice for 5 minutes each and floated on a 65°C water bath for 60 minutes to inactivate cellular phosphatases. The slides were then rinsed with 100 mmol/L Tris-HCl, pH 9.5, 100 mmol/L NaCl, and 5 mmol/L MgCl2. To stain the slides, the ProtoBlot NBT and BCIP color development system containing 0.17 mg/mL BCIP and 0.33 mg/mL NBT in 100 mmol/L Tris-HCl, pH 9.5; 100 mmol/L NaCl, and 5 mmol/L MgCl2 was added to each slide for up to 72 hours.

**Proliferation Assays**

Rb-1 cells were seeded at 1000 cells per well in a 96-well plate and grown to 10% to 20% confluence. Cells were incubated for 24 hours in serum-free DMEM to allow synchronization of the cell cycle. Preliminary experiments established optimum proliferation conditions as DMEM with 10% FCS and 20 ng/mL HGF for a 96-hour incubation period. The cells were therefore incubated in DMEM plus 10% FCS and HGF (20 ng/mL), with or without varying concentrations of angiotatin (0 to 2.0 µmol/L) or plasminogen (2.0 µmol/L), for 96 hours. The cells were harvested by trypsinization, followed by counting with a Coulter counter. The results were compared with the proliferation observed in the presence of DMEM and 10% FCS, which was defined as baseline (100%). An additional cell proliferation assay (Cell Titer 96 AQ, Promega) was performed by using the NBT compound that is reduced by living cells to a colored formazan product that can be detected at 490 nm. Each experiment was...
performed 6 times. The effect of angiotatin on apoptosis was
determined using an in situ cell death detection kit (AP, Boehringer
Mannheim; essentially a terminal deoxynucleotidyl transferase–
mediated dUTP-biotin nick-end-labeling assay).

Migration Assays
Migration assays were performed as previously described13–15 using
a Transwell apparatus with an 8-μm pore size polycarbonate
membrane separating the 2 chambers. Trypsinized cells were pel-
leted by centrifugation at 1000 rpm for 5 minutes and resuspended in
fresh medium to eliminate the trypsin. Preliminary experiments
established optimum migration conditions as a combination of
DMEM, 10% FCS, and 50 ng/mL HGF. The DMEM/10% FCS/HGF
(50 ng/mL) mixture, with or without angiotatin (0 to 2.0 μmol/L) or
plasminogen (2 μmol/L), was added to the lower chamber while
5×10^4 cells in DMEM/10% FCS were added to the upper chamber.
Migration was allowed to proceed for 6 hours, a time frame in which
preliminary experiments had demonstrated that there was no signif-
icient effect on cell proliferation. After the cells adherent to the upper
side of the membrane were scraped away, the cells on the lower side
were fixed in 10% neutral buffered formalin overnight and stained
with Diff-Quick. Three random, nonoverlapping high-powered
(200×) fields (HPFs) were manually counted, and the mean of 6
HPFs was analyzed. The results were compared with the number of
cells that migrated in the presence of DMEM/10% FCS, which was
defined as baseline (100%).

Calculations
The data are expressed as mean±SEM. Differences were compared
using the 2-tailed Student’s t test for paired samples.

Results
Analysis of the Fusion Protein PAP/K1-3
PAP activity was measured from the medium of COS-1 cells
transfected with the APtag-4 and PAP/K1-3 vectors by using
p-nitrophenyl phosphate substrate. At 4 days after transfec-
tion, there was ≈5 μg/mL of recombinant protein expressed
in the serum-free medium.

To verify the construction of the secreted fusion protein, an
immunoblot analysis was performed. Detection of the 97-kDa
fusion protein with both anti-plasminogen (Figure 1A) and
anti-PAP (Figure 1B) verified the structure of our fusion
protein containing both PAP and the first 3 kringles of
plasminogen. As expected, the medium from APtag-4 trans-
fecions did not have a visible band with anti-plasminogen
but did have a 67-kDa protein that was detected by anti-PAP,
which represented nonfused PAP (not shown). Construction
of PAP/K1-3 was also verified by sequencing both strands of
the plasmid construct.

PAP/K1-3 Binds to Endothelial Cells and SMCs in
the Coronary Artery
In situ binding assays of the PAP/K1-3 fusion protein to
human coronary arteries localized angiotatin binding sites to
both the intimal and medial regions (Figure 2B), whereas the
protein obtained from the transfection of the vector APtag-4
showed minimal binding (Figure 2A). When compared with
endothelial cells (anti–CD31, Figure 2C) and SMCs (anti–α-
actin, Figure 2D) detected by immunohistochemistry, it is
apparent that most of the binding of the fusion protein
occurred in a distribution mirroring that of SMCs and was not
restricted to endothelial cells. Similar binding patterns of the
fusion protein were observed in arterioles and capillaries of
the adventitia (Figures 3 and 4).

The binding of the fusion protein was nearly completely
blocked with 10 mmol/L EACA (not shown), as well as with
a 50-fold molar excess of plasminogen (Figure 5). These
results indicate that angiotatin binds to a plasminogen
binding site and that the interaction is mediated via lysine
binding sites.

In situ binding analysis performed on atherectomy tissue
also revealed binding of the fusion protein to SMCs, as
identified by the anti–α-actin pattern (Figure 6A and 6B), and
was inhibited by excess plasminogen (Figure 6C). The fusion
protein did not bind to cellular components other than SMCs,
as shown in the hematoxylin-and-eosin–stained sections (Fig-
ure 6D). Binding of concentrated medium from APtag-4 transfections showed that there was no background cellular
phosphatase activity (Figure 6E).

PAP/K1-3 Binds to Human Aortic SMCs
The binding of various concentrations of the fusion protein
PAP/K1-3 to human aortic SMCs in vitro was determined.
Figure 7 shows a concentration-dependent increase in fusion
protein binding, whereas binding of PAP alone was minimal.

Effect of Angiostatin on the Proliferation and
Migration of Rabbit Aortic (Rb-1) SMCs
Rb-1 cells were exposed to various concentrations of HGF,
with or without FCS, for 24, 48, 72, or 96 hours. From these
preliminary experiments, it was found that the most signifi-
cant induction of proliferation occurred at a concentration of
20 ng/mL HGF, in the presence of FCS, at 96 hours (not
shown). Similarly, preliminary experiments demonstrated
that a concentration of 50 ng/mL HGF produced a maximal
effect on Rb-1 cell migration. These cells were allowed to
migrate for 6 hours, a time frame in which preliminary experiments had demonstrated there was no significant cell proliferation.

The effect of angiostatin (Angiogenesis Research Industries; 0.25 to 2.0 \( \mu \text{mol/L} \)) and plasminogen (2.0 \( \mu \text{mol/L} \)) on HGF-induced cell proliferation was determined (Figure 8). HGF (20 ng/mL) induced a 41% increase in cell number at 96 hours (\( P<0.01 \)). Angiostatin at 2.0 \( \mu \text{mol/L} \) significantly inhibited HGF-induced Rb-1 cell proliferation (\( P<0.01 \)). Plasminogen (2.0 \( \mu \text{mol/L} \)) had no effect on HGF-induced Rb-1 cell proliferation. In the absence of HGF, angiostatin at 2.0 \( \mu \text{mol/L} \) produced a 37% inhibition of proliferation (compared with DMEM/10% FCS alone, \( P<0.05 \); data not shown). The inhibitory effect of angiostatin on cell proliferation was confirmed by using the reduction of NBT (data not shown). No effect of angiostatin (2.0 \( \mu \text{mol/L} \)) on Rb-1 cell apoptosis was observed (data not shown).

Similarly, Figure 9 demonstrates the effect of angiostatin on HGF-induced Rb-1 cell migration. HGF (50 ng/mL) increased SMC migration by 239% (\( P<0.01 \)). Angiostatin significantly inhibited HGF-induced migration at a concentration of 0.5 \( \mu \text{mol/L} \) (\( P<0.05 \)), with maximal inhibition at 2.0 \( \mu \text{mol/L} \) (\( P<0.01 \)). Plasminogen (2.0 \( \mu \text{mol/L} \)) had no effect on HGF-induced migration. Angiostatin had no significant effect on migration that occurred in the absence of HGF (data not shown).

Discussion

Our study demonstrates that angiostatin, a novel inhibitor of angiogenesis, binds to SMCs and endothelial cells in the coronary artery. The binding to endothelial cells is consistent with the previous observation that the antiangiogenic effect of angiostatin is at least partially mediated through its inhibition of endothelial cell proliferation. This relatively modest effect (35% at 10 \( \mu \text{g/mL} \)), however, does not appear adequate to account for the dramatic effect that this agent has on inhibiting tumor growth and metastases. This apparent discrepancy led us to consider other potential cellular targets and mechanisms for the effect of angiostatin. The demonstration that the angiostatin fusion protein binds to SMCs and that angiostatin inhibits the HGF-induced proliferation and migra-
tion of SMCs may lead to an appreciation of novel cellular targets and molecular mechanisms for the antiangiogenic effect of angiostatin.

The role of SMCs in vasculogenesis, the embryological development of blood vessels from progenitor cells, has been defined in mice. The recruitment of SMCs to the wall of developing blood vessels is mediated by angiopoietin-1 binding to the TIE2 receptor and inhibited by angiopoietin-2. In contrast, the contribution of SMCs to angiogenesis, or the sprouting of new vessels from an existing network, is less clearly established. One potentially important role for SMCs in this process could be the elaboration of angiogenic factors that act in a paracrine manner to promote endothelial cell proliferation. SMCs are known to secrete a variety of direct and indirect angiogenesis factors, including HGF, vascular endothelial growth factor (VEGF), and transforming growth factor-β1 (TGF-β1). It has been suggested that pericytes and myofibroblasts, cells that play a supporting role in angiogenesis, may be derived from a population of SMCs. Although there is no direct evidence that SMCs are necessary for the formation of capillaries in the early stages of angiogenesis, the histological detection of proliferating SMCs in vessels elicited from a variety of angiogenic stimuli suggest that the formation of larger, mature, conduit-caliber vessels requires the participation of SMCs. This finding also supports the possibility that SMCs could be a target for antiangiogenic therapy. In addition to the present study showing that angiostatin inhibits SMC proliferation and migration, octreotide and TNP-470, known angiogenesis inhibitors, have both been demonstrated to have an antiproliferative effect on SMCs.

The inhibition of endothelial cell proliferation by angiostatin, but not plasminogen, suggests that there could be a specific angiostatin receptor on endothelial cells. Although preliminary studies are consistent with a specific, saturable endothelial receptor, this putative receptor has yet to be identified [note added in proof: Moser et al (Proc Natl Acad Sci U S A. 1999;96:2811–2816) have shown that angiostatin binds to the αβ ATP synthase in HUVECS]. Our studies suggest that angiostatin binds to a plasminogen receptor on SMCs, because the binding of the fusion protein could be inhibited by excess plasminogen and EACA. The effective concentration of angiostatin in our studies was 1 to 2 μmol/L, which is similar to its concentration in plasma. Even higher levels could be achieved during therapeutic administration of angiostatin. However, it should be noted that our studies do not exclude the possibility of additional binding interactions with other types of receptors or even with extracellular matrix proteins.

By occupying plasminogen receptor(s), such as annexin II and α-enolase, on the SMC surface, angiostatin might block the binding of plasminogen and its subsequent activation to plasmin. Plasmin is an important proteinase in angiogenesis by virtue of its ability to directly degrade matrix components and by its ability to activate matrix metalloproteinases. Plasmin releases proteolytically processed...
soluble products of VEGF-165 and -189 isoforms that retain mitogenic and vascular permeability-enhancing activity. Plasmin appears to cleave these forms of VEGF at the carboxy termini, thereby eliminating the heparin binding sites that are responsible for their retention to heparan sulfate proteoglycan reservoirs in the extracellular matrix or on plasma membranes. Plasmin can also release other direct or indirect modulators of angiogenesis from the extracellular matrix, such as basic fibroblast growth factor, or from cell surface receptors, like TGF-β. In addition to releasing angiogenic growth factors from the extracellular matrix, plasmin also proteolytically activates 2 precursor forms of growth factors to the active state, HGF and TGF-β1. As an example of the reciprocal cooperation between growth factors and plasmin formation in angiogenesis, VEGF, basic fibroblast growth factor, and angiogenin induce plasminogen activator expression and enhance pericellular plasmin generation by endothelial cells. Plasmin generation appears necessary for SMC migration, and therefore, the lack of pericellular plasmin activity could limit the ability of SMCs to participate in angiogenesis.

It is also possible that angiostatin inhibits both endothelial cell and SMC proliferation and migration by mechanisms that are independent from its ability to displace binding to plasminogen receptors. One potential mechanism would involve binding to c-met, the receptor for HGF, which is known to be expressed on both endothelial cells and SMCs. This theory is attractive because HGF is a potent angiogenic factor, inducing endothelial cells and SMCs to proliferate and migrate. Furthermore, HGF is strikingly similar to plasminogen, with 38% identity at the amino acid level and conservation of the kringle motifs, as well as the presence of a nonfunctional serine proteinase-like domain. In addition, the internal cleavage site that gives rise to the disulfide-linked, 2-chain, active form of HGF is identical to that of plasminogen. Thus, it is possible that angiostatin displaces the structurally homologous angiogenic factor HGF from binding to its cell surface receptor c-met on both endothelial cells and SMCs. The finding in the present study that angiostatin inhibits HGF-induced SMC proliferation and migration is consistent with this theory. Because HGF and c-met are expressed in vascular tissue, they interact in an autocrine and paracrine fashion, and angiostatin might inhibit the proliferation of SMCs in response to endogenous vascular levels of HGF. Indeed, in the present study, there...
was a trend toward angiostatin-induced inhibition of SMC proliferation and migration, even in the absence of exogenously added HGF.

In summary, these data demonstrate that angiostatin binds to SMCs in the coronary artery. Furthermore, angiostatin inhibits the effects of HGF, a stimulant of angiogenesis, on SMC proliferation and migration in vitro. These studies suggest that the SMC should be considered a potential cellular target for the effects of angiostatin and other inhibitors of angiogenesis.

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