Deficiency of the Cysteine Protease Cathepsin S Impairs Microvessel Growth


Abstract—During angiogenesis, microvascular endothelial cells (ECs) secrete proteinases that permit penetration of the vascular basement membrane as well as the interstitial extracellular matrix. This study tested the hypothesis that cathepsin S (Cat S) contributes to angiogenesis. Treatment of cultured ECs with inflammatory cytokines or angiogenic factors stimulated the expression of Cat S, whereas inhibition of Cat S activity reduced microtubule formation by impairing cell invasion. ECs from Cat S–deficient mice showed reduced collagenolytic activity and impaired invasion of collagens type I and IV. Cat S–deficient mice displayed defective microvessel development during wound repair. This abnormal angiogenesis occurred despite normal vascular endothelial growth factor and basic fibroblast growth factor levels, implying an essential role for extracellular matrix degradation by Cat S during microvessel formation. These results demonstrate a novel function of endothelium-derived Cat S in angiogenesis. (Circ Res. 2003;92:493-500.)

Key Words: angiogenesis ■ cathepsin S ■ wound healing ■ endothelial cells

Angiogenesis contributes importantly to tumor invasion, embryogenesis, and atherosclerotic plaque growth. This process depends on tightly controlled extracellular matrix (ECM) degradation, which permits endothelial cells (ECs) to penetrate the vascular basement membrane and bore into the surrounding interstitium, key steps in capillary growth. Both matrix metalloproteases (MMPs) and serine proteases participate in these processes. A recent study by Bergers et al1 has suggested involvement of MMP-9 in the switch from vascular quiescence to angiogenesis by the release of vascular endothelial growth factor (VEGF)-A, an angiogenic factor, from an ECM-bound reservoir. The absence of MMP-9 reduces bone growth plate angiogenesis in mice because of the delayed release of a stimulator of angiogenesis from hypertrophic cartilage.2 Serine proteases play a similar role in angiogenesis. Deficiency of the urokinase plasminogen activator has demonstrated severely impaired postinfarction myocardial angiogenesis due to impaired EC invasion.3 However, MMPs and serine proteases can play dual roles in angiogenesis, and considerable evidence points to the operation of non-MMP in this process. MMPs generate molecules with antiangiogenic activity (eg, endostatin and angiotatin). Thus, MMP inhibition might stimulate as well as inhibit angiogenesis. A recent study has revealed that mice null for integrin α1, an inhibitor of MMP synthesis, exhibit reduced tumor vascularization due to their increased plasma levels of angiotatin.4 Similarly, although plasminogen deficiency severely impaired keratinocyte migration in mice, these mice display no differences in inflammatory cell infiltration, fibroblast migration, or neovascularization, suggesting that angiogenesis in this setting does not depend on the plasminogen activator or plasmin.5 Deficiency in the various components of the urokinase plasminogen activator system does not alter the reendothelialization of denuded vessels and tumor vascularization in mice.6

Because these data suggest angiogenic pathways independent of MMPs or serine proteases, we considered in the present study the hypothesis that cysteine proteases participate in angiogenesis. Numerous studies have demonstrated the expression of cysteine proteases in malignant carcinoma,7 but heretofore no direct evidence has indicated their function in vessel growth. By using genetically altered mice, the present study demonstrates evidence of an important role for cathepsin (Cat) S in aspects of angiogenesis both in vitro and in vivo.

Materials and Methods

EC Culture

Human saphenous vein ECs were isolated and subcultured at passages 2 to 3 in medium 199. Cells were stimulated with interferon-γ (IFN-γ, 400 U/mL, Becton Dickinson), interleukin-1β (IL-1β, 10 ng/mL, Becton Dickinson), tumor necrosis factor-α (TNF-α, 10 ng/mL, Becton Dickinson), basic fibroblast growth factor (bFGF, 5 ng/mL, R&D Systems), or VEGF (10 ng/mL, . . .
Endogen) overnight and then used for immunoblot analysis, RNA isolation, [3H]-JPM labeling, and cysteine protease immunoprecipitations as described. Bovine aortic ECs (BAECs) were established in culture from the thoracic aorta of the fetal calf (passages 3 to 6) and were cultured in 10% FBS DMEM.

**Mouse EC Isolation**

Mouse ECs were isolated as previously described. Briefly, mouse lung lobes were minced and incubated with collagenase (2 mg/mL, Calbiochem) at 37°C in DMEM for 45 minutes, followed by separation with 60 μL of Dynabeads (Dynal Biotech) coated with anti-mouse CD31 (Pharmingen). Cells on beads were separated using a Dynal M-PC-1 magnetic separator and cultured on 0.1% gelatin–coated plates. After the cells reached confluence (5 to 9 days), they were separated again with rat anti-mouse CD102 (ICAM-2, Pharmingen) Dynabeads. The phenotype and morphology of these ECs remained stable over 10 passages in culture, consistent with previously reported data.

**In Vitro Angiogenesis Assays**

Established 2D and 3D cultures of BAECs were used to determine whether Cat S plays a role in EC tubule formation in vitro. For the 2D cultures, BAECs suspended in 10% FBS DMEM were plated on type I collagen gels. After 24 hours of incubation, bFGF (10 ng/mL) was added in the presence or absence of morpholinurea leucine-homophenylalanine-vinylsulfone-phenyl (LHVS, 8 nmol/L, a Cat S–selective inhibitor), papain family cysteine protease inhibitor E64d (20 μmol/L), a Cat S selective inhibitor, or tissue inhibitor of metalloproteinase-1 (TIMP-1) (100 ng/mL, Oncogen). The media were replaced every other day.

For the 3D cultures, BAECs were suspended in a neutralized solution of collagen type I (1.5 mg/mL) in 10× DMEM. After gel formation, DMM containing 10% FBS with or without bFGF (10 ng/mL) in the presence or absence of the endogenous Cat S inhibitor cystatin C (0.5 μg/mL, 1 μg/mL, Biotrend), LHVS (8 nmol/L, E64d (20 μmol/L), or the MMP inhibitor TIMP-1 (100 ng/mL) was added and incubated for 6 days. Six microscopic fields were randomly chosen to determine the tubule length by use of an NIH Image Analyzer. To examine the structure of these tubules, collagen gels were fixed in 10% formaldehyde and embedded in OCT, and 10-μm cross sections were stained with hematoxylin and eosin.

**Cell Adhesion Assay**

The 96-well plates were coated with collagen type I (5 μg/50 μL in 0.02% acetic acid per well), fibronectin (1 μg/50 μL PBS per well), or vitronectin (1 μg/50 μL PBS per well), and nonspecific binding was blocked with 0.3% BSA. A BAEC suspension in serum-free DMEM containing 0.3% BSA in the presence or absence of LHVS (10 nmol/L) or E64d (20 μmol/L) was plated at 4×10^4 cells per well. After 1 hour of incubation, the number of attached cells for each condition was counted in six randomly chosen fields of duplicate wells.

ECs isolated from mouse lungs were seeded onto 96-well plates (2×10^4 cells per well) coated with either fibronectin (1.0 μg/cm²), collagen IV (2.0 μg/mL), or BSA (1% [wt/vol]) in PBS. After 20 minutes, adhered cells were stained with crystal violet. Adhesion was quantified by the 590-nm optical density of the extracted crystal violet dye.

**Cell Proliferation Assay**

BAEC proliferation was assessed with the Cell Titer 96A Q Assay kit according to the instructions of the manufacturer (Promega). BAECs were plated on collagen-coated 96-well plates at 5000 cells in 100 μL of 0.3% BSA DMEM per well. After 2 hours of incubation, medium was replaced with DMEM containing 10% FBS or bFGF (10 ng/mL) in the presence or absence of LHVS (10 nmol/L) or E64d (20 μmol/L), followed by another 3 days in culture. Mouse ECs were diluted from 10⁶ to 10² per well on 96-well plates with DMM and cultured overnight at 37°C. Then 20 μL of a mixture of tetrazolium compound and phenazine methosulfate was added, and the absorbance was determined at 492 nm.

**Migration and Invasion Assays**

BAEC migration across a polycarbonate membrane containing 8-μm pores was performed with Transwell (Costar) 12-well plates. The membrane was coated with type I collagen (50 μg/mL). BAECs were seeded on the inner chamber at 3×10⁴ cells per 100 μL in 0.3% BSA DMMEM. BAEC suspension containing LHVS (10 nmol/L) or E64d (20 μmol/L) was plated in the upper chambers. The inner chamber was placed into the outer chamber containing 600 μL of 0.3% BSA DMMEM supplemented with 0.25% FBS or bFGF (30 ng/mL) in the presence or absence of LHVS or E64d and incubated for 6 hours. The cells that migrated onto the outer side of the membrane were fixed and stained with Dif Quick stain (American Hospital Supply Corp.). The number of the migrated cells was counted in six to eight randomly chosen fields. The invasion assay was conducted in a similar fashion but with a coating of fibrillar collagen and 12 hours of incubation.

The mouse EC invasion assay used Matrigel and type-I collagen gels. ECs were resuspended in serum-free medium at 1×10⁶/mL. Invasion chambers were coated with growth factor–reduced Matrigel (100 μg/cm², Collaborative Research) for 4 hours at 37°C or with polymerized type I collagen gels overnight at room temperature, followed by seeding with 0.5 mL of 1×10⁶/mL cell suspension. The lower chamber was supplied with 0.8 mL of complete medium. The invasion assay was carried out at 37°C for 24 hours. Cells were stained by crystal violet, extracted into 0.75 mL ethanol, and quantified by measuring the optical density at 590 nm.

**Mouse EC Elastase and Collagenase Assays**

For elastase activity assay, ECs from either wild-type or Cat S–deficient (Cat S−/−) mice were seeded on 24-well plates and cultured in DMM until confluence. Fresh medium with 300 μg [14C]elastin was added to each well in the presence or absence of mouse bFGF (5 ng/mL) or IFN-γ (500 U/mL). After 2 days of incubation at 37°C, culture media were collected to measure degraded elastin as described. For collagenase assays, mouse ECs were seeded on 24-well plates precoated with [14C]collagen type IV (1 μCi/mL, NEN). Culture media were collected in 2 days, and soluble radioactivity was measured to determine collagenolysis. Three independent experiments were performed for both elastase and collagenase assays.

**Cysteine Protease, MMPs, and Plasminogen Expression in Cat S−/− ECs**

To examine whether the deficiency of Cat S affects other protease expression in ECs, Cat S−/− and wild-type mouse ECs were stimulated with IFN-γ (500 U/mL) for 24 hours. Cells were collected into 1 mL PBS and incubated with 1 μL of [3H]-JPM to visualize cysteine protease expression. To assess MMP and plasminogen expression, ECs were lysed in radioimmunoprecipitation (RIPA) buffer. Total proteins (40 μg) were separated on 10% SDS-PAGE for immunoblot with the use of rabbit anti-rat MMP-2 monoclonal antibody (1:1000, Oncogen). Identical loading was used on separate gels to determine membrane-type (MT)1 MMP, MT2-MMP, and plasminogen expression (1:1000, Oncogen).

**Wound-Healing–Associated Neovascularization**

Cat S−/− mice on a C57/B16 background were generated by backcrossing the C57/B16/129S Cat S−/− mice with C57/B16 mice for >11 generations. Wild-type mice (C57/B16, Jackson Laboratory, Bar Harbor, Me; n=20) and Cat S−/− mice (C57/B16 background, n=20) were anesthetized and injured with a 6-mm biopsy punch as described. After 10 days of healing, skin was removed, OCT-embedded, sectioned, and immunostained with antibodies against CD31 to visualize microvessels around the incisions. CD31-positive microvessels were counted under a light microscope centered at the wound region (2-mm² grid, ×10 magnification). To examine the effect of Cat S on VEGF and bFGF activation and signaling,
wounded skin sections were pulverized and either lysed in RIPA buffer for 1 hour or incubated in serum-free DMEM for 24 hours. Both VEGF and bFGF were measured by ELISA according to the manufacturer (R&D Systems).

Immunohistochemistry
Serial cryostat sections (6 μm) of wounded mouse skin were used to colocalize Cat S with cell types. Cell type–specific antibodies (rat anti-murine MAC-3 [1:1000] and CD-4 [1:100] from Pharmingen and rabbit anti-murine keratin-1 [1:250] from R&D Systems) were used for the first staining, followed by appropriate secondary antibody and avidin-biotin complex (ABC) conjugated with horseradish peroxidase. To detect Cat S expression in these cells, the same sections were treated with a blocking kit (Vector). Rabbit anti-mouse Cat S antibody (1:100) was applied, followed by anti-rabbit secondary antibody and ABC conjugated with alkaline phosphatase. Staining was developed with Fast Blue. To colocalize Cat S to ECs in microvessels at the site of wound healing, double immunofluorescence staining was performed using 6 μm frozen sections. Cat S antibody (1:200), biotinylated secondary antibody, and Texas red–conjugated streptavidin (Amersham) were used. After application of the blocking kit (Vector), anti-mouse CD31 monoclonal antibody (1:600, Pharmingen), biotinylated secondary antibody, and streptavidin-FITC (1:100, Amersham) were used. Cell nuclei were stained with bisbenzimide (blue fluorescence, Calbiochem).

Results
Expression of Cat S in ECs
Primary cultured human saphenous vein ECs had negligible levels of Cat S mRNA. Exposure to IFN-γ, IL-1β, or TNF-α caused accumulation of Cat S transcript. However, the angiogenic factors VEGF and bFGF had limited effects on Cat S transcription (Figure 1A). For detection of Cat S protein in ECs, [125I]JPM ethyl ester, the cell-permeable active site probe for cysteine proteases, was used (Figures 1B and 1C). This irreversible probe labels Cat S immediately on activation and does not affect Cat S immunoreactivity.8 IFN-γ increased EC secretion of active Cat S 20-fold (mean of three independent experiments by densitometry measurement; Figure 1B, left). Cell-associated Cat S increased in parallel (Figure 1C). VEGF and bFGF also induced Cat S secretion up to 20- and 3-fold, respectively (mean of three independent experiments; Figure 1B, right). Compared with other stimuli, TNF-α and bFGF substantially reduced the production and secretion of the endogenous Cat S inhibitor cystatin C by ECs (Figure 1D). ELISA of EC culture medium for cystatin C protein corroborated these observations (data not shown).

We further studied Cat S expression in BAECs. In contrast to human ECs, quiescent BAECs contained Cat S message, as shown by Northern blots probed with human Cat S cDNA (94% sequence shared with bovine Cat S).13 TNF-α or VEGF induced Cat S transcript levels by 3- and 6-fold, respectively (mean of two independent experiments by densitometry analysis, Figure 2A). TNF-α–treated BAECs secreted twice as much active Cat S protein as did control cells (mean of
Cat S Facilitates Formation of Microtubes by ECs

In the presence of bFGF, BAECs formed capillary-like tube structures inside the gel in 6 days (Figure 3A). Cross sections of the collagen gel demonstrated the tube structure of these preparations (Figure 3B). This tubule formation likely involves ECM remodeling by proteases, including Cat S, consistent with elevated Cat S production and secretion by ECs in response to bFGF (Figures 1 and 2). To define the role of Cat S in this process, recombinant cystatin C was added to the cultures. Cystatin C inhibits a spectrum of cysteine proteases but has the highest affinity for Cat S. In this in vitro assay, cystatin C inhibited bFGF-induced tubule formation in a concentration-dependent manner (Figures 3A and 3C). At a physiological concentration (1 μg/mL), cystatin C blocked >80% of tubule formation compared with control conditions. Heat-inactivated cystatin C (1 μg/mL) showed no inhibitory effect (Figure 3C). In addition, E64d (20 μmol/L) and LHVS (8 nmol/L, a concentration that retains Cat S selectivity) reduced tubule formation by >75% and >50%, respectively. Compared with LHVS, E64d (an inhibitor for a broader spectrum of cysteine proteases) inhibits tubule formation by BAECs to a greater extent (Figure 3C). In contrast to results from several in vivo assays, we demonstrated little effect of TIMP-1 (100 ng/mL) on bFGF-induced BAEC tubule formation under our conditions (Figure 3C). Our data agree with the results of Anand-Apte et al.

For study of the molecular mechanism by which Cat S facilitates microtubule formation, BAECs were used for cell adhesion, proliferation, and migration and invasion assays. Compared with untreated control BAECs, LHVS (8 nmol/L)-treated BAECs demonstrated no significant difference in adhesion to type IV collagen (P = 0.796), fibronectin (P = 0.218), or vitronectin (P = 0.797) in six independent experiments. Similarly, BAEC proliferation showed no difference in cells treated with or without 8 nmol/L LHVS either in the presence (P = 0.980) or absence (P = 0.111) of bFGF. LHVS treatment also did not significantly change cell migration under either condition studied (10% FBS [P = 0.2] or 30 ng/mL bFGF [P = 0.8]), nor did treatment with E64d affect cell adhesion, proliferation, or migration (data not shown). However, both LHVS (8 nmol/L) and E64d (20 μmol/L) significantly impaired BAEC invasion through a polymerized type I collagen–coated membrane in the presence of either 0.25% FBS or 10 ng/mL bFGF (Figure 3D).

BAECs also formed capillary-like tube structures on 2D collagen gels in the presence of bFGF within 6 days. The profile of protease inhibition in this 2D assay resembled those in the 3D assay (data not shown).

Reduced Elastolytic and Collagenolytic Activities in ECs From Cat S−/− Mice

Cat S is one of the most potent elastases known. To determine whether the deficiency of Cat S affects EC elastolytic and/or collagenolytic activities, we isolated ECs from both Cat S−/− and wild-type mouse lungs. ECs from Cat S−/− and K−/− mice showed significantly reduced degradation of [3H]elastin. In agreement with the data from Figures 1 and 2, treatment of mouse ECs with bFGF and IFN-γ significantly augmented the elastolytic activity in ECs from wild-type but not Cat S−/− mice, suggesting an important role for Cat S in this process (Figure 4A). Compared with ECs from wild-type mice, ECs from Cat S−/− mice showed similarly reduced degradation of [3H]type IV collagen (Figure 4B), although Cat S has rather weak collagenolytic activity compared with Cat L or K. Interestingly, ECs from Cat S−/− mice did not show augmented collagenolysis or elastolysis in response to angiogenic peptides (Figures 4A and 4B).
Impaired Matrigel and Collagen Gel Invasion of ECs From Cat S<sup>−/−</sup> Mice

As described for BAECs, ECs from mouse lungs were used for cell adhesion assays atop type IV collagen, fibronectin, and BSA. ECs from Cat S<sup>−/−</sup> mice and control mice adhered poorly to BSA-coated plates and showed no differences in proliferation or adhesion on either type IV collagen–coated or fibronectin-coated plates (data not shown).

However, compared with wild-type ECs, Cat S<sup>−/−</sup> ECs showed a lower ability to invade Matrigel ($P<0.003$, Figures 5A through 5C). Compared with control cells, $<$30% of Cat S<sup>−/−</sup> cells invaded the Matrigel. Similar to the observations involving BAECs, ECs from Cat S<sup>−/−</sup> mice showed impaired invasion into type I collagen gel (Figure 5D), although to a lesser extent than into Matrigel (Figure 5C). This difference probably reflects the relatively weak collagenolytic activity of Cat S. Therefore, deficiency of Cat S clearly impairs EC invasion of the basement membrane as well as interstitial matrix. We further tested the possibility that Cat S deficiency affects expression of other proteases in ECs that may influence ECM degradation and invasion. Cysteine protease active site labeling (Figure 6A) showed a deficiency of Cat S but not Cat B or L in Cat S<sup>−/−</sup> compared with wild-type mice. Western blot analysis of mouse EC lysates for plasminogen and MMPs (MMP-2, MT1-MMP, and MT2-MMP) showed no effect of the lack of Cat S on the expression of any of these enzymes (Figures 6B through 6E). Therefore, the reduced invasion (Figure 5) and impaired elastase/collagenase activity (Figure 6) in Cat S<sup>−/−</sup> ECs result mainly from the lack of Cat S, suggesting an important role of this protease in ECM degradation and microvessel growth.

Impaired Microvessel Development During Wound Healing in Cat S<sup>−/−</sup> Mice

The healing of experimental wounds furnishes an established method for the study of angiogenesis in vivo. Skin can be...
injured by incision or by biopsy punches,\textsuperscript{15} permitting ready measurement of microvessel growth in the neodermis and neoeipidermis. Compared with unwounded skin, wounded skin showed considerable Cat S expression (Figure 7A). Control experiments using nonimmune serum or experiments probing Cat S\textsuperscript{−/−} mouse skin sections have verified the specificity of the mouse Cat S antibody (data not shown). Double immunolabeling with cell type–specific antibodies and Cat S antibody showed that all or almost all macrophages and CD4-positive T lymphocytes as well as a small fraction of keratinocytes showed immunoreactive Cat S (purple, Figure 7B). Furthermore, we detected no difference in leu-

**Figure 6.** Expression of cysteine proteases, MMPs, and plasminogen by ECs from Cat S\textsuperscript{−/−} and Cat S\textsuperscript{+/+} mouse lungs. A, Cysteine protease \([^{125}\text{I}]\text{JPM}\) active site labeling of bFGF stimulated mouse ECs. Deficiency of Cat S did not affect the expression of Cat B or L. B through E, Western blot analysis of mouse EC lysates for MMP-2, MT1-MMP, MT2-MMP, and plasminogen, respectively. Molecular weight markers are indicated on the left.

**Figure 7.** Reduction of wound-healing–associated neovascularization in Cat S\textsuperscript{−/−} mice. A, Skin wounding induces Cat S expression. The expression of this protease in skin was localized to the wounded area. B, Cat S expression in areas of wound healing. Double immunostaining with cell type–specific antibodies (red) and rabbit anti–Cat S antibody (blue) showed colocalization (purple) of Cat S with most macrophages (Mac-3, MΦ), T lymphocytes (CD4), and a small fraction of keratinocytes (keratin-1). The specificity of Cat S immunostaining was confirmed using normal rabbit IgG (NI-IgG, upper right). Magnification is indicated. C, CD31-positive microvessels were significantly impaired in Cat S\textsuperscript{−/−} mice (left) compared with control mice (right). Bar graph shows quantitative analysis of microvessel formation in area of wound healing. Cat S\textsuperscript{−/−} mice developed significantly fewer microvessels than did Cat S\textsuperscript{+/+} mice (n=20 per group, \(P<0.005\)). D, Colocalization (orange) of Cat S (red) and microvessel ECs (CD31, green). EC nuclei were stained with bisbenzimide (blue).
kocyte infiltration or the number of keratinocytes in healing wound regions in Cat S−/− mice versus control mice. These observations may explain the unchanged wound-healing rates of these mice. However, consistent with the in vitro tube formation assays, Cat S−/− mice compared with wild-type mice had significantly impaired microvessel development at sites of wound healing (Figure 7C). Furthermore, immunofluorescent staining of wounded skin sections demonstrated that microvascular ECs at the site of wound healing do express immunoreactive Cat S (Figure 7D). We observed an 80% reduction in microvessels in healing wounds associated with Cat S−/− mice compared with control mice (n = 20/group, \(P < 0.005\); Figure 7C).

The defective microvessel growth found in Cat S−/− mice may result from either impaired ECM remodeling or altered VEGF and bFGF activation or release from the ECM due to the absence of Cat S. Latent forms of VEGF and bFGF bind to extracellular matrix, and proteolytic activation can release them.\(^20\) To examine the involvement of Cat S in VEGF or bFGF activation and release, sections of wounded skin were lysed in cell lysis buffer or incubated in serum-free cell culture medium to detect both total growth factors and their released forms. Total VEGF (75.2 ± 16.6 versus 70.0 ± 23.1 pg/mg protein), total bFGF (179.4 ± 22.3 versus 156.6 ± 43.7 pg/mg protein), and released VEGF (52.7 ± 15.5 versus 48.8 ± 24.7 pg/mg protein) did not differ significantly between wild type and Cat S−/− mice.

**Discussion**

Neovessel formation and growth requires local degradation of the endothelial basement membrane, followed by penetration of the ECM in the perivascular interstitium. Both MMPs and serine proteases may participate in these processes. Although these proteases can regulate angiogenesis both positively and negatively, evidence reviewed above suggests that other proteolytic pathways also play a role in angiogenesis. In this regard, the role of lysosomal cysteine proteases remains uncertain.\(^7\)

Cat S has several attributes that render it a good candidate for matrix degradation during angiogenesis. First, extracellular elastin has been associated with angiogenesis and tumor growth. Elastin degradation products promote angiogenesis in vivo,\(^21\) and elastin–cancer cell interaction is important for tumor progression.\(^22\) Extracellular collagen is a major constituent of basement membranes and participates in the initiation and maintenance of angiogenesis.\(^23\) Cat S possesses potent elastolytic and nonfibrillar collagenolytic activity. It retains activity at a neutral pH that prevails in the extracellular milieu.\(^11\) Recent evidence has indicated that cells may produce vacuolar-type H⁺-ATPase components to acidify the pericellular milieu, augmenting the ability of lysosomal cysteine proteases to break down ECM proteins locally.\(^24\) Second, human ECs markedly increase their Cat S expression and secretion when stimulated by inflammatory cytokines and angiogenic factors. Notably, either VEGF or bFGF augmented Cat S expression more potently than IFN-γ in human or bovine ECs with stimulation between 5- and 20-fold (Figures 1B and 2B).

The present study used a gene-targeting strategy to establish a novel role for Cat S in neovascularization. We provide both in vitro and in vivo evidence that Cat S promotes EC invasion and further neovessel growth. BAECs form tubule structures in and on type I collagen gels in response to bFGF (Figures 3A and 3B).\(^10,11\) We chose BAECs for the collagen lattice gel assay because these cells express substantial active Cat S in vitro in the absence of cytokines, unlike human ECs, which require stimulation with cytokines such as IFN-γ. The net effect of IFN-γ on angiogenesis results from a balance of opposing effects.\(^25\) We proved the involvement of Cat S in tube formation by showing that synthetic cysteine protease inhibitor E64d, natural Cat S inhibitor cystatin C, and selective Cat S inhibitor LHVS all reduced tubule length (Figures 3A and 3C). In contrast to Cat S inhibitors and in agreement with earlier findings,\(^18\) the MMP inhibitor TIMP-1 did not block microtubule formations in vitro (Figure 3C). Others have suggested the involvement of MT-MMPs sensitive to TIMP-2 or -3 in tube formation.\(^18\) Although beyond the focus of the present study, these data suggest that multiple proteases may mediate EC microtubule formation in this model.

One of the earliest events of angiogenesis is the degradation of vascular basement membrane. Skin wounds not only provide a well-established angiogenesis model but also allow us to evaluate the function of Cat S, inasmuch as inflammatory cytokines and angiogenic factors at play during wound healing regulate the expression and activity of this protease. Granulation tissues in healing wounds contain numerous microvessels. In agreement with our in vitro observations, Cat S deficiency profoundly impairs wound-healing–associated microvessel development (Figure 7C), although wound healing occurred at similar rates in wild-type and Cat S−/− mice. Precedents exist for the dissociation of these two processes.\(^26\)

These observations suggest that Cat S degrades the capillary basement membrane and paves the way for neovessel formation in angiogenesis. LHVS or E64d treatment limits the ability of BAECs to invade a collagen matrix (Figure 3D). ECs from Cat S−/− mice showed an impaired ability to degrade elastin and type IV collagen (Figure 4) and a markedly reduced invasion across the Matrigel or type I collagen gel membranes (Figure 5). Our data demonstrate that inhibition or deficiency of Cat S has no effect on EC proliferation, migration, adhesion, membrane-bound VEGF and bFGF release/activation, and other protease expression (Cat B, Cat L, MMPs, and plasminogen; Figure 6), suggesting a likely role for Cat S in mediating mouse EC invasion through Matrigel or collagen matrix. It is possible that Cat S may have complex roles in angiogenesis, which are well appreciated in the case of MMPs. Just as MMPs may produce angiogenic inhibitory peptides such as angiotatin and endostatin from plasminogen and collagen, the cysteine protease Cat L also produces endostatin from collagen.\(^27\) Therefore, a potential dual role of Cat S in microvessel formation merits consideration. Regardless, our in vitro and in vivo observations demonstrate a novel function for cysteine proteases in aspects of angiogenesis. Cat S was localized in atheroma\(^8\) and malignant tissues\(^7\) (both processes involve
substantial neovascularization), rendering it plausible that inhibition of Cat S may impair pathogenesis. In view of the ready availability of small molecule inhibitors of cysteine proteases, these findings have implications for cancer therapy and the regulation of wound healing as well as atherosclerotic plaque progression and complication.

Acknowledgments
This study was supported by grants from the National Heart, Lung, and Blood Institute to Drs Shi (HL-60942), Chapman (HL-04055 and HL-48261), and Libby (HL-34636). The authors thank Karen Williams for reading the manuscript. We thank Maria Muszynski, Eugenia Shvartz, and Irina Chulsky for their technical assistance.

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Circ Res. 2003;92:493-500; originally published online February 6, 2003;
doi: 10.1161/01.RES.0000060485.20318.96
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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