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Matrix Metalloproteinases Generate Angiostatin: Effects on Neovascularization

Lynn A. Cornelius,* Leslie C. Nehring,* Elizabeth Harding,‖ Mark Bolanowski,‖ Howard G. Welgus,* Dale K. Kobayashi,¶ Richard A. Pierce,* and Steven D. Shapiro †‡§¶

Angiostatin, a cleavage product of plasminogen, has been shown to inhibit endothelial cell proliferation and metastatic tumor cell growth. Recently, the production of angiostatin has been correlated with tumor-associated macrophage production of elastolytic metalloproteinases in a murine model of Lewis lung cell carcinoma. In this report we demonstrate that purified murine and human matrix metalloproteinases generate biologically functional angiostatin from plasminogen. Macrophage elastase (MMP-12 or MME) proved to be the most efficient angiostatin-producing MMP. MME was followed by gelatinases and then the stromelysins in catalytic efficiency; interstitial collagenases had little capacity to generate angiostatin. Both recombinant angiostatin and angiostatin generated from recombinant MME-treated plasminogen inhibited human microvascular endothelial cell proliferation and differentiation in vitro. Finally, employing macrophages isolated from MME-deficient mice and their wild-type littermates, we demonstrate that MME is required for the generation of angiostatin that inhibits the proliferation of human microvascular endothelial cells. The Journal of Immunology, 1998, 161: 6845–6852.

It has long been recognized that the removal of certain primary tumors, in both experimental models (1–3) and clinical practice (4), can be followed by the rapid growth of dormant metastases. In searching for a factor that may mediate this response, O’Reilly recently isolated an angiogenesis inhibitor termed angiostatin, a fragment of plasminogen containing kringle regions 1–4 (3). Using a murine model of Lewis lung cell carcinoma (LLC),3 these investigators demonstrated that s.c. implanted LLC tumors generated angiostatin, which prevented the neovascularization of lung metastases and maintained them in a dormant state. Upon removal of the primary tumor, presumably the source of angiostatin, lung metastases resumed angiogenesis and grew rapidly.

In addition to these findings, angiostatin was shown to be produced in vitro upon exposure of plasminogen to pancreatic elastase; the source of endogenous proteolytic activity for the in vivo generation of angiostatin in the carcinoma was not identified. In recent in vitro studies, human prostate carcinoma cells were found to possess serine proteolytic capacity capable of converting plasminogen to angiostatin (5). Further work identified two components present in carcinoma cells that provided sufficient protease activity to generate angiostatin: urokinase and free sulfhydryl donors (6).

Recently, however, Dong et al. (7) demonstrated that generation of angiostatin in the LLC model was not caused by tumor cell proteases but, rather, was associated with the presence of macrophages in the primary tumor. Furthermore, they found that angiostatin activity, as measured by inhibition of endothelial cell proliferation, was correlated with the presence of EDTA-inhibitable elastolytic activity, presumably the matrix metalloproteinase murine macrophage elastase (MME or MMP-12) (8–10). Along these lines, a recent communication (11) described the angiostatin-converting enzyme activities of MMP-7 (matrilysin) and MMP-9 (gelatinase B, 92-kDa gelatinase). The biologic activity of the MMP-generated products was not determined, however.

MMPs represent a family of structurally related enzymes with catalytic activity that is dependent upon coordination of zinc and with catalysis that is specifically inhibited by the tissue inhibitors of metalloproteinases (TIMPs) (12, 13). Although MMPs possess a broad capacity to degrade extracellular matrix components, these proteases are capable of cleaving nonmatrix proteins that regulate a variety of biologic processes. For example, MME has been shown to cleave α1-antitrypsin, releasing a 4-kDa fragment that is chemoattract for neutrophils (14). MMPs have also been shown to possess the capacity to cleave latent TNF-α, releasing the biologically active 17-kDa TNF-α from the surface of cells (15). In this report we have studied the relative capacities of several MMPs to act upon plasminogen to generate angiostatin, and most importantly, we have investigated the effect of this product on human endothelial cell proliferation and in an in vitro assay of angiogenesis.

Materials and Methods

Reagents

Purified MMPs were obtained from the following sources. Recombinant mouse (MME) (9) and human (HME) (10, 16) macrophage elastase and MMP-7 (17) were expressed and purified from Escherichia coli to homogeneity in our laboratory. E. coli-derived enzymes were spontaneously active; that is, only their catalytic domains were expressed. Native MMP-1 (18) and MMP-9 (19) were purified in our laboratories (H.G.W. and

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3 Abbreviations used in this paper: LLC, Lewis lung carcinoma; MME, murine macrophage elastase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; HME, human macrophage elastase; K1–4, K1–3, K4, human plasminogen kringle regions 1–4, 1–3, and 4; HPg, human plasminogen; MECs, human dermal microvascular endothelial cells; EBM, endothelial cell basal medium; bFGF, basic fibroblast growth factor.
S.D.S.); MMP-3 was a gift from V. M. Baragi, Parke-Davis (New York, NY). MMP-8 and MMP-13 were provided by Monsanto-Searle (St. Louis, MO). Native enzymes were activated with aprotinin (final concentration, 100 kallikrein inhibitor units/ml) or followed by SDS-PAGE. In separate incubations, the serine proteinase inhibitor aprotinin (1 mg/ml) was added to the reaction mixture at 37°C for various times as indicated. The reaction was stopped by addition of Ac-Pro-Leu-Gly-S-Leu-Leu-Gly-OEt. Hydrolysis of this thiopeptidyl substrate (Bachem Bioscience, King of Prussia, PA) by metalloproteinases was determined as described previously (23). The accuracy and correspondence of these methods were confirmed further by reverse phase HPLC amino acid analysis of the purified protein. MMPs were used at equimolar concentrations (5 × 10⁻⁷ M) based on the TIMP inhibition assay.

Acute inflammatory granulocytes (kneige region 1–4, K1–4) were distinguished in murine cell cultures by the use of specific antibodies to CD11b or CD11c (Novocastra, Newcastle-Upon-Tyne, UK). The purity of the HPg was determined by acetic acid/urea PAGE. The purity of the HPg was >98%. Glu-plasminogen and <2% Lys-plasminogen, as determined by acetic acid/urea PAGE. The purity of the HPg was further verified in our laboratory by submitting it to fast protein liquid chromatography and confirming a sole peak on elution that, by protein electrophoresis, corresponded to HPg at 90 kDa.

Cleavage of plasminogen by MMPs

Each active MMP was incubated with 10 µg of HPg (final concentration, 4 µM) in buffer (300 mM Tris, 60 mM CaCl₂, and 90 mM NaCl, pH 7.5) at 37°C for various times as indicated. The reaction was stopped by addition of 25 mM DTT in protein running buffer (400 mM Tris (pH 7.4), 1.5% glycglycerol, 1% gelatin, and 2% SDS). Proteins were then transferred to a polyvinylidene difluoride membrane and blocked with 5% nonfat dry milk in TBS buffer at 4°C for 1 h. Blots were then incubated with specific primary antibodies and, following washing, with biotinylated secondary antibodies and then horseradish peroxidase-labeled streptavidin. Films were exposed to film. N-terminal sequence analysis of the first 15 amino acids confirmed the purity and presentation of K₁.

The purity of the HPg was further verified in our laboratory by submitting it to fast protein liquid chromatography and confirming a sole peak on elution that, by protein electrophoresis, corresponded to HPg at 90 kDa.

N-terminal amino acid sequence analysis of plasminogen cleavage products

Amino acid sequence analysis was performed on the major protein bands produced by purified MME cleavage of HPg. Ten nanograms of HPg was incubated with 250 ng of MME for 3 h at 37°C and subsequently resolved by 10–12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Applied Biosystems, Foster City, CA), visualized with 0.1% Coomassie blue, excised, and sequenced by automated Edman degradation using an Applied Biosystems 473 Sequenator.

Isolation and culture of human microvascular endothelial cells (MECs)

MECs were isolated from neonatal foreskins by a method modified from that of Kubota et al. (25). Briefly, neonatal foreskins were incubated overnight with 500 U/ml of Dispase II (Collaborative Biomedical, Bedford, MA), the epidermis was removed, and gentle pressure was exerted on the remaining dermis with the plunger of a tuberculin syringe, releasing vascular fragments. The vascular fragments were then centrifuged, and the pellet was resuspended in human endothelial cell basal medium (EBM; Clonetix, San Diego, CA) containing 10% FCS (Irvine Scientific, Irvine, CA), 10 ng/ml epidermal growth factor (Clonetix), 1 µg/ml hydrocortisone acetate (Sigma), 5 × 10⁻⁵ M dibutyryl cAMP (Sigma), 2 × 10⁻³ M glucose (Irvine, and 100 µM penicillin and 100 µg/ml streptomycin (Sigma). Cells were cultured at 37°C in 5% CO₂ in gelatin-coated tissue culture plastic. Endothelial cell cultures were identified as having cobblestone cell morphology.

Human microvascular endothelial cell proliferation assay

MECs, isolated as described above, were maintained in culture and used at passages 2–5. Cells were plated onto gelatin-coated 24-well tissue culture plates at 1.25 × 10⁴ cells/well and incubated overnight in EBM containing 10% FCS, epidermal growth factor, hydrocortisone, cAMP, and antibiotics. The medium was then replaced with EBM containing 5% FCS, basic fibroblast growth factor (bFGF; 1 ng/ml), and either buffer (300 mM Tris, 60 mM CaCl₂, and 90 mM NaCl, pH 7.5), MME (240 nM), HPg (170 nM), and MME-generated cleavage products of HPg (170 nM; 18-h incubation at 37°C) or recombinant HPg K₁–4 (475 nM). Total protein synthesis was unaffected (data not shown). The cells were trypsinized, pelleted, and counted using a hemocytometer and trypan blue exclusion following 72-h incubation at 37°C in 5% CO₂.

Cell supernatants from peritoneal macrophages, harvested from mice deficient in MMP-2 by targeted mutagenesis (MMP-2−/−) (24) and their wild-type littermates (MMP-2+/+), were also used in an endothelial cell proliferation assay. Briefly, MME−/− and MME−/− mice received an i.p. injection of 1 ml of thiglycolate. Three days following the injection, peritoneal macrophages were harvested and plated into 24-well tissue culture plates at 1 × 10⁵ cells/well in serum-free EBM. After 5 days of culture at 37°C in 5% CO₂, cells were treated with 50 µg/ml of HPg or buffer control for 72 h. Cell supernatants were harvested, and 100 µl of MME−/− or MME−/− supernatant was added to MECs plated onto 24-well tissue culture plates at 1.25 × 10⁵ cells/well containing 10% FCS and bFGF (1 ng/ml).

Western blot analysis

Cell supernatants harvested from buffer control and HPg-treated (50 µg/ml) MME−/− and MME−/− macrophages were subjected to SDS-PAGE following addition of 25 mM DTT in protein running buffer at 100°C for 5 min. Proteins were then transferred to a nylon membrane and blocked with 5% nonfat dry milk in PBS overnight at 4°C. The membrane was then incubated with rabbit anti-mouse IgG MME-specific Ab (1/4000 dilution) (9, 24) for 2 h at 37°C, followed by goat anti-rabbit horseradish peroxidase (Amersham, Arlington Heights, IL) and autoradiography. Control and HPg-treated MME−/− and MME−/− macrophage supernatants were similarly subjected to Western analysis for angiotatin using a polyclonal rabbit anti-human angiotatin Ab (Enzyme Research Laboratories).

Endothelial cell in vitro tube formation assay

MECs were isolated and cultured as described above. Following trypsinization, the cells were plated onto two-chambered Tissue-Tek polystyrene slides (Nunc, Naperville, IL) coated with Matrigel (Collaborative Biomedical Products) diluted 1/1 with EBM containing buffer (300 mM Tris, 60 mM CaCl₂, and 90 mM NaCl, pH 7.5), MME (280 nM), HPg (170 nM), MME/HPg product (170 nM), or K₁–4 (475 nM). MECs plated on this basement membrane matrix differentiate into “tubes” within 24 h of plating as described previously (25). The cells were plated in 500 µl of EBM with 1% FCS containing the same additives, and the formation of tubes was assessed at 24 h. To quantify tube formation, cells were fixed in 100% methanol at 4°C for 7 min, rinsed four times with PBS, and incubated overnight at 20°C with rabbit polyclonal anti-human factor VIII-related Ag (G) (von Willibrand factor; Dako, Glostrup, Denmark). The next day, the cells were counterstained with goat anti-rabbit IgG Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA). Endothelial cells were then visualized using an Olympus microscope equipped with a fluorescence filter and Apochromat objectives linked to a Pentium 100-MHz computer containing a frame-grabber board and Optimus Image Analysis software (Bothell, WA). Five separate random fields for each experimental condition were visualized using Macintosh II with the program Paxit (Midwest Information Systems, Chicago, IL), and analysis was performed using the Optimus program to quantify total endothelial cell area and total area encompassed by complete tubes with intact cell-cell contacts, using the mean area of the five fields. Tube formation was quantitated as follows: total area encompassed by endothelial cell tubes/total endothelial cell surface area. Employing this analysis, efficient and complete tube formation is represented by a larger number; inefficient, discontinuous tube formation yields a smaller number.

Results

Human plasminogen is susceptible to cleavage by MMPs

Pancreatic elastase (39 M; Sigma) and all MMPs tested (final concentration, 5 × 10⁻⁷ M), including mouse and human macrophage elastase (MME and HME); MMP-3 (stromelysin); the gelatinases MMP-2 and MMP-9; collagenases MMP-1, MMP-8, and...
MMP-13; and matrilysin (MMP-7), were incubated with 4 μM HPg (final concentration) for 1 or 18 h. As reported previously (3), pancreatic elastase cleaved HPg into smaller protein species (Fig. 1A). As shown in Fig. 1, A and B, each of the MMPs tested also cleaved HPg to several protein products with varying efficiencies and in a time-dependent manner. Specifically, mouse and human macrophage elastase (MME and HME) produced the most rapid and efficient cleavage of HPg to prominent 14-, 35-, and 38-kDa bands (arrows, Fig. 1A), molecular mass migrations consistent with angiostatin and other angiostatic HPg kringle regions as previously described (3, 26). Catalytic efficacy was then followed by MMP-9, MMP-3, and MMP-7. Therefore, macrophage elastase produced the most efficient cleavage of HPg to the 38-kDa protein product corresponding to angiostatin, followed by gelatinase and stromelysin, with the collagenases exhibiting weak, if any, angiostatin-generating capacity.

Separate experiments were then performed to examine the effects of a serine protease inhibitor, aprotinin, as well as an MMP inhibitor, SC 44463, on this catalytic process (Fig. 1C). Aprotinin did not inhibit MME cleavage of HPg (Fig. 1C), in contrast to recent reports of cleavage of HPg to angiostatin by a serine protease in prostate tumor cells (5, 27). In contrast, the MMP inhibitors SC 44463 (a hydroxymate) and TIMP-1 protein totally blocked MME cleavage of HPg (Fig. 1C). These data indicate that HPg processing to angiostatin is metalloproteinase dependent and that the generation of plasmin does not play a role in MMP-mediated formation of angiostatin and other plasminogen fragments in this system.

To more closely examine the MME cleavage of HPg, the catalysis was examined over time (0.5–18 h). As shown in Fig. 2, MMP cleavage of HPg began within 30 min and was complete by 18 h. In fact, >50% generation of angiostatin by 1–2 h by MME at submicromolar enzyme concentrations suggests potential physiologic relevance and perhaps greater catalytic potential than that of serine elastases such as pancreatic elastase.

**MMP cleavage of plasminogen generates angiostatin (K1–4) and K4**

To determine the sites of cleavage of HPg by MMPs, we incubated MME (1 × 10⁻⁶ M) with 5 μg of HPg (2 μM) at 37°C for 18 h. The products were subjected to gel electrophoresis and transferred onto a Pro Blot nylon membrane, and the 14-, 35-, and 38-kDa protein bands produced by MME cleavage of HPg were excised and subjected to N-terminal sequence analysis. The amino-terminal analysis of both the 35- and the 38-kDa bands yielded an identical sequence of KVLSECKTGN (Fig. 3B), representing the N-terminus of K1. The molecular mass of the 38-kDa band corresponds to K1–4 (26). Since HPg is not glycosylated and both the 35- and 38-kDa species have the same N-terminal sequence,
FIGURE 3. MME cleavage of human plasminogen yields angiostatin and other kringle products. A, Amino acid sequence of human plasminogen kringle regions as described (see text), with the N terminus of K1 at amino acid 97 and the N terminus of K4 at amino acid 373. B, HPg (2 μM) (final concentration) was incubated with MME (1 × 10⁻⁶ M) at 37°C for 18 h, the products were subjected to electrophoresis on a 10% reducing gel and electroblotted onto a Pro Blot nylon membrane. The 14-, 35-, and 38-kDa protein bands produced by MME cleavage of HPg were excised and subjected to N-terminal sequence analysis by automated Edman degradation. The amino-terminal sequence analysis of both the 35- and 38-kDa bands produced an identical amino acid sequence of KVYLSECKTN, corresponding to the N terminus of K1. N-terminal sequence analysis of the 14-kDa band produced the sequence VVQDCYHGDT, corresponding to the N terminus of K4.

C-terminal processing is likely for the 35-kDa product. Interestingly, in previous reports (26), K1–3 migrates at a molecular mass of 35 kDa. N-terminal amino acid sequence analysis of the 14-kDa band produced the sequence VVQDCYHGDT corresponding to K1–4 (38-kDa), K1–3 (35-kDa), and K4 (14-kDa) fragments.

Basic FGF-stimulated endothelial cell proliferation is inhibited by MME-generated plasminogen products and recombinant K1–4

To determine whether MME-generated angiostatin and kringle regions altered endothelial cell proliferation, primary MECs were plated onto gelatin-coated 24-well tissue culture plates (Costar, Cambridge, MA) at 1.25 × 10⁴ cells/well containing 5% FCS and 1 ng/ml bFGF. Cells were incubated with buffer (300 mM Tris, 60 mM CaCl₂, and 90 mM NaCl, pH 7.5), HPg (170 nM), MME (240 nM), the MME cleavage products of HPg (170 nM), or K1–4 (475 nM). After 72 h, MECs were trypsinized, and cell counts were performed using trypan blue exclusion. As shown in Fig. 4A (n = 12), MME-generated HPg protein products inhibited bFGF-induced endothelial cell proliferation by 58% (p < 0.001); K1–4 inhibited proliferation by 44% (p < 0.01). Of note, MME-generated kringle regions did not inhibit human dermal fibroblast proliferation (Fig. 4B), demonstrating the specificity of this effect for endothelial cells.

MME is required for the generation of angiostatin and the inhibition of bFGF-stimulated endothelial cell proliferation

Mouse peritoneal macrophages harvested from MME⁺/⁺ and MME⁻⁻/⁻ mice were incubated in the absence or the presence of HPg (Sigma) for 48 h. Western analysis for MME showed a marked induction and activation of MME by the MME⁺/⁺ macrophages exposed to HPg as previously described (28) (Fig. 5A). As expected, control macrophages did not produce active MME, and HPg-treated MME⁻⁻/⁻ macrophages did not produce MME. HPg-treated MME⁺/⁺ and MME⁻⁻/⁻ macrophage supernatants were then subjected to Western analysis for angiostatin using a polyclonal human plasminogen Ab directed against K1–3 (Enzyme Research Laboratories). Cleavage of plasminogen, in a time-dependent manner, to the prominent 38-kDa protein band of angiostatin was demonstrated by the MME-competent macrophages (Fig. 5B); this cleavage was inhibited by the hydroxymate MMP inhibitor SC 44463 (data not shown). MME-deficient macrophages were incapable of degradation of plasminogen to angiostatin (Fig. 5B). In light of recent reports identifying urokinase activity in conjunction with free sulfhydryl donors as a mechanism of angiostatin generation in prostate carcinoma cells (6), we determined the presence of macrophage-associated urokinase in our MME⁺/⁺ and MME⁻⁻/⁻ macrophages by Western analysis. We found no difference in urokinase production between MME-competent and MME-deficient macrophages (data not shown).

MME⁺/⁺ and MME⁻⁻/⁻ macrophage-conditioned media were then used to determine their capacity to inhibit bFGF-stimulated endothelial cell proliferation. The proliferation of MECs exposed to MME-deficient macrophage-conditioned media had no effect on endothelial cell proliferation even in the presence of HPg.

MME-generated and recombinant angiostatin inhibit endothelial cell tube formation in vitro

To assess the effect of MME-generated kringle regions and recombinant angiostatin on endothelial cell differentiation, in vitro angiogenesis experiments were performed. MECs were plated on growth factor-reduced Matrigel in EBM with 1% FCS containing PBS (Fig. 7A), MMP buffer (300 mM Tris, 60 mM CaCl₂, and 90 mM NaCl, pH 7.5; Fig. 7C), recombinant K1–4 (475 nM; Fig. 7B), HPg (170 nM; Fig. 7D), MME (288 nM; Fig. 7E), or the MME
cleavage products of HPg (170 nM; Fig. 7F). The cells were examined after 24 h of incubation on Matrigel, which induces in vitro differentiation of the endothelial cells and subsequent formation of tube-like structures. As shown in Fig. 7, many of the tubes that were formed by the cells exposed to K1–4 (Fig. 7B) and MME-generated kringle regions (Fig. 7F) were discontinuous compared with those formed in buffer (Fig. 7, A and C), HPg (Fig. 7D), or MME (Fig. 7E) alone. To quantify the degree of tube formation, the cells were immunostained for factor VIII-related Ag (von Willebrand factor), and the total area encompassed by endothelial cell tubes/total endothelial cell surface area was calculated. Exposure to angiotatin (K1–4) resulted in a >50% decrease (p < 0.01) in tube formation, reflecting the reduction in total tube area and hence endothelial cell differentiation. Since Fig. 7F demonstrates similar tube formation inhibition, a comparable reduction is implied following exposure to MME/HPg-generated kringle regions.

Discussion

The capacity of both primary and metastatic tumors to grow beyond the limits of oxygen diffusion requires the establishment of a neovasculature (29, 30). The isolation of angiotatin by O’Reilly et al. (3) demonstrated that tumors may limit metastatic growth by generation of antiangiogenic agents. Dong et al. (7) further demonstrated that production of antiangiogenic activity by tumors was associated with the production of host macrophage elastolytic enzyme(s). Additionally, Patterson et al. (11) demonstrated the in vitro proteolytic activity of MMP-7 and MMP-9 to generate angiotatin from plasminogen. We have extended these findings to directly demonstrate that both mouse and human macrophage elastase and to varying extents other human MMPs proteolytically generate angiotatin that inhibits the proliferation of human microvascular endothelial cells. In murine macrophage-conditioned medium, where MME accounts for most extracellular matrix-degrading activities (24), MME is required for the production of angiotatin and the subsequent inhibition of endothelial cell
proliferation. In fact, the HPg-MMP interaction is even more complex. Plasmin is capable of MMP activation (31).

The mechanism of action of the antiproliferative and angiostatic effects of angiostatin remains unclear. As proposed by Cao et al. (26), endothelial cell proliferation may be blocked through the binding of kringle regions to a receptor that is up-regulated in or exclusive to proliferating endothelial cells. As also suggested by this group, angiostatin may be binding to an integrin that is not only increased in response to an angiogenic endothelial cell mitogen such as bFGF (32) but also important in the establishment of angiogenesis, as is αvβ3 (33). Our own investigations (unpublished observations) as well as those previously reported (25) have established that endothelial cell proliferation does not contribute to the formation of cords or tubes when these cells are seeded on Matrigel. Therefore, our additional findings of the inhibition of tube formation on Matrigel by angiostatin suggest that the in vivo effect of angiostatin may not solely be an inhibition of endothelial cell proliferation but may also be an effect on a later stage of angiogenesis that is represented by the in vitro tube-forming assay. The identities of cell surface molecules that may mediate angiostatin-endothelial cell binding remain under investigation.

The concept that MMPs generate angiostatin, potentially limiting tumor neovascularization, is particularly intriguing given the

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Mouse macrophage-derived MME is required for the inhibition of bFGF-induced proliferation of human dermal MECs. One hundred microliters of cell supernatants from MME+/+ and MME+/- peritoneal macrophages exposed to 50 μg/ml of HPg or buffer were added to MECs plated onto 24-well tissue culture plates at 1.25 x 10^4 cells/well containing 200 μl of EBM with 5% FCS and bFGF (1 ng/ml; n = 6). MECs not exposed to macrophage-conditioned media served as controls for proliferation. Cell counts were performed after 72 h using trypan blue exclusion. The proliferation of MECs exposed to cell supernatant from HPg-treated MME+/+ macrophages was inhibited by 55% compared with that in control media (n = 3; p < 0.05). MME+/- macrophage-conditioned media had no effect on endothelial cell proliferation even in the presence of HPg. Bars represent the SEM.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** MME-generated kringle regions and recombinant angiostatin inhibit endothelial cell tube formation in vitro. MECs were plated on Matrigel in EBM containing 1% FCS and PBS (A; control), recombinant K1–4 (B; 475 nM), MMP buffer (C; 300 mM Tris, 60 mM CaCl, and 90 mM NaCl, pH 7.5), HPg (D; 170 nM), MME (E; 288 nM), or MME cleavage products of HPg (F; 170 nM). Endothelial cells formed complete tubes in control buffers (A and C), but cell-cell contacts were disrupted in recombinant angiostatin-treated cells (B) and also in cells treated with MME-generated kringle regions in a dose-responsive manner (F). Magnification: A and B, ×200; C–F, ×600.
fact that tumor-derived MMPs have been recognized as promoters of tumor growth both by degrading matrix barriers and by enhancing angiogenesis (34, 35). Host-derived MMPs, such as macrophage-specific MME, may also contribute to the generation of other antiangiogenic molecules from certain parent serum or matrix molecules, such as thrombospondin or type XVIII collagen, i.e., the angiostatic fragment of thrombospondin (36) or enostatin (37), respectively. Just as MMPs may be pro- or antiangiogenic, macrophages are intimately involved in both initiating and halting angiogenesis (7, 38, 39). It is well recognized that macrophages release an array of angiogenic and angiostatic cyto- kine and growth factor secretory products (40). Mediators such as bFGF, granulocyte-macrophage CSF, and IL-8 function as stimul- ators of endothelial cell migration and mitosis. In contrast, inhib- itors of angiogenesis produced by macrophages include thrombospondin and IFNs. As for most biologic systems, the contribution of macrophages to angiogenesis must then depend upon the fine regulation and balance of proangiogenic and antiangiogenic factors. This applies to physiologic angiogenesis such as wound healing as well as to the pathologic angiogenesis observed in tumor growth.

In light of our findings as well as those recently reported (6, 11, 27), the biologic properties of MMPs, plasminogen, and angiostatin appear to be tightly linked. We have shown that MME act on plasminogen to generate not only angiostatin (K1–4), but also K4 and probably K1–3. Although the combined effects of the three plasminogen products generated by MME (K1–3, K1–4, and K4) have not been studied relative to each individual kringle region, the formation of multiple kringle region products is particularly relevant in light of the recent work establishing synergism between certain plasminogen kringle regions in the in vitro inhibition of endothelial cell proliferation (26, 41). Moreover, plasmin is known to activate pro-MMPs into their active forms (31) and has long been speculated to be a critical MMP activator in vivo. Indeed, macrophages of u-PA mice secrete only pro-MME, while wild- type mice process MME to the active 22-kDa form (28).

The tumor-derived mechanisms of angiotatin production reported to date have been serine proteinase dependent (5, 6, 27). The presence of urokinase activity in MME (5, 6, 27) and MME macrophages was not sufficient to generate angiostatin in our sys- tem. Possibly the requirement for free sulfhydril groups as re- ported in carcinoma cells (6) is the limiting factor in this mecha- nism of angiostatin production. Our findings of MME-angiostatin generation in the presence of a serine proteinase inhibitor (aproti- nin; Fig. 1C) together with the recent findings that LLC cells gen- erate angiostatin through an EDTA-inhibitable elastase (7) dem- onstrate a distinctly metallproteinase-dependent mechanism of angiostatin generation. In fact, our findings that macrophage-generated angiotatin requires MME expression demonstrate a specific host-derived mechanism of angiostatin generation that is MME dependent. Additionally, we have found that both human mono- cytes and alveolar macrophages are capable of generating an- giostatin from HPg. The proteinases responsible for cleavage are presently under investigation.

Nonselective synthetic MMP inhibitors are currently in clinical trials in patients with metastatic cancer. However, we have presented data that provide a strong rationale for specific and selective targeting of MMP inhibition in cancer therapy. The more we learn about the capacities of individual MMPs to gen- erate angiogenic as well as angiostatic molecules, the greater our potential ability to pharmacologically maximize the inhibi- tion of tumor growth.

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