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Matrix Metalloproteinases Regulate Neovascularization by Acting as Pericellular Fibrinolysins

Nobuaki Hiraoka,* Edward Allen,* Ingrid J. Apel,* Margaret R. Gyetko,† and Stephen J. Weiss*‡

*Division of Hematology/Oncology
Department of Internal Medicine and
University of Michigan
Comprehensive Cancer Center
Ann Arbor, Michigan 48109
†Division of Pulmonary Medicine
Department of Internal Medicine
Ann Arbor Veterans Affairs Medical Center and
University of Michigan Medical Center
Ann Arbor, Michigan 48109

Summary

During angiogenesis, endothelial cells penetrate fibrin barriers via undefined proteolytic mechanisms. We demonstrate that the fibrinolytic plasminogen activator (PA)-plasminogen system is not required for this process, since tissues isolated from PA- or plasminogen-deficient mice successfully neovascularize fibrin gels. By contrast, neovessel formation, in vitro and in vivo, is dependent on fibrinolytic, endothelial cell-derived matrix metalloproteinases (MMP). MMPs directly regulate this process as invasion-incompetent cells penetrate fibrin barriers when transfected with the most potent fibrinolytic metalloproteinase identified in endothelium, membrane type-1 MMP (MT1-MMP). Membrane display of MT1-MMP is required, as invasion-incompetent cells expressing a fibrinolytically active, transmembrane-deleted form of MT1-MMP remain noninvasive. These observations identify a PA-independent fibrinolytic pathway wherein tethered MMPs function as pericellular fibrinolysins during the neovascularization process.

Introduction

Proangiogenic signals associated with wound healing, inflammation, or tumor growth induce rapid increases in vascular permeability in surrounding blood vessels (Dvorak et al., 1986; Eitzman et al., 1996; Senger, 1996; Risau, 1997). In turn, the blood-clotting protein, fibrinogen, leaks out of the vascular bed where it is polymerized and cross-linked enzymatically into a dense fibrin gel (Dvorak et al., 1995; Senger, 1996). This deposited fibrin network then serves as a temporary scaffolding, or provisional matrix, for ingressing endothelial cells that subsequently engage morphogenetic programs to generate patent vessels (Gailit and Clark, 1994; Folkman and D'Amore, 1996; Risau, 1997). While the fibrin matrix serves as a structural support for neovascularization, its highly cross-linked structure also presents a major barrier to cell movement (Dvorak et al., 1995; Senger, 1996). To invade fibrin networks, migrating endothelial cells are believed to mobilize proteolytic enzymes whose activities are limited to pericellular compartments (Pepper et al., 1996; Werb, 1997). In this manner, sufficient fibrin is degraded to allow for the formation of endothelial-lined tunnels, while the bulk of the provisional matrix is preserved to accommodate an expanding network of new blood vessels and accessory cell populations (Gailit and Clark, 1994; Pepper et al., 1996).

To date, the ability of endothelial cells to mediate fibrinolytic activity has been largely attributed to a triad of serine proteinases, which includes tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and plasminogen (Montesano et al., 1990; Carmeliet et al., 1994; Bugge et al., 1995, 1996; Ploplis et al., 1995; Koolwijk et al., 1996; Sabapathy et al., 1997). While neither tPA nor uPA degrades fibrin directly, both enzymes catalyze the processing of plasminogen to the powerful fibrinolysin, plasmin. Consistent with the fibrinolytic potential of the plasminogen activator (PA) system, numerous cell types (including keratinocytes, smooth muscle cells, and inflammatory cells) display major defects in their ability to invade or degrade fibrin deposits in PA- or plasminogen-deficient states in vivo (Carmeliet et al., 1994, 1997a, 1997b, 1998; Bugge et al., 1996a, 1996b; Romer et al., 1996). However, in these same animals, gross alterations in either the neovascularization of fibrin-rich tissues or the reendothelialization of damaged vessel walls have not been noted (Bugge et al., 1996a, 1996b, 1997; Romer et al., 1996; Carmeliet et al., 1997b, 1998). Consequently, increased interest has focused on the possibility that endothelial cells do not rely on the PA-plasminogen system to breach fibrin barriers in vivo and that alternate, but uncharacterized, fibrinolytic proteinases exist (Bugge et al., 1996b, 1997).

We now report the identification of a PA- and plasminogen-independent proteolytic system that allows endothelial cells to invade and neovascularize fibrin-rich tissues both in vitro and in vivo. Unexpectedly, the endothelial cell-associated fibrinolytic activity associated with invasive activity is not mediated by serine, cysteine, or aspartate proteinases, but instead by an enzyme belonging to the matrix metalloproteinase (MMP) gene family. Further studies demonstrate that membrane type-1 matrix metalloproteinase (MT1-MMP), a member of a new subclass of membrane-anchored MMPs (Basbaum and Werb, 1996; Werb, 1997), can degrade cross-linked fibrin gels and directly mediate invasive as well as tubulogenic programs. Together, these studies identify matrix metalloproteinases as a required, and previously unsuspected, component of the PA- and plasminogen-independent fibrinolytic program engaged by endothelial cells during angiogenic processes.

‡To whom correspondence should be addressed (e-mail: sjweiss@umich.edu).

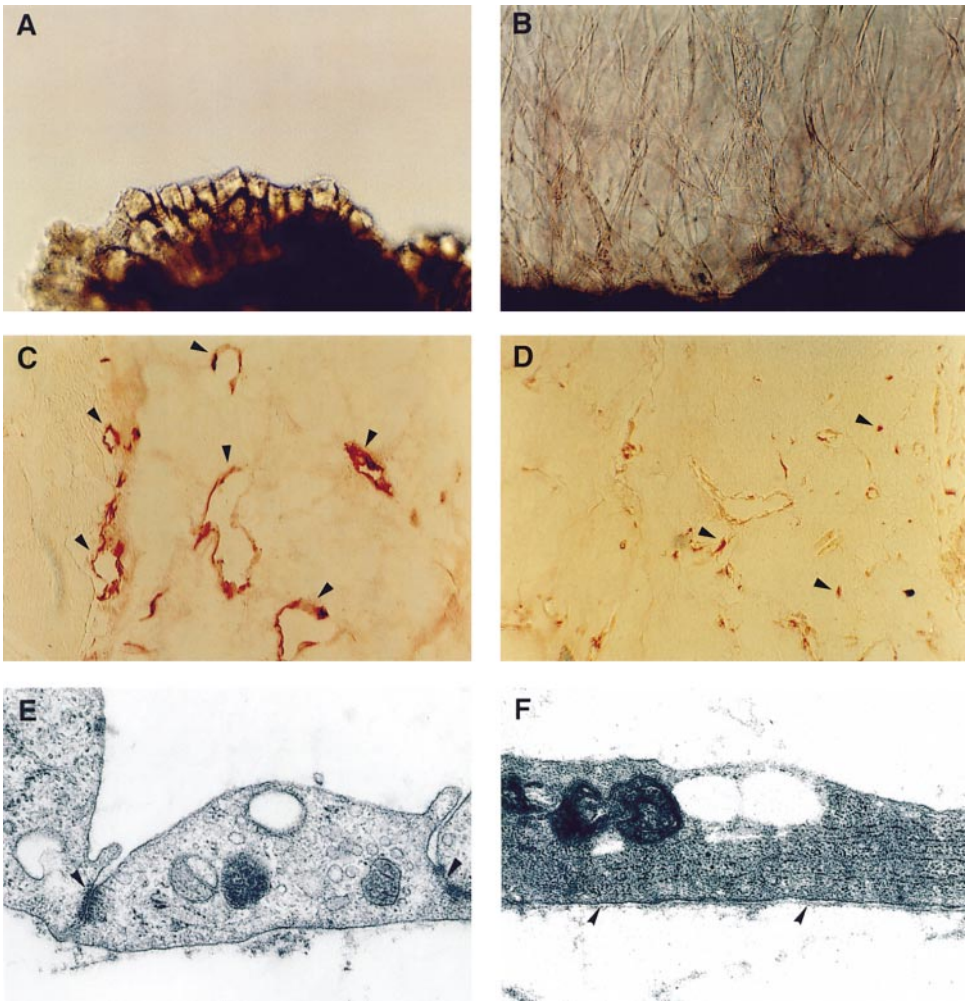

Figure 1. Neovessel Formation by Fibrin-Embedded Murine Explants

(A and B) Rat tissue explants were embedded in a transparent fibrin gel and examined by phase contrast microscopy after 1 day (A) or 12 days (B) in culture at which time an anastomosing network of neovessels had formed (×120).

(C and D) Transverse light sections of 12-day-old cultures stained with endothelial cell-specific, anti-RECA-1 monoclonal antibody (C) or anti-α-smooth muscle cell actin monoclonal antibody (D). Arrows indicate RECA-1-positive vessels (C) and α-smooth muscle cell actin-positive fibroblasts/myofibroblasts (D) (×300). Weak staining of endothelial cells for α-smooth muscle cell actin was observed occasionally. Transmission electron micrographs of 12-day-old neovessels depict the formation of tight junctions (E, ×32,000) and a basement membrane-like subendothelial matrix (F, ×45,000). Arrowheads indicate tight junctions (E) or basement membrane (F).

Results

PA-Plasminogen Deficiency Does Not Affect Fibrin Neovascularization

To characterize the proteolytic mechanisms mobilized during neovessel formation, an ex vivo model of neovessel formation was developed wherein tissue explants from normal or transgenic animals were embedded in uniform fibrin gels prepared at physiologically relevant concentrations (Figure 1A). During the first 4 days of culture, fibroblast-like mesenchymal cells alone migrate into the gel (data not shown). Subsequently, patent tubules begin to form on day 5 with peak neovascularization occurring by day 12 where 141 ± 21 tubules/explant were formed (mean ± 1 SD of 10 experiments; Figure 1B). At this time, an anastomosing network of patent, endothelial-lined neovessels was formed and surrounded by α-smooth muscle actin-positive, fibroblast-like cells (Figures 1C and 1D). As observed in vivo (Gailit and Clark, 1994), the endothelial cells lining the vessels expressed junctional complexes and deposited a basement membrane-like matrix (Figures 1E and 1F).

To determine whether the remodeling of the fibrin matrix that accompanied endothelial cell invasion and tubulogenesis was dependent on either of the PA systems or plasminogen, explants were cultured in the presence of (1) neutralizing antibodies directed against murine tPA or uPA, (2) soluble recombinant uPA receptor (to competitively inhibit uPA binding to the uPA receptor), or (3) recombinant plasminogen activator inhibitor-2 (PAI-2; an inhibitor of two-chain uPA and tPA) (Pepper et al., 1996; Yebra et al., 1996). However, regardless of whether

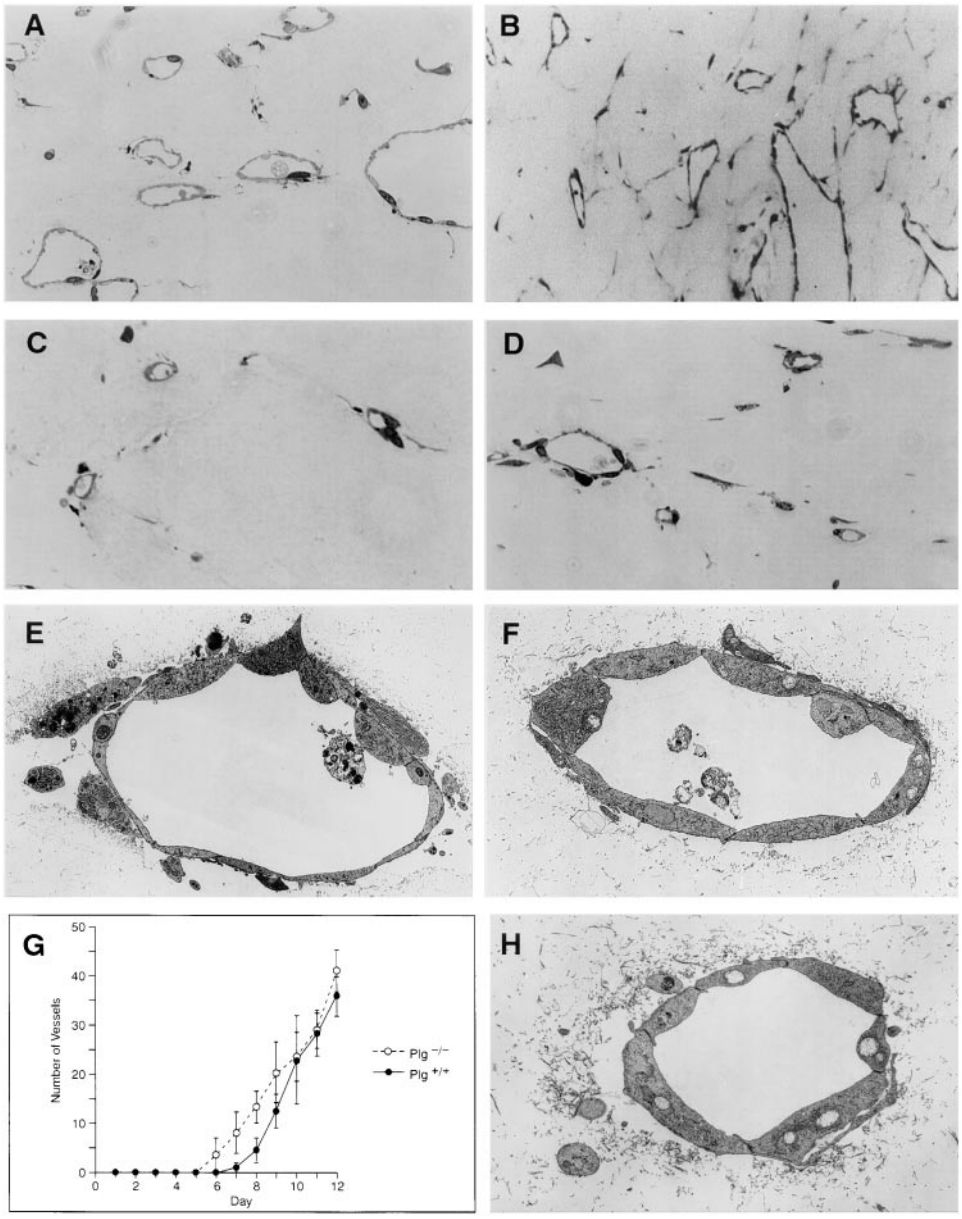

Figure 2. The PA-Plasminogen Axis in Neovessel Formation

(A and B) Rat muscle explants were cultured in fibrin gels alone (A) or with a mixture of anti-tPA (20 μg/ml), anti-uPA (5 μg/ml), soluble recombinant uPA receptor (0.5 μg/ml), recombinant PAI-2 (20 μg/ml), and aprotinin (200 μg/ml) for 12 days (B). In the presence of these inhibitors, the fibrinolytic activity of isolated rat endothelial cells was inhibited by 99%. No differences were detected in neovessel formation as assessed in transverse light sections (×300).

(C–F) Neovessel formation was compared in fibrin-embedded explants isolated from wild-type or uPA$^{-/-}$/tPA$^{-/-}$ and cultured for 12 days in their respective autologous sera (10% final). In transverse light sections (×400) or transmission electron micrographs (×25,000) of wild-type (C and E) or uPA$^{-/-}$/tPA$^{-/-}$ (D and F) explants, no differences in neovessel formation or structure were observed.

(G and H) Neovessel formation by plasminogen$^{-/-}$ explants cultured in 10% autologous sera proceeded at a rate indistinguishable from controls (G). Results are expressed in the mean number of neovessels formed ± 1 SEM (n = 4). Neovessel structure in plasminogen$^{-/-}$ explants (H) was also comparable to controls as assessed by transmission electron microscopy (×25,000).

these inhibitors were added individually or in combination, neovessel formation progressed in a manner indistinguishable from that observed in control explants (Figures 2A and 2B).

To determine whether critical quantities of uPA or tPA escaped inhibition in sequestered environments formed at the endothelial cell–fibrin interface, tissue explants from mice with combined deficiencies in uPA and tPA were embedded in fibrin gels and the angiogenic response monitored. Wild-type mouse explants generated fewer neovessels than rat explants (36 ± 6 neovessels/explant; n = 5), but no significant alterations in vessel

number, length, diameter, or ultrastructure were observed when the responses of wild-type or uPA$^{-/-}$/tPA$^{-/-}$ tissues were compared directly (Figures 2C–2F). Of note, neovessel lumens were clear of fibrin deposits despite the absence of a functional PA system (Figures 2C and 2F). Similar results were obtained when the fibrin concentration was increased from 3 mg/ml to 6 mg/ml.

While uPA and tPA act as the primary activators of plasminogen, alternative pathways exist that might support PA-independent plasmin formation (Bugge et al., 1996b). Thus, explants from plasminogen$^{-/-}$ mice were also examined for their ability to neovascularize fibrin gels. Consistent with the results obtained with uPA$^{-/-}$/tPA$^{-/-}$ tissues, the angiogenic response was unaffected by the absence of plasminogen (Figures 2G and 2H). Similarly, potent inhibitors of plasmin-mediated fibrinolysis (i.e., aprotinin or ϵ-aminocaproic acid) (Pepper et al., 1996) did not affect neovessel formation by either wild-type, uPA$^{-/-}$/tPA$^{-/-}$, or plasminogen$^{-/-}$ explants (data not shown). Thus, neither uPA, tPA, nor plasminogen played a required role in the neovascularization process.

Matrix Metalloproteinases Regulate Neovessel Formation In Vitro and In Vivo

To identify alternate proteinases that mediated fibrin-invasive or tubulogenic activity, wild-type explants were first cultured in the presence of broad-spectrum inhibitors directed against serine proteinases (soybean trypsin inhibitor and α1 proteinase inhibitor), cysteine proteinases (E-64), or aspartate proteinases (pepstatin A) (Reddy et al., 1995). However, none of these inhibitors alone or in combination affected neovessel formation (data not shown).

Endothelial cells, as well as other mesenchymal cell populations, can also synthesize a variety of secreted and membrane-anchored proteinases belonging to the MMP gene family (Basbaum and Werb, 1996; Werb, 1997). Like the PA-plasminogen axis, MMPs have also been implicated in matrix remodeling events, but neither endothelial cells nor any other cell type have been shown to use this class of proteinases to penetrate fibrin barriers. To determine whether MMPs were expressed during the neovascularization of fibrin gels, total RNA was extracted from either freshly isolated or 6- to 12-day-old fibrin-embedded explants and assessed by RT-PCR. While gelatinase A/MMP-2 and membrane-type matrix metalloproteinase-1 (MT1-MMP) were detected in both control and neovascularizing explants, tissues actively engaged in the angiogenic response additionally expressed gelatinase B/MMP-9, stromelysin-1, stromelysin-2, stromelysin-3, matrilysin, and metalloelastase.

Given the active regulation of a complex MMP program during the angiogenic response, we sought to determine whether MMP inhibitors would alter neocapillary development in the explant model. As shown in Figure 3, tissue inhibitor of metalloproteinases-2 (TIMP-2) or the hydroxyamate-based synthetic inhibitor, BB-94 (Basbaum and Werb, 1996; Botos et al., 1996), completely blocked neovessel formation in the course of a 12 day culture period in fibrin gels (Figures 3A–3D) or in plasma clots (data not shown). However, nonendothelial mesenchymal cells (i.e., predominately fibroblasts and myofibroblasts as defined by smooth muscle cell actin staining; Nicosia and Tuszynski, 1994) retained the ability to populate the surrounding matrix in the presence of MMP inhibitors (Figures 3B and 3D). When either wild-type, uPA$^{-/-}$/tPA$^{-/-}$, or plasminogen$^{-/-}$ explants were substituted for rat tissues, neovessel formation, but not fibroblast/myofibroblast migration, was similarly blocked by BB-94 or TIMP-2.

The ability of MMP inhibitors to selectively block endothelial cell invasion suggested that MMPs regulate fibrin-invasive activity in a cell-specific manner. To confirm this possibility, explant-derived fibroblasts and myofibroblasts, or endothelial cells were isolated and reembedded separately in fibrin gels. In the presence of MMP inhibitors, explant-derived fibroblasts and myofibroblasts underwent a neuritic-like change in cell shape but continued to express an invasive phenotype (Figure 3F). However, under the same conditions, the addition of MMP inhibitors completely blocked the invasion of the isolated endothelial cells into the surrounding fibrin gel (Figures 3G and 3H). Thus, while the ability of fibroblasts/myofibroblasts to mount a PA-, plasminogen-, or MMP-independent migratory response is consistent with a nonproteolytic, mechanical process (Brown et al., 1993), endothelial cell invasion was dependent on MMP activity.

Because ex vivo studies supported a primarily role for MMPs rather than the PA-plasminogen axis in regulating endothelial cell invasion and tubulogenesis, a requirement for MMPs in the neovascularization of fibrin matrices was tested in vivo. Thus, fibrin gels or plasma clots were mixed with a cocktail of proangiogenic factors (see Experimental Procedures) in the absence or presence of TIMP-2, sealed in perforated plastic chambers and implanted subcutaneously in wild-type mice. After a 10 day incubation period, the chambers were recovered and the angiogenic response assessed in tissue sections. Macroscopically, both the TIMP-free and TIMP-containing chambers were surrounded by large numbers of neocapillaries in situ. However, when the chambers were freed of surrounding connective tissues and opened, striking differences in the vascularization of the implanted fibrin gels were noted (Figure 4). As shown at both the light and TEM levels, while TIMP-free chambers were infiltrated with large numbers of neovessels, no blood vessels were identified in the presence of TIMP-2 in either fibrin implants (Figures 4C–4F) or plasma clots (data not shown). As observed in the in vitro explant model, nonendothelial mesenchymal cells retained the ability to infiltrate the fibrin gels in the presence of the MMP inhibitor (Figures 4D and 4F). Taken together, these data support the contention that MMPs play a required role in the neovascularization of fibrin-rich tissues in vitro as well as in vivo.

Endothelial Cell MMPs Express PA- and Plasminogen-Independent Fibrinolytic Activity

If endothelial cells use MMPs as direct-acting fibrinolysins, (1) the onset of invasive activity should coincide

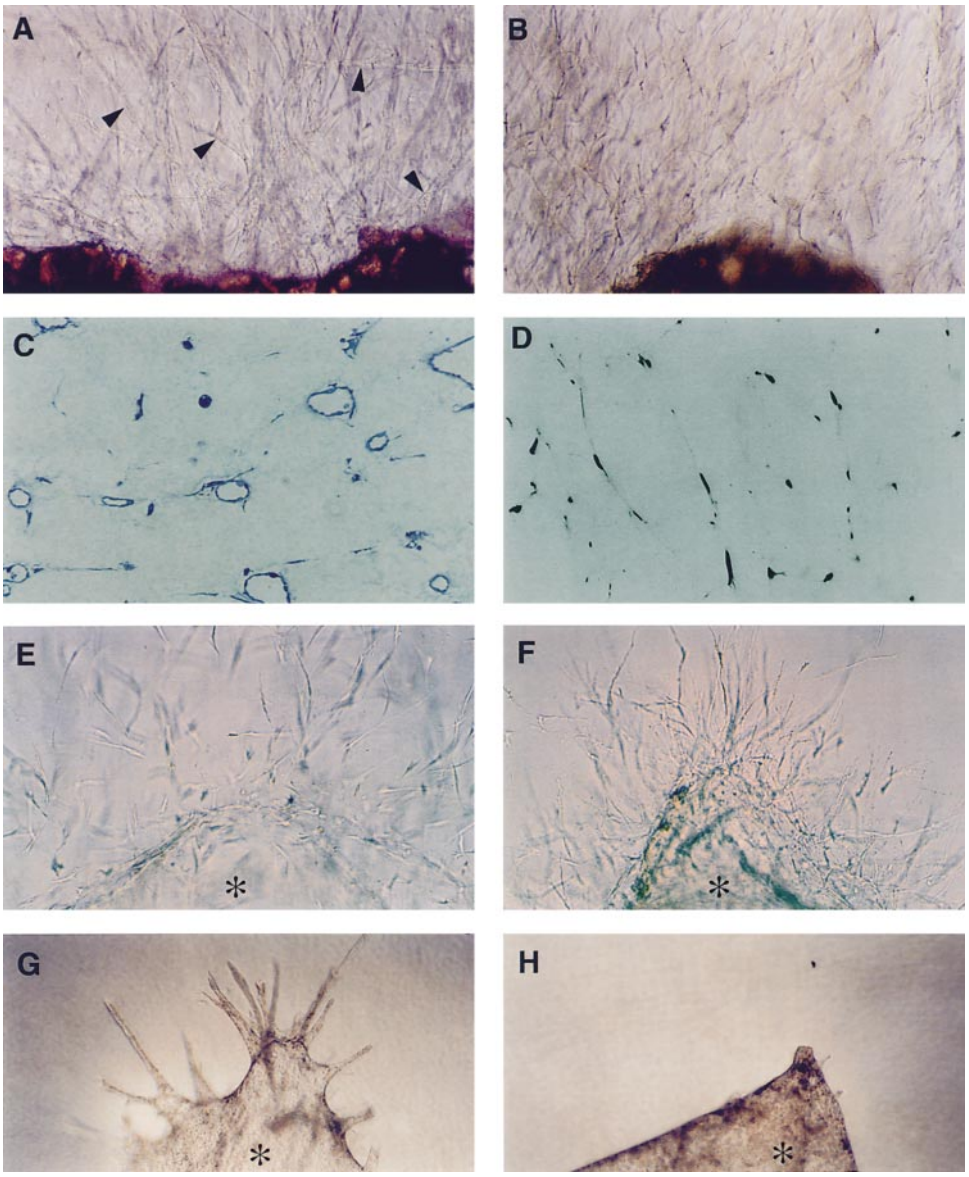

Figure 3. Effect of MMP Inhibitors on Neovessel Formation In Vitro

(A–D) Rat tissue explants were cultured in fibrin gels for 12 days in the absence (A and C) or presence of 20 μg/ml TIMP-2 (B and D). Phase contrast microscopy (×120) demonstrated that neovessel formation was completely blocked by the MMP inhibitor (A versus B). Transverse light sections (×300) confirmed the presence of patent tubules in control, but not TIMP-2-treated cultures (C versus D). In the presence of TIMP-2 or BB-94, infiltrating populations of RECA-1-negative/α-smooth muscle actin-positive fibroblasts/myofibroblasts were detected (data not shown).

(E–H) Isolated, rat explant-derived fibroblasts/myofibroblasts (E and F) or rat endothelial cells (G and H) were embedded in fibrin gel in the absence (E and G) or presence of 5 μM BB-94 (F and H) and examined by phase contrast microscopy (×100). Migration from fibrin gel implants (marked by asterisks) into the surrounding fibrin matrix is shown after a 7 day incubation period. While the MMP inhibitor altered the shape of the migrating fibroblasts/myofibroblasts, motility was preserved (E and F). In contrast, endothelial cell outgrowth from implants (marked by asterisks) was inhibited completely by BB-94 (G and H).

with the MMP-dependent degradation of the fibrin substratum and (2) one or more of the subset of MMPs expressed by the invading endothelial cells should display fibrinolytic activity under cell-free conditions. In the course of a 12 day incubation, growth factor–stimulated endothelial cells invaded an underlying [^{125}I]-labeled fibrin gel (Figures 5A–5D) and increased the solubilization of the substratum by 3-fold in the presence of aprotinin (i.e., from 642 ± 195 to 1596 ± 468 cpm; mean ± 1 SEM of three experiments). However, when endothelial cells were stimulated in the presence of either BB-94 or TIMP-2, endothelial cell-mediated fibrinolysis was reduced to resting levels (i.e., 98% ± 3% and 94% ± 12% inhibition of fibrinolysis; n = 3) and endothelial cell invasion of the underlying fibrin gel was inhibited completely (Figures 5E and 5F). Invasion was similarly inhibited by BB-94 or TIMP-2 when endothelial cells were stimulated to invade fibrin gels cultured in the absence of aprotinin (Figures

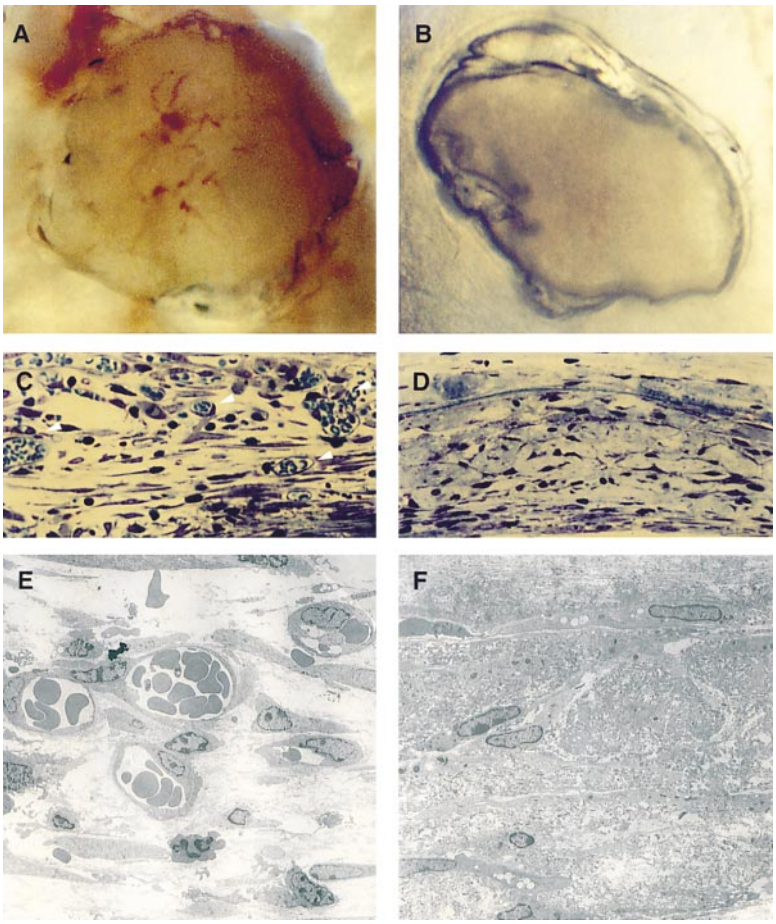

Figure 4. Effect of MMP Inhibition on Neovascularization In Vivo

(A–F) Fibrin gels containing a cocktail of angiogenic factors (see Experimental Procedures) with or without TIMP-2 were sealed in perforated plastic chambers and implanted subcutaneously in wild-type mice for 10 days. Chambers were removed and processed for microscopy. Vascularized implants neovessels enter the perforated chambers in the control implants (A, ×30) and displayed normal ultrastructure by light (C, ×320) or electron microscopy (E, ×7350). No blood vessels were detected in TIMP-2-containing fibrin gels (B, D, and F). In the presence of TIMP-2, nonendothelial mesenchymal cells retained invasive activity (D and F). Results are representative of four independent experiments. Arrowheads indicate the position of neovessels in (A).

5G and 5H). While MMP inhibitors may have affected invasion by modifying cell motility or proliferation (Corcoran and Stetler-Stevenson, 1995; Pilcher et al., 1997), neither BB-94 nor TIMP-2 inhibited endothelial cell migration across fibrin-coated surfaces (i.e., control cells stimulated with angiogenic factors migrated 1.1 ± 0.1 mm in the absence of inhibitors and 1.4 ± 0.1 and 1.1 ± 0.1 mm in the presence of BB-94 or TIMP-2, respectively; n = 3, mean ± 1 SD) or endothelial cell proliferation in the presence of angiogenic factors (i.e., after a 3 day incubation, endothelial cell numbers increased from 1.0×10^6 cells to $7.5 \pm 1.4 \times 10^6$, $7.0 \pm 1.0 \times 10^6$, and $9.1 \pm 0.2 \times 10^6$ when cultured alone, with BB-94, or TIMP-2, respectively; n = 3, mean ± 1 SD).

Coincident with the invasive process, isolated endothelial cells expressed a limited subset of the MMPs detected in the more complex ex vivo model, which included collagenase-1, stromelysin-1, MT1-MMP, and gelatinase A/MMP-2 (Figure 6A). To determine whether these enzymes exhibit fibrinolytic activity, collagenase-1, stromelysin-1, gelatinase A/MMP-2, or a soluble, transmembrane-deletion mutant of MT1-MMP (Pei and Weiss, 1996) were incubated with [^{125}I]-labeled, cross-linked fibrin gels and solubilization quantified. Under cell-free conditions, each of the MMPs solubilized the fibrin gels via a process that could be completely blocked by BB-94 or TIMP-2 (Figure 6B). SDS-PAGE analysis of fibrin(ogen) following incubation with soluble MT1-MMP, the most potent fibrinolytic MMP identified, demonstrated that whereas the Aα and Bβ chains of fibrinogen were sensitive to proteolytic attack, α-fibrin and the high molecular weight α-polymers were preferentially degraded in the cross-linked gels (Figure 6C). Qualitatively similar results were obtained with stromelysin-1, collagenase-1, or gelatinase A (data not shown).

MT1-MMP Regulates Fibrin-Invasive Activity and Supports Tubulogenesis

Given that stromelysin-1 and MT1-MMP expressed the strongest fibrinolytic activity under cell-free conditions, the ability of each of the enzymes to regulate cellular invasion through fibrin matrices was examined. Taking advantage of the recent generation of stromelysin-1$^{-/-}$ mice (Mudgett et al., 1998), tissue explants derived from these animals were embedded in fibrin gels and neovessel formation monitored. Significantly, stromelysin-1$^{-/-}$ explants initiated an angiogenic response equivalent to stromelysin-1$^{+/+}$ control explants when assessed quantitatively or by morphologic criteria (Figure 7A).

As stromelysin-1 was not required for neovessel formation, we next sought to determine if MT1-MMP could play a direct role in pericellular fibrinolysis by endowing an invasion-null cell with invasive or tubulogenic properties via de novo expression of MT1-MMP. Thus, control

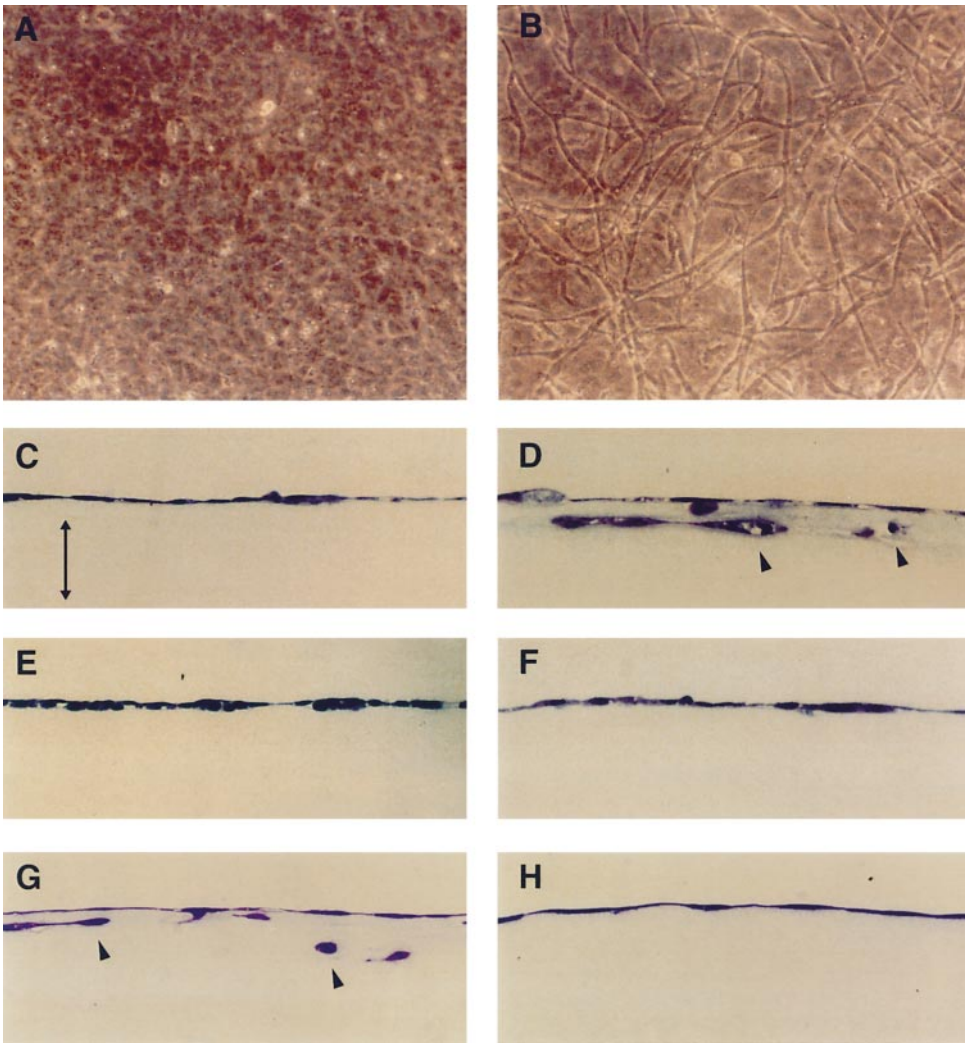

Figure 5. Effect of MMP Inhibitors on the Fibrin-Invasive Activity of Isolated Endothelial Cells

(A and B) Human endothelial cells were cultured atop fibrin gels for 12 days with aprotinin in the absence (A) or presence of angiogenic factors (B) and examined by phase contrast microscopy (×100). Sprouts of invading endothelial cells are formed below the plane of focus of the monolayer in stimulated cultures only (B).

(C–H) Transverse light sections (×200) of unstimulated monolayers (C) or cultures stimulated with growth factors in either the absence or presence of BB-94 (D and E, respectively) or TIMP-2 (F). Endothelial cell cultures stimulated with growth factors in the absence of aprotinin (G) similarly displayed invasive activity, which was blocked completely by TIMP-2 (H). Double-headed arrow indicates the position of the underlying gel in (C) while arrowheads highlight invading endothelial cells in (D) and (G). Results are representative of five or more experiments. The mean distances migrated in (C) and (H) are 35.3 ± 9.7 μm and 37.8 ± 4.1 μm (mean ± 1SEM, n = 5). In the presence of MMP inhibitors, mean invasion was 0.0 ± 0.0 μm.

or MT1-MMP stable transfectants were seeded atop fibrin gels and stimulated with the motogen scatter factor in order to elicit a motile phenotype. As shown in Figure 7B, control MDCK cells were unable to invade the fibrin gels following a 6 day incubation period. In marked contrast, MT1-MMP transfectants invaded the underlying fibrin matrix and underwent a tubulogenic response reminiscent of that observed with endothelial cells (Figure 7C). Furthermore, the invasive/tubulogenic response obtained with MT1-MMP-transfected cells was blocked completely by either BB-94 (Figure 7D) or TIMP-2 (data not shown). While MT1-MMP-transfected cells process progelatinase A/MMP-2 to its active form (Sato et al., 1994; Butler et al., 1998), the MCDK clones used in these studies do not express gelatinase A/MMP-2 and invasion proceeded normally in the presence of gelatinase A–depleted serum (Figure 7E).

As MT1-MMP contains both transmembrane and cytosolic domains that do not play a direct role in controlling proteolytic activity (Pei and Weiss, 1996; Ohuchi et al., 1997), the ability of wild-type MT1-MMP to regulate fibrin-invasive activity was directly compared to mutant forms of the MMP in which either the cytosolic domain (MT1-MMP Δcyt) or transmembrane domain was deleted (MT1-MMP ΔTM). Similarly, MT1-MMP Δcyt expressed an invasive phenotype indistinguishable from

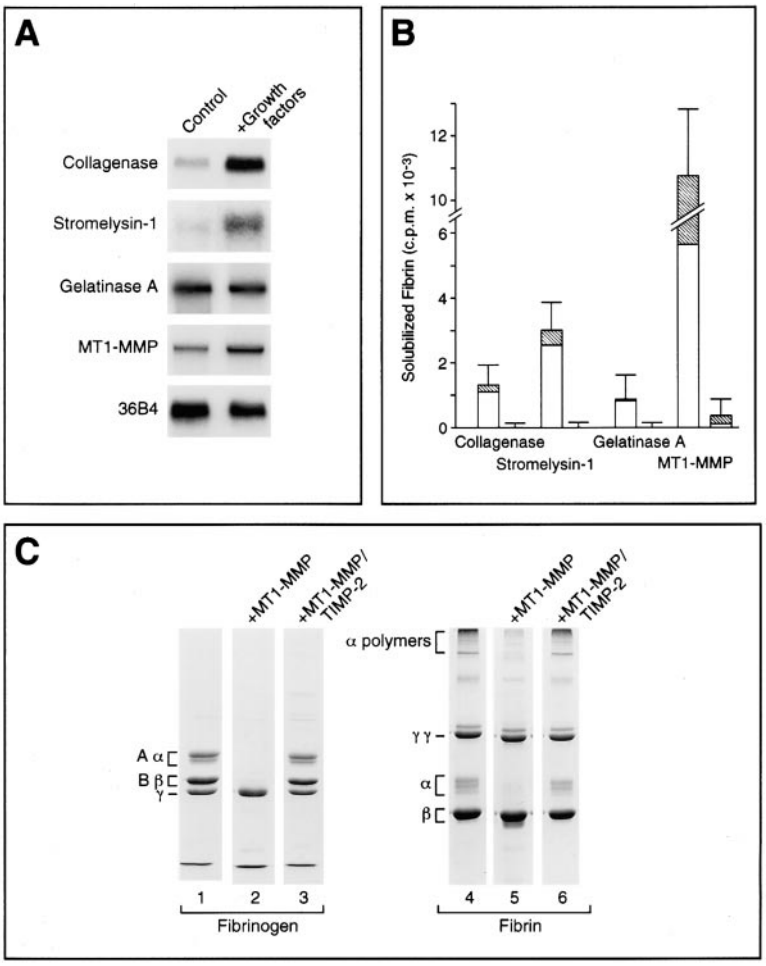

Figure 6. MMP Expression in Endothelial Cells and Fibrinolytic Activity

(A) Northern blot analysis of MMP expression in human endothelial cells after a 3 day incubation with vascular endothelial growth factor, TGFα, hepatocyte growth factor, endothelial cell growth supplement, and heparin.

(B) Fibrinolytic activity of collagenase-1, stromelysin-1, gelatinase A, or a soluble MT1-MMP transmembrane deletion mutant in the absence or presence of BB-94 (values obtained with the inhibitor are the bars shown to the right in each pair). The clear and stippled portion of each bar represents the number of counts recovered in the media or urea extract, respectively. Results are expressed as the mean ± 1 SEM cpm released from [^{125}I]-fibrin-labeled gels (n = 3).

(C) SDS-PAGE analysis of fibrinogen or fibrin (20 μg each) after an 18 hr incubation alone or with soluble MT1-MMP (2.5 pM) in the absence or presence of TIMP-2 (1 μg). Aα, Bβ, and γ chains of fibrinogen and the α polymer, γγ, α, and β chains of fibrin are indicated to the left of each gel.

the full-length enzyme (Figure 7F). However, despite the fact that MT1-MMP ΔTM-transfected cells secrete large amounts of the fully active enzyme extracellularly (Pei and Weiss, 1996), the soluble enzyme was unable to direct the invasive phenotype under conditions permissive for either of the membrane-anchored forms of the proteinase (Figure 7G). Thus, the ability of MT1-MMP to direct invasive activity was critically dependent on its localization to the cell surface.

Discussion

The deposition of a fibrin-rich matrix is tightly linked to the initiation and propagation of angiogenic responses in most, if not all, postdevelopmental states (Dvorak et al., 1995; Senger, 1996). Nonetheless, the proteolytic processes mobilized during neovessel formation have remained the subject of uncertainty (Bugge et al., 1996b, 1997; Pepper et al., 1996; Romer et al., 1996). In order for the fibrin matrix to serve as a scaffolding for ingressing blood vessels, invading endothelial cells should restrict fibrinolytic activity to the pericellular milieu so that the bulk of the fibrin matrix is retained as a structural support. At the cell surface, PAs not only may regulate adhesion, migration, and gene expression (Chapman, 1997) but also catalyze the formation of plasmin. In turn, plasmin efficiently degrades fibrin (Bugge et al., 1995, 1996a, 1996b; Romer et al., 1996) and extracellular matrix components (Chen and Strickland, 1997) as well as processes MMP zymogens to active forms (Carmeliet et al., 1997a, 1998). Consequently, this proteolytic system had been posited to play an indispensable role in regulating fibrin-invasive activity. However, our ex vivo data, coupled with in vivo observations made in PA$^{-/-}$ or plasminogen$^{-/-}$ mice (Bugge et al., 1996a, 1996b, 1997; Romer et al., 1996) and plasminogen-deficient humans (Schuster et al., 1997), all support the existence of an alternate fibrinolytic program critical to the angiogenic process.

Following screening with class-specific antiproteinases, the ability of endothelial cells to invade fibrin gels was found to be dependent solely on the expression of MMP activity in vitro as well as in vivo. Invading endothelial cells were subsequently found to express a number of secreted MMPs, but the cells also synthesized MT1-MMP, a member of a new subclass of membrane-anchored MMPs (Werb, 1997). Until recently, the primary function assigned to MT1-MMP was its ability to process the gelatinase A/MMP-2 zymogen to a series of enzymatically active proteinases (Sato et al., 1994; Butler et al., 1998). Because MMP-2 has been linked indirectly to angiogenic states and can bind to cell surfaces via the $\alpha_v\beta_3$ integrin (Brooks et al., 1996, 1998), we initially considered its role as a potential cell-associated

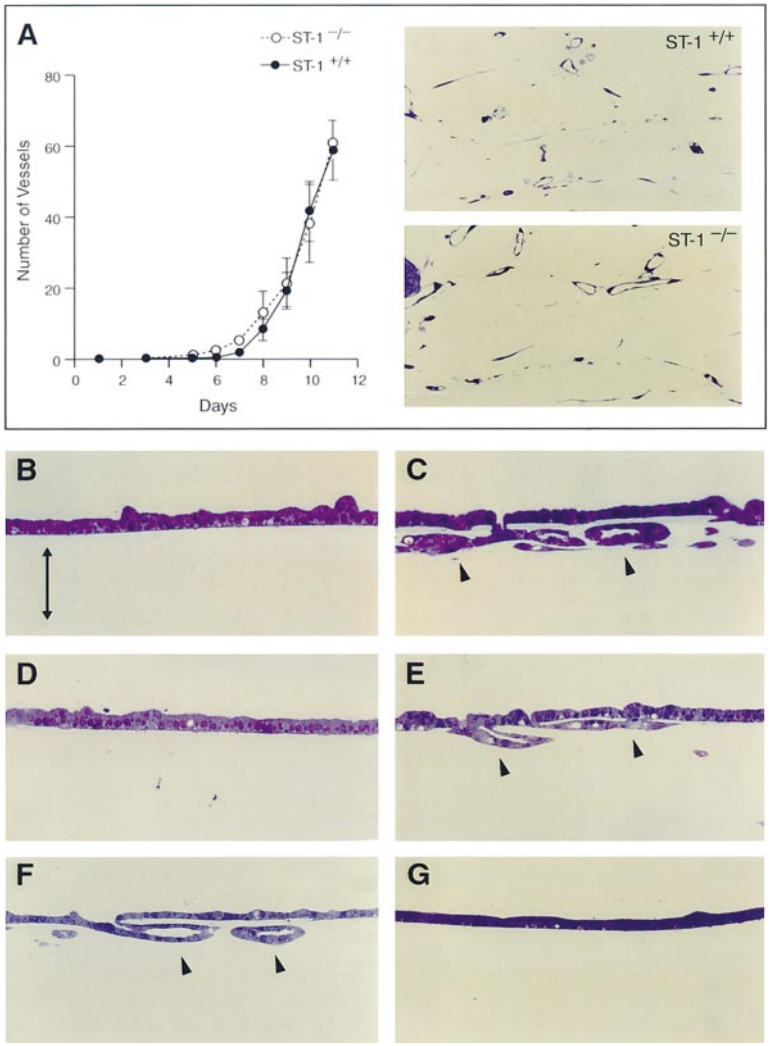

Figure 7. Regulation of Neovessel Formation and Fibrin-Invasive Activity by Stromelysin-1 and MT1-MMP

(A) Neovessel formation by stromelysin-$1^{-/-}$ or stromelysin-$1^{+/+}$ explants as a function of time. Results are expressed as the mean ± 1 SEM of ten experiments with no discernible morphologic differences observed between control or null animals as shown in transverse light sections (×150).

(B–G) MDCK cells transfected with control vector did not invade fibrin gels during a 4 day incubation (B), but cells overexpressing MT1-MMP invaded the underlying fibrin and generated tubules (C) via a BB-94-sensitive process (D). Depleting the sera of gelatinase A/MMP-2 did not alter the invasive phenotype (E). MDCK cells overexpressing MT1-MMP Δcyt, but not MT1-MMP ΔTM, retained fibrin-invasive activity (F and G, respectively). The double-headed arrow in (B) marks the position of the underlying fibrin gel while the arrows in (C), (E), and (F) indicate cells invading the fibrin gel. Results are representative of three or more experiments. The mean distances migrated in (C), (E), and (F) are 104.3 ± 10.9 μm, 120.1 ± 12.5 μm, and 120.0 ± 14.9 μm, respectively (mean ± 1 SEM; n = 3). In (B), (D), and (G), mean distances migrated were all 0.0 ± 0.0 μm.

fibrinolysin. However, gelatinase A/MMP-2 did not exhibit efficient fibrinolytic activity under cell-free conditions nor did it play a required role in regulating fibrin-invasive activity. Significantly, all of these results are consistent with reports that MMP-$2^{-/-}$ mice develop normally and can successfully neovascularize fibrin-rich wound sites in vivo (Itoh et al., 1997, 1998).

While a MT1-MMP·gelatinase A/MMP2 couple was not a required participant in fibrin-invasive activity, recent studies have demonstrated that MT1-MMP can directly cleave extracellular matrix components (Pei and Weiss, 1996; Ohuchi et al., 1997). Given that pericellular proteolysis is most efficiently catalyzed by membrane-associated enzymes (Basbaum and Werb, 1996; Werb, 1997), we considered the possibility that MT1-MMP might function directly as the PA/plasminogen-independent fibrinolysin. MT1-MMP had not previously been known to express fibrinolytic activity, but portions of its catalytic domain bear strong homology to fibrinolysins belonging to a family of hemorrhagic venom metalloproteinases (Bode et al., 1993; Gomis-Ruth et al., 1993; Bini et al., 1996). Consistent with this structural relationship, a soluble transmembrane-deletion mutant of MT1-MMP was shown to efficiently degrade cross-linked fibrin. Furthermore, while neither MT1-MMP-deficient mice nor MT1-MMP-specific inhibitors have been developed, we demonstrated that cells incapable of penetrating fibrin matrices need only express MT1-MMP to acquire invasive as well as tubulogenic activity. The importance of displaying the metalloproteinase on the cell surface was underlined by the fact that cells overexpressing soluble MT1-MMP lost all invasive activity. A recent report has suggested that the cytosolic domain of MT1-MMP may affect the ability of the host cell to focus the enzyme to invasive fronts (Nakahara et al., 1997), but an absolute requirement for the 20-amino acid tail could not be established in our system. As such, anchoring the active enzyme to the cell surface was the sole determinant needed to transfer fibrin-invasive activity to a null cell population. Nonetheless, it seems likely that the other MMPs coexpressed in tandem with MT1-MMP play accessory roles in the final sculpting of the mature vessel in vivo (Hanahan, 1997).

Given a requirement for MMPs in vitro and in vivo during the neovascularization of fibrin gels, the question arises as to when and where plasmin itself participates in fibrin turnover. First, it should be stressed that while MT1-MMP is expressed in endothelial cells in vitro and in vivo (Nomura et al., 1995; Zucker et al., 1995; Ueno et al., 1997), the enzyme is not ubiquitously expressed

by all cell types that confront fibrin barriers in vivo. Indeed, the inability of keratinocytes to efficiently reepithelialize fibrin-rich wounds in plasminogen$^{-/-}$ animals correlates with their inability to express MT1-MMP in vivo and stresses the importance of plasmin in regulating invasive activity in this cell population (Bugge et al., 1996b; Romer et al., 1996; Okada et al., 1997). Thus, a reliance on MMP- versus plasmin-dependent fibrinolytic processes may be dictated by the repertoire of proteinases available to the invading cell population. Second, while all of our in vitro and in vivo studies underlined a primary role for MMPs in endothelial cell-fibrin interactions, plasmin may play important roles under other pathophysiologic conditions. For example, individual growth factors can drive distinct angiogenic programs that rely on different sets of expressed integrins (Brooks et al., 1994). Because our in vitro and in vivo models were all dependent on vascular endothelial growth factor (unpublished data), other angiogenic programs might be more dependent on the PA-plasminogen axis. Indeed, Koolwijk et al. (1996) have reported that isolated endothelial cells can invade fibrin gels in vitro via a uPA-dependent process. Nonetheless, it should be stressed that defects in the neovascularization of fibrin-rich deposits in PA- or plasminogen-null animals have not been reported (Bugge et al., 1996a, 1996b, 1997; Romer et al., 1996).

Finally, regardless of the importance of MMPs in regulating pericellular fibrinolysis, the PA-plasminogen axis has been demonstrated to play the critical role in the bulk proteolysis of fibrin-rich thrombi (Carmeliet et al., 1994; Ploplis et al., 1995; Bugge et al., 1996a). Likewise, following neovascularization in vivo, the remaining fibrin scaffolding is normally resorbed by a plasminogen-dependent process and coordinately replaced by a permanent collagen-based matrix (Gailit and Clark, 1994). If proteolysis of the provisional matrix is delayed by perturbing the PA-plasminogen axis, tissue reparative processes are interrupted and fibrotic responses are precipitated (Eitzman et al., 1996; Kitching et al., 1997; Xiao et al., 1997). A role for MMPs in bulk fibrin clearance has not been examined, but it is noteworthy that intravascular as well as extravascular fibrin deposits are eventually cleared, albeit with altered kinetics, in PA- or plasminogen-deficient states (Bugge et al., 1996a, 1996b; Romer et al., 1996). These findings raise the possibility that MMPs could participate secondarily in the bulk degradation of fibrin deposits in vivo.

Membrane proteinases appear to play an increasingly important role in the proteolytic remodeling of the extracellular matrix (Werb, 1997). From this perspective, it is important to note that MT1-MMP is only one of four members of the membrane-anchored MMPs (Werb, 1997). Unlike some MMPs, which may rely on plasmin for processing to active forms (Carmeliet et al., 1997a, 1998), each of the MT-MMPs are encrypted with an Arg-X-Lys/Arg-Arg motif upstream of their respective catalytic domains (Basbaum and Werb, 1996; Pei and Weiss, 1995, 1996; Maquoi et al., 1998). This basic tetrad can act as a recognition sequence for intracellular proprotein convertases, which then activate the cognate MMP zymogen by removing its regulatory prodomain (Pei and Weiss, 1995, 1996; Maquoi et al., 1998). Thus, the proprotein convertase-dependent activation of metalloproteinase zymogens may have evolved as a PA/plasminogen-independent, proteolytic-processing system. Because the substrate specificity of membrane-anchored metalloproteinases extends beyond fibrin to include a range of extracellular matrix components (Pei and Weiss, 1996; Ohuchi et al., 1997), these enzymes are likely to act as critical mediators of invasive activity by normal as well as neoplastic cells through multiple structural barriers in vivo. Whether MT1-MMP uniquely regulates fibrin-invasive activity in vivo or whether this function can be complimented by other membrane-type MMPs awaits the generation of deficient mice. Nevertheless, therapeutic interventions aimed at MT1-MMP and related membrane-anchored proteinases (Blobel, 1997; Werb, 1997) may prove useful not only in regulating angiogenesis, but also the pathologic remodeling of the extracellular matrix in tissue-destructive disease states as well.

Experimental Procedures

Tissue Explant Model

Abdominal muscle fragments (1.5 mm) were isolated from Fischer rats, uPA$^{-/-}$/tPA$^{-/-}$, plasminogen$^{-/-}$, stromelysin-1$^{-/-}$, or background-matched wild-type mice (Carmeliet et al., 1994; Ploplis et al., 1995; Mudgett et al., 1998) and suspended in a solution (0.9 ml) of 3 mg/ml of PA- and plasminogen-free human fibrinogen (Calbiochem). Copolymerized gels were formed following the addition of 15 milliunits bovine thrombin (Sigma) (Brown et al., 1993) and the explants cultured for 12 days at 37°C in MCDB-131 (Clonetics) supplemented with either 20% fetal bovine serum (FBS) or 10% mouse serum collected from control or null animals. Where indicated, the fibrin gels and media were supplemented with neutralizing polyclonal antisera directed against murine uPA (5 μg/ml) or tPA (30 μg/ml), soluble rat uPA receptor (0.5 μg/ml; all from American Diagnostica), recombinant plasminogen activator inhibitor-2 (20 μg/ml; a gift from M. Baker, University of Wollongong, Australia), ϵ-aminocaproic acid (500 μg/ml; Sigma), aprotinin (200 μg/ml; Sigma), E-64 (100 μM; Sigma), pepstatin A (50 μM; Sigma), recombinant TIMP-2 (20 μg/ml; Amgen), or BB-94 (5 μM; British Biotechnology).

Fibrin Invasion Assays

Rat aorta endothelial cells or rat explant-derived fibroblasts and myofibroblasts were isolated as described (Nicosia and Tuszynski, 1994) and cultured in MCDB-131 supplemented with 20% FBS atop or within fibrin gels in Transwell culture dishes (Costar). To analyze the invasive properties of fibrin-embedded cells, rat aorta endothelial cells or rat explant-derived fibroblasts and myofibroblasts were cultured atop fibrin gels, fragments excised, and then embedded in fibrin gels as described above. Endothelial cell motility was monitored by culturing cells atop fibrin gels whose surface was partially covered with glass cloning chips. After monolayers were formed, the chips were removed leaving denuded areas wherein the distance migrated by the advancing front of cells was measured during a 24 hr incubation.

Fibrin invasion in vivo was assessed by placing fibrin gels (3 mg/ml) or plasma clots containing a mixture of 100 ng/ml vascular endothelial growth factor (Genentech), 10 ng/ml TGFα (Biosource), 50 ng/ml hepatocyte growth factor (Genentech), 75 μg/ml endothelial cell growth supplement (Collaborative Research), and 100 μg/ml heparin in the absence or presence of TIMP-2 (20 μg/ml) in polycarbonate chambers (Dvorak et al., 1987). The chambers were then sealed, one end of the chamber perforated to allow cell ingress and implanted subcutaneously in wild-type mice for 10 days.

MMP-Dependent Fibrinolysis

Human umbilical vein endothelial cells were isolated as described (Huber et al., 1991) and cultured in M-199 supplemented with 20% human serum atop fibrin gels prepared with [^{125}I]-labeled fibrinogen

(3×10^5 cpm total; Amersham) in the absence or presence of 200 μg/ml aprotinin, 5 μM BB-94, or 20 μg/ml TIMP-2. Monolayers were stimulated to invade with a mixture of angiogenic factors (as described above), which were added to the lower compartment of the Transwell system. Solubilized fibrin contained in the media and in a 5 M urea extract of the fibrin gel were pooled, recovered following centrifugation (10,000 × g for 10 min), and quantitated by γ-scintillation counting. Human endothelial cells were used in place of murine cells for these studies given the availability of purified and recombinant human enzymes (see below).

For cell-free studies, fibrinogen or fibrin gels (20 μg each) were incubated with 10 μg/ml aprotinin and 2.5 pM of organomercurial-activated human fibroblast collagenase, human stromelysin-1, recombinant human gelatinase A (active-site titrated as described by Butler et al., 1998) or a catalytically active, transmembrane-deletion mutant of human MT1-MMP (Pei and Weiss, 1996). Following a 48 hr incubation in the absence or presence of BB-94 or TIMP-2, proteolysis was assessed by SDS-PAGE (7.5%). To monitor fibrinolytic activity, radiolabeled fibrinogen was incorporated into the fibrin gel (1×10^5 cpm) and solubilized fibrin quantified as described above.

Northern Blot and RT-PCR Analysis

Total RNA was isolated using TRIzol reagent (Life Technologies), electrophoresed in formaldehyde-agarose gels (5 μg/lane), transferred to nylon filters, and hybridized with [α-^{32}P]dCTP-labeled cDNA probes for human collagenase-1, stromelysin-1, gelatinase A/MMP-2, MT1-MMP, or 36B4. Reverse transcription was performed using an oligo dT primer and AMV reverse transcriptase. cDNAs of matrix metalloproteinases were amplified by PCR using specific oligonucleotide primers for gelatinase A (Genbank accession no. X71466), MT1-MMP (X83537), metalloelastase (M82831), gelatinase B (U36476), stromelysin-1 (X02601), stromelysin-2 (X64020), and matrilysin (Z11887). The identity of the isolated cDNAs was confirmed by sequence analysis as described (Pei and Weiss, 1995). Stromelysin-3 expression was determined by Northern blot analysis using 5 μg total RNA and a human cDNA probe (Pei et al., 1994).

MT1-MMP-Transfected Cell Lines

MDCK cells (ATCC) were stably transfected with a control vector or full-length (amino acid residues 1–582), cytosol-deleted (residues 1–563), or transmembrane-deleted form (residues 1–508 or 1–538) of MT1-MMP as described previously (Pei and Weiss,1996). MDCK transfectants were cultured atop fibrin gels in the presence of 20% fetal calf serum in Transwell dishes as described above and motility stimulated with 50 ng/ml hepatocyte growth factor. Fetal calf serum was depleted of gelatinase A/MMP-2 by gelatin-sepharose affinity chromatography and confirmed by zymography (Pei et al., 1994).

Histology

Tissues were fixed with 2% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4). After postfixation with 1% osmium tetroxide and dehydration in ethyl alcohol, samples were embedded in epoxy resins for light (stained with 1% toluidine blue) or electron microscopy (stained with uranyl acetate and lead citrate). For immunohistochemistry, frozen sections were stained with monoclonal anti-rat RECA-1, an endothelial cell-specific antigen (Serotec Ltd.), or monoclonal anti-α-smooth muscle cell actin (Sigma) as described (Nicosia and Tuszynski, 1994). Staining was performed with a peroxidase detection kit (Vector Laboratories).

Animals

Animal care at the University of Michigan Department of Laboratory Animal Medicine was in accordance with the American Association for Accreditation of Laboratory Animal Care.

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References

Basbaum, C.B., and Werb, Z. (1996). Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. Curr. Opin. Cell Biol. *8*, 731–738.

Bini, A., Itoh, Y., Kudryk, B.J., and Nagase, H. (1996). Degradation of cross-linked fibrin by matrix metalloproteinase 3 (stromelysin 1): hydrolysis of the γ Gly 404-Ala 405 peptide bond. Biochemistry *35*, 13056–13063.

Blobel, C.P. (1997). Metalloprotease-disintegrins: links to cell adhesion and cleavage of TNFα and notch. Cell *90*, 589–592.

Bode, W., Gomis-Ruth, F.-X., and Stockler, W. (1993). Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS Lett. *331*, 134–140.

Botos, I., Scapozza, L., Zhang, D., Liotta, L.A., and Meyer, E.F. (1996). Batimastat, a potent matrix metalloproteinase inhibitor, exhibits an unexpected mode of binding. Proc. Natl. Acad. Sci. USA *39*, 2749–2754.

Brooks, P.C., Clark, R.A.F., and Cheresh, D.A. (1994). Requirement of vascular integrin αvβ3 for angiogenesis. Science *264*, 569–571.

Brooks, P.C., Stromblad, S., Sanders, L.C., von Schalscha, T.L., Aimes, R.T., Stetler-Stevenson, W.G., Quigley, J.P., and Cheresh, D.A. (1996). Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin αvβ3. Cell *85*, 683–693.

Brooks, P.C., Silletti, S., von Schlascha, T.L., and Friedlander, M. (1998). Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. Cell *92*, 391–400.

Brown, L.F., Lanir, N., McDonagh, J., Tognazzi, K., Dvorak, A.M., and Dvorak, H.F. (1993). Fibroblast migration in fibrin gel matrices. Am. J. Pathol. *142*, 273–283.

Bugge, T.H., Flick, M.J., Daugherty, C.C., and Degen, J.L. (1995). Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. Genes Dev. *9*, 794–807.

Bugge, T.H., Flick, M.J., Danton, M.J.S., Daugherty, C.C., Romer, J., Dano, K., Carmeliet, P., Collen, D., and Degen, J.L. (1996a). Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc. Natl. Acad. Sci. USA *93*, 5899–5904.

Bugge, T.H., Kombrinck, K.W., Flick, M.J., Daugherty, C.C., Danton, M.J.S., and Degen, J.L. (1996b). Loss of fibrinogen rescues mice from the pleiotropic effects of plasminogen deficiency. Cell *87*, 709–719.

Bugge, T.H., Kombrinck, K.W., Xiao, Q., Holmback, K., Daugherty, C.C., Witte, D.P., and Degen, J.L. (1997). Growth and dissemination of Lewis lung carcinoma in plasminogen-deficient mice. Blood *89*, 4522–4531.

Butler, G.S., Butler, M.J., Atkinson, S.J., Will, H., Tamura, T., van Westrum, S.S., Crabbe, T., Clements, J., d'Ortho, M.-P., and Murphy, G. (1998). The TIMP2 membrane type 1 metalloproteinase "receptor" regulates the concentration and efficient activation of progelatinase A. J. Biol. Chem. *273*, 871–880.

Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., De Vos, R., van den Oord, J.J., Collen, D., and Mulligan, R.C. (1994). Physiological consequences of loss of plasminogen activator gene function in mice. Nature *368*, 419–424.

Carmeliet, P., Moons, L., Lijnen, R., Baes, M., Lemaitre, V., Tipping, P., Drew, A., Eeckhout, Y., Shapiro, S., Lupu, F., and Collen, D. (1997a). Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. Nat. Genet. *17*, 439–444.

Carmeliet, P., Moons, L., Ploplis, V., Plow, E.F., and Collen, D. (1997b). Impaired arterial neointima formation in mice with disruption of the plasminogen gene. J. Clin. Invest. *99*, 200–208.

Carmeliet, P., Moons, L., Dewerchin, M., Rosenberg, S., Herbert, J.-M., Lupu, F., and Collen, D. (1998). Receptor-independent role of urokinase-type plasminogen activator in pericellular plasmin and matrix metalloproteinase proteolysis during vascular wound healing in mice. J. Cell Biol. *140*, 233–245.

Chapman, H.A. (1997). Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. Curr. Opin. Cell Biol. *9*, 714–724.

Chen, Z.-L., and Strickland, S. (1997). Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. Cell *91*, 917–925.

Corcoran, M.L., and Stetler-Stevenson, W.G. (1995). Tissue inhibitor of metalloproteinase-2 stimulates fibroblast proliferation via a cAMP-dependent mechanism. J. Biol. Chem. *270*, 13453–13459.

Dvorak, H.F., Harvey, V.S., Estrella, P., Brown, L.F., McDonagh, J., and Dvorak, A.M. (1987). Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. Lab. Invest. *57*, 673–686.

Dvorak, H.F., Brown, L.F., Detmar, M., and Dvorak, A.M. (1995). Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol. *146*, 1029–1039.

Eitzman, D.T., McCoy, R.D., Zheng, X., Fay, W.P., Shen, T., Ginsburg, D., and Simon, R.H. (1996). Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. J. Clin. Invest. *97*, 232–237.

Folkman, J., and D'Amore, P.A. (1996). Blood vessel formation: what is its molecular basis? Cell *87*, 1153–1155.

Gailit, J., and Clark, R.A.F. (1994). Wound repair in the context of extracellular matrix. Curr. Opin. Cell Biol. *6*, 717–725.

Gomis-Ruth, F.-X., Kress, L.F., and Bode, W. (1993). First structure of a snake venom metalloproteinase: a prototype for matrix metalloproteinases/ collagenases. EMBO J. *12*, 4151–4157.

Hanahan, D. (1997). Signaling vascular morphogenesis and maintenance. Science *277*, 48–50.

Huber, A.R., Kunkel, S.L., Todd, III, R.T., and Weiss, S.J. (1991). Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science *254*, 99–102.

Itoh, T., Ikeda, T., Gomi, H., Nakao, S., Suzuki, T., and Itohara, S. (1997). Unaltered secretion of β-amyloid precursor protein in gelatinase A (matrix metalloproteinase 2)-deficient mice. J. Biol. Chem. *272*, 22389–22392.

Itoh, T., Tanioka, M., Yoshida, H., Yoshioka, T., Nishimoto, H., and Itohara, S. (1998). Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Res. *58*, 1048–1051.

Kitching, A.R., Holdsworth, S.R., Ploplis, V.A., Plow, E.F., Collen, D., Carmeliet, P., and Tipping, P.G. (1997). Plasminogen and plasminogen activators protect against renal injury in crescentic glomerulonephritis. J. Exp. Med. *185*, 963–968.

Koolwijk, P., van Erck, M.G.M., de Vree, W.J.A., Vermeer, M.A., Weich, H.A., Hanemaaijer, R., and van Hinsbergh, V.W.M. (1996). Cooperative effect of TNFα, βFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. J. Cell Biol. *132*, 1177–1188.

Maquoi, E., Noel, A., Frankenne, F., Angliker, H., Murphy, G., and Foidart, J.-M. (1998). Inhibition of matrix metalloproteinase 2 maturation and HT1080 invasiveness by a synthetic furin inhibitor. FEBS Lett. *424*, 262–266.

Montesano, R., Pepper, M.S., Mohle-Steinlein, U., Risau, W., Wagner, E.F., and Orci, L. (1990). Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. Cell *62*, 435–445.

Mudgett, J.S., Hutchinson, N.I., Chartrain, N.A., Forsyth, A.J., McDonnell, J., Singer, I.I., Bayne, E.K., Flanagan, J., Kawka, D., Shen, C.F., et al. (1998). Susceptibility of stromelysin 1-deficient mice to collagen-induced arthritis and cartilage destruction. Arthritis Rheum. *41*, 110–121.

Nakahara, H., Howard, L., Thompson, E.W., Sato, H., Seiki, M., Yeh, Y., and Chen, W.-T. (1997). Transmembrane/cytoplasmic domain-mediated membrane type 1-matrix metalloprotease docking to invadopodia is required for cell invasion. Proc. Natl. Acad. Sci. USA *94*, 7959–7964.

Nicosia, R.F., and Tuszynski, G.P. (1994). Matrix-bound thrombospondin promotes angiogenesis in vitro. J. Cell Biol. *124*, 183–193.

Nomura, H., Sato, H., Seiki, M., Mai, M., and Okada, Y. (1995). Expression of membrane-type matrix metalloproteinase in human gastric carcinomas. Cancer Res. *55*, 3263–3266.

Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. (1997). Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. J. Biol. Chem. *272*, 2446–2451.

Okada, A., Tomasetto, C., Lutz, Y., Bellocq, J.-P., Rio, M.-C., and Basset, P. (1997). Expression of matrix metalloproteinases during rat skin wound healing: evidence that membrane type-1 matrix metalloproteinase is a stromal activator of pro-gelatinase A. J. Cell Biol. *137*, 67–77.

Pei, D., and Weiss, S.J. (1995). Furin-dependent intracellular activation of the human stromelysin-3 zymogen. Nature *375*, 244–247.

Pei, D., and Weiss, S.J. (1996). Transmembrane-deletion mutants of the membrane-type matrix metalloproteinase-1 process progelatinase A and express intrinsic matrix-degrading activity. J. Biol. Chem. *271*, 9135–9140.

Pei, D., Majmudar, G., and Weiss, S.J. (1994). Hydrolytic inactivation of a breast carcinoma cell-derived serpin by human stromelysin-3. J. Biol. Chem. *269*, 25849–25855.

Pepper, J.S., Montesano, R., Mandriota, S.J., Orci, L., and Vassalli, J.-D. (1996). Angiogenesis: a paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. Enzyme Protein *49*, 138–162.

Pilcher, B.K., Dumin, J.A., Sudbeck, B.D., Krane, S.M., Welgus, H.G., and Parks, W.C. (1997). The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. J. Cell Biol. *137*, 1445–1457.

Ploplis, V., Carmeliet, P., Vazirzadeh, S., Van Vlaenderen, I., Moons, L., Plow, E., and Collen, D. (1995). Effects of disruption of the plasminogen gene on thrombosis, growth and health in mice. Circulation *92*, 2582–2593.

Reddy, V.Y., Zhang, Q.-Y., and Weiss, S.J. (1995). Pericellular mobilization of the tissue-destructive cysteine proteinases, cathepsins B, L, and S, by human monocyte-derived macrophages. Proc. Natl. Acad. Sci. USA *92*, 3849–3853.

Risau, W. (1997). Mechanisms of angiogenesis. Nature *386*, 671–674.

Romer, J., Bugge, T.H., Pyke, C., Lund, L.R., Flick, M.J., Degen, J.L., and Dano, K. (1996). Impaired wound healing in mice with a disrupted plasminogen gene. Nat. Med. *2*, 287–292.

Sabapathy, K.T., Pepper, M.S., Kiefer, F., Mohle-Steinlein, U., Tacchini-Cottier, F., Fetka, I., Breier, G., Risau, W., Carmeliet, P., Montesano, R., and Wagner, E.F. (1997). Polyoma middle T-induced vascular tumor formation: the role of the plasminogen activator/ plasmin system. J. Cell Biol. *137*, 953–963.

Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994). A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature *370*, 61–64.

Schuster, B., Mingers, A.-M., Seidenspinner, S., Nussgens, Z., Pukrop, T., and Kreth, H.W. (1997). Homozygous mutations in the plasminogen gene of two unrelated girls with ligneous conjunctivitis. Blood *90*, 958–966.

Senger, D.R. (1996). Molecular framework for angiogenesis. Am. J. Pathol. *149*, 1–7.

Ueno, H., Nakamura, H., Inoue, M., Imai, K., Noguchi, M., Sato, H., Seiki, M., and Okada, Y. (1997). Expression and tissue localization of membrane-types 1, 2, and 3 matrix metalloproteinases in human invasive breast carcinomas. Cancer Res. *57*, 2055–2060.

Werb, Z. (1997). ECM and cell surface proteolysis: Regulating cellular ecology. Cell *91*, 439–442.

Xiao, Q., Danton, M.J. S., Witte, D.P., Kowala, M.C., Valentine, M.T.,

Bugge, T.H., and Degen, J.L. (1997). Plasminogen deficiency accelerates vessel wall disease in mice predisposed to atherosclerosis. Proc. Natl. Acad. Sci. USA *94*, 10335–10340.

Yebra, M., Parry, G.C.N., Stromblad, S., Mackman, N., Rosenberg, S., Mueller, B.M., and Cheresh, D.A. (1996). Requirement of receptor-bound urokinase-type plasminogen activator for integrin avb5-directed cell migration. J. Biol. Chem. *271*, 29393–29399.

Zucker, S., Conner, C., DiMassmo, B.I., Ende, H., Drews, M., Seiki, M., and Bahou, W.F. (1995). Thrombin induces the activation of progelatinase A in vascular endothelial cells. Physiologic regulation of angiogenesis. J. Biol. Chem. *270*, 23730–23738.