MT1-MMP–dependent neovessel formation within the confines of the three-dimensional extracellular matrix

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During angiogenesis, endothelial cells initiate a tissue-invasive program within an interstitial matrix comprised largely of type I collagen. Extracellular matrix–degradative enzymes, including the matrix metalloproteinases (MMPs) MMP-2 and MMP-9, are thought to play key roles in angiogenesis by binding to docking sites on the cell surface after activation by plasmin- and/or membrane-type (MT) 1-MMP–dependent processes. To identify proteinases critical to neovessel formation, an ex vivo model of angiogenesis has been established wherein tissue explants from gene-targeted mice are embedded within a three-dimensional, type I collagen matrix. Unexpectedly, neither MMP-2, MMP-9, their cognate cell-surface receptors (i.e., integrin and CD44), nor plasminogen are essential for collagenolytic activity, endothelial cell invasion, or neovessel formation. Instead, the membrane-anchored MMP, MT1-MMP, confers endothelial cells with the ability to express invasive and tubulogenic activity in a collagen-rich milieu, in vitro or in vivo, where it plays an indispensable role in driving neovessel formation.

Introduction

Angiogenesis is a specialized form of branching morphogenesis wherein endothelial cells detach themselves from the existing vasculature, invade surrounding tissues, and reorganize into patent tubules (Carmeliet and Jain, 2000; Pepper, 2001; Davis et al., 2002; Lubarsky and Krasnow, 2003). In the postnatal state, new blood vessels routinely traverse a dense connective tissue matrix largely comprised of type I collagen, the major extracellular protein found in mammals (Hay, 1991). Although the triple-helical molecule is resistant to almost all forms of proteolytic attack, endothelial cells engaged in the angiogenic process are believed to negotiate this structural barrier by mobilizing proteolytic cascades that converge on the matrix metalloproteinases (MMPs) progelatinase A (MMP-2) and progelatinase B (MMP-9; Pepper, 2001; Davis et al., 2002; Heissig et al., 2003).

Like all members of the MMP family, MMP-2 and MMP-9 are synthesized as latent enzymes (Egeblad and Werb, 2002, Seiki, 2002). However, coincident with the expression of tissue-invasive or morphogenic processes, secreted MMP-2 and MMP-9 are activated. With specific regard to angiogenesis, the serine proteinase plasmin has been proposed to act as an activator of both MMP-2 and MMP-9, whereas multiple members of the membrane-anchored family of MMPs (i.e., membrane-type [MT]1-, 2-, 3-, 4-, 5-, and 6-MMP) can process the MMP-2 zymogen to its active form (Pepper, 2001; Davis et al., 2002; Seiki, 2002). Surface localization of the activated metalloproteinases may be critical for allowing cells to migrate within dense connective tissues, as binding interactions between MMP-2 and the αβ integrin, and between MMP-9 and the cell surface proteoglycan CD44, have been associated with invasive/angiogenic phenotypes (Brooks et al., 1996, 1998; Yu and Stamenkovic, 1999; Silletti et al., 2001).

Despite the wealth of evidence supporting a role for MMP-2, MMP-9, plasminogen, or MT1-MMP activity in angiogenic events in vivo (Brooks et al., 1998; Itoh et al., 1998; Bergers et al., 2000; Zhou et al., 2000; Heissig et al., 2003), the means by which these proteinases exert their effects remain largely undefined. Long thought to confine their activity to degrading ECM components, increasing evidence suggests that MMPs may modulate endothelial cell function indirectly by activating latent cytokines, cleaving membrane-anchored targets, releasing matrix-bound growth factors, or generating...
bioactive neopeptides (Egeblad and Werb, 2002; Heissig et al., 2003). Consequently, it remains unclear whether MMP-2, MMP-9, their cognate receptors, or the upstream proteinases responsible for their activation, directly support the crucial collagen-remodeling events necessary to drive the morphogenic programs associated with angiogenesis.

To identify the proteolytic systems required for neovessel formation within a physiologically relevant interstitial matrix, three-dimensional (3-D) gels of cross-linked type I collagen were seeded with tissue explants or endothelial cells isolated from mice harboring inactivating mutations in either the plasminogen, MMP-2, MMP-9, β3 integrin, CD44, or MT1-MMP genes. Unexpectedly, we demonstrate that neovessel formation proceeds in unperturbed fashion in the absence of either plasminogen, MMP-2, MMP-9, the β3 integrin, or CD44. Instead, the membrane-anchored collagenase MT1-MMP plays a required role in conferring endothelial cells with the ability to both proteolytically remodel type I collagen and express a collagen-invasive phenotype critical to the tubulogenic process.

Results

3-D neovessel formation engages a collagenolytic phenotype

To determine whether neovessel formation is linked to a collagen remodeling process, mouse tissue explants were embedded in a 3-D type I collagen gel, stimulated with a growth factor cocktail, and monitored for vessel outgrowth as well as collagenolysis. After the egress of fibroblast-like cells during the first two days of ex vivo culture (Fig. 1 A, a), capillary sprouts emerge and coalesce to form an anastomosing network of PECAM-1–positive vessels surrounded by pericyte-like cells at day 7 (Fig. 1 A, b–f). Coincident with the expression of the tubulogenic program, type I collagen degradation products appear in the pericellular environment, as detected with antibodies specific for either (1) the COOH-terminal neopeptide of the three-quarter fragment of cleaved type I collagen or (2) denatured collagen products that have lost their triple-helical integrity (Fig. 1 B). Though the formation of capillary-like structures is associated with type I collagenolytic activity, the degradative phenotype is coordinated with the neodeposition of type IV collagen (Fig. 1 B) and laminin (not depicted) as maturing neovessels initiate basement membrane synthesis (Nicosia and Madri, 1987).

Although multiple proteinases have been implicated in the collagenolytic process (Davis et al., 2002; Heissig et al., 2003; Shi et al., 2003), the formation of patent vessels is unaffected by the broad-spectrum cysteine proteinase inhibitor E-64d or the aspartyl proteinase inhibitor pepstatin A (not depicted). In contrast, cellular outgrowth, as well as tubulogenesis, is almost completely inhibited by the peptidomimetic MMP inhibitor BB-94 (Fig. 1 C). As plasmin is thought to play a key role in type I collagen turnover by virtue of its ability to participate in the processing of latent MMPs to catalytically active forms (Pepper, 2001; Davis et al., 2002), neovessel outgrowth was monitored in tissue explants isolated from plasminogen-null mice that were cultured in plasminogen-null serum. Interestingly, in the absence of plasminogen, an angiogenic response indistinguishable from that displayed by wild-type controls is observed, as determined on morphologic grounds (Fig. 1 C) or by sprout density and length, respectively (6.0 ± 2.6 sprouts...
per field with a mean sprout length of $1,272 \pm 83$ μm in wild-type explants vs. $6.7 \pm 2.1$ sprouts per field and a sprout length of $1,359 \pm 22$ μm for plasminogen-null explants; $n = 3$).

**Capillary morphogenesis proceeds independently of the MMP-2–αβ3 or MMP-2–CD44 axes**

The association of catalytically active MMP-2 with the αβ3 integrin has been reported to regulate the angiogenic response (Brooks et al., 1996, 1998; Silletti et al., 2001), but the role that this complex plays in directing invasive/tubulogenic programs has not been defined. To determine the relative roles of MMP-2 and αβ3 in neovessel formation, we monitored vessel outgrowth and morphology in explants isolated from MMP-2–null or αβ3-null mice. Although wild-type explants expressed both MMP-2 and β3, as assessed by RT-PCR (not depicted), MMP-2–null and wild-type littermate explants mounted an indistinguishable tubulogenic response, without significant differences in mean capillary length or density (Figs. 2 A and 3 C). Furthermore, morphogenesis proceeded in normal fashion, with a ring of endothelial cells circumscribing a patent lumen (Fig. 2 A). Consistent with an MMP-2–independent tubulogenic program, neither vessel outgrowth nor vessel morphology was inhibited in the absence of the β3 integrin (Figs. 2 B and 3 C).

In a fashion similar to that described for MMP-2, MMP-9 has also been linked to tissue-invasive events and angiogenesis, in part, by associating with its cell surface–binding partner, CD44 (Yu and Stamenkovic, 1999; Davis et al., 2002). However, capillary outgrowth and vessel morphogenesis were not affected by the deletion of either MMP-9 or CD44 (Fig. 3).
MMP-2 TIMP-2. Bar, 50 μm was insensitive to 3-MMP-9–null mice are shown after a 5-d incubation period with VEGF–cells per high powered field (hpf) in two experiments.

Ala-TIMP-2 mutant. Results are shown as the mean number of invading

precisely with sites subjacent to overlying endothelial cells as shown in
merged images of MMP-2–null, phalloidin-stained cells (top, right). Col-
lagenolytic activity by control littermate and null cells is completely blocked
when explants of lung, myocardium, or skin are recovered from MT1-MMP–null mice and tested ex vivo (unpublished data). To determine whether MT1-MMP controls endothelial cell–collagen interactions, MT1-MMP–null aortic explants or microvascular endothelial cells were recovered from the knockout mice in order to assess their ability to (1) mount a tubulogenic program, (2) mediate subjacent collagenolysis, and (3) express a collagen-invasive phenotype.

In contrast with wild-type explants, tissues isolated from MT1-MMP+/− mice are completely unable to generate neovessels during a 7-d culture period (Fig. 5 A). Co-cultures of MT1-MMP–null explants with wild-type aorta rings demonstrate that soluble inhibitors of capillary formation are not released from the knockout tissues and that wild-type tissues do not generate soluble factors that are able to rescue the null phenotype (Fig. 5 A). Similar, if not identical, results are obtained when explants of lung, myocardium, or skin are recovered from MT1-MMP–null mice and tested ex vivo (unpublished data). Furthermore, although neovessel formation by control aortic explants resulted in the release of 6.2 ± 1.1 μg hydroxyproline, MT1-MMP+/− explants released only 1.2 ± 0.6 μg hydroxyproline in the course of a 7-d culture period. In the presence of TIMP-2, collagenolysis by wild-type and MT1-MMP+/− explants was inhibited completely (0.4 ± 0.3 μg and 0 ± 0 μg hydroxyproline released, respectively; n = 3). Though MT1-MMP has been postulated to regulate cell function by activating latent TGFβ or generating denatured collagen products that mediate integrin signaling (Heissig et al., 2003), neither the addition of active TGFβ nor that of proteolyzed collagen affected the MT1-MMP+/− phenotype (unpublished data).

Consistent with the inability of MT1-MMP+/− tissue explants to mount a tubulogenic program, isolated MT1-MMP+/− endothelial cells were unable to degrade subjacent collagen

The tubulogenic response mounted by tissue explants involves multiple cell types (e.g., Nicosia and Madri, 1987) that might conceivably mask or compensate for an endothelial cell–specific defect in collagenolytic or invasive activity. Hence, microvascular endothelial cells were isolated from MMP-2 and MMP-9–null mice, and their ability to degrade a subjacent bed of type I collagen fibrils or invade 3-D type I collagen gels was assessed. As shown in Fig. 4 A, wild-type endothelial cells stimulated with a VEGF–hepatocyte growth factor (HGF) mixture proteolyzed a subjacent film of rhodamine-labeled collagen in an area confined to the boundaries of the overlying cell by a process sensitive to either tissue inhibitor of MP (TIMP) 2 or BB-94 (not depicted). Of note, subjacent collagenolytic activity was not affected by targeting either MMP-2 or MMP-9 (Fig. 4 A). Furthermore, consistent with the collagen-degradative phenotypes displayed by either population of null-endothelial cells, MMP-2 or MMP-9 knockout cells retained the wild-type capability to invade 3-D collagen gels (Fig. 4, B and C). Invasion, like collagen degradation, was also blocked completely by both BB-94 and wild-type TIMP-2, but was not affected by TIMP-1, a potent inhibitor of MMP-2 and MMP-9 (Hotary et al., 2003; Fig. 4 C). Although TIMP-2 can affect cell function independently of its ability to block MMP activity (Seo et al., 2003), endothelial cell invasion was not inhibited by an Ala–TIMP-2 mutant devoid of anti-MMP activity (Fig. 4 C).

**MT1-MMP regulates capillary sprouting, collagenolysis, and endothelial cell invasion in a type I collagen-specific fashion**

Although neovessel formation, endothelial cell invasion, and collagenolytic activity proceeded in an unperturbed manner in the absence of either MMP-2 or MMP-9, recent studies indicate that MT1-MMP can display collagenolytic activity directly (Ohuchi et al., 1997; Atkinson et al., 2001). To determine whether MT1-MMP controls endothelial cell–collagen interactions, MT1-MMP–null aortic explants or microvascular endothelial cells were recovered from the knockout mice in order to assess their ability to (1) mount a tubulogenic program, (2) mediate subjacent collagenolysis, and (3) express a collagen-invasive phenotype.

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(Fig. 5 B) or invade 3-D collagen gels (Fig. 5 C). A requirement for MT1-MMP during tissue-invasive activity was further confirmed by the inability of MT1-MMP–null endothelial cells to penetrate an explant of collagen-rich human dermal tissue ex vivo (Fig. 5 D). Despite the fact that MT1-MMP has been posited to regulate cell adhesion or migration by proteolyzing integrins, cadherins, or surface enzymes (Gálvez et al., 2002; Seiki, 2002), MT1-MMP–/– endothelial cells did not express defects in their two-dimensional interactions with collagen, as the cells migrated across type I collagen–coated surfaces at a rate indistinguishable from that of controls (i.e., 29 ± 1 μm/24 h vs. 29 ± 1 μm/24 h; n = 3). Likewise, although MT1-MMP serves as a necessary collagenolysin in endothelial cells, fibrin-invasive and -degradative activity can be conferred by other members of the membrane-anchored MMP family, including MT3-MMP (Hotary et al., 2002). Given the ability of vessel explants as well as isolated endothelial cells to express MT3-MMP (unpublished data), morphogenesis and invasion through 3-D fibrin...
Figure 7. Retroviral reconstitution of the collagenolytic and type I collagen-invasive activity of MT1-MMP-null endothelial cells. (A) Although MT1-MMP−/− endothelial cells transduced with a control cDNA (top, left) were unable to degrade a subjacent collagen film after a 7-d culture period with VEGF–HGF in the presence of 5% mouse serum, retroviral reconstitution of active MT1-MMP restores the collagenolytic activity of MT1-MMP−/− endothelial cells (top, middle). Cells transduced with a catalytically inactive MT1-MMPΔcat, mutant or a transmembrane-deleted, soluble form of MT1-MMP (MT1-MMPΔTMD) do not display collagen-degradative activity. Whereas the collagenolytic activity of MT1-MMP−/− endothelial cells could be rescued by expressing MT2-MMP, MT3-MMP-transduced cells did not display a collagenolytic phenotype. Bar, 50 μm. (B) Collagen-invasive activity of MT1-MMP−/− cells cultured atop a 3-D gel of type I collagen (2.2 mg/ml) for 7 d in the presence of VEGF–HGF and 20% FBS is rescued after transduction with MT1-MMP or MT2-MMP, but not EGFP, MT1-MMPΔcat, MT1-MMPΔTMD, or MT3-MMP. The inset for MT1-MMP shows the protein level of endogenous MT1-MMP in wild-type cells (lane 1), MT1-MMP−/− null endothelial cells (lane 2), and MT1-MMP-transduced-null cells (lane 3). The insets for MT2-MMP and MT3-MMP show the expression of MT2-MMP and MT3-MMP, respectively, in wild-type cells (lane 1), and MT1-MMP-null endothelial cells after retroviral transduction with MT1-MMP, MT2-MMP, or MT3-MMP (lanes 2–4). (C) Immunofluorescent micrographs of 11-d-old CAM cross sections depict a dense interstitial matrix composed of types I and III collagen (top, left; bar, 20 μm). MT1-MMP+/+ or MT1-MMP−/− endothelial cells labeled with fluorescent microbeads (green) invaded 144 ± 55 μm and 53 ± 13 μm, respectively, into the CAM interstitium after a 3-d incubation [the CAM surface is marked by the black arrowheads to the right; top, middle and right, and bottom; bar, 100 μm]. Retroviral gene transfer of GFP highlights the morphology of a group of invading MT1-MMP−/− cells (MT1−/−; middle row, left and center; bar, 20 μm), which form tubular structures (left, arrows). The section of the micrograph bounded by the dashed square is enlarged (middle) and shows a group of elongated endothelial cells [arrowheads] forming a tube with the nuclei highlighted by Hoechst staining. Morphology of GFP-labeled MT1-MMP+/+ endothelial cells [MT1+/+] is shown [middle row, right]. After retroviral gene transfer of MT1-MMP or MT2-MMP into MT1-MMP−/− endothelial cells, invasion increased to 147 ± 46 μm and 123 ± 34 μm, respectively. After retroviral gene transfer of MT1-MMP or MT2-MMP into MT1-MMP−/− endothelial cells, invasion increased to 147 ± 46 μm and 123 ± 34 μm, respectively (mean ± 1 SD, n = 3). The invasive activity of MT3-MMP-transduced cells was not affected relative to the MT1-MMP−/− cells [i.e., 47 ± 22 μm, n = 3].

Structural requirements for MT1-MMP-dependent endothelial cell collagenolysis and invasion in vitro and in vivo

To define the structural features that underlie the ability of MT1-MMP to confer endothelial cells with collagen-degradative and -invasive activity, MT1-MMP−/− endothelial cells were transduced with wild-type MT1-MMP or MT1-MMP variants harboring either an inactivating point mutation in the catalytic domain or a deletion of the transmembrane domain (Horany et al., 2003). Although full-length MT1-MMP bestowed MT1-MMP−/− endothelial cells with both collagenolytic and invasive activity, catalytically inactive MT1-MMP did not rescue the null phenotype (Fig. 7, A and B). Similarly, transduction of MT1-MMP−/− cells with a catalytically active, but soluble, form of MT1-MMP failed to confer collagenolytic or invasive activity, presumably because the recipient cell loses its ability to focus proteolytic activity to the subjacent compartment (Fig. 7, A and B).

Together, these results suggest that pericellular collagenolysis, as well as invasive activity, minimally requires the membrane display of a tethered collagenase. As such, MT1-MMP activity could conceivably be compensated by other members of the MT-MMP family that likewise express type I collagen-degradative activity. Interestingly, recent studies raise the possibility that MT2-MMP, though not normally expressed in mouse endothelial cells (unpublished data), can act as an alternate membrane-anchored collagenolysin (Horany et al., 2000). Indeed, when MT1-MMP−/− endothelial cells were transduced with an MT2-MMP expression vector, subjacent proteolysis was restored fully and in tandem with collagen-invasive activity (Fig. 7, A and B).
of the MT-MMP family that displays little if any collagenolytic activity, MT3-MMP (Horay et al., 2000), was unable to rescue the invasion-null phenotype (Fig. 7, A and B).

Finally, we sought to determine whether the ability of MT1-MMP or MT2-MMP to confer invasive activity to MT1-MMP+/− endothelial cells could be extended from homogenous 3-D constructs of type I collagen in the in vitro setting to a more complex interstitial barrier in vivo. Hence, wild-type or MT1-MMP-null endothelial cells transduced with control, MT1-MMP, MT2-MMP, or MT3-MMP retroviral expression vectors were labeled with either fluorescent microbeads or GFP and cultured atop the type I/III collagen–rich chick chorioallantoic membrane (CAM; Fig. 7 C). Consistent with the results obtained in vitro, wild-type, but not MT1-MMP–null endothelial cells, were able to penetrate deeply into the CAM interstitium. Furthermore, although GFP-labeled wild-type endothelial cells adopted an elongated phenotype and displayed morphogenic properties by generating tubular structures in collagen-rich environments in vitro and in vivo.


discussion

Angiogenesis, like the more generalized program of branching morphogenesis, requires tissue-infiltrating cells to negotiate an ECM rich in collagen (Fisher et al., 1994; Pepper, 2001; Seandel et al., 2001; Davis et al., 2002; Lubarsky and Krasnow, 2003). Proteolytic remodeling of type I collagen has been posited to play a necessary role in allowing numerous cell types to generate a passageway through the matrix, migrate across the newly exposed collagen substratum, and then engage tubulogenic programs (Fisher et al., 1994; Tournier et al., 1994; Lelongt et al., 1997; Haas et al., 1998; Milalles et al., 1998; Zhu et al., 2000; Davis et al., 2001; Seandel et al., 2001; Kheradmand et al., 2002). Although multiple proteolytic systems have been implicated in angiogenic events in vivo (Carmeliet and Jain, 2000; Pepper, 2001; Davis et al., 2002), attempts to identify enzymes critical to tissue remodeling per se have been complicated by the more recent appreciation that the substrate repertoire of MMPs extends to a diverse array of growth factors, cytokines, chemokines, and cell adhesion molecules (Egeblad and Werb, 2002).

Accumulating evidence supports an angiogenic scheme in which endothelial cell–associated MMP-2 and MMP-9 drive endothelial migration, invasion, or tubulogenesis (Haas et al., 1998; Koivunen et al., 1999; Lyden et al., 1999; Xu et al., 2001). Because MT1-MMP proteolyzes the MMP-2 zymogen to its active form which, in turn, can activate MMP-9 (Seiki, 2002; Toth et al., 2003), we speculated initially that all three MMPs would form a collaborative network to drive the angiogenic response. Likewise, reports documenting the ability of α,β1, and CD44 to localize MMP-2 and MMP-9, respectively, to the migrating front of tissue-invasive cells are consistent with a proteolytic model in which integrins and transmembrane glycoproteins act as docking sites for the assembled proteinases (Brooks et al., 1996; Yu and Stamenkovic, 1999; Rolli et al., 2003). Nonetheless, despite the appeal of such schemes, neither MMP-2, α,β1, MMP-9, nor CD44 played a required role in neovessel formation, endothelial cell collagen invasion, or collagenolytic activity ex vivo. Likewise, although plasmin has been linked to the angiogenic process (Carmeliet and Jain, 2000; Pepper, 2001), plasminogen-null explants displayed no defects in our ex vivo model. In contrast, tissue explants recovered from MT1-MMP−/− mice were completely unable to mount a tubulogenic response when suspended in collagen gels. Furthermore, consistent with the proposition that collagenolytic activity is required to support endothelial cell invasion within a type I collagen–rich environment (Fisher et al., 1994; Haas et al., 1998; Seandel et al., 2001; Davis et al., 2002), MT1-MMP-null endothelial cells were unable to degrade subjacent collagen in a serum-containing milieu. Apparently, MT1-MMP is the major, if not sole, collagenolytic operational in mouse endothelial cells that is capable of mediating the pericellular dissolution of type I collagen under physiologic conditions. Given that the MT1-MMP zymogen undergoes efficient processing to its active form via a proprotein convertase–dependent process (Yana and Weiss, 2000), plasmin-mediated processing of MT1-MMP does not play a required role in this system. Although mouse endothelial cells express soluble collagenases (e.g., MMP-13, MMP-2, and MMP-8) and can use plasmin to activate these proteinases, these MMPs were unable to mount a focal collagenolytic effect in the presence of serum antiproteinases (unpublished data). Hence, in the absence of MT1-MMP activity, mouse endothelial cells fail to degrade collagen or negotiate collagenous barriers. As human endothelial cell tubulogenesis is also sensitive to TIMP-2, but not TIMP-1 (Lafluer et al., 2002; Collen et al., 2003), we posit that MT1-MMP and/or MT2-MMP play(s) a dominant role in directing angiogenesis in humans as well.

Despite the correlation between collagenolytic and invasive activity, MT1-MMP hydrolyzes not only type I collagen, but also a variety of noncollagenous targets (Seiki, 2002; Egeblad and Werb, 2002). Consequently, we cannot eliminate the possibility that MT1-MMP cleaves type I collagen in tandem with other substrates in a fashion necessary to affect an invasive and/or tubulogenic program. Our data do, however, rule out recently proposed models wherein MT1-MMP drives invasion by either processing the α,β1 integrin or cleaving CD44 (Deryugina et al., 2000; Kajita et al., 2001; Mori et al., 2002). Furthermore, a more generalized defect in cell adhesion, migration, or invasion that might be consistent with MT1-MMP–dependent proteolysis of integrins, cadherins, growth factors, or surface enzymes is not supported by our observations that MT1-MMP−/− cells migrate at normal rates across collagen-coated surfaces and invade cross-linked fibrin barriers. Though recent studies have suggested that MT1-MMP can regulate endothelial cell migration (Galvez et al., 2002), these conclusions are founded...
on the use of neutralizing antibodies directed against MT1-MMP. Because these antibodies do not recapitulate the MT1-MMP−/− phenotype and, in independent studies, affected the activity of multiple membrane-anchored as well as secreted MMPs (unpublished data), caution should be exercised in assuming their specificity or utility as MT1-MMP inhibitors.

The striking defects in collagenolytic and invasive activity, as well as in neovessel formation, displayed by MT1-MMP−/− tissues seem at odds with the fact that the null animals develop normally (Holmbeck et al., 1999; Zhou et al., 2000). In this regard, it is interesting to note that during embryogenesis, as well as perinatally, the type I collagen content of most tissues is low relative to the content in the postnatal state (Van Exan and Hardy, 1984; Carver et al., 1993). As a possible consequence, newborn null animals appear normal and only begin to display serious skeletal and connective tissue abnormalities after the first week of birth (Holmbeck et al., 1999; Zhou et al., 2000). Hence, although the ECM composition of the developing animal may afford MT1-MMP−/− mice a protected status, we posit that the increased deposition of type I collagen that arises as a consequence of the mechanical demands of adult life initiates the onset of the pathologic states observed in the knockout mice. Indeed, though null animals display an apparently normal vasculature at birth, Zhou et al. (2000) have reported that angiogenic responses in 15-d-old MT1-MMP−/− animals are abrogated in collagen-rich corneal tissues. Nonetheless, even in collagen-replete tissues, our results suggest that angiogenesis in fibrin-rich fields (e.g., wounds) may proceed normally in MT1-MMP–null mice because MT3-MMP, though largely devoid of collagenolytic activity, can function as an efficient fibrinolysin (Hotary et al., 2002).

Together, our observations appear to be at variance with other in vitro or in vivo studies concluding that plasminogen, MMP-2, or MMP-9 plays a required role in the angiogenic process (Carmeliet and Jain, 2000; Brodsky et al., 2001; Pepper, 2001). However, it should be noted that defects in angiogenesis have not been observed uniformly in either plasminogen−/−, MMP-2−/−, or MMP-9−/− mice in response to wounding (Romer et al., 1996; Itoh et al., 1998) or tumor growth (Bergers et al., 2000; Hamano et al., 2003). Furthermore, αβ3−/− mice mount an exaggerated angiogenic response in a range of pathophysiologic settings (Reynolds et al., 2002). In cases where MMP-2 deficiency has been shown to affect neovascularization, a partial reduction in the angiogenic response (~30%) has been most frequently described (Itoh et al., 1998; Berglin et al., 2003; Guedez et al., 2003). In contrast, MMP-9–deficient mice can, in some cases, display more significant defects in angiogenesis in vivo, but this effect has been ascribed largely to the ability of the metalloproteinase to release matrix-bound forms of VEGF (Bergers et al., 2000). Indeed, vascular defects in these animals are reversed by the exogenous application of VEGF, despite the continued absence of MMP-9 (Engsig et al., 2000; Heissig et al., 2003). Nonetheless, under defined circumstances, plasminogen, MMP-2, MMP-9, α3β1, or CD44 can play an important role in modifying angiogenic events in vivo (Carmeliet and Jain, 2000; Pepper, 2001; Davis et al., 2002; Heissig et al., 2003; Oh et al., 2004), but based on our results, these effects are more likely exerted in a fashion independent of the matrix remodeling events associated strictly with invasion or tubulogenesis within the 3-D interstitium. As the ex vivo models used herein largely obviate a required role for endogenous growth factors, chemokines, or immune cell populations, it may well develop that these latter players serve as preferred targets for plasminogen, MMP-2, or MMP-9 in vivo. Similarly, the ex vivo model may circumvent a requirement for basement membrane proteolysis, a key step in the initiation of the angiogenic process, though it should be noted that angiogenesis can proceed in a normal fashion in vivo even in the combined absence of MMP-2 and MMP-9 (Baluk et al., 2004). Clearly, a mounting number of observations describing normal, or near normal, tubulogenic programs in MMP-2−/− or MMP-9−/− null animals (Andrews et al., 2000; Bergers et al., 2000; Wise et al., 2003) strongly suggest that tubulogenic programs—including angiogenesis—can proceed in the absence of these downstream MMPs. As such, the rules we have established for generating patent neovessels in collagen-rich tissues raise the possibility that MT1-MMP and perhaps MT2-MMP play dominant roles in driving a variety of tubulogenic programs in the in vivo, postnatal setting.

Materials and methods

Ex vivo angiogenesis assay

Mouse aortic ring assays were performed with minor modifications of protocols described previously (Nicosia and Madri, 1987; Hirooka et al. 1998). Mouse aortas were isolated from 4- to 5-wk-old male mice deficient in either plasminogen, MMP-2, MMP-9, MT1-MMP, β3 integrin, or CD44, or their respective littermate controls. Mice carrying null mutations for plasminogen (provided by T. Bugge, National Institutes of Health, Bethesda, MD), β3 (provided by S. Teitelbaum [Washington University, St. Louis, MO]) and R. Hynes (Massachusetts Institute of Technology, Cambridge, MA), MMP-2 (provided by S. Itohara, RIKEN Brain Science Institute, Saitama, Japan), or CD44 (provided by C. Doebersch [Rainbow Babies and Children’s Hospital, Case Western Reserve University, Cleveland, OH] and T. Mak [Ontario Cancer Institute, University of Toronto, Toronto, Ontario, Canada]) were backcrossed into the C57BL/6J background for at least 10 or more generations into the Black Swiss or 129SvEv backgrounds, respectively (Romer et al., 1996; Itoh et al., 1998; Holmbeck et al., 1999; Andrews et al., 2000; Reynolds et al., 2002; Wang et al., 2002). Isolated aortas were sectioned (in 1-mm square fragments) and embedded in 2.2 mg/ml reconstituted type I collagen gels or 3.0 mg/ml cross-linked fibrin (Hotary et al., 2000, 2002) in 24-well plates and cultured in RPMI 1640 (GIBCO BRL) supplemented with either 20% FBS or 5% mouse serum (prepared from wild-type littermate or null animals) in the presence of 100 ng/ml recombinant human VEGF-165, 50 ng/ml recombinant HGF (both provided by Genentech, Inc., San Francisco, CA), and 100 pg/ml human TGF-β (R&D Systems). In selected experiments, fragments were cultured in the presence of 100 μg/ml apronin (Sigma-Aldrich), 10 μM E64d (Sigma-Aldrich), 5 μM pepstatin (Sigma-Aldrich), 5 μM BB94 (British Biotechnology), endotoxin-free recombinant human TIMP-1 or TIMP-2 (both manufactured by Fujij, or Ala-TIMP-2 (provided by W. Stetler-Stevenson, National Cancer Institute, Bethesda, MD).

After 7 d in culture, the collagen gel–embedded fragments were fixed, the endothelial cells were immunostained with anti–mouse CD31 antibody (BD Biosciences) followed with Alexa Fluor 488 anti–rat IgG secondary antibody (Molecular Probes), and the nuclei were visualized with DAPI (Vector Laboratories). Samples were mounted in Vector Shield (Vector Laboratories) and fluorescent or light images were captured by a microscope (DMLB; Leica; 5×/0.7 NA or 10×/0.7 NA objective lens) equipped with a SPOT RT camera (Diagnostic Instruments). Neovessel length was determined with SPOT software (Diagnostic Instruments) after calibration, using an objective micrometer. For light and transmission electron microscopic studies, gels were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% OsO4 in 0.1 M cacodylate buffer, dehydrated through a graded ethanol series, and embedded in Spurr’s resin. Ultrathin sections were contrasted with 1% uranyl acetate and lead citrate and viewed with a transmission electron microscope (JEOL 1000EX; JEOL USA, Inc.) equipped with a 3.0-MV field emission gun and a goniometer mount designed for helical reconstructions.
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Endothelial cell invasive activity
Mice-derived endothelial cells were isolated as described by Murphy et al. [1998]. The isolated cells were cultured on gelatin-coated dishes in RPMI 1640 media with 5% mouse serum and supplemented with 100 μg/ml endothelial cell growth supplement (BD Biosciences). The endothelial characteristics of the isolated cells were confirmed by von Willebrand factor, CD31, and VE-cadherin staining (Murphy et al., 1998). Dil-Ac-LDL uptake was >99%. To assess invasive activity, confluent monolayers of microvascular endothelial cells were cultured atop type I collagen gels (2.2 mg/ml), fibrin gels (3.0 mg/ml), or acellular explants of human dermis (Alloederm; Life Cell) in the upper compartment of Transwell dishes (Costar) and exposed to a chemotactic gradient of VEGF (100 ng/ml) and HGF (50 ng/ml).

Collagen film degradation assay
Type I collagen gel films (~100 μg) prepared on glass coverslips were labeled with tetracetylated fluorescein isothiocyanate (Molecular Probes) for 45 min. The microvascular endothelial cells were cultured atop the collagen gels in the presence of 100 ng/ml VEGF, 50 ng/ml HGF, and 5% autologous serum. After 7 d, the samples were fixed, polymerized actin was visualized with Alexa Fluor 488 phalloidin (Molecular Probes), and fluorescent images were obtained by a laser scanning fluorescence microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.) equipped with acquisition software (Version 3.2; Service Pack 2) using either 40× or 63× ×1.4-immersion objectives lenses at 25°C. Samples were mounted in Vector Shield (Vector Laboratories).

Chick CAM assays
Endothelial cell invasion, before or after retroviral transduction, was determined in vivo using 11-d-old chick chorioallantoic membrane (CAM). Murine Tim1-MMP–null endothelial cells were seeded atop the CAM for 3 d (Cameron et al., 2000). To visualize endothelial cells, 105 cells were either labeled with 0.05 μm fluorescein carboxylate microspheres (Polysciences) or transduced with a GFP retrovector. The mean depth of endothelial invasion was determined by measuring the distance from the CAM surface to the leading front of two or more invading endothelial cells in 10 or more randomly selected fields, as assessed with a microscope (DMLB; Leica)/SPOT RT camera system in at least two experiments.

Retroviral gene transfer
HA-tagged human MT1-MMP cDNA; soluble MT1-MMP (Met1-Gly535 that lacks the COOH-terminal transmembrane and cytosolic domains of the wild-type proteinase, and MT1-MMPΔ[VA]) is catalytically inactive fulldensity form of MT1-MMP that harbors an E247K to A substitution in its catalytic domain (MT1-MMPΔ[VA]) or mouse cDNA clones for MT2-MMP and MT3-MMP (provided by M. Seki, University of Tokyo, Tokyo, Japan) were subcloned into pRRET2 retrovector derived from the Moloney murine leukemia virus-based Moloney retroviral backbone (Morita et al., 2001). The control pRRET vector carried an EGFP cDNA. Subconfluent monolayers of the isolated endothelial cells were cultured in the retroviral supernatant for 12 h in the presence of 100 μg/ml endothelial cell growth supplement and collagen invasion and degradation assays were performed 24 h later. The expression of MT1-MMP, MT2-MMP, and MT3-MMP was confirmed by Western blot analysis using polyclonal anti–MT1-MMP antibody (Yano and Weiss, 2000) and monoclonal anti–mouse MT2-MMP and MT3-MMP antibodies (Calbiochem), respectively.

RT-PCR analysis
Endothelial cells were cultured atop type I collagen gels in the presence of 100 ng/ml VEGF and 50 ng/ml HGF in 10% serum, and total RNA was isolated using TRIzol reagent (GIBCO BRL). RT-PCR was performed using One-Step RT-PCR System reagent (Life Technologies). The identities of the PCR products were confirmed by sequence analysis.

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