Plasmin-dependent and -independent effects of plasminogen activators and inhibitor-1 on ex vivo angiogenesis

SERGEY BRODSKY,1 JUN CHEN,1 ALBERT LEE,1 KATERINA AKASSOGLOU,3 JILL NORMAN,5 AND MICHAEL S. GOLIGORSKY1,2,4
Departments of 1Medicine, 2Physiology, and 3Pharmacology, and the 4Program in Biomedical Engineering, State University of New York, Stony Brook, New York 11794-8152; and 5Department of Medicine, Royal Free and University College Medical School, London WC1B 6JJ, United Kingdom

Received 12 October 2000; accepted in final form 6 July 2001

Plasminogen activator (PA) inhibitor-1 (PAI-1) has been recognized as a surrogate marker of endothelial dysfunction in diseases associated with impaired angiogenesis, including atherosclerosis, diabetic vasculopathy, and nephropathy. To establish the necessary and sufficient components of the PA system [PAI-1, urokinase-type PA (uPA), or tissue-type PA (tPA), and plasminogen (Plg)] for angiogenesis, we examined angiogenic competence of vascular explant cultures obtained from mice deficient in PAI-1, tPA, uPA, and Plg. To gain insight into the requirement for different matrix-degrading systems during endothelial cell migration across plasmin-degradable basement membranes compared with profibrotic systems during endothelial cell migration through the plasmin-degradable basement membrane and through the plasmin-nondegradable collagen 1 in the interstitium. Therefore, by contrasting ex vivo angiogenesis in three-dimensional (3-D) collagen I to that in three dimensional (3-D) Matrigel, one could gain insight into the importance of ECM degradation by plasmin for endothelial cell migration.

Considering the potentially important role of the PAI-1-PA-Plg system in defective angiogenesis and the availability of genetically engineered mice lacking various components of this system, we used knockout mice to study the ex vivo angiogenic potential to establish the necessary and sufficient components of the PA system for angiogenesis. The data demonstrate that the tPA-Plg system plays an essential role in supporting capillary sprouting ex vivo. The involvement of PAs in the regulation of angiogenesis is not limited to their ability to convert Plg to plasmin, strongly suggesting additional modes of action on remodeling of the vascular network. Finally, we found that the activity of MMP-9 and -1 are undetectable in angiogenesis-incompetent explant cultures from tPA and Plg knockout mice.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
mice, suggesting the role of these MMPs in supporting angiogenesis.

METHODS

Materials. The following reagents were used in these studies: collagen I (Vitrogen) from Cohesion, growth factors-depleted Matrigel (Becton Dickinson), PAI-1 (American Diagnostica), tPA (provided by Dr. S. Strickland, SUNY, Stony Brook, NY), uPA (Calbiochem), plasminogen (American Diagnostica), Ulex europaeus (Sigma), and antibodies against CD31 (PharMingen). Endothelial basal medium-2 (EBM-2) culture medium (Clonetix) was supplemented with 2% fetal bovine serum and growth factors and used in experiments with explant cultures.

Experimental animals. Studies were carried out in the following animals: wild-type (C57BL/6J) and PAI-1 knockout (C57BL/6J Planh1tm1) mice obtained from Jackson Laboratory, tPA knockout mice (C57BL/6J Platm1) obtained from Dr. S. Strickland (SUNY), uPA knockout (C57BL/6J-Pluarm1), and plasminogen knockout (C57BL/6J-Pigtm1), both kindly provided by Dr. J. Degen (University of Cincinnati, Cincinnati, OH). The animals were housed in a room kept at 20–22°C with a relative humidity of 50–50% with 12:12-h light-dark cycle and were allowed free access to food and water throughout the study. The protocol for the animal studies was approved by the University Committee for Animal Studies.

Explant aortic cultures. Thoracic aortas were obtained from 6- to 8-wk-old mice. Mice were anesthetized by intraperitoneal injection with ketamine-xylazine, and a thoracotomy was performed. The full length of the thoracic aorta was aseptically removed and immediately placed into ice-cold EBM-2 medium. After removal of the periaortie fibroblastic tissue with fine microsurgical forceps under a dissecting microscope, the vessels were placed in fresh EBM-2 medium as described previously (23–26). The aortas were then cross-sectioned with 1-mm interval, and the resulting aortic rings were embedded in 3-D collagen I or Matrigel gels in culture chambers (Nalge Nunc), as previously described (23–26).

Quantitative analysis of angiogenesis in explant cultures was performed under an inverted fluorescence microscope (Nikon) equipped with a CCD camera (Hamamatsu Photonics). Vascular sprouts were counted along the perimeter of each explant by two independent observers blinded to the origin of explant cultures, under ×10–100 magnification, with images captured using a SONY XC-77 camera and displayed on a video monitor. Newly formed capillary cords in explant cultures were counted daily for 7 days. Comparative analysis of vascular sprouts within an experimental series and between the experimental groups was performed using ANOVA for multiple comparisons followed by Tukey posttest. P values <0.05 were considered statistically significant.

Preparation of 3-D lattices. Collagen I gels were prepared according to Montesano et al. (20). Briefly, 7 vol of Vitrogen (Cohesion) were quickly mixed at 4°C with 1 vol 10× EBM-2 and 2 vol of 11.7 mg/ml sodium bicarbonate. Gelation was allowed to take place by incubating the cultures at 37°C for 30 min. After gelation, 500 µl of EBM-2 medium containing 2% serum were added to each well, and the chambers were placed in 95% air–5% CO2 incubator at 37°C. Similar procedures were followed in preparing attached Matrigel lattices.

Immunohistochemistry. For immunohistochemistry, the 3-D explants were fixed for 10 min in 4% paraformaldehyde. The preparations were incubated with lectin U. europaeus, used at concentration 10 µg/ml, or with CD31 antibodies used in a 1:100 dilution, as specified in RESULTS, followed by Alexa Fluor 594 goat anti-mouse antibody (1:100 dilution, Molecular Probes; Eugene, OR), according to the previously described techniques (33). Fluorescence microscopy was performed using a Nikon Diaphot or a laser confocal microscope (Olympus).

Gelatin zymography. The MMPs activity was measured in the culture medium conditioned by aortic explants. The samples were mixed with the appropriate volume of 5× sodium dodecyl sulfate (SDS) sample buffer and subjected to electrophoresis in 8.5% SDS-PAGE containing 0.1% gelatin at 4°C (37). After being washed with 2.5× Triton X-100 to remove SDS, gels were washed for 30 min in 50 mM Tris, 0.2 M NaCl, 5 mM CaCl2, and 0.02% Triton X-100, pH 7.6 (developing buffer), transferred to fresh developing buffer, and incubated overnight at 37°C with gentle agitation. Gels were then stained with 0.1% Coomassie brilliant blue G250 solution in 10% acetic acid and 30% methanol and air-dried. Images were scanned, and the intensities of gelatinolytic bands corresponding to MMP-2 and MMP-9 were measured using computer software (Scion Image, National Institutes of Health).

RESULTS

Validation of the model. Daily counting of sprouting endothelial cells in cultured aortic rings derived from C57BL/6J mice showed a progressive increase in the number of capillary cords emanating from the explant vessel (see below). Staining the sprouting cells for the expression of endothelial cell markers CD31/PECAM and U. europaeus lectin confirmed that these cells were endothelial in origin (Fig. 1).

Ex vivo capillary sprouting in PAI-1-deficient mice. Because our previous data demonstrated that PAI-1 suppressed branching of cultured endothelial cells in 3-D lattices (8), we sought to extend these observations to an ex vivo system devoid of endogenous PAI-1. Ex vivo cultures of aortic explants obtained from PAI-1−/− mice showed that these vessels were endowed with the capacity to form capillary sprouts at a higher rate than their wild-type counterparts (as illustrated in Fig. 2, A and B). The capillary cords from PAI-1−/− aortic rings formed a branched network. Quantitative analysis showed that a significant difference in the density of capillary sprouting from the PAI-1−/− and wild-type C57BL/6J mice was readily detectable on day 4 and persisted throughout the experimental period (Fig. 3A, showing an almost twofold increase by day 7). Furthermore, when a constitutively active PAI-1 (10 ng·ml−1·day−1) was added to the culture medium beginning on day 4, PAI-1−/− vessels showed inhibition of angiogenesis by day 6 (Fig. 3A) to the level found in control vessels (43.9% decrease compared with nontreated explants). However, higher concentrations of exogenous PAI-1 (from 10 to 50 ng/ml) showed no additional inhibition of angiogenesis by PAI-1 (Fig. 3B), suggesting that the range of PAI-1 inhibition of angiogenesis was limited. Experiments were then performed in a model of the plasin-degradable matrix, growth factor-depleted Matrigel. This ECM supported ex vivo angiogenesis better than collagen I (Fig. 3C), and the lack of PAI-1 resulted in a further enhance-
ment of capillary sprouting (by 42.7%), consistent with the role of plasmin degradation in angiogenesis.

**Ex vivo capillary sprouting in PA-deficient mice:** tPA is indispensable for capillary sprouting. Because PAI-1 is a physiological inhibitor of the tPA and uPA, we next attempted to characterize the role of each PA in capillary sprouting. Experiments were performed in aortic rings obtained from the tPA and uPA−/− mice. Mice deficient in urokinase exhibited a defect in capillary sprouting (Fig. 4A), but were able to sprout at two-third of the control level in collagen lattices. In contrast, mice deficient in tPA showed an almost complete angiogenic failure (Fig. 4B) in this plasmin-nondegradable matrix. Vascular explant cultures in Matrigel lattices showed that vessels obtained from uPA−/− mice (Fig. 4C) exhibited only marginal inhibition of angiogenesis compared with wild-type vessels, whereas tPA−/− vessels (Fig. 4D) continued to exhibit a profound defect in capillary sprouting.

**Ex vivo capillary sprouting in Plg-deficient mice.** Plg is the main, but not the only, substrate of PAs. Therefore, in the next series of experiments, aortic explants were derived from Plg-deficient mice (Plg−/−). These explants were characterized by an almost complete lack of capillary sprouting in collagen lattices (Fig. 5A). Interestingly, vessels from heterozygous animals (Plg+/−) also failed to form capillary sprouts. Addition of Plg (1 μg·ml−1·day−1) partially restored angiogenic competence to these vessels (Fig. 5A). The most intriguing, however, were the results obtained in cultures of Plg−/− explants supplemented with either uPA (1 U·ml−1·day−1) or tPA (10 ng·ml−1·day−1). These PA-treated vessels partially regained the ability to form capillary sprouts (Fig. 5B). Plg−/− vessels (but not Plg+/−) cultured in Matrigel also showed a dramatic reduction in endothelial sprouts (Fig. 5C), which was completely restored by the administration of Plg (1 μg·ml−1·day−1) to the level found in Plg+/− mice. Treatment with uPA or tPA also conferred angiogenic competence on Plg−/− explants in Matrigel (Fig. 5D), suggesting the existence of an alternative system(s) for degrading both plasmin-degradable and nondegradable matrices in the absence of plasmin. These findings prompted the investigation of MMP activity, as summarized below.

**Activity of MMP-2 and -9.** In an attempt to detect potential differences in the activity of secreted MMPs in these groups of animals, zymography of MMP-2 and -9 activity was performed. Culture medium was sampled from explant cultures on day 7, and all individual experiments in each group were pooled and analyzed. Despite the fact that PAI-1−/− explants were characterized by the stimulation of capillary sprouting, the expression and activity of MMP-2 and MMP-9 were maximal in media obtained from wild-type vessels (Fig. 6A). On the other hand, angiogenesis-deficient explants cultured in collagen lattices were characterized by the poorly detectable expression of MMP-9 (tPA, uPA, and Plg knockout animals). These same explants cultured in Matrigel expressed MMP-9 at a comparable level, suggesting that this MMP was not directly responsible for the lack of capillary sprouting seen in tPA−/− and Plg−/− vessels cultured in the basement membrane-like extracellular matrix. Furthermore, despite the fact that uPA−/− vessels regained the ability to sprout capillaries in Matrigel, their MMP-2 and MMP-9 activity was indistinguishable from that seen in Plg−/− vessels, which remained angiogenesis deficient. Moreover, the expression and activity of MMP-2 and MMP-9 in the culture media from Plg−/− vessels treated with Plg, uPA, or tPA, all of which restored capillary sprouting, did not show any substantial differences (Fig. 6B). Hence, it appears that the basal MMP-9 activity is necessary for angiogenesis in the nonplasmin-degradable collagen I matrix, whereas it is not required for the Matrigel. However,
significant differences in the activity of MMP-1 (and/or colocalized with it MMP-3), represented by the lower band, were detected: it was absent in Plg$^{-/-}$ samples, but reappeared in vessels treated with exogenous Plg, tPA, or uPA. It is possible that the MMP-1 (and/or MMP-3) is responsible for the observed angiogenic rescue of vessels treated with exogenous Plg, tPA, or uPA.

DISCUSSION

The data presented herein demonstrated that ex vivo cultured vessels obtained from mice lacking PAI-1 exhibited an enhanced angiogenic potential, which was partially inhibited by the addition of PAI-1 to the culture medium. Whereas uPA-deficient vascular explants displayed a diminished ability to sprout, tPA-deficient vessels were characterized by an almost complete failure of angiogenesis. A similar angiogenic failure was observed in vessels lacking Plg. These findings, which to the best of our knowledge represent the first systematic study of ex vivo angiogenesis in PAI-1, PAs, or Plg knockout animals, suggested that both Plg and tPA are indispensable for ex vivo angiogenesis in 3-D collagen I and Matrigel lattices.

To gain insights into the requirement for different proteolytic matrix-degrading systems during endothelial cell migration across plasmin-degradable basement membranes as opposed to profibrotic areas containing plasmin-nondegradable collagen, we contrasted vascular sprouting in collagen with Matrigel lattices. Indeed, vascular explants cultured in Matrigel exhibited a much higher propensity for angiogenesis than their counterparts growing in 3-D collagen I. It is interesting that Plg$^{-/-}$ explants behaved similarly to Plg$^{-/-}$ vessels when cultured in collagen lattices, but in Matrigel, they showed a dramatic gain in capillary sprouting, compared with Plg$^{-/-}$. These findings are consistent with the role of plasmin-induced degradation of ECM for angiogenic sprouting. However, all other defects in angiogenesis seen in collagen gels remained qualitatively conserved in Matrigel: both Plg$^{-/-}$ and tPA$^{-/-}$ vessels showed a profound inhibition of sprouting, uPA$^{-/-}$ explants were characterized by an intermediate degree of the inhibition of angiogenesis, whereas PAI-1$^{-/-}$ explants exhibited a significant stimulation of angiogenic sprouting. Hence, the data suggest that plasmin activity is indispensable for endothelial cell invasion of matrix and angiogenesis, even when angiogenesis occurs in plasmin-nondegradable matrix, such as a collagen gel.

Perhaps the most intriguing observation was made in Plg$^{-/-}$ explants supplemented with tPA or uPA. In either case, previously angiogenesis-deficient explants acquired the ability to sprout; this was demonstrated in both collagen and Matrigel lattices. The above finding suggests that, in addition to the conversion of the zymogen Plg to plasmin, both PAs exhibit a plasmin-independent stimulation of angiogenesis and the lack of Plg could be partially compensated by supplemental PAs. In fact, it has been appreciated that uPA can exert its effects on cell migration independent of the catalytic activity of urokinase (9, 27). Furthermore, it has been demonstrated that urokinase fragments containing only the receptor-binding domain are able to stimulate cell migration (11, 12). The role of uPA interaction with its cognate receptor uPAR in cell migration is further supported by the fact that uPAR-deficient neutrophils in patients with paroxysmal nocturnal hemoglobinuria are chemotaxis defective (28). The emerging paradigm of plasmin-independent action of urokinase ascribes to...

**Fig. 2.** Comparison of sprouting capillary cords in aortic explants derived from wild-type or plasminogen activator inhibitor-1 (PAI-1) knockout (PAI-1$^{-/-}$) mice. Micrographs depict representative images of vascular explants (portions of the vessel rings are shown in the right corner) obtained from C57BL/6J littermates (A) and PAI-1-deficient mice (B) and cultured for 6 days in collagen lattices. Because of the thickness of the preparations, not all cells appear in focus. The number of capillary sprouts was counted at different focal planes daily by two independent observers. To facilitate counting, images were captured by a camera and viewed on the screen of a monitor, as detailed in METHODS.
the uPA-uPAR complex an important role in cell migration, binding to vitronectin and α\textsubscript{v}β\textsubscript{3} or β\textsubscript{1}-integrin receptors (6). Such an explanation, however, appears to be incomplete, judged from the finding that not only uPA, but also tPA addition was able to rescue angiogenesis in Plg\textsuperscript{−/−} vessels. It is possible, therefore, that the role of the tPA receptor annexin-II in endothelial cell migration and angiogenesis, so far neglected, needs reevaluation. This calcium- and phospholipid-binding protein, in addition to Plg and plasmin, binds tPA with high affinity [dissociation constant (K\textsubscript{d}) = 25 nM] and increases the catalytic activity of tPA by ~60-fold (14). Sato et al. (30) have previously demonstrated that tPA is indispensable for capillary formation by microvascular endothelial cells. These authors demonstrated that a neutralizing tPA antibody or a serine protease inhibitor aprotinin blocked EGF-induced tube formation by human endothelial cells, and this response could be restored by the addition of tPA to the culture medium. With these data and our own findings on the role of tPA in capillary sprouting, the question that needs to be answered is: What is (are) the mechanism(s) of Plg-independent angiogenic effects of t-PA? It has been proposed that the angiogenic action of PAs may depend on their ability to mobilize matrix-bound growth factors and activate the latent form of transforming growth factor-β1 (34), which per se can activate angiogenesis (6). However, this is unlikely to be applicable to our experimental setting utilizing growth-factor-depleted Matrigel. The very fact that tPA partially restores capillary sprouting of Plg\textsuperscript{−/−} explants cultured in Matrigel strongly suggests that this effect may be due to a nonproteolytic action of tPA. The possibility of a nonproteolytic action of tPA has previously been alluded to in neuronal and microglial cell activation (16, 29), as detailed below.

Our data demonstrate that in Matrigel, a plasmin-degradable matrix, endogenous tPA partially rescues Plg deficiency. In Fig. 4D we demonstrate that tPA is necessary for the formation of capillary sprouts, because tPA\textsuperscript{−/−} mice show on average 12 capillary sprouts compared with 90 sprouts of tPA\textsuperscript{+/+} mice. Figure 5D shows that Plg\textsuperscript{−/−} mice have, on average, 30 capillary sprouts. Our observations cannot be explained by defects in tPA or uPA expression in Plg\textsuperscript{−/−} mice, because it has been reported that no differences are found in plasma levels of uPA or tPA based on gel zymography of whole plasma of Plg\textsuperscript{−/−} mice (2). Therefore, our results on Matrigel show that the endogenous tPA of the Plg\textsuperscript{−/−} mice can partially, but not entirely, rescue the Plg deficiency. However, endogenous tPA cannot rescue the Plg deficiency on collagen (Fig. 5B). This result suggests that the ability of endogenous tPA to rescue angiogenesis depends on the substrate.

**Fig. 3.** Dynamics of capillary sprouting from wild-type (PAI\textsuperscript{+/+}) and PAI-1 knockout (PAI\textsuperscript{−/−}) mice in collagen (A and B) and Matrigel (C) lattices. Vascular sprouts were counted around the perimeter of each explant, under \times100 magnification, with images captured using a SONY XC-77 camera and displayed on a video monitor. The number of capillary cords emanating from PAI-1\textsuperscript{−/−} aortic explants was consistently higher than in control wild-type explants. A separate group of PAI-1\textsuperscript{−/−} explants was treated daily with a constitutively active recombinant PAI-1 (10 ng·ml\textsuperscript{−1}·day\textsuperscript{−1}), commencing on day 4. The rate of vascular sprouting decreased to the level observed in control within 48 h of PAI-1 restoration. B: increased concentration (10–50 ng·ml\textsuperscript{−1}·day\textsuperscript{−1}) of PAI-1 supplementation did not show a dose-dependent suppression of capillary sprouting in PAI-1\textsuperscript{−/−} vessels. C: dynamics of capillary sprouting from PAI-1\textsuperscript{−/−} and PAI-1\textsuperscript{+/+} vessels in Matrigel lattices. Replenishing PAI-1 (10 ng·ml\textsuperscript{−1}·day\textsuperscript{−1}) suppressed capillary sprouting to the level observed in wild-type vessels. *P < 0.05 vs. PAI-1\textsuperscript{+/+} explants; #P < 0.05 vs. PAI-1\textsuperscript{−/−}.
Fig. 4. Dynamics of vascular sprouting in aortic explants from urokinase-type PA (uPA) (A and C) and tissue-type PA (tPA) (B and D) knockout mice and cultured in 3-D collagen (A and B) or Matrigel (C and D). A and C: capillary sprouting from uPA−/− vessels was inhibited in collagen but not in Matrigel. B and D: severe defect of capillary sprouting in vessels obtained from tPA−/− mice was independent of the extracellular matrix used. *P < 0.05 vs. wild-type explants.

Fig. 5. Profound defect of angiogenesis in explants derived from plaminogen (Plg)−/− mice and cultured in 3-D collagen (A and C) or Matrigel (C and D). A: an almost complete cessation of angiogenesis from vascular rings lacking (−/−) or deficient (+/−) in Plg and a partial restoration of angiogenic competence associated with supplementing culture medium with 1 μg·ml−1·day−1 Plg. B: treatment of Plg−/− explants with daily administration of uPA or tPA (1 U·ml−1·day−1 and 10 ng·ml−1·day−1, respectively) partially restored capillary sprouting. C: lack of Plg was characterized by a significant suppression of angiogenesis only in Plg−/− vessels (but not in Plg+/− vessels). This defect was almost completely restored by the administration of Plg (1 μg·ml−1·day−1) to the level found in Plg+/− mice. D: treatment with uPA (1 U·ml−1·day−1) or tPA 10 ng·ml−1·day−1) has also conferred angiogenic competence on Plg−/− explants. *P < 0.05 vs. nontreated Plg−/− vessels.
possible explanation could come either from the composition of the two substrates or from their dependence on MMP-9 activity. Interestingly, the basal MMP-9 activity seems necessary for the nonplasmin-degradable collagen I matrix, whereas it is not necessary for the Matrigel (Fig. 6). Therefore, if the tPA-plasmin system utilizes two distinct mechanisms to regulate capillary sprouting: 1) a mechanism dependent on plasmin-mediated activation of MMPs; and 2) a mechanism independent of plasmin and, acting probably through a nonproteolytic effect of tPA, endogenous tPA might be able to rescue Plg deficiency when MMP-9 activity is not necessary for the process. Therefore, in the MMP-9 independent matrix, as the Matrigel, endogenous tPA might be sufficient to rescue angiogenesis, whereas in the MMP-9-dependent matrix endogenous, tPA is not sufficient to rescue Plg deficiency. Addition of exogenous tPA at pharmacological levels, which might be higher than the endogenous levels of tPA at the capillaries, might overcome the plasmin-dependent mechanism and be sufficient to induce capillary sprouting independent of the requirements of the substrate. The concept of two distinct pathways, one controlled by PAs and the other by plasmin-induced activation of MMPs, has also been reported in endothelial cell organization, where the effects of serine protease inhibition are additive to those of MMP inhibitors, thus suggesting that the two classes of proteases modulate endothelial cell formation by distinct mechanisms (31). We also suggest that these two mechanisms coexist in vivo, and the local composition of the ECM combined with the local expression pattern of PAs and MMPs determines which mechanism will be dominant.

Fig. 6. Gelatinolytic activity in the culture medium from vascular explants maintained in collagen or Matrigel lattices. A: zymograms of conditioned culture media obtained from wild-type, PAI-1, uPA, tPA, and Plg knockout mice. Position of MMP-2 and MMP-9 is shown on left. An additional band, the fastest-migrating, represents MMP-1 (or a comigrating MMP-3). This band was attenuated in all three groups of angiogenesis-incompetent vessels. Results of densitometric analysis of these bands are presented in right. B: zymograms of conditioned culture media obtained from Plg−/− vessels treated with Plg, uPA, or tPA (see details in RESULTS). All three treatments, which improved capillary sprouting, resulted in the activation of MMP-9, MMP-2, and MMP-1 in collagen, but showed no detectable differences in Matrigel. Results of densitometric analysis of these bands are presented in right.
From the observed plasmin-independent effects of both PAs, it is important to emphasize that mice lacking either tPA or uPA are characterized by normal development, fertility, and life span, despite the reduced thrombolytic potential and occasional spontaneous fibrin deposition in normal or inflamed tissues (6). Double knockout mice also survive but suffer postnatal growth retardation, reduced fertility, and shortened life span, perhaps due to more extensive spontaneous thrombotic events (7). Indeed, it has been demonstrated in mice lacking individual components of the PA-Plg system that the fibrinolytic properties of this cascade are not required for angiogenesis (15) and that angiogenesis in fibrin matrices, as a model of angiogenic remodeling during wound healing, depends on the fibrinolytic properties of the membrane type 1 MMP. In contrast to angiogenesis in collagen or Matrigel lattices emulating interstitial vascular remodeling, as described herein, in fibrin gels Hiraoka et al. (15) did not observe any defect in angiogenic sprouting from abdominal muscle explants that were obtained from Plg$^{-/-}$ or combined uPA$^{-/-}$ and tPA$^{-/-}$ mice. Compared with our data, angiogenesis in fibrin clots was significantly delayed (initial sprouts appeared on days 6–7 as opposed to days 2–3 in our series), despite the high concentration of autologous sera (10% final). These distinct findings suggest that the molecular mechanisms of angiogenesis in wound healing (fibrin clot model) and at the sites of interstitial collagen deposition or across the basement membrane (collagen and Matrigel models, respectively) are characterized by profound differences. Alternatively, angiogenic properties of aortic explants vis-à-vis the vasculature of abdominal muscle have profound differences. These differences notwithstanding, recent findings established the transcriptional activation, expression, and activity of membrane type 1 MMP in endothelial cells cultured in 3-D collagen I lattices (13), thus supporting the participation of this particular proteolytic system in in vitro angiogenesis models. In addition to this, our gelatin zymography studies (Fig. 6) demonstrated that all three groups of angiogenesis-deficient explants (tPA, uPA, and Plg$^{-/-}$) showed a dramatic decrease in MMP-9 activity in collagen and probably MMP-1 activity in collagen and Matrigel. Whereas MMP-1 and MMP-3 are substrates for plasmin activation, MMP-2 and -9 are not activated by plasmin (21). It is possible that several yet unexplained phenomena observed in Plg$^{-/-}$ and tPA$^{-/-}$ angiogenic assays could be related to the cascades of activation by different MMPs.

There is a growing awareness that PAI-1, a surrogate marker of endothelial dysfunction (32, 36), may have pathophysiological implications in the course of atherosclerosis and diabetic vasculopathy and nephropathy. A direct correlation exists among PAI-1, hemoglobin, Alc, insulin level, and intima-media thickness in 40- to 70-yr-old nondiabetic patients with familial history of type II diabetes mellitus (32). Accelerated atherosclerosis and thrombosis are the major causes of morbidity and mortality in kidney transplant recipients. In cyclosporine-A-treated patients, PAI-1 activity was increased, suggesting a contribution to thrombogenicity (34). Recent studies by Kimura et al. (17) have demonstrated a 4/5-guanine tract polymorphism in the promoter region of the PAI-1 gene in diabetic patients experiencing macroangiopathy and showed that the PAI-1 4G4G genotype (which leads to elevated levels of circulating PAI-1 activity compared with 5G5G genotype) is an independent risk factor for development of macroangiopathy. Expression of PAI-1 promotes antifibrinolysis and accumulation of collagen (1, 35). It has been reported that advanced glycation end products-modified albumin induces PAI-1 mRNA concomitant with the increase in abundance of this serine protease inhibitor and its activity in human microvascular endothelial cells (34). Furthermore, serial analysis of gene expression in endothelial cells exposed to atherogenic stimuli revealed 56 differentially expressed genes, PAI-1 being one of them (10). Murphy et al. (22) observed the induction of several genes, including PAI-1, in mesangial cells incubated in media containing high glucose concentration. High glucose and hyperosmolality have also been incriminated in PAI-1 induction in human umbilical vein endothelial cells (18). Our own data on PAI-1 induction by a glycated matrix protein demonstrated an early response of this gene in endothelial cells to environmental cues reminiscent of the diabetic microenvironment in the interstitium undergoing fibrogenesis and link it to the defective branching angiogenesis in vitro (8). Cumulatively, these findings provide a solid foundation to the conclusion that the PAI-1-PA-Plg system plays an important role in vascular remodeling, although the contribution of specific elements of this cascade to angiogenesis may vary depending on the properties of the matrix:angiogenesis in the process of fibrogenesis and in the process of wound healing.

The authors are indebted to Dr. J Degen for providing uPA- and Plg-deficient mice used in these studies. A. Lee was a high-school student conducting an Intel project. These studies were supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-54602 and DK-45462.

REFERENCES


