Thrombin Induces the Activation of Progelatinase A in Vascular Endothelial Cells

PHYSIOLOGIC REGULATION OF ANGIOGENESIS*

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Angiogenesis requires degradation of vascular basement membrane prior to migration and proliferation of endothelial cells; proteinases are essential ingredients in this process. Because of thrombin’s multiple effects on endothelium, we have examined its role in matrix metalloproteinase activation using human umbilical vein endothelial cells. Gelatin zymography of endothelial cells conditioned media revealed a prominent 72-kDa gelatinolytic band which is consistent with the activation of progelatinase A; thrombin had no effect in the absence of cells. This effect requires the proteolytic site of thrombin since progelatinase A activation was abolished by specific inhibitors of thrombin. Matrix metalloproteinase inhibitors diminished thrombin-induced activation of progelatinase A. Pretreatment of endothelial cells with excess tissue inhibitor of metalloproteinase-2 or a COOH-terminal fragment of progelatinase A abrogated thrombin-mediated activation of progelatinase A presumably by competing with the COOH terminus of native progelatinase A for interaction with an activator site on endothelial plasma membranes. Although membrane-type matrix metalloproteinase was demonstrated in endothelial cells by Northern and Western blotting, the receptor function of this molecule in thrombin-induced activation of progelatinase A needs to be clarified. Progelatinase A activation did not require intracellular signal transduction events mediated by the thrombin receptor. These data demonstrate that 1) endothelial cells express a novel activation mechanism for progelatinase A, 2) proteolytically active thrombin regulates this activation mechanism, and 3) activation occurs independently of the functional thrombin receptor.

Whereas the effect of thrombin (EC 3.4.21.5) on production of fibrin and activation of platelets has been intensively studied over many years, interest in the role of thrombin on endothelial function has lagged. Recent studies have indicated that thrombin affects post-dotting events involved in angiogenesis (1). The vascular endothelium actively binds coagulation proteins, resulting in cell-surface generation of thrombin that can persist within the protected environment of a clot (2). A unique G-protein-coupled thrombin receptor (3) is known to functionally expressed by endothelial cells (4). Interaction of thrombin with an endothelial cell-surface thrombin receptor(s) results in a multiplicity of effects including cell retraction and permeability, generation of phosphoinositides and prostaglandin, and secretion of Von Willebrand factor, tissue plasminogen activator, and platelet-derived growth factor (5–7). The role of individual thrombin receptors and endothelial signal transduction events on thrombin-mediated cell activation phenomena remain incompletely characterized (4).

Neoangiogenesis, the formation of new blood vessels from preexisting vessels, requires the degradation of underlying basement membranes prior to migration and proliferation of endothelial cells, and ultimately the formation of new capillary sprouts (8). Matrix metalloproteinases, specifically gelatinase A (72-kDa type IV collagenase, MMP1-2, EC 3.4.24.24), gelatinase B (92-kDa type IV collagenase, MMP-9, EC 3.4.24.35), and interstitial collagenase (MMP-1, EC 3.4.24.7), are released by endothelial cells and play an important role in turnover of basement membrane type IV collagen, type I collagen, laminin, and fibronectin during angiogenesis (9, 10). A delicately orchestrated balance between production, activation, and inhibition of MMPs is considered to be essential in maintenance of blood vessel integrity (11–13). A similar role in angiogenesis has been proposed for plasminogen activators and inhibitors with evidence that these two proteolytic systems may be interconnected (11, 14).

The physiologic mechanism controlling the activation of MMPs, especially progelatinase A, is not well understood (15). Plasmin and stromelysin are capable of activating latent gelatinase B and interstitial collagenase, but the alignment of propeptides in progelatinase A lacks the appropriate proteinase cleavage sites in the amino-terminal domain of the mole-

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‡ The abbreviations used are: MMP, matrix metalloproteinase; HUVEC, human umbilical vein endothelial cells; MT-MMP, membrane-type matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; TR, thrombin receptor; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethylketone 2·HCl; APMA, aminophenyl mercuric acetate; PMSF, phenylmethylsulfonyl fluoride; CT1399, N4-hydroxy-N1-[1-(5-((morpholinosulfonyl)aminoethyl)-amino-carbonyl)2-cyclohexyl-ethyl]-2-(R)-(4-chlorophenyl)propyl)succinamide; PAGE, polyacrylamide gel electrophoresis; E-64, L-transepoxysuccinylleucyl-amido(4-guanido)-butane; PCR, polymerase chain reaction.
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Cell Culture and Transfection Experiments

Endothelial cells were isolated from pooled primary cultures of human umbilical vein (HUVEC) as described previously (4) and used in passage 2-5. Cells were routinely cultivated on gelatin-coated plates in M199 media supplemented with 10% fetal calf serum, 100 μg/ml endothelial cell growth factor (Collaborative Research, Bedford, MA), 100 μg/ml porcine intestinal heparin, penicillin (100 units/ml), and streptomycin (100 μg/ml), in 5% CO2 at 37 °C. To obtain conditioned media for gelatinase studies, HUVEC were propagated in gelatin-coated 24-well dishes (Becton Dickinson, Lincoln Park, NJ) at a concentration of 7.5 × 104 cells/well. After reaching ~ 75% confluence, cells were serum starved for 4 h and subsequently exposed to M199 media with or without other additives for 2-24 h before harvesting spent conditioned media. Endothelial cell viability remained intact, but cell replication did not occur in serum-starved M199 media. In preliminary experiments, we determined that 200 μl of media/well (containing subconfluent endothelial cells) reproducibly resulted in detectable gelatinolytic bands on zymography without requiring concentration of the media.

For functional expression studies of the thrombin receptor, the full-length construct encompassing the thrombin receptor open reading frame (4) was cloned into the Bam HI site of pCdNA (Invitrogen Corp., San Diego, CA) which contained MT-MMP. The activated MT-MMP-TIMP-2 complex in turn acts as a receptor for progelatinase A by binding the carboxy end domain of secreted progelatinase A.

Based on thrombin's known proliferative effects on various cell types (28), including endothelial cells (29), and the subsequent activation of MMPs occurring during angiogenesis, we hypothesized that thrombin may, in part, be responsible for the activation of endothelial cell progelatinase. We now demonstrate that thrombin treatment of endothelial cells results in the activation of progelatinase A; this occurs independently of signal transduction events mediated by the G-protein-coupled thrombin receptor, but is dependent on the presence of an uncharacterized plasma membrane activation mechanism. We propose that thrombin generated during the hemostatic process induces remodeling of the endothelium as a component of neovascularogenesis.

EXPERIMENTAL PROCEDURES

Reagents and Peptides

N-Phenylalanyl-L-prolyl-L-arginine chloromethylketone 2 HCl (PPACK) was purchased from Calbiochem (La Jolla, CA). Fibronectin, hirudin, laminin, gelatin, aprotonin, aminophenyl mercuric acetate (APMA), EDTA, phenylmethylsulfonyl fluoride (PMSF), leupeptin, N-ethylmaleimide, l-transpeptidylcycloleucyl-amidotri-guanidino)butane (E-64), α2-antiplasmin, pepstatin, and 1,10-phenanthroline were purchased from Sigma. Purified human α-thrombin (~ 3,500 units/mg, 1 mg of 0.1 unit/ml, 95% pure) was kindly supplied by Dr. J. Jesty (State University of New York at Stony Brook). The peptide ligands representing the new NH2 termini of the thrombin receptor after thrombin cleavage (TR42–55, SFLLRNPDKYEPF and TR42–47, SFLLRN) have been previously described (27). Described a multistep process in which activated MT-MMP acts as a cell surface TIMP-2 receptor. The activated MT-MMP-TIMP-2 complex in turn acts as a receptor for progelatinase A by binding the carboxy end domain of secreted progelatinase A.

The proteolytic effects of 100 nM thrombin on recombinant TIMP-1 (NOVEX, San Diego, CA), which contains MT-MMP, was assessed by Western blotting and zymography at 48 h and examining the effect on gelatinolytic bands as described previously (34). Recombinant human progelatinase A, NH2-terminal gelatinase (truncated COOH-terminal domain deletion mutant of progelatinase A lacking amino acids 418–631 (36 kDa) (18), COOH-terminal gelatinase A lacking amino acids 1–414 (functionally inactive 31 kDa), an inactive recombinant progelatinase A mutant with glutamic acid residue (E159) replaced by alanine (proE159→A) capable of binding TIMP-2 (30), recombinant TIMP-1 and TIMP-2 (18), and CT1399 (€−hydroy-N-[1-

Western blotting was performed using Tris-glycine SDS sample and running buffers as described by the manufacturer (NOVEX, San Diego, CA). After electrophoresis, SDS was replaced by Triton X-100 (2.5%), thus renaturing gelatinases. Astable system (4) was cloned into the BamHI site of pCdNA (Invitrogen Corp., San Diego, CA) which contained MT-MMP. The activated MT-MMP-TIMP-2 complex in turn acts as a receptor for progelatinase A by binding the carboxy end domain of secreted progelatinase A.

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Identification of MT-MMP

Northern Blotting—Total cellular RNA from HUVEC, the monocytic cell line (U937), human erythroleukemia cell line (HEL), Philadelphia chromosome-positive cell line (K562), the promyelocyte cell line (HL-60), and Rat-2 cells was isolated by guanidine solubilization, phenol/chloroform extraction, and serial precipitation, essentially as described previously (38). Oligonucleotide primers encompassing the open reading frame of the membrane-bound MT-MMP were synthesized on an Applied Biosystems single-channel synthesizer: forward primer (5'-3': CAGGAATTCGAGATGTGCTCCGCGGAGA; reverse primer (5'-3': CAGGAATTCGAGATGTGCTCCGCGGAGA; underlined)). cDNA synthesis was performed by denaturing gel electrophoresis followed by Northern transfer to nylon membranes (Schleicher and Schuell). Blots were hybridized to the 32P-radiolabeled MT-MMP insert at 68°C overnight using a solution containing 6× SSC without formamide and 50% formamide.

RESULTS

Thrombin-induced Activation of HUVEC Progelatinase A—Gelatin zymography of conditioned media isolated from endothelial cells revealed the progressive release of 72 and 92-kDa gelatinolytic bands (72 > 92 kDa), consistent with progelatinase A and progelatinase B, respectively. Thrombin treatment of endothelial cells resulted in a dose-dependent (100 > 10 > 1 nM) generation of gelatinolytic bands of 64 and 62 kDa accompanied by a relative decrease in 72-kDa progelatinase A over 24 h which is consistent with the activation of progelatinase A (Fig. 1A and B). Whereas thrombin treatment of endothelial cells appeared to result in an early increase in total gelatinase A secretion as measured by zymography, gelatinase A secretion was not significantly increased by thrombin at 24 h as measured by either zymography or enzyme-linked immunosorbent assay (Fig. 1B, Table I). Thrombin did not activate 72-kDa latent gelatinase A (HUVEC-conditioned media) during 24 h of incubation at 37°C under cell-free conditions; this observation has been previously reported (16). Thrombin did not affect HUVEC release or activation of progelatinase B. Weak bands of gelatinolytic activity at 54 and 52 kDa were also noted, which were not affected by thrombin treatment; these bands probably represent either interstitial collagenase or stromelysin-1 which have a similar molecular mass (39). Immunoblot of 4–24 h HUVEC-conditioned media revealed low levels of stromelysin-1 release (0.07–0.11 nM), with no significant response to thrombin treatment (data not shown). Interstitial collagenase release was below the level of detection by immunoblot. A weak ~200 kDa gelatinolytic band was also
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TABLE I
Quantification of gelatinase A, TIMP-1, and TIMP-2 released by endothelial cells cultivated in the presence or absence of thrombin

| TIME | GLA, no thrombin | GLA + thrombin (10 nM) | GLA + thrombin (100 nM) | TIMP-1, no thrombin | TIMP-1 + thrombin (10 nM) | TIMP-1 + Thrombin 100 nM | TIMP-2, no thrombin | TIMP-2 + thrombin (100 nM) |
|------|------------------|------------------------|------------------------|--------------------|-------------------------|--------------------------|-------------------|--------------------------|
| h    |                  |                        |                        |                    |                         |                          |                   |                          |
| 4    | 1.43 ± 0.16      | 1.18 ± 0.10            | 1.55 ± 0.16            | 0.10 ± 0.02        | 0.06 ± 0.01              | 0.08 ± 0.01              | 0.38 ± 0.03       | 0.33 ± 0.04               |
| 8    | 1.79 ± 0.27      | 1.64 ± 0.18            | 1.72 ± 0.81            | 0.25 ± 0.05        | 0.22 ± 0.01              | 0.21 ± 0.02              | 0.63 ± 0.05       | 0.65 ± 0.08               |
| 24   | 3.98 ± 0.44      | 3.81 ± 0.26            | 3.72 ± 0.27            | 1.31 ± 0.08        | 1.70 ± 0.20              | 1.32 ± 0.11              | 0.97 ± 0.09       | 1.05 ± 0.12               |

noted inconsistently in endothelial conditioned media within 2-4 h of incubation and was enhanced in thrombin-treated cells (compare Figs. 1A and 4B). Thrombin treatment of HUVEC did not affect the release of TIMP-1 or TIMP-2 as measured by immunoassay (Table I).

To document the class of proteinases identified on zymography, the gels were incubated with low molecular weight proteinase inhibitors (Fig. 2A). Incubation of the gelatin-impregnated gels with 1,10-phenanthroline or EDTA (chelators of metal ions) completely blocked the appearance of the endothelial-conditioned media gelatinolytic bands at 200, 92, 72, 64, and 62 kDa; PMSF (inhibitor of serine proteinases), leupeptin (inhibitor of serine proteinases and cysteine proteinases), and N-ethylmaleimide (inhibitor of cysteine proteinases) had no effect on these lytic bands. This inhibition profile demonstrates that these gelatinolytic bands are bona fide metalloproteinases.

To confirm the identification of the gelatinolytic bands released by thrombin-treated endothelial cells, progelatinase A (72 kDa) and activated gelatinase A (64-62 kDa) were identified on immunoblots using specific rabbit anti-human antibodies (Fig. 2B). Additional gelatinase A immunoreactive bands were noted at 52, 44, and 30 kDa which is consistent with inactive species of gelatinase A (no gelatinolytic activity on zymography) as described previously (40). TIMP-2 immunoreactive bands were noted at 21, 62, and 72 kDa which is consistent with free TIMP-2, TIMP-2 bound to activated gelatinase A, and TIMP-2 bound to latent gelatinase A, respectively (35). TIMP-1 immunoreactive bands were noted at 28 and 62 kDa which is consistent with free TIMP-1 and TIMP-1 bound to activated gelatinase A, respectively.

A direct method for examining the degree of gelatinase activation was to incubate conditioned media (20 × concentrated) from non-treated endothelial cells versus thrombin-treated cells with [3H]gelatin for 2 h. As noted in Fig. 3, conditioned media from untreated HUVEC degraded 0.2 ± 0.1% gelatin/mg protein; preincubation of this conditioned media with APMA (an activator of MMPs) resulted in 433 ± 34% gelatin degradation/mg. In contrast, conditioned media of thrombin-treated endothelial cells degraded 374 ± 28% gelatin/mg protein (p < 0.001 as compared to non-thrombin treatment); preactivation of this conditioned media with APMA resulted in 466 ± 69% substrate degradation/mg indicating that most of the gelatinase A was activated after incubation of HUVEC with thrombin. Incubation of conditioned media from non-thrombin-treated endothelial cells with 100 nM thrombin for an additional 24 h (in absence of cells) resulted in minimal enhancement of substrate degradation (p > 0.2). This result indicates that endothelial cells are essential in thrombin-induced activation of progelatinase A. The ability of TIMP-1 to inhibit the functional biological activity of activated gelatinase A was also examined. Incubation of APMA-activated conditioned media from thrombin-treated and -untreated cells with TIMP-1 resulted in almost total inhibition of substrate degradation.

Activation of Progelatinase A: Effect of Inhibition of Protein Synthesis and of Proteinase Inhibitors—Incubation of thrombin-treated endothelial cells with cycloheximide (1 μM final concentration), an inhibitor of protein synthesis, resulted in 45% decreased gelatinase A production, but did not alter the activation of gelatinase A (Fig. 4A); 3 μM cycloheximide resulted in 90% decrease in protein synthesis but did not diminish the activation of progelatinase A (data not shown). The requirement for the proteolytic activity of thrombin in activation of progelatinase A was evaluated by adding the specific inhibitor PPACK (2 μM) to thrombin-treated endothelial cells; PPACK produced 92% inhibition of progelatinase A activation and reduced the secretion of the ~200 kDa gelatinolytic band (Fig. 4B). A different type of thrombin inhibitor, hirudin (1 unit/ml), also inhibited thrombin-induced activation of progelatinase A.
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Fig. 3. Histogram showing the effect of thrombin treatment of endothelial cells on activation of progelatinase as measured in a soluble [3H]gelatin degradation assay. Conditioned media was collected from buffer-treated HUVEC (left-hand panel) and 100 nM thrombin-treated HUVEC (right-hand panel) after 18 h of incubation in M199 media. Conditioned media was concentrated from untreated cells (final protein concentration 1343 μg/ml) and thrombin-treated cells (1357 μg/ml), incubated with [3H]gelatin (200 μg/ml) for 2 h and [3H]gelatin degradation was measured as described under "Experimental Procedures." Degree of substrate degradation is expressed as percent gelatin degradation/milligram of protein, to account for differences in protein concentration between samples. Thrombin-induced endothelial cell progelatinase A activation was highly significant as compared to buffer-treated cells (p < 0.001). Addition of thrombin to conditioned media of untreated cells (no cells present; left-hand panel) did not result in significant activation of progelatinase A. APMA was used to activate latent MMPs.

Fig. 4. Gelatin zymograms demonstrating the effect of serine, cysteine, and aspartate proteinase inhibitors and of a protein synthesis inhibitor on thrombin-mediated activation of progelatinase A in endothelial cells. HUVEC were incubated at 37°C with or without thrombin; proteinase inhibitors were added to endothelial cells 1 h prior to addition of thrombin. Conditioned media was collected after 18 h. A demonstrates the effect of cycloheximide, soybean trypsin inhibitor, aprotinin, E-64, pepstatin, COOH-terminal gelatinase A, and NH2-terminal progelatinase A on thrombin-induced endothelial cell activation of progelatinase A. HUVEC were incubated for 18 h without (CONT, lane 1) or with 20 nM thrombin (lanes 2-9) in the presence of 1 μM cycloheximide (CYCL, lane 3), 2 μM soybean trypsin inhibitor (SBTI, lane 4), 1 μM aprotinin (APR, lane 5), 1 μM E-64 (lane 6), 1 μM pepstatin (PEPS, lane 7), 0.3 μM COOH-terminal gelatinase A (C-GL, lane 8), and 0.3 μM NH2-terminal gelatinase A (N-GL, lane 9). B demonstrates the activation of progelatinase A (lane 1, no thrombin added) by the addition of thrombin (20 nM) to endothelial cells (lane 2). PPACK (2 μM) inhibited progelatinase A activation by 80%; α2-antiplasmin (α2AP, 1 μM) had no inhibitory effect on activation.

Mechanism (Fig. 4, A and B). Addition of plasminogen (2 μM) to HUVEC cultures did not enhance spontaneous activation of progelatinase A (data not shown), further negating a role for plasminogen activators and plasmin in activation of progelatinase A. The cysteine proteinase inhibitors E-64 (1 μM) did not inhibit thrombin-mediated activation of progelatinase A, nor did the aspartate proteinase inhibitor pepstatin (1 μM). (Fig. 4A). In contrast, non-toxic concentrations of CT1399 (10–100 nM), a hydroxamic acid-based inhibitor of MMPs (M, 630) designed with enhanced specificity for gelatinase A and gelatinase B as compared to stromelysin-1, collagenase, or matrilysin, induced a dose-dependent inhibition of progelatinase A activation (Fig. 6A).

Thrombin-induced Signal Transduction Events Are Not Sufficient for Activation of Progelatin A—To determine if the thrombin effect on HUVEC progelatinase A activation is mediated through the G-protein-coupled thrombin receptor, HUVEC were stimulated with the thrombin receptor activating peptide TR42-55, this ligand did not mimic native thrombin activation of progelatinase A (Fig. 5). The thrombin receptor activation peptide TR42-47 likewise did not activate HUVEC progelatinase A (data not shown). Furthermore, the inhibitory thrombin receptor antibody (anti-TR1-160) (4) did not interfere
with thrombin-induced activation of progelatinase A (Fig. 5). These data suggest that signal transduction events mediated by the functional thrombin receptor were unrelated to progelatinase A activation, which was confirmed using Rat-2 fibroblasts. As demonstrated in Fig. 7A, Rat-2 fibroblasts expressing the functional thrombin receptor (Rat-TR) displayed typical elevations in intracellular calcium transients when activated by either thrombin or TR42–55. No activation is seen using wild-type cells. We then studied the role of this receptor in recapitulating thrombin-induced progelatinase A activation. As demonstrated in Fig. 7B, the conditioned media of Rat-2 cells expressing the thrombin receptor contained latent gelatinase A exclusively; incubation of these cells with 40 nM thrombin did not result in the activation of progelatinase A. Incubation of non-TR-transfected Rat-2 cells (wild-type) with thrombin also did not result in the activation of progelatinase A. In contrast, incubation of Rat-2 cell-conditioned media (no HUVEC present) with APMA, an activator of latent MMPs, resulted in activation of progelatinase A confirming the latency of the secreted enzyme.

Cell Surface-related Events in the Activation of Progelatinase A—We next evaluated whether a cell surface activation mechanism was responsible for thrombin's effect on progelatinase A. Preincubation of endothelial cells with the COOH-terminal component of gelatinase A (300 nM) prior to the addition of thrombin resulted in 80% inhibition (as determined by densitometry) of thrombin-induced activation of gelatinase A (Fig. 4A). Similar inhibition of progelatinase A activation was achieved using a nonfunctional mutant proE475->A progelatinase A (data not shown). In contrast, preincubation of endothelial cells with a COOH-terminal domain deletion mutant of gelatinase A (containing an intact NH2-terminal region but lacking amino acids 418–631) had no inhibitory effect on thrombin-induced activation of progelatinase A in Rat-TR is demonstrated. Rat-2 cells and Rat-TR cells were cultivated in serum-free media with or without thrombin. Lanes 1, 2, and 5 contain conditioned media collected after 24 h of incubation in M199 of parent Rat-2 cells not transfected with TR cDNA. Lanes 3, 4, and 6 contain conditioned media of Rat-TR cells expressing the thrombin receptor. Lanes 1, 3, and 5 contain conditioned media of untreated cells; lanes 2, 4, and 6 contain conditioned media supplemented with 40 nM thrombin. Lanes 5 and 6 contain conditioned media, which following separation from cells, was subsequently treated for 2 h with 1 μM APMA to active progelatinase A. The 68 and 70 kDa doublet band of gelatinolytic activity is characteristic of rat gelatinase A. Metalloproteinase activation with APMA generated the characteristic 62-kDa active gelatinase A.

Based on the well known capacity of TIMP-2, but not TIMP-1, to bind to the COOH-terminal domain of progelatinase A (stabilization site) and inhibit membrane induced activation of progelatinase A (17), we compared the effect of these inhibitors on thrombin activation of MMPs. Addition of TIMP-2 (24 nM final concentration) to endothelial cell cultures was able to totally abrogate the activation of 72-kDa progelatinase A to 64 and 62 kDa gelatinolytic bands. TIMP-1 (24 nM) had no inhibitory effect on progelatinase A activation; a 40-fold higher concentration of TIMP-1 (960 nM) was required to produce 55% inhibition of progelatinase A activation (Fig. 6B). TIMP-2 (24 nM), but not TIMP-1 (24 nM), also inhibited spontaneous acti-

![Image](97x140)
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MT-MMP protein was identified by immunoblotting in both HUVEC and Rat-2 fibroblast lysates as a 63-kDa protein using monoclonal antibody 113-587 (Fig. 8B). Staining intensity of MT-MMP bands were equivalent between wild-type and TR-transfected Rat-2 cells. The 63-kDa MT-MMP protein was also identified in HUVEC and Rat-2 cells using a rabbit polyclonal antibody to MT-MMP (data not shown).

Proteolytic Effect of Thrombin on TIMP-1 and TIMP-2—To determine whether the activation of progelatinase A was related to an indirect proteolytic effect of thrombin on MMP inhibitors, thrombin (100 nM) was incubated for 24 h with recombinant TIMP-1 and TIMP-2. As examined by SDS-PAGE, there was no evidence of thrombin digestion of the 28- or 21-kDa recombinant proteins, respectively (data not shown).

**DISCUSSION**

It has been proposed that protease activity is involved at three discrete points during angiogenesis: 1) local degradation of basement membrane allowing migration of endothelial cells out of the existing vessel, 2) migration of endothelial cells through stroma, and 3) remodeling of the basement membrane as the new vessel forms (12). Matrix metalloproteinases and plasminogen activators appear to have important roles in these remodeling events. Early studies indicated that endothelial cells secrete relatively high concentrations of immunoreactive, but functionally inactive metalloproteinases (41). Difficulty in demonstrating metalloproteinase activity was attributed to endothelial cell production of large amounts of TIMP-1 and TIMP-2 which form complexes with and inhibit MMP activity. Endothelial cells secrete gelatinase A and gelatinase B selectively in a basal direction further supporting a role for these proteases in turnover of basement membrane components during angiogenesis (9). Experiments examining the function of gelatinase A in endothelial cell tube formation demonstrated that exogenous TIMP-1 or TIMP-2 were able to inhibit and exogenous gelatinase A was able to stimulate vessel formation, thereby emphasizing the requirement for balanced production of gelatinases and inhibitors in the early stage of angiogenesis (12, 14, 42). Specific pharmacologic inhibitors of MMPs have also been demonstrated to decrease angiogenesis in experimental animals (43).

Based on the central role of thrombin in diverse aspects of endothelial cell function (2, 4, 5), and the potential importance of gelatinases in angiogenesis (12, 14, 39, 41, 42), we considered the possibility that thrombin may be involved in the regulation of matrix metalloproteinases in endothelial cells. We herein report that within 2–4 h of addition of thrombin to endothelial cells, increased amounts of activated gelatinase A were detected in conditioned media; progelatinase B was not activated under these conditions. The total concentration of gelatinase A (latent plus activated), released by endothelial cells as measured by a specific immunoassay, was not significantly increased by thrombin treatment suggesting that thrombin initiates the progelatinase A activation mechanism without affecting progelatinase A synthesis. This was confirmed by showing that treatment of endothelial cells with cycloheximide, an inhibitor of protein synthesis, did not prevent activation of progelatinase A, although it did decrease total gelatinase A production. In addition, a 200-kDa gelatinase was induced by thrombin treatment of HUVEC. A similar 200 kDa gelatinolytic band has been identified in human plasma (35) (see Fig. 2A) and in the conditioned media from cytokine treated endothelial cells (39) and keratinocytes (44).

To explore the mechanism of thrombin activation of progelatinase A, we pretreated HUVEC with protease inhibitors
prior to the addition of thrombin to endothelial cell cultures. Pretreatment with PPACK or hirudin, specific inhibitors of thrombin, abrogated thrombin-induced activation of progelatinase A indicating that the proteolytic activity of thrombin is an essential component of the process. To determine if the thrombin effect is mediated through the recently described functional thrombin receptor (4), the anti-thrombin receptor antibody (4), which inhibits numerous endothelial cell responses to thrombin, was tested; neither this antibody nor the thrombin receptor activating peptides TR42–55 or TR42–47 displayed effects on endothelial cell activation of progelatinase A. These results indicate that the thrombin-tethered ligand receptor mechanism is not involved in activation of progelatinase A, which lends support to the possible existence of a second unidentified cell receptor for thrombin (4). To more specifically evaluate the role of the thrombin receptor in progelatinase activation, the cDNA encompassing the open reading frame of TR was stably transfected into Rat-2 fibroblasts. Addition of thrombin to transfected cells resulted in the anticipated calcium flux response, but not activation of progelatinase A, suggesting that activation of progelatinase A occurs independent of this signal transduction event mediated by the thrombin receptor.

The observation that thrombin is unable to activate recombinant progelatinase A in a cell-free system (16) indicates that the endothelial cell is an essential component of the thrombin-induced progelatinase A activation mechanism. Two types of data from our experiments support the hypothesis that the endothelial plasma membrane is responsible for activation of progelatinase A: 1) addition of 24 nm TIMP-2 (20-fold excess compared to endogenous secreted TIMP-2), but not 24 nm TIMP-1, to endothelial cells abrogated thrombin-induced activation of progelatinase A; and 2) addition of the COOH-terminal domain of gelatinase A, but not a progelatinase A mutant lacking the COOH-terminal domain (30), abrogated thrombin activation of endothelial progelatinase A. The inhibitory effect of 24 nm TIMP-2, but not TIMP-1, is explained by the fact that excess TIMP-2 binds to latent gelatinase A near the COOH-terminal domain of the molecule producing a stabilized complex which may interfere with the interaction between the plasma membrane and latent gelatinase A required for the activation of progelatinase A (18, 45). Similarly, the competitive inhibitory effect on progelatinase A activation of the intact COOH-terminal region of gelatinase A, but not a mutant gelatinase A with a deleted COOH-terminal domain, suggests that binding of the COOH-terminal region of gelatinase A to the plasma membrane activator mechanism is required for metalloproteinase activation. Strongin et al. (20) have also demonstrated that the plasma membrane-dependent activation of 72-kDa progelatinase A is followed by conversion to a 64 kDa intermediate and subsequently a 62-kDa active enzyme. Inhibition of 72-kDa progelatinase A is followed by conversion to a 64 kDa intermediate and subsequently a 62-kDa active enzyme. The lack of inhibitory effect of low dose TIMP-1 may be due to the fact that TIMP-1 can only bind to activated gelatinase A at a site closer to the NH₂-terminal domain of gelatinase A and cannot bind to the COOH-terminal region of latent gelatinase A. Of relevance to this hypothesis, Murphy et al. (13) demonstrated that TIMP-2, but not TIMP-1, inhibited proliferation of endothelial cells and may limit neovascularization through this mechanism.

Recent data of Strongin et al. (27) suggest that activation of progelatinase A requires the assembly of a trimolecular complex on the cell surface. According to this hypothesis, endothelial cell secretion of TIMP-2 in low concentration which does not exceed the receptor capacity of MT-MMP, is followed by cell surface binding of TIMP-2 to MT-MMP, and subsequent binding of the COOH-terminal domain of the progelatinase A molecule to the TIMP-2-MT-MMP complex. Generation of this trimolecular complex culminates in activation of progelatinase A. Our Northern and Western blotting data, demonstrating expression and synthesis of MT-MMP by endothelial cells, is consistent with a role for MT-MMP in progelatinase A activation. Inhibition of protein synthesis with cycloheximide, however, was not accompanied by abrogation of thrombin-induced activation of progelatinase A, suggesting that in endothelial cells the activation mechanism does not require induction of MT-MMP synthesis. Our demonstration of MT-MMP expression and synthesis by Rat-2 fibroblasts which lack a thrombin-induced plasma membrane activation mechanism for progelatinase A further suggests that other factors are required for cell surface activation of progelatinase A. Whether serine proteinases such as plasmin and possibly thrombin are involved in the proteolytic processing of endothelial 63-kDa MT-MMP to the 60 kDa activated form as suggested by Strongin et al. (27), will require additional study.

In conclusion, these experiments indicate that endothelial cells and smooth muscle cells (data not shown) possess a unique capacity for thrombin-induced activation of progelatinase A that may facilitate angiogenesis.

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REFERENCES
1. Tspanopoglou, N. E., Pipili-Syntos, E. & Maragoudakis, M. E. (1993) Am. J. Physiol., 264, C1302–C1307
2. Stern, D., Nawroth, P., Handle, D. & Kisiel, W. (1985) Proc. Nat. Acad. Sci. U.S.A., 82, 2523–2527
3. Vu, T. K., Hung, D. T., Wheaton, V. I. & Coughlin, S. R. (1991) Cell, 64, 1057–1068
4. Bahou, W., Collier, B. S., Cotter, C. L., Norton, K. J., Kutok, J. L. & Goligorsky, M. S. (1993) J. Clin. Invest., 91, 1406–1413
5. Goligorsky, M., Menton, D., Laszlo, A. & Lumm, H. (1990) J. Biol. Chem., 264, 16771–16775
6. Garcia, J. G. N., Painter, R. G., Fenton, J. W. I., English, D. & Callahan, K. S. (1990) J. Cell. Physiol., 142, 186–193
7. Jaffe, E. A., Grulich, J., Weksler, B. B., Hampel, G. & Watanabe, K. (1987) J. Biol. Chem., 262, 8557–8562
8. Folkman, J. (1986) Cancer Res., 46, 467–473
9. Unemori, E., Boughan, K. S. & Werb, Z. (1990) J. Biol. Chem., 265, 445–451
10. Fisher, C., Gilbertson-Beadling, S., Powers, E. A., Petzold, G., Poorman, R. & Mitchell, M. A. (1994) Dev. Biol., 162, 499–511
11. Mignatti, P., Tsuboi, R., Robbins, E. & Rifkin, D. B. (1989) J. Cell Biol., 108, 671–682
12. Schaper, H. W., Grant, D. S., Stetler-Stevenson, W. G., Fridman, R., D’Orazi, G., Murphy, A. N., Bird, R. E., Hoyth, M., Fuerst, T. R., French, D. L., Quigley, J. P. & Kleinman, H. K. (1993) J. Cell. Physiol., 157, 351–358
13. Pepper, M. S. & Montesano, R. (1990) Cell Differ. Dev., 32, 319–328
14. Stetler-Stevenson, W. G., Krutzsch, H. C., Wacher, M. P., Margules, I. M. & Liotta, L. A. (1989) J. Biol. Chem., 264, 1353–1356
15. Okada, Y., Morodomi, T., Enghild, J. J., Suzuki, K., Yau, A., Nakashima, I., Salvesen, G. & Nagase, H. (1990) Eur. J. Biochem., 194, 721–730
16. Ward, R. V., Atkinson, S. J., Slocombe, P. M., Docherty, A. J. P., Reynolds, J. J. & Murphy, G. (1991) Biochim. Biophys. Acta, 1079, 242–246
17. Murphy, G., Willenbrock, F., Ward, R. V., Cockett, M. I., Eaton, D. & Docherty, A. J. P. (1992) Biochem. J., 283, 637–641
18. Brown, P. D., Kleiner, E. D., Unsworth, E. J. & Stetler-Stevenson, W. G. (1993) Kidney Int., 43, 160–170
19. Strongin, A. Y., Marmer, B. L., Grant, G. A. & Goldberg, G. I. (1993) J. Biol. Chem., 268, 14033–14039
20. Overall, C. M. & Sodek, J. (1995) J. Biol. Chem., 268, 11-2151
21. Kalebic, T., Gabrisa, S., Glaser, B. & Liotta, L. A. (1983) Science, 220, 281–284
22. Zucker, S., Wiesman, J. M., Lysik, R. M., Wilkie, D., Ramanarayun, N. S., Golub, L. & Lane, B. (1987) Cancer Res., 47, 1608–1614
23. Chen, W. T. (1990) Cell Differ. Dev., 32, 329–336
24. Emondor, H., Renadice, A. G., Noel, A. C., Grinard, J. A., Stetler-Stevenson, W. G. & Foidart, J. M. (1992) Cancer Res., 52, 5845–5848
25. Sato, H., Takino, T., Okada, Y., Cao, J. & Shinga, Y., A., Yamamoto, E. & Seki, M. (1994) Nature, 370, 61–65
26. Strongin, A. Y., Collier, I., D’Orazi, G., Morro, L., Grant, A. G. & Goldberg, G. I. (1995) J. Biol. Chem., 270, 5311–5338
27. Chambard, J., Paris, S., Allaincar, G. & Pouyssegour, J. (1987) Nature, 326, 230–235
28. Belloni, P. A., Carney, D. H. & Nicolson, G. L. (1992) Microvasc. Res., 43, 20–45
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30. Crabbe, T., Zucker, S., Cockett, M. I., Willenbrook, F., Tickle, S., O’Connell, J. P., Scothern, J. M., Murphy, G. & Docherty, A. J. P. (1994) Biochemistry 33, 6684–6690
31. Chen, C. & Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
32. Kleiner, D. E. & Stetler-Stevenson, W. G. (1994) Anal. Biochem. 218, 325–329
33. Zucker, S., Mancuso, P., DiMassimo, B., Lysik, R. M., Conner, C. & Wu, C.-L. (1994) Clin. & Exp. Metastasis 12, 13–23
34. Hurewitz, A. N., Zucker, S., Mancuso, P., DiMassimo, B. & Moutsakis, D. (1992) Chest 102, 1808–1813
35. Moutsakis, D., Mancuso, P., Krutzsch, H., Stetler-Stevenson, W. & Zucker, S. (1992) Connect. Tissue Res. 28, 213–230
36. Zucker, S., Lysik, R. M., Malik, M., Bauer, B. A., Caamano, J. & Kleinszanto, A. J. P. (1992) Int. J. Cancer 52, 1–6
37. Cooksley, S., Hipkiss, J. P., Tickle, S. P., Holmes-Ievers, E., Docherty, A. J. P., Murphy, G. & Lawson, A. D. G. (1990) Matrix 10, 285–291
38. Bahou, W., Campbell, A. & Wicha, M. (1992) J. Biol. Chem. 267, 13986–13992
39. Hanemaaijer, R., Koolwijk, P., Le Clercq, L., De Vree, W. J. A. & Van Hinsbergh, V. W. M. (1993) Biochem. J. 296, 803–809
40. Kleiner, D. E., Tuuttila, A., Tryggvason, K. & Stetler-Stevenson, W. G. (1993) Biochemistry 32, 1583–1592
41. Herron, G. S., Banda, M. J., Clark, E. J., Gavrilovic, J. & Werb, Z. (1986) J. Biol. Chem. 261, 2814–2818
42. Mignatti, P. & Rifkin, D. B. (1993) Physiol. Rev. 73, 161–195
43. Galardy, R., Grobelny, D., Foelmer, H. G. & Fernandez, L. A. (1994) Cancer Res. 54, 4715–4718
44. Salo, T., Lyons, J. G., Rahentullia, F., Birkedal-Hansen, H. & Larjava, H. (1991) J. Biol. Chem. 266, 11436–11441
45. Fridman, R., Fuerst, T., Bird, R. E., Hoyhtya, M., Oelkuc, M., Kraus, S., Komrarek, D., Liotta, L. A., Berman, M. L. & Stetler-Stevenson, W. G. (1992) J. Biol. Chem. 267, 15398–15405