Activation of pro-(matrix metalloproteinase-2) (pro-MMP-2) by thrombin is membrane-type-MMP-dependent in human umbilical vein endothelial cells and generates a distinct 63 kDa active species

Marc A. LAFLEUR*, Morley D. HOLLENBERG†, Susan J. ATKINSON*, Vera KNÄUPER*, Gillian MURPHY* and Dylan R. EDWARDS*[1]

*School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K., and †Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Thrombin, a critical enzyme in the coagulation cascade, has also been associated with angiogenesis and activation of the zymogen form of matrix metalloproteinase-2 (MMP-2 or gelatinase-A). We show that thrombin activated pro-MMP-2 in a dose- and time-dependent manner in cultured human umbilical-vein endothelial cells (HUVECs) to generate a catalytically active 63 kDa protein that accumulated as the predominant form in the conditioned medium. This 63 kDa thrombin-activated MMP-2 is distinct from the 62 kDa species found following concanavalin A or PMA stimulated pro-MMP-2 activation. Hirudin and leupeptin blocked thrombin-induced pro-MMP-2 activation, demonstrating that the proteolytic activity of thrombin is essential. However, activation was also dependent upon membrane-type-MMP (MT-MMP) action, since it was blocked by EDTA, *o*-phenanthroline, hydroxamate metalloproteinase inhibitors, tissue inhibitor of metalloproteinase-2 (TIMP-2) and TIMP-4, but not TIMP-1. Thrombin inefficiently cleaved recombinant 72 kDa pro-MMP-2, but efficiently cleaved the 64 kDa MT-MMP-processed intermediate form in the presence of cells. Thrombin also rapidly (within 1 h) increased cellular MT-MMP activity, and at longer time points (> 6 h) it increased expression of MT1-MMP mRNA and protein. Thus signalling via proteinase-activated receptors (PARs) may play a role in thrombin-induced MMP-2 activation, though this does not appear to involve PAR1, PAR2, or PAR4 in HUVECs. These results indicate that in HUVECs the activation of pro-MMP-2 by thrombin involves increased MT-MMP activity and preferential cleavage of the MT-MMP-processed 64 kDa MMP-2 form in the presence of cells. The integration of these proteinase systems in the vascular endothelium may be important during thrombogenesis and tissue remodelling associated with neovascularization.

Key words: proteinase, gelatinase-A, proteinase inhibitors, angiogenesis, thrombosis.

INTRODUCTION

Thrombin is a multifunctional serine proteinase involved in multiple cellular and enzymic processes [1]. Among its cellular effects, thrombin can act as a mitogenic factor and platelet-aggregation agent, as well as an angiogenic factor [2] by signalling via the proteinase-activated receptors (PARs) which belong to the seven-transmembrane-G-protein-coupled receptor family. Of the four presently known PARs, PAR1, PAR3 and PAR4 are known to be activated by thrombin [3]. PAR activation requires proteolytic processing of its N-terminal domain, unmasking a sequence that acts as a tethered ligand capable of binding a recognition site on the receptor [4]. Independent of its PAR-mediated actions, thrombin is a critical component of the blood-clotting cascade by virtue of its proteolytic processing of fibrinogen to fibrin [2–4].

Angiogenesis, the formation of new blood vessels from the pre-existing microvasculature, comprises several steps, namely basement-membrane degradation, endothelial-cell migration, proliferation and tube formation [5]. Matrix metalloproteinases (MMPs), a family of zinc-binding, calcium-dependent endopeptidases which degrade all of the proteins in the extracellular matrix [6], have been shown to play a role in angiogenesis, specifically the gelatinases (MMP-2 and MMP-9), owing to their ability to degrade components of the basement membrane such as type IV collagen and fibronectin [7]. Due to the fact that thrombin itself can induce angiogenesis *in vivo* [8] and generate active pro-MMP-2 *in vitro* [9], the link between these two processes warrants further investigation.

Enzymes demonstrated to play a role in the activation process of pro-MMP-2 include members of the membrane-type MMPs (MT-MMPs), specifically MT1-, MT2-, MT3-, MT5- and MT6-MMP [10–14], plasmin [15] and thrombin [9]. The current model for pro-MMP-2 activation via the MT-MMPs involves a ternary complex formed between the MT-MMP, tissue inhibitor of metalloproteinase-2 (TIMP-2) and pro-MMP-2 at the cell surface. Once this complex is formed, a TIMP-2-free MT-MMP molecule at the cell surface can then cleave the propeptide from the pro-MMP-2 (72 kDa) in the ternary complex, generating the intermediate 64 kDa species of MMP-2 [16]. This intermediate is then converted into the fully active 62 kDa form via an autocatalytic mechanism [17]. The plasminogen activator–plasmin system has also been reported to activate pro-MMP-2, but recent evidence indicates that this is via cleavage of the 64 kDa MT-MMP-processed intermediate form to generate the fully active species [15].

Thrombin-induced pro-MMP-2 activation has been studied by several groups, but the exact mechanism of its action still remains unclear, owing to conflicting results in the literature [9,18,19]. In particular, one recent study has argued against the dependency

Abbreviations used: BCA, bicinchronic acid; con A, concanavalin A; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical-vein endothelial cell; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; PAR, proteinase-activated receptor; RT, reverse transcriptase; TIMP, tissue inhibitor of metalloproteinase.

[1] To whom correspondence should be addressed (e-mail dylan.edwards@uea.ac.uk).

of thrombin-mediated pro-MMP-2 activation upon MT1-MMP in endothelial cells [19]. In the present work we report that, in HUVECs, thrombin can only efficiently cleave the MT-MMP-generated intermediate form of MMP-2 to produce an active species of slightly higher molecular mass than the conventional 62 kDa mature form. Signalling via the cell-surface thrombin receptors (PARs) may also play a role, both by rapidly up-regulating MT-MMP activity and increasing MT1-MMP production over a longer time scale. Thrombin can partially activate pro-MMP-2 in a cell-independent fashion, but requires the presence of cells for efficient activation of pro-MMP-2 (acting predominantly on the 64 kDa species). This pro-MMP-2 activation model therefore links thrombin, the MT-MMP subfamily, and MMP-2, all of which have been shown to play a role during angiogenesis.

EXPERIMENTAL

Cell culture and reagents

Primary HUVECs were obtained from TCS Biologicals (Botolph Claydon, Buckingham, Bucks., U.K.) and grown on type-I-collagen (60 μg/ml) coated-tissue-culture plates, in the medium supplied by the manufacturer. All experiments were performed on cells between passage number 1 and 5 and maintained at 37 °C and 5 % (v/v) CO_2. During each experiment in which the conditioned medium was analysed, HUVECs were seeded on type-I-collagen (60 μg/ml)-coated 24-well plates at a density of 1×10^5 cells/well. The following day, cells were washed twice with serum-free medium and incubated in fresh serum-free medium with or without stimulation for the indicated period of time. The conditioned medium was then collected, centrifuged at 5200 ***g*** for 10 min to remove any cells in suspension, and 50 mM Tris, pH 8.0, was added and stored at −20 °C. PMA, thrombin, hirudin, aprotinin, pepstatin, leupeptin, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), 6-aminohexanoic acid, EDTA, *o*-phenanthroline and concanavalin A (con A) were all obtained from Sigma–Aldrich (Poole, Dorset, U.K.). The hydroxamate metalloproteinases BB94 and BB3113 were obtained from British Biotech Pharmaceuticals (Oxford, U.K.). The PAR1 agonist peptide [TFLLR-NH_2 (with an amide group at the N-terminus)] and PAR2 agonist peptide (SLIGRL-NH_2) were synthesized by solid-phase methods at the Peptide Synthesis Facility (University of Calgary, Faculty of Medicine, Calgary, Alberta, Canada). The composition and purity of both peptides were confirmed by HPLC analysis, MS analysis and quantitative amino-acid analysis. Stock solutions, prepared in 25 mM Hepes buffer, pH 7.4, were standardized by quantitative amino-acid analysis to verify peptide concentration and purity. The selectivity of the two peptides for activating PAR1 (TFLLR-NH_2) and PAR2 (SLIGRL-NH_2) has been previously described [20]. The PAR4 agonist peptide (AYPGKF-NH_2) was synthesised at the Advanced Biotechnology Centre, Imperial College School of Medicine, London, U.K.

Recombinant pro-MMP-2 incubation with thrombin

Eppendorf tubes (0.5 ml) were precoated with a solution of 1 % (w/v) BSA in PBS for 1 h at room temperature (to prevent adhesion of recombinant pro-MMP-2 to the plastic) and washed thoroughly with PBS. A 5 ng portion of recombinant pro-MMP-2 was either left untreated, or treated with 0.1, 1, 10 or 100 units/ml of thrombin in a total reaction volume of 20 μl. This reaction was incubated at 37 °C for 24 h and then stopped by the addition of SDS/PAGE sample-loading buffer without reducing agents. Gelatin zymography was then performed on these samples.

HUVEC-conditioned-medium incubation with thrombin and PMA

HUVECs were grown to confluency on type-I-collagen (60 μg/ml)-coated 24-well plates after which time the cells were washed twice with serum-free medium and incubated with fresh serum-free medium with or without PMA (0.1 μM) for 24 h at 37 °C and 5 % (v/v) CO_2. The conditioned medium was then collected and centrifuged twice at 5200 ***g*** for 10 min to remove any cells in suspension. The conditioned medium was then either left untreated, or treated with thrombin (10 units/ml) for 24 h at 37 °C. Gelatin zymography was then performed on these samples.

Gelatin zymography

MMP activity in the conditioned medium of cultured HUVECs was analysed by substrate-gel electrophoresis (zymography), using a 10 % PAGE co-polymerized with 1mg/ml gelatin (Sigma). Equal amounts of samples were mixed with 1 % (w/v) SDS-sample buffer under non-reducing conditions and loaded on to the gel (resolving gel: 17.5 cm × 10.5 cm). Gels were run using a V15-17 vertical-gel-electrophoresis apparatus (Gibco BRL), at 20 mA until the Bromophenol Blue dye traversed into the resolving gel. The current was then increased to 30 mA and the gels were electrophoresed until 1 h after the Bromophenol Blue dye had run off the gel. The gels were then washed in 50 mM Tris/HCl (pH 8.0)/5mM $CaCl_2$/2.5 % (v/v) Triton X-100 overnight and then incubated in 50mM Tris/HCl, pH 7.5, 5 mM $CaCl_2$ for 18 h at 37 °C. Gels were stained with Coomassie Blue and destained in 10 % (v/v) acetic acid/10 % (v/v) propan-2-ol. Gelatinolytic activity appears as a clear band on a blue background.

RNA extraction

HUVECs were grown to confluency in T175 cm^2 flasks (Life Technologies, Paisley, Renfrewshire, Scotland, U.K.). These cells were washed twice in serum-free medium and fresh serum-free media was added along with thrombin at a concentration of 10 units/ml. RNA was then either extracted immediately, or after a 3, 6, 12 and 24 h period of thrombin stimulation. Total RNA from HUVECs was harvested using RNAzol B (Biogenesis, Poole, Dorset, U.K.) according to the manufacturer's instructions.

Reverse transcriptase (RT) reaction

Each reaction mixture contained 2 μg of total RNA, 1 × PCR buffer (10 mM Tris/HCl, pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$), 1 mM each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 20 units of placental ribonuclease inhibitor (RNAguard; Pharmacia), 100 pmol of random hexamer oligodeoxynucleotides and 200 units of Moloney murine leukaemia virus reverse transcriptase (Superscript I; Gibco BRL). The final reaction volume was 20 μl. Each reaction mixture was pre-incubated at 20 °C for 10 min and the RT reaction was performed at 42 °C for 50 min. Each sample was then heated to 95 °C for 5 min in order to terminate the RT reaction and then cooled to 4 °C and samples stored at −20 °C. A PTC-200 Peltier thermal cycler (MJ Research, Watertown, MA, U.S.A.) was used for all reactions.

Table 1 Primers for RT-PCR

Target gene	Primer sequence (5′–3′)	Target accession number	Position (bp)	Product size (bp)	PCR cycle no.
MT1-MMP (MMP-14)	GCCCATTGGCCAGTTCTGGCGGG CCTCGTCCACCTCAATGATGATC	NM004995.1	1178–1200 1707–1685	530	30
GAPDH	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTGGTGAAGAC	M33197	78–101 384–361	307	23

PCR

Each reaction mixture contained 1.5 μl of cDNA from the above RT reaction, 1 × PCR buffer (10 mM Tris/HCl, pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$), 80 μM of each four deoxynucleotides (dATP, dGTP, dCTP and dTTP), including the dNTP left over from the RT reaction for a final approximate concentration of 180 μM, and 20 pmol of each 5′- and 3′-primer (Table 1). Two units of *Taq* DNA polymerase (Gibco BRL) was added to each reaction mixture during the first denaturation step ('hot start'). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′- and 3′-primers (20 pmol) were added at the appropriate cycle number by the 'primer dropping method' [21]. The PCR reactions were performed in 50 μl volumes in thin-walled 0.5 ml volume PCR tubes. The PCR cycles for each reaction were as follows: heat-denaturation step at 94 °C for 1 min, primer-annealing step at 55 °C for 30 s and primer-extension step at 72 °C for 1 min. Reaction mixtures were then cooled to 4 °C and stored at −20 °C. All PCR reactions were carried out in a PTC-200 Peltier thermal cycler. Aliquots of each PCR reaction were run on a 2% (w/v) agarose gel containing 0.2 μg/ml ethidium bromide and equalized in order to give equivalent signals with an internal GAPDH control. DNA on the agarose gels was revealed under UV light.

Western-blot analysis

Cell lysates from HUVECs either untreated or treated with thrombin (10 units/ml) for 24 h were collected by lysing the cells with the following lysis buffer: 10 mM Tris/HCl, pH 7.6, 10 mM NaCl, 3 mM $MgCl_2$, 1% (v/v) Nonidet P40, and 100 μM PMSF. This suspension was then centrifuged at 3800 ***g*** for 1 min to pellet the nuclei, and the supernatant collected. Total cellular protein was quantified using the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, U.S.A.). A 5 μg portion of total protein (with 100 mM dithiothreitol as a reducing agent and boiled for 5 min) was run on a SDS/10%-PAGE and transferred to PVDF membrane. The membrane was then probed with a sheep polyclonal anti-(human MT1-MMP) antibody. This primary antibody was then detected using a horseradish-peroxidase-conjugated donkey anti-sheep secondary antibody (Jackson ImmunoResearch Laboratories, Luton, Beds., U.K.) and the ECL®plus (enhanced chemiluminescence) system (Amersham Pharmacia Biotech).

[^{14}C]Gelatin degradation assay

HUVECs were grown to confluency on type-I-collagen-(60 μg/ml)-coated 10 cm-diameter dishes. The cells were washed twice in serum-free medium and 5 ml of fresh serum-free medium was added and the cells either left untreated, or stimulated with 50 μg/ml con A or 10 units/ml thrombin, and left for 24 h at 37 °C and 5% (v/v) CO_2. The conditioned medium was then collected and centrifuged at 192 ***g*** for 10 min to remove cells in suspension and passed through a gelatin–agarose (Sigma) column. The column was then washed thoroughly with wash buffer [50 mM Tris/HCl, pH 7.8, 1 M NaCl, 10 mM $CaCl_2$, 0.02% (w/v) sodium azide and 0.05% (v/v) Brij 35 (Sigma–Aldrich, Poole, Dorset, U.K.)], and then eluted with several 100 μl aliquots of wash buffer as above with 10% (v/v) DMSO.

Gelatin was made by denaturing type I collagen from rat skin at 60 °C for 20 min. Each reaction (performed in triplicate) contained 100 μl of [^{14}C]gelatin (1 mg/ml), 50 μl of Tris assay buffer [200 mM Tris/HCl, pH 7.9, 60 mM $CaCl_2$ and 0.02% (w/v) sodium azide], 15 μl of eluted sample (or 15 μl of elution buffer as a negative control) and 85 μl of water in a Microfuge tube for a total reaction volume of 250 μl. A positive control was also included by substituting 10 μl of trypsin (1 mg/ml) and 5 μl of water for the 15 μl eluted sample. Samples were left at 37 °C for 20 h and then placed on ice for 30 min. Cold 90% (v/v) trichloroacetic acid (50 μl) was then added to each sample and left for a further 30 min on ice. Samples were then centrifuged at 10200 ***g*** for 15 min at 4 °C to pellet undigested gelatin. The supernatant (200 μl) of each sample was then carefully removed and placed in a liquid-scintillation vial with 2 ml of scintillation fluid and counted for radioactivity.

RESULTS

Thrombin activates pro-MMP-2 in HUVECs in a dose- and time-dependent manner

Thrombin induced cleavage of the pro-form of MMP-2 in HUVECs in a dose-dependent manner, as seen in Figure 1(a). Thrombin also caused cleavage of pro-MMP-2 as early as 1 h post-stimulation, the amount of active MMP-2 further increasing with time (Figure 1b). A similar profile was seen using PMA as a stimulus, but the increase in active enzyme was seen initially after 3 h, the amount of active enzyme also increasing with time. PMA stimulation of HUVECs resulted in the generation of the intermediate 64 kDa form (mediated by an MT-MMP [22,23]) and the mature, fully activated 62 kDa MMP-2, while thrombin treatment leads to the accumulation of a single MMP-2 form that migrates with a size of 63 kDa. This distinction in the size of the activated forms can be most clearly seen in the 3 h and 6 h stimulation panels (Figures 1b and 1c), where amounts of the active species are relatively low. It has not been seen previously [9,18,19], but it is apparent using the optimized zymographic conditions that we have employed, allowing us to further resolve the MMP-2 processed forms (see also Figures 2–4 below). We also looked at the cell-associated species of MMP-2 after PMA and thrombin stimulation. In the cell lysates of unstimulated, PMA- and thrombin-stimulated HUVECs, we saw the 72 kDa pro-MMP-2 form and both the 64 kDa and 62 kDa forms in all time points analysed (Figure 1c). Levels of cell-associated 64 kDa and 62 kDa forms were increased following both PMA and thrombin treatment compared with unstimulated HUVECs even after treatments of 1 h. The presence of the 63 kDa thrombin-generated form in cell lysates was low and variable between experiments. We also noticed that PMA induced production of

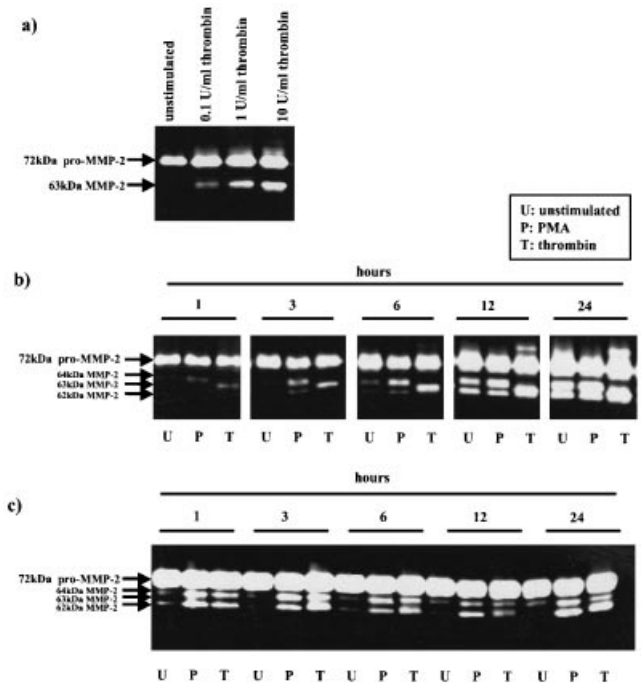

Figure 1 Thrombin activates pro-MMP-2 in a dose- and time-dependent manner

(**a**) HUVECs were cultured in serum-free medium and stimulated with increasing concentrations of thrombin for a 24 h period. The conditioned medium was then analysed by gelatin zymography. (**b**) and (**c**) HUVECs were cultured in serum-free medium and stimulated with PMA (0.1 μM) or thrombin (10 units/ml) for a period of either 1, 3, 6, 12, or 24 h. (**b**) Conditioned medium and (**c**) total cell lysates were then analysed by gelatin zymography. Pro-MMP-2 has a molecular mass of 72 kDa, the intermediate form of MMP-2 generated by PMA migrates at 64 kDa, while the fully active zymogen migrates at 62 kDa. The thrombin-generated active form of MMP-2 migrates slightly faster (63 kDa) than the intermediate form of MMP-2 generated by PMA.

MMP-9 in HUVECs, although this was variable between batches of HUVECs and was not observed in the HUVECs used for these experiments. MMP-9 was never found, however, to be induced by thrombin in HUVECs.

These observations suggest that thrombin contributes to pro-MMP-2 activation through at least two mechanisms in HUVECs. Firstly, just as with PMA stimulation, increased amounts of the MT-MMP-processed 64 kDa form and the mature 62 kDa species are seen in cell lysates following thrombin treatment. This suggests that one component of thrombin's action involves stimulation of cell-associated MT-MMP activity. In the conditioned medium of PMA stimulated cells, the 64 kDa and 62 kDa MMP-2 forms accumulate over time, suggesting that both forms are relatively long-lived and are released from cells in parallel with the zymogen form of MMP-2. However, in the conditioned medium of thrombin-treated cells, a single 63 kDa form dominates, suggesting that a second contribution of thrombin is the generation of this species, which could occur either directly from the pro-form of MMP-2, or via the 64 kDa MMP-2 processed form.

Thrombin-generated 63 kDa MMP-2-processed form is catalytically active

In order to verify that the 63 kDa thrombin-generated form of MMP-2 was indeed catalytically active, we performed a

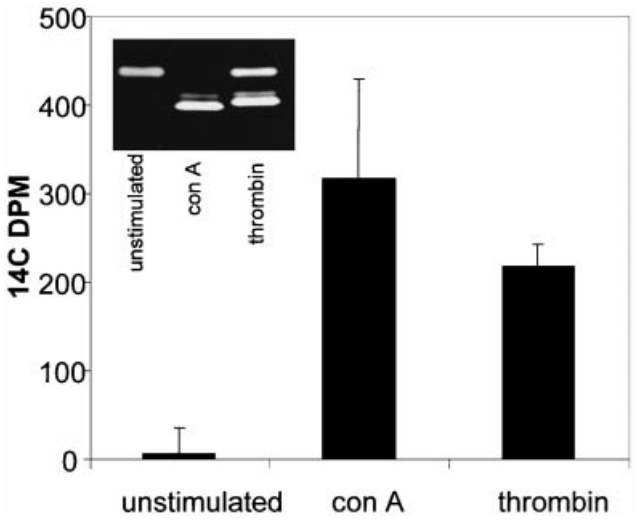

Figure 2 The 63 kDa MMP-2 species is catalytically active

Confluent HUVEC monolayers were incubated in serum-free medium without or with con A (50 μg/ml) or thrombin (10 units/ml) stimulation for 24 h. The conditioned media of each sample was then passed through a gelatin–agarose column and equal volumes of eluates were incubated with [^{14}C]gelatin at 37 °C overnight. The samples were then centrifuged to pellet undigested gelatin, and the supernatant containing the degraded gelatin was transferred to scintillation vials for counting the radioactivity. Each sample was processed in triplicate and the average value is shown above. A gelatin zymogram was also performed on equal volumes of eluates to demonstrate the relative amount of enzyme present.

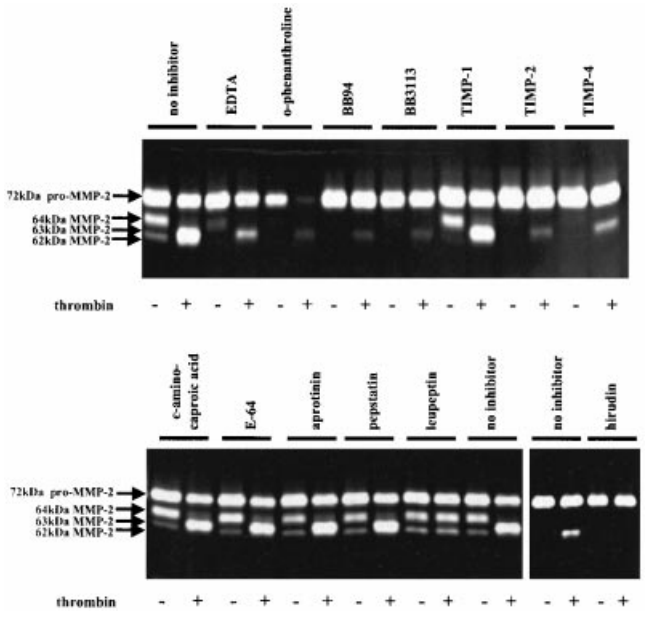

Figure 3 Pro-MMP-2 activation is sensitive to metalloproteinase inhibitors and thrombin proteinase inhibitors

HUVECs were cultured in serum-free medium with the addition of the following proteinase inhibitors: EDTA (1 mM), *o*-phenanthroline (0.3 mM), BB94 (5 μM), BB3113 (5 μM), TIMP-1 (5 μg/ml), TIMP-2 (5 μg/ml), TIMP-4 (5 μg/ml), 6-aminohexanoic acid (ϵ-amino-caproic acid; 50 mM), E64 (50 μM), aprotinin (20 μg/ml), pepstatin (5 μg/ml), leupeptin (1 mM) and hirudin (10 units/ml). Thrombin was then subsequently added where indicated at a concentration of 10 units/ml and incubated for 24 h at 37 °C. Gelatin zymography was then performed on these samples.

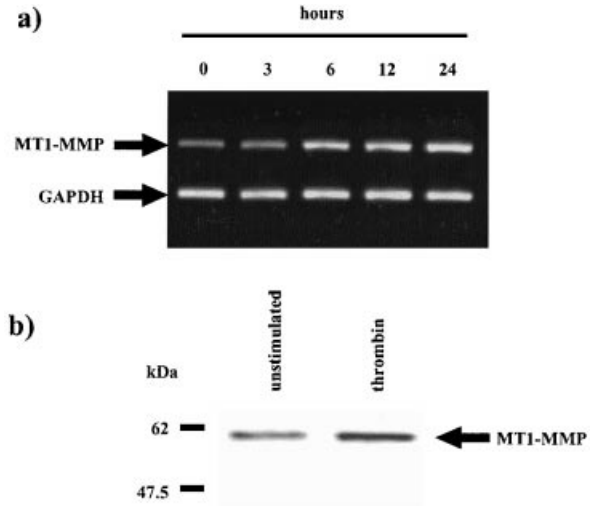

Figure 4 Thrombin increases MT1-MMP transcription and expression

(**a**) HUVECs were cultured in serum-free medium and stimulated with 10 units/ml thrombin for 0, 3, 6, 12, and 24 h, after which times total cellular RNA was harvested. RT-PCR was then performed on these RNA samples using specific primers for MT1-MMP and standardized to GAPDH levels, which were amplified in the same reaction. The MT1-MMP PCR product has a length of 530 bp and GAPDH 307 bp. (**b**) HUVECs were cultured in serum-free medium with and without thrombin stimulation (10 units/ml) for 24 h. Total cellular lysates were then collected from these cells and standardized according to total protein using the BCA protein quantification assay. A Western blot was then performed using 5 μg of total protein from each sample and probed for MT1-MMP. The 60 kDa band corresponds to the active form of MT1-MMP.

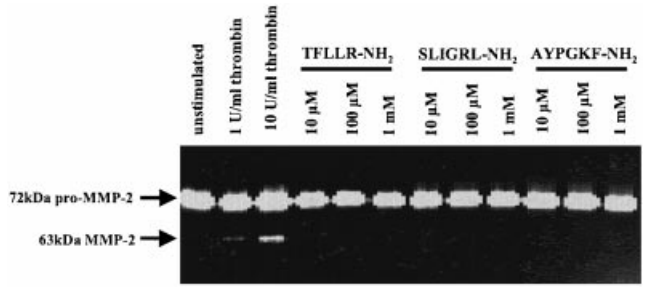

Figure 5 PAR1-, PAR2-, and PAR4-agonist peptides do not activate pro-MMP-2

HUVECs were cultured in serum-free medium and either unstimulated or stimulated with thrombin, PAR1-agonist peptide (TFLLR-NH_2), PAR2-agonist peptide (SLIGRL-NH_2), or PAR4-agonist peptide (AYPGKF-NH_2) where indicated for a 24 h period. Gelatin zymography was then performed on the conditioned medium of these samples as described in Figure 1.

[^{14}C]gelatin-degradation assay. Conditioned media from HUVECs either unstimulated or stimulated with con A (50 μg/ml) or thrombin (10 units/ml) were collected and passed through a gelatin–agarose column to remove any traces of thrombin, which itself has gelatinolytic activity. Equal volumes of column eluates corresponding to equal amounts of total MMP-2 (as calibrated by zymography) were then incubated with [^{14}C]gelatin to assess net gelatinolytic activity. The 72 kDa, 64 kDa, 63 kDa, and 62 kDa species of MMP-2 were the only gelatinolytic enzymes present in the eluates as assessed by gelatin zymography. No MMP-9 or 45 kDa (lacking the C-terminal domain) MMP-2 were found in the elutions. Figure 2 demonstrates that the thrombin-mediated 63 kDa MMP-2 processed form had proteolytic activity against gelatin, equivalent to that of the 62 kDa fully-active species generated by con A. The 63 kDa thrombin-activated MMP-2 is therefore not an inactive-processed species.

MT-MMPs are involved in thrombin-mediated pro-MMP-2 activation

In order to clarify the involvement of other proteinases in the MMP-2-activation mechanism induced by thrombin, we used a panel of metallo-, serine-, aspartic-, and cysteine-proteinase inhibitors, as well as a specific thrombin inhibitor, to study the role of these proteinases during pro-MMP-2 activation. Figure 3 demonstrates that the specific thrombin inhibitor hirudin completely blocked pro-MMP-2 activation induced by thrombin. The proteolytic activity of thrombin is thus essential for pro-MMP-2 activation. The inhibitory action of hirudin also indicated that other proteinases, which may have been present as minor contaminants in the thrombin preparation, were not responsible for the observed activation. The acidic-proteinase inhibitor pepstatin had no effect on pro-MMP-2 activation. Likewise, the serine-proteinase inhibitors aprotinin and 6-amino-hexanoic acid, as well as the cysteine-proteinase inhibitor E64, had no effect on pro-MMP-2 activation. Leupeptin, however, a broad-spectrum serine- and cysteine-proteinase inhibitor that can inhibit thrombin, suppressed thrombin-induced pro-MMP-2 activation. Chelating agents (EDTA and *o*-phenanthroline), synthetic MMP inhibitors (BB94 and BB3113) and natural MMP inhibitors (TIMP-2 and TIMP-4) were also capable of blocking pro-MMP-2 activation induced by thrombin. The concentration of *o*-phenanthroline (0.3 mM) that we used was slightly toxic to the cells, which probably explains the lower levels of MMP-2 present on the zymogram in Figure 3. This inhibition, however, was not complete, as there was always some residual active 63 kDa MMP-2 present. TIMP-1 had no inhibitory effect on thrombin-induced pro-MMP-2 activation. These results indicate that two classes of proteinases may be involved during thrombin-mediated pro-MMP-2 activation: a serine proteinase (likely thrombin itself) and a MMP. The insensitivity of thrombin-mediated activation to TIMP-1 is consistent with the involvement of a MT-MMP [24].

Since PMA induced pro-MMP-2 activation involves the up-regulation at both the mRNA and protein levels of MT1-MMP [25,26], we decided to look at the transcription and expression profiles for MT1-MMP in HUVECs stimulated with thrombin. As demonstrated in Figure 4(a), RT-PCR results show increased MT1-MMP mRNA levels in a time-dependent manner in response to thrombin, the rise being detectable at 6 h and increasing to 24 h after stimulation. This increase was reflected in Western-blot analysis of MT1-MMP protein levels in Figure 4(b).

To assess the contribution of PAR-mediated signalling to thrombin-induced pro-MMP-2 activation, we treated HUVECs with a PAR1-agonist peptide (TFLLR-NH_2), a PAR2-agonist peptide (SLIGRL-NH_2), and a PAR4-agonist peptide (AYPGKF-NH_2). No peptide had any effect on activation, suggesting that signalling via PAR1, PAR2, or PAR4 is not involved (Figure 5). Since endothelial cells express PAR3 [27], it is possible that this receptor may be involved in the rapid rise in cellular MT-MMP activity.

Thrombin-mediated activation of MMP-2 involves the MT-MMP-processed intermediate

We next asked whether thrombin could directly cleave recombinant 72 kDa pro-MMP-2 in the absence of other factors.

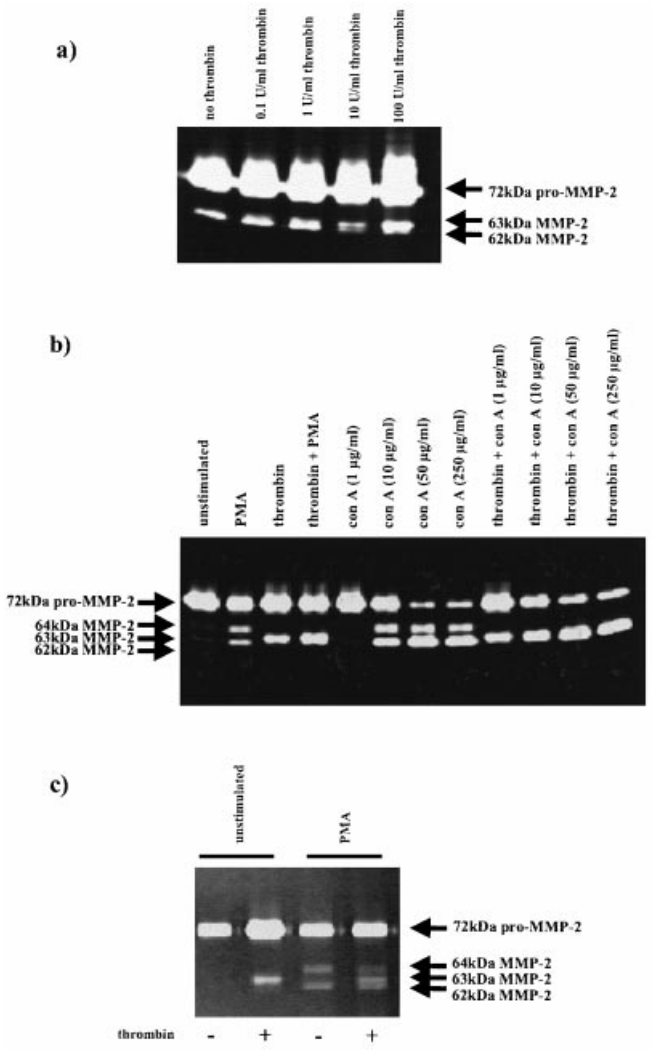

Figure 6 Thrombin inefficiently cleaves recombinant 72 kDa pro-MMP-2, but efficiently cleaves the 64 kDa intermediate MMP-2 form in the presence of cells

(**a**) Recombinant pro-MMP-2 (5 ng) was incubated without or with 0.1, 1, 10 or 100 units/ml thrombin at 37 °C for 24 h. Samples were then analysed by gelatin zymography. Pro-MMP-2 has a molecular mass of 72 kDa, while the fully active zymogen migrates at 62 kDa. The thrombin generated active form of MMP-2 migrates at 63 kDa. (**b**) HUVECs were grown in serum-free medium and stimulated with either PMA (0.1 μM), thrombin (10 units/ml), con A, or a combination of these factors as indicated for a 24 h period. Samples were then analysed by gelatin zymography. (**c**) Confluent HUVECs were cultured in serum-free medium and either left unstimulated, or stimulated with PMA (0.1 μM) for 24 h at 37 °C. The conditioned medium was then collected and centrifuged twice at 5200 ***g*** to pellet any cells in suspension. The cell-free supernatants were then either not treated or treated with thrombin (10 units/ml) for a 24 h period. Gelatin zymography was then performed on all samples.

Figure 6(a) shows that thrombin can cleave recombinant pro-MMP-2, but is extremely inefficient at doing so directly, requiring a very high amount of enzyme to observe noticeable cleavage. Since thrombin could not efficiently cleave recombinant pro-MMP-2, we thought that either thrombin has a cleavage bias towards the 64 kDa intermediate, or that thrombin might require another factor (produced by the cells) in order to elicit proper cleavage of pro-MMP-2.

We first tested the hypothesis that thrombin has a cleavage bias towards the 64 kDa intermediate form of pro-MMP-2 generated by MT1-MMP. We used two agents (PMA and con A) known to increase the amounts of 64 kDa intermediate MMP-2 via MT1-MMP in HUVECs, along with thrombin to see if the latter efficiently cleaves the 64 kDa intermediate form of MMP-2. Figure 6(b) illustrates that both PMA and con A substantially increased the amounts of intermediate 64 kDa MMP-2 in HUVEC-conditioned medium, and when cells were co-stimulated with thrombin, there was a corresponding augmentation of the amount of 63 kDa species generated with a correlative disappearance of the 64 kDa form.

In order to test the second hypothesis, that another factor produced by the cells is required in order to elicit proper cleavage of pro-MMP-2 by thrombin, we added thrombin to cell-free conditioned medium from HUVECs. This led to an inefficient generation of the 63 kDa MMP-2 form, where the amount of 63 kDa enzyme was lower compared with treatment of cells with thrombin. Also, in these conditions (the absence of cells), the 64 kDa band persisted (Figure 6c), which was never observed in the presence of cells. Essentially identical results were also obtained when using HUVEC-derived, gelatin–agarose-purified MMP-2 incubated with thrombin in the absence of cells. The conditioned media from unstimulated and PMA-stimulated HUVECs were passed through a gelatin–agarose column and the eluates containing purified MMP-2 were incubated with thrombin. In both cases, thrombin was able to generate the 63 kDa form, but was very inefficient at doing so. Furthermore the 64 kDa form persisted in the PMA-stimulated eluates incubated with thrombin (results not shown). We therefore conclude from these experiments that thrombin has a cleavage preference for the intermediate 64 kDa MMP-2 form generated by an MT-MMP rather than the 72 kDa pro-MMP-2 form. Furthermore, thrombin cannot efficiently cleave the 64 kDa MMP-2 form in solution, but requires the presence of cells to do so.

DISCUSSION

In this study and previous work [9,18,19] it has been demonstrated that thrombin can activate pro-MMP-2 in endothelial and smooth-muscle cells. We observed this activation in HUVEC conditioned medium in a dose- and time-dependent manner as early as 1 h post-thrombin stimulation. This differs from PMA-induced-pro-MMP-2 activation, which became evident after 3 h with the generation of the 64 kDa MT-MMP-generated MMP-2 intermediate, followed by autocatalytic processing to the 62 kDa fully mature species. Thrombin-mediated MMP-2 activation generates a single 63 kDa MMP-2 form that is catalytically active. We suggest that the 64 kDa intermediate generated via a member of the MT-MMP family is rapidly processed to the 63 kDa form by thrombin.

Alone, thrombin was extremely inefficient at processing recombinant pro-MMP-2 (and purified HUVEC-derived pro-MMP-2) directly *in vitro*, implying that an additional event or another factor is required for optimal activation. Analysis of a series of proteinase inhibitors reveals that two classes of proteinases are involved during thrombin-mediated pro-MMP-2 activation. Firstly, thrombin's proteolytic activity was essential, since the process was completely inhibited by hirudin, a potent and specific thrombin inhibitor [28,29]. Leupeptin (a serine- and cysteine-proteinase inhibitor) also blocked pro-MMP-2 activation, most likely via inhibition of the proteolytic activity of thrombin itself. Other serine-proteinase inhibitors (6-aminohexanoic acid and aprotinin) had no inhibitory effect against thrombin. Cysteine and aspartic proteinases did not appear to be involved in this activation process. MMP inhibitors such as chelating agents,

synthetic MMP inhibitors, TIMP-2 and TIMP-4 also inhibited thrombin-mediated pro-MMP-2 activation, though TIMP-1 had no effect. Thus proteolytic co-operativity appears to be responsible for thrombin-mediated pro-MMP-2 activation involving thrombin's proteolytic activity and an MMP. In HUVECs this MMP is likely an MT-MMP, either MT1-, MT2-, MT3-, or MT5-MMP owing to their sensitivity to TIMP-2 but insensitivity to TIMP-1 [11,24]. Our results are supported by work from two other groups that have demonstrated sensitivity of thrombin-mediated pro-MMP-2 activation to MMP inhibitors [9,18].

We then tested the hypothesis that thrombin-mediated pro-MMP-2 activation involves thrombin cleavage of the 64 kDa intermediate form of MMP-2 generated by MT-MMPs, by increasing the amount of this intermediate in the conditioned medium of HUVECs. Augmentation of the level of the 64 kDa intermediate form by treatment of cells with PMA or con A (both of which increase the expression of MT1-MMP [25,30]), led to a corresponding increase in the 63 kDa species in medium from cells exposed to thrombin together with either con A or PMA as compared with HUVECs treated with thrombin alone. The 64 kDa species was not observed in the conditioned medium of HUVECs stimulated with thrombin, or combinations of thrombin and PMA or con A. Thus the MT-MMP-processed 64 kDa MMP-2 appears to be superior to the zymogen form with respect to ability to be activated by thrombin. The 64 kDa form did persist, however, when conditioned medium of HUVECs (in the absence of cells) was treated with thrombin. From this we conclude either that thrombin preferentially cleaves the 64 kDa intermediate-MMP-2 form when bound to the cell surface (perhaps bound to the MT-MMP–TIMP-2 complex), or an additional protein, perhaps regulated by thrombin signalling via the PARs, assists in thrombin-mediated cleavage of the extracellular 64 kDa intermediate MMP-2. The 63 kDa form was barely detectable in cell lysates compared to the 64 kDa and 62 kDa forms and accumulated as a single species in the conditioned medium, suggesting that it is stable and does not undergo further autolysis. A similar mechanism has been proposed by Baramova et al. [15], where the uPA–plasmin system was shown to be involved during the second step of MMP-2 activation in HT1080 cells by selectively cleaving the 64 kDa intermediate form to the 62 kDa mature form. There is only one potential thrombin cleavage site (XPR$^{\downarrow}$XXX, where X is a non-acidic residue) [31] in pro-MMP-2 that would be consistent with generation of a 63 kDa protein, namely the KPR$^{72\downarrow}$C^{73}GN in the cysteine switch region. The precise identity of the 63 kDa form remains to be established, and it will also be interesting to determine whether this form displays activities that are different from the conventional 62 kDa autoproteolytically processed form, where the cleavage occurs at Asn$^{80\downarrow}$Tyr^{81}.

Treatment of HUVECs with either PMA or thrombin led to increased levels of cell-associated 64 kDa and 62 kDa MMP-2 forms as early as 1 h after treatment, suggesting that, like PMA, thrombin may cause rapid activation of MT-MMPs on the cell surface, or trafficking to specific cell-surface domains. It is clear that thrombin-mediated cellular signalling involves the PARs, a family of proteinase-activated G-protein-coupled receptors. Of the four presently known PARs, PAR1, PAR3, and PAR4 have been shown to be activated by thrombin, while PAR2 is mainly activated by trypsin [32]. We tested three agonist peptides (for PAR1, PAR2, and PAR4) for their ability to induce pro-MMP-2 activation in HUVECs. These agonist peptides activate their respective PAR without the need for proteolytic activity [33], and therefore aid in the identification of signalling versus direct proteolytic activities of thrombin. Since all three agonist peptides failed to induce activation of pro-MMP-2, we can conclude that signalling via PAR1, PAR2, or PAR4 was not essential for pro-MMP-2 activation in HUVECs. These results support observations by Zucker et al. [9] with the PAR1-activating peptide. Since there is no suitable peptide agonist for PAR3 at the present time, we cannot confirm the involvement of PAR3; however, transcripts for PAR3 can be detected in endothelial cells by RT-PCR (M. D. Hollenberg, unpublished work).

We observed increased MT1-MMP mRNA and protein expression in HUVECs in response to thrombin, which may be due to signalling events mediated by the PARs. If this increase was due to signalling via PAR1, PAR2 or PAR4, then we would have expected to see an increase in the 64 kDa intermediate form of MMP-2 in response to the agonist peptides tested, but we did not observe this. Therefore the signalling events responsible for the increase in MT1-MMP expression might be occurring via PAR3 or some other unknown mechanism possibly involving a cell-regulatory domain of thrombin distinct from its catalytic site [34]. Induced expression occurred over a longer time scale (after 6 h) than the increase in cell-associated MT-MMP activity that was inferred from the presence of the 64 kDa and 62 kDa MMP-2 forms. This is similar to effects of PMA and other angiogenic stimuli on MT1-MMP expression [25,26,35], suggesting that the primary effect of thrombin-induced cell signalling may involve intracellular trafficking of pre-existing MT-MMPs to the cell surface. Over the longer time scale these signalling events may enhance the activation of pro-MMP-2 by increasing the expression of MT1-MMP and thereby generating more 64 kDa intermediate MMP-2, the optimal MMP-2 substrate for thrombin. It is not clear whether the cell-signalling aspects of thrombin's action involve a particular MT-MMP, as HUVECs express at least five of the six known MT-MMP family members (M. A. Lafleur and D. R. Edwards, unpublished work). It is therefore possible that the rapid up-regulation of MT-MMP activity on the cell surface by thrombin and the delayed increase due to induced expression may involve different types of MT-MMP.

Another recent study [19] has argued that thrombin activation of pro-MMP-2 in human foreskin microvascular endothelial cells is independent of MT1-MMP. In part, these differences may simply reflect differences in the cell types, since HUVECs spontaneously generate a low level of intermediate and fully active forms of MMP-2 without stimulation. In human foreskin microvascular endothelial cells, activation by PMA was inhibited by TIMP-2 at 500 ng/ml (24 nM) and 50 μM *o*-phenanthroline, consistent with the involvement of a MT-MMP-dependent step. However, thrombin-mediated activation was resistant to the same concentrations of these inhibitors [19]. In the experiments presented here we used 5 μg/ml TIMP-2, which completely blocked both thrombin and PMA-induced pro-MMP-2 activation. However, in HUVECs we have observed that effective suppression of the effects of thrombin required a TIMP-2 concentration greater than 1 μg/ml, whereas PMA-induced activation was suppressed at this lower level (M. A. Lafleur and D. R. Edwards, unpublished work). A possible explanation for this difference is that the added TIMP may be cleaved and inactivated by thrombin, and indeed by reverse zymography we have observed reduced recovery of TIMP-2 in conditioned media of thrombin-treated cells compared with cells stimulated with PMA (results not shown). However, there may be other factors, including the possibility that the type of MT-MMP and its localization may differ in thrombin-stimulated pro-MMP-2 activation compared with PMA-stimulated pro-MMP-2 activation.

With the results obtained in the present work we propose the following model for maximal thrombin-mediated pro-MMP-2 activation in HUVECs which supports and expands upon results put forward by other groups [9,18,36]. Thrombin rapidly (within

1h) stimulates MT-MMP activity and cleaves the intermediate 64 kDa MMP-2 form on the cell surface to the proteolytically active 63 kDa form. The 63 kDa active species does not accumulate on the cell surface and remains largely as a soluble, stable enzyme. A very small proportion of the 72 kDa pro-MMP-2 can also be directly cleaved by thrombin to generate the 63 kDa form. Signalling events through the PARs (possibly PAR3) may increase the expression of MT1-MMP thus enhancing the generation of the 64 kDa intermediate form that is then rapidly processed by thrombin to the 63 kDa active form. This activation model encompasses three factors; thrombin, an MT-MMP, and MMP-2, all of which have been shown to play a role during the angiogenic process [8,37,38]. MMP-2 and the MT-MMPs may therefore be important players in endothelial cell basement membrane and extracellular matrix degradation during thrombin-induced angiogenesis and vascular remodelling. Furthermore, it has recently been reported that active MMP-2 can stimulate platelet aggregation [39]. Platelets aggregate at sites of blood-vessel injury in order to limit blood loss by the formation of a mechanical plug. Since angiogenesis is a prerequisite for wound healing, the activation of pro-MMP-2 by thrombin at the wound site may help in both promoting angiogenesis and potentiation of platelet activation.

This work was supported by awards from the Medical Research Council (Canada) to D.E. and M.H., and the Medical Research Council (U.K.) to G. M. M. L. was supported by the Natural Sciences and Engineering Research Council (Canada). D.E. is grateful for support from the Norfolk and Norwich Big C Appeal (U.K.) and the Cancer Research Campaign (U.K.).

REFERENCES

1 Tesfamariam, B. (1994) Distinct receptors and signalling pathways in α-thrombin- and thrombin-receptor-peptide-induced vascular contractions. Circ. Res. **74**, 930–936
2 Tsopanoglou, N. E., Pipili-Synetos, E. and Maragoudakis, M. E. (1993) Thrombin promotes angiogenesis by a mechanism independent of fibrin formation. Am. J. Physiol. **264**, C1302–C1307
3 Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H. and Coughlin, S. R. (1999) Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J. Clin. Invest. **103**, 879–887
4 Vu, T. K., Hung, D. T., Wheaton, V. I. and Coughlin, S. R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell **64**, 1057–1068
5 Folkman, J. (1986) How is blood vessel growth regulated in normal and neoplastic tissue? Cancer Res. **46**, 467–473
6 Coussens, L. M. and Werb, Z. (1996) Matrix metalloproteinases and the development of cancer. Chem. Biol. **3**, 895–904
7 Okada, Y., Morodomi, T., Enghild, J. J., Suzuki, K., Yasui, A., Nakanishi, I., Salvesen, G. and Nagase, H. (1990) Matrix metalloproteinase 2 from human rheumatoid synovial fibroblasts. Purification and activation of the precursor and enzymic properties. Eur. J. Biochem. **194**, 721–730
8 Haralabopoulos, G. C., Grant, D. S., Kleinman, H. K. and Maragoudakis, M. E. (1997) Thrombin promotes endothelial cell alignment in matrigel *in vitro* and angiogenesis *in vivo*. Am. J. Physiol. **273**, C239–C245
9 Zucker, S., Conner, C., DiMassmo, B. I., Ende, H., Drews, M., Seiki, M. and Bahou, W. F. (1995) Thrombin induces the activation of progelatinase A in vascular endothelial cells. J. Biol. Chem. **270**, 23730–23738
10 Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A. and Goldberg, G. I. (1995) Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. J. Biol. Chem. **270**, 5331–5338
11 Butler, G. S., Will, H., Atkinson, S. J. and Murphy, G. (1997) Membrane-type-2 matrix metalloproteinase can initiate the processing of progelatinase A and is regulated by the tissue inhibitors of metalloproteinases. Eur. J. Biochem. **244**, 653–657
12 Matsumoto, S., Katoh, M., Saito, S., Watanabe, T. and Masuho, Y. (1997) Identification of soluble type of MT-MMP-3 formed by alternatively spliced mRNA. Biochim. Biophys. Acta **1354**, 159–170
13 Llano, E., Pendás, A. M., Freije, J. P., Nakano, A., Knäuper, V., Murphy, G. and López-Otín, C. (1999) Identification and characterization of human MT5-MMP, a new membrane-bound activator of progelatinase A overexpressed in brain tumors. Cancer Res. **59**, 2570–2576
14 Velasco, G., Cal, S., Merlos-Suárez, A., Ferrando, A. A., Alvarez, S., Nakano, A., Arribas, J. and López-Otín, C. (2000) Human MT-matrix metalloproteinase: identification, progelatinase A activation, and expression in brain tumors. Cancer Res. **60**, 877–882
15 Baramova, E. N., Bajou, K., Remacle, A., L'Hoir, C., Krell, H. W., Weidle, U. H., Noel, A. and Foidart, J. M. (1997) Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation. FEBS Lett. **405**, 157–162
16 Imai, K., Ohuchi, I., Aoki, T., Nomura, H., Fuji, Y., Sato, H., Seiki, M. and Okada, Y. (1996) Membrane-type matrix metalloproteinase 1 is a gelatinolytic enzyme and is secreted in a complex with tissue inhibitor of metalloproteinases 2. Cancer Res. **56**, 2707–2710
17 Atkinson, S. J., Crabbe, T., Cowell, S., Ward, R. V., Butler, M. J., Sato, H., Seiki, M., Reynolds, J. J. and Murphy, G. (1995) Intermolecular autolytic cleavage can contribute to the activation of progelatinase A by cell membranes. J. Biol. Chem. **270**, 30479–30485
18 Galis, Z. S., Kranzhofer, R., Fenton, II, J. W. and Libby, P. (1997) Thrombin promotes activation of matrix metalloproteinase-2 produced by cultured vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. **17**, 483–489
19 Nguyen, M., Arkell, J. and Jackson, C. J. (1999) Thrombin rapidly and efficiently activates gelatinase A in human microvascular endothelial cells via a mechanism independent of active MT1 matrix metalloproteinase. Lab. Invest. **79**, 467–475
20 Kawabata, A., Saifeddine, M., Al-Ani, B., Leblond, L. and Hollenberg, M. D. (1999) Evaluation of proteinase-activated receptor-1 (PAR1) agonists and antagonists using a cultured cell receptor desensitization assay: activation of PAR2 by PAR1-targeted ligands. J. Pharmacol. Exp. Ther. **288**, 358–370
21 Wong, H., Anderson, W. D., Cheng, T. and Riabowol, K. T. (1994) Monitoring mRNA expression by polymerase chain reaction: the 'primer-dropping' method. Anal. Biochem. **223**, 251–258
22 Foda, H. D., George, S., Conner, C., Drews, M., Tomplins, D. C. and Zucker, S. (1996) Activation of human umbilical vein endothelial cell progelatinase A by phorbol myristate acetate: a protein kinase C-dependent mechanism involving a membrane-type matrix metalloproteinase. Lab. Invest. **74**, 538–545
23 Lewalle, J. M., Munaut, C., Pichot, B., Cataldo, D., Baramova, E. and Foidart, J. M. (1995) Plasma membrane-dependent activation of gelatinase A in human vascular endothelial cells. J. Cell. Physiol. **165**, 475–483
24 Will, H., Atkinson, S. J., Butler, G. S., Smith, B. and Murphy, G. (1996) The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation. J. Biol. Chem. **271**, 17119–17123
25 Hofmann, A., Laue, S., Rost, A. K., Scherbaum, W. A. and Aust, G. (1998) mRNA levels of membrane-type I matrix metalloproteinase (MT1-MMP), MMP-2, and MMP-9 and of their inhibitors TIMP-2 and TIMP-3 in normal thyrocytes and thryoid carcinoma cell lines. Thyroid **8**, 203–214
26 Li, L., Eisen, A. Z., Sturman, E. and Seltzer, J. L. (1998) Protein tyrosine phosphorylation in signalling pathways leading to the activation of gelatinase A: activation of gelatinase A by treatment with the protein tyrosine phosphatase inhibitor sodium orthovanadate. Biochim. Biophys. Acta **1405**, 110–120
27 Schmidt, V. A., Nierman, W. C., Maglott, D. R., Cupit, L. D., Moskowitz, K. A., Wainer, J. A. and Bahou, W. F. (1998) The human proteinase-activated receptor-3 (PAR-3) gene. Identification within a Par gene cluster and characterization in vascular endothelial cells and platelets. J. Biol. Chem. **273**, 15061–15068
28 Harvey, R. P., Degryse, E., Stefani, L., Schamber, F., Cazenave, J. P., Courtney, M., Tolstoshev, P. and Lecocq, J. P. (1986) Cloning and expression of a cDNA coding for the anticoagulant hirudin from the bloodsucking leech, *Hirudo medicinalis*. Proc. Natl. Acad. Sci. U.S.A. **83**, 1084–1088
29 Markwardt, F. (1985) Pharmacology of hirudin: One hundred years after the first report of the anticoagulant agent in medicinal leeches. Biomed. Biochim. Acta **44**, 1007–1013
30 Sato, T., Kondo, T., Fujisawa, T., Seiki, M. and Ito, A. (1999) Furin-independent pathway of membrane-type 1 matrix metalloproteinase activation in rabbit dermal fibroblasts. J. Biol. Chem. **274**, 37280–37284
31 Stone, S. R. and Le Bonniec, B. F. (1998) in Handbook of Proteolytic Enzymes (Barrett, A. J., Rawling, N. D. and Woessner, J. F., eds.), pp. 168–174, Academic Press, London
32 Coughlin, S. R. (1999) How the protease thrombin talks to cells. Proc. Natl. Acad. Sci. U.S.A. **96**, 11023–11027
33 Saifeddine, M., Roy, S. S., Al-Ani, B., Triggle, C. R. and Hollenberg, M. D. (1998) Endothelium-dependent contractile actions of proteinase-activated receptor-2-activating peptides in human umbilical vein: release of a contracting factor via a novel receptor. Br. J. Pharmacol. **125**, 1445–1454
34 Herbert, J. M., Dupuy, E., Laplace, M. C., Zini, J. M., Bar Shavit, R. and Tobelem, G. (1994) Thrombin induces endothelial cell growth via both a proteolytic and a non-proteolytic pathway. Biochem. J. **303**, 227–231

35 Teti, A., Farina, A. R., Villanova, I., Tiberio, A., Tacconelli, A., Sciortino, G., Chambers, A. F., Gulino, A. and Mackay, A. R. (1998) Activation of MMP-2 by human GCT23 giant cell tumour cells induced by osteopontin, bone sialoprotein and GRGDSP peptides is RGD and cell shape change dependent. Int. J. Cancer **77**, 82–93
36 Zucker, S., Mirza, H., Conner, C. E., Lorenz, A. F., Drews, M. H., Bahou, W. F. and Jesty, J. (1998) Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase A activation and cell proliferation. Int. J. Cancer **75**, 780–786
37 Hiraoka, N., Allen, E., Apel, I. J., Gyetko, M. R. and Weiss, S. J. (1998) Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. Cell **95**, 365–377
38 Itoh, T., Tanioka, M., Yoshida, H., Yoshioka, T., Nishimoto, H. and Itohara, S. (1998) Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Res. **58**, 1048–1051
39 Sawicki, G., Salas, E., Murat, J., Miszta-Lane, H and Radomski, M. W. (1997) Release of gelatinase A during platelet activation mediates aggregation. Nature (London) **386**, 616–619

Received 7 July 2000/13 November 2000; accepted 20 April 2001