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Three-dimensional collagen matrices induce delayed but sustained activation of gelatinase A in human endothelial cells via MT1-MMP

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Abstract

Gelatinase A, a member of the matrix metalloproteinase (MMP) family, plays an important role during angiogenesis. It is constitutively expressed by human endothelial cells as a latent enzyme and requires activation. Thrombin is the only described physiological inducer of gelatinase A in human endothelial cells. In this study, we investigated the mechanisms of gelatinase A activation by another physiological inducer, collagen. Endothelial cells were cultured on various ECM components for 24 h and the conditioned media were assessed for gelatinase A activity using gelatin zymography. The results demonstrated that type I collagen matrix specifically activates gelatinase A after 24 h in human umbilical vein and 48 h in neonatal foreskin endothelial cells. In contrast, thrombin activated gelatinase A after only 2 h. Activation by collagen was sustained over long periods of time in culture (96 h). Unlike thrombin-induced activation, collagen required active membrane type 1-MMP (MT1-MMP) on the endothelial cell surface to activate gelatinase A. In addition, collagen-induced activation of gelatinase A was inhibited by antibodies to the integrin receptor, $\alpha_2\beta_1$, but not $\alpha_3\beta_1$. Our findings, that collagen can provide long-term activation of gelatinase A are likely to be relevant to endothelial cell invasion during angiogenesis.

Keywords: Human endothelial cells; Gelatinase A; Type I collagen matrix; Membrane-type matrix metalloproteinase; Thrombin

1. Introduction

Angiogenesis, the formation of new capillaries, occurs in physiological processes such as wound healing and certain diseases, such as cancer and arthritis. An essential event that occurs during angiogenesis is the invasion of the collagen-rich extracellular matrix by endothelial cells. Matrix metalloproteinases (MMPs) are crucial for this process [1]. One member of this family, gelatinase A, is known for its ability to degrade the col-

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lagens present in the basement membrane [2]. However, this enzyme also contributes to fibrillar collagen degradation by (a) activating interstitial collagenase [3], (b) degrading the cleaved collagen fragments after collagenase digestion [4] and (c) directly cleaving intact type I collagen [5]. Gelatinase A is constitutively expressed by human endothelial cells as a latent enzyme and can be activated by membrane-type-MMPs (MT-MMPs) on the cell surface [6,7]. To date, four MT-MMPs have been reported, although MT1-MMP appears to be the most efficient activator of gelatinase A [6]. Activation of gelatinase A can be induced in human endothelial cells by non-physiological agents, such as phorbol myristate acetate (PMA) via upregulation of MT1-MMP [8,9], resulting in the generation of an intermediate active 62 kD and fully active 59 kD species. Thrombin is the only physiological agent known to induce gelatinase A activation in human endothelial cells [10]. We have recently shown that thrombin induces the fully active species via a mechanism independent of MT1-MMP [11]. This activation occurs rapidly (<2 h) and ceases by 10 h.

Type I collagen can activate gelatinase A in certain cell types. Human skin fibroblasts induce the activation of gelatinase A when cultured on type I collagen [12]. Gilles et al. [13] have shown that type I collagen induces the activation of gelatinase A and concomitantly increased the MT1-MMP mRNA levels in invasive human breast carcinoma cell lines, but not in noninvasive cell lines. Recently, Haas et al. [14] have shown that a type I collagen matrix can activate gelatinase A in rat capillary endothelial cells. However, endothelial cells from different sources are heterogeneous [15]. There are differences between animal and human cells as well as cells from large vs small vessels. We have recently shown that there are marked differences in the expression of MMPs between different endothelial cells [16]. There have been no reports on the effect of type I collagen on gelatinase A activation in human endothelial cells. In the current report we show that type I collagen can activate gelatinase A in human endothelial cells by a mechanism different to thrombin-induced activation. This activation is sustained over extended time periods, requires the proteolytic activity of MT1-MMP and is inhibited by antibodies to the integrin receptor, $\alpha_2\beta_1$.

2. Materials and methods

2.1. Materials

Antibodies to $\alpha_2\beta_1$ (CD49b) and $\alpha_3\beta_1$ (CD49c) specific for the α_2-subunit and the α_3-subunit, respectively, were purchased from Dako, Carpinteria, CA, USA. These antibodies block the attachment of cells to collagen (manufacturer's literature and unpublished observation). TIMP-1 and TIMP-2 were purchased from Oncogene Science, Uniondale, NY, USA. Thrombin-α was purchased from ICN Biochemicals, Aurora, OH, USA.

2.2. Cells

Human umbilical vein endothelial cells (HUVE) and human neonatal foreskin endothelial cells (FSE) were isolated and maintained as previously described [17,18]. FSE were grown and maintained in Biorich medium (ICN Biomedicals) containing 30% normal pooled serum (derived from 10 healthy volunteers) plus 100 μg/ml endothelial cell growth supplement (ECGS) (Sigma, St Louis, MO, USA) and 50 μg/ml heparin (Sigma). HUVE were grown in Biorich containing 20% fetal calf serum plus 50 μg/ml ECGS and 50 μg/ml heparin. Cells were used at passage four. Cell viability was measured using the CellTiter 96 Aqueous One Cell Proliferation assay (Promega, Madison, USA).

2.3. Matrix preparation

Type I collagen (Collaborative Research, Bedford, MD, USA) matrix was prepared by adding 100 μl per well of a mixture containing $5\times$ medium 199, 0.1 M sodium bicarbonate buffer, bovine type I collagen (3 mg/ml), distilled water (2:1:4:2) and allowed to gel at 37°C for 30 min. For adsorbed matrix coatings, 50 μl per well of

either gelatin (0.2 g/100 ml), fibronectin (10 μg/10 μl), poly-L-lysine (50 μg/ml), type I collagen (12.5 μg/2.5 μl) or type IV collagen (12.5 μg/10 μl) (Sigma) was added for 3 h at room temperature. The solution was removed and the cells added immediately.

2.4. Experimental protocol

Endothelial cells were added to various matrix coatings at 30,000 cells/well in 96-well plates (Nunc, Kamstrup, Denmark). Cells were plated down in growth medium for up to 24 h to allow for cell adhesion. They were washed twice with Hank's balanced salt solution and pre-incubated for 6 h in basal medium (Biorich plus 1% normal pooled serum, which was stripped of gelatinases by running through a gelatin-sepharose column) (Pharmacia, Sweden). The culture medium was then replaced with fresh basal medium and experiments performed.

2.5. Gelatin zymography

Gelatinase A was detected using zymography [19]. Gelatin (1 mg/ml, BDH Chemicals, Poole, UK) was incorporated into 10% polyacrylamide mini-gels. Samples were mixed with an equal volume of 2 × sample buffer [0.25 M Tris–HCl, pH 6.8, 10% (v/v) glycerol, 0.05% bromophenol blue, 5% (w/v) SDS] and electrophoresed through the substrate gel. The gels were renatured in 2.5% (v/v) Triton X-100 for 1 h at room temperature and incubated for 16 h at 37°C in developing buffer [50 mM Tris–HCl, 100 mM NaCl, 10 mM $CaCl_2$, 0.02% (w/v) NaN_3, pH 7.5]. The gels were then stained with 0.2% Coomassie blue R-250 in 50% (v/v) ethanol and 10% (v/v) acetic acid and destained in 30% ethanol, 10% acetic acid. The gels were scanned into an IBM PC using an AVR 8800 scanner (AVR Technology, San Jose, CA, USA) and the intensity of the bands was semi-quantitated using Scion Image (Meyer Instruments, Houston, TX, USA).

2.6. Northern blotting

HUVE cultured on collagen matrix were harvested by digesting the collagen matrix with bacterial collagenase D [0.25% (w/v)] (Boehringer Mannheim, Germany) for 30 min at 37°C. The extraction of total RNA was performed using the acid guanadine thiocyanate–phenol–chloroform method of Chomczynski and Sacchi [20]. Ten micrograms of total RNA was run on a 1% agarose gel containing 1.25 M formaldehyde. The RNA was transferred to a Hybond-N+ nylon membrane (Sigma) and cross-linked by UV irradiation. The membrane was pre-hybridised for 1 h at 65°C in 1 M $NaHPO_4$, 10 mM EDTA, pH 7.2 (hybridisation buffer), 7% (w/v) SDS and 100 μg/ml sheared salmon sperm DNA. Probe DNA was radioactivity labelled using the random primer method. Probe for MT1-MMP was generously provided by Professor Paul Basset (Illkirch, France). Hybridisation was carried out overnight at 65°C in hybridisation buffer. The nylon membrane was washed for 15 min in 2 × SSC (0.3 M NaCl, 0.3 M sodium citrate, pH 7) and 0.1% (w/v) SDS at room temperature, then washed in 0.5 × SSC and 1% SDS for 10 min at 70°C, with vigorous agitation. The nylon membrane was sealed in a plastic bag and measurement was performed using a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results

3.1. Collagen matrix specifically activates gelatinase A

HUVE were plated onto various ECM substrates for 24 h and the conditioned medium was analysed using gelatin zymography. Results are shown in Fig. 1. When the cells were plated onto the inert attachment agent, poly-L-lysine, they secreted substantial amounts of latent gelatinase A, and a small amount of the 62 kD intermediate active enzyme (5.9% of total gelatinase A activity as determined by scanning densitometry) [Fig. 1(B)]. There was a similar pattern in gelatinase A secretion when the cells were plated onto adsorbed type I collagen, type IV collagen, gelatin or fibronectin. However, when grown on or embedded in type I collagen matrix for 24 h,

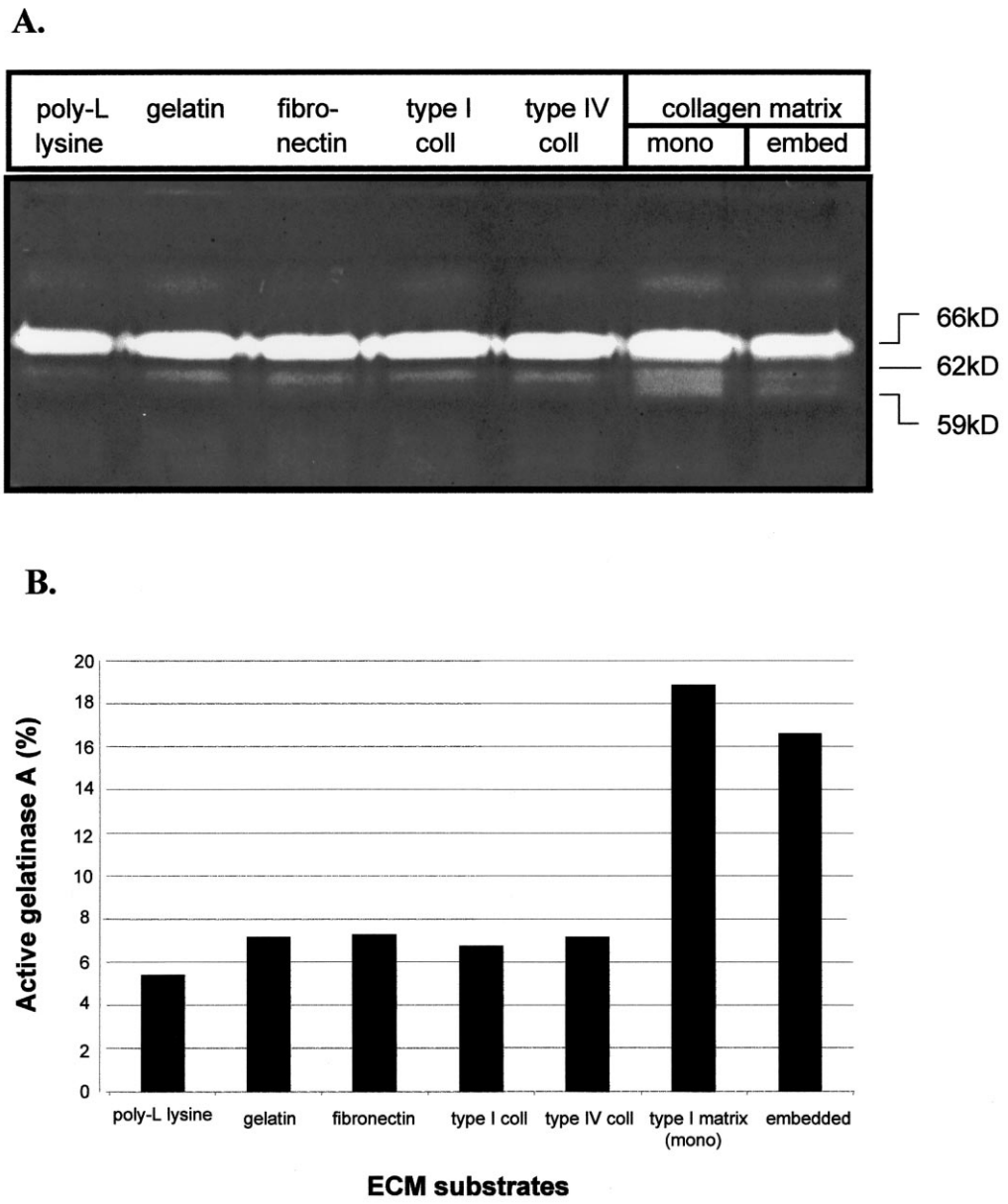

Fig. 1. Effect of ECM components on activation of gelatinase A. (A) HUVE were pre-incubated in growth medium for 2 h on adsorbed matrix coatings or type 1 collagen matrix, followed by pre-incubation for 6 h in basal medium (Biorich plus 1% gelatinase-free normal pooled serum). The cells were then incubated in fresh basal medium for 24 h either on adsorbed poly-L-lysine, gelatin, fibronectin, type I collagen (*coll*), type IV collagen (*coll*), type 1 collagen matrix (*mono*) or embedded (*embed*) in type 1 collagen matrix. Zymographic analysis was performed on conditioned media as described in Section 2. (B) Scanning densitometry was used to semi-quantitate gelatinase A activity. The results are shown as percentage of total active gelatinase A over total gelatinase A activity.

HUVE enhanced processing of the latent enzyme into both the intermediate form and the 59 kD fully active forms (18.7% of total gelatinase A activity). There was minimal overall increase in the synthesis of gelatinase A in response to a collagen matrix. When HUVE were cultured on Matrigel, activation did not occur, indicating that the activation was specific to a collagen matrix (data not shown). In the absence of cells, there was no gelatinase activity detected in the conditioned medium from the collagen matrices, thus excluding the possibility that activation was performed by the collagen itself (data not shown).

3.2. Activation by collagen matrix is sustained over time

Longer time course experiments performed on HUVE revealed that the secretion of the 59 kD fully active form of gelatinase A, in response to collagen progressively increased up to 96 h (Fig. 2). In contrast, there was substantially less fully active form when cells were cultured on adsorbed gelatin. Scanning desitometry disclosed that after 96 h, the fully active species of gelatinase A represented 10 and 42% of total gelatinase A on adsorbed gelatin and collagen, respectively. Further time points were not examined, as after 96 h, the endothelial cell monolayer began to become disrupted (data not shown).

Further time-course experiments were performed on FSE. Unlike HUVE, FSE do not activate gelatinase A when cultured on gelatin [16]. We compared the time course of thrombin- and collagen-induced activation (Fig. 3). As previously described [11], thrombin activated gelatinase A within 2 h and generated only the 59 kD fully active species, not the 62 kD intermediate species [Fig. 3(A)]. Activation continued to occur up to 10 h after which, thrombin had no further effect. After this time the active band diminished, even though secretion of the latent form of gelatinase A was still increasing [Fig. 3(B)]. In contrast, collagen did not induce activation until 48 h in FSE, at which time both the 62 and 59 kD active forms appeared [Fig. 3(C)]. As with HUVE, activation increased up to 96 h (data not shown). Similar results were obtained on four cell lines each of HUVE and FSE.

3.3. Collagen-induced activation involves MT1-MMP

We have recently shown that thrombin-induced activation of gelatinase A is not mediated by MT1-MMP. Here, we examined whether MT1-MMP was required for collagen-induced activation. To determine whether protein synthesis

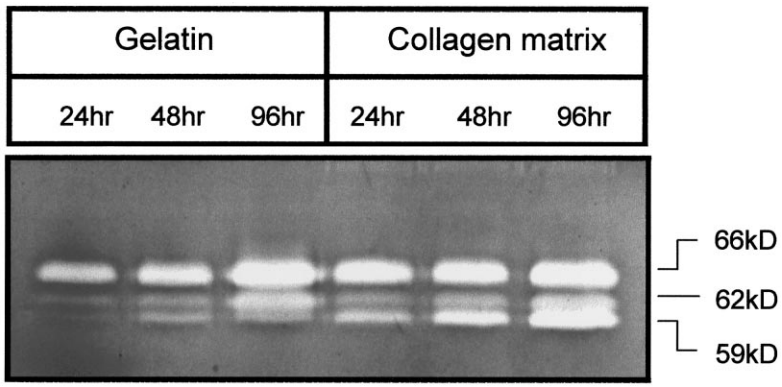

Fig. 2. Sustained activation of gelatinase A by type I collagen matrix. HUVE were pre-incubated in growth medium for 2 h on adsorbed gelatin (*Gelatin*) or type 1 collagen matrix (*Collagen*), followed by pre-incubation for 6 h in basal medium (Biorich plus 1% gelatinase-free normal pooled serum). The cells were then incubated with fresh basal medium for 24, 48 or 96 h. Zymographic analysis was performed on conditioned media.

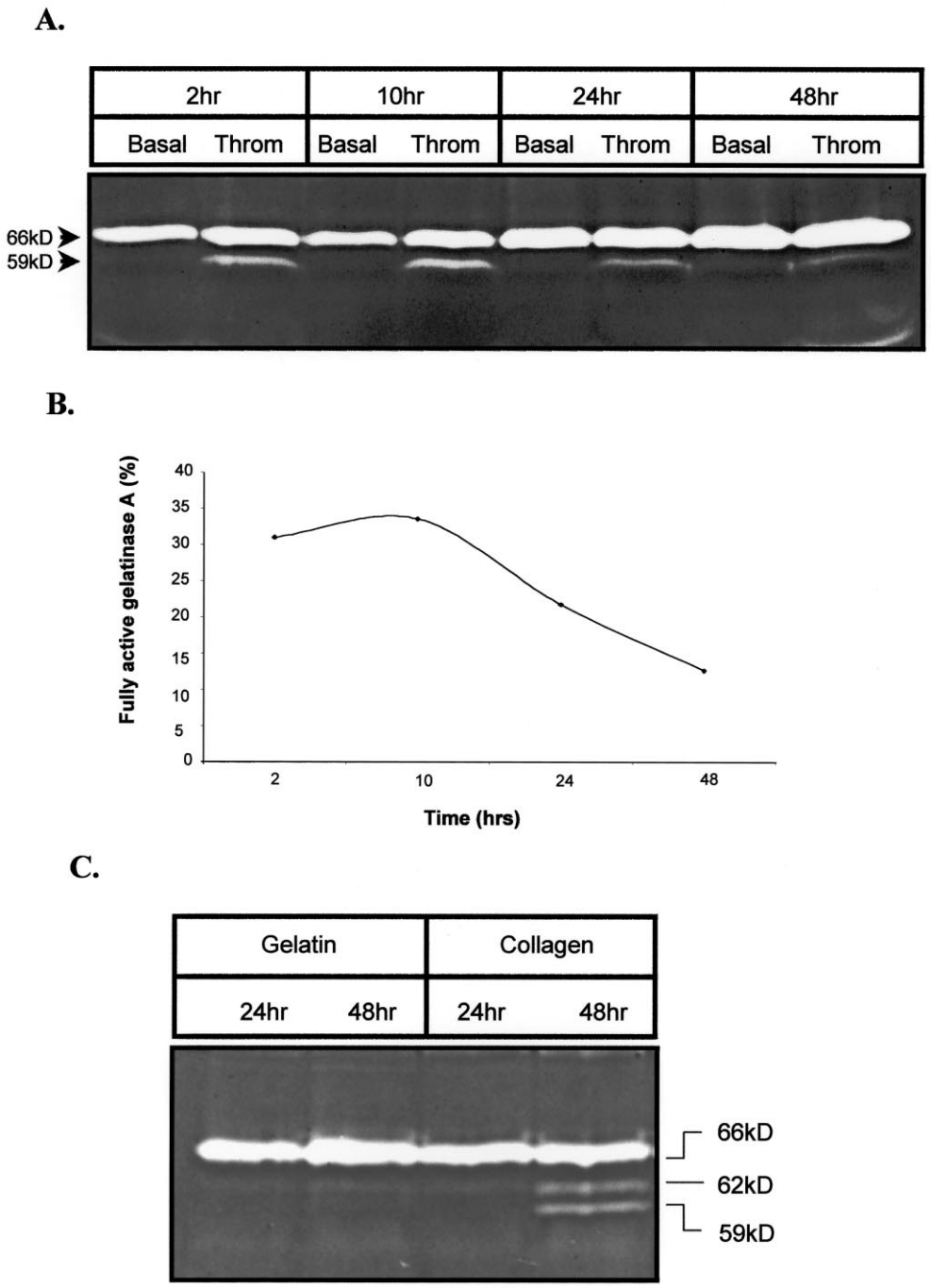

Fig. 3. Activation of gelatinase A by thrombin or collagen. FSE were pre-incubated in growth medium for 2 h on either adsorbed gelatin or type 1 collagen matrix, followed by pre-incubation for 6 h in basal medium (Biorich plus 1% gelatinase-free normal pooled serum). (A) Cells cultured on adsorbed gelatin were incubated in the presence of 100 nM thrombin (*Throm*) or no test agent (*Basal*) for 2, 10, 24 or 48 h. Zymographic analysis was performed on conditioned media. (B) Scanning densitometry was used to semi-quantitate gelatinase A activity. The results are shown as percentage of the 59 kD fully active gelatinase A over total gelatinase A activity. (C) Cells cultured on adsorbed gelatin (*Gelatin*) or type 1 collagen matrix (*Collagen*) were incubated in fresh basal medium for 24 or 48 h. Zymography analysis was perfomed on conditioned media.

or MMP activity was required for the activation, HUVE were cultured on collagen matrix for 24 h in the presence of a protein synthesis inhibitor, cycloheximide, or the MMP inhibitor, 1,10-phenanthroline. At concentrations of 1 and 10 μg/ml respectively, cycloheximide and 1,10-phenanthroline substantially inhibited the activation of gelatinase A (data not shown), suggesting that protein synthesis and MMP activity were required for collagen-induced gelatinase A activation.

Previous workers have shown that an excess of TIMP-2, but not TIMP-1, can inhibit the membrane-associated activation of gelatinase A induced by MT1-MMP [21]. We added exogeneous TIMP-1 or TIMP-2 (0.25, 0.5 and 1 μg/ml) to HUVE plated on type I collagen matrix for 24 h. TIMP2 prevented the activation of gelatinase A in a dose-dependent fashion (Fig. 4). At 1 μg/ml, TIMP-2 inhibited the production of both the intermediate and fully active forms. Together, the levels of these active forms were inhibited by approx. 78% compared to control, as determined by scanning densitometry. TIMP-1 slightly reduced the levels of the active forms secreted, although its inhibitory effect was substantially less marked than TIMP-2.

We next measured the level of MT1-MMP mRNA expressed by HUVE when grown on gelatin or type I collagen. Northern analysis revealed that mRNA for MT1-MMP was detected when the cells were grown on gelatin. Cells cultured on type 1 collagen for 24 h caused a two-fold increase in the expression of mRNA for MT1-MMP compared to cells grown on collagen (Fig. 5).

3.4. $\alpha_2\beta_1$ inhibits collagen-induced activation

The integrin receptor, $\alpha_2\beta_1$, plays an important role in mediating the adhesion of endothelial cells to collagen. To examine whether gelatinase A activation was dependent on the adhesion of endothelial cells to collagen, HUVE were treated with a blocking antibody to the integrins $\alpha_2\beta_1$ or $\alpha_3\beta_1$ for 1 h at 37°C. The cells were then plated onto or embedded in a collagen gel for 48 h in the presence of the antibody. As previously reported [22], treatment with anti-$\alpha_2\beta_1$ at 10 μg/ml completely inhibited the adhesion of cells cultured on collagen gel, whereas $\alpha_3\beta_1$ allowed the cells to adhere (data not shown). When the cells were embedded in the gel, the expression of the active species of gelatinase A was inhibited by the addition of anti-$\alpha_2\beta_1$ for 24 h [Fig. 6(A)]. This inhibition was dose responsive to antibody concentrations between 1 and 10 μg/ml. In contrast, anti-$\alpha_3\beta_1$, a receptor which does not med-

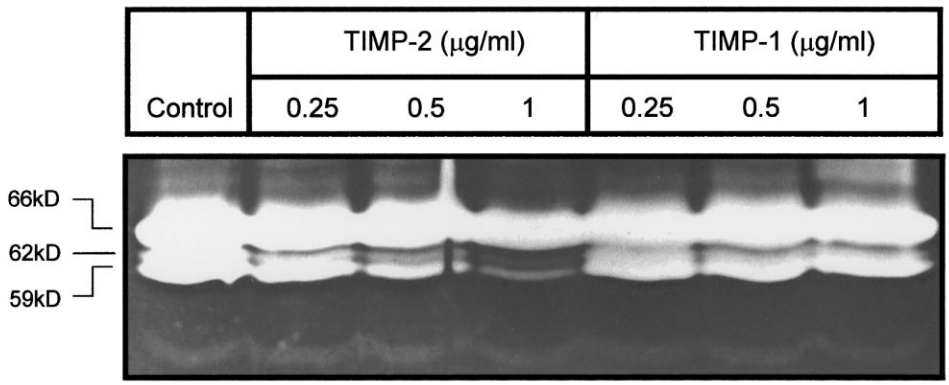

Fig. 4. Effects of TIMP-1 and TIMP-2 on gelatinase A activation by collagen. HUVE were pre-incubated in growth medium for 2 h on type 1 collagen matrix, followed by pre-incubation for 6 h in basal medium (Biorich plus 1% normal pooled serum). The cells were then incubated for 24 h with fresh basal medium in the absence (*Control*) or presence of 0.25, 0.5 or 1 μg/ml of TIMP-2 or TIMP-1. Zymographic analysis was performed on conditioned media.

iate cell attachment, did not prevent gelatinase A activation when used within the same concentration range [Fig. 6(B)].

4. Discussion

We have demonstrated that a type I collagen matrix activates gelatinase A in human endothelial cells. Until now, thrombin was the only known physiological activator of gelatinase A in human endothelial cells. Our results showed that activation by collagen matrix was different to that by thrombin. Whereas thrombin activated gelatinase A within 2 h, collagen exhibited a delayed effect, taking 24 h for HUVE and 48 h for FSE. The difference between the two cell types probably reflects the constitutive difference in their ability to activate gelatinase A. We have previously shown that HUVE activate gelatinase A under basal conditions, whereas FSE do not [16]. There was also a marked difference in the duration of activation between thrombin and collagen. Whereas thrombin stopped activating gelatinase A after 10 h, collagen sustained activation for a period of 96 h in culture. It was not possible to study time points beyond 96 h as the endothelial monolayer began to become disrupted under our experimental conditions. Previous workers have shown that, in the absence of angiogenic factors, collagen induces apoptosis in cultured endothelial cells [23]. However, it is likely that in vivo, the presence of angiogenic agents would prevent apoptosis and allow long-term activation of gelatinase A by interstitial collagen.

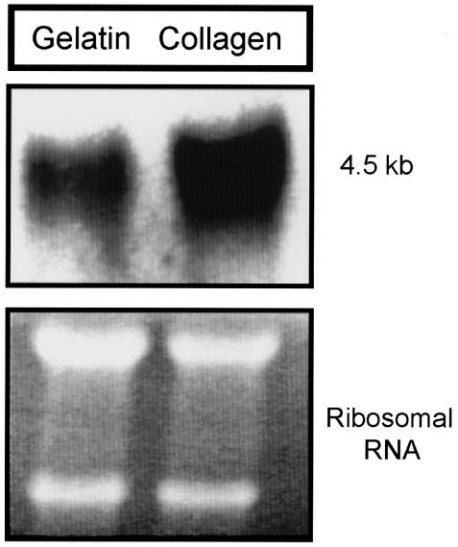

Fig. 5. Northern analysis for MT1-MMP. HUVE were pre-incubated in growth medium for 2 h on either adsorbed gelatin (Gelatin) or type 1 collagen matrix (*Collagen*), followed by pre-incubation in basal medium (Biorich plus 1% gelatinase-free normal pooled serum) for 6 h. The cells were then incubated in fresh basal medium for 24 h. Total RNA was extracted, as described in Section 2, and hybridised with a ^{32}P-labelled MT1-MMP probe. The 4.5-kb band represents MT1-MMP. Ribosomal RNA was used to verify equal loading.

There are at least two mechanisms by which latent gelatinase A can be activated on the human endothelial cell surface. The first, involving active MT1-MMP, works via a two-step process by cleaving the Asn^{37}–Lys^{38} bond in the propeptide domain to generate the intermediate 62 kD form, followed by intermolecular autocatalytic conversion to the fully active 59 kD species [24,25]. PMA upregulates MT1-MMP and induces activation of gelatinase A in this manner [8]. The second mechanism, which is independent of MT1-MMP, generates only the fully active species and can be induced by thrombin [11]. In the current report, we have presented five lines of evidence that MT1-MMP is involved in collagen-induced gelatinase A activation. First, collagen induced not only the 59 kD fully active species but also the 62 kD intermediate species, which is generated by MT1-MMP. Second, our finding that activation was inhibited by TIMP-2, but not TIMP-1, implicates a role for MT1-MMP. This is based on the evidence that excess TIMP-2 blocks free MT1-MMP active sites and prevents gelatinase A activation [21,26,27]. Third, cycloheximide prevented activation, suggesting that induction of protein synthesis is required. Fourth, the MMP inhibitor 1,10-phenanthroline, inhibited activation indicating that active MMP(s) is required. Finally, in agreement with other workers who used invasive human breast carcinoma cell line [13] or rat endothelium [14],

collagen-induced activation was accompanied by a co-ordinated upregulation of MT1-MMP mRNA.

Human endothelial cells constitutively express the collagen-binding integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ [28], however, they preferentially use $\alpha_2\beta_1$ to bind to type 1 collagen. We have demonstrated in this study that by blocking the $\alpha_2\beta_1$ integrin receptor, the activation of gelatinase A is inhibited, whereas blocking ligand interaction to $\alpha_3\beta_1$ had no effect. These data demonstrate that binding of the $\alpha_2\beta_1$ integrin to collagen is required for the induction of gelatinase A activation in human endothelial cells. Whether this interaction directly induces gelatinase activation is unclear. It is possible that the prevention of attachment of endothelial cells to collagen may compromise cell function and thus have a secondary effect on gelatinase A activation. This is unlikely as the cells continued to constitutively secrete latent gelatinase A in the presence of $\alpha_2\beta_1$ antibody [Fig. 6(A)]. Ingber et al. [29] have proposed that the mechanical forces generated by a three-dimensional extracellular matrix is transferred to the cells through integrin-mediated focal adhesion complexes causing a transduction of intra-

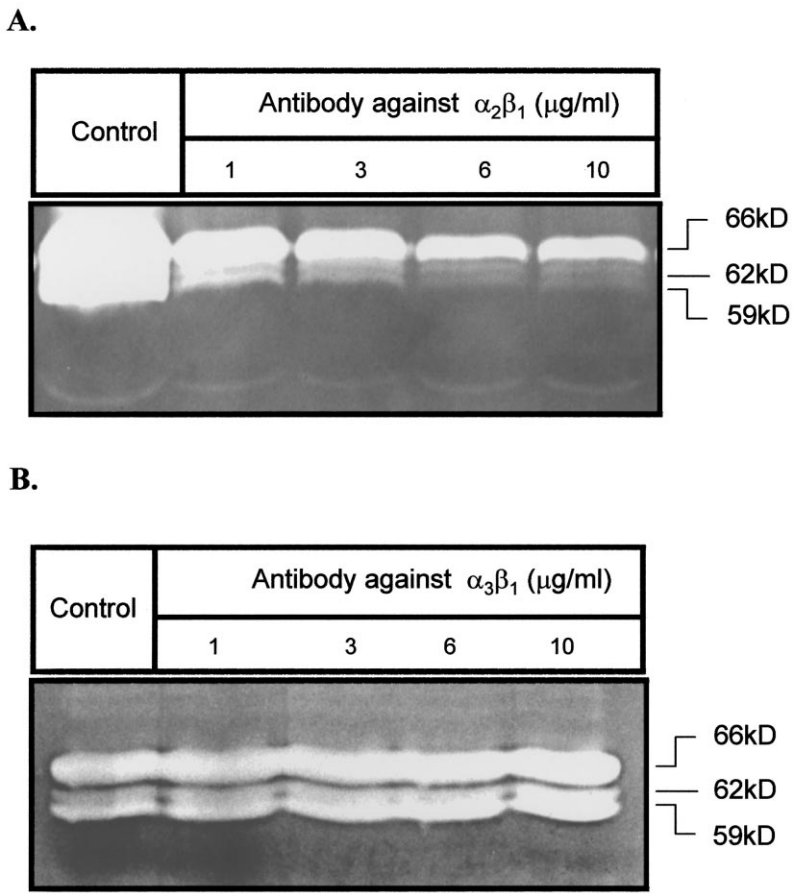

Fig. 6. Effects of antibodies against $\alpha_2\beta_1$ and $\alpha_3\beta_1$ on gelatinase A activation by collagen. HUVE were preincubated with various concentrations of antibodies against $\alpha_2\beta_1$ or $\alpha_3\beta_1$ for 1 h at 37°C. The cells were then embedded in collagen matrix and incubated for 48 h in basal medium (Biorich plus 1% gelatinase-free normal pooled serum) in the absence (*control*) or presence of 1, 3, 6 or 10 μg/ml of antibodies against $\alpha_2\beta_1$ (A) or $\alpha_3\beta_1$ (B) Zymographic analysis was performed on conditioned media.

cellular signals. This phenonenom, known as tensegrity, may be involved in collagen-induced gelatinase A activation. This is further evidenced by our finding that only three-dimensional matrices of collagen and none of the ECM components which were adsorbed to the cell culture surface, including type I collagen, induced gelatinase A activation.

Active gelatinase A plays a unique role during angiogenesis. Not only can it degrade the basement membrane, but it can also breakdown the interstitial components by directly cleaving type I collagen and/or degrading type I collagen after cleavage by collagenase [4]. Crabbe et al. [3] have also reported that gelatinase A can directly degrade type I collagen. We propose a hypothesis to explain the mechanism of gelatinase A activation during angiogenesis. Thrombin, which is present at high levels in angiogenic situations, acts as an initial stimulus and rapidly activates latent gelatinase A causing disruption of an existing capillary bed. The effect of thrombin is likely to be short-lived as it is readily incorporated into fibrin clots, immobilised in the subendothelial basement membrane or inactivated by agents such as antithrombin III or heparin [30]. Nonetheless, we have shown that thrombin is an efficient activator of gelatinase A [11] and its action is likely to be sufficient for basement membrane breakdown. This would allow the endothelial cells to make contact with type I collagen, which is the predominant protein in the interstitial stroma. Collagen then induces the upregulation of MT1-MMP expression and activates gelatinase A. This activation can be sustained for long periods of time and may at least partly explain the extended duration of angiogenesis in diseases, such as cancer. Other matrix-degrading enzymes, such as collagenase, are also likely to be involved. Activation of gelatinase A would persist until the newly formed capillary secretes its basement membrane. The latter then acts as a barrier to prevent further contact with type I collagen. Thus, the presence of type I collagen in the interstitial stroma could perpetuate its own degradation by endothelial cells, allowing for endothelial migration and subsequent angiogenesis.

Acknowledgements

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