The extracellular matrix produced by bovine corneal endothelial cells contains progelatinase A

Suzanne Menashi[b,*], Israel Vlodavsky[a], Rivka Ishai-Michaeli[a], Yves Legrand[b], Rafael Fridman[c]

[a]*Department of Oncology, Hadassah Hebrew University Hospital, Jerusalem 91120, Israel*
[b]*Unité 353 INSERM, Hospital Saint Louis, 1 Ave Claude Vellefaux, 75010 Paris, France*
[c]*Department of Pathology, Wayne State University, Detroit, MI 48201, USA*

Received 22 December 1994; revised version received 2 February 1995

Abstract Progelatinase A is a matrix metalloproteinase involved in the turnover of extracellular matrix (ECM). We report that the ECM produced by bovine corneal endothelial (BCE) cells contains progelatinase A free of tissue inhibitor of metalloproteinase (TIMP2). The matrix-bound progelatinase A can be activated by APMA to generate a 62 kDa and a 45 kDa species with enzymatic activity and is inhibited by TIMP2. The bound progelatinase can be released after treatment of the ECM with gelatinase B. These studies suggest that the ECM can function as a reservoir for progelatinase A which may be readily available for cells in processes such as metastasis, angiogenesis, inflammation and wound healing.

Key words: Matrix metalloproteinase; Extracellular matrix; Gelatinase; Corneal endothelial cell; Protease

1. Introduction

The ECM plays an active and complex role in regulating the morphogenesis of cells that contact it, influencing their development, migration, proliferation and metabolic functions [1,2]. It is now evident that the ECM provides a storage depot for growth factors (e.g. bFGF, TGFβ) [3,4], enzymes (e.g. tPA, uPA) [5,6], enzyme inhibitors (e.g. PAI1) [7] and plasma proteins (e.g. plasminogen) [8]. Some of the biological effects of the ECM can be attributed to the combined action of structural adhesive macromolecules and ECM-immobilized molecules that are thereby protected and stabilized [9,10].

The 72-kDa progelatinase A (MMP-2, 72-kDa type IV collagenase) and the 92-kDa progelatinase B (MMP-9, 92-kDa type IV collagenase) are two matrix metalloproteinases thought to play a role in the degradation and remodeling of ECM during development, metastasis formation and angiogenesis [11]. Like other members of the MMP family, the gelatinases are secreted as inactive zymogens requiring activation to attain enzymatic activity [11] and are specifically inhibited by the tissue inhibitors of metalloproteinases (TIMPs) [11]. The physiological mechanisms of activation of the progelatinases are not completely understood but may involve the action of other proteases including MMPs. For example, a recently described membrane-associated matrix metalloproteinase has been shown to activate progelatinase A [12]. The localization and binding of progelatinase A to the surface of certain tumor cells may facilitate its activation by the membrane MMP [13,14]. The present study was designed to analyse whether the gelatinases may also bind to the ECM. These proteinases may bind to the collagenous components of ECM through their fibronectin-like gelatin binding domain which is only present in the gelatinases [11]. We report on the presence of progelatinase A in the subendothelial ECM produced by cultured bovine corneal endothelial (BCE) cells. These cells produce an underlying extracellular matrix (ECM) that closely resembles the subendothelial basement membrane deposited in vivo in its morphological appearance and molecular composition.

*Corresponding author. Fax: (33) (1) 42490733.

2. Materials and methods

2.1. Recombinant enzymes and inhibitors

Human recombinant progelatinase A, progelatinase B, TIMP-1 and TIMP-2 were all expressed in mammalian cells using a recombinant vaccinia virus expression system (Vac/T7) as previously described [15–17]. Proenzymes were purified from serum-free medium by gelatin-affinity chromatography [18]. TIMP-2 was purified by affinity chromatography using a mouse mAb against TIMP-2, as described previously [16,17]. TIMP-1 was purified from the media of infected cells using a lentil lectin-Sepharose 4B (Sigma) [19]. The concentration of the purified enzymes and TIMPs was determined by amino acid analysis.

2.2. Cells

Cultures of bovine corneal endothelial (BCE) cells were established from steer eyes as previously described [20]. Stock cultures were maintained in DMEM (1 g/l glucose) supplemented with 10% heat-inactivated newborn calf serum (NCS), 5% heat inactivated FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a 10% CO_2 humidified incubator. Partially purified brain-derived bFGF (100 ng/ml) was added every other day during the phase of active cell growth.

2.3. ECM-coated dishes, ECM extracts and serum-free conditioned media

BCE cells were dissociated from stock cultures (second to fifth passage) with trypsin/EDTA and plated into culture dishes. After reaching confluence, BCE cells were maintained for additional 7 days in complete medium containing 5% dextran T-40 and without addition of bFGF. The complete medium was then aspirated, the cells washed (4×) with PBS and 0.25 ml of serum-free DMEM were added to each dish (35 mm) to obtain conditioned media. Twenty-four hours afterwards the serum-free medium was collected, centrifuged to remove cell debris and stored at −20°C until used. The subendothelial ECM was exposed by dissolving (5 min, room temperature) the cell layer with sterile PBS containing 0.5% Triton X-100 and 20 mM NH_4OH, followed by four washes in PBS. The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue culture dish [5,21,22]. ECM extracts for zymography were prepared by scrapping the ECM-coated culture dishes (35 mm) with the aid of a rubber policeman in 250 μl of Laemmli sample gel buffer (10% SDS, 30% glycerol, 0.25 M Tris-HCl, pH 6.8, 0.1% Bromophenol blue) [23] diluted 1 : 3 with water.

2.4. Zymography and enzymatic assays

Gelatin SDS-substrate gels (zymograms) were prepared as previously described [24]. Briefly, 1 mg/ml of gelatin was incorporated into the standard Laemmli SDS-10% polyacrylamide gels. Aliquots (25 μl) of

SSDI 0014-5793(95)00125-5

either solubilized ECM, serum-free conditioned media or purified enzymes were subjected to gelatin-SDS-PAGE without heating or reduction. After electrophoresis, the gels were washed twice with 2.5% Triton X-100 with gentle shaking for 30 min at room temperature. The gels were then rinsed with distilled water and incubated in assay bufffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 0.02% NaN_3) at 37°C for 24 h. For reverse zymography, the zymograms were incubated (37°C, 12 h) with assay buffer containing APMA-activated recombinant gelatinase B (1 μg/ml). The gels were then stained for 1 h with 2% Coomassie blue in R-250, 30% isopropyl alcohol and 10% acetic acid, followed by destaining in 30% isopropyl alcohol and 10% acetic acid.

Gelatinolytic activity was assayed using [^{3}H]gelatin as substrate as described previously [25]. The ECM of 35 mm culture dishes was scraped with a rubber policeman into 50 mM Tris pH 7.5, 5 mM $CaCl_2$ buffer (250 μl/dish). Aliquots (25 μl) were incubated with 0.5 mM APMA (30 min, 37°C) prior to assay. Pure recombinant APMA-activated gelatinase A (10 ng/reaction) was assayed in parallel. Samples were incubated for 3 h at 37°C with 100 μg [^{3}H]gelatin (50,000 cpm/mg) in a total volume of 250 μl of 50 mM Tris-HCl buffer pH 7.6, 5 mM $CaCl_2$ and 0.01% Brij 35. The reaction was stopped by the addition of ice-cold trichloroacetic acid to a final concentration of 12% (w/v) and samples were incubated at 4°C for 1 h. After centrifugation at 15,000 × g for 15 min, the radioactivity in 100 μl of supernatant was determined by liquid scintillation spectrometry.

2.5. Immunoblot analysis

Samples of purified enzymes, TIMPs, ECM extracts and conditioned medium were dissolved in SDS-PAGE sample buffer and electrophoresed on 10% SDS-PAGE under reducing conditions followed by transfer onto a nitrocellulose membrane. After blocking with 3% bovine serum albumin and 3% non-fat dry milk in 50 mM Tris-HCl pH 7.5, the blots were incubated with either anti-gelatinase A (CA-801), anti-gelatinase B (CA-809) or anti-TIMP-2 (CA-101) mouse monoclonal antibodies (5 μg/ml), or with anti TIMP-1 rabbit polyclonal antibodies (1:500 dilution) diluted in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20. Immunoreactivity was visualized by successive incubations with alkaline phosphatase-conjugated goat anti-mouse (or anti-rabbit for TIMP-1) IgG and color developing substrates. The preparation of mAb against human progelatinase A, progelatinase B and TIMP-2 were previously described [26,27]. The rabbit polyclonal antiserum against human TIMP-1 was kindly provided by Dr. Chua (Wayne State University).

2.6. Release of ECM-bound gelatinase A

ECM-coated wells (16 mm) were incubated at 37°C for various time periods with either bacterial heparinase I (0.05 U/ml), human placental heparanase (0.6 mg/ml), chondroitinase ABC (0.2 U/ml), gelatinase B (5 μg/ml) with or without 0.5 mM APMA or with 0.5 mM APMA alone. The incubation was carried out in 50 mM Tris pH 7.5, 50 mM NaCl, 1 mM $CaCl_2$ (250 μl/well), except in the case of placental heparanase where 10 mM citrate-phosphate buffer pH 6.0 with 50 mM NaCl and 1 mM $CaCl_2$ was used. At the end of the incubation, the supernatants were collected, centrifuged, and 25 μl analyzed by zymography as described above.

3. Results

3.1. The subendothelial ECM of BCE cells contains progelatinase A

Extracts of BCE-ECM and conditioned media were analyzed by gelatin-zymography (Fig. 1A) and immunoblotting (Fig. 1B) for the presence of gelatinases. Purified human recombinant progelatinase A and B were used as positive controls. As shown in Fig. 1A and B, both assays demonstrated that the BCE-ECM extract and the conditioned media contained only progelatinase A. In contrast, progelatinase B could not be detected by these assays in both the ECM and the conditioned media of BCE cells.

The ECM and conditioned medium were examined for the presence of TIMP-1 and TIMP-2 by immunoblot analysis using a mAb against human TIMP-2 and a rabbit polyclonal antibody against human TIMP-1. Purified human recombinant TIMP-1 and TIMP-2 were used as positive controls. It was found that BCE cells do not secrete detectable TIMPs into the ECM and conditioned medium (not shown). To rule out the possibility that the lack of detection of TIMPs was due to a lack of antibody cross-reactivity, the ECM and conditioned medium were also analyzed by reverse zymography. This technique allows the detection of MMP inhibitors. Neither a 30-kDa nor a 21-kDa inhibitory bands corresponding to TIMP-1 and TIMP-2, respectively, could be detected by this method, indicating that cultured BCE cells under this conditions do not secrete detectable TIMP-1 and TIMP-2.

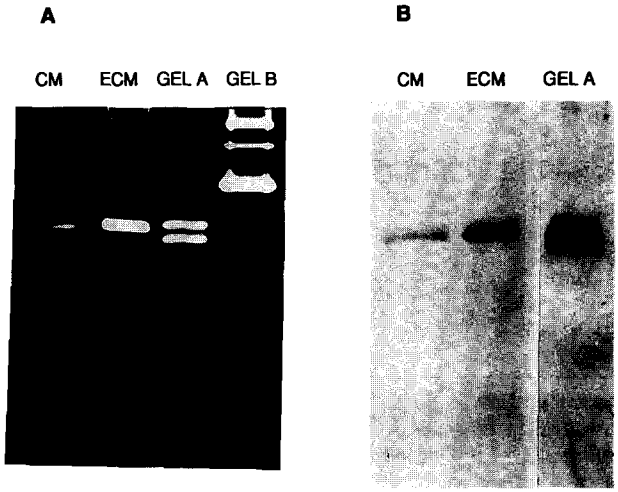

Fig. 1. Zymography and immunoblot of ECM and conditioned media of BCE cells. ECM extracts (ECM) and serum-free conditioned media (CM) of BCE cells were prepared as described in section 2 and analyzed by gelatin zymography (A) and immunoblot (B). Gelatin zymogram: 25 μl of serum-free conditioned medium (CM); 25 μl solubilized ECM; 20 ng human recombinant progelatinase A (GEL A); 20 ng human recombinant progelatinase B (GEL B). Immunoblot: 50 μl of serum-free conditioned medium (CM); 50 μl solubilized ECM; 100 ng human recombinant progelatinase A (GEL A). For immunoblots, samples were subjected to SDS-PAGE under reducing conditions followed by transfer onto nitrocellulose paper. The blots were incubated with an mAb against human progelatinase A (5 μg/ml) followed by alkaline phosphatase conjugated goat anti-mouse IgG.

Since the 72 kDa enzyme present in ECM extract was in the latent form, we examined its activation with APMA. For this purpose, ECM was incubated (30 min, 37°C) with 0.5 mM APMA followed by zymography. As shown in Fig. 2A, APMA treatment of the BCE-ECM resulted in the conversion of progelatinase A into a major active form of 62 kDa and an additional minor active species of 45 kDa. A faint 32–35 kDa active form was also detected. Both the 45- and the 35-kDa species were recognized by a mAb to progelatinase A indicating that these fragment were derived from progelatinase A (not shown). Addition of TIMP-2 (0.5 μg/35 mm dish) to the ECM prior to the addition of APMA, inhibited the activation of the ECM-bound progelatinase A, as indicated by a strong reduction of the 62-kDa form and disappearance of the smaller active fragments. This effect is probably due to the ability of TIMP-2 to form a complex with the latent gelatinase A in solubilized ECM. These results are consistent with previous studies with purified enzyme [16,17] showing that the complex cannot be fully activated by APMA. Since the ECM-bound gelatinase appears to be free

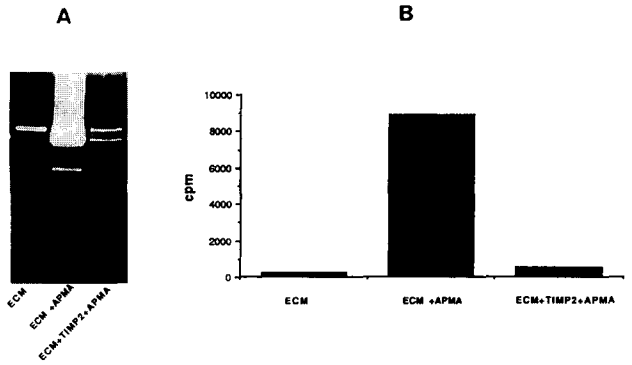

Fig. 2. Activation of ECM-bound progelatinase A and effect of TIMP-2. BCE-ECM from a 35 mm culture dish was scraped with a rubber policeman into 250 μl of 50 mM Tris pH 7.5, 5 mM $CaCl_2$ buffer. Scraped ECM was incubated (30 min, 37°C) with either buffer alone (ECM), buffer with 0.5 mM APMA (ECM + APMA) or with buffer containing 0.5 μg of human recombinant TIMP-2 for 10 min prior to the addition of APMA (ECM + APMA + TIMP-2). The samples (25 μl) were then analyzed by gelatin zymography (A) and for activity against gelatin (B) as described in section 2. This experiment was repeated 3 times with similar results. Each data point represents the average of triplicate dishes and the standard deviation did not exceed 15%.

of TIMP-2, the pattern of APMA activation shown in Fig. 2A is consistent with studies using purified TIMP-2-free gelatinase A.

APMA treatment of the BCE-ECM generated enzymatic activity against soluble [^{3}H]gelatin which was inhibited by the addition of TIMP-2, suggesting that the gelatinase can be activated while bound to the ECM (Fig. 2B). However, the contribution to this activity by other MMPs which may be present in the ECM, such as stromelysin, cannot be ruled out since these enzymes can also be inhibited by TIMP-2.

3.2. Release of ECM-bound progelatinase A

Since progelatinase A associates with the ECM, we investigated its release using various ECM-degrading enzymes. Spontaneous release of progelatinase A into the incubation buffer alone was small (Fig. 3). APMA treatment of the ECM caused release of activated gelatinase A, as indicated by the appearance of the 62- and the 45-kDa forms in the incubation medium. Incubation of the ECM with APMA-activated gelatinase B caused a release of the 72-kDa proenzyme form of gelatinase A, together with the two (62 and 45 kDa) active forms. The appearance of the active gelatinase A forms after treatment with gelatinase B may be due to the effect of the APMA as seen after treatment of the ECM with APMA alone. Since the latent ECM-bound gelatinase A was not released into the buffer after treatment of ECM with APMA alone, this result suggests that the release of the latent gelatinase A after treatment of ECM with the APMA-activated gelatinase B may be a consequence of the action of the gelatinase B. The higher molecular weight bands seen correspond to the added gelatinase B. These results suggest that the ECM component involved in binding of progelatinase A is sensitive to the proteolytic action of the gelatinases. When ECM-coated dishes were incubated with either placental heparanase (0.6 mg/ml), bacterial heparinase I (0.05 U/ml) or with chondroitinase ABC (0.2 U/ml), none of these enzymes released progelatinase A from its association with the BCE-ECM as the gelatinolytic activity released was similar to that released in buffer alone. Thus, degradation of heparan sulfate, dermatan sulfate and chondroitin sulfate does not promote the release of the ECM bound progelatinase A.

4. Discussion

In the present study we show that the ECM of BCE cells contains progelatinase A. The ECM bound enzyme was found in the latent form and free of TIMPs as determined by immunoblot analysis and reverse zymography. These findings are in agreement with a previous [6] study showing that matrigel, a reconstituted basement membrane, contains progelatinase A and B but undetectable TIMP activity in reverse zymography. Here we have shown that ECM-bound progelatinase A was activated by APMA generating enzymatic activity and forming the 62-kDa active species and an additional minor active fragment of 45 kDa, a pattern of activation consistent with a proenzyme form free of TIMP-2 as previously reported [17,28]. Preincubation of the ECM with TIMP-2 inhibited the activation of the ECM-bound enzyme in agreement with previous studies using soluble enzyme and inhibitor [17]. We have recently reported that formation of the 45-kDa species involves an autolytic cleavage of both the N-terminal profragment and the C-terminal domain (Fridman et al., in preparation). Since the C terminal end is required for the efficient inhibition of gelatinase A by TIMP-2, enzyme species like the 45-kDa form, if present in vivo, may escape the regulatory control of TIMP-2 [16,29]. Furthermore, we have found that both the 62- and the 45-kDa species can activate progelatinase B. Thus, formation of these species in the ECM may play a role in the degradation of ECM by directly degrading matrix proteins, or indirectly by activating other MMPs. An interesting observation of this study was the specific release of ECM-bound progelatinase by treatment of the ECM with gelatinase B which can be brought to the matrix by migratory cells such as polymorphonuclear

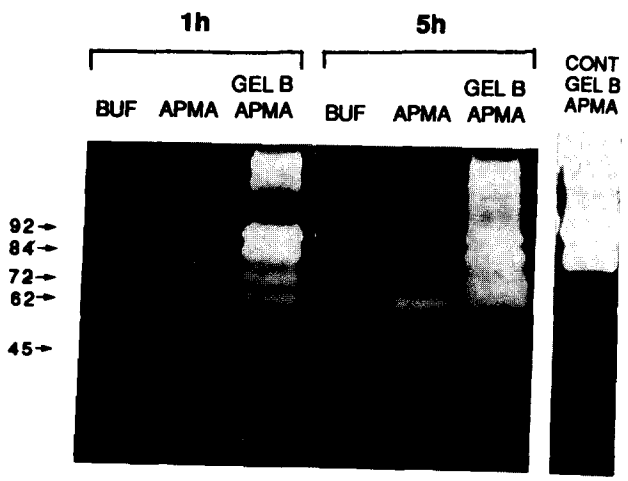

Fig. 3. Release of ECM-bound progelatinase A. ECM-coated wells (16 mm) were incubated (1 h or 5 h, 37°C) with 250 μl/well of either buffer alone (50 mM Tris pH 7.5, 50 mM NaCl, 2 mM $CaCl_2$) (BUF); or buffer containing 0.5 mM APMA (APMA) or human recombinant gelatinase B (5 μg/ml) with 0.5 mM APMA (GEL B APMA). Aliquots (25 μl) of the supernatant were centrifuged (5 min, 15,000 × g) and assayed by zymography. Control of gelatinase B (5 μg/ml) with 0.5 mM APMA was assayed in parallel (CONT GEL B APMA).

leukocytes, macrophages and tumor cells. Other ECM-degrading enzymes, in contrast, failed to release progelatinase A from the matrix to the supernatant. This suggests that the ECM component/s involved in the association of progelatinase A with the ECM may be susceptible to the proteolytic attack of gelatinase B. Although the precise substrate specificity of gelatinase B is not known, it is somewhat different from that of gelatinase A [30,31]. The binding of progelatinase A to the ECM may be mediated by its fibronectin-like gelatin binding domain [18,30] to the collagen component of the ECM.

The presence of progelatinase A in the ECM may be compared to that of active tissue-type and urokinase-type plasminogen activators [5]. ECM-bound plasminogen, for example, was found to be a better substrate for tissue plasminogen activator (t-PA) than soluble plasminogen and it was protected from inhibition by plasmin inhibitor [8]. Likewise, ECM-bound thrombin was protected from inactivation by its physiological inhibitor anti-thrombin III [32]. However the significance of the binding of progelatinase A to the ECM remains to be determined. Since progelatinase A is mainly produced by stromal cells surrounding tumors [33–35], the proenzyme may be sequestered by the ECM, awaiting activation by the appropriate signal. Recently a membrane bound matrix metalloproteinase which induced specific activation of progelatinase A was identified on the surface of invasive tumor cells [12]. This enzyme may trigger invasion, tumor cell proliferation and angiogenesis by activating stromal and ECM-bound progelatinase A at the invasive edge of tumor cell nests.

Acknowledgements: This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) and by the Ligue Nationale Contre le Cancer.

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