The sensitivity of versican from rabbit lung to gelatinase A (MMP-2) and B (MMP-9) and its involvement in the development of hydraulic lung edema

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Abstract Large chondroitinsulphate-containing proteoglycan (versican) isolated from rabbit lung was cleaved by purified gelatinase A (MMP-2) and gelatinase B (MMP-9), as well as by crude enzyme extract from rabbit lung with hydraulic edema. Gelatine zymography, performed after purification of gelatinases by affinity chromatography, demonstrated that the enzyme extract contained two main gelatinolytic bands at about 92 kDa and 72 kDa, identified by specific antisera as the latent proMMP-9 and proMMP-2, respectively. Moreover, enzyme extract from edematous lung showed an increased amount of the proteolytically activated forms of both gelatinases with respect to normal controls. These results suggest that MMP-2 and MMP-9 are involved in the breakdown of versican occurring in rabbit lung during the development of hydraulic edema.

Key words: Gelatinase; MMP-2; MMP-9; Proteoglycan; Versican; Lung edema

1. Introduction

The ECM of lung interstitium provides a strong and expandable framework for the thin alveolar epithelial-capillary intersection and consists mainly of collagen, elastic fibers and PGs, which are largely responsible for tensile strength, elastic recoil and tissue compliance, respectively [1]. The integrity of ECM native architecture depends on the balance between the regulation of synthesis and degradation of its components and an excess of proteolytic activity over the inhibitory capacity of the lung is likely to lead to parenchymal destruction.

The turnover and remodeling of lung ECM involves a family of structurally related zinc-dependent endopeptidases, that are known as MMPs, are active at neutral pH and can collectively degrade almost all components of ECM [2,3]. In particular, the MMP subfamily of gelatinases (gelatinase A, MMP-2 and gelatinase B, MMP-9) degrades denaturated collagens such as gelatin, type IV collagen, fibronectin and elastin [2,3] and was found to be involved in some pathological processes, including pulmonary emphysema [4–6], interstitial lung diseases [7] and hyperoxia-induced acute lung injury [8–10].

Recently, we found that PGs were broken down during the development of lung edema, induced in rabbits by i.v. saline loading [11] and that PG breakdown involved mainly the versican family of the ECM [12]. On this basis, the aim of this study was to determine: (1) whether gelatinases may recognize as substrate the core protein of lung interstitium PGs; (2) whether increased activities of gelatinases are involved in the development of hydraulic pulmonary edema, accounting also for PG breakdown.

We found that large PG (versican) extracted and purified from rabbit lung was cleaved by purified MMP-2 and MMP-9, as well as by an enzyme extract from rabbit lung with hydraulic edema. This enzyme extract contained both MMP-2 and MMP-9 as demonstrated by gelatine zymography and by specific antibodies. Moreover, the amount of the proteolytically activated forms of both gelatinases was increased in edematous lung relative to control.

2. Materials and methods

2.1. Materials

^{125}I and iodo beads were purchased from Dupont-NEN (Boston, MA, USA) and Pierce Chemicals (Rockford, IL, USA), respectively. Seikagaku (Tokyo, Japan) supplied the depolymerising C-O lyase chondroitinase ABC from *Proteus vulgaris*. Sigma (St. Louis, MO, USA) supplied standard disaccharides employed in capillary electrophoresis analysis. Gelatin-Sepharose resin was from Pharmacia (Uppsala, Sweden). Purified proMMP-2 and proMMP-9 (from human neuroblastoma cell cultures) were supplied by Chemicon (Temecula, CA, USA). Specific sheep antisera against MMP-2 and MMP-9 and PVDF were from The Binding Site (Birmingham, UK) and BioRad (Hercules, CA, USA), respectively. The other biochemicals and reagents were commercial products of the highest purity.

2.2. Experimental protocol

The experiments were performed on adult New Zealand rabbits anesthetized and tracheotomized to allow spontaneous breathing [11]. The control group of rabbits (C) did not receive any treatment, whereas the other group of animals was let to develop lung edema by slow infusion of saline solution into jugular vein (hydraulic edema, H)[11]. Animals were killed by an overdose of anesthetic [11] after 180 min, then the lungs were excised, pooled into two groups (C and H) and then cut into small slices [11]. Aliquots of the pooled lung slices, representing about 10% of the total sample, were used to determine the water content, by measuring wet weight to dry weight ratio (W/D) [11]. The W/D ratios were: 5.2 ± 0.2 for C ($n = 8$) and 6.0 ± 0.4 ($n = 4$) for H, in agreement with previous determinations of lung W/D ratio in control rabbits and in animals affected with hydraulic edema, respectively [11].

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Abbreviations: ECM, extracellular matrix; MMP, matrix metalloproteinase; PG, proteoglycan; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; GuHCl, guanidine hydrochloride; PVDF, polyvinylidene difluoride

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2.3. Isolation, characterization and radiolabelling of large proteoglycans from normal rabbit lung

PGs were extracted from pooled lung slices of group C with 0.4 M GuHCl and purified as previously reported [11]. Versican, the large chondroitin sulphate-containing PG of the ECM, was then isolated by gel-filtration on Sepharose CL-4B under dissociative conditions [11]. Versican identification was confirmed by capillary electrophoresis analysis of unsaturated disaccharides released by chondroitinase ABC digestion [12]. The analysis of molecular size distribution of native GAG chains was performed by gel-filtration in HPLC, after papain digestion of PG core protein and removal of protease-resistant peptides by β-elimination [13]. This analysis was also performed on PGs extracted from edematous lung specimens to evaluate whether GAG chains have been degraded during the development of hydraulic edema.

Versican was then radiolabelled with ^{125}I [13].

2.4. Assay of versican sensitivity to gelatinases

Aliquots of ^{125}I-versican (70 000 cpm) were incubated with 1 μg of either purified MMP-2 or MMP-9 in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$, for 18 h at 37°C. ^{125}I-versican degradation was then evaluated by gel-filtration chromatography on Sepharose CL-4B column (50×1 cm, flow rate 6 ml/h) under dissociative conditions [11], determining the changes in elution pattern of the radioactive material in comparison to the control untreated sample.

2.5. Preparation of crude enzyme extracts from lungs and assay of versican degrading enzyme activities

Lung specimens were washed carefully, homogenized at 4°C in 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 0.002% Tween-20, (buffer A) [14] and cleaned by centrifugation at 12 000×*g* for 10 min. Aliquots (100 μg protein) of the crude enzyme extract from H group were incubated with ^{125}I-versican (70 000 cpm) for 18 h at 37°C in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$. Versican degradation was then evaluated by gel-filtration chromatography on Sepharose CL-4B as reported above.

2.6. Radioactivity measurements

^{125}I-radioiodinated materials were monitored by a Cobra II γ-counter (Canberra Packard).

2.7. Purification of gelatinases by affinity chromatography

Aliquots of the crude enzyme extracts from C and H groups were incubated with gelatin-Sepharose resin (100 μg protein/100 μl resin) for 1 h at 4°C under gentle mixing to isolate gelatin degrading enzymes [14]. The resin was then recovered, washed carefully with buffer A, containing 5 mM $CaCl_2$ and 10 mM Na_2-EDTA, resuspended in 4×Laemli sample buffer and used for electrozymograms.

2.8. SDS-PAGE zymography

SDS-PAGE was performed using 10% (w/v) gel copolymerized with gelatin (1 mg/ml) [14] to identify proteins with gelatinolytic activities. After washing and overnight incubation of the gel at 37°C in an activating buffer (50 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl and 5 mM $CaCl_2$), the gel was stained with 0.5% (w/v) Coomassie Brilliant Blue R-250 [14]. Proteolytic activities were detected as clear bands against the blue background, indicating areas where gelatin was degraded by the enzymes. The area of the gelatinolytic bands was evaluated by a GS 700 densitometer (BioRad) and showed a linear increase with increasing amounts of standard MMPs (from 0.2 to 1 μg, regression coefficient (r^2) = 0.90 ± 0.04). The molecular weights of gelatinolytic bands were estimated by comparing their electrophoretic migration to that of protein standards (BioRad). To analyze metalloproteinase inhibition, the gels were incubated in the presence of 20 mM EDTA.

Immunoblot analysis was performed after SDS-PAGE, carrying out Western transfer to PVDF using a semi-dry transfer cell (BioRad) at 15 V/cm^2 for 20 min. The identification was then performed with specific polyclonal sheep antibodies (1:5000), which reacted with both the zymogen latent forms and the proteolytically activated forms of these enzymes. The positive bands were revealed by chemiluminescence (Super Signal Pierce) after incubation with antisheep donkey antibodies conjugated with horseradish peroxidase.

3. Results

3.1. Versican degradation by purified gelatinases and by crude enzyme extract from edematous rabbit lung

Gel-filtration chromatography of ^{125}I-versican isolated from normal rabbit lung on Sepharose CL-4B under dissociative conditions showed that the radioactive material was eluted in the void volume of the column (Fig. 1, panel C). The incubation with purified MMP-2, MMP-9 and crude enzyme extract from edematous rabbit lung resulted in a fragmentation of ^{125}I-versican, as the amount of radioactive material eluted in the void volume of the column was markedly reduced (−65.4%, −50.1% and −78.5% relative to control C, respectively) (Fig. 1, panels MMP-2, MMP-9 and H). The degradation products were likely represented by peptidoglycans. In fact, gel-filtration HPLC analysis of native GAG chains from control and edematous lungs showed that the chain size was dispersed within the same range of values. Low molecular size material referable to oligosaccharides was not detected in these GAG preparations, indicating that the breakdown of PGs during the development of hydraulic edema depended only on the degradation of core protein.

3.2. SDS-PAGE zymography

Gelatin zymography revealed different gelatinolytic bands in the purified enzyme extracts from C and H groups (Fig. 2, panel A, lanes 3 and 4). The bands at about 72 kDa (band a) and 92 kDa (band b) corresponded to standards of latent proMMP-2 and proMMP-9, respectively (Fig. 2, panel A, lane 2) and showed enlarged areas in H group with respect to C (compare lane 4 and lane 3). The two minor, faster migrating bands (bands a′ and b′) likely represented the proteolytically activated forms of the enzymes and were more evident in H group.

The bands of higher molecular mass are known to be included in the typical zymography pattern of MMP-9 from neutrophils [4,5,15,16]. The 135 kDa band (band c) was

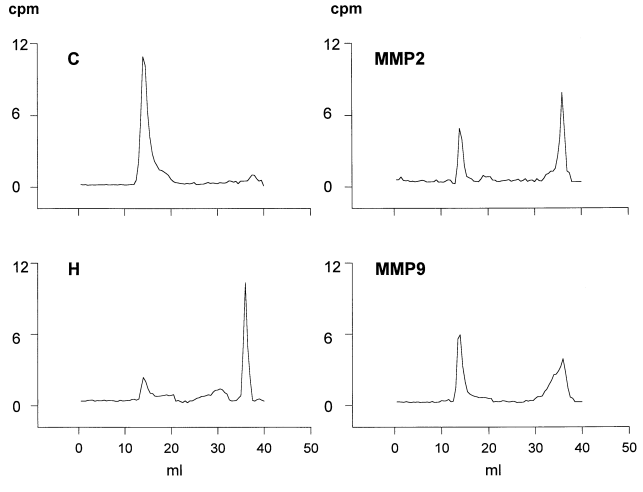

Fig. 1. Sepharose CL-4B dissociative gel-filtration chromatography of ^{125}I-versican extracted and purified from normal rabbit lung. ^{125}I-versican incubated with buffer only (panel C); ^{125}I-versican incubated with purified MMP-2 (panel MMP-2); ^{125}I-versican incubated with purified MMP-9 (panel MMP-9); ^{125}I-versican incubated with enzyme extract from edematous lung (panel H). Determination of cpm is expressed in thousands.

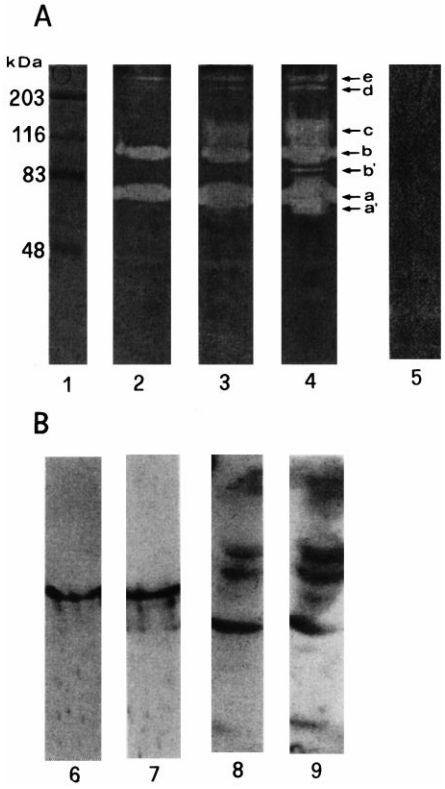

Fig. 2. Zymography (panel A) and immunoblotting identification (panel B) of MMP-2 and MMP-9. Molecular weight markers (lane 1). Standard latent proMMP-2 and proMMP-9 from human neuroblastoma cell cultures (lane 2). Affinity chromatography purified enzyme extract from control lung (lane 3) and from edematous lung (lane 4). The small letters on the right identify the gelatinolytic bands as described in Section 3. Zymogram incubated in the presence of 20 mM EDTA (lane 5). Immunoblotting identification of MMP-2 in affinity chromatography purified enzyme extract from control lung (lane 6) and from edematous lung (lane 7). Immunoblotting identification of MMP-9 in affinity chromatography purified enzyme extract from control lung (lane 8) and from edematous lung (lane 9).

shown to be a complex of 92 kDa MMP-9 and a 25 kDa protein, named neutrophil gelatinase-associated lipocalin [16], whereas the larger ones (>200 kDa, bands d and e) consisted probably of multiple forms of the enzyme [4,5,16]. Also these bands were more evident in H group with respect to C.

No gelatinolytic bands were detectable when the gels were incubated in the presence of 20 mM EDTA, a typical inhibitor of MMPs (Fig. 2, panel A, lane 5).

The identification of the gelatinolytic bands was confirmed by specific antisera in the purified enzyme extract from C and H groups. In the case of immunoblotting detection of MMP-2, the antibody reacted with the band of 72 kDa and with some lower molecular mass degradation products (Fig. 2, panel B, lanes 6 and 7). The antibody against MMP-9 recognized both the zymogen latent form and the proteolytically activated form of the enzyme, as well as its higher molecular mass forms and some degradation products (Fig. 2, panel B, lanes 8 and 9).

4. Discussion

This study provides direct evidence that large chondroitin-sulphate-containing PG (versican) purified from lung interstitium is sensitive to MMP-2 and MMP-9 activity. Both MMP-2 and MMP-9 were previously shown to cleave the large PG of cartilage (aggrecan) [17–20], the activity of MMP-9 being lower [17]. In agreement with these findings, we found that versican degrading activity of MMP-2 was higher than that of MMP-9 (Fig. 1).

Gelatinases are expressed in normal adult tissues and an immunohistochemical study of normal lung showed that MMP-2 and MMP-9 are localized in ciliated cells, endothelial cells, pneumocytes, macrophages and smooth muscle cells, whereas fibroblasts show a strong reaction only for MMP-2 [21]. The enzyme extract from edematous rabbit lung was able to cleave versican from lung samples of the same animal and displayed both MMP-2 and MMP-9 activities, indicating that gelatinases recognize as a substrate in vivo also the large PG family of lung interstitium.

An upregulation of gelatinases was shown to be involved in both chronic and acute lung injury [4–10]. Our data indicate that an increase of both MMP-2 and MMP-9 activity is involved in the development of pulmonary edema induced by saline infusion, sharing the massive fragmentation of versican occurring in this type of edema [11,12].

The pattern of gelatine zymography of lung enzyme extracts included the 135 and >200 kDa forms, which are characteristic of MMP-9 of neutrophil origin [4,5,15,16]. A large body of evidence implicates neutrophils in the acute respiratory distress syndrome that often follows systemic inflammation [22]. Our finding indicates that neutrophils are likely to take part in damaging pulmonary interstitium PGs during the development of hydraulic edema.

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