Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury

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Abstract The effect of neutrophil elastase on the functional status of gelatinases was studied in an hamster model developed by intratracheal administration of lipopolysaccharide followed by in situ cell activation with phorbol myristate acetate. This resulted in the production in bronchoalveolar lavage fluids, in addition to the matrix metalloproteinase MMP-9, of a 75 kDa gelatinase associated with collagenolytic activity. Treatment in vivo with an elastase inhibitor abolished the latter activity. Since, in addition, elastase activates in vitro purified MMP-9 gelatinase into a similar 75 kDa entity, these data suggest that elastase may be a physiological activator of MMP-9 in vivo.

Key words: Acute lung injury; Elastase; Gelatinase; S 18465; Metalloproteinase; MMP-9; Zymography

1. Introduction

Extracellular matrix (ECM) degradation by specific enzymes is thought to play a causative role in lung diseases as diverse as emphysema, fibrosis and acute respiratory distress syndrome (ARDS) [1]. Neutrophils may contribute to acute and chronic lung injury by releasing numerous factors especially proteolytic enzymes which are able to destroy important structural proteins of the lung [2]. Considering the massive influx of neutrophils and the increased elastolytic activity in bronchoalveolar lavage (BAL) fluid from patients with ARDS, it has been suggested that neutrophil elastase may play an important role in the pathogenesis of high-permeability lung oedema [3]. In addition to elevated levels of neutrophil elastase, neutrophil-derived collagenase has also been detected in BAL fluid from patients with ARDS, and MMP-9 gelatinase (a matrix metalloproteinase) levels in BAL have been shown to increase during the course of ARDS [4]. MMP-9 also might be a major factor in facilitating migration of neutrophils across basement membranes [5]. Gelatinase activities have also been demonstrated in different animal models of acute lung injury. For example, increases in gelatinase (MMP-2 and MMP-9) activities were observed in the BAL from rats exposed to ozone [6]. Similarly, when lipopolysaccharide (LPS) is used to induce acute lung injury in a guinea pig model, a total increase in MMP-9 gelatinase proform expression was observed [7]. In a rat model of acute alveolitis induced by intra-pulmonary deposition of immune complexes, when recombinant TIMP-2 (tissue inhibitor of matrix metalloproteinase-2) is instilled intratracheally, lung damage is significantly reduced [8]. This suggests a functional role of MMPs in the process.

MMPs must be activated to ensure extracellular matrix substrate degradation. Although products of the respiratory burst, such as hypochlorous acid, can activate MMPs, including MMP-9 [9], the in vivo relevance of this activation mechanism remains speculative [10]. Therefore, the use of animal models of acute lung injury might be helpful to identify the physiological mechanisms leading to MMP activation.

To further investigate the potential functional role of the MMP-9 gelatinase in lung injury, a model of massive PMN recruitment followed by an exogenous cell activation was chosen. Neutrophil (PMN) recruitment and activation were achieved by treating hamsters intratracheally with LPS followed by phorbol myristate acetate (PMA). Gelatinolytic activities and their functional status were studied in this model, with a particular emphasis on the potential role of neutrophil elastase as a gelatinase activator.

2. Materials and methods

2.1. Animal treatment

Ten-week-old male Syrian golden hamsters (Harlan, Zeist, The Netherlands), weighing 110–130 g were challenged for 30 min with aerosolised lipopolysaccharide (LPS, *E. coli* 026:B6, Difco, Detroit, MI), 500 μg/ml dissolved in 0.9% saline, delivered by a compressed air nebulizer at 11 l/min in a 10 l chamber. Control group inhaled saline only. Twenty-four hours after LPS inhalation, hamsters, under isoflurane anaesthesia, were instilled intratracheally with the S 18465 elastase inhibitor [11] at the concentration of 300 nmol/hamster, or with saline, under 0.2 ml. Three hours after this injection, the animals were further injected, again intratracheally, with 0.1 ml PMA (500 μg/kg) in 20% DMSO. A bronchoalveolar lavage was performed 3 h after PMA administration.

2.2. BAL recovery and analyses

Lungs were washed gently using a single 3 ml aliquot of 0.9% saline in a 5 ml syringe, yielding a final volume of approximately 2.5 ml of BAL fluid from each animal.

The amount of blood in each BAL sample was determined by using the Boehringer Mannheim Diagnostica kit for haemoglobin. BAL was centrifuged at 200×*g* for 10 min at 4°C. The cell pellets were counted using a Coulter Counter (Coultronic, Margency, France) and differential cell counts from at least 200 leukocytes were performed after cytocentrifugation with a Cytospin (Shandon, Runcorn, UK). The supernatants were routinely stored at −25°C since this procedure was not found to alter the enzymatic profiles as compared to freshly analysed samples.

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Abbreviations: ARDS, acute respiratory distress syndrome; APMA, 4-aminophenylmercuric acetate; BAL, broncho alveolar lavage; DNP, 2,4-dinitrophenyl; ECM, extracellular matrix; MMP, matrix metalloproteinase; Nma, *N*-methylanthranilic acid; r(h) recombinant (human); TIMP, tissue inhibitor of metalloproteinase

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2.3. Zymography

Gelatinolytic or caseinolytic activities were detected by zymography, as described [12]. Briefly, aliquots of BAL fluid underwent electrophoresis in polyacrylamide gels containing either 1 mg/ml gelatin or 1.5 mg/ml casein (both from Sigma) in the presence of SDS under non-reducing conditions. After electrophoresis, gels were washed twice in 2.5% Triton X-100 for 1 h, rinsed briefly in 50 mM Tris-HCl (pH 7.4), and incubated at 37°C for 24 h in a buffer containing 50 mM Tris-HCl (pH 7.4) and 5 mM $CaCl_2$. The gels were then stained with Coomassie Brillant Blue R250. Zones of enzymatic activity were detected as clear bands against a blue background.

2.4. Type IV collagen degradation assay

Collagenolytic activity was measured with ^{3}H-labelled type IV collagen (NEN) (37 Kbq/1 μg) as substrate [13]. BAL and inhibitors were added to solutions containing the radiolabelled substrate and incubated for 20 h at 37°C in 0.5 M Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0,02% Brij35, pH 7.5. Substrate hydrolysis was analysed by SDS PAGE (gradient 4–20%) and autoradiography.

2.5. Fluorogenic enzymatic assay

The peptidomimetic substrate Dnp–Pro–Cha–Gly–Cys(Me)–His–Ala–Lys(Nma)NH2 (BACHEM, Voisins-le-Bretonneux, France) which is cleaved between amino acids Gly and Cys by MMP-9 [14], was used to assay the functional activity of purified human recombinant MMP-9 (from Dr. Gillian Murphy, Strangeways Laboratory, Cambridge, UK). MMP-9 at a concentration of 1.3 μg/ml was activated with 2 mM APMA in 0.1 N NaOH at 37°C for 1 h or by 180 ng/ml human elastase (Elastin Products Company, Inc., Owensville, MO) for 24 h.

The assays were performed in 50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, 0.1% Brij35, pH 7.7, containing 130 ng/ml purified MMP-9, started with 20 μM of substrate and incubated at 37°C for 10 min. The fluorescent product of cleavage was measured with a cytofluorimeter (Cytofluor 2350, Millipore, Saint-Quentin en Yvelines, France) equipped with a combination of 340 nm and 440 nm filters for excitation and emission, respectively.

2.6. Elastase activity

The elastase activity of the BAL fluid was measured using a synthetic elastase substrate: methoxy succinyl-Ala–Ala–Pro–Val–*p*-nitroanilide, according to Virca et al. [15]. Briefly 0.1 ml of BAL fluid supernatant was mixed with 0.9 ml of 0.1 M Tris-HCl buffer, pH 7.8, 0.5 M NaCl. The optical density was read at 410 nm during 30 min at 37°C with a Unicam spectrophotometer (Cambridge, UK).

3. Results

3.1. Cytology and haemoglobin content of BAL

BAL fluids of four different groups of hamsters were studied. The first two groups were composed of animals treated (group 2) or not (group 1) intratracheally with PMA 3 h before lavage. The BAL cell population of these animals was essentially composed of macrophages (Table 1).

The third and fourth group of animals were initially treated intratracheally with LPS 24 h before lavage recovery. Massive PMN recruitment accounted in both groups for most of the increase in total cell number observed as compared to control animals (Table 1). The fourth group, in addition to LPS instillation, was further treated intratracheally, 3 h before BAL, with PMA to activate resident and recruited cells. Haemoglobin, a marker of acute lung injury, increased only in the fourth group (Table 1).

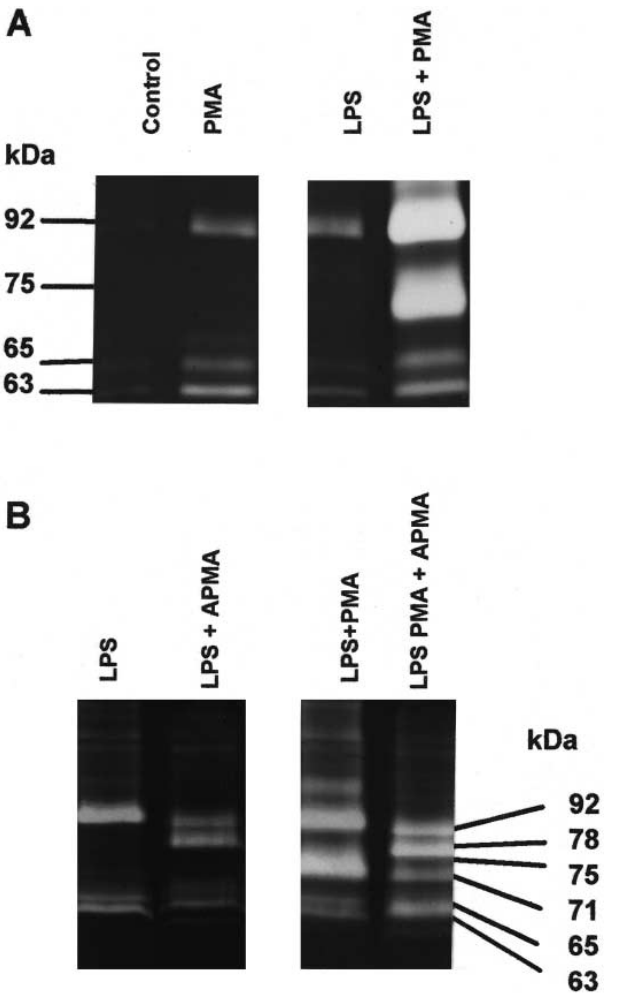

Fig. 1. A: Gelatin zymography of BAL fluids. BAL from a control animal; BAL from one animal treated with PMA 3 h before lavage; BAL from a LPS-treated animal; BAL from a hamster treated sequentially with LPS and PMA. Samples of 10 μl of crude BAL were deposited. B: Zymogram of BAL fluids activated for 1 h with APMA.

3.2. Zymographic analyses of gelatinase activities

Gelatin zymograms of control hamster BAL fluids demonstrated that negligible amounts of gelatinases were secreted, mainly in the 63, 65 and 92 kDa forms (Fig. 1A). BAL fluids from animals treated with PMA only exhibited an increase in 63, 65 and 92 kDa activities, the 92 kDa gelatinase being now clearly detectable (Fig. 1A). After LPS treatment and in the absence of PMA, the gelatinolytic activities detected at 63, 65 and 92 kDa were slightly increased as compared to control

Table 1
Cytology and lung injury parameter

	PMN number × 10^6/BAL	% of total cell population	Haemoglobin g/100 ml/BAL	*n*
Control	0.88 ± 0.13	7 ± 4	0.043 ± 0.001	5
Control PMA	0.65 ± 0.07	37 ± 11	0.093 ± 0.02	5
LPS	9.79 ± 1.73	85 ± 4	0.066 ± 0.013	5
LPS+PMA	3.09 ± 0.81	91 ± 2	0.540 ± 0.165	10

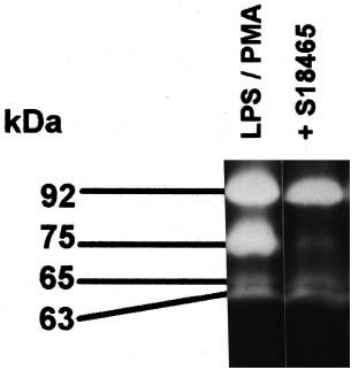

Fig. 2. Effect of in vivo treatment of animals with S 18465 on the gelatinolytic activity profile of LPS plus PMA-treated animals.

hamsters, the 92 kDa activity being predominant (Fig. 1A). After a sequential treatment with LPS and PMA, an overall increase in gelatinotytic activity was observed, in particular at 92 kDa, and a new activity appeared at 75 kDa (Fig. 1A). The observed activities all required divalent cations and were inhibited by EDTA, indicating that they belong to the MMP family (not shown). Zymography was carried out with BAL samples incubated in the presence of APMA to activate the MMPs prior to SDS-PAGE. The observed profile of BAL from LPS-treated animals was suggestive of the activation of the major 92 kDa species into a 78 kDa entity (Fig. 1B). In the sample from LPS plus PMA-treated animals the same 78 kDa activity appeared whereas the 75 kDa activity progressively disappeared (Fig. 1B). No caseinolytic activity could be detected by zymography in a buffer devised to reveal MMP activity, showing that there was no interstitial collagenase (MMP-1) or stromelysin-1 (MMP-3) readily detectable in any of the crude BAL fluids tested.

3.3. *Overall type IV collagenase and elastase activities in BAL samples*

Global type IV collagenase activity in BAL fluid was tested by using radiolabelled type IV collagen as substratum. Only BAL from LPS plus PMA-treated animals fully degraded type IV collagen. To further analyse the type of proteolytic activity involved in this degradation, the assay was carried out in the presence of either an inhibitor of MMPs (batimastat at 100 nM), an inhibitor of serine proteinases (benzamidine at 1 mM), or a specific inhibitor of elastase (S 18465 at 100 nM) added in vitro after BAL fluid recovery. Only batimastat completly inhibited the ex vivo degradation of type IV collagen mediated by BAL from LPS plus PMA-treated animals (not shown).

Elastase activity in BAL fluid only increased significantly in the group of hamsters treated sequentially with LPS and PMA (Table 2). This activity was totally inhibited with the selective neutrophil elastase inhibitor, S 18465. Furthermore, no inhibition was observed with the metal chelator EDTA.

3.4. *Influence of neutrophil elastase inhibition in vivo on ex vivo gelatinase and type IV collagenolytic activities*

Hamsters receiving LPS and PMA were treated intratracheally with S 18465, concomitantly with PMA. In zymography, the 75 kDa gelatinase activity was considerably reduced in the treated group (Fig. 2). No major differences were observed for the other forms of gelatinases. In addition, when the BAL fluids from animals treated with LPS followed by PMA and S 18465 administration were assayed on the degradation of type IV collagen, a total inhibition of this degradation was observed.

Table 2
Elastase levels in BAL fluids

	ΔDO/ml/BAL	*n*
Control	0.0014 ± 0.0002	5
Control PMA	0.0009 ± 0.0003	5
LPS	0.0004 ± 0.0003	5
LPS PMA	0.0227 ± 0.0069	10

3.5. *Elastase can activate 92 kDa pro-MMP-9 into a 75 kDa species*

When recombinant pro-MMP-9 is incubated with purified elastase, a major gelatinolytic activity at 75 kDa is then detected by zymography (Fig. 3A). On the other hand, treatment of pro-MMP-9 with APMA gave two intermediate species at 78 and 71 kDa and a 65 kDa entity. Elastase alone

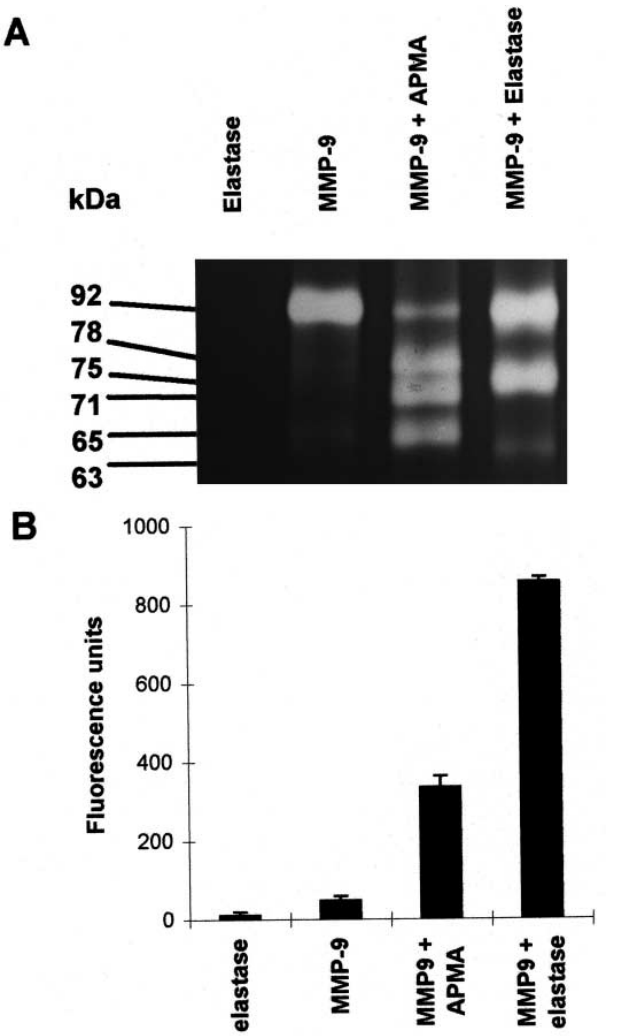

Fig. 3. A: Activation of human recombinant pro-MMP-9 by APMA or elastase as studied by zymography. MMP-9 was incubated with either APMA for 1 h or human elastase for 24 h. B: Peptidomimetic substrate degradation by human pro-MMP-9 activated by APMA or elastase. The activity is expressed as fluorescence units of the cleaved substrate after 10 min incubation.

exhibited no activity. To assay the functional status of elastase-activated pro-MMP-9, a synthetic substrate was used. Incubation with elastase was found to be more potent than APMA in activating the otherwise unreactive pro-MMP-9 (Fig. 3B). Again elastase alone was inactive in this assay.

4. Discussion

The main enzymes involved in the proteolysis of the ECM in acute lung injury are serine proteases, namely leucocyte elastase, and matrix metalloproteinases [2]. In a tentative representative model of acute lung injury we have attempted to delineate the inter-relationship between two proteolytic enzymes produced by the neutrophils, i.e., neutrophil elastase and the 92 kDa gelatinase B or MMP-9. The in vitro and in vivo experiments carried out in this study suggest that neutrophil elastase is a physiological activator of pro-MMP-9 converting it into a 75 kDa species associated with collagenolytic potential.

Acute lung injury in hamster was induced by intratracheal administration of LPS causing a massive PMN recruitment in the lung, followed by an exogenous cell activation with PMA. During the first phase after instillation of LPS, corresponding to the PMN influx, only small amounts of 92, 65 and 63 kDa gelatinases are recovered in BAL fluid. In addition, based on the level of haemoglobin content, this procedure was unable to induce acute lung injury in hamsters. Previous results in a guinea-pig model have demonstrated that the BAL fluid of LPS-treated animals exhibits an increase in MMP-9 mostly in its inactive 92 kDa proform [7]. However, in this model, a global gelatinolytic activity in the BAL fluid was detected 24 h after LPS instillation. In the hamster model, despite massive PMN influx to the lung, we were unable to detect an increase in gelatinolytic activity in the BAL fluid suggesting that PMNs were not activated during recruitment in this species and under our experimental conditions.

Hamster treatment with PMA which can mobilise the tertiary granules of PMNs containing the 92 kDa MMP-9 gelatinase in vitro (data not shown) ensured PMN activation and correlated with a marked overexpression of an elastolytic activity with properties similar to neutrophil elastase, and of a 92 kDa gelatinase with properties similar to pro-MMP-9 along with 65 and 63 kDa gelatinases and the appearance of a 75 kDa gelatinase. Both MMP-9 and elastase are produced by PMNs, and these cells were recruited and activated in our model. Thus the two proteases detected in BAL fluids are likely to be neutrophil-derived.

It is not clear whether the 65 and 63 kDa gelatinolytic activities are related to MMP-2 or not. However, it is clear that the levels expressed did not correlate with an overall collagenolytic activity in BAL fluids.

In contrast, the emergence of a 75 kDa activity — and its level of expression — found in the gelatinolytic profiles after LPS and PMA activation correlated with the ability of the corresponding BAL to degrade type IV collagen ex vivo. Both activities — global or separated on a zymogram — were inhibited by MMP inhibitors.

At this point, at least two hypotheses could be put forward in an attempt to explain the presence of this 75 kDa activity: (i) based on its molecular weight, it may be a gelatinase similar to MMP-2 or MMP-8 although the latter is at least 10 times less potent than MMP-2 and MMP-9 in the degradation of gelatin [16,17] and MMP-2 is not produced by PMNS, or (ii) the 75 kDa species might be a degradation or activation product of the 92 kDa gelatinase. Additionally, assuming that this 75 kDa gelatinase is responsible for ECM degradation, it must be an active form.

Our results and previously published studies suggest an affiliation between the 75 kDa activity and the 92 kDa pro-MMP-9. Two physiological protease activators of MMPs are present in BAL fluids from ARDS patients: tissue kallikrein, a very efficient activator of MMP-9 [18], and elastase [19]. We did not titrate kallilrein levels in our model but elastase is present at high levels in BAL concomitantly with the 75 kDa gelatinase. Secondly, we verified that under our experimental conditions, purified recombinant pro-MMP-9 incubated in vitro with purified leukocyte elastase produces a 75 kDa gelatinase species, and this transformation correlates with a more active and distinct species than after activation by APMA. Finally, in vivo, a potent and specific leucocyte elastase inhibitor, S 18465 [11], prevents the appearance of this 75 kDa gelatinase concomitantly with a loss of ex vivo type IV collagen degradation.

In conclusion, it is suggested that neutrophil-derived elastase induces activation of MMP-9 in this animal model of acute lung injury. In addition to previous in vitro work showing that neutrophil elastase converts stromelysin-1 (MMP-3) [17] and metalloelastase (MMP-12) [19] into active enzymes and allows activation of MMP-9 by selective destruction of its specific endogenous inhibitor TIMP-1 [20], the present study emphasises, using an in vivo model, the interrelationship between neutrophil elastase and the MMP family. Also it suggests that neutrophil elastase may play an important role in in vivo ECM destruction not only by its direct action on lung connective tissue components like elastin, but also by converting pro-MMP-9 into an active form exhibiting type IV collagenase activity. The ability of neutrophils to activate gelatinase by either a reactive oxygen-dependent or independent pathway has already been shown in vitro [9]. We further demonstrate that in vivo, neutrophil elastase might be the reactive oxygen species-independent pathway leading to gelatinase B activation.

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