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Potentiative effects of neutral proteinases in an inflamed lung: relationship of neutrophil procollagenase (proMMP-8) to plasmin, cathepsin G and tryptase in bronchiectasis *in vivo*

R. Sepper*++, Y.T. Konttinen*+, L. Buø#, K.K. Eklund**+, A. Lauhio+, T. Sorsa*, H. Tschesche‡, A.O. Aasen#, H. Sillastu++

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ABSTRACT: We attempted to study the possible relationships between neutrophil-type procollagenase/pro-matrix metalloproteinase (MMP-8) and the serine proteinases plasmin, cathepsin G and tryptase in bronchiectasis.

The presence of the plasmin/plasminogen system and plasmin-, cathepsin G- and tryptase-like activities were compared to the activity of endogenously activated MMP-8 in bronchoalveolar lavage (BAL) fluid in 38 bronchiectasis patients and in 14 healthy controls by means of immunohistochemistry, Western-blot and substrate-based functional assays.

In contrast to cathepsin G- and tryptase-like activities, the plasmin/plasminogen activator system in BAL fluid was observed to have a relatively weak activation stage and no correlation with disease severity. Neither plasmin-like activities nor concentrations of plasminogen activators from the bronchiectatic patients differed significantly from the values of healthy controls. Immunolocation of plasminogen activator inhibitor-1 showed a marked, but not significant, increase in bronchiectatic lung as compared to controls. In contrast to cathepsin G- and tryptase-like activities, with their strong and significant correlation with endogenously activated collagenase (r=0.9; p=0.0001; and r=0.6; p=0.03, respectively), no correlations were observed between plasmin-like and endogenously activated collagenase (r=0.3; p=0.2) in bronchiectasis.

These findings suggest that cathepsin G- and tryptase-like activities may act as potent pro-matrix metalloproteinase-8 activators in patients with bronchiectasis, whereas the plasminogen activator/plasmin cascade was shown to be down-regulated. *Eur Respir J 1997; 10: 2788–2793.*

Depts of *Anatomy and **Medical Chemistry, Institute of Biomedicine, and +Dept of Medicine, University of Helsinki, Helsinki, Finland. ++Dept of Pulmonology, University of Tartu, Estonia. #Institute for Surgical Research, the National Hospital, Oslo, Norway. ‡Dept of Biochemistry, University of Bielefeld, Germany.

Correspondence: R. Sepper
Institute of Biomedicine
Dept of Anatomy
P.O. Box 9
FIN-00014
University of Helsinki
Finland

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Bronchiectasis (BE) is a chronic lung disease characterized by major destruction of airways and lung tissue extracellular matrix (ECM). Active matrix metalloproteinases (MMPs) at the inflammation site are the most powerful degradative agents towards almost all ECM components. Because they are synthesized and released as latent proenzymes, for catalytic action they must be activated extracellularly (for review, see [1]). Activation of latent proMMPs has been shown to occur after cleavage of a disulphide bond between an active site Zn^{2+} and ^{73}Cys-residue in the latent proenzyme molecule [2]. Apart from the direct effect of reactive oxygen metabolites [3], this process can also be mediated by other proteolytic enzymes able to cleave the so-called activation peptide from the N-terminus of the latent proMMP molecule [4, 5]. Activation of interstitial collagenases, neutrophil-type/MMP-8 and fibroblast-type/MMP-1, has been shown to occur *in vitro* by serine proteinases sharing tryptic activities; examples are trypsin [4, 5], cathepsin G, [5], and tryptase [6]. However, the role of tryptic enzymes in activation of proMMPs *in vivo is* not yet clear.

Plasmin has differentiated ability to activate proMMPs; it directly activates the proMMP-1 [7], whereas, as demonstrated by Knäuper *et al*. [8], the indirect activation of the proMMP-8 can occur first after activation of MMP-3/prostromelysin *in vitro*. Plasmin is an active form of cell-bound plasminogen formed after the proteolytic cleavage by plasminogen activators (urokinase- and tissue-type). Both activator enzymes belong to the serine proteinase family, but are encoded by two distinct genes [9]. Tissue-type plasminogen activator (t-PA) is synthesized mainly by endothelial cells, whilst urokinase-type plasminogen activator (u-PA) is a product of epithelial cells and monocyte/macrophages [9]. The t-PA has a major role in thrombolysis, whereas u-PA is associated with cell migration and tissue injury in various pathological conditions, such as inflammation, tumour growth and metastasis [9–11]. Plasmin has multifunctional proteolytic effects toward the ECM components, laminin, fibronectin and various proteoglycans [12, 13]. Catalytic activities of PA-system enzymes are inhibited mainly by specific serpins, the plasminogen-activator inhibitors (PAIs) [14].

Studies of bronchiectasis-like inflammation have demonstrated highly active serine proteinases [15, 16] and specific collagenase of MMP-8 type [17] in bronchoalveolar lavage (BAL) fluid. This study analyses the possible co-operation between two enzyme systems in this chronic destructive inflammation, with particular reference to the eventual role of serine proteinases in the activation of the procollagenase *in vivo*.

Materials and methods

Study groups

The clinical diagnosis of BE patients was based on a prolonged history of intermittent sputum production, persistence of crackles on auscultation, and cystic changes on computed tomographic (CT) scans and/or bronchograms. Patients with cystic fibrosis were excluded.

Based on their clinical characteristics and radiological findings, 38 BE patients were divided into three groups with respect to disease activity: 1) patients with severe BE (n=12) were characterized by frequent pneumonia and bronchitis, complaints of mucopurulent and purulent sputum production, and bilateral, multiple segmental cystic or varicosal changes of the bronchi, with fibrosis of surrounding lung tissue detected by bronchograms and CT scans; 2) patients with moderate BE (n=14) showed mucopurulent sputum with clinically manifest inflammation approximately 2–3 times a year, and by more cylinder-like and cyst-like unilateral deformations of the bronchi; and 3) patients with mild BE (n=12) had mild cylinder-like bronchial changes and infrequent complaints of sputum production. The healthy volunteer group consisted of 14 healthy students, all nonsmokers, with normal results in routine blood analyses and normal baseline spirometric values (table 1).

Tissue specimens were obtained from nine of the 38 patients (five males and four females, mean age 19 yrs, range 13–31 yrs), who had a localized, unilateral lesion of the lung and underwent pulmonary lobectomy or bilobectomy due to BE. Lung tissue specimens from three patients (all males, mean age 39 yrs, range 17–51 yrs) who had been operated on for nonrelated reasons (one patient for inherited pulmonary anomaly, two for benign lung tumour) were included as controls.

The research protocol was approved by the Ethics Committee of the University of Tartu, Estonia. Informed consent was obtained from all patients and volunteers before their enrolment in the study.

Bronchoalveolar lavage

BAL of the healthy volunteer controls was performed in the middle lobe. BAL of the BE patients was performed during a clinically quiescent period in segments where bronchiectatic lesions were located; in cases of widespread bronchiectases, lavages were performed either in the middle or lingular lobe. Five 20 mL aliquots of 0.9% NaCl (+37°C) were instilled into the segmental bronchus. The solution with washed-out material was immediately sucked back after each aliquot, the first aliquot being excluded. The mean±SD volume of liquid recovered by BAL was 62±9 mL. BAL fluid was centrifuged for 20 min at 500×g; the supernatant was divided into 500 mL aliquots and stored at -70°C.

Immunohistochemical staining

Each specimen of lung tissue was individually embedded in ornithine carbamyl transferase (OCT) medium and snap-frozen in liquid nitrogen. Samples were stored at -70°C before being cut into 6 mm thick sections on a cryostat. Slides were fixed in acetone for 10 min at +4°C, air-dried for 2 h and stained by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method, as described by CORDELL *et al*. [18] and modified by BUØ *et al*. [19], by use of monoclonal antihuman urokinase plasminogen activator (25 μg·mL^{-1}, u-PA; Monozyme, Virum, Denmark), antihuman tissue plasminogen activator (25 μg·mL^{-1}, t-PA; Biopool, Sweden), and antihuman plasminogen activator inhibitor (50 μg·mL^{-1}, PAI-1 and PAI-2; Biopool, Sweden) antibodies. For negative controls, a monoclonal antibody layer was omitted, with a nonimmune serum substituted.

Quantitation of immunohistochemical stainings was performed by calculation of the positively stained area as nm^2 per mm^2 area of the tissue, with correction for background by use of a low-light charge-screen-coupled closed circuit display (CCD) camera (Panasonic WV-CD130L; Matsushita Electric Industrial Co., Osaka, Japan) mounted on a Leitz Diaplan (Wetzlar, Germany) lens system and linked to semiautomatic Kontron image analysis (VIDAS 2.1 with colour option; Kontron Bildanalyse GmbH, Munich, Germany) and processing (VIDEOPLAN 2.2; Kontron Elektronik GmbH Munich, Germany) systems. Background particles as nonobjectives were excluded by a pointing device linked to a computer.

Enzyme-immunoassay for measurement of concentration of u-PA and t-PA in BE patients and healthy controls

Enzyme immunoassays (EIAs) were used for measurements of u-PA and t-PA (ng·mL^{-1}) by the TintElize™ system from Biopool, Umeå, Sweden.

Table 1. – Patient characteristics

Group	Subjects n	Sex M/F	Age yrs#	Daily sputum volume mL	Relapses n·yr^{-1}	Operated n	Smokers n
Healthy controls	14	8/6	23 (21–24)	-	-	-	0
Mild bronchiectasis	12	7/5	24 (19–30)	0–50	0–1	7	0
Moderate bronchiectasis	14	6/8	28 (16–44)	50–150	2–3	No	0
Severe bronchiectasis	12	8/4	22 (6–42)	100–250	3–6	2	1

#: mean, and range in parenthesis. M: male; F: female.

Functional assays for serine proteinase activities

Plasmin-, cathepsin G- and tryptase-like activities ($U \cdot L^{-1}$, where one unit expresses cleavage of one nanomole of substrate per second) were measured by spectrophotometry, by use of synthetic peptide substrates HD-Val-Leu-Lys p-nitroanilide (S-2251; Chromogenix AB, Mölndal, Sweden) [20], N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SAAPFNA) [21] and N-Benzoyl-DL-Arginine p-nitroanilide (BAPNA) [22] (both from Sigma Chemical Co., St Louis, MO, USA), respectively.

Measurements of collagenase activity and concentration

Total human leucocyte collagenase/MMP-8 (as latent as active) and total human fibroblast collagenase/MMP-1 (as latent as active) concentrations in BAL fluid were measured by enzyme-linked immunosorbent assay (ELISA), by use of specific antibodies and commercially available kits according to manufacturers' recommendations (Amersham International plc, Little Chalfont, UK). Endogenously-activated collagenase was measured with a native soluble 1.5 µM type I collagen as the substrate. Results were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by laser densitometric scanning, as described in detail elsewhere [17].

Western-blot analysis

Total protein in BAL fluid was measured as described by BRADFORD [23], and all BAL fluid samples for immunochemical analysis contained approximately 10 µg protein. After SDS-PAGE of BAL fluid samples, the gels were blotted onto nitrocellulose membrane (BIO-RAD Lab., Richmond, CA, USA) as described by TOWBIN *et al.* [24]. Blotted membranes were incubated with mouse monoclonal antihuman u-PA (5 $\mu g \cdot mL^{-1}$, Monozyme, Virum, Denmark) and antihuman PAI-1 (5 $\mu g \cdot mL^{-1}$, Biopool, Sweden) antibodies. Sites of peroxidase binding were revealed by the avidin-biotin-enzyme complex (ABC) method, as described by HSU *et al.* [25]. For negative controls, the primary antibody layers were substituted with incubation of membranes by the nonimmune serum.

Data analysis

Kruskal-Wallis one-way analysis of variance (ANOVA) was applied to analyse the data of the groups studied. When appropriate, the Mann-Whitney U-test, with correction for multiple comparisons, was used to compare the difference between the BE patient group and controls. The Spearman rank correlation and linear correlation tests served for correlation analysis. The BMDP package was used (BMDP Statistical Software Inc., Los Angeles, CA, USA). A p-value less than 0.05 was considered statistically significant. Data are expressed as mean±SEM.

Results

The plasmin/plasminogen system in BAL fluid and lung tissue

Immunolocalization of u-PA, t-PA, PAI-1, PAI-2 in BE and control lung tissue. The t-PA, u-PA and PAI-2 immunoreactivities were negligible and appeared to be below the level of quantitation both in control and BE lung tissue (data not shown). In contrast, strong immunoreactivity of the PAI-1 was found intracellularly in the perivascular space and in the mononuclear cells in tissue interstitium in the controls as well as in BE lung tissue. The regions of deformed bronchioli and alveoli exhibited positive immunoreactivity toward PAI-1 (fig. 1). After quantitation of immunoreactive PAI-1 from the BE and control tissue sections, no statistically significant differences appeared between these groups (35,708±1,873 and 16,461±347 $nm^2 \cdot mm^{-2}$, respectively; p=NS).

Activity of plasmin and concentrations of u-PA and t-PA in BAL fluid. Plasmin-like activity ($U \cdot L^{-1}$) was elevated, in severe and moderate cases of BE compared to healthy controls, but this was not statistically significant (table 2). The same tendency was seen in the antigen concentrations of t-PA and u-PA: 1.0±0.4 and 1.1±0.6 $ng \cdot mL^{-1}$ in severe BE; 0.6±0.1 and 0.5±0.1 $ng \cdot mL^{-1}$ in moderate BE; and 0.7±0.4 and 0.2±0.1 $ng \cdot mL^{-1}$ in mild BE; and in the healthy control group 0.3±0.2 and 0.2±0.1 $ng \cdot mL^{-1}$. When molar amounts of plasmin (1 µg of plasmin being equivalent to 0.2 nkat) [26] were compared to molar amounts of u-PA and t-PA (molarity=protein concentration/molecular mass) by a linear regression analysis, the correlation between u-PA and plasmin in BAL fluid (r=0.4; p=0.05) was slightly higher than that between t-PA and plasmin (r=0.3; p=0.09).

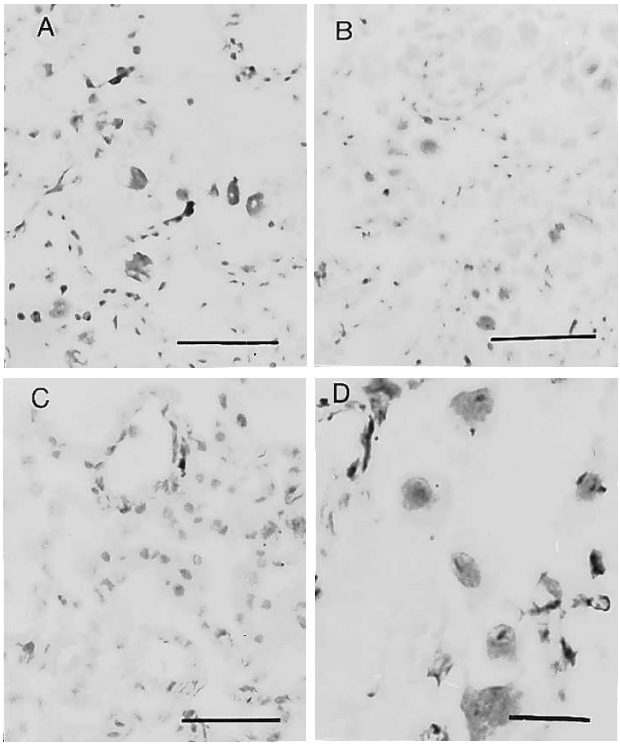

Fig. 1. – Immunohistochemical staining of lung from bronchiectatic and healthy control subjects. Sections (6 mm thickness) were stained with antihuman plasminogen activator inhibitor-1 (PAI-1) antibody by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method. PAI-1 staining is localized in mononuclear cells in: A) control; B) bronchiectatic lung. C) The section adjacent to A was stained with normal serum as a specificity control. D) This section shows positively immunostained mononuclear cells in bronchiectatic lung tissue. Internal scale bars for A–C = 50 µm. Internal scale bar for D = 25 µm.

Table 2. – Summary of proteolytic activities of different serine proteinases in bronchoalveolar lavage fluid of bronchiectasis patients and healthy controls

Group	Plasmin		Cathepsin		Tryptase	
	U·L^{-1}	p-value*	U·L^{-1}	p-value*	U·L^{-1}	p-value*
Healthy controls	0.2±1.7		50.9±16.5		25.1±4.2	
Mild bronchiectasis	0.2±0.2	NS	57.4±20.9	NS	185.6±70.1	0.0004
Moderate bronchiectasis	0.3±2.7	NS	89.2±31.4	0.05	348.1±134.2	0.0002
Severe bronchiectasis	1.4±0.9	NS	139.2±58.0	0.03	961.1±240.1	0.0001

Values are mean±SEM. *: Mann-Whitney U-test, for comparison with controls. NS: nonsignificant.

Characterization of molecular forms of PAI-1 and u-PA in BAL fluid. Western-blot analysis revealed multiple molecular forms of PAI-1 in different BAL fluids. The complexed forms of PAI-1 and its endogenous target enzymes, most probably with u-PA and t-PA with an apparent MW of 65–75 kDa [26], were detected in BAL fluid samples from moderately and mildly diseased patients and healthy controls, but not in BAL fluid from severely ill patients. Moreover, the native/uncomplexed form of inhibitor-protein of 55 kDa [27] was evident only in mild and moderate cases of BE and healthy controls. BAL fluid samples from severely diseased BE patients were found to contain only fragmented parts of the PAI-1 molecule, with apparent MWs of 45 kDa, and of 32–25 kDa, evidently representing proteolytically and/or oxidatively cleaved native PAI-1.

Immunoreactivity to u-PA in BAL fluid was observed only in cases of severe BE, and it was revealed as a single molecular form of 33 kDa, a proteolytic product of the high MW form of u-PA [26, 27]. Immunoreactivity to u-PA was not observed either in BAL fluids from mild and moderate cases of BE or in healthy controls (data not shown).

Other serine proteinase activities in BAL fluid

Catalytic activities (U·L^{-1}) of plasmin-, cathepsin G-, and tryptase-like enzymes are summarized in table 2. Statistically significant differences in enzymatic activities between the healthy control and BE groups were detected for cathepsin G- and tryptase-like activities; plasmin-like activities did not differ significantly between these groups (table 2).

Total and endogenously activated collagenases (MMP-1 and MMP-8) in BAL fluid

To address the cellular origins of interstitial collagenases (MMP-1 and MMP-8) in BAL fluid of BE patients and healthy controls in more detail, total (containing both latent and active enzyme-protein) leucocyte-type/MMP-8 and fibroblast-type collagenase/MMP-1 concentrations were measured. Concentrations of total MMP-8 were: 66.8±10.1 ng·mL^{-1} in mild BE; 126.6±47.0 ng·mL^{-1} in moderate BE; and 450.3±296.1 ng·mL^{-1} in severe BE, and all these values differed significantly from those measured in BAL fluid of healthy controls (27.1±1.2; $p<0.05$). Total concentrations of MMP-1 in BAL fluid of healthy controls (4.6±0.8 ng·mL^{-1}) did not differ significantly as compared with the concentrations measured in BAL fluid in severe (4.7±0.7 ng·mL^{-1}), moderate (4.6±0.4 ng·mL^{-1}), or mild BE (4.6±0.2 ng·mL^{-1}). The amount of endogenously-activated collagenase in BAL fluid, expressed as percentage of cleaved native type I collagen substrate, has been shown to be 8±0.1, 42±0.4, 92±0.2 and 3±0.8% in mild, moderate and severe BE, and in healthy controls, respectively, with significant differences between the healthy and BE groups ($p<0.05$, $p<0.05$ and $p<0.01$, respectively) [17].

Relationships between endogenously-activated collagenase and serine proteinases in BALF

Analysis of the relationships between *in vivo* activated collagenase and serine proteinase activities in BAL fluid showed significant correlations between the amounts of endogenously-activated collagenase and tryptase- ($r=0.9$; $p=0.0001$), and cathepsin G-like ($r=0.6$; $p=0.03$) activities, but not with plasmin-like activities ($r=0.3$; $p=0.2$).

Discussion

This study demonstrated relationships between cathepsin G and tryptase and endogenously-activated collagenase/MMP-8. This indicates potentiating effects of two enzyme families on tissue destructive events in bronchiectatic lung. Tissue injury is a central process in the pathogenesis of BE [15]. Distinct proteinases from the serine- and MMP-families can directly cleave components of lung ECM [1, 28]. In addition, some serine proteinases share an ability to significantly enhance the injury potential in the lung *via* direct or indirect activation of latent proMMPs (for review, see [1]). Recent *in vitro* studies have demonstrated that latent proMMPs can be activated by different serine proteinases [2–8], which suggest that co-operative and potentiative effects could also occur between the MMP- and serine enzymes *in vivo*.

In chronic lung inflammation, a cascade of various highly active serine proteinases, which could be involved in the injury of lung ECM in BE, has been demonstrated. The presence of active elastase and cathepsin G has been well characterized [15, 16]. Recently, we have demonstrated an involvement of mast cell (MC) tryptase [29] and eosinophil cationic protein (ECP) (Sepper *et al*., manuscripts submitted for publication) in the pathogenesis of BE. Because various native human proteins have been described as potential substrates for plasmin, components of the plasmin system were also analysed in BE. However, the PA/plasmin cascade showed relatively low activity in BAL fluid of these patients, with no significant differ-

ences between the data for BE patients and controls. Antigen concentration of the u-PA in BAL fluid, however, showed a weak correlation with BAL fluid plasmin (r=0.4; p= 0.05). This suggests that the u-PA rather than t-PA could be a potential activator of plasminogen in the transformation from a zymogen- into catalytically active plasmin-form in the BE lung. Previous work has also emphasized the participation of u-PA in tissue-destructive processes, whereas t-PA can be synthesized mainly by vascular en-dothelial cells, and probably acts in the intravascular pool [9, 26, 30].

Strong immunoreactivity of PAI-1 both in BE and control tissue, stresses the role of PAI-1 as the predominant local serpin as the counterbalance to PA activity in the inflamed lung. In fact, the Western-blot analysis disclosed the immunoreactive PAI-1, native and complexed with target proteinases, in the compensated, that is the mild and moderate cases of BE, whereas in contrast, the PAI-1 in BAL fluid from patients with severe BE revealed only fragmented particles of the protein molecule. Evidently, as is the case regarding other serpins in the inflamed lung [16, 31], PAI-1 can also be a target of the oxidative and/or proteolytic effects leading to the structural and functional integrity of the serpin molecule. Results of Western-blot analysis for the u-PA confirm the presence of immunoreactive u-PA (but not t-PA) in BAL fluid from the seriously inflamed BE lung alone. Evidently, an excess of PAI-1 in the lung tissue can prevent the action of PAs on plasminogen only in the compensated, not in the severe cases of BE. This evidence is also in accordance with that recently published by PARTON *et al*. [26] and GROSS *et al*. [30], indicating that u-PA and PAI-1 are locally synthesized and released by cells lining the airways and alveoli, but not in the vascular compartment.

From the MMP family, almost all proteinases are known to be able to cleave a wide variety of ECM components in the lung also [1, 17, 28]. In the inflamed BE lung, the predominant collagenase was shown to be of MMP-8 type [17]. Notably, certain cytokine-induced non-polymorphonuclear (PMN)-lineage cells (osteoarthritic joint chondrocytes, rheumatoid synovial fibroblasts, as well as inflamed gingival and periodontal ligament fibroblasts) also produce neutrophil-type collagenase MMP-8, collagenase-2) [32]. Thus, BAL fluid MMP-8, in addition to triggered degranulating PMNs, may also be derived from cytokine-induced mesenchymal cells. Latent proMMP-8 can be activated *in vitro* directly by reactive oxygen metabolites, especially chlorinated oxidants [3], and by serine proteinases, *e.g*. cathepsin G [6], trypsin [4, 5] and MC tryptase [6], and by MMP-3/stromelysin [8]. *In vivo*, the relative efficacy of the potential activating enzyme species in a competitive situation, such as prevails in biological inflammatory fluids, is unknown. Using data showing activities of the endogenously activated collagenase and of potential serine proteinases in BAL fluid, we found the most significant correlation to be between tryptase-like and collagenase activities (r=0.9; p=0.0001). This evidence is in accordance with *in vitro* data by GRUBER *et al*. [6], demonstrating the ability of MC tryptase to activate procollagenase *in vitro*. Such co-dependence may, however, imply *in vivo* an underlying and shared regulatory influence instead of a causal relationship. High activity of cathepsin G in biological fluid can reflect activation/degranulation of PMNs. Furthermore, cathepsin G has been described as a powerful activator of procollagenase/pro-MMP-8 in the oxidizing environment generated by PMNs [3, 5]. The correlation between cathepsin G and endogenously active collagenase (r=0.6; p=0.03) in BAL fluid of BE supports suggestions derived from *in vitro* work. Direct activation of procollagenase by the PA/plasmin system is collagenase-species-specific, being operative on the MMP-1 but not on the MMP-8-type enzyme *in vitro* [5]. Analysis of the relationship between plasmin-like and endogenously active collagenase activities, indicates that plasmin is not directly involved as an activator of latent proMMP-8 in BE *in vivo*. Among the MMP family members, the MMP-8 either from PMNs [3–5, 17] and/or non-PMN-cellular sources [32] in catalytically active form is most efficient in degradation of collagens.

Our findings suggest that cathepsin G- and tryptase-like activities may act as potent pro-matrix metalloproteinase-8 activators in patients with bronchiectasis, whereas the plasminogen-activator/plasmin cascade was shown to be down-regulated.

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