RHEUMATOLOGY

Concise report

Relative α_1-anti-trypsin deficiency in systemic sclerosis

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Abstract

Objective. Neutrophil elastase is secreted by neutrophils during activation and circulates in the plasma where it can play a role in inflammation and fibrosis. This study examines the role of neutrophil elastase in SSc, a systemic CTD that is typified by vascular dysfunction, tissue fibrosis and inflammation.

Methods. Serum neutrophil elastase and α_1-anti-trypsin concentrations were assessed in SSc patients and healthy controls by ELISA. Serum neutrophil elastase activity was assessed by the elastase-dependent conversion of methoxy-succinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide to p-nitroanilide using a colourimetric assay. Elastase concentration and activity were correlated with clinical disease features.

Results. Serum neutrophil elastase concentration and activity were equivalent in patients and controls; however, in SSc serum, there was an increase in elastase activity for each unit of elastase concentration ($P = 0.03$). This was due to a decrease in serum α_1-anti-trypsin concentrations ($P = 0.04$). Serum elastase concentration ($P = 0.03$) and activity ($P = 0.02$) were significantly lower in RNP-positive patients and serum elastase concentrations were lower in ANA-positive patients ($P = 0.003$).

Conclusions. Relative deficiency in serum α_1-anti-trypsin concentrations in SSc could have important and pathogenically relevant effects since elastase has pro-inflammatory and pro-fibrotic roles. Elastase inhibitors are available in clinical practice and could represent potential therapeutic options in SSc.

Key words: Neutrophil elastase, α_1-Anti-trypsin, Systemic sclerosis, Serine protease, Innate immunity, Neutrophil.

BASIC SCIENCE

Introduction

Neutrophil elastase is a serine protease that is stored in the azurophilic granules of neutrophils. It predominantly functions as an intracellular anti-microbial protein and is released into the phagolysosome following phagocytosis to mediate bacterial digestion. However, in addition, it is secreted by the cell. In the extracellular space, neutrophil elastase has additional functions in the regulation of inflammation and it is implicated in inflammatory and fibrotic conditions, including fibrotic lung disease [1, 2]. Elevations in serum elastase have previously been reported in patients with SSc where it was associated with lung disease [3].

Serum elastase activity is regulated by serine protease inhibitors. The main intracellular inhibitor of elastase is serpin peptidase inhibitor clade B member 1 (SERPIN B1), whereas the main extracellular inhibitor of neutrophil elastase is α_1-anti-trypsin and additional inhibition is mediated by α_2-macroglobulin, elafin and secreted leucocyte proteinase inhibitor (SLPI) [4]. Neutrophil elastase can also be expressed on the neutrophil membrane, where it occupies low- and high-affinity binding sites. Membrane expression is increased by neutrophil activation with cytokines such as TNF-α and IL-8 [5]. The increase in membrane expression is more significant than the amount secreted extracellularly during typical activation. Plasma membrane-bound elastase has the same catalytic functions as soluble elastase, but there is some evidence that the membrane-bound enzyme is relatively resistant to α_1-anti-trypsin [6, 7].

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Neutrophils were shown by Hussein *et al*. to be increased in lesional biopsies of SSc patients compared with controls [8]. Others have explored neutrophil function in SSc, in particular their ability to contribute to oxidative stress by the production of reactive oxygen species. The data are contradictory and largely limited by old-fashioned neutrophil isolation procedures that can lead to neutrophil activation [9, 10]. A recent study has, however, shown that neutrophils produce less reactive oxygen species *in vitro* compared with control neutrophils when unstimulated [11]. In agreement with this we have found that neutrophils from patients with SSc are hypofunctional in tests of reactive oxygen species generation and chemotaxis (unpublished data). This may reflect *in vivo* stimulation, and hence *in vitro* exhaustion. Proteomic studies show that SSc neutrophils have increased expression of proteins that are also increased on stimulation with lipopolysaccharide or TNF, again indicative of neutrophil activation *in vivo* (unpublished data).

Since neutrophil elastase has established roles in other inflammatory and fibrotic disorders, we hypothesized that neutrophil elastase could be an important mediator in the pathogenesis of SSc. To explore this hypothesis, the concentration and catalytic activity of neutrophil elastase in SSc serum was compared with controls. In addition, the membrane expression of elastase was measured in SSc neutrophils compared with controls, and clinical correlates were studied.

Methods

The study was approved by the Sefton Local Research Ethics Committee, in accordance with the Declaration of Helsinki. Informed written consent was taken from patients with SSc [12] and from healthy volunteers. Thirty millilitres of heparinized venous blood were taken from the subjects. Peripheral blood was separated into neutrophil and mononuclear cell fractions using Polymorphprep (as described in the manufacturer's instructions). Contaminating erythrocytes were removed using ammonium chloride lysis buffer ($KHCO_3$ 3.4 mM, NH_4Cl 155 mM and EDTA 96.7 μM). Neutrophils were routinely examined for purity using morphological analysis of cytospins after staining with Rapid Romanowsky: purity was >95% immediately after isolation. Neutrophils were resuspended in Roswell Park memorial institute (RPMI) 1640 + 25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) + 2 mM glutamine at a concentration of 5×10^6 cells/ml.

Serum elastase concentrations

Neutrophil elastase and α_1-anti-trypsin levels were measured in SSc patient and control serum using a PMN elastase ELISA (Bender, Vienna) and an α_1-anti-trypsin ELISA (Abnova, Taipei), respectively, according to the manufacturer's instructions. The intra- and interassay coefficients of variation for the PMN elastase ELISA were 4.8 and 5.6%, respectively, with standard range 0.16–10 ng/ml and sensitivity 1.98 ng/ml. The epitope for the PMN elastase ELISA is unknown and it is unknown whether both

active and inactive enzymes are detected using this assay. The intra- and interassay coefficients of variation for the α_1-anti-trypsin ELISA were 4.2 and 7.1%, respectively, with standard range 0.039–40 μg/ml and sensitivity 20 ng/ml.

Neutrophil elastase concentrations in neutrophil culture supernatants

Neutrophils were isolated from SSc and healthy control blood, and were cultured for 6 h with gentle agitation at 37°C at a density of 10^7 cells/ml in RPMI 1640 + 25 mM HEPES + 2 mM glutamine. Cells were precipitated by centrifugation (1000 *g* for 5 min) and the neutrophil elastase concentration in the supernatants was measured by ELISA (Bender, Vienna).

Serum neutrophil elastase enzymatic activity

Following experiments to optimize substrate concentration and length of incubation, serum elastase activity was measured using a colourimetric assay. In 96-well clear plastic plates, 150 μl of elastase buffer (0.1 M HEPES, 0.5 M NaCl, pH 7.5) was added to 50-μl serum samples from patients with SSc and healthy controls. The substrate (15 mM methoxy-succinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide) was added to a final concentration of 750 μM. The reaction was incubated in the dark at 37°C for 6 h. The elastase-dependent conversion of colourless methoxy-succinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide to yellow *p*-nitroanilide was measured as a change of absorbance at 405 nm on a plate reader. Standards of known concentration were used to generate a calibration curve (data not shown). The intra- and interassay co-efficients of variation were 1.2 and 6.8%, respectively.

Clinical data

Clinical data were collected on all patients as a part of routine clinical care and included Rodnan skin score, neutrophil count, autoantibody profile, medications, major organ involvement and disease duration (defined as time since onset of first non-Raynaud's symptom). Pulmonary artery hypertension was confirmed by right heart catheterization. Pulmonary fibrosis was diagnosed according by pulmonary function tests and high resolution computed tomography (HRCT), subdivisions for severity were not applied.

Neutrophil membrane elastase expression

Neutrophil membrane elastase expression was measured by flow cytometry. Neutrophils were isolated from SSc and healthy control blood as previously described. Cells were resuspended in 200 μl of phosphate buffered saline (PBS) 2% BSA, and incubated for 30 min in the dark at 4°C with 2 μl elastase monoclonal antibody raised in rabbit (Calbiochem), following experiments to find the optimal antibody dilution (data not shown). The cells were washed three times in PBS and resuspended in PBS 2% BSA and 2 μl FITC-conjugated anti-rabbit IgG secondary antibody. Cells were incubated for 30 min at 4°C in the dark. Cells were washed three times in PBS, resuspended in PBS and analysed on the flow cytometer on the FITC

channel. Mean fluorescence readings were corrected to a secondary antibody control.

Statistical methods

The data were non-normally distributed and were therefore compared using the Mann–Whitney U-test.

Results

Clinical characteristics

Table 1 shows the clinical characteristics of the SSc patients.

Serum neutrophil elastase levels were equivalent in patients with SSc compared with controls

There was no significant difference between SSc patient and control serum levels of neutrophil elastase ($P = 0.11$) (Fig. 1A).

Neutrophil elastase concentrations in control and SSc neutrophil culture supernatants are equivalent

There was no difference in the levels of neutrophil elastase in supernatants following a 6-h culture of control and SSc neutrophils in RPMI 1640 + 25 mM HEPES + 2 mM glutamine ($P = 0.53$).

Serum neutrophil elastase activity is equivalent in SSc patients and controls

The serum neutrophil elastase activity was measured in SSc patients and controls by the elastase-dependent

TABLE 1 Patient characteristics

Clinical feature	Median Interquartile range
Disease duration, months	40 (21–96)
Neutrophil count × 10^9/l	4 (2.4–4.8)
Rodnan skin score	6 (3–10)
	n/N (%)
Limited SSc	29/33 (88)
Diffuse SSc	4/33 (12)
ANA	26/31 (84)
Anti-centromere	13/31 (42)
Anti-RNP	9/31 (29)
Anti-Scl70	9/31 (29)
Lung involvement	10/33 (30)
Pulmonary artery hypertension	5/33 (15)
DMARDs	14/33 (42)
HCQ	3/33 (9)
MMF	4/33 (12)
MTX	1/33 (3)
CYC	1/33 (3)
PRED	3/33 (9)
AZA	1/33 (3)
Bosentan	1/33 (3)
Sildenafil	3/33 (9)

PRED: prednisolone.

conversion of methoxy-succinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide to *p*-nitroanilide. No difference in serum elastase activity was found between SSc patients and controls ($P = 0.91$) (Fig. 1B). However, there was a proportion of patients [6/18 (33%)] that had high serum elastase activity (>0.15 U/ml) compared with 1/9 (11%) controls. These patients had either early disease <36 months or a higher Rodnan skin score of >9. There was a significant decrease in the ratio of serum elastase concentration: activity ($P = 0.03$) (Fig. 1C). There were no distinguishing clinical features in the patients with a low serum elastase concentration:activity ratio ($<50\,000$).

Clinical correlations with serum neutrophil elastase concentration and activity

Disease subtype, disease duration, major organ involvement, neutrophil count or DMARD use did not correlate with either serum neutrophil elastase concentration or activity. Serum elastase concentration was significantly lower in RNP and ANA-positive patients compared with antibody-negative patients ($P = 0.03$ and $P = 0.003$, respectively). In addition, serum elastase activity was significantly lower in RNP antibody-positive patients ($P = 0.02$).

Membrane expression of neutrophil elastase

No difference was found in the membrane expression of neutrophil elastase between SSc and control neutrophils.

Serum α$_1$-anti-trypsin

Serum α$_1$-anti-trypsin concentrations were significantly lower in SSc patients compared with controls ($P = 0.04$, $n = 20$) (Fig. 1D).

## Discussion

In this study, we identified no difference in serum elastase concentration or catalytic activity in SSc patients compared with controls. This contradicts a previous study by Hara *et al.* [3], which observed an increase in serum elastase concentration in both limited and diffuse SSc patients. However, an examination of their data reveals that the serum elastase levels measured in this study were similar in magnitude and variation to their observations in SSc patients. In this study, however, higher serum elastase concentrations and greater variance in concentration were found in the control cohort, whereas the previous study showed consistently low levels in all controls. The previous study did not examine elastase activity.

Hara *et al.* [3] also reported that serum elastase levels were more likely to be outside the normal range in patients with joint involvement, and they observed that most patients who were ACA positive were likely to have normal levels of elastase. We did not record joint involvement as a clinical outcome in our cohort. It is interesting to note, however, that we observed lower serum elastase levels in RNP-positive patients, since these patients would be expected to have higher rates of joint involvement. Hara *et al.* [3], did not record RNP antibody status in their study.

Elastase and α_1-anti-trypsin were measured in SSc and control serum by ELISA, serum elastase activity was measured by the elastase-dependent conversion of methoxy-succinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide to *p*-nitroanilide. No difference was found in serum elastase concentration (**A**) or activity (**B**). There was a significant decrease in the ratio of elastase concentration : elastase activity in SSc serum compared with controls (**C**): this was due to a decrease in serum α_1-anti-trypsin concentration in SSc serum compared with controls (**D**).
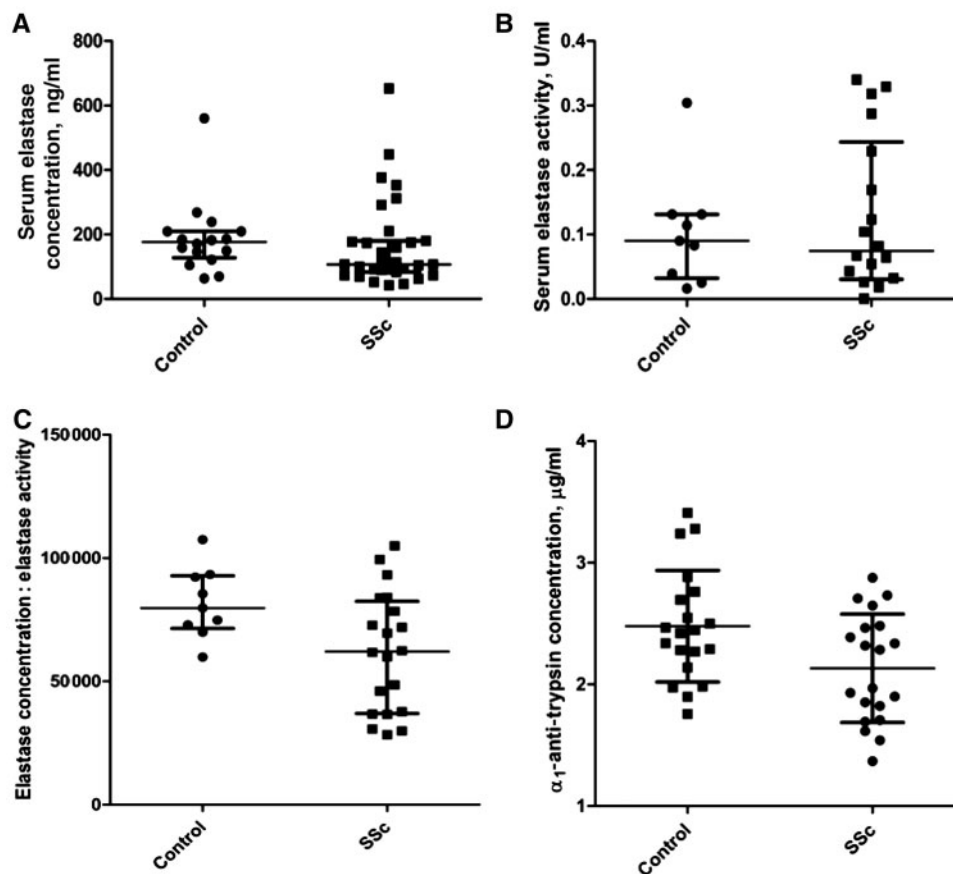
It would be interesting to correlate the data with anti-RNA polymerase III expression as this antibody can be associated with inflammatory skin disease; however, we do not routinely perform this autoantibody on our patients.

It may seem somewhat surprising that Hara *et al.* [3] described such an increase in serum elastase levels, since only 2% of serum elastase is released during neutrophil activation and raised serum levels are usually only found in situations where there is pronounced neutrophil infiltration, frustrated phagocytosis or excess neutrophil apoptosis, which overwhelms phagocytic clearance [5, 13, 14]. As none of these is implicated in SSc, we would not expect to find elevated serum levels, an expectation confirmed in our studies. Certainly, direct observations do not show significant neutrophilic infiltration and there is no evidence of excessive neutrophil apoptosis.

CRP at the time the assays were collected was normal in all but five patients. The mean CRP was <5 (where 5 is the lower limit of detection in the assay used). Of the five patients with elevated CRP, none of these had an elevated serum elastase nor were they patients with a low serum α_1-anti-trypsin. One of these patients, with a CRP of 8, did

have a low serum elastase concentration:activity ratio (<50 000); however, the others (CRP 8–19) did not.

We did not observe any increase in the membrane expression of elastase on SSc neutrophils. Secretion of azurophilic granule contents during degranulation or activation by cytokines results in the increased expression of neutrophil elastase at the membrane. Therefore, this observation argues against significant degranulation of the azurophilic granules in SSc neutrophils [5].

We did observe a decrease in the ratio of elastase concentration : activity in SSc serum. This may imply a decrease in the serum concentration or activity of neutrophil elastase inhibitors. This was confirmed by a decrease in serum α_1-anti-trypsin concentrations in SSc patients, which is the main inhibitor of elastase function in the serum. In addition, serum elastase inhibitors can be oxidized in the presence of ROS and this decreases their affinity for elastase, reducing their inhibitory capacity [15, 16]. There is evidence for increased oxidative stress in SSc [17].

The stoichiometry of the interaction between α_1-anti-trypsin is 1 : 1 and the inhibition of elastase is linear for

molar ratios of α₁-anti-trypsin:elastase of up to 2. Simultaneous measurements of α₁-anti-trypsin and elastase were not taken in this study, and therefore the molar ratios in these patients cannot be derived. However, in this disease, it is unlikely to be the serum molar ratios that are relevant rather the local tissue levels. Tissue levels of α₁-anti-trypsin are likely to be dictated by the serum concentration since it is produced by the liver and not produced distally [4]; however, the molar concentration of elastase is likely to be dictated locally where, for instance, interaction of neutrophils with activated endothelium and pro-inflammatory cytokines may lead to elastase release.

Neutrophil elastase has pleiotropic roles in the extracellular environment including profound effects on inflammation [1, 2]. Neutrophil elastase activates and degrades inflammatory cytokines, regulates neutrophil attachment, activates proteinase-activated receptor 2 (PAR-2) leading to the release of IL-8 and macrophage chemotactic factor-1, activates toll-like receptor-4 leading to the release of IL-8, cathepsin B and MMP-2. Elastase can cleave TNF p75 receptor and IL-6R from cells leading to an increased pool of soluble receptor. This in turn leads to inactivation of soluble TNF-α but increased IL-6 trans-signaling. Elastase can cleave the phosphatidylserine receptor, leading to defects in the phagocytic clearance of apoptotic cells and can enter endothelial cells, where it cleaves nuclear factor kappa B leading to endothelial cell apoptosis.

Although the role of neutrophil elastase in fibrosis is well established, the mechanism remains unclear. Certainly, elastase-deficient mice are resistant to bleomycin-induced fibrosis and treatment with elastase inhibitors also abolished fibrosis in this model [18–21]. Neutrophil elastase can cleave TGF-β-binding protein leading to release of latent TGF-β from extracellular matrix stores [22]. It has also been shown to release PDGF and VEGF from stores by a similar mechanism [23]. All of these cytokines are implicated in the pathogenesis of SSc; TGF-β and PDGF are pro-fibrotic and VEGF is pro-angiogenic. Neutrophil elastase has other roles in promoting the activity of TGF-β. Intra-tracheal instillation of elastase in mice leads to a time-dependent increase in the TGF-β content of the bronchoalveolar lavage fluid [24]. In elastase-deficient mice, the resistance to bleomycin is associated with an inability to activate TGF-β [18].

It is interesting that serum elastase concentrations and activity are lower in RNP-positive patients. This may imply that there is a different pathological process involved in RNP-positive patients and that neutrophil elastase is unlikely to be a significant mediator in these patients. In fact, serum elastase concentrations were lower in RNP-positive patients than controls ($P = 0.008$). This may represent an accelerated loss of neutrophil elastase in RNP-positive patients.

Analysis of the literature shows that neutrophil elastase could be an attractive mediator in SSc. It can promote chronic inflammation and TGF-β-dependent fibrosis, and cause endothelial cell apoptosis. Serum deficiency in elastase inhibitors could lead to a localized excess of elastase activity despite normal serum concentrations. This could have potential therapeutic implications as neutrophil elastase inhibitors already exist in clinical practice and could be used in SSc patients.

> **Rheumatology key message**
> - α₁-Anti-trypsin is decreased in serum of patients with SSc and may contribute to inflammation and fibrosis.

References

1 Pham C. Neutrophil serine proteases fine-tune the inflammatory response. Int J Biochem Cell Biol 2008;40:1317–33.

2 Chua F, Laurent G. Neutrophil elastase. Mediator of extracellular matrix destruction and accumulation. Proc Am Thorac Soc 2006;3:424–7.

3 Hara T, Ogawa F, Yanaba K *et al*. Elevated serum concentrations of polymorphonuclear neutrophilic leukocyte elastase in systemic sclerosis: association with pulmonary fibrosis. J Rheumatol 2009;36:99–105.

4 Fitch P, Roghanian A, Howie S, Sallenave J-M. Human neutrophil elastase inhibitors in innate and adaptive immunity. Biochem Soc Trans 2006;34:279–82.

5 Owen CA, Campbell MA, Boukedes SS, Campbell EJ. Cytokines regulate membrane-bound leukocyte elastase on neutrophils: a novel mechanism for effector activity. Am J Physiol 1997;272(3 Pt 1):L385–93.

6 Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. Cell surface-bound elastase and cathepsin G on human neutrophils: a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. J Cell Biol 1995;131:775–89.

7 Bangalore N, Travis J. Comparison of properties of membrane bound versus soluble forms of human leukocytic elastase and cathepsin G. Biol Chem Hoppe Seyler 1994;375:659–66.

8 Hussein MR, Hassan HI, Hofny ER *et al*. Alterations of mononuclear inflammatory cells, CD4/CD8+ T cells, interleukin 1beta, and tumour necrosis factor alpha in the bronchoalveolar lavage fluid, peripheral blood, and skin of patients with systemic sclerosis. J Clin Pathol 2005;58:178–84.

9 Maslen CL, Hall ND, Woolf AD, Maddison PJ. Enhanced oxidative metabolism of neutrophils from patients with systemic sclerosis. Br J Rheumatol 1987;26:113–7.

10 Stevens TR, Hall ND, McHugh NJ, Maddison PJ. Spontaneous neutrophil activation in patients with primary Raynaud's phenomenon and systemic sclerosis. Br J Rheumatol 1992;31:856.

11 Foerster J, Storch A, Fleischanderl S *et al*. Neutrophil respiratory burst is decreased in scleroderma and normalized by near-infrared mediated hyperthermia. Clin Exp Dermatol 2006;31:799–806.

12 LeRoy EC, Medsger TA Jr. Criteria for the classification of early systemic sclerosis. J Rheumatol 2001;28: 1573–6.

13 Owen C. Leukocyte cell surface proteinases: regulation of expression, functions and mechanisms of surface localization. Int J Biochem Cell Biol 2008;40:1246–72.

14 Liszt F, Schnittker-Schulze K, Stuhlsatz HW, Greiling H. Composition of proteoglycan fragments from hyaline cartilage produced by granulocytes in a model of frustrated phagocytosis. Eur J Clin Chem Clin Biochem 1991;29: 123–30.

15 Beatty K, Bieth J, Travis J. Kinetics of association of serine proteinases with native and oxidized alpha-1-proteinase inhibitor and alpha-1-antichymotrypsin. J Biol Chem 1980; 255:3931–4.

16 Nobar SM, Zani ML, Boudier C, Moreau T, Bieth JG. Oxidized elafin and trappin poorly inhibit the elastolytic activity of neutrophil elastase and proteinase 3. Febs J 2005;272:5883–93.

17 Sambo P, Baroni S, Luchetti M *et al*. Oxidative stress in scleroderma. Arthritis Rheum 2001;44:2653–64.

18 Chua F, Dunsmore SE, Clingen PH *et al*. Mice lacking neutrophil elastase are resistant to bleomycin-induced pulmonary fibrosis. Am J Pathol 2007; 170:65–74.

19 Nagai A, Aoshiba K, Ishihara Y *et al*. Administration of alpha 1-proteinase inhibitor ameliorates bleomycin-induced pulmonary fibrosis in hamsters. Am Rev Respir Dis 1992;145:651–6.

20 Mitsuhashi H, Asano S, Nonaka T, Hamamura I, Masuda K, Kiyoki M. Administration of truncated secretory leukoprotease inhibitor ameliorates bleomycin-induced pulmonary fibrosis in hamsters. Am J Respir Crit Care Med 1996;153:369–74.

21 Taooka Y, Maeda A, Hiyama K, Ishioka S, Yamakido M. Effects of neutrophil elastase inhibitor on bleomycin-induced pulmonary fibrosis in mice. Am J Respir Crit Care Med 1997;156:260–5.

22 Taipale J, Lohi J, Saarinen J, Kovanen PT, Keski-Oja J. Human mast cell chymase and leukocyte elastase release latent transforming growth factor beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. J Biol Chem 1995;270:4689–96.

23 Wada Y, Yoshida K, Tsutani Y *et al*. Neutrophil elastase induces cell proliferation and migration by the release of TGF-alpha, PDGF and VEGF in esophageal cell lines. Oncol Rep 2007;17:161–7.

24 Buczek-Thomas JA, Lucey EC, Stone PJ *et al*. Elastase mediates the release of growth factors from lung in vivo. Am J Respir Cell Mol Biol 2004;31:344–50.