Elevated Serum Concentrations of Polymorphonuclear Neutrophilic Leukocyte Elastase in Systemic Sclerosis: Association with Pulmonary Fibrosis

TOSHIHIDE HARA, FUMIHIDE OGAWA, KOICHI YANABA, YOHEI IWATA, EIJI MUROI, KAZUHIRO KOMURA, MOTOI TAKENAKA, KAZUHIRO SHIMIZU, MINORU HASEGAWA, MANABU FUJIMOTO, and SHINICHI SATO

ABSTRACT. ***Objective.*** To determine the serum concentrations and clinical association of polymorphonuclear neutrophilic leukocyte (PMN) elastase in patients with systemic sclerosis (SSc).

Methods. Serum PMN elastase levels from 21 patients with limited cutaneous SSc (lSSc) and 32 with diffuse cutaneous SSc (dSSc) were examined by ELISA.

Results. Serum PMN elastase levels were elevated in patients with SSc, especially dSSc, compared to healthy controls. SSc patients with elevated serum PMN elastase levels had more frequent presence of pulmonary fibrosis, arthritis, contracture of phalanges, and diffuse pigmentation. Anticentromere antibody was detected less frequently in SSc patients with elevated serum PMN elastase levels than in controls. Consistently, serum PMN elastase levels also correlated positively with serum levels of KL-6 and surfactant protein-D, serological markers for pulmonary fibrosis. Serum PMN elastase levels were also associated with levels of serum 8-isoprostane, an oxidative stress marker in SSc.

Conclusion. Serum PMN elastase levels were elevated in patients with SSc, and it was more prominent in patients with pulmonary fibrosis, suggesting that serum PMN elastase is a novel serological marker for SSc-related pulmonary fibrosis. (First Release Oct 15 2008; J Rheumatol 2009; 36:99–105; doi:10.3899/jrheum.080269)

Key Indexing Terms:

POLYMORPHONUCLEAR NEUTROPHILIC LEUKOCYTE ELASTASE
SYSTEMIC SCLEROSIS PULMONARY FIBROSIS OXIDATIVE STRESS

Systemic sclerosis (SSc) is a multisystem disorder of connective tissue characterized by sclerotic changes in the skin and other visceral organs with an autoimmune background. The central event in SSc is an abnormal accumulation of extracellular matrix components[1-3]. Although the pathogenesis of SSc remains unknown, interactions among lymphoid cells, endothelial cells, and fibroblasts are likely to be central to the pathogenesis of the disease. In early skin lesions of SSc, mononuclear cell infiltration, microvascular lesions, and increased extracellular matrix synthesis are first seen around small vessels in the dermis[4]. Recent studies suggest that leukocyte migration is the first step of the multistep cascade involving cell adhesion molecules and chemokines[5]. Serine proteases, released from granulocytes and monocytes, are implicated directly or indirectly in the excessive deposition of extracellular matrix, being involved in marked conversion of latent matrix metalloproteinases (MMP) to lower molecular weight forms, active MMP that contribute to matrix remodeling[6]. Thus, collaborative interactions between serine proteases and MMP are generally presumed to contribute to the tissue destruction and extracellular matrix damage[6,7].

Polymorphonuclear neutrophilic leukocyte (PMN) elastase is one of the serine proteases found in the azurophilic granules of neutrophils[8]; it is capable by itself of degrading several different structural components such as collagen, fibronectin, proteoglycan, heparin, and cross-linked fibrin[7]. Studies have suggested that PMN and their products are involved in endothelial injury, inflammatory processes, and fibrosis[7-11]. The activation of PMN leads to the release of multiple microbicidal products, including reactive oxygen species (ROS), cationic peptides, eicosanoids, and proteolytic enzymes[8]. PMN also release growth factors, cytokines, and chemokines, which can enhance the inflammatory response[12]. Further, PMN elastase can enhance

From the Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki; Department of Dermatology, The Jikei University School of Medicine, Tokyo; and Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan.

T. Hara, MD; F. Ogawa, MD, PhD, Assistant Professor; Y. Iwata, MD; E. Muroi, MD, PhD; K. Komura, MD, PhD; M. Takenaka, MD, PhD, Assistant Professor; K. Shimizu, MD, PhD, Associate Professor; S. Sato, MD, PhD, Professor, Chair, Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences; K. Yanaba, MD, PhD, Department of Dermatology, The Jikei University School of Medicine; M. Hasegawa, MD, PhD, Assistant Professor; M. Fujimoto, MD, PhD, Associate Professor, Department of Dermatology, Kanazawa University Graduate School of Medical Science.

Address reprint requests to Dr. S. Sato, Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. E-mail: s-sato@nagasaki-u.ac.jp

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PMN migration by inducing the secretion of granulocyte-macrophage colony-stimulating factor, interleukin 6, and interleukin 8 from epithelial cells[13,14]. A recent study suggests that an impairment in the ability of PMN to control hypoxia and inflammation could relate to the increased production of free oxygen radicals and consequent oxidative damage in SSc[15]. In addition, serine proteases and ROS have been suggested to be frequent companions at the sites of inflammation[9,16].

Passively released or actively secreted elastase from PMN has been linked to the pathologic processes of a variety of inflammatory diseases, including pulmonary fibrosis[17-20], rheumatoid arthritis[21], sarcoidosis[22,23], adult respiratory distress syndrome[24,25], and cystic fibrosis[14,26]. Pulmonary surfactant apoproteins are also digested by PMN elastase. Studies have reported that the number of PMN is elevated in bronchoalveolar lavage (BAL) fluid from SSc patients with active interstitial lung disease[18,27]. Moreover, PMN elastase-deficient mice are resistant to bleomycin-induced fibrosis[28,29]. Therefore, PMN and their products may be involved in either endothelial injury or inflammatory and fibrotic processes in SSc.

However, the prevalence or clinical significance of serum PMN elastase in SSc has not been clarified. We investigated whether PMN elastase was elevated in serum samples from patients with SSc, and whether serum PMN elastase levels correlated with the activity or severity of pulmonary fibrosis and other clinical symptoms in patients with SSc.

MATERIALS AND METHODS

Serum samples. Blood samples were obtained from 53 Japanese patients with SSc (44 women, 9 men; age 50 ± 16 yrs). All patients fulfilled the criteria proposed by the American College of Rheumatology[30]. These patients were grouped according to the classification system proposed by LeRoy, *et al*[31]: 21 patients (19 women, 2 men; age 52 ± 13 yrs) had limited cutaneous SSc (lSSc) and 32 patients (25 women, 7 men; age 48 ± 18 yrs) had diffuse cutaneous SSc (dSSc). The disease duration of lSSc and dSSc patients was 7.4 ± 8.2 and 3.0 ± 2.9 years, respectively. The duration of disease was calculated from time of onset of the first clinical event (other than Raynaud's phenomenon) that was a clear manifestation of SSc. No patient had been treated with calcium antagonist, corticosteroid, D-penicillamine, or other immunosuppressive therapy at the evaluation. Antinuclear antibody was determined by indirect immunofluorescence using HEp-2 cells as the substrate, and autoantibody specificities were further assessed by enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation. Anticentromere antibody (ACA) was positive for 17 patients (2 dSSc and 15 lSSc), anti-topoisomerase I antibody for 26 (22 dSSc and 4 lSSc), anti-U1RNP antibody for 2 (all dSSc), anti-U3RNP antibody for 1 (dSSc), anti-RNA polymerases I and III antibody for 6 (all dSSc), and anti-Th/To antibody for 1 (lSSc). Twenty-five age and sex-matched healthy Japanese individuals served as controls. Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at –70°C prior to use.

The protocol was approved by the Kanazawa University Graduate School of Medical Science, and informed consent was obtained from all patients.

Clinical assessment. Complete medical histories, physical examinations, and laboratory tests were conducted for all patients at their first visit. Skin score was measured by the modified Rodnan total skin thickness score (modified Rodnan TSS)[32]. The 17 anatomic areas were rated as 0 (normal skin thickness), 1+ (mild but definite skin thickening) 2+ (moderate skin thickening), and 3+ (severe skin thickening); and the modified Rodnan TSS was derived by summing the scores from all 17 areas (range 0–51). Organ system involvement was defined as described[33]: lung = bibasilar fibrosis on chest radiography and high resolution computed tomography; esophagus = hypomotility shown by barium radiography; joint = inflammatory polyarthralgias or arthritis; heart = pericarditis, congestive heart failure, or arrhythmias requiring treatment; kidney = malignant hypertension and rapidly progressive renal failure without any other explanation; and muscle = proximal muscle weakness and elevated serum creatine kinase. Pulmonary function tests, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLCO), were also done. When the DLCO and VC were < 75% and < 80%, respectively, of predicted normal values, they were considered to be abnormal.

ELISA. Specific ELISA kits were used for measuring serum PMN elastase levels (Bender MedSystems, Vienna, Austria), according to the manufacturer's protocol. Each sample was tested in duplicate. Serum levels of ACA or anti-topoisomerase I antibody were assessed using specific ELISA kits (Medical & Biological Laboratories, Nagoya, Japan), according to the manufacturer's protocol. To assess oxidative stress, serum levels of 8-isoprostane, a reliable biomarker of oxidative stress[34,35], were also examined using a specific ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA), according to the manufacturer's protocol. Serum levels of KL-6 and surfactant protein-D (SP-D), both serological markers of pulmonary fibrosis, were investigated using specific ELISA kits (Eitest KL-6, Eisai, Tokyo, Japan, and SP-D kit, Yamasa, Chiba, Japan, respectively), according to the manufacturer's protocol.

Statistical analysis. Statistical analysis was performed by Mann-Whitney U test for comparison of serum PMN elastase levels, Fisher's exact probability test for comparison of frequencies, and Bonferroni's test for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between 2 continuous variables. A p value < 0.05 was considered statistically significant.

RESULTS

Serum PMN elastase levels in SSc. The levels and presence of PMN elastase in serum samples from SSc patients and healthy controls were assessed by ELISA (Figure 1a). Serum PMN elastase levels in dSSc patients were significantly higher than those in controls ($p < 0.0001$). Similarly, lSSc patients exhibited significantly elevated PMN elastase levels compared with controls ($p < 0.0005$). Optical density values higher than the mean + 2 SD (0.9 ng/ml) of control serum samples were considered to be elevated. Elevated serum PMN elastase levels were observed in 58% (31/53) of all SSc patients. Regarding the disease subsets, serum PMN elastase levels were increased in 69% (22/32) of dSSc patients, while 43% (9/21) of lSSc patients had elevated PMN elastase. In contrast, only 4% (1/25) of controls showed elevated PMN elastase levels. Thus, PMN elastase levels were elevated in SSc patients, especially in dSSc patients.

Clinical correlation of PMN elastase levels in SSc. Next we assessed clinical correlations of serum PMN elastase levels in SSc patients. Patients with elevated PMN elastase levels had more frequent presence of pulmonary fibrosis ($p < 0.05$), arthritis ($p < 0.05$), contracture of phalanges ($p < 0.01$), and diffuse pigmentation ($p < 0.005$) than those with

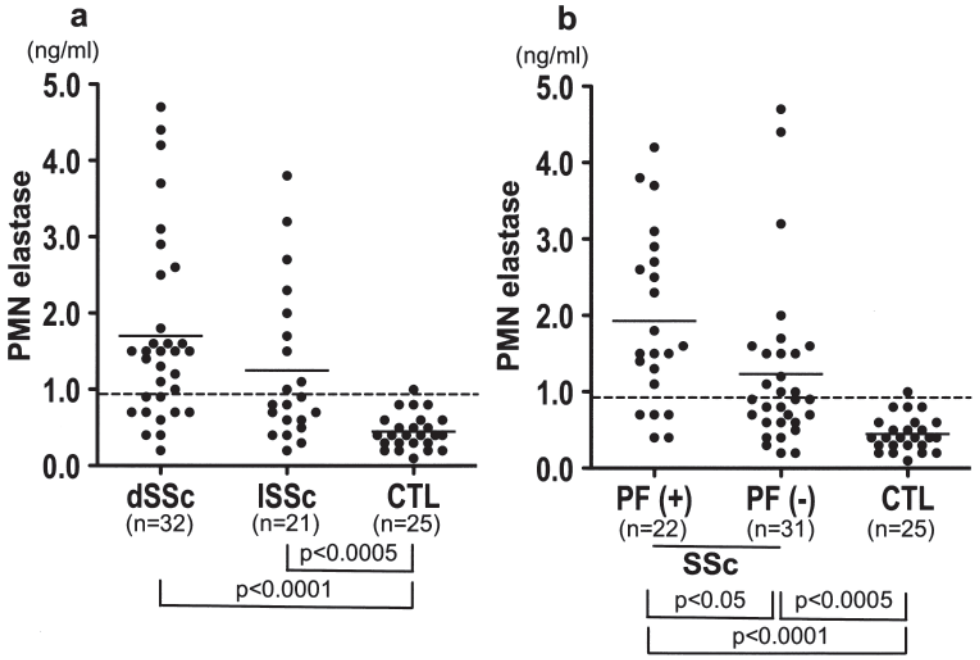

Figure 1. a. Serum PMN elastase levels in dSSc patients, lSSc patients, and healthy controls (CTL). b. Serum PMN elastase levels in SSc patients with pulmonary fibrosis [PF(+)], without pulmonary fibrosis [PF(–)], and controls. Serum PMN elastase levels were determined by a specific ELISA. Short bar indicates the mean value in each group. Broken line indicates the cutoff value (mean + 2 SD of control samples).

normal PMN elastase levels (Table 1). ACA was detected less frequently in SSc patients with elevated PMN elastase levels (18%) than those with normal levels (58%; $p < 0.005$). Conversely, the prevalence of anti-topoisomerase I antibody positivity tended to be higher in patients with elevated PMN elastase levels than those with normal levels (56% vs 37%; $p = 0.07$). Consistent with these findings, elevated PMN elastase levels were detected more frequently in dSSc patients (71%) than lSSc patients (29%; $p < 0.05$). To determine the correlations of serum PMN elastase levels with disease duration, we classified SSc patients into 2 groups: the early disease population (within 2 yrs from onset) and late disease population (more than 2 yrs from onset). Serum PMN elastase concentrations in the early disease population were significantly elevated compared with the late disease population (1.8 ± 1.0 vs 1.3 ± 1.4 ng/ml, respectively; $p < 0.05$). Although serum PMN elastase levels in dSSc patients tended to be higher than those in lSSc patients, in both early and late disease populations (data not shown), these differences were not statistically significant. Moreover, SSc patients with pulmonary fibrosis exhibited significantly elevated serum PMN elastase levels compared to SSc patients without pulmonary fibrosis ($p < 0.05$) and controls ($p < 0.0001$; Figure 1b). Similarly, serum PMN elastase levels in SSc patients without pulmonary fibrosis were significantly higher than those in controls ($p < 0.0005$). In addition, serum PMN elastase levels tended to correlate inversely with %DLCO ($r = –0.28$, $p = 0.07$) in SSc patients with pul-

Table 1. Clinical and laboratory data of patients with SSc showing elevated serum PMN elastase levels. Values of clinical features and organ involvement are percentages.

	Elevated Serum PMN Elastase, n = 34	Normal Serum PMN Elastase, n = 19
Age at onset, yrs, mean ± SD	48 ± 17	47 ± 16
Sex, male:female	7:28	2:17
Disease duration, yrs, mean ± SD	4.0 ± 5.6	5.5 ± 6.4
Clinical features		
dSSc	71*	42
lSSc	29	58*
Pitting scars	47	37
Contracture of phalanges	59**	21
Diffuse pigmentation	68***	26
Organ involvement		
Lung	54*	26
Decreased % VC	24	40
Decreased % DLco	64	86
Pulmonary hypertension	17	7
Esophagus	53	50
Heart	21	11
Kidney	12	33
Joint	32*	16
Muscle	26	21
Autoantibodies		
Antitopoisomerase I	56	37
Anticentromere	18	58***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs SSc patients with normal PMN elastase levels.

monary fibrosis. Remarkably, serum PMN elastase in SSc patients showing markedly reduced VC (< 60%) was associated with decreased %VC (r = –0.643, p < 0.05) and the presence of pulmonary fibrosis (p < 0.01). Further, we investigated correlations of serum PMN elastase levels with serum levels of KL-6, which is a mucin-like high molecular-weight glycoprotein[36] and a marker of pulmonary fibrosis[37], and SP-D, which is produced and secreted by alveolar type II pneumocytes in alveoli and Clara cells and is a marker of pulmonary fibrosis[38]. Serum PMN elastase levels correlated positively with serum KL-6 levels (p < 0.05, r = 0.517) and SP-D levels (p < 0.05, r = 0.447) determined by ELISA (Figure 2). Serum levels of 8-isoprostane, a reliable biomarker of oxidative stress, also correlated positively with serum PMN elastase levels (p < 0.05, r = 0.329). Thus, elevated PMN elastase in SSc patients was generally associated with disease severity, especially with the extent of pulmonary fibrosis.

DISCUSSION

Our study is the first to show that serum PMN elastase levels were elevated in patients with SSc, especially in patients with dSSc. Interestingly, serum PMN elastase levels in SSc correlated with the presence of pulmonary fibrosis. Consistent with these findings, serum PMN elastase levels correlated positively with the serum levels of KL-6 and SP-D, markers for monitoring the activity of pulmonary fibrosis[38,39]. In addition, serum levels of PMN elastase in SSc patients were associated with more frequent presence of arthritis, contracture of phalanges, and diffuse pigmentation. Further, serum PMN elastase levels correlated positively with serum levels of 8-isoprostane, a reliable biomarker of oxidative stress. These results indicate that serum PMN elastase is a novel serological marker for disease severity, especially for SSc-related pulmonary fibrosis.

Pulmonary complications are the leading cause of mortality in SSc[40,41]. Early diagnosis and immunosuppressive treatment of pulmonary fibrosis may improve the prognosis of SSc patients[42]. Many studies have suggested that serum PMN elastase levels may be an indicator of disease activity of pulmonary interstitial disease[19,25]. Consistently in our study, increased levels of serum PMN elastase were observed in SSc patients with pulmonary fibrosis. In addition, PMN elastase levels in BAL fluid are reported to be elevated in patients with pulmonary fibrosis[18-20], sarcoidosis[22,23], adult respiratory distress syndrome[24,25], cystic fibrosis[14,26], and SSc-related pulmonary fibrosis[27], and correlate with serum levels of PMN elastase[20,43]. Although elevated levels of serum PMN elastase overall were associated with presence and severity of pulmonary fibrosis in SSc, the correlations between PMN elastase and the results of pulmonary function tests were not statistically significant. The recognition of interstitial lung manifestation in SSc may be delayed, because the clinical signs are often mild and onset is insidious[44,45]. In some patients, lung function may remain stable for years, even in the later disease stage, while others

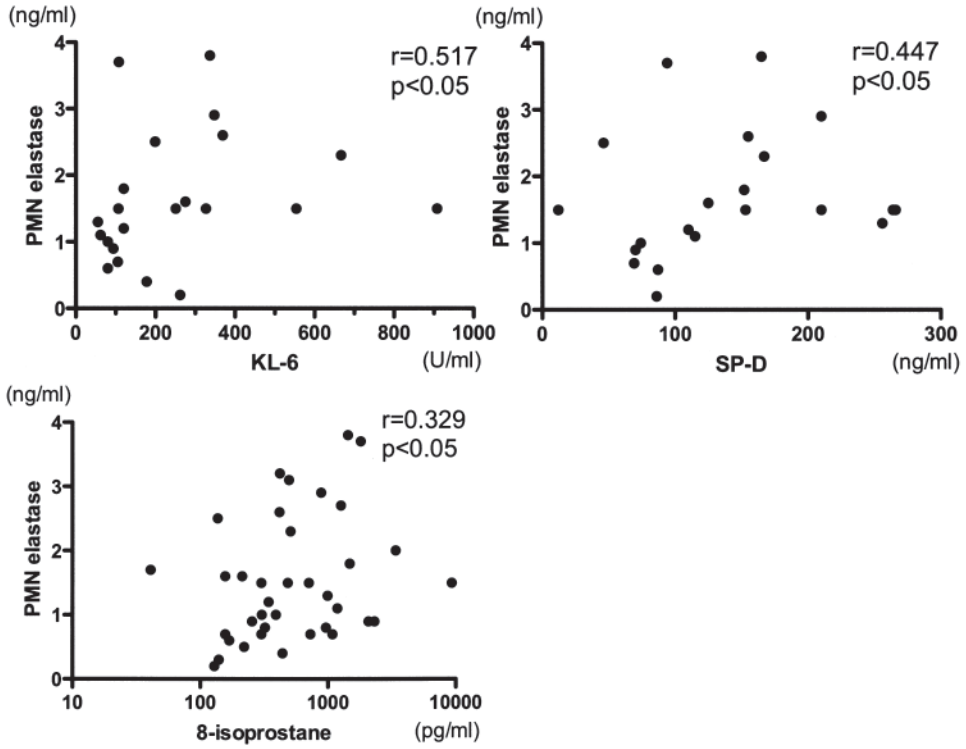

Figure 2. Correlation of PMN elastase levels against serum KL-6 levels, SP-D levels, and 8-isoprostane levels in patients with SSc. Serum PMN elastase, KL-6, SP-D, and 8-isoprostane concentrations were determined by ELISA.

may experience fatal progressive pulmonary fibrosis associated with SSc[46,47]. These may be the reasons for the discrepancy between the extent of pulmonary fibrosis and the pulmonary function tests in SSc patients. At the same time, serum PMN elastase levels correlated positively with serum levels of KL-6 and SP-D, which are reported to be markers of pulmonary fibrosis, and the concentrations were associated with the activity of pulmonary fibrosis[38]. Further, serum PMN elastase levels correlated positively with serum 8-isoprostane levels. A study has reported that serum 8-isoprostane levels correlate with the severity of pulmonary fibrosis, suggesting that serum 8-isoprostane is a useful serological marker for severity of lung fibrosis[35]. Collectively, these results suggest that elevated PMN elastase is a novel serological marker for the severity of pulmonary fibrosis in patients with SSc.

SP-D plays important roles in innate immunity, and its expression increased rapidly following many types of lung injury[48,49]. Additionally, SP-D levels were strongly correlated with KL-6 levels in sera from SSc patients[38]. Recent studies reported that PMN elastase cleaved SP-D at sites of inflammation, with potential deleterious effects on its biological functions[50,51]. SP-D-deficient mice show an exaggerated neutrophil response to viral and bacterial challenge[52]. Thus, SP-D could interact directly with PMN that infiltrate the lung in response to acute inflammation and infection. Moreover, PMN elastase increases oxidative stress in lung cells[9]. Antiproteinases including α1-proteinase inhibitor are sensitive to inactivation by oxidants released from activated neutrophils[53]. Therefore, the toxic effect of PMN elastase is greatly enhanced by increased oxidative stress[54]. Recently, increased adenosine deaminase activity and reduced adenosine receptor activity have been described in PMN from SSc patients, suggesting that impairment in the ability of PMN to control hypoxia and inflammation could relate to the increased production of free oxygen radicals and consequent oxidative damage in SSc[15]. Indeed, ischemia and reperfusion injury following Raynaud's phenomenon can generate ROS that may result in vascular endothelial damage[55,56]. ROS could explain the tissue lesions in SSc, as free radicals can induce endothelial cell injury through peroxidation of lipid components of cell membrane, and may stimulate fibroblasts to proliferate and to produce increased amounts of collagen[57]. Consistently in our study, serum PMN elastase levels were positively correlated with levels of serum 8-isoprostane, a marker of oxidative stress generated through cell-membrane oxidation[34,35]. Thus, PMN elastase may participate in development of SSc by enhancing oxidative stress.

In addition, PMN elastase was also associated with more frequent presence of arthritis and contracture of phalanges. Previous studies showed that PMN elastase was elevated in destructive joints in RA[21]. In inflammatory lesions, MMP play a key role in matrix destruction[58,59]. Serine proteases such as PMN elastase, trypsin, plasminogen, plasminogen activator, and cathepsin G help activate these MMP[60,61]. Studies have also suggested that these serine proteinases themselves, such as proteoglycanase, may cause cartilage matrix destruction[62]. Although the relationship between serine proteinases and MMP and the contribution of each factor to matrix destruction remain unclear, PMN elastase may play a role in damage to articular cartilage.

One study reported low elastase activity in circulating granulocytes of SSc patients[63]. Although the reasons for this discrepancy remain unclear, it may be due to the differences in the patient population and treatment: the study[63] included SSc patients treated with a calcium antagonist (80%) that has antioxidant effects and antielastase activity[64,65], while such patients were excluded in our study. Nevertheless, these results suggest that elevated PMN elastase levels reflect the disease severity, especially pulmonary fibrosis, in patients with SSc. Future prospective and longitudinal studies are required to determine whether the changes in serum PMN elastase concentrations are correlated with new onset or deterioration of pulmonary fibrosis. The pathogenesis of SSc remains unknown — PMN elastase may play an important role in the initiation and development of this disease.

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