

Function Blocking Autoantibodies Against Matrix Metalloproteinase-1 in Patients with Systemic Sclerosis

Shinichi Sato, Ikuko Hayakawa, Minoru Hasegawa, Manabu Fujimoto,* and Kazuhiko Takehara

Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan; *Department of Regenerative Medicine, Research Institute, International Medical Center of Japan, Tokyo, Japan

Systemic sclerosis is characterized by fibrosis and systemic autoimmunity; however, roles of autoantibodies in the development of fibrosis remain unknown in systemic sclerosis. The net accumulation of extracellular matrix is dependent on the balance between the synthesis and degradation of extracellular matrix components, the latter process regulated by matrix metalloproteinases. Matrix metalloproteinase-1 (interstitial collagenase-1) can initiate degradation of collagen types I–III that are major extracellular matrix constituents in affected skin of systemic sclerosis. In this study, we tested the hypothesis that systemic autoimmunity in systemic sclerosis induced anti-matrix metalloproteinase-1 autoantibodies that inhibited matrix metalloproteinase-1 activity, resulting in collagen accumulation. Enzyme-linked immunosorbent assay using human recombinant matrix metalloproteinase-1 revealed that IgG anti-matrix metalloproteinase-1 autoantibody levels were significantly elevated in sera from patients with systemic sclerosis, but not patients with active systemic lupus erythematosus or dermatomyositis, relative to normal controls. IgG anti-matrix

metalloproteinase-1 autoantibody levels were significantly higher in patients with diffuse cutaneous systemic sclerosis than those found in patients with limited cutaneous systemic sclerosis. Furthermore, IgG anti-matrix metalloproteinase-1 antibody levels significantly correlated with the extent of fibrosis in the skin, lung, and renal blood vessels. The presence of IgG anti-matrix metalloproteinase-1 autoantibody in sera from systemic sclerosis patients was confirmed by immunoblotting analysis. Remarkably, IgG anti-matrix metalloproteinase-1 autoantibody in sera from systemic sclerosis patients inhibited matrix metalloproteinase-1 collagenase activity. Collectively, the results of this study suggest that anti-matrix metalloproteinase-1 autoantibody contributes to the development of fibrosis by inhibiting matrix metalloproteinase-1 collagenase activity and reducing the extracellular matrix turnover and suggest that the presence of anti-matrix metalloproteinase-1 autoantibody in systemic sclerosis is the link between systemic autoimmunity and fibrosis. *Key words: autoimmunity/collagenase/extracellular matrix/fibrosis. J Invest Dermatol 120:542–547, 2003*

Systemic sclerosis (SSc) is a connective tissue disorder that is characterized by fibrosis and vascular changes in the skin and other visceral organs, with an autoimmune background. The central event in the pathogenesis of SSc is an abnormal accumulation of extracellular matrix (ECM) components, predominantly types I and III collagens (Lovell *et al*, 1979; Jimenez *et al*, 1996). Consistently, fibroblasts cultured from the affected skin of patients with SSc display a morphologically activated phenotype producing increased amounts of various connective tissue components, mainly of type I collagen (LeRoy, 1974; Jimenez *et al*, 1996). Various cytokines or growth factors that are produced partly by inflammatory cells infiltrating the affected tissues may be involved in the initiation or development of fibrotic process (Jimenez *et al*, 1996; Sato, 1999).

The net accumulation of ECM in tissue is dependent on the balance between the synthesis and the degradation of ECM components. The latter process is regulated by matrix metalloproteinases (MMP), a family of zinc-dependent endopeptidases that collectively can digest all ECM components (Kähäri and Saarialho-Kere, 1997; Nagase and Woessner, 1999). MMP-1 (interstitial collagenase-1) is able to initiate degradation of interstitial collagen types I–III that are major constituents of the ECM in both normal skin and affected skin of SSc (Lovell *et al*, 1979; Jimenez *et al*, 1996; Kähäri and Saarialho-Kere, 1997). The expression of MMP is induced in response to exogenous signals, such as various cytokines or growth factors, cell–matrix interactions, and cell–cell interactions (Kähäri and Saarialho-Kere, 1997; Nagase and Woessner, 1999). To prevent excessive function of MMP, the proteolytic activity of MMP is inhibited by nonspecific inhibitors, including α_2 -macroglobulin, as well as specific inhibitors of tissue inhibitors of metalloproteinases (TIMP) (Gomez *et al*, 1997; Kähäri and Saarialho-Kere, 1997; Nagase and Woessner, 1999). Thus, the balance between MMP and TIMP levels governs connective tissue homeostasis.

The abnormal regulation of MMP and TIMP expression has been reported in SSc. Takeda *et al* (1994) have shown that SSc fibroblasts exhibit decreases in MMP-1 activity and production compared with normal fibroblasts. By contrast, another recent study has shown that fibroblasts from patients with early stage

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Reprint requests to: Dr Shinichi Sato, Department of Dermatology, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan. Email: s-sato@med.kanazawa-u.ac.jp

Abbreviations: DLco, diffusion capacity for carbon monoxide; dSSc, diffuse cutaneous SSc; lSSc, limited cutaneous SSc; MMP, matrix metalloproteinase; SSc, systemic sclerosis; TIMP, tissue inhibitor of metalloproteinase; TSS, total skin thickness score; VC, vital capacity.

SSc display higher levels of MMP-1 and MMP-3 than those from normal individuals, whereas levels of MMP-1 and MMP-3 decrease in fibroblasts from SSc patients with mid-stage SSc (Kuroda and Shinkai, 1997). Expression levels of TIMP-1, -2, and -3 by fibroblasts from SSc patients are increased relative to normal fibroblasts (Kirk *et al*, 1995; Kikuchi *et al*, 1997; Kuroda and Shinkai, 1997; Mattila *et al*, 1998). In addition, elevated serum levels of TIMP-1 or TIMP-2 have been reported in patients with SSc in correlation with disease activity (Kikuchi *et al*, 1995; Yazawa *et al*, 2000; Young-Min *et al*, 2001). Thus, the altered balance between MMP and their inhibitors appears to play an important part in the development of fibrosis in SSc.

Systemic autoimmunity is another central feature of SSc as anti-nuclear antibodies are detected in more than 90% of patients with SSc (Okano, 1996); however, roles of these autoantibodies in disease expression, such as fibrosis, remains unknown in SSc. We hypothesized that autoimmune background in SSc induced pathogenic autoantibodies that inhibited MMP-1 activity and thereby contributed to the collagen accumulation. To test this possibility, the presence or levels of anti-MMP-1 antibodies, their clinical correlation, and their functional significance were investigated in this study. The results of this study suggest that anti-MMP-1 autoantibodies detected in patients with SSc play a part in the development of fibrosis by directly inhibiting MMP-1 collagenase activity.

MATERIALS AND METHODS

Serum samples Serum samples were obtained from 57 Japanese patients with SSc (51 females and six males). All patients fulfilled the criteria proposed by the American College of Rheumatology (Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee 1980). These patients were grouped according to the classification system proposed by LeRoy *et al* (1988): 33 patients (32 females and one male) had limited cutaneous SSc (lSSc) and 24 patients (five females and 19 males) had diffuse cutaneous SSc (dSSc). The age of patients with SSc (mean \pm SD) was 44 ± 16 . Patients with dSSc were 42 ± 19 y old, whereas those with lSSc were 46 ± 15 y old. The disease duration of patients with lSSc and dSSc was 7.7 ± 8.7 and 4.7 ± 6.7 y, respectively. None of the SSc patients were treated with steroids, D-penicillamine, or immunosuppressive therapy. Anti-nuclear antibodies were determined by indirect immunofluorescence using HEp-2 cells as substrate and autoantibody specificities were further assessed by enzyme-linked immunosorbent assay (ELISA) specific for antibodies against topoisomerase I, centromere, or U1RNP. Anti-topoisomerase I antibody was positive for 23 (dSSc, 18 and lSSc, five), anti-centromere antibody for 24 (dSSc, one and lSSc, 23), and anti-U1RNP antibody for three (dSSc, one and lSSc, two). Seven patients (dSSc, four and lSSc, three) had anti-nuclear antibodies, but their specificities were not identified by autoantibody-specific ELISA. Of these seven patients, two patients had anti-RNA polymerases I and III antibody and anti-U3RNP antibody by immunoprecipitation, respectively; however, autoantibody specificities in the remaining five patients were not still identified by immunoprecipitation. Nineteen patients with systemic lupus erythematosus (SLE), who fulfilled the American College of Rheumatology criteria (Tan *et al*, 1982), were also examined as disease control in this study. These patients had active SLE as determined by the SLE Disease Activity Index (Bombardier *et al*, 1992). In addition, 16 patients with dermatomyositis that fulfilled Bohan and Peter criteria (Bohan and Peter, 1975a; Bohan and Peter, 1975b) were included. Age- and sex-matched 30 Japanese healthy persons (three males and 27 females; age, 43 ± 10 y) were used as normal controls. Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at -70°C prior to use.

Clinical assessment Complete medical histories, physical examinations, and laboratory tests were conducted for all patients. Skin score was measured by scoring technique of the modified Rodnan total skin thickness score (modified Rodnan TSS) (Clements *et al*, 1993). 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 summation of the scores from all 17 areas (range 0–51). Organ system involvement was defined as described previously (Steen *et al*, 1988; Sato *et al*, 1994): 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. Renal vascular damage was determined as a pulsatility index by color-flow Doppler ultrasonography of both kidneys (Warshauer *et al*, 1988; Nishijima *et al*, 2001). The pulsatility index, which represents vascular impedance, was calculated as $A-B/\text{mean}$, where A is the peak systolic frequency, B is the end diastolic frequency, and the mean is the time-averaged frequency. The pulsatility index was calculated as an average value obtained with eight waveforms on the renal interlobar arteries of both kidneys. Pulmonary function test, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLco), was also tested. When the DLco and VC were $<75\%$ and $<80\%$, respectively, of the predicted normal values, they were considered to be abnormal. There were no patients with pulmonary hypertension without pulmonary fibrosis. The protocol was approved by the Kanazawa University Graduate School of Medical Science and Kanazawa University Hospital, and informed consent was obtained from all patients.

ELISA for anti-MMP-1 antibodies ELISA were conducted as previously described (Sato *et al*, 2000). Ninety six-well plates were coated with human recombinant MMP-1 (1 μg per ml; Techne Corp., Minneapolis, MN) at 4°C overnight. The serum samples diluted to 1 : 100 were added to triplicate wells for 90 min at 20°C . After washing, the bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG or IgM antibodies (Cappel, Durham, NC), using p-nitrophenyl phosphate (Sigma-Aldrich, St Louis, MO) as substrate. The optical density (OD) of the wells was subsequently determined. Relative levels of autoantibodies were determined for each group of patients and normal controls using pooled serum samples as previously described (Sato *et al*, 2000). The sera were diluted at log intervals (1 : 10^{-1} : 10^3) and assessed for relative autoantibody levels as above except the results were plotted as OD vs dilution (log scale). The dilutions of sera giving half-maximal OD values were determined by linear regression analysis, thus generating arbitrary unit per milliliter values for comparison between sets of sera.

Immunoblotting Human recombinant MMP-1 (0.1 μg per lane; Techne Corp.) was subjected to electrophoresis and electrotransferred to nitrocellulose sheets. The nitrocellulose strips were incubated overnight with serum samples diluted 1 : 50 and were then incubated for 1.5 h with alkaline phosphatase-conjugated goat anti-human IgG antibody (Cappel). Color was developed using an amplified alkaline phosphatase Immuno-Blot assay kit (Bio-Rad Laboratories Inc., Hercules, CA). Ten SSc patients positive for IgG anti-MMP-1 antibody by ELISA, nine SSc patients positive for either anti-topoisomerase I antibody, anti-centromere antibody, or anti-U1RNP antibody, but not for IgG anti-MMP-1 antibody by ELISA, and six healthy individuals were evaluated.

MMP-1 collagenase activity assay IgG was purified from serum samples using magnetic beads coated with recombinant protein G covalently coupled to the surface (DynaL Inc., Lake Success, NY). Final IgG concentration was measured by spectrophotometer (Gene Quant II, Amersham Biosciences Inc., Piscataway, NJ). MMP-1 collagenase activity was determined by a collagenase activity kit (Chemicon International, Inc., Temecula, CA), according to the manufacturer's protocol. Briefly, 4.5 ng of MMP-1 activated by p-aminophenylmercuric acetate was incubated with 40 μg of purified IgG for 20 min at 20°C . The enzymatic activity of MMP-1 was measured using biotinylated bovine native collagen as substrate. The cleaved biotinylated fragments of collagen by MMP-1 were transferred to triplicate wells of biotin-binding 96-well microtiter plates and detected by streptavidin-peroxidase complex and enzyme substrate. The OD of the wells was subsequently determined. MMP-1 that was activated by p-aminophenylmercuric acetate and untreated with IgG served as positive control. Ten SSc patients positive for IgG anti-MMP-1 antibody by ELISA, 10 SSc patients positive for positive for either anti-topoisomerase I antibody, anti-centromere antibody, or anti-U1RNP antibody, but not for IgG anti-MMP-1 antibody by ELISA, and 10 healthy individuals were assessed.

Statistical analysis Statistical analysis was performed using the Mann-Whitney U test for determining the level of significance of differences between sample means, 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 two continuous variables. $p < 0.05$ was considered statistically significant.

RESULTS

Anti-MMP-1 autoantibody levels in SSc by ELISA The presence and levels of anti-MMP-1 autoantibodies in serum samples from patients with collagen diseases and normal controls were assessed by ELISA (Fig 1). The dilution of sera giving half-maximal OD values in ELISA was also determined to generate arbitrary units per milliliter that could be directly compared between patients and normal controls. Pooled sera from patients with dSSc had mean IgG and IgM anti-MMP-1 antibody levels that were significantly 6.2-fold and 2.0-fold higher than those found in normal controls ($p < 0.0001$ and $p < 0.05$, respectively). Patients with ISSc exhibited mean IgG anti-MMP-1 antibody levels that were significantly 56% higher than those in normal controls ($p < 0.05$), but had almost normal IgM levels of anti-MMP-1 antibody. IgG anti-MMP-1 antibody levels in patients with dSSc were significantly increased compared with patients with ISSc ($p < 0.001$), those with active SLE ($p < 0.0005$), or those with dermatomyositis ($p < 0.0005$). There were no significant differences, however, in IgM anti-MMP-1 antibody levels between patients with dSSc and those with ISSc, SLE, or dermatomyositis. In total patients with SSc, IgG anti-MMP-1 antibody levels were significantly higher than those found in normal controls ($p < 0.001$), patients with SLE ($p < 0.05$), or those with dermatomyositis ($p < 0.01$), whereas IgM anti-MMP-1 antibody levels were similar for total patients with SSc, normal controls, and patients with SLE or dermatomyositis. IgG anti-MMP-1 antibody levels correlated positively with IgM anti-MMP-1 antibody levels in total patients with SSc ($r = 0.361$, $p < 0.0001$). IgG and IgM anti-MMP-1 antibody levels, however, did not correlate with serum total IgG and IgM levels, respectively (data not shown). In addition, IgG or IgM anti-MMP-1 antibody levels were not associated with levels of other autoantibodies, including antibodies against topoisomerase I, centromere, and U1RNP (data not shown). Thus, IgG anti-MMP-1 autoantibody levels were increased in SSc, especially

dSSc, but not in other collagen diseases, including SLE and dermatomyositis.

Frequency of anti-MMP-1 antibody positivity and clinical correlation in SSc Absorbance values higher than the mean + 2 SD (0.59 for IgG anti-MMP-1 antibody and 0.78 for IgM anti-MMP-1 antibody) of the control serum samples were considered positive in this study (Fig 1). IgG or IgM anti-MMP-1 antibody was detected in 75% of patients with dSSc, whereas only 30% of patients with ISSc were positive (Table I). In total patients with SSc, IgG or IgM anti-MMP-1 antibody was found in 49%. By contrast, anti-MMP-1 antibody was detected in only one healthy individual (3%). In dSSc, all seven patients with IgM anti-MMP-1 antibody were also positive for IgG anti-MMP-1 antibody, whereas the remaining 11 patients had IgG anti-MMP-1 antibody alone. In ISSc, both IgM and IgG anti-MMP-1 antibodies were positive in one patient, IgM anti-MMP-1 antibody alone in five patients, and IgG anti-MMP-1 antibody alone in four patients. IgG or IgM anti-MMP-1 antibody was positive in 57% (13 of 23) of SSc patients with anti-topoisomerase I antibody and 67% (two of three) of those with anti-U1RNP antibody, whereas it was found in 38% (nine of 24) of those with anti-centromere antibody. One patient with anti-RNA polymerases I and III had IgG anti-MMP-1 antibody, whereas one patient with anti-U3RNP antibody did not have anti-MMP-1 antibody. Concerning clinical correlation, SSc patients with IgG anti-MMP-1 antibody had lung fibrosis more frequently than those without IgG anti-MMP-1 antibody (14 of 23, 61% vs 10 of 34, 29%, $p < 0.05$). Frequency of IgG anti-MMP-1 antibody positivity, however, was not associated with the presence or absence of other organ involvement, including joint, muscle, esophagus, kidney, and heart (data not shown). Frequency of IgM anti-MMP-1 antibody positivity did not correlate with the presence or absence of any organ involvement (data not shown).

Then, the direct correlation of anti-MMP-1 antibody levels with the extent of skin sclerosis, renal vascular damage, and lung fibrosis was assessed. IgG anti-MMP-1 antibody levels correlated positively with the modified Rodnan TSS, the semiquantitative measure of skin sclerosis (Clements *et al*, 1993) ($p < 0.0002$; Fig 2A). Similarly, the positive association of IgG anti-MMP-1 antibody levels with renal vascular resistance, which was determined as the pulsatility index value in the renal interlobar arteries by color-flow Doppler scans (Warshauer *et al*, 1988; Nishijima *et al*, 2001), was observed ($p < 0.001$; Fig 2B). Furthermore, IgG anti-MMP-1 antibody levels correlated inversely with %DLco ($p < 0.005$; Fig 2C) or %VC ($p < 0.01$; Fig 2D), both of which reflect the severity of lung fibrosis (Silver, 1996). Any significant association of IgM anti-MMP-1 antibody levels with clinical parameters described above, however, was not observed (data not shown). Thus, IgG anti-MMP-1 antibody levels correlated with the extent of fibrosis in the skin, lung, and blood vessels.

Table I. Frequency of anti-MMP-1 antibody positivity in collagen diseases and normal controls^a

	Anti-MMP-1 antibody		
	IgG ^b	IgM	IgG or IgM
SSc (n = 57)	23 (40)	13 (23)	28 (49)
ISSc (n = 33)	5 (15)	6 (18)	10 (30)
dSSc (n = 24)	18 (75)	7 (29)	18 (75)
SLE (n = 19)	1 (5)	2 (11)	3 (16)
Dermatomyositis (n = 16)	1 (5)	1 (6)	2 (13)
Normal (n = 30)	1 (3)	0	1 (3)

^aValues are the number (%) of patients with anti-MMP-1 antibody that was determined by ELISA using human recombinant MMP-1.

^bIsotypes (IgG or IgM) of anti-MMP-1 antibody were determined using isotype-specific anti-human immunoglobulin antibodies.

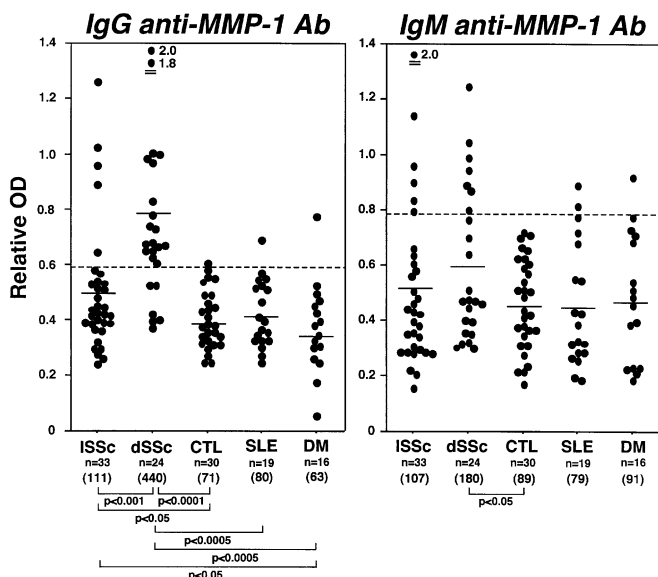


Figure 1. Anti-MMP-1 antibody levels were elevated in serum samples from patients with SSc, especially dSSc. IgG or IgM anti-MMP-1 antibody levels were determined by ELISA using human recombinant MMP-1 in patients with ISSc, those with dSSc, those with SLE, those with dermatomyositis, and normal controls (CTL). The short bar indicates the mean value in each group. A broken line indicates the cut-off value (mean + 2 SD of the control samples). Values in parenthesis represent the dilutions of pooled sera giving half-maximal OD values in ELISA, which were determined by linear regression analysis to generate arbitrary units per milliliter that could be directly compared between each group of patients and normal controls.

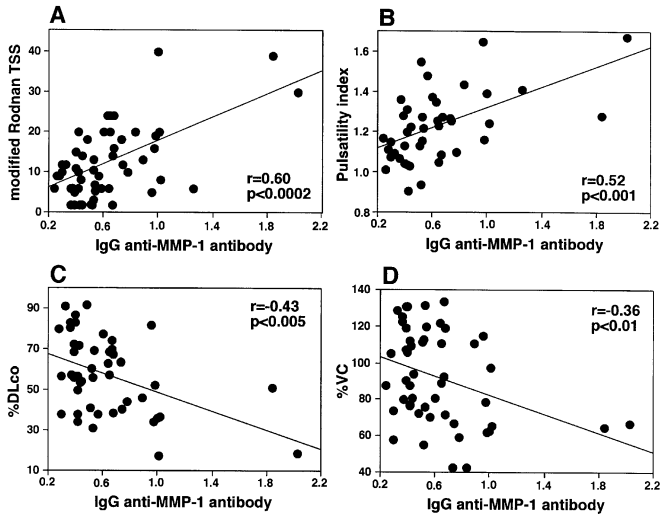


Figure 2. IgG anti-MMP-1 antibody levels correlated with the extent of fibrosis in the skin, renal blood vessels, and lung in patients with SSc. We correlated IgG anti-MMP-1 antibody levels (relative OD) with modified Rodnan TSS (A), pulsatility index (B), %DLco (C), or %VC (D). Anti-MMP-1 antibody levels were determined by ELISA using human recombinant MMP-1. The extent of skin sclerosis was measured by modified Rodnan TSS. The 17 anatomic areas were rated as 0 (normal), 1+ (mild skin thickening), 2+ (moderate), and 3+ (severe), and the modified Rodnan TSS was derived by summation of the scores from all 17 areas. The pulsatility index is a parameter for renal vascular resistance determined by color-flow Doppler ultrasonography of the renal interlobar arteries of both kidneys. The extent of lung fibrosis was evaluated by a pulmonary function test, including %DLco and %VC.

Immunoblotting analysis for anti-MMP-1 antibody The presence of anti-MMP-1 antibody was evaluated by immunoblotting analysis using human recombinant MMP-1. All tested serum samples from SSc patients positive for IgG anti-MMP-1 antibody by ELISA exhibited reactivity with MMP-1 (≈ 52 kDa) by immunoblotting (lanes 2–4, Fig 3). By contrast, any reactivity with MMP-1 was not observed using serum samples with either anti-topoisomerase I, anti-centromere antibody, or anti-U1RNP antibody, but without IgG anti-MMP-1 antibody by ELISA (lane 5 and data not shown). Furthermore, serum samples from healthy individuals did not react with MMP-1 (lane 6). Thus, the presence of anti-MMP-1 autoantibody in patients with SSc was confirmed by immunoblotting analysis.

Inhibition of MMP-1 collagenase activity by anti-MMP-1 antibody To determine the functional relevance of anti-MMP-1 antibody *in vivo*, it was assessed whether anti-MMP-1 autoantibody was able to inhibit MMP-1 collagenase activity. MMP-1 collagenase activity was determined using biotinylated bovine native collagen as substrate. The amount of cleaved biotinylated fragments of collagen by MMP-1 was measured by ELISA. MMP-1 collagenase activity was not inhibited by IgG isolated from healthy individuals (Fig 4). By contrast, IgG isolated from serum samples of SSc patients positive for IgG anti-MMP-1 antibody by ELISA significantly inhibited MMP-1 collagenase activity by 77% compared with normal control ($p < 0.001$). This inhibitory activity was not due to the presence of autoantibodies other than anti-MMP-1 antibody as MMP-1 collagenase activity was not inhibited by IgG isolated from serum samples that contained autoantibodies against topoisomerase I, centromere, or U1RNP, but not IgG anti-MMP-1 antibody. Serum samples from ISSc patients positive for IgM anti-MMP-1 antibody alone did not block MMP-1 activity compared with sera from normal individuals (data not shown). Thus, IgG anti-MMP-1 antibody from patients with SSc was able to inhibit MMP-1 collagenase activity.

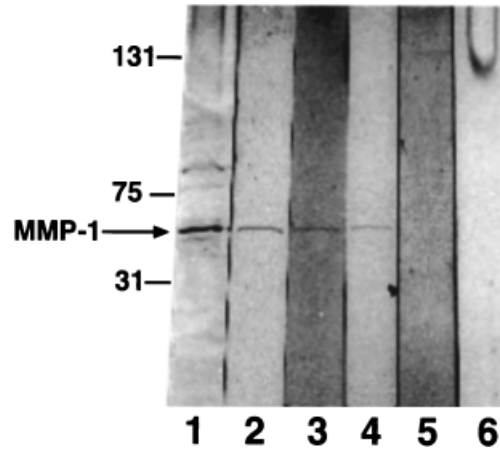


Figure 3. The presence of IgG anti-MMP-1 antibody in sera from patients with SSc was confirmed by immunoblotting. Representative immunoblotting of human recombinant MMP-1 with sera from patients or a normal control is shown. Lane 1, colloidal gold-stained MMP-1; lanes 2–4, serum samples from patients with SSc positive for IgG anti-MMP-1 antibody by ELISA; lane 5, a serum sample from the SSc patient positive for anti-topoisomerase I antibody, but not for IgG anti-MMP-1 antibody by ELISA; and lane 6, a normal human serum. Markers for molecular weights (kDa) are shown to the left. The results represent those obtained with 10 SSc positive for IgG anti-MMP-1 antibody by ELISA, nine SSc patients positive for either anti-topoisomerase I antibody, anti-centromere antibody, or anti-U1RNP antibody, but not for IgG anti-MMP-1 antibody by ELISA, and six healthy individuals.

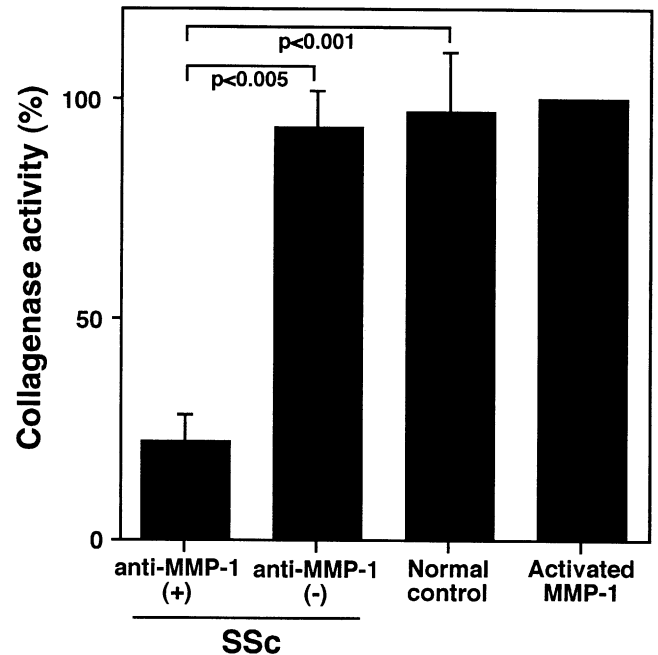


Figure 4. IgG anti-MMP-1 antibody from patients with SSc inhibited MMP-1 collagenase activity. IgG was purified from serum samples of SSc patients positive for IgG anti-MMP-1 antibody by ELISA [anti-MMP-1 (+)], those positive for either anti-topoisomerase I antibody, anti-centromere antibody, or anti-U1RNP antibody, but not for IgG anti-MMP-1 antibody by ELISA [anti-MMP-1 (-)], and normal control. Purified IgG was incubated with MMP-1 activated by p-aminophenylmercuric acetate and MMP-1 collagenase activity was determined using biotinylated bovine native collagen as substrate. The amount of cleaved biotinylated fragments of collagen by MMP-1 was measured by ELISA using a biotin-binding microtiter plate. MMP-1 enzymatic activity is shown as a percentage of p-aminophenylmercuric acetate-activated MMP-1 that was defined as 100% (activated MMP-1). Each histogram shows the mean (\pm SD) results obtained for 10 persons of each group.

DISCUSSION

In this study, ELISA revealed that IgG anti-MMP-1 autoantibody levels were significantly elevated in sera from patients with SSc relative to normal controls (Fig 1). Anti-MMP-1 autoantibody levels were associated with a more severe form of SSc as IgG anti-MMP-1 antibody levels were significantly higher in dSSc patients than those found in lSSc patients (Fig 1). Consistent with this finding, IgG anti-MMP-1 antibody levels significantly correlated with the extent of fibrosis in the skin, lung, and renal blood vessels (Fig 2). Furthermore, elevated levels of anti-MMP-1 autoantibody were specific for patients with SSc as anti-MMP-1 antibody levels in patients with active SLE or dermatomyositis were similar to those in normal controls (Fig 1). The presence of anti-MMP-1 antibody in sera from patients with SSc was also confirmed by immunoblotting analysis (Fig 3). Remarkably, IgG anti-MMP-1 antibody in sera from SSc patients inhibited MMP-1 collagenase activity (Fig 4). Collectively, the results of this study suggest that anti-MMP-1 autoantibody contributes to the development of fibrosis by inhibiting MMP-1 collagenase activity and reducing the turnover of the ECM.

The presence of autoantibodies is a central feature of SSc as specific autoantibodies are detected in more than 90% of patients (Okano, 1996). In addition, anti-topoisomerase I antibody levels closely correlate with disease activity and severity in SSc (Sato et al, 2001); however, the pathogenic relationship between systemic autoimmunity and the clinical manifestations of SSc, including skin and visceral fibrosis, remains unknown as autoantigens are generally intracellular components critical for cell mitosis and autoantibodies are not thought to contribute to tissue damage in SSc (Okano, 1996). This study suggests that systemic autoimmunity is linked to the development of fibrosis through the production of anti-MMP-1 antibody in SSc. Consistently, critical roles of pathogenic autoantibodies in disease expression have been demonstrated in a murine model of systemic autoimmunity: arthritis is provoked in healthy animals that received arthritogenic autoantibody from K/BxN mice, a model for human rheumatoid arthritis (Korganow et al, 1999; Matsumoto et al, 2002). In addition, a recent study has shown that the presence of anti-fibrillin 1 autoantibodies in SSc may be the link between fibrosis and systemic autoimmunity as normal fibroblasts treated with anti-fibrillin 1 antibodies display an activated phenotype overexpressing fibrillin 1 as well as some other ECM components.¹ Furthermore, we have recently reported that the elimination of autoantibody production results in decreased skin fibrosis in a tight-skin mouse, a genetic model for human SSc (Saito et al, 2002). Taken together, the presence of anti-MMP-1 autoantibody may be a clue for a role of systemic autoimmunity in the disease expression of SSc.

Although anti-MMP-1 autoantibody was detected in 75% of patients with dSSc, it was found only in 30% of patients with lSSc (Fig 1, Table I). This finding suggests that these autoantibodies deteriorate or develop the fibrosis rather than initiate the fibrosis. Although early studies reported decreased production of MMP-1 and MMP-3 by SSc fibroblasts (Bou Gharos et al, 1994; Takeda et al, 1994), recent studies of fibroblasts from patients with early dSSc (disease duration < 1 y) have shown that mRNA levels of MMP-1 and MMP-3 increase relative to normal fibroblasts (Kuroda and Shinkai, 1997). Consistently, recent cDNA microarray studies of dermal fibroblasts have shown increased levels of MMP-1 transcripts in SSc fibroblasts compared with control fibroblasts.² This augmented expression of MMP-1 is not due to the promoter single nucleotide polymorphism, but to secondary

factors, such as cytokines or growth factors, including transforming growth factor- β and interleukin-1 β , or other post-transcriptional events (Johnson et al, 2001). Therefore, as SSc has distinct autoimmune background, it is possible that the upregulation of MMP-1 expression in the early phase of dSSc induces anti-MMP-1 autoantibodies that may contribute to the development of fibrosis.

Important roles of TIMP in the pathogenesis of fibrosis have been suggested. Specifically, fibroblasts from SSc patients express elevated mRNA levels of TIMP-1, TIMP-2, and TIMP-3 (Kirk et al, 1995; Kikuchi et al, 1997; Kuroda and Shinkai, 1997; Mattila et al, 1998). In addition, patients with SSc exhibit elevated serum levels of TIMP-1 and TIMP-2 that correlate with the extent of skin sclerosis and lung fibrosis (Kikuchi et al, 1995; Yazawa et al, 2000). Like TIMP, IgG anti-MMP-1 antibody levels correlated with the extent of skin sclerosis, lung fibrosis, and vascular damage (Fig 2). Furthermore, IgG, but not IgM, anti-MMP-1 antibody was able to inhibit MMP-1 collagenase activity (Fig 4, and data not shown). Therefore, it is likely that IgG anti-MMP-1 antibody and TIMP cooperatively inhibit collagenase activity, which may result in the accumulation of ECM in SSc. The role of reduced type I collagen turnover by MMP inhibitors in the development of skin fibrosis is further supported by studies with transgenic mice in which the collagenase cleavage site in type I collagen is mutated rendering type I collagen resistant to cleavage by collagenase (Liu et al, 1995). These transgenic mice developed marked dermal fibrosis similar to human SSc (Liu et al, 1995). In addition to roles of MMP in the turnover of ECM, MMP are clearly implicated in angiogenesis as MMP inhibitors reduce angiogenic responses both *in vitro* and *in vivo* (Stetler-Stevenson, 1999). Vascular damage that results in scleroderma renal crisis and digital ulceration is an important manifestation in SSc. Anti-MMP-1 antibody may also contribute to SSc vasculopathy through inhibition of angiogenesis as well as vascular damage due to fibrosis. Thus, anti-MMP-1 antibody may be related to various aspects of SSc disease expression, including skin sclerosis, lung fibrosis, vasculopathy, and autoimmunity. In addition, IgG anti-MMP-1 antibody may be a useful, serologic marker for dSSc as they were detected in 75% of dSSc patients, whereas they were negative in 85% of lSSc patients. Collectively, the results of this study suggest that MMP inhibitors, including anti-MMP-1 antibody, would be potential therapeutic targets for SSc.

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