Type III procollagen N-terminal propeptide, soluble interleukin-2 receptor, and von Willebrand factor in systemic sclerosis

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Abstract

Objective

To evaluate the blood concentration of type III procollagen N-terminal propeptide (PIIINP), soluble interleukin-2 receptor (sIL-2R), and von Willebrand factor (vWF) in systemic sclerosis (SSc) patients.

Methods

PIIINP, sIL-2R, and vWF were measured in the sera and plasma of 29 SSc patients and 29 sex- and age-matched healthy controls. Serum PIIINP was determined by radioimmunoassay. Both serum sIL-2R and plasma vWF were measured by enzyme-linked immunosorbent assay (ELISA). Associations between concentrations and clinical and laboratory features were evaluated.

Results

Serum levels of PIIINP and sIL-2R were significantly higher in the SSc group than in the control group ($p < 0.01$ for both). No differences in serum PIIINP and sIL-2R levels were found between the limited and diffuse cutaneous subsets. However, PIIINP concentrations were significantly higher in anti-Scl-70 positive SSc patients compared with those of anti-Scl-70 negative patients ($p = 0.01$). Serum PIIINP levels were significantly higher in SSc patients with restrictive pulmonary function (FVC < 80%) than in patients with normal pulmonary function ($p < 0.05$). The correlation between PIIINP levels and FVC ($p < 0.05$) was negative, but the correlation between PIIINP levels and modified Rodnan skin scores ($p < 0.05$) was positive. sIL-2R levels were not correlated with skin and pulmonary involvement of SSc. There was no difference in vWF levels between those of the SSc patients and those of the control groups.

Conclusion

These results suggest that serum PIIINP serves as a biologic marker for the extent of skin and pulmonary involvement in systemic sclerosis. Increased serum levels of sIL-2R in SSc patients support a role for T lymphocyte activation in the pathogenesis of systemic sclerosis.

Key words

PIIINP, sIL-2R, vWF, systemic sclerosis.

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Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterized by fibrosis of the skin and internal organs such as the esophagus, the lungs, the heart, or the kidneys. The etiology of SSc is still unknown but excessive fibrosis, lymphocyte activation, and endothelial damage may play a major role in the pathogenesis. Type III procollagen N-terminal propeptide (PIIINP), soluble interleukin-2 receptor (sIL-2R) and von Willebrand factor (vWF), respectively, have been considered as markers representative of these components (1).

PIIINP is an aminopropeptide released during the synthesis of type III collagen. In SSc patients, it was reported that the levels of PIIINP were increased in serum or bronchoalveolar lavage fluid (2-7) and were related to the total skin scores or survival (2-5, 8). However, the results of studies concerning the association between PIIINP levels and lung involvement were not consistent. Some authors reported that they were not correlated (7,8), but others observed some correlation between them (9,10).

Activated CD4+ T cells in tissue specimens, a raised CD4+ /CD8+ ratio and increased levels of serum IL-2 or sIL-2R suggest that activated T cells may play an important role in the pathogenesis of SSc (11-13). However, different results were reported in the relationship between sIL-2R and clinical features of SSc (14-17).

vWF is released from damaged vascular endothelial cells and was reported to have increased levels in SSc patients (18, 19). Some reported a correlation between the level of vWF and internal organ involvement (20, 21), renal function (22), pulmonary perfusion, or pulmonary arterial pressure (22, 23).

In this study, we determined the concentrations of PIIINP, sIL-2R and vWF in 29 SSc patients and age- and sex-matched healthy controls, evaluating the correlation between their levels and clinical features of SSc.

Materials and methods

Patients

Twenty-nine SSc patients who took neither steroids nor immunosuppressive drugs were enrolled. All patients fulfilled the preliminary American Rheumatism Association criteria for classification of SSc (24) and did not have liver disease. Patients were classified as having diffuse (skin involvement in the extremities proximal to the elbows or knees and/or trunk) or limited (skin involvement in the extremities distal to the elbows and knees and/or face) cutaneous SSc (25). Fifteen patients had limited cutaneous involvement, while 14 had diffuse cutaneous involvement. None of the patients had any overlap with other connective tissue diseases. The control group consisted of 29 age- and sex-matched healthy persons who visited our hospital for medical checkups. There were 2 smokers in the patient group and 3 in the control group.

Clinical assessment

The clinical characteristics of the patient group were evaluated as follows: onset of systemic sclerosis, medications, Raynaud's phenomenon, digital ulcer or pitting scar, arthralgia, telangiectasia, sclerodactyly, subcutaneous calcification, modified Rodnan skin score and basilar crackle on auscultation. Laboratory tests included a blood cell count, Westergren ESR, serum creatinine, and creatinine clearance. Pulmonary involvement was assessed by chest x-ray, forced vital capacity (FVC), forced expiratory volume in one second (FEV1), and carbon monoxide diffusing capacity divided by the alveolar volume (DLco/VA). Restrictive pulmonary dysfunction was defined as FVC < 80% and FEV1/FVC > 80% and a decrement in the diffusing capacity was defined as DLco/VA < 80%. Interstitial lung disease was defined as the plain radiographic appearance of bibasilar interstitial fibrosis and/or honeycombing.

Autoantibodies

We investigated antinuclear antibody (ANA), anticentromere antibody (ACA), and anti-Scl-70 antibody in the patient group. ANA and ACA were detected by an indirect immunofluorescence technique using HEp-2 cell as

substrate. Anti-Scl-70 antibody was determined by double immunodiffusion (Immunovision, Springdale, AR).

PIIINP, sIL-2R, and vWF
Serum and plasma were stored at -70°C until the determination of PIIINP, sIL-2R and vWF.
Serum PIIINP was measured by radioimmunoassay (Orion Diagnostica Co., Espoo, Finland). Serum sample and ^{125}I-labelled PIIINP were mixed to compete for rabbit anti-human PIIINP antibody. Anti-PIIINP antibody were added and the mixture was incubated for 2 hours at 37°C. Separation reagent was added and the tubes were centrifuged for precipitation at 2,000 g at 4°C. The supernat was decanted and residual radioactivity was measured with a gamma counter. The final concentration of the samples was determined by interpolation from the standard curve.
Serum sIL-2R was assayed by ELISA (DAKO Co., Glostrup, Denmark). The serum sample was added to microplates pre-coated with mouse anti-human IL-2R antibody. Peroxidase-conjugated anti-sIL-2R antibody was added for sandwich formation. The microplate was incubated for 2 hours at room temperature using a mixing apparatus. Tetramethylbenzidine solution was added as chromogenic substrate and absorbance was measured at 450 nm. Concentrations of sIL-2R in the specimens were determined by interpolation from the standard curve.
Plasma vWF was determined by ELISA (Diagnostica Stago Co., Chausson, France). The plasma sample was added into wells pre-coated with rabbit anti-human vWF antibody and then incubated for 2 hours at room temperature. Peroxidase-conjugated anti-vWF antibody was used as a secondary antibody. Color was developed by the addition of ortho-phenylenediamine and absorbance was measured at 492 nm. Each measurement was duplicated.

Statistics
Statistical analyses were performed by SPSS for Windows (version 9.0). The Wilcoxon signed-rank test was used for the comparison of PIIINP, sIL-2R, and vWF levels in SSs with those in the control group. The Mann-Whitney test was carried out for continuous variables and Fisher's exact test for dichotomous variables. Correlation was calculated using Spearman's correlation coefficient. Each measured value was described as the median and range. P values less than 0.05 were considered significant.

Results
Patients
The patient group consisted of 24 females and 5 males. The median age was 44 [21 - 71] years and the median disease duration was 90 [11 - 280] months. The clinical features of the diffuse and limited cutaneous subsets are summarized in Table I. There was no significant difference between the two groups except for their modified Rodnan skin scores. Eleven patients (37.9%) had interstitial fibrosis or honeycombing on chest x-ray: 5 of 15 patients (30.0%) in the limited cutaneous subset and 6 of 14 patients (42.9%) in the diffuse cutaneous subset. Eighteen patients (66.7%) had restrictive pulmonary dysfunction; 8 (53.3%) in the limited cutaneous subset and 10 (71.4%) in the diffuse cutaneous subset. Pulmonary involvement was not associated with the cutaneous subsets. In the limited cutaneous subset, 11 patients had early (< 5 years) and 4 had late disease. In the diffuse cutaneous subset, 12 had early (< 2 years) and 2 had late disease. The distribution of early and late disease was not significantly different for the two subsets and there was no difference in clinical features in early and late disease.

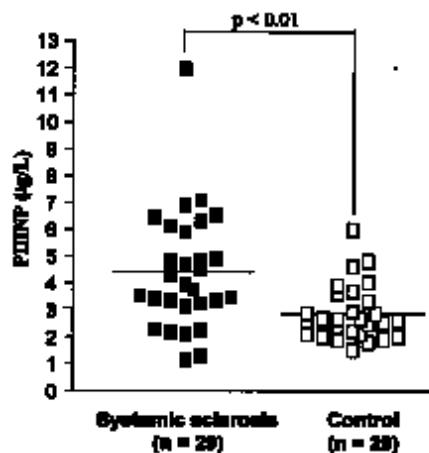*Levels of PIIINP, sIL-2R, and vWF*
The serum concentration of PIIINP levels were significantly increased in SSc (3.94 [1.15 - 11.96] g/L) compared to those of the control group (2.59 [1.51 - 6] g/L; p < 0.01, Fig. 1). Eleven patients had serum PIIINP levels above the 95th percentile (4.74 g/L) of the control group. The characteristics of

Table I. Clinical features and laboratory findings of systemic sclerosis group.
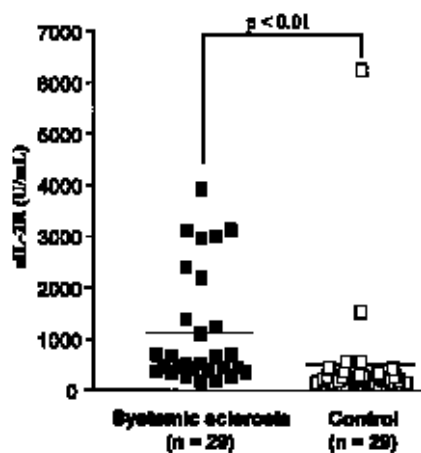
	All patients (n = 29)		Limited cutaneous subset (n = 15)		Diffuse cutaneous subset (n = 14)	
Age (years)*	44	[21~71]	49	[36~64]	40	[21~71]
Sex (M : F)	5:24		2 : 13		3:11	
Disease duration (months)*	90	[11~280]	90	[12~210]	69	[11~280]
Total skin score#*	10	[2~44]	9	[2~14]	22	[8~22]
Raynaud's phenomenon	29	(100%)	15	(100%)	14	(100%)
Sclerodactyly	29	(100%)	15	(100%)	14	(100%)
Digital pitting scar	20	(69.0%)	11	(73.3%)	9	(64.3%)
Arthralgia	11	(37.9%)	6	(40.0%)	5	(35.7%)
Telangiectasia	7	(24.1%)	2	(13.3%)	5	(35.7%)
Subcutaneous calcification	2	(6.9%)	1	(6.7%)	1	(7.1%)
Bibasilar rale	9	(31.0%)	5	(33.3%)	4	(28.6%)
Westergren ESR (mm/hr)*	19	[2~110]	22	[2~110]	18	[4~83]
Creatinine clearance (mL/min)*	80	[62~159]	80	[68~159]	84	[62~124]
Interstitial lung disease	11	(37.9%)	5	(30.0%)	6	(42.9%)
FVC less than 80%	18	(66.7%)	8	(53.3%)	10	(71.4%)
DLco/V$_A$ less than 80%	7	(25.9%)	4	(26.7%)	3	(25.0%)
Antinuclear antibody	28	(97.0%)	15	(100%)	13	(92.9%)
Anti-Scl-70 antibody	20	(69.0%)	10	(66.7%)	10	(71.4%)
Anticentromere antibody	2	(6.9%)	2	(13.3%)	0	(0.0%)

# p < 0.01 when comparing the limited cutaneous subset with the diffuse cutaneous subset; *median [range].
FVC = forced vital capacity, DLco/V$_A$ = carbon monoxide diffusing capacity divided by alveolar volume.
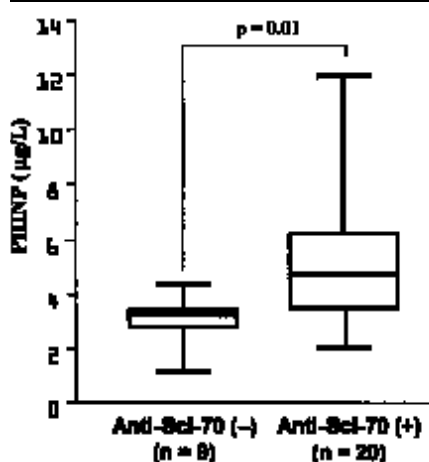
Fig. 1. Serum levels of PIIINP in systemic sclerosis patients and healthy controls. The patient group had significantly higher serum levels of PIIINP than the control group (p < 0.01). The horizontal line indicates the median for each group.
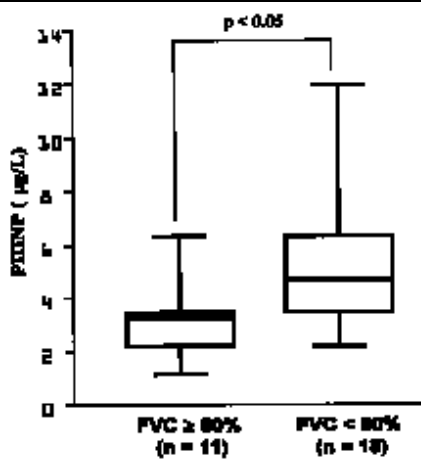

Fig. 2. Serum sIL-2R concentrations in the systemic sclerosis and healthy control groups. Serum sIL-2R concentration was significantly higher in the patient group when compared with the control group (p < 0.01). The horizontal line indicates the median for each group.

these patients showed a higher incidence of anti-Scl70 (100% versus 61.1%; p < 0.05) and a higher level of IL-2R (1243.1 [3923.8 - 262.7] versus 443.4 [3104.8 - 171.4] U/mL; p< 0.05), when compared to those with a serum PIIINP level below the 95th percentile. sIL-2R levels were significantly increased in SSc (525.5 [171.4 - 3923.8] U/mL) compared to those of the control group (248 [117.5 - 6246.1] U/mL; p < 0.01, Fig. 2). There was no difference in plasma vWF levels between the SSc and control groups (106% [93% -

252.4%] and 106 % [64% - 108%], respectively). There was no difference in PIIINP, sIL-2R and vWF levels between the limited and diffuse cutaneous subsets (3.45 [1.32 - 11.96] versus 4.51 [1.15 -7.1]   g/L, 529.4 [178.3 - 3119.2] versus 612.46 [171.4 - 3932.8] U/mL, and 106 [101 - 148.6] versus 106 [93 - 252.4] %, respectively). Those concentrations were not different in early and late SSc. Serum PIIINP levels were significantly increased in anti-Scl-70 positive patients compared to those of anti-Scl-70 negative patients

(4.76 [2.12 - 11.96]   g/L and 3.20 [1.15 - 4.83]   g/L, respectively; p = 0.01, Fig. 3). PIIINP levels were significantly higher in patients with restrictive pulmonary dysfunction than in those with normal pulmonary function (4.68 [2.17- 11.96]   g/L and 3.26 [1.05 - 6.30]   g/L, respectively; p < 0.05, Fig. 4). But, PIIINP, vWF, and sIL-2R levels were not increased in patients with interstitial lung disease on chest x-ray.

Correlations between the levels of PIIINP, sIL-2R and vWF and clinical features
There was a significant negative correlation between FVC and serum PIIINP concentrations (p < 0.05, r = -0.40195, by Spearman's correlation test, Fig. 5A) and a positive correlation between the modified Rodnan skin scores and serum PIIINP concentrations (p < 0.05, r = 0.36753, Fig. 5B). Serum levels of sIL-2R were correlated with the Westergren ESR (p < 0.05, r = 0.410). However, other clinical variables were not correlated with the levels of serum sIL-2R and plasma vWF.

Discussion
Previous studies reported that the serum levels of PIIINP were higher in SSc patients than in those with normal persons and were correlated with the extent of skin involvement (2-5, 8). Some reports demonstrated that the serum PIIINP levels were higher in the diffuse cutaneous subset than in the limited cutaneous subset (3, 8) and were not related to anti-Scl-70 or anti-centromere antibody (4). However, Scheja *et al*. showed no difference in PIIINP levels between the diffuse and limited cutaneous subsets of SSc (5).
Our study showed that serum PIIINP levels in SSc patients were higher than those in healthy controls and that the levels were positively correlated with skin scores. A significant difference in PIIINP levels was not observed between the limited and diffuse cutaneous subsets, whereas anti-Scl-70 positive patients had higher levels of serum PIIINP than did anti-Scl-70 negative patients. The relationship of anti-Scl-70 antibody and serum PIIINP levels has

Fig. 3. Serum PIIINP levels in systemic sclerosis patients with or without anti-Scl-70 antibody. Anti-Scl-70 positive patients had significantly higher PIIINP levels than anti-Scl-70 negative patients (p = 0.01). The box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th percentile) and whiskers show the range of the data.
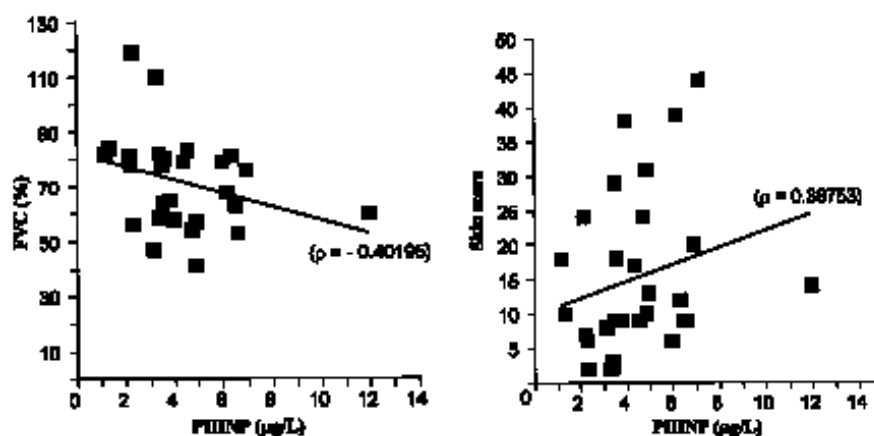

Fig. 4. Serum PIIINP levels in systemic sclerosis patients with FVC < 80% versus patients with FVC   80%. The PIIINP levels were significantly higher in patients with FVC < 80% than in patients with FVC   80% (p < 0.05). The box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th percentile) and whiskers show the range of the data.

Fig. 5. Correlation of serum PIIINP levels with FVC and the modified Rodnan skin scores in systemic sclerosis patients. (**A**) There was significant negative correlation between serum PIIINP levels and FVC (p < 0.05, by Spearman's correlation test). (**B**) There was significant positive correlation between serum PIIINP levels and the modified Rodnan skin scores (p < 0.05).   (rho) indicates the Spearman coefficient.

not yet been reported. Anti-Scl-70 positivity has been described in patients in the diffuse cutaneous subset and/or with pulmonary involvement. In this study, however, patients with diffuse cutaneous SSc did not show a higher frequency of anti-Scl-70 positivity or pulmonary involvement compared to limited cutaneous subset.

Serological profiles are known to have a predictive value in the determination of the patient's course; anti-Scl-70 positive patients have rapid progression, low survival rates, and higher incidences of pulmonary fibrosis (26, 27). Patients with progression were reported to have high serum PIIINP levels (2-4). Scheja *et al*. found that the highest serum PIIINP concentration was seen in patients who died within 2 years (5). Thus, serum PIIINP has been suggested as a prognostic marker in systemic sclerosis.

Interestingly, two clinical subsets in our study did not have significantly different distributions of anti-Scl-70. One possible explanation is that the limited cutaneous patients may have been recruited for this study late in their course but at one time might have had diffuse cutaneous involvement earlier in their disease. However, there was no significant difference in disease duration between the limited and diffuse cutaneous patients and none in the limited cutaneous subset had widespread skin involvement in their history. Another possibility is that patients who later

developed diffuse cutaneous SSc were initially classified as having limited SSc. Classification error may be less likely in view of the natural course; in this study, the median duration was 90 months in the limited cutaneous subset and 68.5 months in the diffuse cutaneous subset. The third explanation could be the small patient number or racial differences. It was reported that there were racial differences in the frequencies of autoantibodies in systemic sclerosis (28). Kang *et al*. reported that anti-Scl-70 was comparably distributed in two cutaneous subsets in 56 Korean SSc patients. He found the association of anti-Scl-70 positive SSc with HLA-DRB1*1502 and anti-Scl-70 negative limited SSc with HLA-DRB1*1501 (29). The positivity of anti-centromere antibody was 13.3% in the limited cutaneous subset in this study and it has been reported that 15-37% of Japanese patients with limited cutaneous involvement have the anti-centromere antibody (30, 31). Therefore, Asian SSc patients may have a lower incidence of anti-centromere antibody than Caucasians (50-90%).

Lung involvement in SSc manifests as pulmonary fibrosis or hypertension and is a leading cause of death in SSc patients. Although pulmonary function tests, high-resolution computed tomography, bronchoalveolar lavage, and echocardiography are generally used for the diagnosis (27), they have limited utility in the early detection of pul-

monary involvement. A simple serological marker has still not been found. We showed that SSc patients with restrictive pulmonary dysfunction had higher PIIINP levels and that PIIINP levels were negatively correlated with FVC. Valat *et al*. and Diot *et al*. reported that SSc patients with lung involvement had higher PIIINP levels than those without lung involvement (9,32). Kuhn *et al*. showed that collagen III histologically increased in the extracellular matrix of pulmonary fibrosis (33). These findings suggest that serum PIIINP may be a serologic marker of pulmonary involvement in SSc patients.

Increased serum levels of sIL-2R imply T cell activation. We showed that sIL-2R levels were higher in SSc patients than in healthy controls. Silver reported that peripheral lymphocytes in SSc, when stimulated by IL-2, were cytotoxic to vascular endothelial cells (34). Hawrylko *et al*. observed that synthesis of IL-2 increased in peripheral blood mononuclear cells when stimulated by type I collagen (35). In addition, Bruns *et al*. reported that serum IL-6 levels, co-stimulators of T cells, were positively correlated with serum PIIINP levels (13). However, we did not observe any correlation between sIL-2R and PII-INP. In previous studies concerning the association of serum sIL-2R levels with clinical features - skin scores (15, 17), internal organ involvement (14, 16) or cutaneous subsets (11, 14, 17) - the results were not constant. We did not find any relationship between serum sIL-2R levels and skin scores, FVC, DLco/VA, disease duration, or serological status. Therefore, increased levels of sIL-2R can imply that activated T cells are relevant to the pathogenesis of SSc and serum sIL-2R is not thought to be a useful marker in the clinical assessment of SSc.

Plasma vWF levels were not elevated in SSc patients when compared with those of control patients. This is in contrast with previous results (18-23) and may result from the absence of pulmonary hypertension or azotemia in our patient population. In this study, there was no patient with a normal FVC and inappropriately low DLco/VA and no patient was clinically suspected of hav-

ing pulmonary vascular disease. Scheja *et al.* reported that plasma levels of vWF were correlated with renal function and pulmonary perfusion (22). Matucci-Cerinic *et al.* found a correlation between plasma vWF concentration and pulmonary arterial pressure (23).

In conclusion, we found that SSc patients had higher concentrations of PIIINP and sIL-2R. Serum PIIINP was associated with anti-Scl-70 positivity, restrictive pulmonary dysfunction, and skin scores. These results indicate that serum PIIINP can be a serologic marker of pulmonary and skin involvement in SSc patients. Increased sIL-2R concentration supports the important role of T lymphocytes in the pathogenesis of SSc.

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