Is soluble CD40 ligand an indicator of immunopathological disturbance in silicosis patients?

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ABSTRACT Silicosis patients (SIL) develop respiratory fibrosis caused by inhaled silica particles, and autoimmune disorders such as rheumatoid arthritis and systemic sclerosis (SSc). Silica is known as one of the most important environmental factors leading to the development of SSc. Various soluble cytokines such as interleukin (IL)-2 receptor (sIL-2R) and CD155/CD40 ligand (sCD40L), soluble endoglin, von Willebrand factor antigen (vWFAg), and soluble Fas show elevated levels in SSc. Among these parameters, sCD40L was targeted as a possible subclinical parameter in SIL. Factor analysis using the data of ten SIL showed that sCD40L was correlated only with sFas, whereas sCD40L formed a factor showing immunological alteration (without respiratory progression) with several other immunopathological parameters such as the titer of anti-nuclear antibodies (ANA) and immunoglobulin (Ig) G. However, sCD40L assays using ten healthy donors (HD), ten SIL and ten SSc patients did not show a higher level for SSc, although ANA and sIL-2R levels were correlated with a progressive ranking (HD as 1, SIL as 2, SSc as 3). The overall results suggest that sCD40L in SSc should be examined in regard to the state of the disease. At present, sCD40L is not considered a good indicator of subclinical disturbance in SIL.

(Accepted on January 1, 2009)

Key words: **Silicosis, Soluble CD40 ligand, Autoimmunity, Systemic sclerosis**

INTRODUCTION

Silica is known to cause pulmonary fibrosis and autoimmune diseases such as rheumatoid arthritis (RA), systemic sclerosis (SSc) and systemic lupus erythematosus (SLE)[1,2]. RA complicated with silicosis (SIL) is well known as Caplan's syndrome[3]. SSc is caused by some environmental substances such as silica and vinyl chloride[4,5]. These substances are also associated with other autoimmune disorders such as systemic vasculitis

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and pemphigus vulgaris complicated with SIL[6, 7]. Moreover, various autoantibodies have been detected in the sera of SIL without any clinical manifestations of autoimmune disorders, i.e., antibodies against desmoglein, Fas, and caspase 8 molecules[8-10].

On the other hand, there have been reports of many molecules related to immunoreactions such as soluble interleukin (IL)-2 receptor (sIL-2R), soluble CD40 ligand (sCD40L), soluble endoglin, von Willebrand factor antigen (vWFAg), and soluble Fas[11-17] in SSc.

We have been investigating the possibility of using several serum markers as preclinical parameters for SIL with immunopathological disturbances and mechanisms involved in silica-induced dysregulation of autoimmunity[18-22]. In particular, the following aspects of Fas and Fas-related molecules have been investigated: (i) elevated levels of serum-soluble Fas (sFas) in SIL[23], (ii) excessive expression of soluble rather than wild-type Fas genes in peripheral blood mononuclear cells (PBMCs) from SIL[24], (iii) excessive detection of variant messages of Fas transcripts and sFas[25], (iv) excessive expression of decoy receptor 3 (DcR3) in PBMCs from SIL[26], (v) reduced expression of intracellular anti-apoptotic molecules related to Fas-mediated apoptosis in PBMCs from SIL[27, 28], and (vi) detection of functional (apoptosis inducible) serum anti-Fas autoantibody in SIL[9]. These Fas-related parameters were isolated as immunological markers from other respiratory clinical parameters such as percent vital capacity (%VC), profusion rate (PR), which is a category pertaining to radiological findings according to the International Labor Office (ILO) guideline[29], and the percentage of forced expiratory volume in 1 second (FEV1.0%)[30].

As mentioned above, sCD40L has been reported as a serum marker for SSc. The CD40/CD154 (CD40L) interaction is important for activation of humoral and cellular responses[31-39]. CD40 and its ligand belong to the tumor-necrosis factor (TNF) receptor superfamily, and both molecules are transmembrane glycoproteins. However, both can be cleaved easily by proteolytic activity. Activated T cells express CD40L and interact with CD40 on the surface of B cells. During this reaction, the cleavage of CD40L occurs and a soluble form of CD40L with biological activities is produced. The sCD40L can bind with CD40 and enhances the reaction. Thus, it appears that sCD40L reflects the degree of T cell activation in autoimmune diseases, and elevated serum sCD40L levels in SSc have been reported[11-17].

We recently reported that sIL-2R is a marker for immunopathological disturbance in SIL without clinical manifestation of autoimmune diseases[40]. We then considered the possibility that sCD40L is also a clinical parameter for immunopathological disturbance in SIL. In this article, details of the measurement of sCD40L in HD, SIL and SSc patients are presented and the clinical role of sCD40L in SIL is discussed.

SUBJECTS AND METHODS

Subjects and clinical parameters

All subjects were Japanese. Ten HD (age (mean ± standard deviation (SD)) = 60.6 ± 5.8, male:female = 6:4), ten SIL (age = 74.4 ± 8.7, M:F=7:3) and ten SSc patients (age = 50.8 ± 13.3, M:F=2:8) were investigated in this study. All SIL were brickyard workers in Bizen City, Okayama Prefecture, Japan, and were monitored at Kusaka Hospital. The amount of free silica inhaled by these patients was estimated as high as 40 to 60% as determined from their work environment. The subjects were diagnosed with pneumoconiosis according to the ILO 2000 guideline[29]. They showed no clinical symptoms of autoimmune diseases, including sclerotic skin, Raynaud's phenomenon, facial erythema or arthralgia. The SSc patients were monitored by the Department of Dermatology,

Kawasaki Medical School Hospital, Japan. Eight of the ten SSc patients were administered oral corticosteroids. Heparinized peripheral blood was drawn from the cubital vein from all subjects. Serum samples from all subjects were analyzed for anti-nuclear antibodies (ANA) using the Enzyme-Linked ImmunoSorbent Assay (ELISA)-based MESACUP ANA Test Kit (MBL Co. Ltd., Nagoya, Japan), serum immunoglobulin (Ig) G concentration, complement (C) 4, IL-2 concentration, sFas, titer of Anti-Scl-70 antibody (Ab), titer of anti-centromere (CM) Ab, sIL-2R and sCD40L (all parameters were measured using individual ELISA kits, MBL). The ANA titer performed in this study included several recombinant proteins such as RNP, SS-A/Ro, SS-B/La, Scl-70, Jo-1 and Ribosomal P *in vitro* transcribed U1 RNA and CENP-B protein, and purified antigens (Sm, SS-A/Ro, Scl-70m Histone and DNA). All measurements were performed according to the manufacturer's instructions, and ANA, anti-Scl-70 and anti-CM Abs measurements are presented as index values. Clinical data (exposure years, FEV1.0%, %VC, PR (numbered according to the ILO guideline, 1 to 4) and subjective dyspnea (numbered as 1: slight, 2: mild, 3: moderate, and 4: severe, according to the Hugh-Jones classification) were provided by Kusaka Hospital. All specimens were taken only when informed consent had been obtained. This study was approved by the Ethics Committees of Kawasaki Medical School and Kusaka Hospital.

Statistical analysis

Correlations in SIL for all respiratory and immunological parameters with age were initially analyzed. Factor analysis was performed to determine clinical parameters related to the other parameters. Parameters showing factor loading values greater than ±0.4 were regarded as meaningful for factor configuration. The data can therefore be used to determine (i) parameters that contribute to the factor, (ii) how the values of contributing parameters (values near 1.0 are strongly related to the formation of the factor, whereas minus (or plus) values can be interpreted as reflecting a lower (or higher) contribution by this parameter) explain the tendency of the factor, and (iii) the contribution ratio of factors[30].

To determine whether the immunological parameters (including C4, Ig G, IL-2, ANA, anti-Scl-70 Ab, anti-CM Ab, sIL-2R and sCD40L) were related to the progression of altered autoimmunity, differences between HD, SIL and SSc patients were analyzed for each parameter. The statistical differences among the three groups were analyzed using the Mann-Whitney test. In addition, after defining HD = 1, SIL = 2 and SSc =3 as metric variables for immunological progression, the correlation between these numbers and each parameter value was analyzed. All statistical analyses were performed using StatView software version 5.0 (SAS Institute Inc. Cary, NC, USA) and StatFlex version 5.0 software for Windows (Artech Co. Ltd., Osaka, Japan), which yield results that are compatible with SPSS software.

RESULTS

Correlation of clinical parameters in SIL

As shown in Table 1, reciprocal correlations among all clinical parameters investigated in this study in SIL were analyzed. First of all, age was related negatively with %VC and positively with IL-2. These findings may reflect the physiological age-related changes of both parameters. Among the respiratory parameters, there was a reasonable negative correlation between dyspnea and FEV1.0%. On the other hand, analysis of assumed immunological parameters revealed that Ig G was correlated positively with ANA, sIL-2R, anti-Scl-70 Ab and anti-CM Ab, and negatively with C4. The results also showed a negative correlation between C4 and ANA, and positive correlations between

Table 1. Correlation between various clinical parameters among silicosis patients

	Age (years)	Duration of exposure (years)	PR (numbered)	Subjective Dyspnea (numbered)	%VC (%)	FEV1.0 % (%)	Ig G (mg/dl)	C4 (mg/dl)	ANA (Index Value)	IL-2 (pg/ml)	sIL-2R (pg/ml)	sFas (ng/ml)	Anti-Scl-70 Ab (Index Value)	Anti-CM Ab (Index Value)	sCD4oL (pg/ml)
Age (years)		.061 .8813	-.190 .3367	.385 .2828	**-.695** **.0233**	-.407 .2534	.289 .4304	-.109 .7721	.609 .0612	**.652** **.0392**	.324 .3739	**.604** **.0868**	-.161 .6684	.333 .9601	**.600** **.0669**
Duration of exposure (years)			.500 .1788	-.158 .6960	.248 .5352	-.238 .5529	-.135 .7396	-.055 .8921	-.148 .7145	-.140 .7308	-.154 .7046	.271 .5339	.095 .8163	-.163 .6870	-.055 .8919
PR (numbered)				.379 .2908	-.116 .7579	-.028 .9420	-.212 .6588	**.679** **.0287**	-.503 .1434	.027 .9437	-.133 .7233	-.401 .2979	-.228 .5387	.131 .7280	.081 .8301
Subjective Dyspnea (numbered)					-.473 .1742	**-.753** **.0096**	-.167 .6553	.316 .3865	-.040 .9162	.204 .5848	-.058 .8771	-.184 .6476	-.288 .4333	.002 .9541	.019 .9595
%VC (%)	**-.695** **.0233**					.306 .4033	-.107 .7760	-.185 .6203	-.325 .3723	**-.706** **.0199**	-.122 .7464	-.204 .6126	.075 .8427	-.141 .7080	-.295 .4205
FEV1.0% (%)				**-.753** **.0096**			.455 .1934	-.171 .6480	.038 .9201	-.348 .3367	.391 .2750	.079 .8468	.460 .1885	.384 .2846	.146 .6978
Ig G (mg.dl)								**-.658** **.0369**	**.766** **.0076**	.238 .5204	**.988** <.0001	.407 .2901	**.794** **.0042**	**.823** **.0020**	.151 .6870
C4 (mg/dl)			**.679** **.0287**				**-.658** **.0369**		**-.775** **.0063**	-.173 .6435	**-.586** **.0757**	.124 .7594	**-.604** **.0640**	-.203 .5858	.266 .4709
ANA (Index Value)							**.766** **.0076**	**-.775** **.0063**		.538 .1112	**.745** **.0110**	.526 .1523	.463 .1844	.518 .1287	.152 .6854
IL-2 (pg/ml)	**.652** **.0392**				**-.706** **.0199**						.218 .5569	.081 .8429	.123 .7427	.102 .7875	.004 .9908[
sIL-2R (pg/ml)							**.988** <.0001		**.745** **.0110**			.418 .2753	**.750** **.0100**	**.886** **.0002**	.203 .5857
sFas (ng/ml)													-.337 .3899	.472 .2037	**.715** **.0281**
Anti-Scl70 Ab (Index Value)							**.794** **.0042**				**.750** **.0100**			.529 .1189	-.376 .2951
Anti-CM Ab (Index Value)							**.823** **.0020**				**.886** **.0002**				.464 .1840
sCD4oL (pg/ml)												**.715** **.0281**			

In each cell in the upper-right half of the table, the upper value represents ρ and the lower entry is the p value. Significant correlations are listed in the lower-left half of the table. Correlations showing p < .01 are presented in bold and significant (p<.05) correlations are shaded in gray.

sIL-2R and ANA, anti-Scl-70 Ab, or anti-CM Ab. These correlations are reasonable and it is thought that several immunopathological parameters showed similar directions in the development of disturbance. Results concerning sCD40L showed that only the sFas level was positively correlated. Thus, sCD40L may not be a strong marker of immunopathological progression in SIL. In addition, a positive correlation between C4 and PR and a negative correlation between IL-2 and %VC were observed. Although the latter may be interrupted by age, the former was not interrupted to a considerable degree. However, this finding may be coincidental.

Factor analysis

Factor analysis was performed to determine clinical parameters related to the other parameters. Parameters showing factor loading values greater than ± 0.4 were regarded as meaningful for factor configuration and are shaded in gray in Table2. The data can therefore be used to determine (i) parameters that contribute to the factor, (ii) how the values of contributing parameters (values near 1.0 are strongly related to the formation of the factor, whereas minus (or plus) values can be interpreted as reflecting a lower (or higher) contribution by this parameter) explain the tendency of the factor, and (iii) the contribution ratio of factors[30].

According to results shown in Table 2, factor 1 consists of several respiratory variables (FEV1.0, %VC and subjective dyspnea) and several immunological variables (sFas, IL-2, ANA and anti-Scl-70 Ab). FEV1.0, %VC and v25/Ht having minus-loading values were recognized as representing a worse respiratory function. Similarly, dyspnea with a plus-loading value indicated a worse respiratory disease status. In regard to immunological variables, the plus-loading values of IL-2 and ANA are thought to represent a worse immunological status, whereas minus values of Scl-

Table 2. Factor analysis

Parameter	Factor 1	Factor 2	Factor 3
Age (years)	0.97916	0.27601	0.06296
Exposure Years (years)	-0.05027	-0.01221	0.38142
FEV1.0% (%)	-0.62279	0.49622	0.20688
%VC	-0.70821	-0.14560	0.20396
PR (Radiological Classification)	-0.16950	0.12272	-0.89780
Subjective Dyspnea (numbered)	0.49768	-0.23287	-0.71221
Serum Ig G (log) (mg/d)	-0.20556	0.90564	0.06658
C4 (mg/dl)	0.00646	0.43799	-0.73200
Serum-soluble Fas(ng/ml)	0.42936	0.57232	0.36309
Serum IL-2 (pg/ml)	0.88604	-0.34320	0.07629
Serum-soluble IL-2R (pg/ml)	-0.05009	0.95234	-0.29647
Serum ANA titer (index)	0.74471	0.00927	0.47323
Serum Anti-Scl-70 Ab (index)	-0.78648	-0.09121	0.13032
Serum Anti-Centromere-Ab (index)	0.01392	0.85914	-0.40600
sCD40L (ng/ml)	0.39918	0.83605	-0.00505
Contribution Ratio	29.82693	28.15242	18.12474

Factor analysis was performed to determine clinical parameters related to the other parameters. Parameters showing factor loading values greater than ± 0.4 were regarded as meaningful for factor configuration and are shaded in gray. The data can therefore be used to determine (i) parameters that contribute to the factor, (ii) how the values of contributing parameters (values near 1.0 are strongly related to the formation of the factor, whereas minus (or plus) values can be interpreted as reflecting a lower (or higher) contribution by this parameter) explain the tendency of the factor, and (iii) the contribution ratio of factors. According to the analysis shown here, factor 1 consists of several respiratory variables (FEV1.0, %VC and subjective dyspnea) and several immunological variables (sFas, IL-2, ANA and anti-Scl-70 Ab). FEV1.0, %VC and v25/Ht parameters with minus values were recognized as representing a worse respiratory function. Similarly, dyspnea with a plus-loading value indicated a worse respiratory disease status. In regard to immunological variables, plus-loading values of IL-2 and ANA are thought to represent a worse immunological status, whereas minus values of Scl-70 do not. Overall, factor 1 should be interpreted as representing patients having a tendency for both a respiratory and immunological worse status, with 30% of patients possessing this tendency.

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The sCD40L parameter was involved in the formation of factor 2. This factor consisted mostly of immunological parameters such as Ig G, C4, sFas, sIL-2R and anti-CM Ab, with a weak contribution by FEV1.0%. As the plus- but weak loading values of C4 and FEV1.0% were considered unimportant, this factor can be viewed as an immunological factor. The patients (28%) associated in this factor are considered to have progressive immunopathology without the development of respiratory alterations. It is important that sCD40L was involved in this factor because this supports the assertion that sCD40L in SIL also represents an immunopathological marker.

Factor 3 was associated with better PR and dyspnea, and worse C4 in 18% of patients. It is difficult to interpret this factor; however, 18% of patients did not show any worse respiratory progression with slight disturbance of immunopathology.

Statistical differences between several immunopathological parameters in HD, SIL and SSc patients

ANA, Ig G, sIL-2R and sCD40L values were compared among HD, SIL and SSc patients. As shown in Fig. 1, the titer of ANA in HD was lower than that of SIL and SSc. The titer of ANA in SIL was also lower than that of SSc. Thus, ANA increased significantly from HD to SIL to SSc. The levels of Ig G and sIL-2R in SSc were higher than those of HD. However, levels in SIL did not show any differences from those of HD or SSc. Surprisingly, sCD40L in SSc was lower than that of HD or SIL. These results are the reverse of those showing higher levels of sCD40L in SSc[11-17]. This finding may be dependent on the therapeutic status of SSc in this study. Eight of the ten SSc patients were administered glucocorticoids for medication. However, other immunopathological parameters such as ANA, Ig G and sIL-2R showed

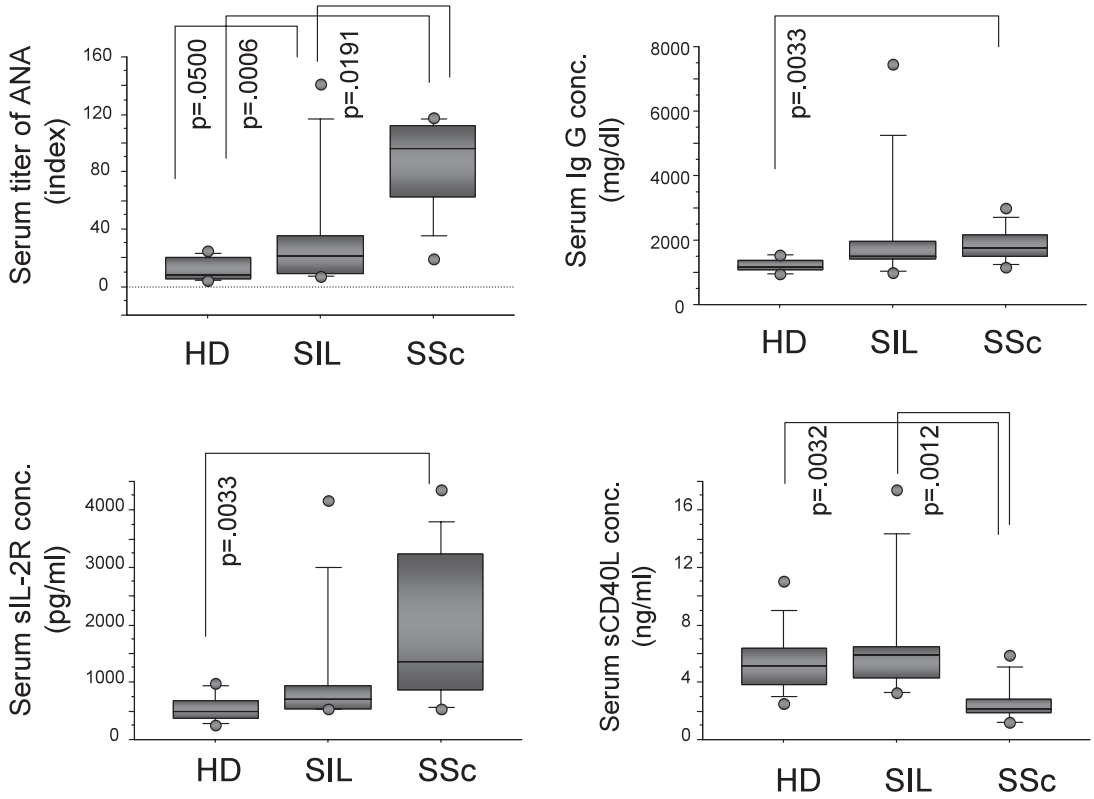

Fig. 1. Analysis of statistical differences between index values of serum titers of anti nuclear antibodies analyzed using the Enzyme-Linked ImmunoSorbent Assay (ELISA)-based MESACUP ANA Test Kit (MBL Co. Ltd., Nagoya, Japan), serum immunoglobulin (Ig) G level, serum soluble interleukin (IL)-2 receptor (sIL-2R) level analyzed by ELISA (MBL), and serum soluble CD40 ligand (sCD490L) level assayed by ELISA (MBL) among healthy donors (HD), silicosis patients (SLI) and patients with systemic sclerosis (SSc). Statistical significance was examined using the Mann-Whitney test. Values of $p \leq 0.5$ are shown in the figure.

significantly higher values. It is possible that levels of sCD40L fluctuated in response to the degree of disease activity. However, higher numbers of SSc patients should be examined with respect to disease activities reported previously[11–17].

The statistical analysis indicated that ANA is the only candidate for the detection of immunopathological alteration in SIL without any clinical manifestation of autoimmune disorders. However, in order to determine early subclinical markers for immunopathological status in SIL, the correlations between these parameters and hypothetical immunopathological progression status were assayed, using HD as 1, SIL as 2, and SSc as 3. These results are presented in Fig. 2 and show that serum levels of ANA and sIL-2R exhibited significant positive correlations with disease status, whereas levels of Ig G and sCD40L showed no such correlation. These findings indicate that levels of ANA and sIL-2R were the only good markers of autoimmune dysfunction in SIL, although factor analysis showed that sCD40L formed an immunological factor with other immunopathological parameters, including sIL-2R.

DISCUSSION

In order to analyze immunopathological dysfunction in SIL, it is important to consider the preclinical status of autoimmune disorders such as SSc and SLE[1, 2]. Reports of alterations in Fas and

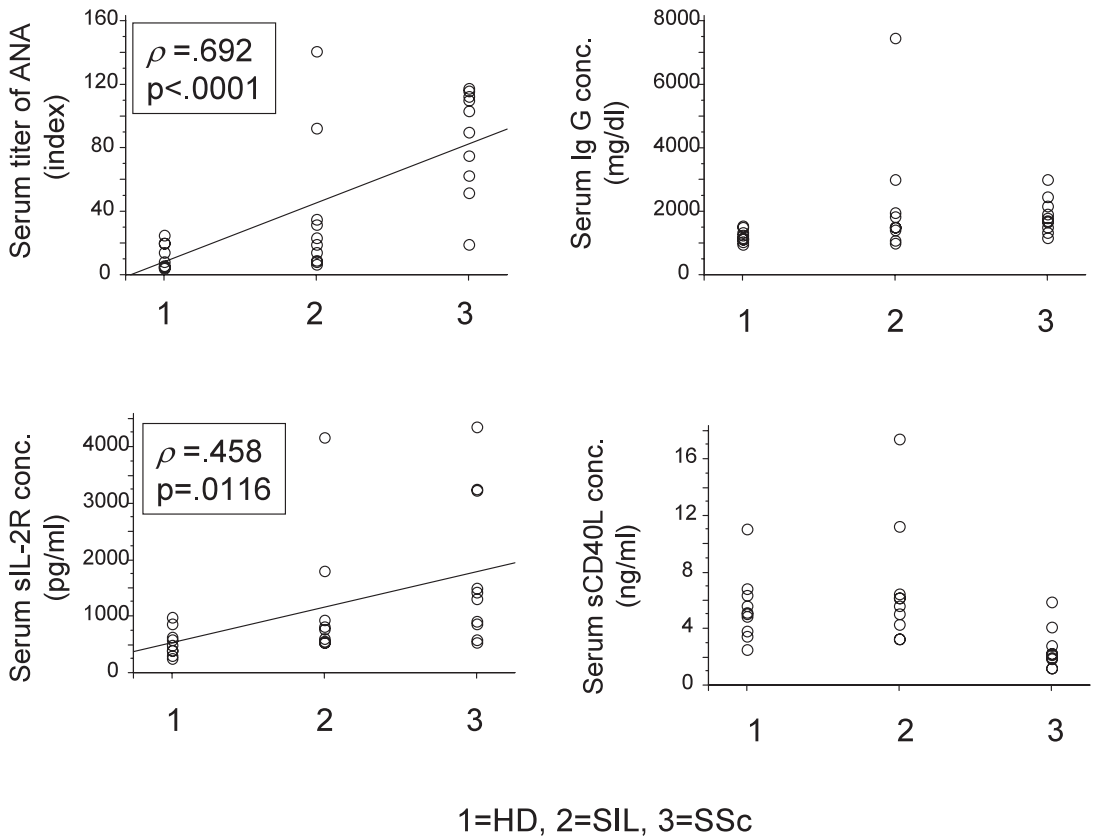

Fig. 2. Correlations between levels of clinical parameters such as ANA, Ig G, sIL-2R and sCD40L and ranked immunopathological progression (HD as 1, SIL as 2, SSc as 3) were analyzed using Fisher's z transformation ranking test.

related molecules have been compared with similar observations found in autoimmune diseases[19-28], and it has been concluded that the dysfunction of autoimmunity and cellular and molecular processes caused by silica is similar in SIL and generalized autoimmune diseases[1,2,6,7]. In addition, our group revealed the reduced function of $CD4^{+}25^{+}$ peripheral T cells, in which regulatory T (Treg) cells are included[41]. Treg cells are known to possess the expression of a lineage-specific gene, FoxP3, and are important for control of the activation status of effector T cells. If there is interference with the size and function of Treg, the T cell reaction against foreign and self antigens will be over-activated and causes allergies and autoimmune disorders. In contrast, if the size and function of Treg are enhanced, tumor immunity, host defense against foreign microorganisms, and immunity to transplanted tissues may all be reduced[42-44]. Thus, it is reasonable that SIL showed reduced Treg function[41].

In the search for clinical parameters useful for the detection of subclinical immunopathological disorders in SIL, our group has found many autoantibodies, alterations of Fas-related molecules, and sIL-2R[8-10,18-22,40]. In this study, a similar role for sCD40L was examined because sCD40L was detected as a marker of disease activity for SSc and other autoimmune diseases[11-17]. If sCD40L is bound to membrane CD40 on B cells, continuous production of antibodies against self-antigens may result even after cessation of the activation of effector T cells[31-39]. However, various issues were raised in this study. Firstly, and contrary to

expectation, sCD40L levels in SSc were lower than those recorded in HD and SIL. Although SSc patients included in this study were medicated with corticosteroids and exhibited stable disease activities, other parameters such as Ig G, ANA and sIL-2R were significantly higher than those of HD. It was shown that sCD40L is sensitive to disease activity; however, the significance of this finding is undermined by the fact that this study only included ten patients. Thus, a prospective study in relation to disease activity should be performed with a higher number of SSc patients.

The second issue raised by this study concerns the role of sCD40L as a subclinical immunopathological parameter in SIL. Although factor analysis showed that sCD40L formed factor 2 with most of the other immunopathological parameters, there was no significant correlation when immune dysfunction was viewed as a progression from HD ranked as 1, SIL as 2, and SSc as 3. Of course, no correlation between sCD40L and the progression ranking is dependent on the issue mentioned above, and SSc patients did not show a higher level of sCD40L compared to HD.

The overall results support the conclusion that the sCD40L level is altered with the progression of immunological dysfunction in SIL, but at present it is not considered a clinical parameter for the detection of subclinical immunopathological alterations found in SIL. Further studies should be performed to elucidate the role of sCD40L in autoimmune diseases such as SSc, and then its role in SIL should be re-analyzed.

ACKNOWLEDGMENTS

Authors thank all the members of the Department of Hygiene for their experimental assistance. Part of this work was performed by a research grant from the Project for Young Investigators 2007 from the Japanese Society of Hygiene, and Kawasaki Medical School Project Grant 20-410I.

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