Review of Regulation for the Fas-mediated Apoptotic Pathway in Silicosis Patients

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ABSTRACT. The past several years, we have been investigating immunological aspects of silicosis focusing on Fas-mediated apoptosis. We found elevated serum level of soluble Fas (sFas) molecule, higher gene expression of sFas and decoy receptor 3 (DcR3) genes in peripheral blood mononuclear cells (PBMC) than healthy volunteers, and various alternatively spliced Fas transcripts in PBMC. The factor analysis using these results indicated that there were a small number of patients who developed immunological diseases without presenting with respiratory disorders. In addition, we discuss the mechanism involved in the development of autoimmune disorders found in silicosis patients.

Key words : silicosis — apoptosis — Fas — autoimmune diseases

Dysregulation of apoptosis, particularly in the Fas/Fas ligand (FasL) pathway, has been considered to play a role in the pathogenesis of autoimmune diseases such as SLE.[1-4] Mutations of the Fas and FasL genes, which lead to defects in apoptosis, have been indicated in autoimmune strains of mice ; i.e. *lpr* mice for Fas and *gld* mice for FasL,[5,6] and human sutoimmune lymphoproliferative syndrome (ALPS) in childhood.[7-10] Several alternatively spliced variants of the Fas gene have been reported. One of these variants, deleted exon 6 in the transmembrane domain, is generally known as soluble Fas (sFas).[11,12] sFas inhibits membrane Fas (mFas)/FasL binding through competition, thereby preventing the apoptosis of cells. Similarly, the decoy receptor 3 (DcR3) molecule, which binds FasL and inhibits FasL-induced apoptosis like sFas, had been identified.[13-15] Since the DcR3 gene has been reported to be amplified and overexpressed in primary lung and colon tumors, it has been suggested that certain tumor cells which over-express the DcR3 molecule may escape FasL-dependent immune-cytotoxic attack. Since DcR3 seems to function in a manner similar to sFas, an alteration of DcR3-expression may also be involved in the acquisition of autoimmunity.

Silicosis is clinically characterized not only by respiratory disorders but by immunological abnormalities such as the appearance of autoantibodies

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and complications of autoimmune diseases,[16-19] typically progressive systemic sclerosis (PSS)[20-23] and systemic lupus erythematosus (SLE).[24-26] However, little is known about how occupational, chronic and recurrent exposure to silica compounds causes abnormalities in autoimmunity.

The past several years, we have been investigating immunological aspects of silicosis focusing on Fas-mediated apoptosis.[27-35] In this review, we show the results of study *in vitro* using a polyclonal T cell line and clinical results on the serum levels of sFas and soluble FasL (sFasL), gene expression and a mutation analysis of Fas and related genes, and a factor analysis of silicosis patients. In addition, we discuss the mechanism involved in the development of autoimmune disorders found in silicosis patients.

STUDIES IN PATIENTS WITH SILICOSIS

Patients studied

All the silicosis patients studied were workers of the brickyards in Bizen city and Hinase town, Okayama prefecture, Japan. Therefore, the content of free silica, which these patients inhaled, has been estimated as relatively high level such as 40 to 60%. They showed no clinical symptoms of autoimmune disease, including sclerotic skin, Raynoud's phenomenon, facial ertythema or arthlargia, or no malignant tumors.

Fas, Fas ligand and sFas

As shown in Table 1, serum sFas levels were higher in silicosis patients than in healthy volunteers, although serum sFasL livels did not differ between these two groups.[29,32] In addition, we examined sFas expression in PBMCs derived from silicosis patients by the semiquantitative reverse transcription-polymerase chain reaction (RT-PCR).[31] When a primer set was designed to cover the transmembrane domain known to be deleted in the sFas molecule by alternative splicing, the soluble form was amplified as well as the membrane (will-type) form as shown in Fig 1-A. Thus, the ratio of both products (soluble and membrane forms) was expressed as a soluble/membrane Fas expression ratio (s/m FER). As shown in Fig 1-B, silicosis patients had a significantly higher s/m FER than the volunteers.[31]

TABLE 1. Comparison of molecules and genes related to Fas-mediated apoptosis in silicosis patients and healthy volunteers

	Items	Silicosis		Helathy Volunteers	Statistical Significance
Serum	sFas (ng/ml)	2.51±0.75	>	1.97±0.56	p<.005
	sFasL (ng/ml)	0.16±0.07		0.16±0.07	
PBMCs (relative expression ratio)	soluble/membrane Fas expression	1.58±0.62	>	0.48±0.16	p<.0001
	DcR3	0.41±0.12	>	0.36±0.11	p=.0298
	TOSO	0.85±0.47		0.87±0.45	
	Sentrin	1.07±0.12	<	2.07±0.47	p=.0003
	I-Flice	0.75±0.24	<	1.01±0.08	p=.0069
	CPAN/DFF40	0.25±0.12		0.32±0.08	
	DFF45	0.34±0.17	<	0.48±0.15	p=.0143

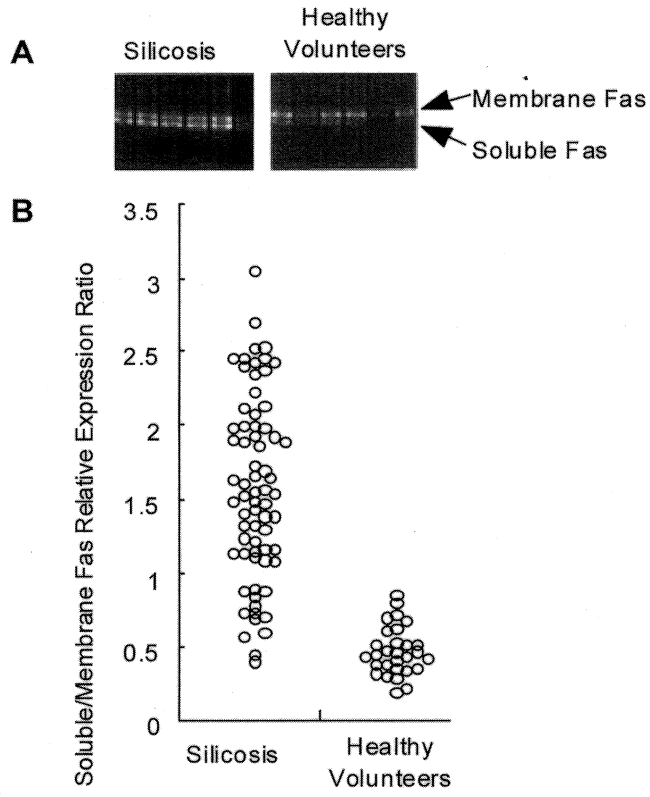

Fig 1. [A] Representative amplified membrane Fas and soluble Fas RT-PCR products in peripheral blood mononuclear cells (PBMCs) derived from silicosis patients and healthy volunteers. [B] Comparison of relative expression ratios od soluble/membrane Fas among patients with silicosis (1.58 ± 0.62) and healthy volunteers (0.48 ± 0.16) ($p<.0001$).

It has been reported that variant messages of the Fas gene differing from the typical sFas message are produced by alternative splicing.[40-43] When the primers for RT-PCR were designed to amplify the entire coding sequence of the Fas message, several alternatively spliced variants as well as the typical sFas message were detected and the variants were more frequent and stronger in their intensity in silicosis patients than healthy volunteers. The cloning of a number of these spliced variants, revealed that there are several variant messages which conserve signal peptide to bind with FasL, delete the transmembrane domain, and possess novel amino acid sequences, as shown in Fig 2. It was assumed that these variant messages are secreted into the extracellular spaces and function as a competitor of membrane Fas to bind with FasL. However, although the results of a mutational screening of the Fas and FasL coding sequences failed to detect any substitution of amino acid in PBMCs derived from silicosis patients, there were several base-substitutions which have not been reported as polymorphisms. Taken together, the alternative splicing and bese-substitutions might be due to the affinity of the silica compounds to DNA and future investigations should examine this issue.

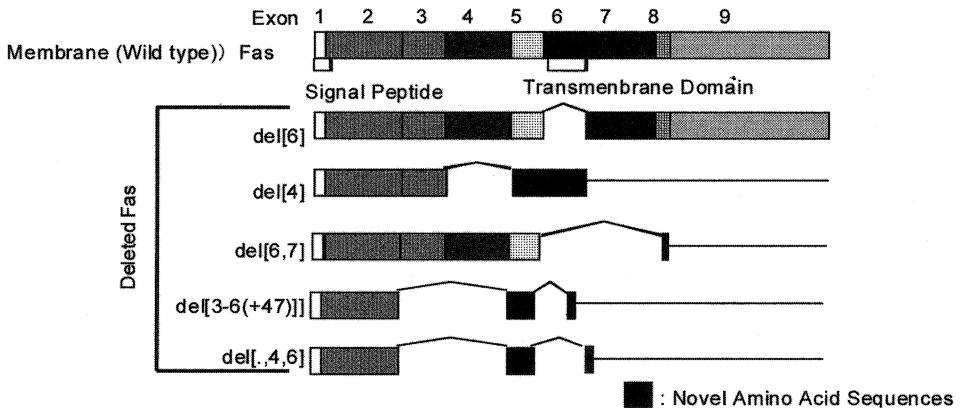

Fig 2. Schematic manifestation of spliced variants of Fas genes found in a silicosis patient. All of the variant messages, with the exception of typical soluble Fas (Del [6]), formed truncated novel amino acid (AA) sequences. All of these variant messages lost the transmembrane domain but retained the signal peptide. Black boxes show novel AA sequences which do not correspond to the wild-type Fas protein.

DcR3 gene

As mentioned in the Introduction, the DcR3 gene is also soluble and exhibits inhibitory effects on Fas-mediated apoptosis by competing with the binding of FasL to mFas.[13-15] Therefore, we analyzed the gene expression of DcR3 in PBMCs from silicosis patients, healthy volunteers, and patients with autoimmune diseases using multiplex RT-PCR methods which amplify the target gene and β-actin gene in the same PCR and compared them.[28,44] As shown in Fig 3-A, both the DcR3 and β-actin genes were amplified and the relative expression level of the DcR3 gene was calculated as the intensity of DcR3 product divided by that of β-actin from the same reaction. Fig 3-B demonstrates that the relative expression ratio of the DcR3 gene was significantly higher in the silicosis and SLE patients than in the healthy voluntees. In addition, the REL of the DcR3 gene showed a positive correlation with serum sFas levels. These results indicated that both the sFas and DcR3 molecules might act to inhibit the Fas-mediated apoptosis in silicosis patients.

Inhibitory and regulatory genes of Fas-mediated apoptosis

As shown in Table 1, we also examined the REL of the TOSO,[45] Sentrin,[46] I-Flice,[47] CPAN/DFF40,[48] and DFF45[49] genes, which are known to have inhibitory and regulatory functions in Fas-mediated apoptosis, in PBMCs derived from silicosis patients.[35] The RELs of Sentrin, I-Flice and DFF45 were significantly lower in the silicosis patients than in the healthy volunteers.

Clinical evaluation

To evaluate whether or not parameters related to Fas-mediated apoptosis such as serum sFas and sFasL levels, and the s/m FER are indicating of the immunological disorders found in silicosis patients, we performed a factor analysis using various respiratory and immunological

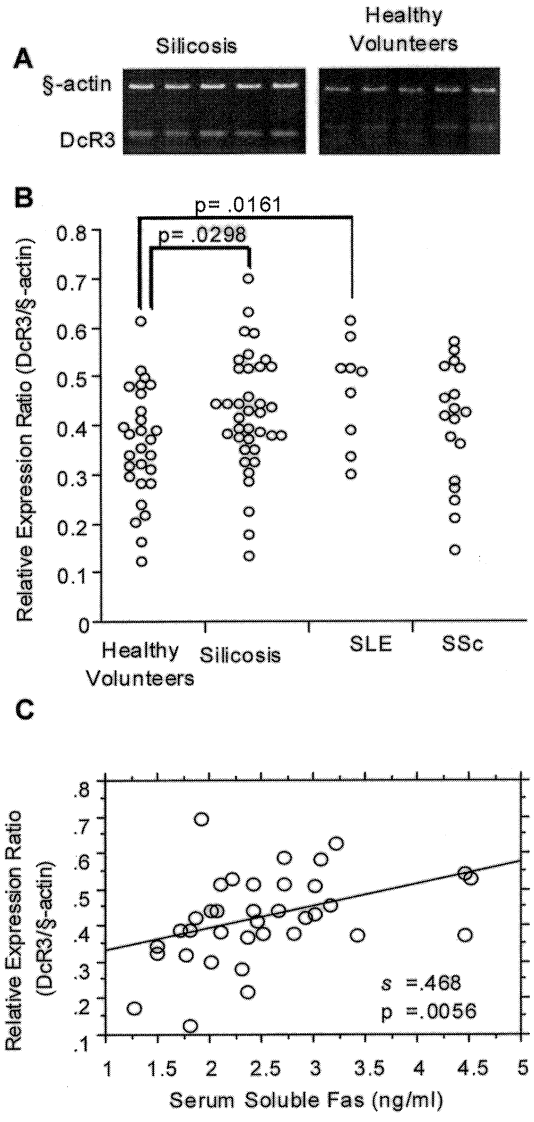

Fig 3. [A] Representative multiplex RT-PCR products of DcR3 and β-actin genes in PBMCs derived from silicosis patients (SIL) and healthy volunteers (HVs). [B] The plotting as a ratio of the expression of the DcR3 gene in HVs, relative to that in SIL, SLE, or SSc/PSS. Significant differences were detected between HVs (0.360 ± 0.114) and SIL (0.423 ± 0.120) ($p = .0298$), and between HVs and SLE (0.469 ± 0.108) ($p = .0161$).

parameters including those listed in Table 2.[30] It was clear that serum sFas, serum sFasL, and s/m FER together with IgG from a group of parameters different from PO_2, A-aDO_2, the duration of exposure and symptomatic dyspnea and that presence of the former group of parameters indicated immunological abnormalities. In addition, plotting of the value of factor 1 (respiratory score) and factor 2 (immunological score) in individual patients, revealed that there was a small group which showed abnormalities

TABLE 2. Extraction of common factors

Parameters	Factor loading after VARIMAX rotation and contribution score	
	Factor 1	Factor 2
Duration of exposure (years)	0.3721*	−0.0527
Symptomatic dyspnea (numbering)	−0.4846**	−0.2358
PR (X-ray clossification) (numbering)	−0.0184	−0.0287
PO_2 (torr)	0.9698**	0.0853
PCO_2 (torr)	−0.3861**	−0.1730
A-aDO_2 (torr)	−0.6991**	0.0586
IgG (mg/dl)	−0.0614	0.6546**
ANA titer (numbering)	−0.0919	0.3832*
mFas (%)	−0.1961	0.0940
sFas (μg/ml)	0.1855	0.6627**
sFasL (μg/ml)	0.2511	0.4012**
s/m FER (ratio)	−0.1021	0.3511*
Contribution Score	17.5878	11.7286

PR, profusion rate; ANA, antinuclear antibody; mFas, membrane Fas expression in peripheral blood lymphocytes analyzed by a flow cytometer; sFas, serum soluble Fas level analyzed by ELISA, sFasL, serum soluble Fas ligand level analyzed by ELISA; s/m FER, soluble/membrane Fas gene expression ratio analyzed by the RT-PCR and mentioned in the text.

**, parameters which showed a factor loading greater than ±0.4, which means they contributed significantly to the extraction of factors positively or negatively.
*, parameters which had a factor loading of between ±0.3 and ±0.4. These parameters also contributed to the extraction of factors but less than those with**.

for factor 2 with little disturbance of factor 1.[30] These results indicated that there were a small number of patients who developed immunological diseases without presenting with respiratory disorders after exposure to silica compounds.

***In vitro* analysis**

As shown in Fig 4, peripheral blood mononuclear cells (PBMCs) derived from healthy volunteers undergo typical apoptotic changes when co-cultured with 50 μg/ml of chrysotile B, an asbestos.[33,34,36-38] These apoptotic changes were also demonstrated when the cells were cultured with man-made mineral fibers, which have been used as asbestos substitutes,[33] such as ceramic fibers, mullite fibers, glass wool, and rutile in whisker form (all provided by the Japan Fibrous Material Research Association; JFMRA). Based on these studies, it has been suggested that a Fas-mediated apoptotic pathway may play an important role in the silica-induced apoptosis of PBMCs, because of the disappearance of cells that express Fas following apoptosis,[34] and alteration of gene expression related to the Fas-mediated apoptosis. These results also suggested that the analysis of Fas-mediated apoptosis in silicosis patients may shed light on the mechanism involved in the immunological disturbances found in silicosis patients.

However, it should be noted that immunological disorders may be the

result of occupational, chronic and recurrent exposure to silica compounds. Therefore, we have been trying to establish an *in vitro* model using a polyclonal T cell line, MT-2.[39] The MT-2 line was selected its

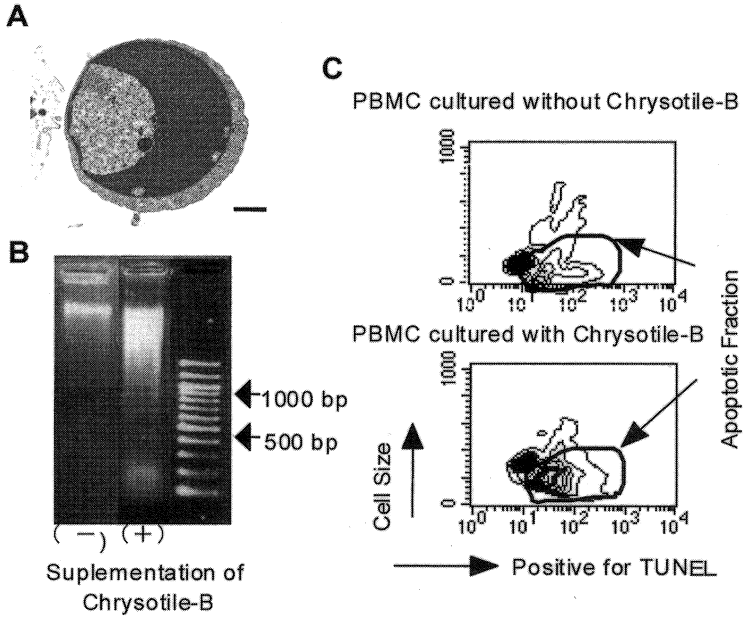

Fig 4. Apoptosis of PBMCs derived from healthy volunteers was detected when the cells were cultured with Chrysotile-B (50 μg/ml) for three days ultrastructurally [A], by DNA ladder formation [B], and by the TUNEL method using flow cytometry [C].

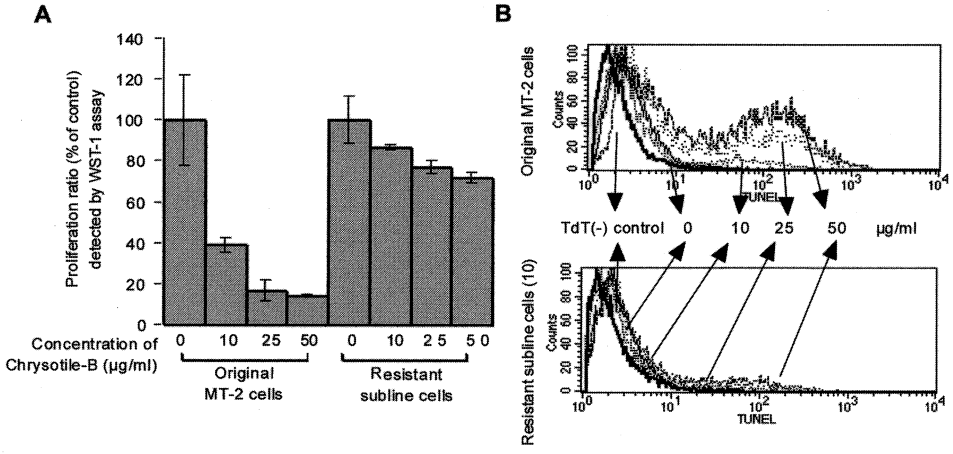

Fig 5. A human polyclonal T cell line, MT-2. showed dose-dependent growth inhibition [A, left] and a dose-dependent increase of TUNEL-positive apoptotic cells [B, upper panel], when cultured with various concentrations of Chrysotile-B for two days. However, after continuous exposure to 10 μg/ml of Chrysotile-B for eight months, a Chrysotile-B-resistant subline, which showed no Chrysotile-B-induced growth inhibition [A, right] and the appearance of an apoptotic fraction [B, lower panel], was developed.

characteristics to proceed apoptosis when cultured with chrysotile B among various lymphoid cell lines including T cell leukemias, B cell lymphomas and polyclonal B cell lines. MT-2 cells underwent apoptosis when cultured with 50 μg/ml of chrysotile B similar to PBMCs, as shown in Figure 4. Then, we exposed the cells to a relatively low concentration of chrysotile B (10 μg/ml) approximately for nine months, and analyzed the appearance of apoptosis induced by 10 to 50 μg/ml of chrysotile B, monthly. After eight months of low-dose exposure, a chrysotile B-resistant MT-2 subline appeared, as shown in Fig 5. This acquistion of resistance to silica-induced apoptosis in the MT-2 subline might be due to the expansion of resistant clone(s). In the future, efforts should be made to find differentially expressed genes and differences in clonality between the parent MT-2 line and the resistant subline.

DISCUSSION

Based on the results presented in this review, it seems that levels of inhibitory molecules such as sFas and DcR3 are higher in silicosis patients than healthy volunteers. In addition, a T cell clone, resistant to the silica-induced apoptosis mediated by the Fas apoptotic pathway, has gradually proliferated during long-term low-dose exposure to silica compounds *in vitro*. These results indicate that a T cell clone resistant to silica-induced apoptosis has appeared and survived for some time along with occupational, chronic and recurrent exposure to silica compunds *in vivo*.

The expression levels of inhibitory genes for the Fas-mediated pathway were lower in PBMCs from silicosis patients. In addition, the PBMCs from healthy volunteers underwent Fas-mediated apoptosis when co-cultured with asbestos *in vitro*. These results suggest that even in silicosis patients who have been exposed to silica compounds chronically, there is a sub-fraction of cells which undergoes apoptosis induced by silica exposure via the Fas-

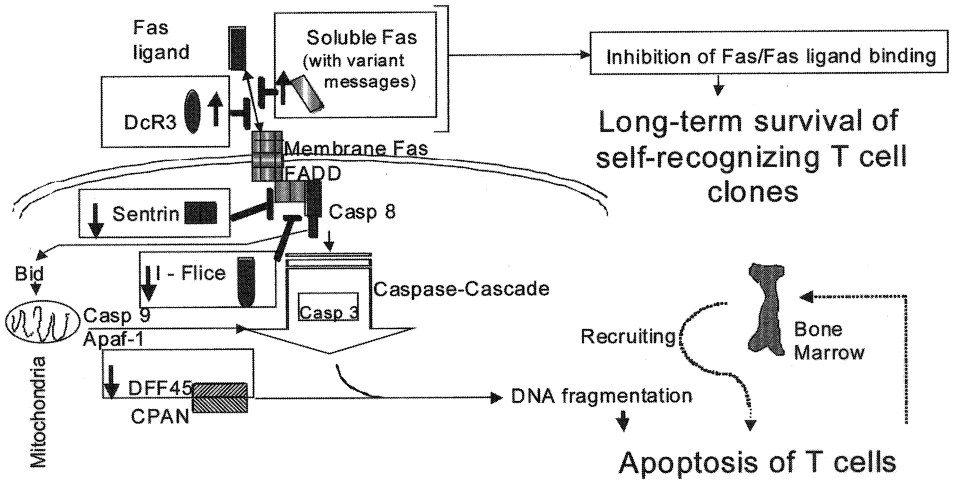

Fig 6. Schematic model of dysregulation of the Fas-mediated apoptotic pathway in patients with silicosis.

mediated pathway.

As shown schematically in Fig 6, there may be two sub-fractions of lymphocytes in silicosis patients. One is a fraction which survives longer and includes auto-recognizing T cell clones. This fraction may help to produce sFas and overexpress DcR3. The other is a fraction which proceeds to undergo silica-induced and Fas-mediated apoptosis and is recruited from the bone marrow.

Further investigation is required to characterize the two assumed fractions of lymphocytes in silicosis patients. In addition, if several important genes were to be discovered through *in vitro* studies, the mechanism behind the appearance of the immunological disorders found in silicosis might be resolved in the near future.

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