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Soluble Fas mRNA is dominantly expressed in cases with silicosis

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SUMMARY

Although it is well known that cases with silicosis exhibit various immunological abnormalities, the mechanisms involved in the occurrence of immuno-dysfunction or dysregulation induced by silica compounds have not yet been determined. Fas is a well-known cell surface molecule that is involved in the apoptosis pathway that belongs to the tumour necrosis factor-receptor family. Soluble Fas (sFas) is produced as an alternatively spliced product of the Fas gene and protects cells from apoptosis due to antagonization of the binding between membrane form of the Fas gene (mFas) and the Fas ligand. To determine the role of the Fas/Fas ligand system in silica-induced immunological abnormalities, we investigated Fas and Fas-ligand message expression levels using the multiplex reverse transcription–polymerase chain reaction (RT–PCR) method with peripheral blood mononuclear cells from silicosis cases with no clinical symptoms of autoimmune diseases. Although the relative expression levels of the Fas or Fas-ligand genes were not remarkably altered in these cases, we observed the sFas message was dominantly expressed compared with mFas expression. These results suggest that self-recognizing clones in cases with silicosis survive for decades, escaping the exclusion mechanisms induced by apoptosis. Then they cause the appearance of autoantibodies and the acquisition of autoimmune diseases sequentially.

INTRODUCTION

It is well known that cases of silicosis are characterized by not only pulmonary lesions but also various immunological abnormalities such as hypergammaglobulinaemia, and the appearance of autoantibodies or complications of autoimmune diseases, typically, progressive systemic sclerosis (PSS).[1–5] However, the mechanisms involved in the occurrence of immuno-dysfunction or dysregulation induced by silica compounds have not yet been determined.

We have found that silica compounds such as chrysotile, crocidolite, anthophyllite, and amosite asbestos act as superantigens to activate human T cells polyclonally *in vitro*.[6] Silica compounds have been found to induce increase in intracellular calcium concentrations,[7,8] enhancement of interleukin-2 (IL-2) secretion and activation of particular T-cell receptor (TCR) Vβ 5.3,[9,10] in human peripheral blood T cells.

Fas is a well known cell surface molecule involved in the apoptosis pathway which belongs to the the tumour necrosis factor-receptor family.[11–14] Several alternative spliced variants of the Fas gene have been reported previously.[14–16] Among those variants the deletional form of exon 6 in the transmembrane domain is generally known as soluble Fas (sFas) This sFas inhibits membrane Fas (mFas)/Fas ligand binding by competition,[16–18] subsequently preventing the apoptosis of cells. Several investigations of elevated serum sFas levels in systemic lupus erythematosus (SLE),[18–20] rheumatoid arthritis,[21–23] and haematological malignancies[24] suggest that dysregulation of competition between sFas and mFas to bind the Fas ligand might be one of the most important mechanisms for acquiring autoimmunity.[16,25–28] Recently, we also studied serum sFas levels in cases of silicosis and observed elevation of these levels.[29]

In the present study, we analysed Fas and Fas ligand message expression levels using the multiplex reverse transcription–polymerase chain reaction (RT–PCR) method with peripheral blood mononuclear cells from silicosis cases with no clinical symptoms of autoimmune diseases. Although the relative expression levels of the Fas or Fas ligand genes were not altered, we noted that the sFas message was dominantly expressed in these cases as compared with mFas expression when a modified RT–PCR method was used. Moreover, we recently reported that serum sFas levels in cases with silicosis were significantly higher than those in healthy volunteers (HV), or cases with PSS.[29] The dysregulation of the Fas gene in silicosis cases may cause self-recognizing clones to survive for decades, escaping the exclusive mechanisms induced by apoptosis. Then, they cause the appearance of autoantibodies and acquisition of autoimmune diseases sequentially.

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Abbreviations: sFas, soluble Fas; PSS, progressive systemic sclerosis; RT–PCR, reverse transcriptase–polymerase chain reaction; mFas, membrane Fas; SLE, systemic lupus erythematosus; HV, healthy volunteers; SQ-MPL-PCR, semi-quantitative multiplex RT–PCR; PR, radiological profusion grades.

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MATERIALS AND METHODS

Cases studied

Sixty-nine cases with silicosis (63 men and 6 women, average age: 68 ± 7 years old) without any clinical symptoms of autoimmune diseases such as sclerotic skin, Raynaud's phenomenon, facial erythema or arthralgia, or any malignant tumours, 11 cases with SLE (all women, average age: 39 ± 14 years old), 19 cases with PSS (all women, average age: 56 ± 13 years old), and 30 HV (20 aged and 10 young) were the subjects of this study. The aged HV consisted of 12 men and 8 women, average age 64 ± 8 years old, and the young HV were all women, average age 25 ± 2 years old. In all cases, the subjects were Japanese and they gave informed consent according to the guidelines of the Institutional Review Board of Kawasaki Medical School or Kusaka Hospital.

RNA extraction and cDNA synthesis

Peripheral blood mononuclear cells (PBMC) were isolated from 10 ml of heparinized blood from each case by the Ficoll –Hypaque density centrifugation method (Lymphoprep®, Nycomed Pahrma As, Oslo, Norway). Total cellular RNA was extracted using a TRIzol® reagent (Gibco BRL Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions. One microgram of total RNA and oligo(dT)$_{18}$ primer (final concentration; 1 µM) in 12·5 µl of diethyl pirocarbonate (DEPC)-treated H_2O was heated to 70° for 2 min followed by cooling on ice for 1 min. cDNA synthesis was initiated using 200 units of recombinant MMLV (Moloney-murine leukaemia virus) reverse transcriptase (Clonthech Lab. Inc., Palo Alto, CA) under conditions recommended by the manufacturer, and the reaction was allowed to proceed at 42° for 60 min. The reaction was terminated by heating at 94° for 5 min and then it was diluted to a final volume of 100 µl by adding 80 µl of DEPC-treated H_2O.

RT – PCR for the Fas, Fas ligand, and β-actin genes

The primers for the Fas, Fas ligand, and β-actin genes were as follows:

primer set #1 for β-actin,
1-F; 5′-TGACGGGGTCACCCACACTGTGCC-CATCTA-3′
1-R; 5′-CTAGAAGCATTTGCGGTGGACGA-TGGAGGG-3′
primer set # for Fas,
2-F; 5′-CTTTCACTTCGGAGGATTGC-3′
2-R; 5′-AGCCATGTCCTTCATCACAC-3′
primer set # for Fas ligand,
3-F; 5′-GGTCAATCTTGCAACAACCTG-3′
3-R; 5′-ACAACATTCTCGGTGCCTG-3.

The primer set for Fas (#2) was designed to generate the signal peptide domain of the Fas gene cDNA sequences within exons 1 and 2, because no alternatively spliced variant messages deleting this domain have been reported.[15–19] Each reaction of RT–PCRs contained 1/100 of cDNA (equivalent to a cDNA amount from 10 ng of initial total RNA), 200 nM concentrations of each primer, 200 µM of deoxynucleotide triphosphates, 10 mM of Tris–HCL (pH 8·8), 1·5 mM of $MgCl_2$, 50 mM of KCL, 0·08% Nonident P40, and one unit of recombinant *Thermus aquaticus* DNA polymerase (MBI Fermentas, Vilnius, Lithuania) in a final volume of 20 µl. Reactions to detect RT–PCR products by radioactive labelling using a BAS-2000II image analyser (Fuji Film Inc., Tokyo, Japan) were performed under the same conditions described above except for the inclusion of 1 µCi of [α-^{32}P]dCTP (specific activity, 3000 Ci/mmol) and 10 µl of final volume. After an initial denaturation for four minutes at 94°, 15 to 35 cycles of denaturation (94° for 1 min), annealing (57° for 1 min), and extension (72° for 1 min) were performed on a DNA thermal cycler (Applied Perkin Elmer, Norwark, CT). The final extension time was 7 min. Standard RT–PCR products were visualized by 1·2% agarose gel stained with ethidium bromide or an exact 1/10 volume of radioactive products was applied onto 6% polyacrylamide gel containing 10% glycerol, after which gel electrophoresis was performed at 40–60 W for 3–5 hr.

Semi-quantitative multiplex RT–PCR for the Fas, Fas ligand, and β-actin genes

A semi-quantitative multiplex RT–PCR (SQ-MPL-PCR) was designed to compare RT–PCR products of the Fas and Fas ligand genes with β-actin gene products to analyse the relative expression levels of Fas and the Fas ligand in the cases described above. To obtain a phase in which all three genes would be amplified logarithmically, each reaction of the SQ-MPL-PCRs contained the same reagents shown above except for the addition of 200, 100, and 20 nM of primers to Fas, the Fas ligand, and β-actin, respectively, and radioactive dCTP. An initial trial for the SQ-MPL-PCR proceeded for 27 to 32 cycles under the same conditions described above after the initial denaturations. Finally, 29 cycles of SQ-MPL-PCR were employed as the screening procedure for all of the samples from the cases with silicosis, SLE, or PSS, and HV after the analysis of SQ-MPL-PCR products from a number of cycles (27 to 32) using several silicosis cases and HVs resulted in a logarithmical amplification of all three genes with a similar ratio of each product for Fas, the Fas ligand, and β-actin.

RT–PCR for mFas and sFas amplification

A primer set to generate mFas and sFas was designed including the transmembrane domain of the Fas gene. The sequences were as follows:

primer set #4
2-F; 5′-AAGGAGTACACAGACAAAGCCC-3′
2-R; 5′-GGTGATATATTTACTCAAGTC-3′.

Each reaction for the mFas and sFas RT–PCRs contained the same reagents for Fas, the Fas ligand, or β-actin amplification. RT–PCR products were demonstrated on 1·2% agarose gel stained by ethidium bromide. In addition, to estimate the ratio of PCR products for mFas and sFas, 19 to 35 cycles of the RT–PCR including radioactive dCTP were performed and the amounts of incorporated [α-^{32}P]dCTP in the PCR products were analysed using a BAS-2000II image analyser after running polyacrylamide gel electrophoresis. Twenty-seven RT–PCR cycles of mFas and sFas were employed as the screening method for all of the samples from the cases with silicosis, SLE, or PSS, and HV after analysis of RT–PCR products from sequential cycles using several silicosis cases and HV resulted in a logarithmical amplification with a similar ratio of each product for the mFas and sFas genes. Semi-quantitative sFas expression was calculated as the radioactivity of the sFas product divided by the mFas product (s/m Fas expression ratio) in each case.

Comparison and correlation of s/m Fas expression ratio with clinical parameters

The s/m Fas expression ratio obtained from each case with silicosis was compared and analysed with several clinical parameters such as Po_2 and Pco_2 values, duration of exposure to silica dust, severity of subjective dyspnea, radiological profusion grades (PR), and serum immunoglobulin G(IgG) level.

Statistical analysis

Statistical significance was calculated using Fisher's protected least-significance difference (PLSD) test.

RESULTS

No significant differences in Fas and Fas ligand gene expression among cases with silicosis, SLE, or PSS, or healthy volunteers analysed by the SQ-MPL-PCR

The SQ-MPL-PCR was employed to estimate the relative expression levels of the Fas and Fas ligand genes among cases with silicosis, SLE, or PSS, and HV, and to compare them. Fas and Fas ligand semi-quantitative expression levels were calculated as the ratio of radioactivities of each product divided by that of the β-actin product derived from the same case sample. The distributions of the Fas/β-actin expression ratio in cases with silicosis, SLE, or PSS, and HV were $0{\cdot}93 \pm 0{\cdot}51$, $0{\cdot}89 \pm 0{\cdot}55$, $0{\cdot}77 \pm 0{\cdot}34$, and $0{\cdot}93 \pm 0{\cdot}62$, and those of the Fas ligand/β-actin expression ratio were $0{\cdot}66 \pm 0{\cdot}47$, $0{\cdot}52 \pm 0{\cdot}60$, PSS $0{\cdot}94 \pm 1{\cdot}62$, and $0{\cdot}89 \pm 0{\cdot}77$, respectively. There were no significant differences in Fas/β-actin or Fas ligand/β-actin expression ratios among these groups. These results suggest that the Fas and Fas ligand genes are expressed similarly in cases with silicosis, SLE, or PSS, or age-matched HV at least on the mRNA level.

RT–PCR for membrane and the soluble Fas gene

Using a primer set overhanging the transmembrane domain of the Fas gene, two RT–PCR products were generated, by agarose gel electrophoresis (Fig. 1a) and by polyacrylamide gel electrophoresis (Fig. 1b) using radioactive dCTP. The larger one was 684 bp derived from a wild type Fas (mFas) cDNA sequence. The other (the smaller one) was 621 bp from which 63 nucleotides of transmembrane domain of the Fas gene (most of exon 6) were deleted. This product is known as the soluble Fas gene and was spliced alternatively after confirmation of both sequences with a Dyeterminator sequencing kit (Perkin Elmer) (data not shown).

In Fig. 1a and b, there is an apparent tendency in the amount of both products (sFas and mFas) for silicosis and HV. More sFas products (smaller products) were generated than mFas (larger products) ones in silicosis cases, whereas, HV showed inverse amplifications. To estimate this tendency, the 27-cycle RT–PCR including radioactive dCTP was used as a screening method for all of the samples including cases with silicosis, SLE, or PSS, and HV. Semiquantitative sFas expression was calculated as the radioactivity of the sFas product divided by the mFas product (s/m Fas expression ratio) in each case and compared among all case groups.

The s/m Fas expression ratios in the cases with silicosis, SLE, or PSS, and HV were $1{\cdot}58 \pm 0{\cdot}62$, $0{\cdot}69 \pm 0{\cdot}37$, $0{\cdot}55 \pm 0{\cdot}33$, and $0{\cdot}48 \pm 0{\cdot}16$, respectively. As shown in Fig. 2, The s/m Fas expression ratio for the silicosis group was significantly higher than those for the SLE, PSS, and HV groups. These results suggest that the soluble Fas message is dominantly expressed in cases with silicosis as compared with HV or cases with autoimmune diseases such as SLE or PSS, even though total Fas expression is not altered as described above.

Comparison and correlation of s/m Fas expression ratio with clinical parameters

There were no significant correlations between s/m Fas expression ratio and clinical parameters studied with an exception of serum IgG levels. The weak direct correlation ($\sigma = +0{\cdot}355$, $\rho = 0{\cdot}0279$) between serum IgG levels in cases with silicosis and s/m Fas expression ratio was detected.

DISCUSSION

Dysregulation of apoptosis, particularly in the Fas/Fas ligand pathway, has been considered to possibly be involved in the pathogenesis of autoimmune diseases.[19,25,27,28] Mutations of the Fas and Fas ligand genes, which lead to defects in apoptosis, have been identified in autoimmune strains of mice such as *lpr* mice for Fas and *gld* mice for the Fas ligand[26,30,31] and in human autoimmune lymphoproliferative disorders in childhood.[32–35] Moreover, several alternatively spliced variant messages of the Fas gene have been detected in human peripheral blood T cells activated by phytohaemagglutinin (PHA)[15–19] and these variants functionally protect cells from apoptosis caused by antagonization of membrane (wild type) Fas to bind Fas ligand. Soluble Fas, in which 63 nucleotides are deleted from the transmembrane domain in exon 6,[19] in particular has been thoroughly investigated as a competitor of the membrane Fas gene in binding of its ligand and switching of apoptosis signals into intracellular molecules.[14]

In addition, it is also well known that cases with silicosis show various immunological abnormalities such as hypergammaglobulinaemia, the appearance of autoantibodies, or complications of autoimmune diseases, typically PSS.[1–5] However, the mechanisms involved in immuno-dysfunction in silicosis have not yet been recognized. Positioning silicosis in a pre-autoimmune status, we recently found that serum sFas levels in cases with silicosis were significantly higher than those in HV, or even in cases with PSS.[29] Furthermore, in *in vitro* studies we have reported,[6–10] we have suggested that silica compounds activate human T cells polyclonally and induce apoptosis if the T cells are exposed transiently. Because these results strongly suggest that dysregulation in the Fas/Fas ligand pathway in apoptosis might play a major role in silicosis, which is caused by recurrent and chronic exposure to silica compounds, we genotypically analysed the message status of the Fas gene in peripheral blood mononuclear cells from cases with silicosis.

Although the total Fas expression levels in silicosis were similar to those in the disease groups studied and the age-matched HV, the s/m Fas expression ratio in cases with silicosis was significantly higher than in the other groups. Furthermore, this genotypical phenomenon correlated with phenotypic serum sFas elevation in silicosis.[29] Therefore, it seems to be more important to consider the expression levels

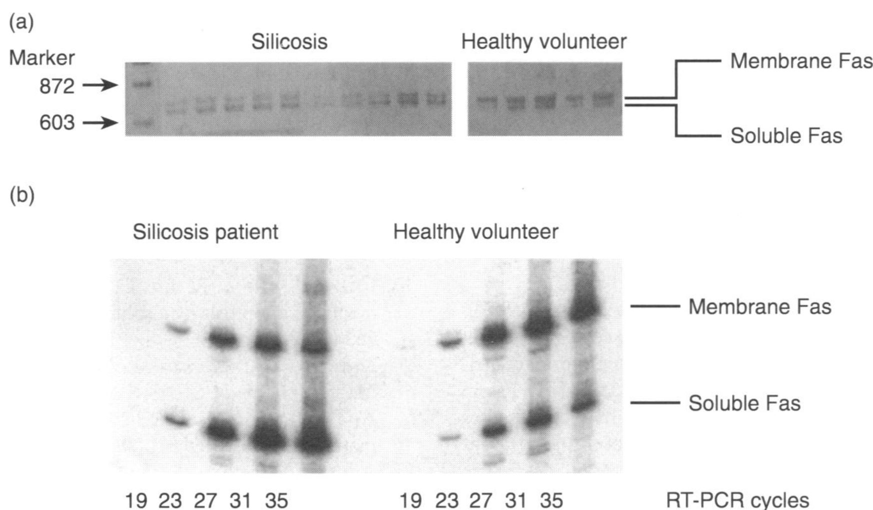

Figure 1. (a) Represented amplified mFas (684 bp) and sFas (621 bp) RT–PCR products using primer set #4 overhanging the transmembrane domain of the Fas gene from cases with silicosis (left side) and HV (right side) electrophoresed on a 1·2% agarose gel and stained with ethidium bromide. For the RT–PCR, initial denaturation and 35 cycles of denaturing, annealing, and extension steps, and final extensions were done as described in Materials and Methods. (b) Sequential (19 to 35 cycles) RT–PCR for mFas and sFas amplification containing radioactive dCTP electrophoresed on polyacrylamide gel.

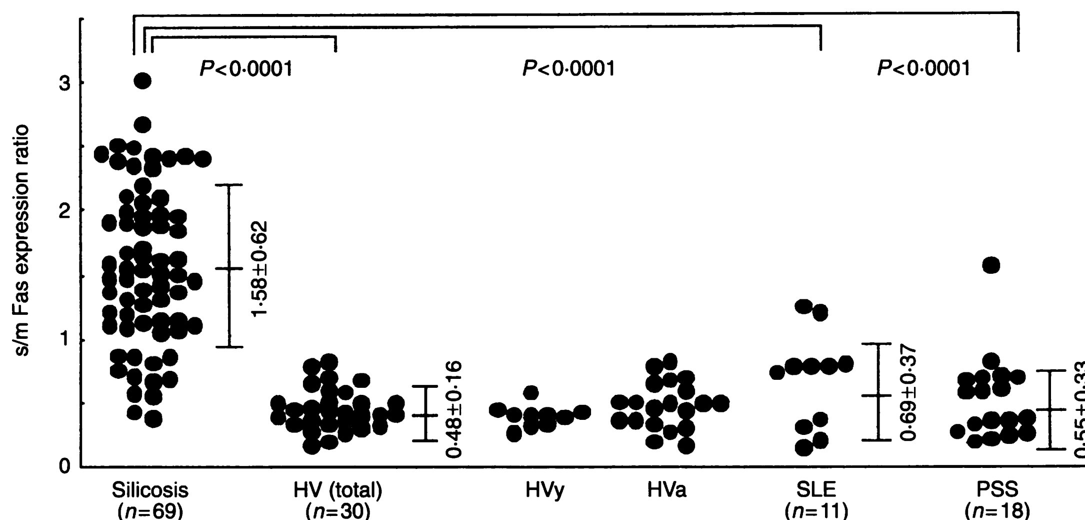

Figure 2. Comparison of the s/m Fas expression ratio among cases with silicosis, SLE, or PSS, and HV. The silicosis group had a significantly higher s/m Fas expression ratio than all other groups.

of soluble or membrane forms of the Fas gene separately than to estimate the total Fas expression levels. Because soluble Fas expression was genotypically and phenotypically higher in silicosis, even though total expression was not altered, apoptosis in peripheral blood mononuclear cells from silicosis patients is probably inhibited by antagonizing mechanisms between the membrane and soluble forms of the Fas gene. As we mentioned above, it is considered that self-recognizing clones exist in polyclonally activated clones exposed recurrently and chronically to silica compounds in the peripheral blood of silicosis cases. These self-recognizing clones may escape from exclusion mechanisms via apoptosis resulting from an inhibitory effect of elevated soluble Fas molecule and survive for decades during occupational exposure to silica. This could be one of the most important mechanisms for explaining why cases with silicosis have immunological abnormalities.

sFas is typical of the several alternatively spliced variant messages of the Fas gene. There have been several reports of studies which demonstrated other variant messages in cDNAs from T cells from healthy volunteers activated by PHA *in vitro.*[14–18] We have also investigated other variant messages of the Fas gene and detected at least four other forms from cases with silicosis. All four variants delete the transmembrane domain and show truncated amino acid sequences, but maintain the signal peptide domain and are expressed more frequently and highly in silicosis than in healthy volunteers (unpublished data). Because all these variant forms, including the so-called sFas gene examined in this study are considered to be generated by alternative splicing, several questions, such as why recurrent and chronic silica exposure causes these enhanced alternative splicings in the Fas gene, whether or not other genes that are expressed in silicosis cases are also

alternatively spliced frequently and highly, and whether or not an enhanced alternative splicing status in certain genes affects respiratory pathophysiology in silicosis, are being posed in consideration of the pathological effects of silica compounds on human beings. It is necessary to investigate the pathophysiological functions of these variant messages and to analyse the mechanisms that produce many alternative splicing variants in order to address these questions.

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