Review

Immunological Effects of Silica and Asbestos

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Silicosis patients (SILs) and patients who have been exposed to asbestos develop not only respiratory diseases but also certain immunological disorders. In particular, SIL sometimes complicates autoimmune diseases such as systemic scleroderma, rheumatoid arthritis (known as Caplan syndrome), and systemic lupus erythematoses. In addition, malignant complications such as lung cancer and malignant mesothelioma often occurr in patients exposed to asbestos, and may be involved in the reduction of tumor immunity. Although silica-induced disorders of autoimmunity have been explained as adjuvant-type effects of silica, more precise analyses are needed and should reflect the recent progress in immunomolecular findings. A brief summary of our investigations related to the immunological effects of silica/asbestos is presented. Recent advances in immunomolecular studies led to detailed analyses of the immunological effects of asbestos and silica. Both affect immuno-competent cells and these effects may be associated with the pathophysiological development of complications in silicosis and asbestos-exposed patients such as the occurrence of autoimmune disorders and malignant tumors, respectively. In addition, immunological analyses may lead to the development of new clinical tools for the modification of the pathophysiological aspects of diseases such as the regulation of autoimmunity or tumor immunity using cell-mediated therapies, various cytokines, and molecule-targeting therapies. In particular, as the incidence of asbestos-related malignancies is increasing and such malignancies have been a medical and social problem since the summer of 2005 in Japan, efforts should be focused on developing a cure for these diseases to eliminate nationwide anxiety.
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Introduction

Silicosis patients (SILs) develop respiratory fibrosis and impaired their pulmonary function. In addition, silica is known as one of the strongest environmental substances causing autoimmunity dysfunction (1-3). SILs often develop immunological complications such as rheumatic arthritis (known as Caplan syndrome (4-6)), systemic sclerosis (SSc), and systemic lupus erythematoses (SLE). The effects of silica on autoimmunity have also been assumed as patients who have undergone plastic surgery with implants containing silicone ($[SiO_2\text{-}O\text{-}]_n$) show frequent complications of autoimmune disorders (7-9). These accumulated findings clearly indicate that crystalline silica causes dysregulation and/or disturbance of the human immune system, particularly autoimmunity.

Regarding asbestos, which is categorized as a silicate (mineralogical complexes containing metals, such as iron and magnesium) including chrysotile, crocidolite, and amosite, patients exposed to asbestos also develop pulmonary fibrosis

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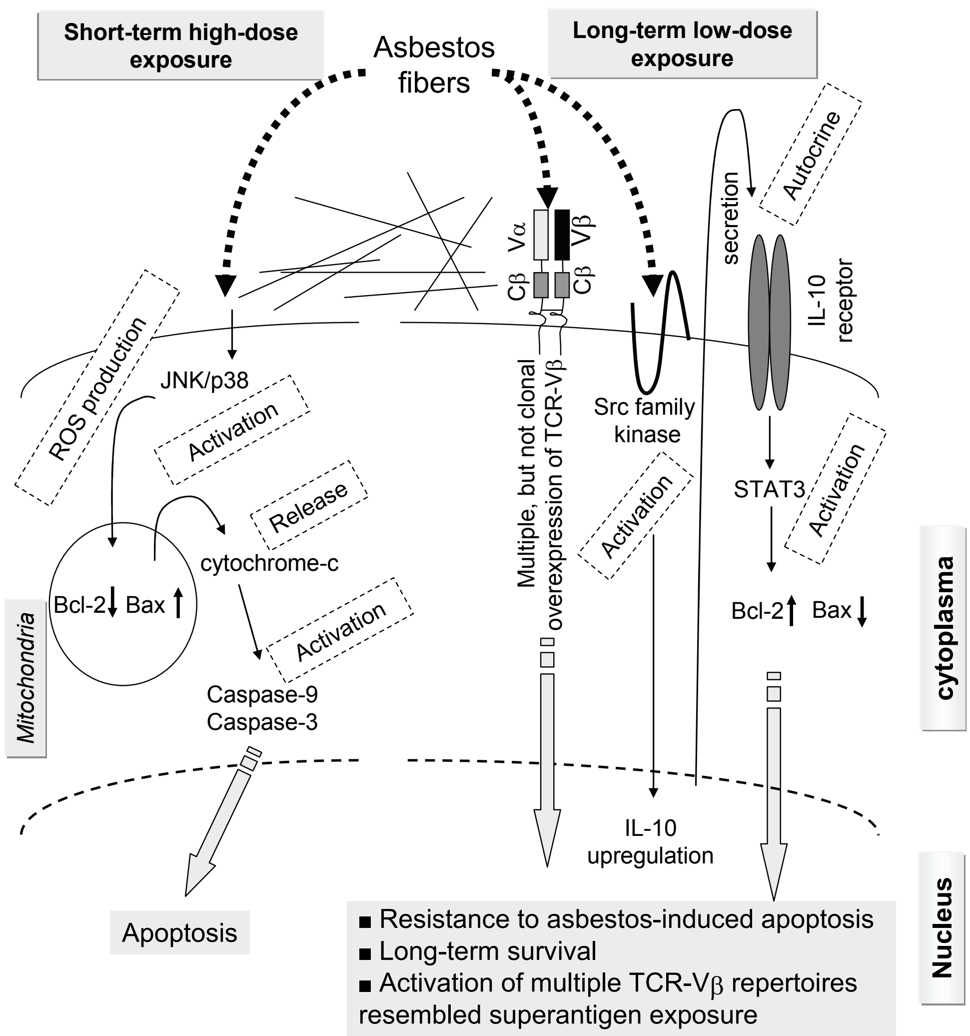

Figure 1. Experimental findings of immunological effects of chrysotile, a form of asbestos, induced by short-term and high-dose exposure (left panel) or long-term and low-dose exposure using MT-2, an HTLV-1 immortalized human polyclonal T cell line.

known as asbestosis, mesothelial plaque, and malignant diseases such as lung cancer and mesothelioma (10-13). Some of these malignancies may be considered a result of a decline in tumor immunity owing to exposure of immuno-competent cells to asbestos.

Silica and silicates may disturb immune functions such as autoimmunity and tumor immunity. In this article, a brief summary of our investigations related to the immunological effects of silica/asbestos is presented. Details of each subject can be found in the references cited.

Immunological effects of chrysotile and asbestos

The International Agency for Research on Cancer (IARC) categorizes both asbestos and crystalline silica as group I carcinogens, because it is well known that asbestos (e.g., chrysotile, crocidolite, and amosite) causes malignant lung cancer or mesothelioma (10-13). According to the IARC classification, asbestos affects alveolar epithelial and mesothelial cells. There have been many studies of asbestos-induced apoptosis of these cells (14-22). In comparison with most other solid tumors, mutation of the *p53* gene is rare. Instead of the alteration of *p53*, loss of $p16^{INK4a}$ expression has been detected in most mesotheliomas and cell lines. In addition, $p14^{ARF}$, a *p53* regulator, is simultaneously deleted (23-25). Under experimental conditions, these cells undergo apoptosis upon high-level, short-term exposure to asbestos as a result of the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) *via* activation of the mitochondrial apoptotic pathway. Furthermore, several non-small-cell lung cancer cell lines constitutively contain the active signal transducer and activator of transcription 3 (STAT3) (26, 27). Moreover, inhibition of tumor-derived interleukin (IL)-10 and IL-10 receptor (IL-10R) interaction by an autocrine/paracrine loop results in a decrease in the expression level of constitutively active STAT3 and the subsequent inhibition of Bcl-2 transcription and expression (28, 29). Thus, it has been considered that during low-level, long-term exposure to asbestos, alveolar epithelial and mesothelial cells escape from the apoptotic pathway due to genetic changes and undergo malignant transformation. Although nuclear factor-κ B (NF-κB) was shown to be involved in the transcriptional activity of anti-apoptotic genes such as *bcl-2*, the role of NK-κB in the carcinogenesis of mesothelioma has not been well investigated. Advancement in genetic analyses related to the oncogenesis of mesothelioma may lead to the discovery of newer target genes for molecular therapy.

We have mainly focused on the immunological effects of chrysotile. Asbestos, chrysotile, polyclonally activated $CD4^+$ T cells and caused activation-induced cell death (30, 31). PBMCs from HD exposed to asbestos in culture underwent apoptosis; however, many patients with asbestosis have had chronic occupational or other recurrent exposure to silicates. Therefore, there seems to be a need to develop an *in vitro* experimental model of chronic exposure to analyze the immunobiological effects of silicates during long-term exposure.

For this purpose, we employed a human T-cell leukemia virus type-1 (HTLV-1)-immortalized human polyclonal T cell line, MT-2, for the development of an *in vitro* model. Upon short-term, high-level exposure to chrysotile, MT-2 cells underwent apoptosis with the production of ROS *via* the activation of the mitochondrial apoptotic pathway with the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) signaling molecules, resulting in a shift of the Bax-dominant Bax/Bcl-2 balance, the release of cytochrome-c from mitochondria into cytosol, and the activation of caspases 9 and 3, as shown on the left side of Figure 1 and as previously reported (32).

Next, we established a chrysotile-B (CB)-induced apoptosis-resistant subline of MT-2 (MT-2Rst), and characterized the cell biological differences between the original MT-2 cell line (MT-2Org) and MT-2Rst. The MT-2Rst cells were characterized by (i) an enhanced expression of *bcl-2*, restoring apoptosis sensitivity with a decrease in the *bcl-2* expression level by siRNA, (ii) excessive IL-10 secretion and expression, and (iii) the activation of STAT3 inhibited by 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolol [3,4-d] pyrimidine (PP2), a specific inhibitor of Src family kinases. These findings suggest that contact between cells and asbestos may affect the human immune system and trigger a cascade of biological events, such as the activation of Src family kinases, enhancement of IL-10 expression, STAT3 activation, and Bcl-2 overexpression, as shown on the right side of Figure 1 and as previously reported (33). This speculation was partially confirmed by the detection of higher *bcl-2* expression levels in $CD4^+$ peripheral blood T cells from patients with malignant mesothelioma than in those from patients with asbestosis or from HDs (33).

In addition, if asbestos possesses the superantigenic potential against T cells, a certain number of the T cell receptor Vβ (TCR Vβ) repertoire may be overexpressed without evidence of clonal expansion, as observed on T cells exposed to superantigens such as staphylococcal enterotoxin B (SEB). Therefore, the expression levels of TCR Vβ on MT-2Org and MT-2Rst were compared. In addition, 23 types of TCR Vβ expression were examined on $CD3^+$ peripheral blood T cells. As a result, MT-2Rst cells overexpressed various TCR Vβ (34). Although TCR Vβ-overexpressing MT-2Org cells underwent apoptosis due to their first contact with chrysotile, MT-2Rst cells showed no significant changes when they again came in contact with CB. The over-expression of various TCR Vβ may be the result of contact between cells and CB, asbestos fiber, during the acquisition of resistance to CB-induced apoptosis caused by long-term and low-dose exposure to CB. To support this interpretation, patients with asbestos-related diseases (ARDs), such as asbestosis and malignant mesothelioma, were compared with SILs as a disease control and with HDs. ARDs showed limited overexpression of TCR Vβ without clonal expansion, whereas SILs showed significant overexpression of TCR Vβ 7.2. These experimental and clinical analyses indicate superantigenic and dysregulation of the autoimmunity-inducing effects of asbestos and silica, respectively (34).

There are still many issues concerning the immunological effects of asbestos, particularly from the viewpoint of tumor immunity. NK cells may also be affected by exposure to asbestos, and Treg, which regulate autoreactions including tumor immunity, may change their function following their exposure to asbestos. In addition, the characterization of immunocompetent cells may be modified not only by asbestos fibers *in vivo*, but also by malignant tumor cells such as mesothelioma cells; however, most of these changes have not been clarified yet. Thus, future investigations should be carried out, and the discovery of biological tools to improve the prognosis of patients with asbestos-related malignancies is anticipated.

Detection of autoantibodies, alterations of Fas-related molecules and $CD4^+CD25^+$ regulatory T cell fraction in SILs

To clarify the status of autoimmunity abnormalities found in SILs, efforts have been made to detect autoantibodies in serum derived from SILs, and autoantibodies against topoisomerase I (35-37), desmoglein (38), caspase-8 (39) and Fas (40) were detected. In particular, the last two autoantibodies may be of interest because the target molecules, i.e., caspase-8 and Fas, have a key role during apoptosis processing in lymphocytes. The functional assay of antibodies against Fas showed that the autoantibody induces Fas-mediated apoptosis of membrane-Fas-expressing cells (40).

Fas (CD95), which is mainly expressed on the cell membrane of lymphocytes, usually exists as membrane type-Fas and forms a trimer after binding with the Fas ligand. The signal-transducing death-domain located in the intra-cellular domain of Fas then recruits Fas-associating death-domain-containing protein (FADD) and procaspase 8 to form the active death-inducing signaling complex (DISC). Thereafter, activated caspase-8 triggers a caspase cascade

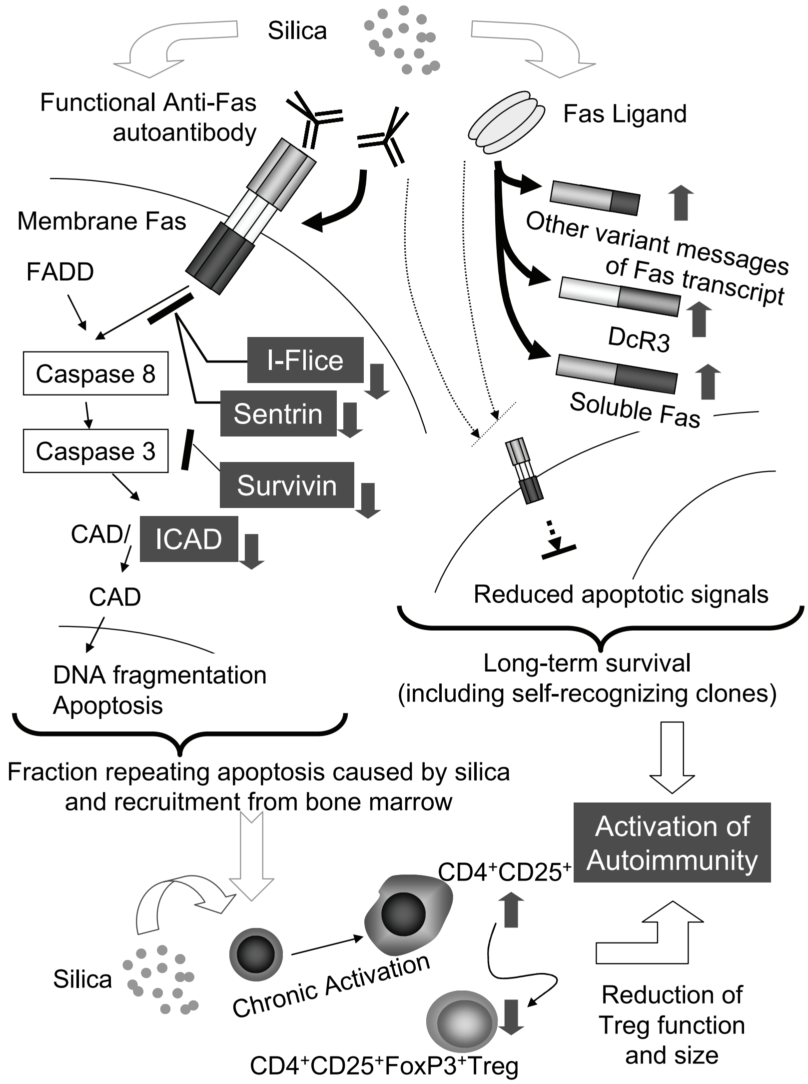

Figure 2. Schematic presentation of the activation mechanisms of autoimmunity found in silicosis patients focusing on the alterations of Fas and Fas-related molecules, and attenuated function of the $CD4^{+}CD25^{+}$ T cell fraction.

involving the activation of CAD/CPAN/DFF40 by removing its inhibitor, ICAD/DFF45, DNA fragmentation, and finally apoptosis (41-45).

The most typical alternatively spliced variant of the wild-type *fas* gene transcript is soluble *fas*. As this variant transcript lacks 63 bp of the transmembrane domain, its product (soluble Fas) is secreted from cells to suppress membrane Fas-mediated apoptosis by blocking the binding between membrane Fas and the Fas ligand in the extracellular region (46, 47). If there is a high level of soluble Fas in the extracellular region, lymphocytes in these regions may avoid apoptosis and survive longer. Indeed, there have been several studies showing elevated levels of serum-soluble Fas in patients with autoimmune diseases (48-51); therefore, we compared cellular and molecular changes in the levels of Fas and Fas-related molecules between SILs and healthy donors (HDs):

The level of serum-soluble Fas was higher in SILs than HDs (52). The level of serum-soluble Fas ligand did not differ between SILs and HDs (53). Although the Fas ligand is usually localized in the membrane of natural killer (NK) cells, activated T cells, and cytotoxic T cells, it is sometimes cleaved by matrix-metalloproteinase-like enzymes and secreted into extracellular spaces (54, 55). Although the percentage of Fas-positive lymphocytes (membrane Fas expression) did not differ between SILs and HDs, the mean fluorescence intensity (MFI) of membrane Fas was lower in SILs than in HDs. In addition, weaker membrane Fas expressers among lymphocytes were identified to be weaker *fas* message expressers (52, 56). The relative gene expression ratio of wild-type and soluble *fas* and various genes related to Fas-mediated apoptosis, such as *decoy receptor 3* (*dcr3*), the apoptosis-accelerating genes *caspase-8*, *-3*, and *-9* and *cpan* (*cad*), and the intracellular apoptosis-inhibitory genes *xiap*,

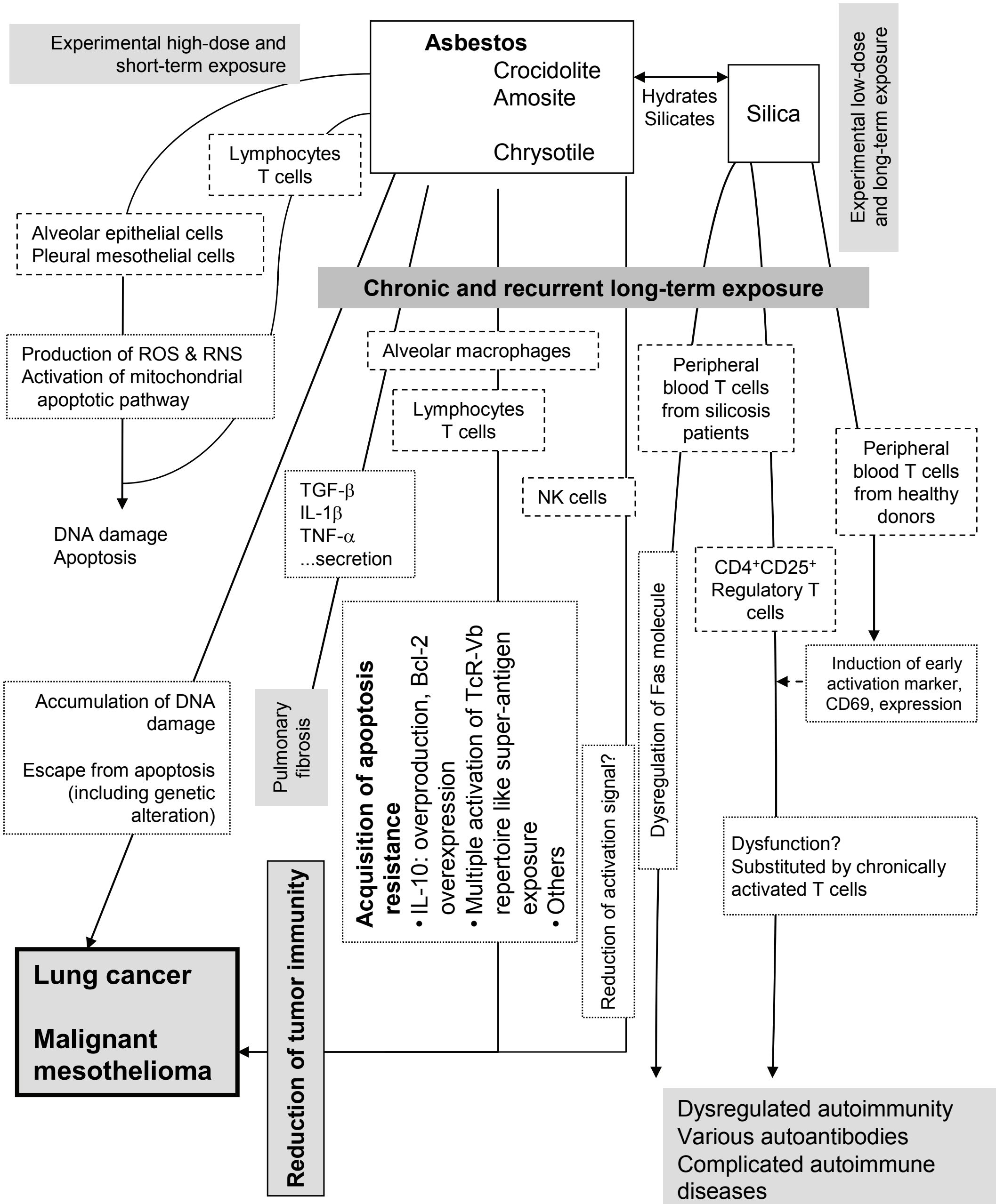

Figure 3. Summary of immunological effects of silica/asbestos.

survivin, *dff45* (*icad*), *toso*, *i-flice*, and *sentrin*, in peripheral blood mononuclear cells (PBMCs) was analyzed (57-60). DcR3 was initially discovered as a protein secreted from lung and colon cancer cells that prevents the Fas ligand from targeting them, and is also expressed on cytotoxic T cells and natural killer cells (61, 62). Thus, DcR3 functions similarly to soluble Fas, namely, it inhibits membrane Fas-mediated apoptosis. The findings were as follows, (i) soluble *fas* mRNA is predominantly expressed in PBMCs from SILs, but not from HDs (57), (ii) the *dcr3* gene expression level is higher in PBMCs from SILs than from HDs (59) and these may induce the inhibition of Fas and Fas ligand binding similar to the cases with a higher level of soluble Fas molecules and (iii) the gene expression levels of intracellular inhibitors of Fas-mediated apoptosis such as *i-flice*, *sentrin*, *survivin*, and *icad* were lower in SILs than in HDs (59, 60). Alternatively spliced variants of *fas* and mutational screening for *fas* and *fas ligand* genes were then detected (63). Although significant mutations in *fas* and *fas ligand* coding sequences were not detected, many alternatively spliced variants were found and analysis of amino-acid translation from detected variants showed that all of these as well as the typical soluble *fas*, possess the binding site of the Fas ligand, but lack the transmembrane domain and death domain. These findings indicate that all these variants may inhibit the binding between membrane Fas and the Fas ligand, similar to soluble Fas and DcR3 molecules (63).

In vitro exposure of T cells derived from HD to silica causes slow but precise activation of these cells, as indicated by the expression of CD69, a typical early marker of T cell activation (64).

The percentage of the peripheral blood $CD4^+CD25^+$ fraction, which includes $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) suppressing excess autoreaction, in the scarce self-recognizing T cell fraction in peripheral blood, was slightly lower in SILs as determined in terms of age-predicted values calculated from the analysis of HD. In addition, the function of this fraction in SILs was less significant than in HDs, as determined by alloreactive mixed lymphocyte reaction (MLR) analysis (65).

From these findings, a hypothesis for activated autoimmunity in SILs has been proposed, as shown in Figure 2 and preliminarily reported previously (56, 66). The findings of the levels of factors in extracellular spaces, such as soluble Fas, DcR3, and products from various alternatively spliced *fas* variants, indicate that apoptosis mediated by membrane Fas seems to interfere with these molecules and Fas-mediated apoptosis is reduced. However, since there was a reduced expression of intracellular molecules for anti-Fas-mediated apoptosis such as *i-flice*, *sentrin*, and *survivin* gene products in SILs compared with those in HDs, it seemed likely that Fas-mediated apoptosis is enhanced in lymphocytes derived from SILs. In addition, the anti-Fas autoantibody found in serum from SILs may contribute to the enhanced apoptosis of lymphocytes, because of the Fas-stimulating function of this antibody. As compared with HDs, in which the apoptosis of lymphocytes is assumed to be neither enhanced nor reduced, it seems that the two fractions of lymphocytes would respectively show enhanced and reduced Fas-mediated apoptosis in SILs.

Thus, there are two populations of $CD4^+$ lymphocytes, the stronger expresser of membrane Fas and the weaker expresser of Fas, in SILs. Weaker expressers may have developed owing to excessive transcription of the alternatively spliced *fas* gene and other variant messages; therefore, these cells may be resistant to the functional anti-Fas autoantibody, because membrane Fas is relatively scarce. Consequently, it is speculated that there is a particular fraction of $CD4^+$ T lymphocytes in SILs that expresses weak levels of membrane Fas, secretes higher levels of soluble Fas, DcR3, and spliced variants, and is resistant to anti-Fas autoantibody-induced apoptosis, as shown in Figure 2 and previous reports (56, 66). As patients with a weaker MFI of membrane Fas have a higher titer of anti-nuclear antigens (ANA), as reported previously (56), self-recognizing clones in SILs may be included in this fraction, because these clones may survive longer and show resistance to apoptosis.

It is possible that Fas-mediated apoptosis occurs to a certain degree in lymphocytes of SILs, because of the observed decrease in the levels of intracellular inhibitors of Fas-mediated apoptosis. This may be explained by the presence of a different fraction of lymphocytes in SILs, which are strongly positive for membrane Fas, sensitive to the anti-Fas autoantibody, and undergo apoptosis; however, this fraction may be recruited from bone marrow after reaching the final stage of cell death. This recruited fraction would not have encountered silica and would be sensitive to silica/silicate-induced apoptosis. As a result, cells in this fraction would be continuously undergoing renewal and apoptosis (56, 66).

In addition, the attenuated function of the $CD4^+CD25^+$ fraction of T cells also activated autoimmunity (67-70). This attenuation may be caused by substitution of the $CD4^+CD25^+$ fraction by chronically activated T cells due to their chronic and recurrent exposure to silica, as shown by our *in vitro* findings of the slower activation of T cells by silica (64).

However, it is necessary to clarify why silica exposure leads to a higher frequency of alternative splicing of *fas* (or other) gene(s), whether weaker expressers of membrane Fas among lymphocytes survive for a significantly long time and include self-recognizing clones, and how silica exposure causes a decrease in $CD4^+CD25^+FoxP3^+$ Treg. Recently, the relationship between the expression level of membrane Fas and Treg function has been noted and investigated (71, 72). This may also be interesting to clarify the mechanisms underlying the dysregulation of autoimmunity caused by silica exposure.

Conclusion

A summary of the findings described in this article is shown in Figure 3. Recent advances in immunomolecular studies led to detailed analyses of the immunological effects of asbestos and silica. Both affect immuno-competent cells and these effects may be associated with the pathophysiological development of complications in silicosis and asbestos-exposed patients such as the occurrence of autoimmune disorders and malignant tumors, respectively. In addition, immunological analyses may lead to the discovery of new clinical tools to modify the pathophysiological aspects of diseases such as the regulation of autoimmunity or tumor immunity using cell-mediated therapies, various cytokines and molecule-targeting therapies. As the incidence of asbestos-related malignancies is increasing and such malignancies have been a medical and social problem since the summer of 2005 in Japan, efforts should be focused on developing a cure for these diseases to eliminate nationwide anxiety.

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