Cellular Characteristics of an Asbestos (Chrysotile-B) Resistant Subline of an HTLV-1-Immortalized Human Polyclonal T Cell Line (MT-2)

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Introduction

There are two common subtypes of pneumoconiosis, silicosis and asbestosis. Patients with silicosis have been exposed to silica and their condition is often complicated by autoimmune disorders such as SLE or SSc (systemic scleroderma)[1-3]. Asbestosis patients have been exposed to asbestos (silicates) in such forms as chrystotile and crocidolite, and may develop not only respiratory diseases but also malignant tumors, including lung cancers and malignant mesotheliomas[4-7]. We have been focusing our investigations on the immunological abnormalities found in silicosis and have found abnormalities of various molecules in the Fas-mediated apoptotic pathway[8-14] and detected various unique auto-antibodies[15-18] . These facts and our findings indicate that silica and silicates affect the human immune system and may cause dysregulation of autoimmunity and reduction of tumor immunity. To explore the immunological cellular and molecular effects of silicates, an HTLV-1 immortalized human polyclonal T cell line, MT-2[19], was employed.

Appearance of Apoptosis Following Short and High Dose Exposure

Before the long-term and low-dose exposure effects of chrysotile on MT-2 cells could be examined in an in vitro model of human asbestosis from the immunological viewpoint, it was necessary for us to clarify what occurs after short and relatively high-dose exposure to chrysotile in MT-2 cells. Therefore, MT-2 cells were cultured with or without 5 to 50 μg/ml of chrysotile-A (CA) for one to four days and cell growth, the appearance of apoptosis (TUNEL method), activation of caspases 3 and 9, the relative balance of Bcl2 and Bax proteins, the release of cytochrome-c to cytoplasma from mitochondria, activation of the p38 and JNK MAP kinases, which stimulate the apoptotic signal, and production of superoxide were analyzed. As shown in Fig.1, MT-2 cells cultured with CA exhibited growth inhibition (Fig. 1-A), and increases in apoptotic fractions (Fig. 1-B and C) in a dose dependent manner. The appearance of apoptosis was also confirmed by the detection of activated caspase 3 and caspase 9 (Fig 2), which are the typical indicators of activation of the mitochondrial apoptotic pathway.

Thereafter, molecular modifications known to take place in the mitochondrial apoptotic pathway were examined by Western blotting. As shown in Fig. 3, cytochrome-c was released from mitochondria (Mi) to cytoplasma (Cy), the relative expression ratio of BAX/Bcl2 increased, and JNK and p38, both known as apoptosis-inducing MAK kinases, were phosphorylated in a dose-dependent manner in MT-2 cells cultured with 0, 10, and 25 mg/ml of CA.

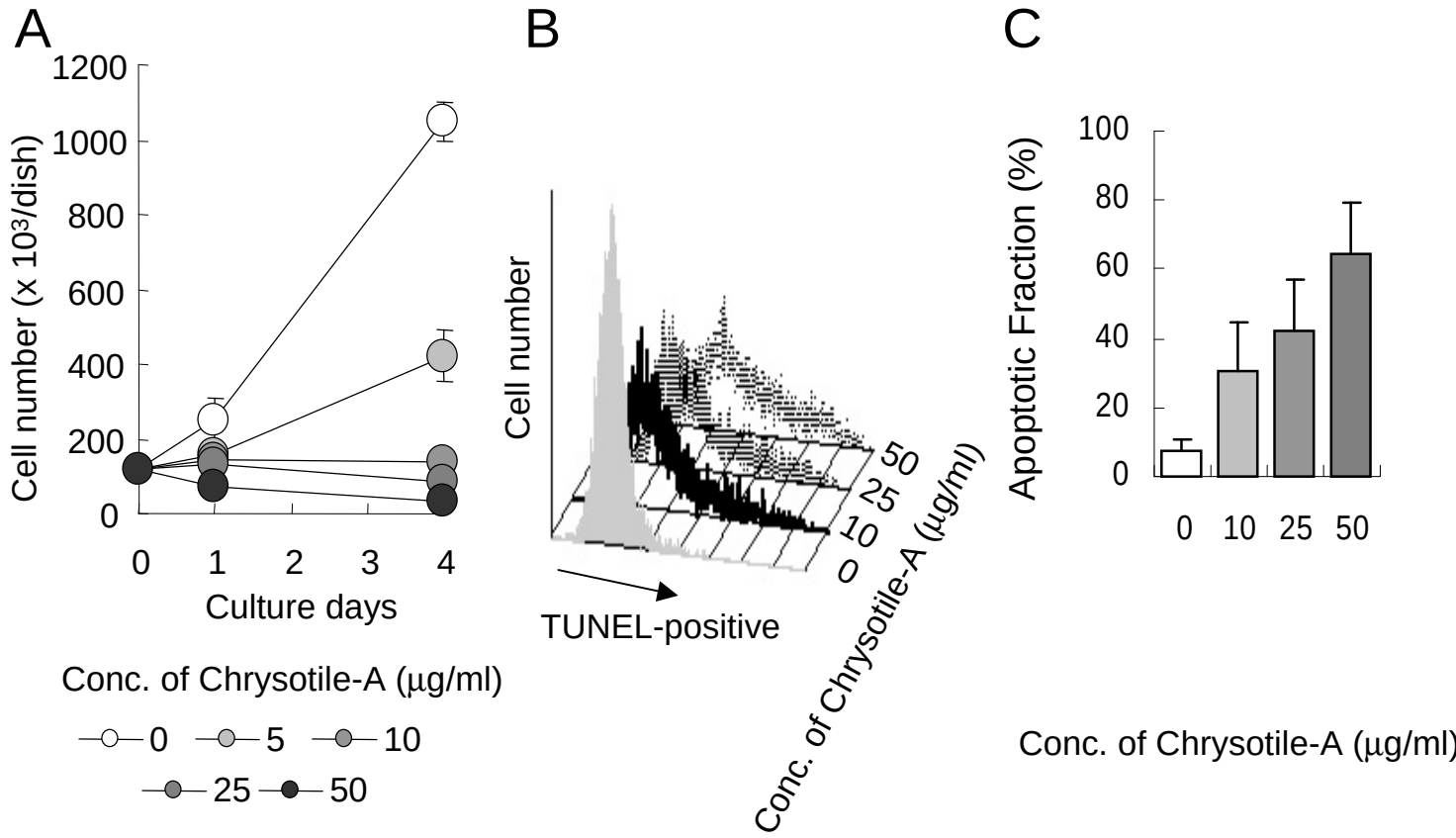

Fig. 1 [A] Growth curve of MT-2 cells cultured with various concentrations of chrysotile-A (CA). [B] TUNEL detection of apoptotic cells in MT-2 cells cultured with CA and increase of its fraction in a dose-dependent manner [C].

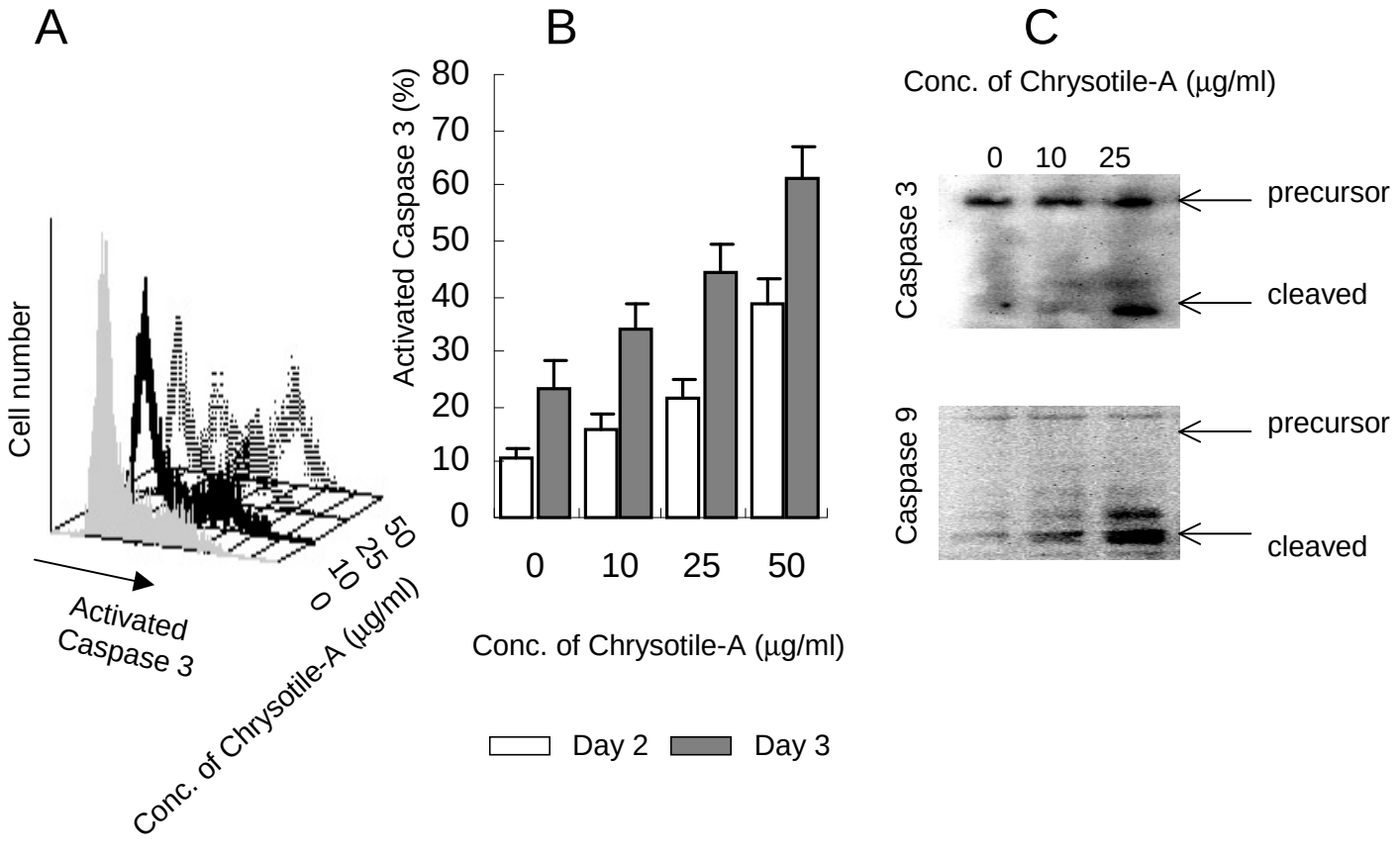

Fig. 2 [A] Activation of caspase 3 analyzed by flow cytometry. [B] Increase of activated caspase 3 fraction in dose and time-dependent manners when MT-2 cells were cultured with various concentrations of CA. [C] Activation of caspase 3 and 9 analyzed by Western blotting. Cleaved forms were observed in a dose-dependent manner.

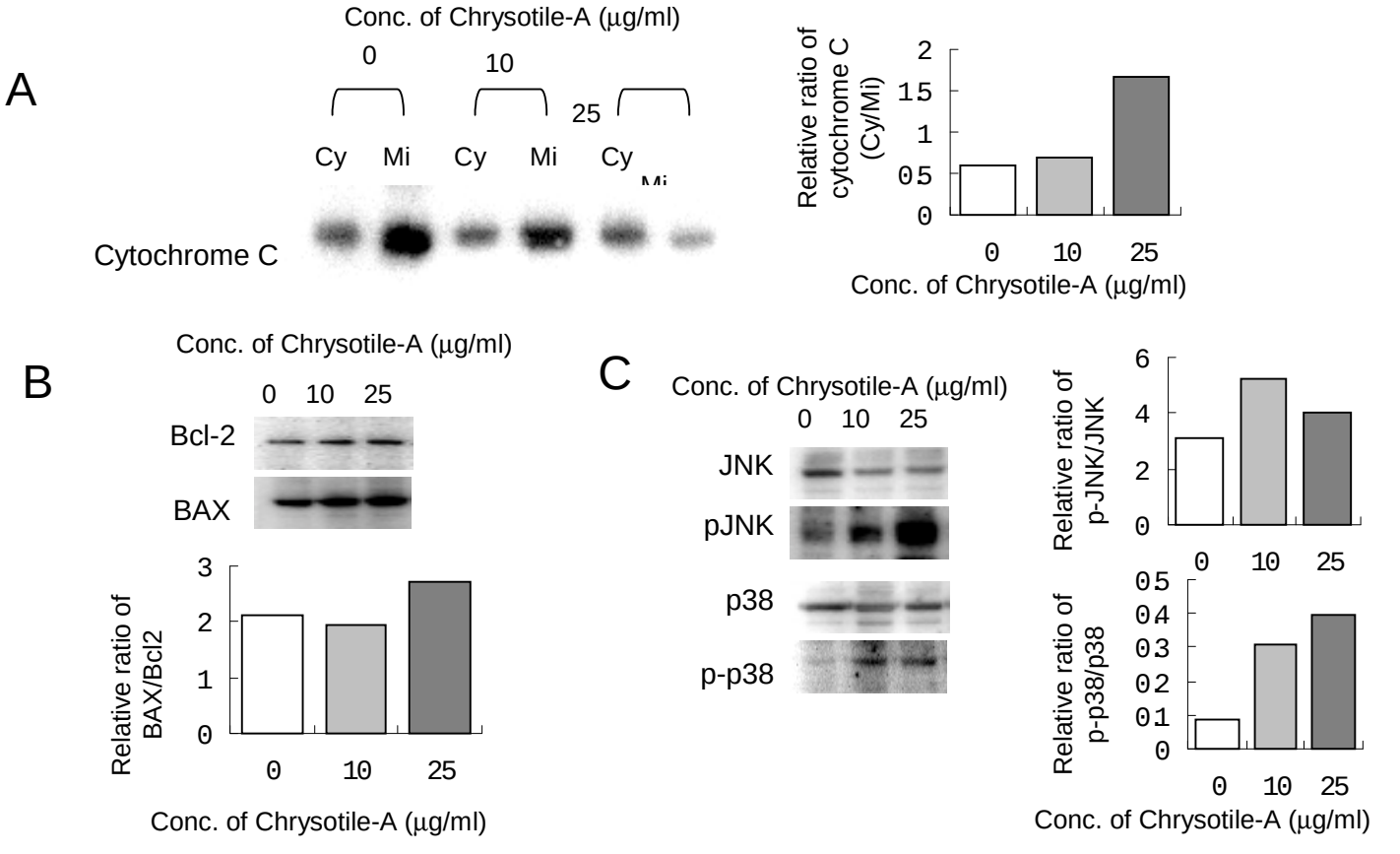

Fig. 3 Western blotting analysis of mitochondrial apoptotic pathway related molecules. When MT-2 cells were cultured with 25 μg/ml of CA, [A] cytochrome-c release to Cy (cytoplasma) from Mi (mitochondria), [B] increase of the Bax/Bcl2 balance, and [C] phosphorylation of apoptosis-stimulating MAP kinases such as JNK and p38 were observed.

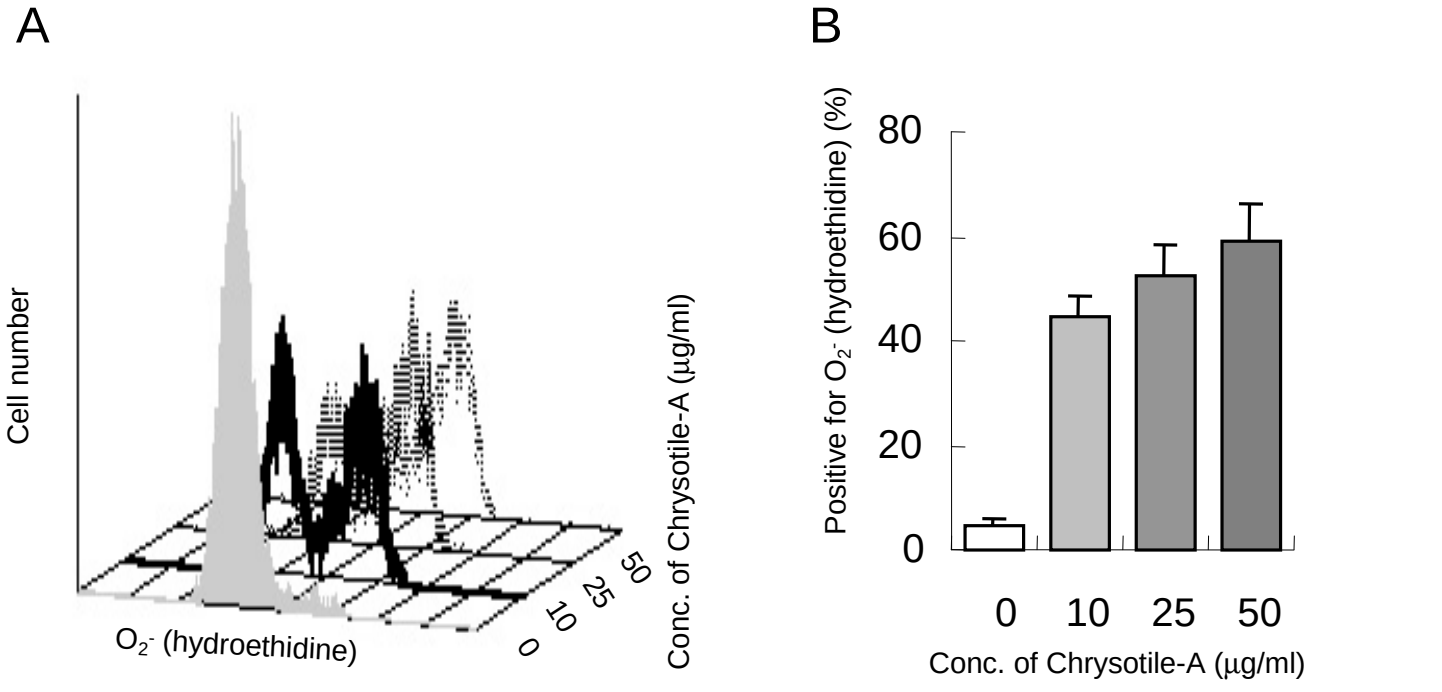

Fig. 4 Production of superoxide in MT-2 cells cultured with CA. [A] Production of superoxide was detected by flow cytometry and [B] The cell percentage of positive for O_{2^-} cells increased in a dose dependent manner.

Since cellular environmental stress or cytotoxic chemical substances induce activation of the mitochondrial apoptotic pathway to cause cell death, the role of ROS (reactive oxygen species) is considered a very important factor. Therefore, the production of superoxide was analyzed using flow cytometry

As shown in Fig. 4, MT-2 cells cultured with 0, 10, 25, and 50 µg/ml of CA showed increased production of superoxide in a dose dependent manner. Together with data showing that anti-oxides significantly reduced CA-induced apoptosis in MT-2 cells, these results indicate that MT-2 cells undergo apoptosis via activation of the mitochondrial pathway when cells are exposed to chrysotile for a short time and in high doses.

Establishment of an In Vitro T Cell Model For Long-Term and Low Dose Exposure to Asbestos

As mentioned above, MT-2 cells proceeded to apoptosis when cultured with 25 to 50 µg/ml of chrysotile for a few days. We considered that if MT-2 cells were continuously exposed to relatively low doses (i.e., 5 or 10 µg/ml) of chrysotile for months to years, these cells might acquire resistance to chrysotile-induced apoptosis.

Although the initial cultures of MT-2 cells with 5 or 10 µg/ml of chrysotile-B (CB) only permitted us to change the whole medium and to keep total cell number in their culture dishes, several months later, they gradually started to grow even with low doses of CB.

Eight months after starting with low-dose continuous exposure, the exposed subline (MT-2Rst) showed a decrease in the appearance of TUNEL positive fractions when cultured with high doses (25 to 50 µg/ml) of CB when compared with the original MT-2 cells (MT-2Org). In addition, the growth of MT-2Rst cultured with high doses of CB was not reduced when assayed by WST-1 (Fig. 5A and B). These results indicated that more than eight months exposure to CB induced resistance to chrysotile-induced apoptosis in MT-2 cells.

The cellular and molecular biological differences between MT-2Org and MT-2Rst should be analyzed to try to determine the mechanisms involved in the silicate/silica induced dysregulation/reduction of the human immune system. As a first step, cDNA microarray analysis was employed to find gene expression differences between these two lines. In Tables 1 and 2, the up- and down-regulated genes are respectively listed in the order of their relative expression ratio (MT-2Org/MT-2Rst) in the gene. Among these genes, there are several genes of interest, such as the chloride channel 5 gene (clcn5), one of the chemokines (scya8), and one of the virus receptors (cxadr) in the upregulated group, and one of the chemokines (scyb13) and the receptor type protein tyrosine phosphatase (ptprn2; this is also an autoantibody found in type I DM patients) in the downregulated group.

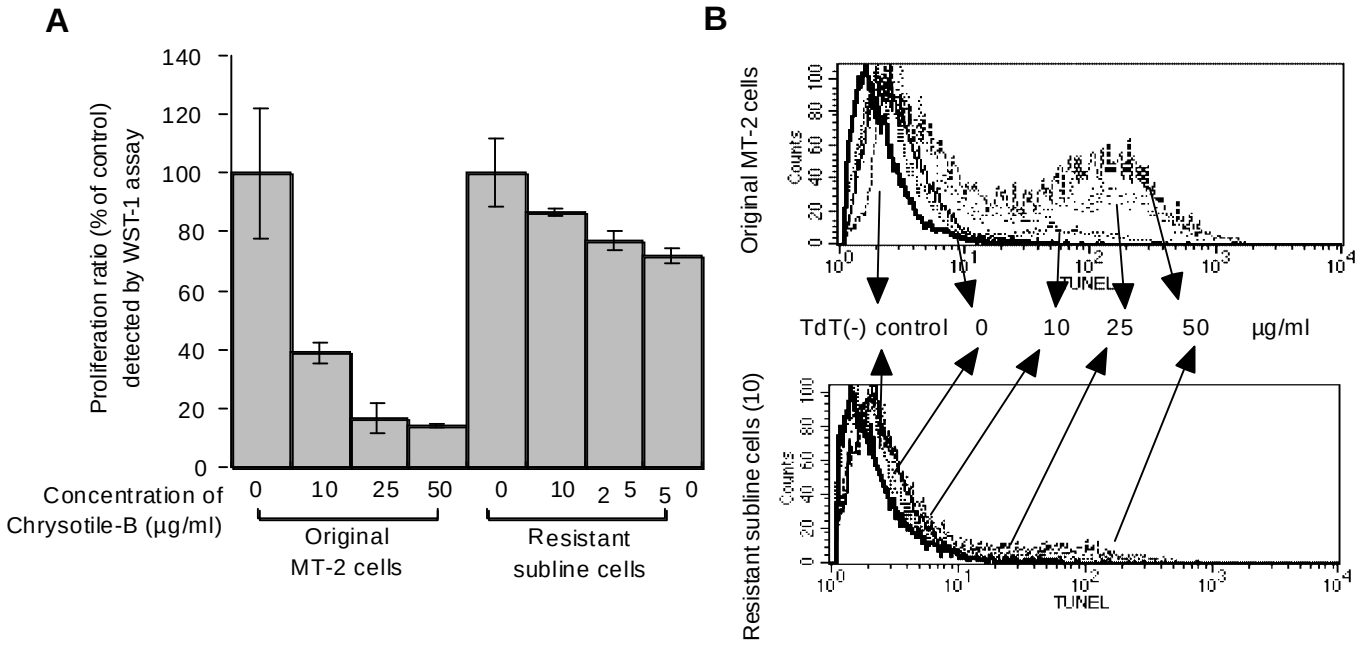

Fig. 5 MT-2, showed dose-dependent growth inhibition [A, left] and a dose-dependent increase in TUNEL-positive apoptotic cells [B, upper panel], when cultured with various concentrations of Chrysotile-B (CB) for two days. However, after continuous exposure to 10 μg/ml of CB for eight months, a CB-resistant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

Table 1 The list of up-regulated genes in the MT-2-Rst line compared with the MT-2-Org line analyzed by cDNA microarray

Name of genes	Ratio: Original/Resistant subline
poly(a) polymerase gamma; papolg	0.091
chloride channel 5; clcn5	0.124
tolloid-like 2; tll2	0.162
coxsackie virus and adenovirus receptor; cxadr	0.179
epoxide hydrolase 1, microsomal (xenobiotic); ephx1	0.202
small inducible cytokine subfamily a (cys-cys), member 8 (monocyte chemotactic protein 2); scya8	0.202
chromobox homolog 8; cbx8	0.208
similar to non-functional folate binding protein loc82903	0.232
ebv-induced g protein-coupled receptor 2; ebi2	0.310
doublecortex; lissencephaly, x-linked (doublecortin); dcx	0.350
chromosome 20 open reading frame 1; c20orf1	0.358
nhp2 non-histone chromosome protein 2-like 1 (s. cerevisiae); nhp2l1	0.378
potassium voltage-gated channel, shaw-related subfamily, member 1; kcnc1	0.391
mad, mothers against decapentaplegic homolog 3 (drosophila); madh3	0.412
chromosome 8 open reading frame 1; c8orf1	0.414
pro2353 predicted protein of hq2353	0.488

Table 2 The list of downregulated genes in the MT-2-Rst line compared with the MT-2-Org line analyzed by cDNA microarray

Name of gene	Ratio:Original/Resistant subline
small inducible cytokine b subfamily (cys-x-cys motif), member 13 (b-cell chemoattractant); scyb13	12.065
protein tyrosine phosphatase, receptor type, n polypeptide 2; ptprn2	4.880
eukaryotic translation initiation factor 4a, isoform 1; eif4a1	4.519
cgi-143 protein; loc51027	4.254
nadh dehydrogenase (ubiquinone) 1 beta subcomplex, 6 (17kd, b17); ndufb6	4.203
ubiquitin-conjugating enzyme e2n (homologous to yeast ubc13); ube2n	3.845
hypothetical protein xp_051150; loc93398	3.783
karyopherin (importin) beta 3; kpnb3	3.231
chaperonin containing tcp1, subunit 4 (delta); cct4	3.091
proteasome (prosome, macropain) subunit, alpha type, 1; psma1	2.994
non-metastatic cells 1 protein; nme1	2.941
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, complete cds; glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	2.886
tata box-binding protein-associated factor 2f; taf7	2.844
pituitary tumor-transforming protein 1; pttg1	2.828
h2a histone family, member z; h2afz	2.801
testican 3; hsaj1454	2.785
cofilin 1 (non-muscle); cfl1	2.746
ran binding protein 1; ranbp1	2.725
splicing factor, arginine/serine-rich (transformer 2 drosophila homolog) 10; sfrs10	2.707
signal recognition particle 14kd (homologous alu rna binding protein); srp14	2.673
tryptophanyl-trna synthetase; wars	2.653
fumarate hydratase; fh	2.650
methionyl aminopeptidase 2; metap2	2.635
hypothetical protein flj11506; flj11506	2.596
trp-related cation influx channel; trpm4	2.530
transcription elongation factor a (sii), 1; tcea1	2.514

A few more resistant sublines should be established to detect common alterations in gene expression profiles and to investigate the biological roles of the detected genes. In addition, proteomics and transcriptome analyses may lead to the discovery of important molecules involved in the mechanisms of asbestos-induced immune dysfunction.

Conclusion

A human polyclonal T cell line, MT-2, was sensitive to asbestos-induced apoptosis, and there was significant involvement of the ROS system in the appearance of apoptosis, as has been noted in other reports which confirmed the role of ROS in asbestos-induced cell death in alveolar and mesothelial cells[20-26] . In addition, cellular and molecular biological comparison of MT-2Org and MT-2Rst should provide us with clues to the mechanisms involved in the occurrence of the immune dysregulation found in asbestosis or silicosis patients.

Acknowledgement

The authors thank Ms Haruko Sakaguchi, Tamayo Hatayama and Satomi Hatada for their skilful technical assistance. This study was supported by a JSPS KAKENHI Grant (16390175) and Kawasaki Medical School Project Grants (16-401M and 16-212S).

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