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Involvement of IL-10 and Bcl-2 in resistance against an asbestos-induced apoptosis of T cells

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Abstract To analyze the possibility that immunological alteration in asbestos-related diseases (ARDs) such as asbestosis (ASB) and malignant mesothelioma (MM) may affect the progression of cancers, a human adult T cell leukemia virus-immortalized T cell line (MT-2Org) was continuously exposed to 10 μg/ml of chrysotile-B (CB), an asbestos. After at least 8 months of exposure, the rate of apoptosis in the cells became very low and the resultant subline was designated MT-2Rst. The MT-2Rst cells were characterized by (i) enhanced expression of *bcl-2*, with regain of apoptosis-sensitivity by reduction of *bcl-2* by siRNA, (ii) excess IL-10 secretion and expression, and (iii) activation of STAT3 that was inhibited by PP2, a specific inhibitor of Src family kinases. These results suggested that the contact between cells and asbestos may affect the human immune system and trigger a cascade of biological events such as activation of Src family kinases, enhancement of IL-10 expression, STAT3 activation and Bcl-2 overexpression. This speculation was partially confirmed by the detection of elevated *bcl-2* expression levels in CD4+ peripheral blood T cells from patients with MM compared with those from patients with ASB or healthy donors. Further studies will be required to verify the role of T cells with enhanced *bcl-2* expression in tumor progression induced by asbestos exposure.

Keywords Asbestos · T cell · IL-10 · Bcl-2 · Apoptosis

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Introduction

Although most developed nations have banned the industrial use of all types of asbestos, an increase in usage has been reported in developing countries, particularly in Asia. In Japan, most asbestos has been banned since October, 2004. However, workers of, housewives of workers of, and residents near asbestos-using factories have been dying from asbestos-related diseases. These issues constitute social and public problems with medical, industrial and political ramifications.

Asbestos (e.g. chrysotile, crocidolite, and amosite) is known to cause malignant lung cancer or mesothelioma [1–5]. The International Agency for Research on Cancer (IARC) categorizes both asbestos and crystalline silica as group I carcinogens [6]. According to the IARC classification, asbestos affects alveolar epithelial and mesothelial cells. There have been many studies concerning asbestos-induced apoptosis in these cells [7–9]. Under experimental coditions, these cells undergo apoptosis upon relatively high-level,

short-term exposure to asbestos as a result of the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) with activation of the mitochondrial apoptotic pathway [10–12]. Furthermore, several non-small-cell lung cancer cell lines contain constitutively active signal transducer and activator of transcription 3 (STAT3) [13]. It is also known that inhibition of tumor-derived IL-10 and IL-10 receptor (IL-10R) interaction by an autocrine/paracrine loop results in a decrease of the constitutively active STAT3 and subsequent inhibition of Bcl-2 transcription and expression [14]. Thus, it has been considered that during low-level, long-term exposure to asbestos in the human body, alveolar epithelial and mesothelial cells escape from the apoptotic pathway due to genetic changes and undergo malignant transformation.

We have also found that asbestos polyclonally activated CD4-positive T cells and caused activation-induced cell death [15–17]. In addition, peripheral blood mononuclear cells (PBMCs) from healthy individuals 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 activation of the mitochondrial apoptotic pathway with phosphorylation of p38 mitogen-activated protein kinase (MAPK) and JNK signaling molecules, shifting to a Bax-dominant Bax/Bcl-2 balance, release of cytochrome-c from mitochondria into the cytosol, and activation of caspases 9 and 3 [18]. In this study, 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. In addition, differences such as overexpression of the *bcl-2* gene were analyzed using peripheral blood CD4-positive T-lymphocytes derived from patients with asbestos-related diseases such as malignant mesothelioma and asbestosis.

Materials and methods

Cell culture

An HTLV-1-immortalized human polyclonal T cell line, MT-2, was maintained in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum plus antibodies at 37°C in a humidified atmosphere [19]. MT-2 cells were cultured with various concentrations of CB and cell viability was assessed by the exclusion of trypan blue dye.

Detection of apoptotic cells

To detect apoptotic MT-2 cells, the terminal deoxynucleotidyl transferase biotin-dUDP nick end labeling (TUNEL) method was performed as reported previously [18].

Cell growth assay

MT-2 cells (1×10^4 cells per well in a 96-well plate) were cultured in 100 μl of medium containing 0, 1, 5, 10, 25, or 50 μg/ml of CB. The growth properties were analyzed using water soluble tetrazolium salt (WST-1) as reported previously [20]. Cell growth was tested using a Premix WST-1 Cell Proliferation Assay System (Takara Biochem., Tokyo, Japan) according to the manufacturer's instructions.

Western blotting

Whole cell lysates from drug-treated MT-2 cells were made with lysis buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate and 0.05% SDS) supplemented with protease inhibitor (Sigma-Aldrich Japan Inc., Tokyo, Japan,) as reported previously [21, 22]. Soluble fractions were boiled in 1 × SDS loading buffer before electrophoresis. Proteins in the gels were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech., Little Chjalfont, Buckinghamshire, UK) and blocked with 1% bovine serum albumin (BSA) in PBS with Tween 20. Blots were probed with primary antibodies against Bcl-2, STAT3, phosphorylated STAT3, (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and GAPDH (Dako-Cytomation Co. Ltd. Kyoto, Japan), followed by horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (Santa Cruz) secondary antibodies, and were visualized with enhanced chemiluminescent (ECL) reaction reagents (Amersham Pharmacia Biotech).

Small interference RNA (siRNA) design, synthesis and transfection

The siRNA sequence used for targeted silencing of human Bcl-2 (Genebank accession number; M13994) was designed by siRNA Design Support System (TaKaRa Bio Inc.). The double-stranded RNA consisted of the sense strand 5′-CAUCGCCCUGUGGAUGACUTT-3′ and the antisense strand 5′-AGUCAUCCACAGGGCGAUGTT-3′. Searches of the human genome database (BLAST) were carried out to ensure that the sequence would not target other gene transcripts. The transfection of siRNA was facilitated by

TransIT-TKO transfection Reagent (Mirus Corporation, Madison, WI, USA), which was used according to the manufacturer's directions [23].

Measurement of cytokines

The levels of interferon γ (IFNγ), TNFα, interleukin (IL)-10, and IL-6 in the culture supernatants from MT-2Org and MT-2Rst cells were measured using the Human Th1/Th2 Cytokine Kit II of the Cytometric Bead Array (CBA) Kit (BD Bioscience, San Jose, CA, U.S.A.). Detection was performed using FACSCalibur flow-cytometry (BD Bioscience) according to the manufacturer's instructions. The data were expressed as the mean of three measurements made using the CBA kit.

Real-time and multiplex reverse transcription-polymerase chain reaction (RT-PCR)

A fluorescence thermocycler (M × 3000P® QPCR System, Stratagene Corporation, La Jolla, CA) was used for real-time RT-PCR experiments following the instructions of the manufacturer. With this technique, the fluorescence-labeled amplification product is measured continuously. Total RNA obtained from MT-2 Org and MT-2Rst cells or CD4+ T cells isolated from PBMCs was extracted using an RNA Bee kit (Tel-Test, Inc., Friendswood, Texas), and 5 μg of RNA was reverse-transcribed with standard methods using a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas International Inc., Ontario, Canada). An amount of cDNA equivalent to 50 ng of RNA served as the template for PCR in a volume of 20 μl (each primer and SYBER Premix Ex Taq, TaKaRa). Multiplex RT-PCR to amplify control (*gapdh*) and target (*bcl-2*) genes in the same reaction tube with an optimal ratio of 2 primer sets was performed to confirm the silencing effect of siRNA for *bcl-2* as reported previously [24, 25]. The primers for *bcl-2* and *gapdh* were added to the same reaction tube at the optimal concentration for each primer set and PCR was done. The primers were as follows: Bcl-2; 5′-TGATGTGAGTCTGGGCTGAG-3′ (Forward: Fw) and 5′-GAACGCTTTGTCCAGAGGAG-3′ (Reverse: Rv), Bax; 5′-AGTAACATGGAGCTGCAGAGG-3′ (Fw) and 5′-ATGGTTCTGATCAGTTCCGG-3′ (Rv), IL-10; 5′-CAAGCCTTGTCTGAGATGATCC-3′ (Fw) and 5′-CATTCTTCACCTGCTCCACG-3′ (Rv), GAPDH; 5′-GAGTCAACGGATTTGGTCGT-3′ (Fw) and 5′-TTGATTTTGGAGGGATCTCG-3′ (Rv).

The relative expression of various target genes such as *bcl-2* and *bax* was calculated as follows when real-time RT-PCR was performed:

A: number of PCR cycles required to reach a certain intensity of fluorescence for the *gapdh* product.

B: number of PCR cycles required to reach the same fluorescent intensity for the target gene product (*bcl-2* or *bax*) derived from the same sample.

The relative level of the target gene is expressed as 1/2[B-A], with *gapdh* expression being 1.0. The PCR products were confirmed to be successfully amplified by standard agarose gel electrophoresis and staining with ethidium bromide.

Isolation of CD4+ T cells

The PBMCs were isolated from heparinized blood of healthy donors, and patients with asbestosis and malignant mesothelioma using a Ficoll-Hypaque density gradient (Separate-L®, Muto Pure Chemicals Co. Ltd., Tokyo, Japan). For the isolation of CD4+ T cells, PBMCs were further separated using Magnetic Cell Separation (MACS) CD4 MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The enriched cells were $>$ 90% pure as determined by flow cytometry.

Specimens were taken from healthy donors and patients from whom informed consent had been obtained. The Institutional Ethics Committee of Kawasaki Medical School, Hyogo College of Medicine, and Okayama Rosai Hospital had approved the project.

Statistical analyses

Comparisons of the results for relative gene expression and proliferation assayed by real-time RT-PCR and WST-1, respectively, were analyzed using Fisher's parametric least significant difference (PLSD) test.

Results

Growth curve and detection of apoptosis in MT-2 cells

As shown in Fig. 1(A), the growth of MT-2 cells was markedly reduced in the presence of more than 10 μg/ml of CB. In addition, among MT-2 cells cultured with 0, 1, 5, 10, 25 and 50 μg/ml of CB for 2 to 4 days, the apoptotic fraction detected by the TUNEL assay increased in a dose- and time-dependent manner (Fig. 1(B)). These results indicate that MT-2 cells are sensitive to CB-induced apoptosis and the appearance of the apoptotic fraction reflects the inhibition of their growth.

Establishment of a CB-induced apoptosis-resistant MT-2 subline (MT-2Rst)

To establish an *in vitro* model of long-term, low-level recurrent exposure to asbestos, MT-2 cells were continuously

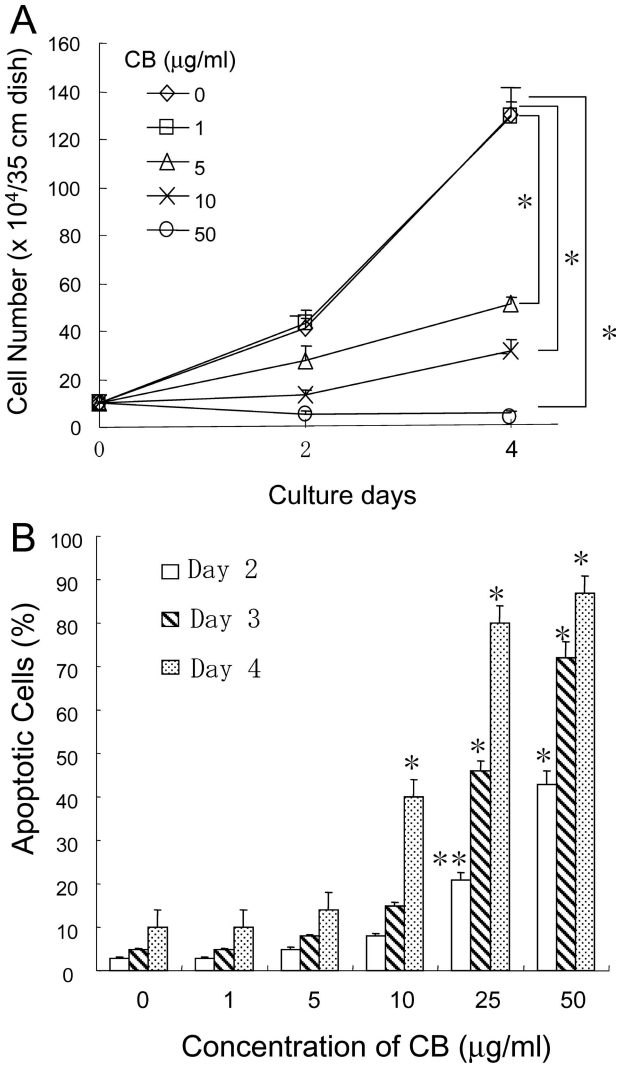

Fig. 1 Chrysotile-B (CB) induced apoptosis in MT-2 cells (A) MT-2 cells were cultured with 0, 1, 5, 10 or 50 μg/ml of CB for 2 or 4 days. The number of viable cells was assayed by exclusion of trypan blue dye. Results are expressed as the mean ± SD of three identical experiments ($^*p < 0.0001$). (B) MT-2 cells were cultured with 0, 1, 5, 10, 25 or 50 μg/ml of CB for 2, 3 or 4 days. The percentage of apoptotic cells was measured by the TUNEL method. Results are expressed as the mean ± SD of four identical experiments. ($^*p < 0.0001$; $^{**}p < 0.005$ compared with untreated cells)

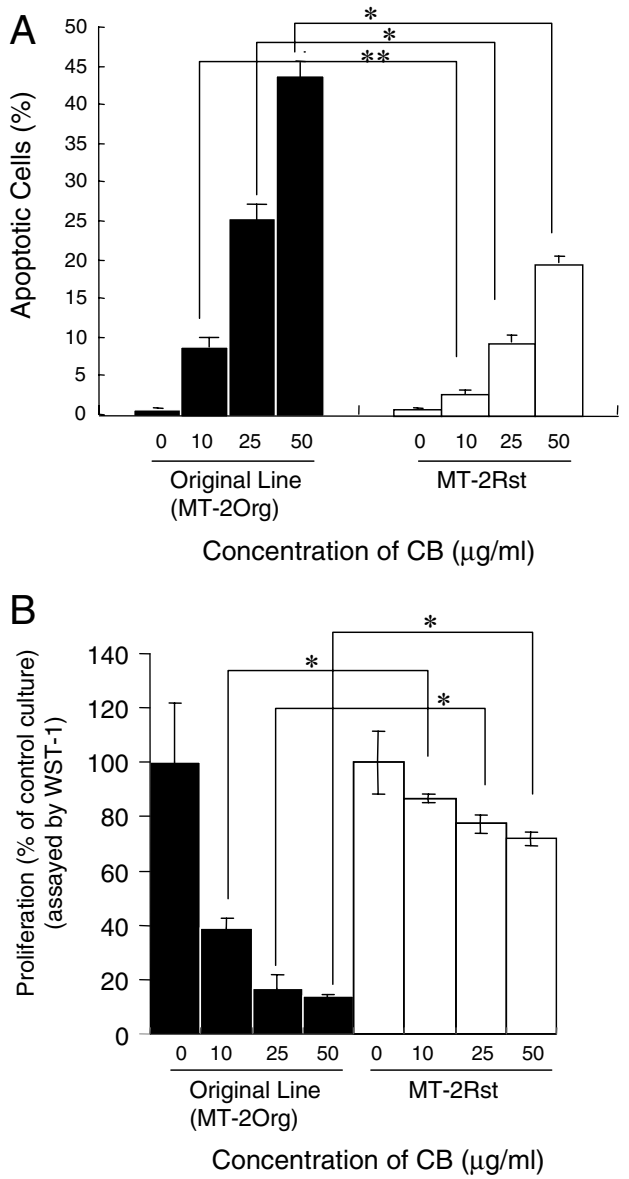

Fig. 2 Establishment of a CB-induced apoptosis-resistant MT-2 subline (MT-2Rst). (A) The original MT-2 cells (MT-2Org) were cultured with 10 μg/ml of CB for more than 8 months with monthly monitoring of apoptosis using the TUNEL method. The data are representative of three identical experiments at 8 months after the start of continuous cultivation. (B) The WST-1 assay was performed in MT-2Org and MT-2 cells continuously exposed to 10 μg/ml of CB (MT-2Rst). MT-2Org cells were exposed to 0, 10, 25 or 50 μg/ml CB and MT-2Rst cells were re-exposed to the same concentration of CB for 48 hours. Results are expressed as the mean ± SD of three identical experiments ($^*p < 0.0001$; $^{**}p < 0.005$)

cultured in RPMI 1640 medium containing 10 μg/ml of CB for more than 8 months. Assays were conducted monthly using the TUNEL method to examine whether or not the fraction of apoptotic cells had changed. Before each monthly assay, CB was removed from the culture using the Ficoll-Hypaque method. Then, MT-2 cells were re-started without CB for 7 days. Thereafter, the TUNEL assay was performed after exposure to 0, 10, 25, or 50 μg/ml of CB for 48 hours. Figure 2(A) shows that the percentage of TUNEL-positive cells was very low among the cells continuously exposed to 10 μg/ml of CB compared with the original (MT-2Org) cells, which have never been exposed to CB. Reflecting the appearance of the TUNEL-positive fraction, the growth of MT-2 cells that had been continuously exposed to 10 μg/ml of CB was not affected by re-exposure to CB, although MT-2Org cells exposed to 10, 25 and 50 μg/ml of CB showed a marked reduction in growth as analyzed using the WST-1 assay (Fig. 2(B)). These results suggest that MT-2 cells continuously exposed to 10 μg/ml of CB gained.

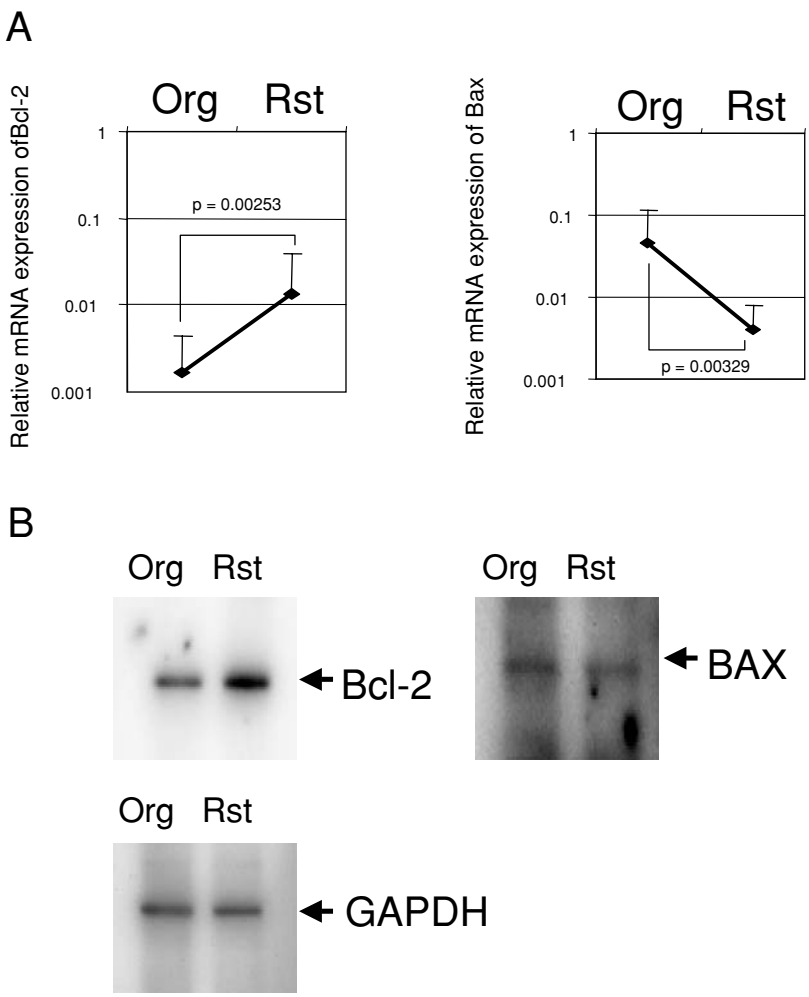

Fig. 3 Comparison of apoptosis-related gene expression between MT-2Org and MT-2Rst cells (A) Relative mRNA expression of *bcl-2* (light panel) and *bax* (right panel) in MT-2Org (Org) and MT-2Rst (Rst) cells. The relative expression of *bcl-2* and *bax* was estimated as described in Materials and Methods using real time RT-PCR with SYBR-G. (B) Western blot analysis of Bcl-2, Bax and GAPDH was performed using whole cell lysates from MT-2Org (Org) and MT-2Rst (Rst) cells. The data are representative of two identical experiments

resistance to CB-induced apoptosis over the course of more than 8 months. This subline was designated MT-2Rst.

Expression of apoptosis-related molecules in MT-2Org and MT-2Rst cells

First, the expression of various apoptosis-related genes such as *dff45, cIAP1, cIAP2, XIAP, survivin, apollon, acinus, smac, i-flice,* and *bid* was compared between the MT-2Org and MT-2Rst cell lines using real-time RT-PCR (data not shown). *bcl-2* and *bax* were the only genes for which the difference in the relative expression ratio exceeded 10 fold. The expression of *bcl-2* mRNA in MT-2Rst cells was approximately 10 fold higher than that in MT-2Org cells, whereas the expression of *bax* mRNA in MT-2Rst was over 10 fold lower than that in MT-2Org (Fig. 3(A)). Consistent with these results, the up-regulation of Bcl-2 expression and the down-regulation of Bax expression were observed using Western blotting in MT-2Rst compared with MT-2Org (Fig. 3(B)). These results suggest that the Bcl-2/Bax balance favoring an anti-apoptotic function may play an important role in the acquisition of resistance to CB-induced apoptosis in MT-2Rst cells.

Next, to investigate whether or not the MT-2Rst cells lose their resistance to CB-induced apoptosis when the expression of Bcl-2 is reduced, 5 or 10 nM of siRNA for the *bcl-2* gene was transfected into MT-2Rst cells as described in Materials and Methods. As shown in Fig. 4(A) and (B), the transfected cells exhibited a significant decrease in *bcl-2* gene expression and Bcl-2 protein production, respectively. The subsequent disappearance of resistance to CB-induced growth reduction in MT-2Rst cells is shown in the middle panel of Fig. 4(C). When MT-2Rst cells were transfected with siRNA for *bcl-2* and treated with 5 and 10 μg/ml of CB for 48 hours, a significant reduction in growth was observed (Fig. 4(C) right panel), indicating that the cells again became sensitive to CB-induced apoptosis. These results suggest that Bcl-2 plays a critical role in the acquisition of resistance to CB-induced apoptosis in MT-2Rst cells.

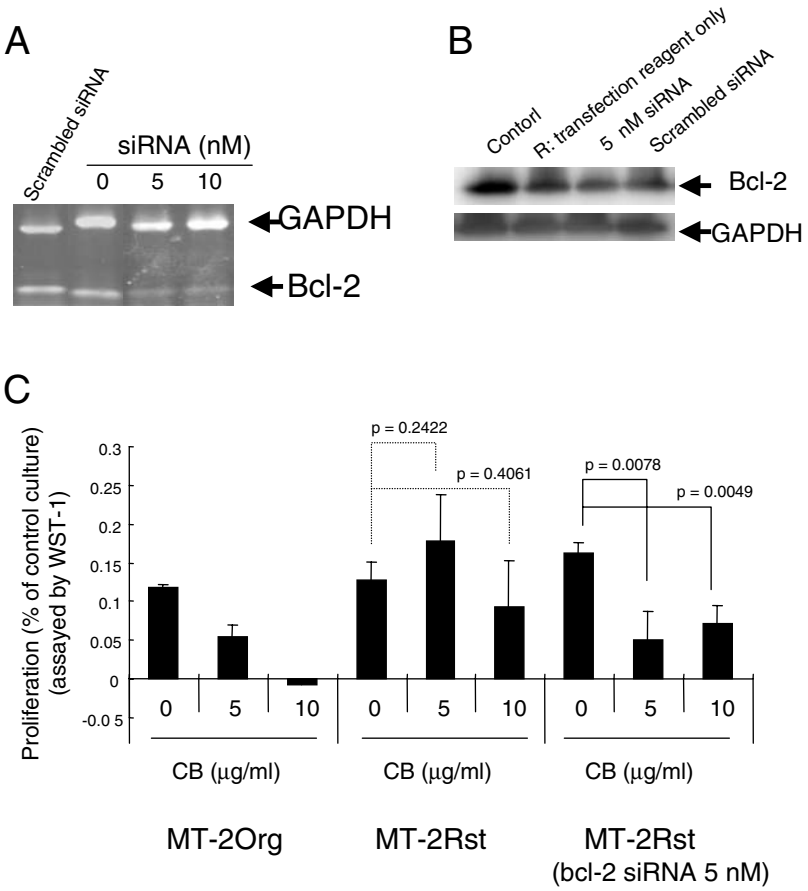

Fig. 4 Abrogation of resistance to CB-induced apoptosis in MT-2Rst cells transfected with siRNA for *bcl-2*. (A) Total RNA from MT-2Rst cells transfected with siRNA for *bcl-2* or scrambled control siRNA was extracted using the RNA Bee kit. Multiplex RT-PCR was performed to assess *bcl-2* and *gapdh* expression. MT-2Rst cells were untreated (0 nM of siRNA) or transfected with 5 or 10 nM of siRNA for 24 h. Scrambled nonsense siRNA was used as a negative control. (B) Western blot analysis for Bcl-2 was performed using whole cell lysate from MT-2Rst cells transfected with 5 nM of siRNA for bcl-2. MT-2Rst cells were untreated (control), transfected with TransIT-TKO transfection Reagent (R: transfection reagent only), transfected with scrambled control siRNA, or transfected with 5 nM of siRNA for 24 h. (C) WST-1 assays were performed in MT-2Org, MT-2Rst, and siRNA-transfected MT-2Rst cells. Each cell line was exposed to 0, 5, or 10 μg/ml of CB for 24 hours. Growth inhibition was induced by CB treatment in MT-2Org cells (left panel) and but not MT-2Rst cells (middle panel). MT-2Rst cells transfected with siRNA became sensitive to CB-induced growth inhibition (right panel)

Cytokine production in MT-2Org and MT-2Rst

To evaluate the other biological differences between MT-2Org and MT-2Rst cells, cytokine production was analyzed using the Human Th1/Th2 Cytokine Kit II of the Cytometric Bead Array. As shown in Fig. 5(A), among the measurable cytokines, the concentrations of IFNγ, TNFα and IL-6 in the supernatant were approximately two-fold lower in MT-2Rst than MT-2Org cells, whereas the concentration of IL-10 was two-fold higher in MT-2Rst. From these results, it was concluded that IL-10-mediated signaling plays an important role in the acquisition of the biological features of MT-2Rst cells. Gene and protein expression of IL-10R was observed in MT-2Org and MT-2Rst cells (data not shown), and thus it is possible that auto/paracrine effects of enhanced production of IL-10 in MT-2Rst may modify the cells' characteristics. The best-characterized IL-10 signaling pathway is the Jak/Stat system. IL-10/IL-10R interaction leads to activation of the cytokine-activated Janus kinase (Jak) family of tyrosine kinases and consequently to activation of several latent transcription factors such as signal transducer and activator of transcription (STAT) 1, STAT3, and STAT5 [26, 27]. Among these factors, STAT3 is used primarily by IL-10 to transduce its signaling into the cell [28]. Because of the biological importance of STAT3, its expression and phosphorylation were analyzed by Western blotting. As shown in Fig. 5(B), MT-2Rst cells showed enhanced STAT3 expression and phosphorylation compared with MT-2Org cells. The relative phosphorylation was digitally analyzed from the data of Western blotting and is represented in Fig. 5(C).

To investigate events upstream of the IL10-mediated signaling pathway, MT-2Org and MT-2Rst cells were treated with 5 or 15 μM of 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3, 4-d] pyrimidine (PP2), a specific inhibitor of

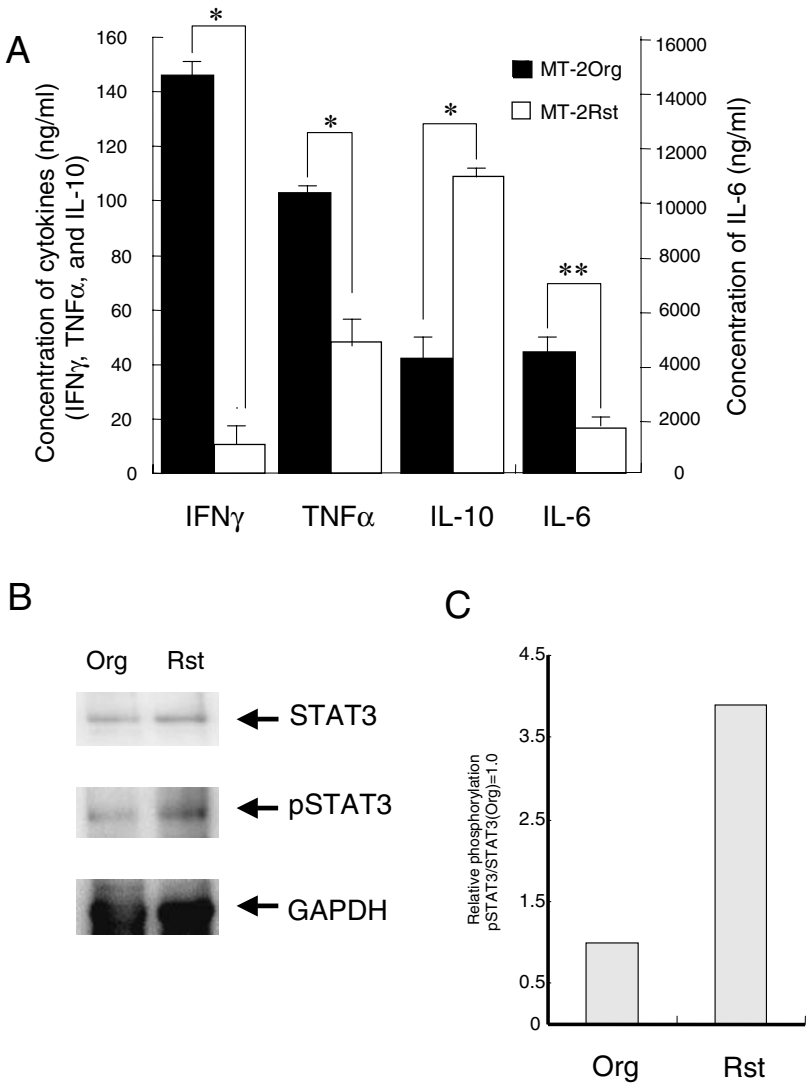

Fig. 5 Cytokine production and STAT3 expression in MT-2Org and MT-2Rst cells (A) Cytokine production was analyzed by assaying the supernatant of MT-2Org and MT-2Rst cells using Human Th1/Th2 Cytokine Kit II. Results are expressed as the mean ± SD of three identical experiments ($^{*}p < 0.0001$; $^{**}p < 0.005$). (B) Western blot analysis for STAT3, phosphorylated STAT3 (pSTAT3) and GAPDH was performed using whole cell lysates from MT-2Org (Org) and MT-2Rst (Rst) cells. (C) All the blots in (B) were analyzed by densitometry and the relative rate of phosphorylation of STAT3 (pSTAT3/STAT3 in Org = 1.0) is shown graphically with each blot. The data in A, B and C are representative of three identical experiments

Src family kinases, since it has been reported that Src family kinases transcriptionally upregulate IL-10 [14]. The gene expression of *Il-10* analyzed by real-time RT-PCR was reduced by PP2 in a dose-dependent manner in both lines (Fig. 6(A)), whereas the basic up-regulation remained in MT-2Rst cells when PP2 was added to the culture (Fig. 6(A)). The secretion of IL-10 was also inhibited by PP2 treatment both in MT-2Org and in MT-2Rst cells (Fig. 6(B)). These results suggest that Src family kinases regulate the mRNA expression and secretion of IL-10 in both MT-2Org and MT-2Rst cells.

Relative expression of bcl-2 mRNA in asbestos-related diseases

The importance of Bcl-2 and IL-10 was demonstrated with the *in vitro* model of long-term, low-level recurrent exposure to asbestos of CD4 lymphocytes, MT-2. Since *bcl-2* is one of the transcriptional targets of activated STAT3 [29, 30], the examination of *bcl-2* expression in CD4+ lymphocytes derived from patients with asbestos-related diseases may provide information about the relationship between occupational exposure to asbestos and clinical occurrence of malignant tumors. As shown in Fig. 7, the relative gene expression of *bcl-2* in CD4+ lymphocytes derived from 17 malignant mesothelioma patients was significantly higher than that in five healthy volunteers and asbestosis patients with no clinical sign of malignant tumors such as lung cancer or mesothelioma. These results suggest that enhanced expression of *bcl-2* in peripheral blood CD4+ lymphocytes may reflect the occurrence of malignant tumors in patients who have been chronically, occupationally, and recurrently exposed to asbestos.

Discussion

The occurrence of asbestos-related diseases in workers of, family members of workers of, and residents of

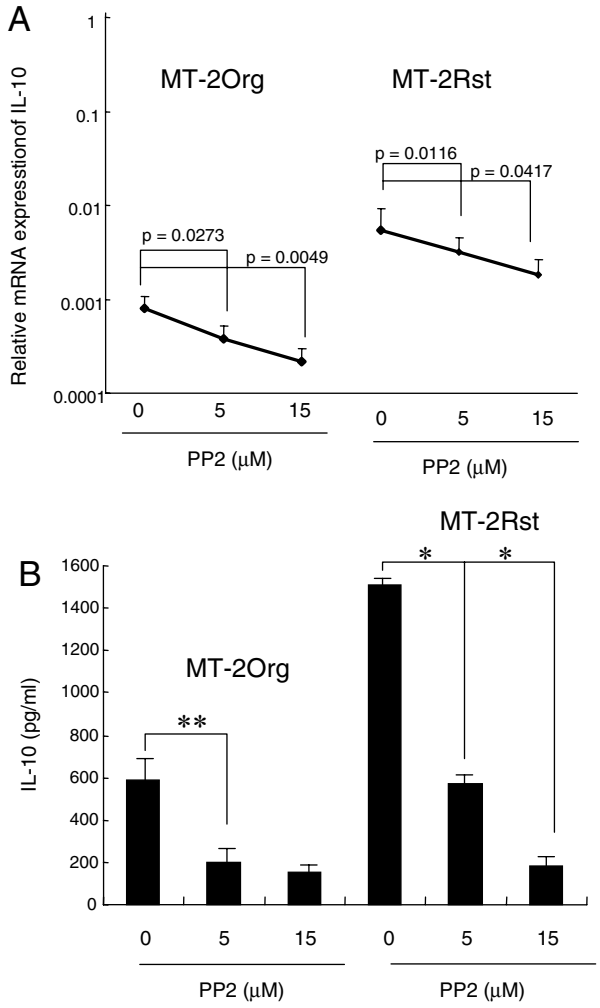

Fig. 6 Regulation of IL-10 transcription and secretion by Src family kinases MT-2Org and MT-2Rst cells were incubated with or without 5 or 10 μM PP2 for 24 h. (A) Relative mRNA expression of *il-10* in MT-2Org (left) and MT-2Rst (right) cells was calculated as described in Materials and Methods with real time RT-PCR. (B) Cytokine production was analyzed by assaying the culture supernatant from PP2-treated or untreated MT-2Org (left) and MT-2Rst (right) cells. Results are expressed as the mean ± SD of three identical experiments ($^{*}p < 0.0001$; $^{**}p < 0.005$)

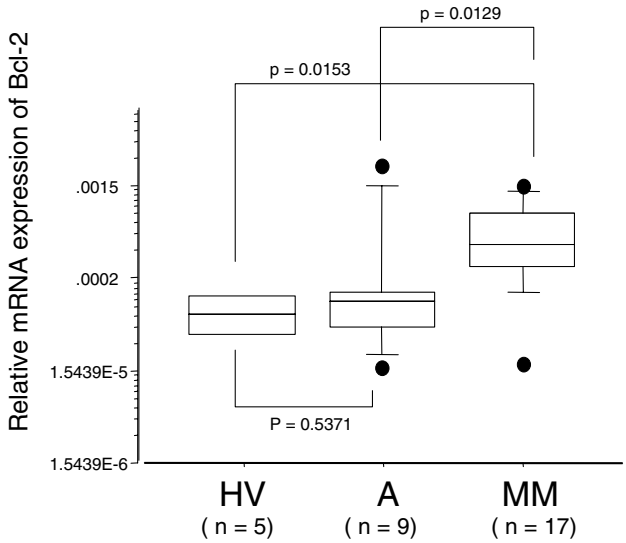

Fig. 7 Comparison of bcl-2 mRNA expression between healthy donors and patients with asbestos-related diseases RNA from healthy volunteers (HV) and patients with asbestosis (A) or malignant mesothelioma (MM) was extracted using the RNA Bee kit. Relative expression of *bcl-2* was calculated as described in Materials and Methods using the real time RT-PCR method with SYBR-G

neighborhoods surrounding asbestos-related factories is currently one of the most important public issues in terms of medical, social and political importance in Japan. Although the mass-media have prompted widespread attention to the problem of asbestos-induced diseases from the medical and preventive standpoints, further advances of medical science will be required to elucidate the cellular and molecular effects of asbestos on various human cells and organs and to establish the best methods for prevention of asbestos-related health disturbances such as asbestosis, lung cancers and malignant mesotheliomas.

We have been investigating the immunological features of patients with silicosis and previously found an aberrant expression of the Fas molecule, the decoy receptor 3 (DcR3) gene, and various auto-antibodies such as anti-topoisomerase I, anti-desmosome, anti-caspase 8, and anti-Fas antibodies in such patients [31–36]. In addition, we have reported the experimental effects of asbestos, mainly chrysotile, on human immune-competent cells and demonstrated that T cells exposed to chrysotile undergo apoptosis via death-receptor and mitochondrial pathways [15, 18]. The activation of the mitochondorial apoptotic pathway in MT-2 cells was accompanied by the phosphorylation of p38 MAPK and JNK, a Bax-dominant shift in the Bcl-2/Bax balance, and the activation of caspases 9 and 3. In addition, ROS were produced and various anti-oxidants inhibited apoptosis when MT-2 cells were cultured with chrysotile [18]. Thus, the apoptosis resistance of MT-2Rst cells may result from long-term exposure to oxidative stress, and be partly due to an increase anti-oxidant defenses. Additional studies will be required to test this possibility. Furthermore, it is considered that asbestos affects human immune-competent cells and may modify the immune system. Considering the occurrence of malignant tumors in individuals exposed long-term to low doses of asbestos, the effects of asbestos on the immune system may cause a reduction in tumor immunity. To analyze the mechanisms involved in the asbestos-induced alteration of the immune system, the establishment of an *in vitro* model for longer and lower-dose exposure was thought to be necessary. MT-2 cells were considered a good candidate for a model of long-term exposure, since their biological changes after short-term exposure to high levels of asbestos have been clarified [18].

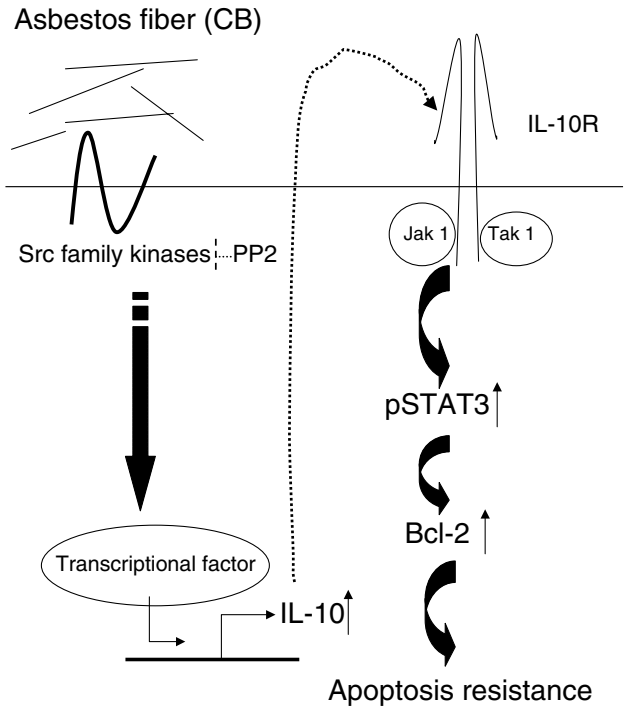

Fig. 8 A proposed model of mechanisms involved in the acquisition of resistance to CB-induced apoptosis in MT-2. Based on the findings reported in this study, the following events are proposed to be involved in the resisitance to CB-induced apoptosis (i) activation of Src (Lyn or Lck) family kinases, (ii) transcription and secretion of IL-10, (iii) auto/paracrine usage of secreted IL-10 with membrane IL-10R, (iv) activation of STAT3, (v) transcriptional activation of bcl-2, and (vi) long-term survival of a particular fraction of CD4+ T lymphocytes

In this study, we established a model for long-term, low-dose asbestos exposure using the MT-2 cell line. After demonstrating the acquisition of resistance to asbestos-induced apoptosis in the newly designated MT-2Rst line, cellular and molecular changes in MT-2Rst cells were analyzed, particularly the expression of apoptosis-related genes and production of cytokines. As shown in Fig. 8, all the data obtained from this study indicate that the cellular and molecular processing after the attachment of asbestos fibers to CD4+ lymphocytes occur as follows: (i) activation of Src (Lyn or Lck) family kinase, (ii) transcription and secretion of IL-10, (iii) auto/paracrine usage of secreted IL-10 with membrane IL-10R, (iv) activation of STAT3, (v) transcriptional activation of *bcl-2*, and (vi) long-term survival of a particular fraction of CD4+ T lymphocytes.

To test this hypothesis, the CD4+ T lymphocytes derived from patients with asbestos-related disorders were analyzed in terms of *bcl-2* expression. Interestingly, CD4+ T lymphocytes from asbestosis patients without any clinical signs of a malignant tumor showed similar relative expression of *bcl-2* to healthy donors. In contrast, CD4+ T lymphocytes from malignant mesothelioma patients exhibited significant up-regulation of *bcl-2* expression. These results indicate that long-term survival of the CD4+ T cell population with increased expression of *bcl-2* seems to be closely related to tumorigenesis rather than chronic exposure to asbestos.

If the enhanced survival of a certain population of CD4+ T lymphocytes affects the formation of tumors in the human body, a particular CD4+ CD25+ regulatory T cell fraction (Treg) may be one of the candidate populations, since it is reported that an increase of Treg cells reduced the tumor immunity and enhanced tumorigenesis [37]. CD4+ T cells from non-asbestos-induced tumor-bearing patients may also show up-regulation of the bcl-2 gene, similar to the increase of *bcl-2* expression in CD4+ T cells from patients with asbestos-related cancer. MT-2Rst cells became apoptosis-resistant during long-term exposure to asbestos. In addition, MT-2 cells express CD4 and CD25 molecules on their surface. These results suggest that MT-2Rst cells may function as Treg and may suppress tumor immunity. TakingMT-2Rst as an *in vitro* model of chronic exposure to asbestos in this study, our findings suggest that enhanced Treg function suppresses tumor immunity in patients with asbestos-related malignant mesothelioma. Furthermore, the MT-2 cell line is an HTLV-1-immortalized polyclonal T cell line and there has been discussion regarding adult T cell leukemia/lymphoma (ATL) as a tumor of Treg (personal communications).

In conclusion, we show that long-term, low-level recurrent exposure to asbestos affects human immune-competent cells, leading to the activation of Src family kinases, IL-10, the STAT3 signaling pathway, and Bcl-2 enhancement, while short-term, high-level transient exposure induces the apoptosis of these cells. To identify one of these cell populations as the Treg fraction is urgently needed. In this regard, immunological modifications such as reduction of the Treg population using *in vivo* siRNA and the regulation of tumor immunity with tumor vaccines should be considered and developed for patients with asbestos-induced malignancies and a sub-clinical population who have been exposed to asbestos, but have no clinical signs of disease.

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