Chapter 14
Reduction of Antitumor Immunity Caused by Asbestos Exposure

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Abstract Asbestos fibers are known to cause not only benign pulmonary and pleural diseases such as asbestosis and pleural plaque, but also malignant tumors such as lung cancer and malignant mesothelioma. In addition to the carcinogenic activities possessed by the fibers themselves, it has been considered that asbestos fibers may affect the human immune system. In this review, a cell culture model using a human T cell line exposed to asbestos fibers continuously and at relatively low doses to mimic exposure of environmentally and occupationally exposed people to these fibers is introduced. Although transient and high-dose exposure caused cell apopto-

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sis, the cell line employed acquired resistance to asbestos-induced apoptosis with continuous exposure as a result of various cellular and molecular changes such as changes in cytokine production and cytoskeletal molecules. On the other hand, changes in various immune cells such as cytotoxic T lymphocytes, natural killer cells, T helper cells, and regulatory T cells by in vitro exposure using certain cell lines as well as freshly isolated peripheral blood immune cells derived from healthy volunteers revealed impairment of antitumor immunity. Thereafter, the findings obtained were confirmed using peripheral blood immune cells derived from asbestos-exposed patients with pleural plaque or mesothelioma. The findings are also shown in this chapter. Further research should explore the effects of asbestos fibers on other immune cells such as Th17, investigate the development of diagnostic markers using altered immune cells, and pursue the identification of physiological substances from plants and other sources that can halt or recover the antitumor immunity caused by asbestos exposure.

Keywords Asbestos · Antitumor immunity · T helper (Th) cell · Cytotoxic T lymphocyte (CTL) · Natural killer (NK) cell · Regulatory T (Treg) cell

14.1 Introduction

Asbestos fibers can cause not only lung fibrosis known as asbestosis, but also malignant tumors such as lung cancer and malignant mesothelioma (MM) [1–4]. Additionally, benign diseases can also occur following asbestos exposure, such as pleural plaque (PP), diffuse pleural thickening, benign pleural effusion, and rounded atelectasis. Among these asbestos-related diseases, malignant tumors are the most important in terms of prognosis and the long latency period that usually ensues following exposure to asbestos. For example, the appearance of MM occurs 30–50 years after initial exposure [1–4]. Once MM occurs, most of the MM tumors progress rapidly, thereby resulting in poorer prognosis of the disease, notwithstanding the development of various novel approaches such as molecular targeting therapies [5, 6] and surgical treatments such as pleurectomy/decortication [7–9].

With regard to the carcinogenicity of asbestos fibers, the most important factor appears to be the iron which is included in amphibole asbestos materials, e.g., crocidolite (Na_2Fe_2+3Fe_3+2$Si_8O_{22}(OH)_2$) and amosite ($Fe_7Si_8O_{22}(OH)_2$) [10]. Although other amphibole materials such as actinolite ($Ca_2(Mg, Fe)_5(Si_8O_{22})(OH)_2$) and anthophyllite ($(Mg, Fe)_7Si_8O_{22}(OH)_2$) contain iron, these fibers have not been used in industry. Other amphibole materials such as tremolite ($Ca_2Mg_5Si_8O_{22}(OH)_2$) do not possess iron. Thus, crocidolite and amosite are considered stronger and potentially more dangerous in terms of asbestos-induced cancers. On the other hand, serpentine fibers such as chrysotile ($Mg_3(Si_2O_5)(OH)_4$) do not contain iron. Thus, although most industrial uses employed chrysotile, its carcinogenicity was considered to be the lowest. However, recent studies have demonstrated the carcinogenicity of chrysotile fibers [10–14]. Animal models showed higher frequencies of

chrysotile-induced mesothelioma compared with other asbestos fibers [15, 16]. Additionally, chrysotile showed a capacity to adhere to red blood cells and cause increased levels of iron in the body by hemolysis [17]. The International Labor Organization (ILO) then declared that "all forms of asbestos, including chrysotile, are considered as known human carcinogens" [12].

The iron yields oxygen stress and produces reactive oxygen species (ROS) that result in genotoxicity to nearby cells. When alveolar macrophages come into contact with asbestos fibers, they are unable to effectively treat the fibers as foreign entities due to the fibers being rigid and long [18–20]. As a result, these macrophages produce ROS and are referred to as "frustrated macrophages." In addition to the aforementioned theories, asbestos fibers can directly attack surrounding cells. Due to their rigid characteristics, fibers can damage chromosomes. Moreover, fibers can adsorb various carcinogenic substances which are inhaled into the lungs such as tobacco smoke and air pollutants [21, 22].

On the other hand, the effects of asbestos fibers on the human immune system have not been well documented, except with the investigation of cellular and molecular mechanisms related to the signaling of these fibers as foreign and a danger by inflammasomes included in alveolar macrophages as antigen presenting cells [23, 24]. In the case of asbestos fibers, the pattern-recognition receptor NALP3 (NACHT, LRR, and PYD domains-containing protein 3) plays a role with ASC (apoptosis-associated speck-like protein containing a CARD) and caspase-1 inflammasome which results in the activation of caspase-1. Thereafter, pro-inflammatory cytokines such as interleukin (IL)-1β and IL-18 are secreted to promote fibrogenic changes in lung fields [25, 26]. However, these recognition processes represent just the initial events following the entry of asbestos fibers into the human body. Given their physical characteristics, fibers are retained in the lung fields as well as related lymph nodes. Then, various circulating lymphocytes may have repeat encountering with fibers. These phenomena may cause cellular and molecular alterations in these lymphocytes.

A number of reports have detailed the use of in vitro experiments to investigate the effects of asbestos on lung alveolar epithelial cells and pleural mesothelial cells in terms of the progression of cells toward a cancerous state [27–30]. Most of these trials demonstrated the importance of ROS production and activation of mitochondrial apoptotic pathways by transient and relatively high-dose exposure. It can be speculated that an accumulation of damage to the genome by ROS might prevail, and that certain cellular mechanisms may be enacted that place cells on a non-apoptotic pathway. Thereafter, these cells may then possess the cellular and molecular characteristics of cancer cells.

These experimental procedures could be utilized to clarify the immunological effects of asbestos fibers, and to establish continuous and relatively low-dose exposure conditions in an effort to delineate the cellular and molecular changes that occur in environmentally and occupationally exposed people to asbestos. Consequently, our strategies to explore the immunological effects of asbestos fibers on various lymphocytes such as T helper (Th) cells, regulatory T cells (Tregs), cytotoxic T lymphocytes (CTLs), and natural killer (NK) cells included the following approaches:

1. Applying cell lines and established cell culture models to continuous and low-dose exposure.
2. Generating an ex vivo model using freshly isolated peripheral blood mononuclear cells (PBMCs) or sorted specific lymphocyte fractions for activation in a cell culture system in the absence or presence of asbestos fibers.
3. If certain changes were observed from the aforementioned approaches, confirming such findings using peripheral blood lymphocytes derived from PP and MM patients, as both are considered to be caused by exposure to asbestos.

These strategies to explore the immunological effects of asbestos fibers have previously been reported [31–33]. In this review, one example will be given of the cellular and molecular alterations found resulting from continuous exposure of the human T cell leukemia virus (HTLV)-1 immortalized polyclonal cell line MT-2 to chrysotile and crocidolite asbestos fibers. Additionally, the effects of asbestos exposure on CTLs, NK cells, Th cells, and a Treg cell line model will also be summarized in terms of antitumor immunity.

14.2 Cellular and Molecular Alterations of the MT-2 T Cell Line Exposed Continuously to Asbestos Fibers

Using the MT-2 cell line, cellular events caused by transient and relatively high-dose exposure to chrysotile or crocidolite were examined [34, 35]. Dependent on iron content, ROS production was higher in cultures with crocidolite compared to chrysotile cultures, whereas growth inhibition and the appearance of apoptosis were slightly greater in chrysotile compared to crocidolite cultures. In both crocidolite and chrysotile cultures, MT-2 cells proceeded toward apoptosis via increased phosphorylation of proapoptotic mitogen-activated protein kinase (MAPK) signaling molecules such as p38 and JUN, increased release of cytochrome c from mitochondria to the cytoplasm, decreased expression ratio of Bcl-2/BAX, and activation of caspase-9 and caspase-3 [34, 35].

After more than 8 months of continuous and low-dose exposure (causes less than half of the cells to proceed toward apoptosis by transient exposure) to chrysotile or crocidolite fibers, MT-2 cells acquired resistance to asbestos-induced apoptosis. The monitoring of apoptosis continued monthly by transient high-dose exposure to fibers after removal of fibers employed for continuous exposure. A number of interesting findings have been found with respect to the cellular and molecular characteristics in all of these sublines (continuously exposed MT-2 cells; there are independently established sublines, three were exposed to chrysotile A, three to chrysotile B, and four to crocidolite). For example, IL-10 was overproduced in the sublines IL-10 was overproduced and regulated by Src kinase. This overproduction of IL-10 activated signal transducer and activator of transcription 3 (STAT3) via phosphorylation and upregulated Bcl-2 located down stream of STAT3 by autocrine usage of IL-10 [36]. This represents one route to apoptosis resistance or enhanced survival. Additionally, sublines showed overproduction of transforming growth factor (TGF)-β. The

autocrine mechanism involving use of TGF-β caused phosphorylation of p38, thereby resulting in increased levels of phosphorylated SMAD3 and decreased levels of phosphorylated SMAD2 [37]. Therefore, these sublines showed resistance to TGF-β-induced growth inhibition found in MT-2 original cells [37].

The other interesting feature of the sublines was reduced expression of forkhead box protein O1 (FoxO1), which regulates various apoptosis-related molecules. Examination of proapoptotic molecules such as p53 upregulated modulator of apoptosis (Puma), Bcl-2 interacting mediator (Bim), and Fas ligand in sublines revealed decreased expression [38]. This represents another route to acquire apoptosis resistance by decreasing proapoptotic signals.

Moreover, various cytoskeletal molecules were altered following long-term continuous exposure to asbestos in MT-2 cells. Additional phosphorylation and increased expression of β-actin were detected in sublines. Furthermore, myosin9, vimentin, and tubulin β2 extracted from sublines showed increased binding capacity to chrysotile fibers [39]. These findings were considered to be reasonable since MT-2 cells are incapable of digesting fibers and repeatedly encounter fibers on their cell surface. Therefore, changes in cytoskeletal molecules occurred as a result of continuous exposure. Although the precise impact on cellular function caused by these changes have yet to be delineated, an examination of the alteration of other molecules on the cell surface is important in determining the effects of fibers on immune cells.

All of these findings are schematically represented in Fig. 14.1.

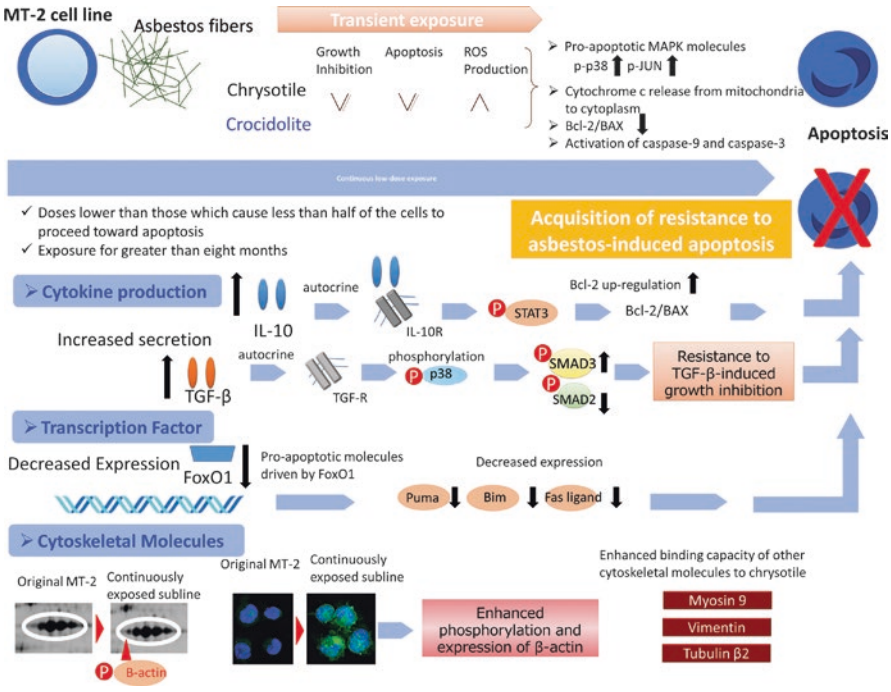

Fig. 14.1 Schematic presentation of cellular and molecular alterations of the MT-2 T cell line continuously exposed to asbestos. Detailed explanations are described in the text

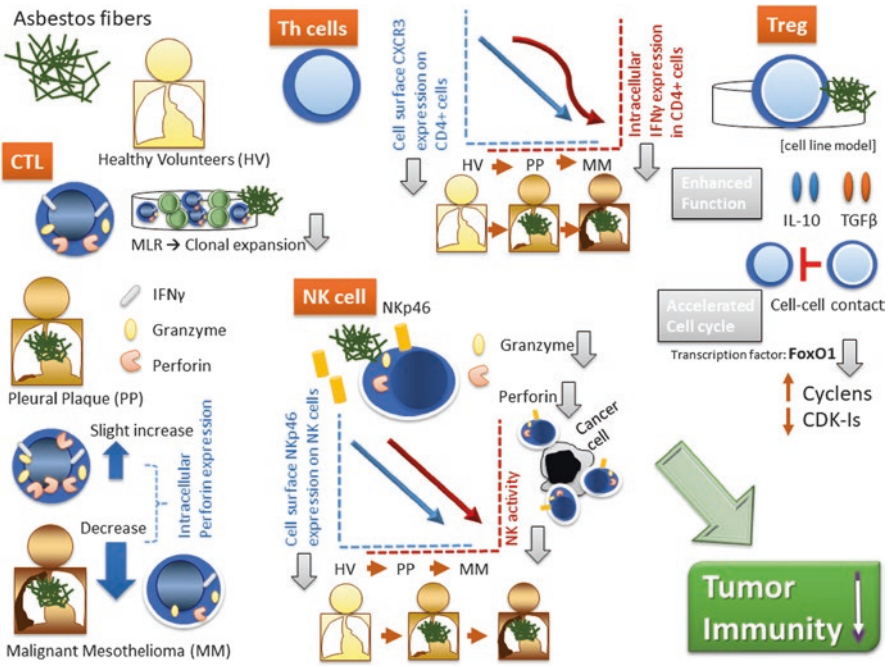

Fig. 14.2 Schematic presentation of the decrease of antitumor immunity caused by exposure to asbestos fibers in CTL, NK, Th, and Treg cells

14.3 Reduced Antitumor Immunity Caused by Exposure to Asbestos Fibers

The experimental results as well as analyses using clinical specimens (peripheral blood immune cells) derived from HV as well as PP and MM patients are schematically summarized in Fig. 14.2.

14.4 CTLs

Since CTLs are important when considering antitumor immunity, the effects of chrysotile on a mixed lymphocyte reaction (MLR) assay to examine the transition of CD8+ naïve T lymphocytes toward CTLs were analyzed in the absence or presence of fibers. As a result, supplemented chrysotile caused a decrease in CD8+ cell proliferation and inhibited cellular transition from naïve to effector/memory type CD8+ cells as determined by examining cell surface molecules such as CD45RA, CD45RO, and CD25. Additionally, intracellular granzyme B and IFN-γ levels were lower compared to the MLR in the absence of supplemented chrysotile. The

supernatant of the MLR showed decreased levels of IFN-γ and tumor necrosis factor (TNF)-α when chrysotile was supplemented in the MLR. All of these findings indicated that asbestos fibers suppress the clonal expansion of CTLs [40, 41].

Thereafter, the intracellular cell-attacking granules, containing cytotoxic molecules such as granzyme and perforin, were examined using PBMCs derived from HV as well as PP and MM patients after overnight stimulation with phorbol myristate acetate (PMA) and ionomycin. Interestingly, the percentage of intracellular granzyme B+ and perforin+ cells in PMA/ionomycin-stimulated CD8+ lymphocytes was higher in PP patients compared to HV. Additionally, intracellular levels of perforin+ cells in stimulated CD8+ cells derived from MM patients were lower compared to those derived from PP patients. These results indicated that MM patients possess impairment of stimulation-induced cytotoxicity of peripheral blood CD8+ lymphocytes, while PP and MM patients possess a common alteration of those lymphocytes, namely, an increase in memory cells (percentage of perforin+ cells and CD45RA− cells in fresh CD8+ lymphocytes of PP and MM groups were higher compared to HV), possibly related to asbestos exposure. It is noteworthy that intracellular perforin levels differed between PP (noncancerous) and MM (cancerous) patients. Thus, the effects of asbestos on CTLs may be altered due to complicated physiopathological conditions [40, 42].

14.5 NK Cells

The effects of asbestos fibers on human NK cells were reported in our previous review "Dysregulation of the immune system caused by silica and asbestos" [43].

Briefly, the human NK cell line YT-CB was employed for studies. Then, ex vivo exposure of freshly isolated and activated NK cells from HV to asbestos fibers was investigated. These experiments showed decreased levels of certain NK cell-activating receptors such as 2B4, NKG2D, and NKp46. Thereafter, the correlation between these activating receptors and the cytotoxic activity of NK cells derived from HV as well as PP and MM patients were examined [44, 45]. As shown in Fig. 14.2, expression of NKp46 and cytotoxicity showed a significant positive correlation, where decreased NKp46 expression resulted in weaker cytotoxic potential of the analyzed NK cells. Moreover, the extent of NKp46 expression on NK cells gradually decreased in the order HV to PP to MM. NK cells from MM patients displayed the lowest expression. Additionally, it was supposed that if the NKp46 expression were examined in PP patients, that higher expression may be present only in PP patients even if they were exposed to asbestos, whereas lower expression may require careful medical evaluations for risk of mesothelioma onset. Additionally, the reduction in cytotoxicity was accompanied with reduced intracellular signaling via receptors, phosphorylation of the extracellular signal-regulated kinase (ERK), and decreased degranulation of cell-killing granules containing perforin [44, 45].

These findings indicated that asbestos exposure results in decreased NK cell cytotoxicity with lower expression of NKp46.

14.6 Th Cells

As described above, the HTLV-1 immortalized polyclonal T cell line MT-2 was exposed to asbestos fibers and continuously exposed sublines were established [35, 36]. From the cDNA microarray data and pathway analysis using those data of the comparison between MT-2 original cells and sublines, there were some interesting findings regarding antitumor immunity [46, 47]. One finding involved the molecular pathway leading to IFN-γ. The expression of many molecules involved in this pathway decreased in sublines. Additionally, and related to this pathway, the expression of CXC chemokine receptor (CXCR) 3 was reduced in sublines.

After confirming the reduced expression of CXCR3 in sublines by real-time RT-PCR as well as using flow cytometry and immunohistochemical assays for a comparison of protein expression between sublines and MT-2 original cells, freshly isolated CD4+ cells from peripheral blood of HV were examined for surface expression of CXCR3 after ex vivo stimulation with anti-CD3 and anti-CD28 antibodies supplemented with IL-2 in the absence or presence of chrysotile fibers. After 4 weeks, surface CXCR3 was significantly reduced when fibers were present; however, other chemokine receptors such as CCR5, which was not detected by cDNA microarray, remained unchanged under these ex vivo conditions. Moreover, intracellular protein and mRNA expression of IFN-γ were also reduced under these ex vivo conditions [46, 47].

Surface expression of CXCR3 on freshly isolated CD4+ cells from HV as well as PP and MM patients were then compared. From the results shown in Fig. 14.2, it can be seen that the extent of CXCR3 expression decreased in the order HV to PP, and then markedly decreased in CD4+ cells derived from MM patients. Additionally, these cells were stimulated with anti-CD3 and anti-CD28 antibodies and IL-2 for 5 days. Then, intracellular IFN-γ positive cells were analyzed. As a result, although there was no difference between CD4+ cells derived from HV and PP patients, the number of intracellular IFN-γ-positive CD4+ cells from MM patients was significantly lower compared to that of HV and PP patients [46, 47].

CXCR3 is considered to be important in facilitating the introduction or movement of cancer-attacking T cells near tumor cells. Additionally, IFN-γ plays an important role in tumor cell damage. Thus, these findings also indicated that asbestos exposure results in reduced antitumor immunity.

14.7 Cell Line Model of Tregs

The MT-2 cell line is considered to possess Treg function ([48]: [49]). Hence, the Treg function associated with suppressing the proliferation of responder T cells was compared between MT-2 original cells and the aforementioned sublines. Freshly isolated peripheral blood CD4+ cells were activated by anti-CD3 and anti-CD8 antibodies with in vitro differentiated auto-dendritic cells. Into this activated culture

were added irradiated MT-2 and one subline (designated as CB1 cells) to represent the role of Tregs. The antiproliferative effects against responder T cells were found to be significantly enhanced in CB1 cells compared to original MT-2 cells. Additionally, as mentioned above, sublines showed increased production of IL-10 and TGF-β [50]. Since these two cytokines are known as typical soluble factors secreted from Tregs and which contribute to the inhibitory function of Tregs, IL-10, or TGF-β was silenced using a siRNA method in the CB1 subline, and the suppressive effects against freshly isolated and activated peripheral CD4+ cells derived from HV were examined using a Transwell culture assay. As a result, it was found that approximately half of the suppressive effects were canceled by silencing IL-10 or TGF-β [50].

Moreover, it is known that FoxO1 regulates several molecules involved in cell cycle progression such as cyclin D1 and cyclin-dependent inhibitors (CDK-I) including INK4 family members such as p16INK4a and p15INK4b and Cip/Kip family CDK-I members including p21Cip1 and p27Kip1. FoxO1 inhibition regulates accelerating molecules, but stimulation regulates braking molecules. As mentioned above, since FoxO1 expression is specifically reduced in continuously exposed sublines of MT-2 [38], the expression of these cell cycle regulators in original MT-2 cells and sublines was determined and compared. As a result, it was found that accelerating molecules were upregulated while CDK-I was downregulated. Thereafter, the S/G1 ratio in cell cycle phases analyzed by flow cytometry was higher in sublines compared to original MT-2 cells [51]. These results indicated that asbestos exposure causes rapid progression of the cell cycle in Tregs and results in increased volume in Tregs.

These results indicated that Tregs exposed to asbestos fibers possess enhanced function via cell–cell contact (including the effects of membrane-bound TGF-β in addition to overproduction of functional cytokines IL-10 and TGF-β. Furthermore, Tregs exposed to asbestos undergo rapid proliferation by altering the regulation of FoxO1. Unfortunately, although the function of peripheral blood or tumor-surrounding Tregs derived from PP and MM patients have yet to be examined, these cell line models also suggest that asbestos exposure causes a reduction of antitumor immunity.

14.8 Conclusion

This review presented experimental approaches examining the immunological effects of asbestos fibers using human cell lines and freshly isolated lymphoid cells derived from HV, and provided detailed confirmation of certain findings using peripheral blood immune cells derived from PP and MM patients exposed to asbestos. Exposure to asbestos altered the cellular and molecular characteristics of various immune cells such as Th, Treg, CTL, and NK cells. Additionally, most of the changes suggested a reduction of antitumor immunity in asbestos-exposed populations. However, detailed examinations regarding the effects of asbestos fibers on

Th17, dendritic cells, small populations of T cells such as γδT and other cells have yet to be undertaken.

One potential clinical use of these findings is to provide a comprehensive assessment of the immune status of high-risk groups such as past (and present) workers involved in asbestos manufacturing, asbestos handling, building demolition, rubble processing, and other asbestos-related activities, rather than just making a diagnosis of PP or other asbestos-related pathological changes by chest X-ray or CT imaging. The immunological findings may be examined by drawing peripheral blood from subjects.

The other possibility is to identify physiological substances from plants and other sources that can halt the reduction of or reduce the antitumor immunity caused by asbestos exposure. If certain substances can be administered orally on a daily basis to the aforementioned high-risk groups, this may assist in the chemoprevention of asbestos-related diseases and the development of malignant tumors.

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