Immunobiology 218 (2013) 1184–1191

Contents lists available at SciVerse ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Alteration of cytoskeletal molecules in a human T cell line caused by continuous exposure to chrysotile asbestos

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ARTICLE INFO

Article history:
Received 1 December 2012
Received in revised form 4 April 2013
Accepted 7 April 2013
Available online 19 April 2013

Keywords:
Asbestos
T cell
Protein expression
β-Actin
Cytoskeleton

ABSTRACT

Among the various biological effects of asbestos such as fibrogenesis and carcinogenesis, we have been focusing on the immunological effects becausesilica (SiO_2) and asbestos chemically is a mineral silicate of silica. Observations of the effects of asbestos on CD4+ T cells showed reduction of CXCR3 chemokine receptor and reduced capacity of interferon γ production. In particular, use of theHTLV-1 immortalized human T cell line, MT-2, and cDNA array analysis have helped to identify the modification of CXCR3. We investigated alteration of protein expression among MT-2 original cells that had no contact with asbestos, and six chrysotile-continuously exposed independent sublines using ProteinChip and two-dimensional gel electrophoresis (2DGE) assays. Further confirmation of the changes in protein expression due to asbestos exposure was obtained after the 2DGE method indicated protein modification of β-actin. β-actin was upregulated in mRNA, as were the levels of protein expression and phosphorylation. Moreover, a binding assay between cells and chrysotile showed that various molecules related to the cytoskeleton such as vimentin, myosin-9 and tubulin-β2, as well as β-actin, exhibited enhanced bindings in asbestos-exposed cells. The overall findings indicate that the cell surface cytoskeleton may play an important role in inducing the cellular changes caused by asbestos in immune cells, since fibers are not incorporated to the cells and how the alterations of cytoskeleton determined cell destiny to cause the reduction of tumor immunity is important to consider the biological effects of asbestos. Further studies to target several cytoskeleton-related molecules associated with the effects of asbestos will result in a better understanding of the immunological effects of asbestos and support the development of chemo-prevention to recover anti-tumor immunity in asbestos-exposed patients.

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Introduction

The cellular and biological effects of asbestos are usually discussed in regard to its fibrogenicity and carcinogenicity due to the development of pulmonary fibrosis and complicated cancers such as malignant pleural mesothelioma (MPM) and lung cancer (Craighead 2008a; Lemen 2006). MPM has been particularly important not only in medical fields, but also in international social and economic arenas (Craighead 2008c; Moazzam and Kratzke 2008).

The carcinogenic potential of asbestos has been considered due to the (1) iron-including asbestos fibers such as crocidolite and amosite that cause reactive oxygen species (ROS) production from tissues near the fibers, and also alveolar macrophages that proceed to apoptosis with insufficient destruction of phagocytozed fibers, resulting in DNA damage in the cells surrounding the fibers and (2) physical chromatin/DNA damage caused by direct interaction between cellular chromatin and the incorporated fibers (Huang et al. 2011; Toyokuni 2009). In addition, recent molecular analyses of MPM cells highlighted several important genes that are responsible for the carcinogenicity of MPM such as p16/ink4a, neurofibromin2 (NF2)/merlin (Sekido 2011), BRCA1 associated protein-1 (BAP1; ubiquitin carboxyl-terminal hydrolase) (Bott et al. 2011; Testa et al. 2011), serine/threonine-protein kinase LATS2 (Murakami et al. 2011), and the Yes-associated protein (YAP1) (Mizuno et al. 2012). However, the interaction between the fragility of these genes and the physiological effects of asbestos

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0171-2985/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.imbio.2013.04.007

fibers has not been examined thoroughly and was relegated to future investigations.

We have been focusing on the immunological effects of silica and asbestos (Maeda et al. 2010; Otsuki et al. 2007, 2009). Patients with silicosis who have been exposed to silica often develop autoimmune diseases (D'Cruz 2000; Steenland and Goldsmith 1995). Our investigations have been clarifying the alteration of CD95/Fas and its related molecules in silicosis patients (Otsuki et al. 2006). Moreover, silica can activate both responder T cells and regulatory T cells (Treg), which are characterized by CD4+ and CD25+ with a forkhead box P3 (FoxP3)-positive state and cause early loss of Treg by activation of CD95/Fas on Treg, including the continuous activation and longer survival of responder T cells and probably self-reactive clones (Hayashi et al. 2010). The question that arises is "What are the immunological effects of asbestos?" To consider this, we focused on anti-tumor immunity and expected it would be reduced by asbestos exposure, since malignant complications associated with asbestos-exposed patients not only include pulmonary lesions, but also other cancers involving the larynx, gastrointestinal tract, and bladder (Craighead 2008b; Lemen 2006; Rolston and Oury 2004). We previously reported reduction of cell-killing activity of natural killer (NK) cells with a decline of surface expression of NKp46, an activation receptor (Nishimura et al. 2009a, 2009b). In regard to T cells, we reported reduction of surface expression of chemokine receptor CXCR3, a G protein-coupled receptor in the CXC chemokine receptor family, in a human T cell line continuously exposed to chrysotile asbestos, and a decreased production potential of interferon (IFN)-γ in this cell line and also for circulating peripheral CD4+ T cells from asbestos-exposed patients such as those with pleural plaque and MPM (Maeda et al. 2011a, 2011b). Thus, asbestos exposure causes a decline of anti-tumor immunity and makes asbestos-exposed individuals sensitive to cancer development (Kumagai-Takei et al. 2011; Matsuzaki et al. 2012).

These results were obtained by utilizing a human adult T cell leukemia virus-1 (HTLV-1) immortalized human polyclonal T cell line, MT-2 (Miyoshi et al. 1981), and six established sublines that exhibited resistance to chrysotile-induced apoptosis and were designated CA 1 to 3 and CB 1 to 3 (Maeda et al. 2011a, 2011b; Miura et al. 2006). These six sublines were independently exposed to chrysotile A (CA) for sublines CA 1 to 3, or chrysotile B for sublines CB 1 to 3, for more than eight months. The cellular mechanism involved in the acquisition of apoptosis-resistance and reduction of CXCR3 expression was reported previously (Maeda et al. 2011a, 2011b; Miura et al. 2006). In particular, CXCR3 alteration was extracted from the results of cDNA microarray analyses using the original MT-2 line (Org) and the sublines. During the establishment of these sublines, we wondered how these sublines changed their cellular features without incorporating fibers into their intracellular spaces. Therefore, this study initially conducted proteomics analysis and 2-dimensional electrophoresis while ensuring continuous asbestos exposure to investigate protein expression in T cells, and then focused on the expression levels of molecules involved in the formation of the cytoskeleton, since fibers are not incorporated to the cells and how the alterations of cytoskeleton determined cell destiny to cause the reduction of tumor immunity is important to consider the biological effects of asbestos.

Materials and methods

Cell lines and asbestos (CA and CB)

To analyze the effects of asbestos on human polyclonal CD4+ T cells, the MT-2 cell line was utilized (Miyoshi et al. 1981). ROS production and apoptosis were investigated following transient and relatively high-dose exposure to CA (Hyodoh et al. 2005). In addition, the establishment of the CB1 subline exposed to CB for more than eight months revealed resistance to CB-induced apoptosis with activation of Src-family kinases, overproduction of interleukin (IL)-10, phosphorylation of signal transducer and activator of transcription 3 (STAT3), and upregulation of BCL-2 (Miura et al. 2006). Five other sublines, CB2, CB3, and CA 1 to 3, were also established, and all six sublines exhibited reduction of CXCR3 surface expression as reported previously (Maeda et al. 2011a, 2011b). Briefly, all independent sublines started their CA or CB exposure with concentrations of 10 μg/ml (we used μg/ml of chrysotile instead of $\mu g/cm^2$, which usually applies to *in vitro* experiments for adherent cells, since all the cells were cultured in suspension), which was expected to cause less than half of the cells to proceed toward apoptosis when CA or CB was exposed transiently for three days. Cultures were treated monthly to remove CA or CB by Ficoll gradient centrifugation and cells released from CA or CB were cultured without fibers for one week. Cells were then re-exposed to CA or CB with a concentration inducing apoptosis for Org cells (original MT-2 cells that were never exposed to asbestos) and monitored for the appearance of apoptosis by Annexin-V, terminal deoxynucleotidyltransferase-dUTP nick end labeling (TUNEL), and flow cytometrical examination of active caspase 3. Thereafter, when the continuously exposed subline showed approximately 80% cell survival with an exposure of 50–100 μg/ml of CA or CB in which Org cells showed more than 95% apoptosis, we defined the sublines as established and they were designated CA 1 to 3 and CB1 to 3. All sublines established independently showed similar gene expression patterns as analyzed by a cDNA microarray assay, comprising overexpression of IL-10 and BCL-2, and reduction of CXCR3 as reported previously (Maeda et al. 2011a, 2011b; Miura et al. 2006).

The mineralogical features of CA and CB used in this study have been reported previously. Both types of asbestos were UICC standards and were obtained from the Japan Asbestos Association (Kohyama et al. 1996). Briefly, and as introduced in referenced manuscript, CA and CB used in this study possess an average length of 2.4 (standard deviation: $\pm$2.3) and 2.6 ± 2.3 μm, and an average width of 0.17 ± 1.8 and 0.15 ± 1.8 μm, respectively. Both fibers were applied after autoclaving, but no destruction was detected by ultrasound. The iron (Fe_2O_3 and Fe) contents in CA and CB were 2.46 and 3.53%, respectively. In addition, CA itself contained 2.46% of iron with 2% of fiber contamination with anthophylite, which possesses 12.07% of iron, according to a previous report (Kohyama et al. 1996).

ProteinChip analysis

Protein extraction from Org, CA1 to 3 and CB1 to 3 cells was performed using a COMPARTMENTAL PROTEIN EXTRACTION KIT (CHEMICON International, Inc. Temecula, CA) according to the manufacturer's instructions. Each cell was lysed in HEPES buffer (pH 7.9) containing $MgCl_2$, KCl, EDTA, sucrose, glycerol, sodium orthovanadate and protease inhibitors, and centrifuged at 20,000 *g* for 20 min.The supernatant was defined as the cytoplasmic fraction. The pellet was washed and lysed in HEPES buffer (pH 7.9) containing $MgCl_2$, NaCl, EDTA, glycerol, sodium orthovanadate and protease inhibitors, and centrifuged at 20,000 *g* for 20 min. The resulting supernatant was defined as the nuclear fraction. The pellet was then washed and lysed in HEPES buffer (pH 7.9) containing $MgCl_2$, KCl, EDTA, sucrose, glycerol, sodium deoxycholate, NP-40, sodium orthovanadate and protease inhibitors, and centrifuged at 20,000 *g* for 20 min. The resulting supernatant was defined as the membrane fraction. The efficiency of subcellular fractionation was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Extracted proteins were detected by Coomassie Brilliant Blue

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