Release of IL-8 from Human Monocytes by Asbestos

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ABSTRACT. It is supposed that neutrophil infiltration into the pulmonary airspace is involved in the pathology of asbestosis development. We investigated interleukin-8 (IL-8) release from monocytes by asbestos exposure and a part of the related mechanisms. Chrysotile A asbestos showed the highest stimulating effect among the particulates to which monocytes were exposed, followed by chrysotile B, crocidolite, and SiO_2. The optimum concentration of chrysotile A was 50 μg/ml. The amount of IL-8 released was dependent on the incubation time. Leaching magnesium (Mg) from chrysotile A by HCl-treatment significantly reduced its stimulating effect. Cycloheximide treatment significantly reduced the amount of IL-8 released. The amount of IL-8 released from monocytes was investigated with uncharged, positively or negatively charged dextran to determine the stimulating effect of the surface charges of asbestos. Our results in this investigation showed that the amount of IL-8 release markedly increased when charged dextran was applied. Among the signal transduction inhibitors used in this study, herbimycin A, H-7, calmidazolium, and EGTA reduced the amount of IL-8 released.

These results suggest that asbestos stimulates monocytes to release IL-8 synthesized de novo, and that the mechanism of this stimulation is involved in the surface charge of asbestos and Mg contained in chrysotile A. It is also suggested that the signal transduction route in this experiment is associated with cytosolic tyrosine kinase, protein kinase C (PKC), Ca^{2+}, and calmodulin.

Key words : IL-8 — monocytes —asbestos — charge — signal transduction

Exposure to asbestos causes a chronic inflammatory reaction and finally leads to asbestosis, a form of lung disease characterized by interstitial pulmonary fibrosis.[1] A common finding obtained from investigation based on biopsy and bronchoalveolar lavage in asbestos-exposed workers is neutrophil infiltration into the alveoli, the extent of which correlates with the duration of asbestos exposure[2] and the stage of asbestosis.[3] Appearance of neutrophils is frequently observed in asbestosis models.[4] Neutrophils release reactive oxygen intermediates and protease resulting in tissue damage, which is deeply involved in the pathology of fibrosis development caused by asbestos.[5,6]

IL-8, a CXC subfamily chemokine, is the most stable and strongest chemotactic factor and an activating factor for neutrophils.[7,8] It also works in the lung as a major chemotactic and activating factor for neutrophils.[9] The neutrophil count and IL-8 level in bronchoalveolar lavage fluid increase in patients with idiopathic pulmonary fibrosis,[10] adult respiratory distress

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syndrome,[11] cystic fibrosis,[12] and chronic bronchitis. A variety of inflammatory mediators including lipopolysacharide (LPS), tumor necrosis factor (TNF), and interleukin-1 (IL-1) stimulate various kinds of cells, such as monocytes, epithelial cells, endothelial cells and fibroblasts, to produce and release IL-8.[8]

Studies of the dynamics of alveolar macrophage populations have shown that monocytic passage from blood to alveoli[13] and in situ proliferation of alveolar macrophages[14] contribute to the recruitment of alveolar macrophage populations in the normal state. However, it is also known that the monocyte influx is dramatically accelerated in pulmonary inflammation, which indicates an intricate involvement of monocytes in inflammation.[15,16]

Asbestos has direct effects on the human pulmonary type II-like epithelial cell line A549, primary human bronchial epithelial cells,[17] and rabbit mesothelial cells[18] in vitro, making them produce IL-8. Alveolar macrophages play a major role in pulmonary inflammation and produce a variety of cytokines from in vitro or in vivo exposure to asbestos.[19] Thus alveolar macrophages are a most likely source of IL-8 in the lung. However, alveolar macrophages from healthy humans do not release IL-8 with the direct stimulus of asbestos exposure.[18,20] We report here an investigation of IL-8 release from monocytes following asbestos exposure and a part of the mechanisms of this phenomenon.

MATERIALS AND METHODS

Particulates

Asbestos (chrysotile A, chrysotile B and crocidolite), the International Union against Cancer (UICC) standard reference sample, was kindly provided to us by the National Centre for Occupational Health, South Africa. Silica particulates were purchased from Nacalai Tesque (SiO_2-L), and JIMRO (SiO_2-S). The particulates were sterilized with a dry heat sterilizer at 180°C for 1 hr or a steam sterilizer for 1 hr.

Isolation and culture of human monocytes

Mononuclear cells from 10 healthy donors were separated by a centrifugation method using Ficoll-Hypaque solution (Separate-L, Muto pure chemicals). The cells were washed, suspended in RPMI-1640 medium supplemented with 10% FCS, and seeded onto plastic dishes prepared for the adherent cell separation method (MSP-P, JIMRO) at 37°C, 5% CO_2 for 30 min. Non-adherent cells were then removed by gently washing the flask twice with Dulbecco's phosphate-buffered saline (PBS, pH 7.4, Mg^{2+}, Ca^{2+} free). Adherent cells were collected by gentle pipetting in cold PBS. The adherent cell population consisted of 85 to 90% monocytes as determined by esterase staining.[21] The viability of the cells, determined by trypan blue exclusion, was above 98%. The adherent cells will be referred to as monocytes. The cells were seeded on 2% agarose coated 96 well-plates to prevent adherence effects[22] with a density of 10^6 cells/ml. They were stimulated in serum-free RPMI-1640 medium with different reagents in the presence or absence of signal transduction inhibitors. The stimuli included were asbestos, silica, dextran (Nakalai Tesque), dextran sulfate (Nakalai Tesque) and diethylaminoethyl-

dextran (DEAE-dextran, Pharmacia). The signal transduction inhibitors used were cycloheximide (Sigma), H-7 (Seikagaku co.), herbimycin A (GIBCO), genisteine (Extrasynthese), calmidazolium (RBI), cholera toxin (GIBCO) and EGTA. The culture supernatant was then collected and stored at $-70℃$.

IL-8 assay
Human IL-8 concentrations were determined by ELISA using a commercially available system (Amersham Life Science). The assays were performed according to the manufacturer's instructions. Briefly, the culture supernatant or standards were added to wells of rigid flat bottom microtiter plates coated with murine monoclonal antibody to human IL-8. After incubation of the culture supernatant or standards and thorough washing of the wells, HRP-conjugated anti-IL-8 antibody was added to the wells. After a second incubation, the excess HRP-conjugated antibody was removed by washing. The HRP substrate was then added, and the color intensity was measured with a microtitre plate reader. The minimum detectable dose of IL-8 in this assay system was 4.7 pg/ml.

Depletion of Mg from asbestos
Chrysotile A was incubated at room temperature for four days in 2N HCl or distilled water. The treated asbestos was washed with distilled water by centrifugation to remove any remaining HCl. At the end of the procedure, the asbestos was dried, weighed and sterilized. The extent of Mg depletion was determined with an analytical transmission electron microscope (Hitachi H-7100) fitted with a kevex energy dispersive X-ray (EDX) microanalyzer (KEVEX DELTA PLUS 2).

Statistics
The mean and standard deviation for each group were calculated and a Mann-Whitney U test was used to determine significance. A p value of less than or equal to 0.05 was considered as significant.

<div align="center">RESULTS</div>

Effects of particulates on IL-8 release
The effects of particulates (50 μg/ml) on the release of IL-8 from monocytes are shown in Fig 1. Human peripheral blood monocytes incubated in medium alone released a negligible amount of IL-8. Particulates induced significant release of IL-8. Of the particulates used, chrysotile A had the greatest effect on IL-8 release, resulting in a 43-fold increase in IL-8 release compared with untreated cells. Particulates induced IL-8 release from monocytes in the following order of magnitude : chrysotile A > chrysotile B > crocidolite > SiO_2. Chrysotile A was chosen for additional studies, as it allowed for a vigorous IL-8 release. To ascertain whether asbestos was stimulating the release of preformed IL-8 or de novo IL-8 synthesis, we treated monocytes with cycloheximide, an inhibitor of protein synthesis. Complete inhibition was obtained with 10 μg/ml of cycloheximide added together with asbestos (Fig 1).
Fig 2 shows the time course of IL-8 release from monocytes exposed to

chrysotile A. The release could be detected within 4 hr of the addition of chrysotile A and continued to accumulate in culture over the length of the incubation period. As shown in Fig 3, release of IL-8 from monocytes exposed to chrysotile A was dependent on the amount of chrysotile A, from 10 μg/ml
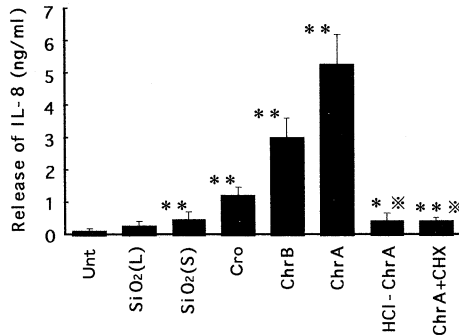
Fig 1. Release of IL-8 from human monocytes 8 hr after exposure to particulates (50 μg/ml). Values represest means $\pm$ SD of five samples. $*$: p<0.05, $**$: p<0.01 (compared with untreated cells). $\divideontimes$: p<0.01 (compared with the cells treated with ChrA). Unt: untreated, SiO$_2$: silica, Cro: crocidolite, ChrB: chrysotile B, ChrA: chrysotile A, HCl-ChrA: HCl-treated chrysotile A, CHX: cycloheximide.
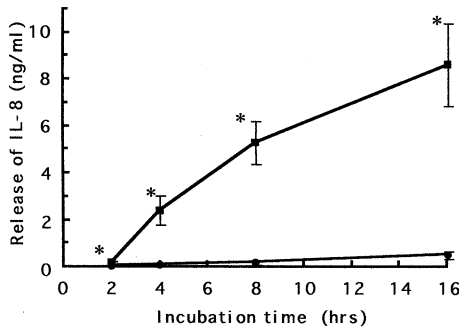
Fig 2. Kinetics of IL-8 release by chrysotile A-treated (50 μg/ml) human monocytes. Values represent means $\pm$ SD of five samples. $*$: p<0.01 (compared with untreated cells). ●-●: untreated, ■-■: chrysotile A.
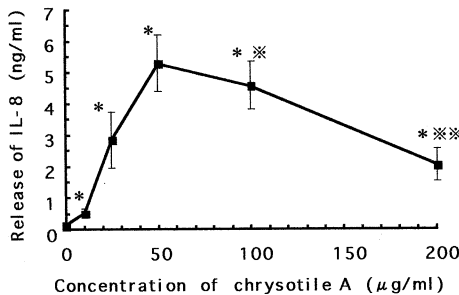
Fig 3. The dose dependent release of IL-8 from human monocytes 8 hr after chrysotile A exposure. Values represent means $\pm$ SD of five sample. $*$: p<0.01 (compared with untreated cells), $\divideontimes$: p<0.05, $\divideontimes\divideontimes$: p<0.01 (compared with the cells treated with 50 μg/ml).

to 50 μg/ml.   However, at a cytotoxic dose of chrysotile A, 200 μg/ml, chrysotile A-induced IL-8 release was remarkably reduced.

Mg depletion from asbestos

The effects of HCl and distilled water on leaching of Mg from the chrysotile A are shown in Fig 4.   HCl-treated asbestos showed a remarkable depletion of Mg, while distilled water-treated asbestos did not show any depletion.   In addition, HCl treatment caused Fe component depletion from the asbestos.   Then, we examined whether the HCl-treated asbestos would provoke monocytes to increase the release of IL-8.   As shown in Fig 1, the HCl-treated asbestos showed only a slight stimulatory effect on monocytes.
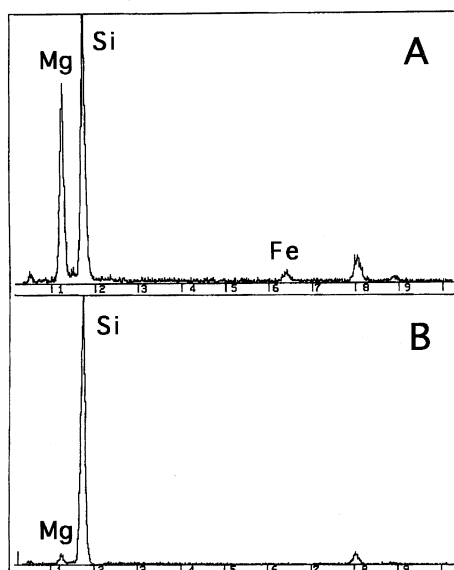

Fig 4.   Energy dispersive X-ray spectra of chrysotile A.
A : distilled water-treated chrysotile A, B : HCl-treated chrysotile A.

Polyanion and Polycation

To determine if the surface charge of asbestos is important in the release of IL-8 from monocytes, we compared the effects of untreated dextran, negatively charged dextran and positively charged dextran on the release of IL-8 from monocytes.   As shown in Fig 5, IL-8 release from monocytes incubated with the anionic dextran or cationic dextran increased 13-to 57-fold as compared with the control cells.   Of the two charged dextrans, dextran sulfate was slightly more potent than DEAE-dextran, resulting in a 57-fold increase in IL-8 release when compared with untreated cells.   In contrast, uncharged dextran did not induce the release of IL-8 at any dose used.

Signal transduction inhibitors

Herbimycin A (an inhibitor of cytosolic tyrosine kinase) used at 5 μg/ml fully inhibited IL-8 release by asbestos-exposed monocytes.   On the other hand, genistein (an inhibitor of membrane receptor tyrosine kinase) used at 5 μg/ml
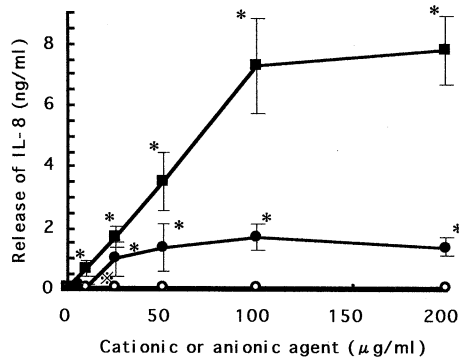
Fig 5. Release of IL-8 from human monocytes 8 hr after treatment with cationic or anionic agent. Values represent means ± SD of five samples. *: p<0.01 (compared with untreated cells). ○-○: dextran, ●-●: DEAE-dextran, ■-■: dextran sulfate.

did not inhibit IL-8 release. H-7 (an inhibitor of PKC activity) at 25 μM, calmidazolium (an inhibitor of calmodulin) at 0.5 μg/ml and EGTA (a calcium chelator) at 5 mM reduced the release of IL-8 by 42.8%, 52.8% and 68.1%, respectively.   However, cholera toxin (an agent that raises the intracellular cAMP level) had no affect on IL-8 release (Table 1).

TABLE 1. Effects of metabolic inhibitors on the asbestos-induced release of IL-8 by human monocytes

Inhibitors	Target of inhibitors	IL-8 (Percentage of inhibition)※
EGTA 5 mM	Extracellular calcium	68.1±9.9※†
Calmidazolium, 0.5 μg/ml	Calmodulin	52.8±14.9†
H-7 25 μM	PKC	42.8±10.2†
Herbimycin A, 5 μg/ml	src tyrosine kinase	86.5±3.7 †
Genistein, 5 μg/ml	Tyrosine kinase	14.6±13.6
Cholera toxin, 5 μg/ml	G protein	−1.4±13.8

The amount of IL-8 released by monocytes incubated for 8 hr with chrysotile A in the presence or absence of metabolic inhibitors was measured by ELISA. ※: Percentage of inhibition was calculated as {1−(asbestos + inhibitor/asbestos alone)}×100. *: Each value represents the meas ± SD of five samples. †: p<0.01 (compared with asbestos alone).

DISCUSSION

Human monocytes release IL-1β and TNF-α by asbestos exposure and IL-1β, TNF-α, and IL-8 by silica exposure.[23,24] In this report, we demonstrated that asbestos, a kind of silicate, stimulates the release of newly synthesized IL-8 rather than preformed one. This finding suggests that monocytes infiltrating into the inflammatory lung could be a source of IL-8 in asbestos-induced alveolitis.   Model studies have reported that a dramatic rise in the influx of monocytes occurs in pulmonary inflammation caused by silica exposure or carbon loading.[15,16] IL-8 is a chemotactic and activating factor for neutrophils. Recently it has been reported that IL-8 works on vascular endothelial cells to

increase vascular permeability,[25] promote proliferation and chemotaxis of these cells, and induce vascularization,[26] which indicates the complex involvement of IL-8 in inflammation. It is known that monocytes are activated when they are allowed to adhere to the surface of the bottom of plastic cultivation dishes.[27] We used agarose plates for monocyte cultivation to minimize the effect of adhesion of monocytes to dishes.

The surface of asbestos is charged totally positive in chrysotile and negative in crocidolite.[28] We showed by the dye-adsorption test that chrysotile contains both positively and negatively charged sites, whereas crocidolite contains only negatively charged ones.[29] In this report, we demonstrated that positively or negatively charged dextran could stimulate monocytes to release IL-8, but uncharged dextran had no stimulating effect. These findings suggest that the status of electric charges may be an important factor for increasing the amount of IL-8 released.

Cationic substances attach themselves to negatively charged sites of cells by electrostatic force to activate neutrophils[30] and lymphocytes.[31] On the other hand, negatively charged substances such as acid phospholipids and dextran sulfate promote macrophage proliferation,[32] and make macrophages and monocytes produce and release urokinase[33] and IL-1β,[34] respectively. The effects of these substances are caused by the association mediated by the positively charged collagenous domain of scavenger receptors.[35] Scavenger receptors have been manifested on monocytes, macrophages, endothelial cells, fibroblasts, and smooth muscle cells.[34,35] A recent report has demonstrated that scavenger receptors bind specifically to crocidolite asbestos.[36] We showed that the amount of IL-8 released from human monocytes is increased by crocidolite exposure, which may start as a result of combining of the negatively charged site of crocidolite with the positively charged site of the scavenger receptor.

In this study, we showed that the monocyte-stimulating effect disappeared in Mg-leaching chrysotile, which suggests the importance of Mg component in chrysotile A. Chrysotile is a phyllosilicate containing Mg ($3MgO \cdot 2SiO_2 \cdot H_2O$), which stimulates lung epithelial cells, monocytes/macrophages and neutrophils to make them release cytokine[17,23,37] and reactive oxygen species.[37,38,39] We also reported that chrysotile asbestos stimulates $CD4^+CD45RA^+$ lymphocytes specifically.[40] There has been a report that the positive charge on the surface of chrysotile is a key element of its stimulating mechanism.[39] We also have obtained an experimental result showing that the positively charged site on chrysotile asbestos is essential in increasing the intracellular Ca^{2+} concentration of lymphocytes by chrysotile (unpublished data). However, as described above, we previously reported that chrysotile has negatively charged sites as well as positively charged ones.[29] It has been reported that the Mg of chrysotile is important for the induction of mucin release from tracheal epithelial cells.[41] Further investigation should be carried out to determine which site is actually involved in promoting IL-8 release from monocytes by chrysotile.

The amount of IL-8 released from monocytes by stimulation of asbestos was markedly decreased by herbimycin A, a cytosolic tyrosine kinase inhibitor, but it was not decreased by genistein, an inhibitor of membrane receptor tyrosine kinase. Reduction of cytokine release by herbimycin A has also been reported in stimulating mouse macrophages by heparan sulfate[42] and A549

lung epithelial cells by asbestos.[43] Tyrosine kinase activity is considered to be involved in nuclear factor-$\varkappa$B (NF-$\varkappa$B) activation.[44] NF-$\varkappa$B activation is found to be associated with asbestos exposure in the lung[45] and is involved in the regulation of a gene encoding for IL-8.[46] This study suggests that the IL -8 production in this experiment may be mainly associated with cytosolic tyrosine kinase-mediated signal transduction system.

ACKNOWLEDGMENTS

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