Increases of Thrombomodulin Activity and Antigen Level on Human Umbilical Vein Endothelial Cells Treated with Asbestos and Man-Made Mineral Fibers

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Abstract: The potential influences of crocidolite asbestos fibers and man made mineral fibers (potassium titanate whisker and magnesium sulfate whisker) on a procoagulant system of human umbilical vein endothelial cells (HUVECs) were investigated by measuring the activity and antigen level of thrombomodulin (TM) on the cell surface. Statistically significant increases in both the TM activity and TM antigen level were observed on HUVECs treated with crocidolite asbestos fibers for 48 h and 72 h compared to untreated cells at low concentrations of the fibers which showed no sign of a cytotoxic effect on the cells. An extensive increase in both the TM activity and TM antigen level was also observed on HUVECs treated with potassium titanate whisker or magnesium sulfate whisker for 48 h and 72 h. A statistical analysis revealed that these fibers had almost the same effects on the increases in both TM activity and the TM antigen level of HUVECs treated with the fibers for 48 h and 72 h, but a treatment of magnesium sulfate whisker at more than 1.25 μg/ml for 24 h was slightly more effective in increasing TM activity on HUVECs compared to other fibers (p<0.05). The [3H]leucine incorporation in HUVECs increased when the cells were treated with crocidolite asbestos or man-made mineral fibers (MMMFs), indicating that the increases in TM activity and the TM antigen level on HUVECs directly exposed to those fibers may not reflect the sole induction of anticoagulant activities, but the general cell damage induced by the fibers.

Key words: Asbestos, Man-made mineral fibers, Human umbilical vein endothelial cell, Thrombomodulin, Procoagulant activity

Introduction

Exposure of humans to asbestos causes inflammation and fibrosis of the pulmonary interstitium and peripheral airspaces1). Various types of man-made mineral fibers (MMMFs) induced bronchoalveolar inflammation and pulmonary fibrosis in rat lungs2–4). At the cellular level, asbestos causes lysis of red blood cells5) and is toxic for macrophages6). Alveolar macrophages are known to play a critical role in pathogenesis of lung fibrosis. Interaction between endothelial cell monolayers and asbestos resulted in rapid phagocytosis of the particulates7) and uptaking of asbestos fibers into endothelial cells, which may represent an in vitro model for in vivo asbestosis8,9). In
vitro studies also demonstrated that active phagocytosis of cytotoxic amosite fibers by human vascular endothelium resulted in cell activation, supporting the concept that these important interstitial cells may be one of the responsive members for the development of pneumoconiosis. Furthermore, it has been reported that procoagulant activity in bronchoalveolar lavage fluid increased after exposure to asbestos. When treated with asbestos, both human alveolar macrophages and endothelial cells increased tissue factor expression, and they represent the potential sources of enhanced procoagulant activity and thus may contribute to the pathogenesis of asbestos-induced pulmonary fibrosis.

To clarify the effects of asbestos and man-made mineral fibers (MMMFs) on the procoagulant activity of endothelial cells, we investigated the changes in thrombomodulin (TM) activity and the TM antigen level on human umbilical vein endothelial cells (HUVECs) treated with asbestos or mineral fibers, because TM is a membrane glycoprotein anchored on the endothelial cells and one of the most important regulatory factors in the anticoagulant system.

**Materials and Methods**

**Reagents**

Heparin (pig intestinal mucosa), thrombin and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Boc-Leu-Ser-Thr-Arg-MCA was obtained from the Peptide Institute, Osaka, Japan. Antithrombin III (AT III) was purchased from Hoechst Japan, Tokyo and Bovine protein C was from Enzyme Research Laboratories, Inc., New York, NY, U.S.A. A TM antigen measurement kit including peroxidase (POD)-conjugated anti-human-TM monoclonal IgG was purchased from Fuji Chemical Industries, Ltd., Toyama, Japan. Culture medium for HUVECs consisted of modified MCDB 131, 2% (v/v) fetal calf serum (FCS), 10 ng/ml of epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF) 4 ng/ml, heparin 25 µg/ml, 1 µg/ml of hydrocortisone, and antibiotics (50 µg/ml of gentamicin sulfate). [4,5-3H]-leucine (2.22-4.44TBq/mmol) was purchased from ICN Biomedicals Inc. Acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-1,3,3',3'-tetramethyl-indo-carbocyanine percholate (Dil-Ac-LDL) was purchased from Biomedical Technologies, Stoughton, MA, U.S.A. An immunohistochemical staining kit for human factor VIII related antigen was obtained from Biomedda Co., Foster, CA, U.S.A. All other reagents were purchased from Wako Pure Chemicals, Osaka, Japan.

**Fibers**

The UICC asbestos, crocidolite, amosite and chrysotile were obtained from Department of National Health and Population Development National Centre for Occupational Health, Johannesburg, South Africa. Potassium titanate whisker (K2Ti6O13) and magnesium sulfate whisker (MgSO4·5Mg(OH)2·3H2O) were kindly presented by Dr. Isamu Tanaka, University Occupational and Environmental Health, Japan.

**Cell culture**

Vascular endothelial cells were isolated from human umbilical cord veins according to the method of Jaffe et al., and grown in gelatin coated 25 cm² flasks containing the culture media in a 5% CO2 atmosphere at 37°C. The endothelial cell monolayer was identified by the typical cobblestone morphology, the presence of von Willebrand factor and Factor VIII complex which was demonstrated by indirect immuno-staining and uptake of Dil-Ac-LDL. The cells in the second passages were grown to confluence in 96-well plates without gelatin coating. These cells were used for experiments 1 to 3 days after confluence.

**Treatment of cells with fibers**

When HUVECs were grown to confluence in wells of 96-well plates, a fresh medium (100 µl) containing asbestos fibers or MMM fibers (200, 100, 50, 25, 12.5, 6.25, µg/ml for cytotoxicity assay 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 µg/ml for TM activity and TM antigen level assays) was poured into each well. The plates were placed in a CO2 incubator at 37°C for the indicated periods until the time of assay.

**Cytotoxicity assay**

The cytotoxicity was tested by the CytoTox96 (Promega Co. Madison, U.S.A.) assay which quantitatively estimates the activity of lactate dehydrogenase (LDH) that has been released upon cell lysis into the culture medium by measuring the conversion of a tetrazolium salt (INT) into a red formazan product. This assay system is based on the fact that the strength of the color formed is proportional to the number of cells lysed.

**Assays for TM activity and TM antigen level**

TM activity and TM antigen levels were determined in 96-well plates by the method of Horie et al. Briefly, for the assay of TM activity, the cells were washed three times with buffer A (100 mM NaCl, 5 mg/ml BSA, 20 mM HEPES,
pH 7.5) and then incubated for 30 min at 37°C in 100 µl of buffer A containing 1 mM CaCl₂, 0.5 NIH U/ml thrombin, and 40 mg/ml protein C. The activity of protein C in the incubation mixture was determined by measuring the cleavage of 50 µM Boc-Leu-Ser-Thr-Arg-MCA, in the presence of 2 U/ml ATIII and 8 U/ml heparin. The concentration of 7-amino-4-methylcoumarin liberated was measured with a Fluorescence Spectrophotometer F-4010 (Hitachi Co., Ibaragi, Japan) with excitation at 380 nm and emission at 460 nm.

For the estimation of the TM antigen level, the cells were washed once with PBS (pH 7.3) and incubated with POD-conjugated anti-human-TM monoclonal IgG (MFTM-6, 0.5 mg/l), for 45 min at room temperature. The cells were rinsed three times with PBS and incubated for 45 min in 150 µl of citrate buffer solution (pH 4.5) containing o-phenylenediamine (0.5 mg/ml) and 0.015% (v/v) H₂O₂. The concentration of the product of the H₂O₂-POD-coupled reaction was measured at 492 nm with an MPR A4 Micro Plate Reader (Toyosoda industry Co., Tokyo, Japan). All data were expressed as percentages of control values.

[³H]Leucine incorporation
HUVECs were cultured in wells of 96-well plates with or without crocidolite fibers (1 µg/ml) or MMM fibers (1 µg/ml) for the indicated times and, further incubated for an additional 3 h in a medium containing [³H]leucine (1 µCi/ml). Then the cells were harvested and radioactivity was determined in a Matrix 9600 Direct Beta Counter, Packard Co. Ltd.

Cell counting
All data were corrected by the number of cells in each well. The number of cells was determined with CellTiter 96 (Promega Co., Madison, U.S.A.) by the tetrazolium/formazan assay which is based on the observation that the 490 nm absorbance of formazan produced from tetrazolium compound (3-[4,5-dimethylthiazol-2-y1]-5-[3-carboxymethoxyphenyl]-2-sulphenyl]-2H-tetrazolum, inner salt; MTS) was correlated to the number of viable cells. In a preliminary experiment, we confirmed that the result of the tetrazolium/formazan assay was well correlated with the number of cells counted with a hemocytometer.

Statistical analysis
Data are reported as the mean ± SE. Student’s two-tailed unpaired t-test for data was used to compare differences between means, with significance at p<0.05.

Results and Discussion

Cytotoxic effects of asbestos and man-made mineral fibers on HUVECs
To estimate the effect of mineral fibers on cell viability, human vein endothelial cells (HUVECs) were exposed to different types of fibers for the periods indicated and lactate dehydrogenase (LDH) release was quantified. Figure 1a shows the dose-dependent cytotoxic effects of asbestos fibers on HUVEC monolayers treated with the fibers for 48 h. Phagocytosis of three types of asbestos by HUVECs occurred as previously reported (data not shown). Three different types of asbestos, amosite, crocidolite and chrysotile, all showed the similar cytotoxicities on HUVECs at a concentration higher than 100 µg/ml. Among the three types of asbestos fibers, chrysotile asbestos comes first, amosite second, and crocidolite third in the strength of the toxic effect on HUVECs at concentrations lower than 50 µg/ml (p<0.05), and crocidolite and amosite asbestos showed no cytotoxicity at less than 12.5 µg/ml (Fig. 1a). Most of the cells died at 200 µg/ml with each of the three types of asbestos. Similar cytotoxic effects of asbestos fibers were observed when treated for 24 h and 72 h (data not shown). Figure 1b shows the dose-dependent cytotoxic effects of potassium titanate whisker (K₂Ti₆O₁₃) and magnesium sulfate whisker (MgSO₄·5Mg(OH)₂·3H₂O) on HUVEC monolayers treated with the fibers for 48 h. Potassium titanate whisker was significantly more cytotoxic than magnesium sulfate whisker at concentrations over 25 µg/ml (p<0.05) and had a cytotoxicity comparable to that of chrysotile asbestos. Most HUVEC cells died at a concentration of 100 µg/ml of potassium titanate whisker, while only 40% of HUVEC cells died when treated with magnesium sulfate whisker at the same concentration.

The solubility of potassium titanate whisker is extremely low (0.06 µg/ml in distilled water); on the other hand, magnesium sulfate whisker fibers are crystals highly soluble in water (30 µg/ml). Light micrographs showed that a large amount of insoluble potassium titanate whisker fibers occurred when the HUVEC monolayer was treated with them at 25 µg/ml and some of them were phagocytized by the cells (data not shown), while most of magnesium sulfate whisker fibers were soluble at the same concentration. The difference in the cytotoxicity of these fibers on HUVECs may reflect the greater physical damage to cells caused by potassium titanate whisker on HUVECs due to their different solubility. The cytotoxic effect of asbestos fibers was similar to that of potassium titanate whisker at over 25 µg/ml, which
may reflect the low solubility of asbestos fibers (less than 0.1 \( \mu g/ml \) in distilled water).

**Effects of crocidolite asbestos fibers on TM activity and TM antigen levels in HUVECs**

Crocidolite asbestos fibers have been reported to have more potent effects on mesotheliomas *in vivo* in rats than chrysotile asbestos \(^6\), on macrophages, however, crocidolite asbestos fibers has less cytotoxic effects than chrysotile asbestos, and almost the same effects as amosite \(^6\). As shown in Figure 1a, crocidolite asbestos fibers had less cytotoxic effects on HUVECs than two other types of asbestos fibers, prompting us to do the present investigation of the specific effects of crocidolite asbestos fibers on TM activity and TM antigen levels of HUVECs. Figure 2 shows the TM activity(a) and TM antigen level (b) which were determined for HUVECs after incubation with crocidolite asbestos fibers at the different concentrations for 24 h, 48 h and 72 h. The increases in TM activity were observed in HUVECs treated with crocidolite asbestos, which reached the maximum (140%) of 1.25 \( \mu g/ml \) for 48 h and 2.5 \( \mu g/ml \) for 72 h, respectively. Augmentations of the TM antigen level were also observed in HUVECs treated with crocidolite asbestos for 48 h and 72 h, which reached the maximum (135-145%) of 1.25 \( \mu g/ml \). No statistical difference was observed between the 48 h and 72 h treatments of crocidolite asbestos in either the TM activities or the TM antigen levels of HUVECs; however, the 48 h and 72 h incubations were more effective than that for 24 h for the increases in TM activities and TM antigen levels (p<0.05). It is noteworthy that 120-130% rises in TM activity and the TM antigen level occurred at very low concentrations of crocidolite asbestos (less than 0.313 \( \mu g/ml \)).

The solubility of crocidolite asbestos was very low (less than 0.1 \( \mu g/ml \) in distilled water). Some of the insoluble crocidolite asbestos fibers were phagocytized, which were assessed by light microscopy (data not shown). Phagocytized asbestos may contribute to the activations of TM activity and the TM antigen level. Increases in both TM activity and the TM antigen level attenuated at higher concentrations of crocidolite asbestos, probably due to their cytotoxic effects, although they were not the concentrations at which they kill cells. Both the TM activity and TM antigen level showed a slight increase in the cells treated with crocidolite asbestos for 24 h.

**Effects of man-made mineral fibers on TM activity and TM antigen level in HUVECs**

HUVECs were exposed to various concentrations of either potassium titanate whisker (Fig. 3) or magnesium sulfate.
whisker (Fig. 4) for the indicated periods, and TM activity and the TM antigen level were determined. TM activity increased to 135% in the cells treated with potassium titinate whisker even at a very low concentration (0.313 µg/ml) with a maximum increase (155%) of 1.25 µg/ml. As for the period of treatment, 72 h was slightly less effective than 48 h at 1.25 µg/ml and 0.625 µg/ml (p<0.05), and 24 h treatment has only a little effect.

A statistically significant increase in the TM antigen level was observed on HUVECs treated with potassium titinate whisker for 24 h (X), 48 h (●) or 72 h (▲), TM activity (a) and the TM antigen level (b) were determined. All data are averages ± SE for triplicate wells of three independent experiments.
whisker for 48 h with a maximum increase (143%) of 1.25 µg/ml. The 72 h incubation also augmented the TM antigen level, which exhibited a maximum increase (133%) of 1.25 µg/ml; however, no statistical difference was observed between 48 h and 72 h treatments of the fibers, except at 2.5 µg/ml. Potassium titanate whisker and crocidolite asbestos had similar activity for the induction of TM antigen in a similar dose dependent pattern for 48 h and 72 h treatments. Both TM activity and the TM antigen level were fairly reduced when treated with potassium titanate whisker at 10 µg/ml for 72 h, probably due to its cytotoxic effect (Fig. 3). A 24 h treatment of HUVECs with potassium titanate whisker induced slight increases (115%) in both TM activity and the TM antigen level, the extent of which was similar to those with crocidolite asbestos.

As for magnesium sulfate whisker, 72 h incubation showed a similar effect as 48 h incubation in the increases in both TM activity and the TM antigen level in the cells as shown in Figs. 4a and 4b. The longer period of incubation with magnesium sulfate whisker at 2.5 µg/ml induced the larger increases in TM activity (152% for 72 h) and TM antigen level (135% for 72 h) compared to 48 h incubation, maybe due to the lower toxicity of this whisker. When compared to the cells treated with either crocidolite asbestos or potassium titanate whisker for 24 h, HUVECs treated with magnesium sulfate whisker for 24 h exhibited slightly larger augmentation of TM activity at more than 1.25 µg/ml of the fiber (p<0.05).

### Table 1. Effects of crocidolite fibers and MMM fibers on the incorporation of [3H]-leucine into HUVECs

<table>
<thead>
<tr>
<th>Fibers</th>
<th>1 µg/ml</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crocidolite asbestos</td>
<td>98 ± 8%</td>
<td>135 ± 10%</td>
<td>125 ± 6%</td>
<td></td>
</tr>
<tr>
<td>Potassium titanate whisker</td>
<td>112 ± 10%</td>
<td>131 ± 9%</td>
<td>122 ± 7%</td>
<td></td>
</tr>
<tr>
<td>Magnesium sulfate whisker</td>
<td>111 ± 6%</td>
<td>158 ± 11%</td>
<td>144 ± 12%</td>
<td></td>
</tr>
</tbody>
</table>

All data are expressed as percentages of control values.

**Effects of crocidolite asbestos and MMM fibers on the incorporation of [3H]-leucine into HUVECs**

To investigate whether crocidolite fibers and MMM fibers induced a specific augmentation of the TM antigen level on HUVECs, incorporation of [3H]-leucine into the cells was determined for HUVECs grown in wells of a 96-well plate treated with 1 µg/ml of crocidolite fibers or 1 µg/ml MMM fibers for the times indicated. When compared with untreated HUVECs, incorporation of [3H]-leucine increased to 135 ± 10% and to 125 ± 6% in HUVECs treated with crocidolite fibers for 48 h and 72 h, respectively as shown in Table 1. Potassium titanate whisker enhanced incorporations of [3H]-leucine in HUVECs to 112 ± 10% after 24 h treatment, to 131 ± 9% after 48 h treatment and to 122 ± 7% after 72 h treatment. As for the treatment of magnesium sulfate whisker,
incorporations of [3H]-leucine increased to 111 ± 6%, 158 ± 11% and 144 ± 12%, after 24 h, 48 h and 72 h treatment, respectively. Despite the differences in the means of data for the incorporation of [3H]-leucine, no statistically significant difference was observed with those different fibers as there was no significant difference in activations of either TM activity or the TM antigen level on HUVECs with different fibers.

It was reported that asbestos exposure resulted in increased lung procoagulant activation in vivo. When HUVECs were directly exposed to asbestos and MMMFs in our assay system, TM activity and the TM antigen level increased, which was just opposite to the results reported earlier because TM plays a role in prohibiting coagulation. Nevertheless, the activation of incorporation of [3H]-leucine in HUVECs was observed when they were treated with crocidolite asbestos or MMM fibers as shown in Table 1, and the time dependent pattern of activation was very similar to that of enhancement of the TM antigen level. As enhancement of incorporation of [3H]-leucine in HUVECs probably in part results in an increase in protein synthesis in the cells, nonspecific and/or broad augmentation of the activity of procoagulant systems may have occurred in HUVECs directly treated with fibers. The augmentations of TM activity and the TM antigen level may therefore not necessarily imply the acceleration of only anticoagulant systems. Since it has been reported that in vivo alveolar macrophages and cytokines released from them as well as endothelial cells played a important role in enhanced procoagulant activity in lung disorders, the result of our present study on the induction of TM activity and TM antigen in HUVECs directly exposed to crocidolite asbestos and MMM fibers may not be compared with lung disorders induced in vivo by asbestos exposure but the increases in TM activity and the TM antigen level on HUVECs treated with those fibers may represent the physiological reaction of cells induced by the fibers.

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References

