Gene Expression of Surfactant Protein-A and Thyroid Transcription Factor-1 in Lungs of Rats Exposed to Silicon-Carbide Whisker in vivo

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Abstract: Gene Expression of Surfactant Protein-A and Thyroid Transcription Factor-1 in Lungs of Rats Exposed to Silicon-Carbide Whisker in vivo: Yasuo Morimoto, et al. Institute of Industrial and Ecological Sciences, University of Occupational and Environmental Health—Intratracheal instillation studies have shown that exposure to silicon carbide whisker (SiCW), an asbestos substitute, produces pulmonary fibrotic changes, suggesting that SiCW might have a fibrogenic potential. It is thought that surfactant protein is a good biomarker of lung injury and pulmonary fibrotic activity. In order to explore whether or not surfactant protein is associated with lung disorder through exposure to SiCW, we examined the expression of SP-A, SP-C and thyroid transcription factor-1 (TTF-1), a common transcription factor of SP-A and SP-C mRNA in lungs exposed to SiCW. Male Wistar rats were administered 2 mg or 10 mg of SiCW suspended in saline by a single intratracheal instillation, and were sacrificed at 3 d, 1 wk, 1 month, 3 months and 6 months after the intratracheal instillation. RNA was subsequently extracted from the lungs, and expression of SP-A, SP-C and TTF-1 mRNA from the lungs was observed by reverse transcription-polymerase chain reaction (RT-PCR). Exposure to 2 mg of SiCW showed a decrease in mRNA of SP-A and TTF-1 at 6 months, but exposure to 10 mg of SiCW showed decreased levels of SP-A and TTF-1 mRNA at 3 d and 6 months. On the other hand, 2 mg of SiCW increased the level of SP-C mRNA from 3 d to 3 months, and 10 mg of SiCW decreased the levels of SP-C mRNA in the rat lungs at 3 d, 1 month and 6 months.

No clear tendency to the expression of SP-C was observed, but the patterns of expression of TTF-1 and SP-A were similar. These data suggest that SP-A and TTF-1 are associated with not only the acute phase but also the chronic phase in lungs exposed to SiCW. (J Occup Health 2003; 45: 307–312)

Key words: SP-A, SP-C, TTF-1, Silicon carbide whisker, RT-PCR, Lung

As occupational and environmental inhalation exposure to asbestos causes pulmonary fibrosis1, a number of man-made mineral fibers (MMMFs) have been developed as a substitute, but, because of their similar physicochemical properties, MMMFs are thought to have adverse biological effects similar to those of asbestos2. Animal studies have shown that silicon carbide whisker (SiCW), which is used in abrasive and refractory materials, and the production of parts for electronic equipment, cause fibrosis3, 4, suggesting that SiCW might have a fibrogenic potential.

Surfactant protein5 produced by mainly type II alveolar epithelial cells acts as a control tower responsible for guiding the secretion and re-uptake of phospholipid which decreases alveolar surface tension, and through the prevention of alveolar collapse, these are thought to play contributory roles in preventing the progress of fibrotic processes6, 7. Decreases in the levels of SP-A and SP-C mRNA have been reported to occur in the broncho-alveolar lavage fluid (BALF) of patients with idiopathic pulmonary fibrosis8, 9, and SP-A is thought to be a good biomarker of lung injury and pulmonary fibrotic activity.

Furthermore, thyroid transcription factor-1 (TTF-1)10, a common transcription factor of SP-A and SP-C mRNA, is involved in tissue-specific gene expression of epithelial
cells and also plays an important role in the regulation of gene expression of SP-A and SP-C. We conducted serial measurements of gene expression of SP-A, SP-C and TTF-1 mRNA in the rat lung after intratracheal instillation of SiCW in rats in order to determine whether SP-A and SP-C are associated with lung remodeling induced by SiCW.

Materials and Methods

Animals

Male Wistar rats (10 wk old) used in this study were purchased from Kyudo (Kumamoto, Japan). They were divided into 2 mg-exposed (n=5), 10 mg-exposed (n=5), and control (n=5) groups in each exposure category. Each rat was anaesthetized, and a tracheal cannula was inserted into the trachea of the rat by means of a laryngoscope. Either saline or SiCW suspension (2 mg or 10 mg/0.4 ml) was administered to the rats intratracheally. The rats were kept for 3 d, 1 wk, 1 month, 3 months and 6 months after the injection. Each rat was injected intraperitoneally with a fatal overdose of Phenobarbital. The rats were handled according to the guidelines described in the Japanese Guide for the Care and Use of Laboratory Animals as approved by the University of Occupational and Environmental Health Animal Care and Use Committee.

Fiber samples

The silicon carbide whisker (SiCW) used in the study is an organic fiber composed of Si and C. The geometric mean diameter was 0.3 µm (SD 1.5) and the geometric mean length was 5.1 µm (SD 2.3). The SiCW was provided by the Japan Fibrous Material Research Association[10, 11].

Table 1. Oligonucleotides of primers of four target genes

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<thead>
<tr>
<th>mRNA species</th>
<th>Primers</th>
<th>NO. of cycles</th>
<th>bp</th>
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</thead>
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<tr>
<td>SP-A</td>
<td>Sense 5'-TTACCCCTCTCTTGACTGT-3'</td>
<td>22</td>
<td>590</td>
</tr>
<tr>
<td></td>
<td>Anitisense 5'-AGCCCCATCCAGTGTTGGA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP-C</td>
<td>Sense 5'-TGGAGAGCCACCGGATTAC-3'</td>
<td>20</td>
<td>521</td>
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<tr>
<td></td>
<td>Anitisense 5'-TGCCAAGACCTTTGGCGGA-3'</td>
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<td></td>
</tr>
<tr>
<td>TTF-1</td>
<td>Sense 5'-AACCTGGGCAAATGACGAGCTG-3'</td>
<td>25</td>
<td>352</td>
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<tr>
<td></td>
<td>Anitisense 5'-ATCTTGACTCGTGTTGTCGAG-3'</td>
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<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense 5'-ATCATGTTTGAGACCTTCAACACC-3'</td>
<td>33</td>
<td>357</td>
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<tr>
<td></td>
<td>Anitisense 5'-TAGCTCTTCTCCAGGAGG-3'</td>
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Preparation of RNA, cDNA Synthesis, and PCR

Total RNA from the lung was prepared in the presence of guanidium thiocyanate[13]. Five hundred nanograms of total RNA was used for the synthesis of single-strand cDNA with moloney murine leukemia virus-derived reverse transcripts (Perkin Elmer, Norwalk, Connecticut). An equal amount of cDNA from each sample, was amplified by specific primers for each gene (Table 1). The amplification was performed with a Thermocycler (Astech) under the following conditions: denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 2 min for target and β-actin genes.

Detection of the fragments amplified by polymerase chain reaction (PCR) was made by electrophoresis on a 2 % agarose gel. PCR products were resolved by gel electrophoresis and visualized by ethidium bromide staining. The gel was photographed with Polaroid Type 665 positive/negative film (Polaroid Corporation, Cambridge, Mass.) under UV light at identical exposure and development times. The bands from the positive film were scanned, and the density of each PCR product was measured with National Institute of Health (NIH) image 1.55 software (written by Wane Rasband at NIH, Bethesda, MD).

Statistical analysis

Values were expressed as the mean ± one standard deviation. We used the non-parametric statistical, Mann-Whitney test, for the two groups. Differences at p<0.05 were considered statistically significant in the test.

Tissue preparation for staining with hematoxylin and eosin

The left lungs were placed at 4°C for 24 h in a refrigerator. Tissue was washed for 10 min per wash in phosphate-buffered saline and dehydrated by immersing in a graded series of ethanol washes, 1 h per wash, before being finally maintained in 100 % ethanol at 4°C. The lung tissue was embedded in paraffin, and sections were cut from the lobe. The samples were then sectioned and
Expression of SP-A mRNA

Exposure to 10 mg SiCW significantly decreased in levels of SP-A mRNA in the lung at recovery for 3 d and 6 months after intratracheal instillation. A significant increase in SP-A mRNA in the exposed groups was obtained compared to control groups at recovery for 3 months. Exposure to 2 mg SiCW did not change the expression of SP-A mRNA except for recovery from 3 d to 3 months, and decreased that only at 6 months.

Expression of SP-C mRNA

Levels of SP-C mRNA also decreased in the 10 mg-exposed lungs at 3 d, 1 month and 6 months. On the other hand, gene expression of SP-C mRNA in the 2 mg-exposed groups was higher than that of the control groups at recovery for 3 d, 1 wk, 1 month and 3 months.

Expression of TTF-1 mRNA

In comparison with the control group, levels of TTF-1 mRNA in rat lungs decreased significantly in the 10 mg-exposed groups at 3 d and 6 months after intratracheal instillation. Levels of TTF-1 mRNA in the lung were significantly lower at 6 months in the 2 mg-exposed groups than in the control (saline-exposed) group. At the other exposure periods, there were no significant differences between the control and the SiCW-exposed groups in transcriptional levels of TTF-1 in the lung.

Pathological findings

We observed maximum infiltration of neutrophils, alveolar macrophages and lymphocytes in the

stained with hematoxylin and eosin.

Results

Expression of SP-A mRNA

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Pathological findings

We observed maximum infiltration of neutrophils, alveolar macrophages and lymphocytes in the
parenchymal tissue of the terminal bronchiole and alveolar duct region at 3 d. The aggregation of inflammatory cells decreased with time. Alveolar and bronchial epithelial cell desquamation and hyperplasia was also observed. Slight amounts of collagenous materials were occasionally deposited in some of the thickened alveolar ducts and adjoining alveoli together with the aggregates of alveolar macrophages.

**Discussion**

In this study we examined whether surfactant protein plays a role in pulmonary fibrosis caused by organic fibers. We observed a decrease in gene expression of SP-A in rat lung tissue not only in the acute phase but also in the chronic phase of the pulmonary fibrotic process. To the best of our knowledge no previous reports have shown gene expression of SP-A mRNA in animal models with pulmonary fibrosis. Awasthi et al. reported that change in mRNA expression of SP-A corresponded to that of the protein level of SP-A in an animal model with chronic lung injury. It was reported that the concentration levels of SP-A decreased in the BALF of IPF patients. We consider that our results coincide with this report given that surfactant protein was secreted in the alveolar spaces.

In our previous study we reported that both inflammation and fibrosis were non-progressive in a SiCW intratracheal-instillation rat model. It has been reported that surfactant has the ability to reduce dynamic surface tension. Taking these into account, the decrease in SP-A may cause the collapse of lung tissue and develop nonprogressive inflammation and nonprogressive fibrosis. In a previous study we observed a decrease in SP-A in lung tissue of rats exposed to potassium octatitanate whisker (PT1) which induces nonprogressive morbid pulmonary changes. Patterns of Inflammation and fibrosis in rat lungs induce by SiCW were similar to that induced by PT-1, suggesting that a similar pattern of the expression of SP-A may be observed between SiCW and a PT-1 induced rat model. It was also reported that alveolar epithelial cell damage plays a role in the reduction of SP-A. Pathological findings in IPF patients showed alveolar epithelial cell reduction, bronchialization and fibrotic honeycomb. In this study we observed
Effects of Mineral Fibers on Surfactant Protein Alveolar and Bronchial Epithelial Cell Desquamation Immediately after Intratracheal Instillation of SiCW.

Epithelial cell damage plays a role in SP-A reduction. We also observed a temporary suspension of the decrease in gene expression of SP-A at 3 months. In a previous study we observed a temporary improvement in gene expression of Clara cell secretory protein (CCSP) and SP-A in a dust exposure rat model. Pathological findings in this study showed increased hyperplasia in the bronchial epithelial cells between the acute and chronic phase, a proliferation of production cells that might have temporarily compensated for the decrease in gene expression of SP-A, because of the presence of bronchial cells with co-expression of CCSP and SP-A. Little hyperplasia was detected at 6 months and gene expression of SP-A had again decreased.

SP-C is a hydrophobic protein produced specifically in type II alveolar epithelial cells. We did not observe a definite tendency to gene expression of SP-C between 2 mg and 10 mg of SiCW exposure which was different from the SP-A expression pattern, but Leukauf et al. reported that both SP-A and SP-C decreased in mice with acute lung injury induced by exposure to nickel sulfate aerosol. We believe that the gene expression patterns of SP-A and SP-C should be similar as their production cells are almost the same, but it is unclear why our results are different. According to Dangio et al., gene expression of SP-A increased, but that of SP-C was unchanged in rabbits exposed to high concentrations of oxygen. SP-A differs from SP-C in that it is also produced in Clara cells whereas SP-C is not. Linking this to the fact that we found damage not only to the alveoli but also to the terminal bronchiole may help to explain the role played in the decrease in the gene expression of SP-A.

We observed a decrease in the TTF-1 gene not only in the acute but also in the chronic phase of the pulmonary fibrotic process. The expression pattern of TTF-1 showed a similar tendency to that of SP-A. We also observed a decrease in the gene expression of not only SP-A but also TTF-1 in the PT1 intratracheal-injection rat model. As TTF-1 is a transcription factor of surfactant protein we consider the decrease in the gene expression of TTF-1 to play a partial role in the decrease in gene expression of SP-A.

Fig. 5. Lung sections from rats exposed to SiCW. Magnification × 100 (A) saline-exposed (control) lung, 3 d from recovery time; (B) SiCW-exposed lung, 3 d from recovery time; (C) SiCW-exposed lung, 1 wk from recovery time; (D) SiCW-exposed lung, 6 months from recovery time.
In summary, we conducted serial measurements of gene expression of SP-A and SP-C mRNA in lung after the intratracheal instillation of SiCW in rats to determine the role of SP-A and SP-C in pulmonary fibrosis induced by SiCW. Levels of SiCW had decreased at 3 d and 6 months of recovery, suggesting that SP-A and SP-C are involved not only in the acute phase but also in the chronic phase of lung injury induced by SiCW.

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References