Gene Expression of Clara Cell Secretory Protein, Surfactant Protein-A and Thyroid Transcription Factor-1 in the Lungs of Rats Exposed to Potassium Octatitanate Whiskers in vivo

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Abstract: Gene Expression of Clara Cell Secretory Protein, Surfactant Protein-A and Thyroid Transcription Factor-1 in the Lungs of Rats Exposed to Potassium Octatitanate Whiskers in vivo: Li Ding, et al. Institute of Industrial and Ecological Sciences, University of Occupational and Environmental Health, Japan—Inhalation studies have shown that exposure to potassium octatitanate whiskers (PT1), an asbestos substitute, produces pulmonary fibrotic changes, suggesting that PT1 might have fibrogenic potential. It has been theorized that Clara cell secretion protein (CCSP) and surfactant protein-A (SP-A) play a critical role in regulating the acute inflammatory response in the lung. The present study was conducted to investigate the time course (3 days, 1 wk, 1 month, 3 months, and 6 months) of the expression of mRNA of CCSP, SP-A and thyroid transcription factor-1 (TTF-1), a common transcription factor of CCSP and SP-A, in lungs exposed to PT1 in vivo. PT1 suspended in saline was administered to male Wistar rats at a dose of 2 mg or 10 mg by single intratracheal instillation, and RNA was then extracted from the lungs. Expression of CCSP, SP-A and TTF-1 mRNA from the lungs was examined by reverse transcription-polymerase chain reaction. Exposure to 2 mg of PT1 did not increase levels of CCSP, SP-A or TTF-1 mRNA. The level of SP-A mRNA in PT1-exposed rats was decreased at 1, 3 and 6 months after a single instillation of 10 mg. Levels of CCSP and TTF-1 mRNA were also decreased at 3 days, 3 and 6 months after a single instillation. These findings suggest that CCSP and SP-A are involved not only in the acute inflammatory response but also in the chronic response of the lung exposed to PT1.

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Key words: CCSP, SP-A, TTF-1, Potassium octatitanate whiskers, RT-PCR, Lung

Occupational and environmental inhalation exposure to asbestos causes pulmonary fibrosis¹. Various types of man-made mineral fibers have been developed as substitutes for asbestos². These fibers are thought to have adverse biological effects similar to those of asbestos due to their similar physicochemical properties. Animal studies have shown that potassium octatitanate whiskers (PT1), which are used in filters, separators and catalyst carriers, cause fibrosis, suggesting fibrogenic potential similar to that of asbestos³.

Clara cell secretory protein (CCSP) produced specifically by Clara cells⁴ and surfactant protein-A (SP-A) produced by type II pneumocytes⁵ may be involved in the fibrotic processes. It has been theorized that CCSP plays a role as a phospholipase A2 inhibitor⁶ in suppressing inflammation and fibrosis, and SP-A acts as a control tower responsible for guiding the secretion and re-uptake of phospholipids, which decrease alveolar surface tension⁷, ⁸, and that these proteins play a contributory role in preventing the progress of fibrosis through the prevention of alveolar collapse. Decreased levels of CCSP and SP-A mRNA have been reported in broncho-alveolar lavage fluid (BALF) from patients with idiopathic pulmonary fibrosis⁴, ⁵, ⁹. A reduction in the levels of these fibrosis-suppressing factors may contribute to pulmonary fibrosis.

Furthermore, thyroid transcription factor-1 (TTF-1)¹⁰,
a common transcription factor of CCSP and SP-A mRNA, is involved in tissue-specific gene expression of CCSP and SP-A mRNA and also plays an important role in the regulation of gene expression. Gene expression of CCSP and SP-A may therefore be influenced by TTF-1.

We made serial measurements of gene expression of CCSP, SP-A, and TTF-1 mRNA in the rat lung following the intratracheal instillation of PT1 in rats to determine the role of CCSP and SP-A in lung remodeling induced by PT1.

**Materials and Methods**

**Animals**

The male Wistar rats (10 wk old) used in this study were purchased from Kyudo (Kumamoto, Japan). Either saline or a PT1 suspension (2 mg or 10 mg/0.4 ml saline) was administered to the animals intratracheally. The rats were maintained for 3 days, 1 wk, 1 month, 3 months, and 6 months after recovery and were then assigned to an exposed group (n=5) and a control group (n=5) for each exposure category. After completion of the exposure time, the rats were killed with an overdose of phenobarbital by intraperitoneal injection. They were handled according to the guidelines in the Japanese Guide for the Care and Use of Laboratory Animals as approved by Animal Care and Use Committee, University of Occupational and Environmental Health, Japan.

**Fiber samples**

The potassium octatitanate whiskers (PT1) used in the study was a crystalline fiber composed of K₂O·8TiO₂. The geometric mean diameter was 0.35 μm (SD 1.51) and the geometric mean length was 6.0 μm (SD 2.04). The PT1 was provided by the Japan Fibrous Material Research Association

**Preparation of RNA, cDNA Synthesis, and polymerase chain reaction**

Total RNA from the lung was prepared in the presence of guanidium thiocyanate. Single-strand cDNA was synthesized with moloney murine leukemia virus-derived reverse transcriptase (Perkin Elmer, Norwalk, Connecticut, USA) using 500 ng of total RNA. An equal amount of cDNA from each sample was amplified by specific primers for each gene (Table 1). Amplification was performed with a thermocycler (Astech, Japan) under the following conditions: denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 2 min for the target and β-actin genes.

The fragments amplified by polymerase chain reaction (PCR) were detected by electrophoresis on 2% agarose gel. The 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, MA, USA) under ultraviolet 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**

We used non-parametric statistical tests: the Kruskal-Wallis test for three groups and the Mann-Whitney test for two groups. Differences at p<0.05 are considered statistically significant in both tests.

**Tissue preparation for immunohistochemistry**

After removal of the right lung, the left lung was inflated and fixed by intratracheal instillation of 4% paraformaldehyde at 25 cm H₂O pressure. The lungs and trachea were resected from the surrounding tissue, and allowed to stand at 4°C for 24 h. The tissue was
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washed for 10 min in phosphate-buffered saline, dehydrated by immersion in a graded series of ethanol washes for 1 h per wash, then maintained in 100% ethanol at 4°C. The lung tissue was embedded in paraffin, and sections were cut out from the lobe. Immunostaining for CCSP was performed on 8 µm sections. Rabbit anti-human urine protein 1 (human CCSP) antibody from Dako (Glostrup, Denmark) was used as the primary antibody and was detected by the avidin-biotin-peroxidase staining method with the DAKO LSAB Kit (Dako Corporation, USA). Tissue sections were counterstained with hematoxylin.

The lung tissue sections were also stained with Van Gieson stain.

Results

Expression of SP-A mRNA (Figs. 1A and 2)

No significant differences in transcriptional levels of SP-A in the lung were found between the control (saline-exposed) and the PT1 2 mg exposed groups at any time.

The SP-A mRNA level was significantly lower after 1, 3 and 6 months recovery in the PT1 10 mg exposed groups than in the control groups. Gene expression of SP-A mRNA was also decreased after 3 days recovery from exposure to PT1 10 mg, but the difference was not significant.

Fig. 1. Ethidium bromide staining of PCR products separated in 2% agarose gel. M: DNA marker (φX174/Hae III-digested). The figure for the groups show the PCR products for 5 rats. (A) SP-A; (B) CCSP; (C) TTF-1; (D) β-actin.
Expression of CCSP and TTF-1 mRNA (Figs. 1B–C, 3 and 4)

There were also no significant differences in transcriptional levels of CCSP and TTF-1 in the lung between the control and the PT1 2 mg exposed groups at any time. Levels of CCSP and TTF-1 mRNA in the lung were significantly lower at 3 days, 3 and 6 months after
intratracheal instillation in the PT1 10 mg exposed groups than in the control group.

**Immunohistochemical staining for CCSP (Fig. 5)**

Anti-CCSP immunostaining in the control group showed staining of the epithelial cells of the proximal and peripheral airways (5A). In the PT1 10 mg exposed groups, CCSP staining of the epithelial cells was less marked at 3 days’ recovery (5B). Hyperplasia of the epithelial cells with anti-CCSP antibodies was observed at 1 month (5C). There was no intense staining of the epithelial cells at 3 or 6 months (5D), but the PT1 2 mg exposed groups are not evidently different from the control groups.

**Van gieson stain (Fig. 6)**

It was observed that inflammatory cells accumulated in alveolar spaces at all times in the PT1 10 mg exposed groups, especially at 3 days’ recovery. From 1 week’s recovery, deposition of collagen was observed and this gradually decreased with time.

**Discussion**

Pulmonary fibrosis frequently occurs when there is an imbalance between factors stimulating and inhibiting inflammation and fibrosis\(^5\). We previously reported that exposure to PT1 increases gene expression of proinflammatory cytokines and growth factors in the rat lung\(^6\). In the present study, expression of CCSP and SP-A mRNA were decreased in rats exposed to PT1 intratraherally. This suggests that reduction in factors which inhibit pulmonary fibrosis is associated with the fibrotic process in the lung. This finding is consistent with the observation that levels of CCSP and SP-A are decreased in the BALF of patients with idiopathic pulmonary fibrosis\(^4,5,9\). As alveolar and bronchial injury can be the first manifestation of pulmonary fibrosis, decreased gene expression of these factors in the lung may be related to epithelial cell injury in the alveolar and bronchial regions. This phenomenon was observed not only in the acute phase but in the chronic phase as well (3 and 6 months after intratracheal instillation). In the
present study pathological examination in the chronic phase revealed accumulation of inflammatory cells, suggesting that decreased gene expression is related to epithelial cell injury in the chronic phase. Nevertheless, levels of both CCSP and SP-A are high in the BALF of patients with silicosis\textsuperscript{17} and asbestosis\textsuperscript{18}, a finding apparently inconsistent with the results of our study and those for patients with idiopathic pulmonary fibrosis. Although the reason for the difference in the fibrotic response is unknown, it might reflect the extent of alveolar and distal airway injury. In our study, intratracheal instillation of 10 mg of PT1 induced severe lung damage, and as a result, far fewer airway cells and alveolar epithelial cells were fully repaired. This may explain why gene expression of CCSP was decreased in our study, as it is in idiopathic pulmonary fibrosis\textsuperscript{19}. Some reports showed a relationship between the extent of pulmonary fibrosis and gene expression of CCSP and SP-A\textsuperscript{19, 20}. Arsalane \textit{et al.} reported that in rats exposed to ozone, the CCSP concentration in serum was dose dependent and correlated with the extent of lung injury as assessed by levels of total protein, LDH and inflammatory cells in BALF\textsuperscript{19}. There was a report that the amount of SP-A in BALF was related to the total levels of proteins and phospholipids\textsuperscript{20}. Gene expression of CCSP and SP-A in the lung in our experiment may also reflect the extent of pulmonary damage and fibrosis.

Lowered gene expression of CCSP mRNA after PT1 exposure temporarily improved 1 wk and 1 month after exposure. Since Clara cells are thought to be progenitors of distal airway epithelial cells\textsuperscript{21}, it is conceivable that Clara cell hyperplasia occurred to regenerate the airway epithelium after airway and alveolar injury caused by the intratracheal instillation of PT1. This may have been why lowered gene expression of CCSP mRNA in the lung tissue temporarily improved. The subsequent differentiation of the Clara cells into other cells would have again lowered CCSP expression. This is supported by the pathologic findings which frequently showed Clara cell hyperplasia in the lung tissue exposed to PT1, mainly in the distal airways, from 1 wk to 1 month after exposure.

This is the first study to examine gene expression of TTF-1, a common transcription factor of CCSP and SP-A, in lung induced by dust. Expression of TTF-1 mRNA

\textbf{Fig. 6.} Van Gieson stain of lung sections from PT1-exposed rats. Magnification \( \times 100 \) (A) saline-exposed (control) lung after 3 days’ recovery; (B) PT1-exposed lung after 3 days’ recovery; (C) PT1-exposed lung after 1 month’s recovery; (D) PT1-exposed lung after 6 months’ recovery.
followed a similar pattern to that of CCSP, in part because the decreases in TTF-1 expression play a role in the reduction of CCSP expression. Nevertheless, TTF-1 expression did not coincide with that of SP-A. It has been speculated that the binding sites of AP-1 transcription factors overlap with those of TTF-1 in the murine alveolar type II cell line, resulting in transcription factor interference. This may have been the reason why control by TTF-1 of SP-A expression was less noticeable than that of CCSP.

In summary, we made serial measurements of gene expression of CCSP and SP-A mRNA in the rat lung after the intratracheal instillation of 10 mg of PT1 to determine the role of CCSP in pulmonary fibrosis induced by PT1. Levels of CCSP decreased at 3 days, 3 months and 6 months after a single dose instillation, and levels of SP-A peaked at 1 wk and decreased at 1, 3 and 6 months, suggesting that CCSP and SP-A are involved not only in the acute phase of lung fibrosis, but also in the chronic phase.

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References