Effects of Mineral Fibers on the Expression of Genes Whose Product May Play a Role in Fiber Pathogenesis

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To determine which factors are useful for the risk assessment of man-made fibers, we examined the gene expression of proinflammatory cytokines, growth factors, manganese superoxide dismutase (MnSOD), and inducible nitric oxide synthase (iNOS) in mineral fiber-exposed rats by means of reverse transcription–polymerase chain reaction (RT–PCR). Male Wistar rats received a single intratracheal instillation of either saline (control) or two types of fibers (2 mg of Union Internationale Contre le Cancer (UICC) chrysotile or alumina silicate refractory ceramic fiber [RCF]). Expression of interleukin-1α (IL-1α), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), platelet-derived growth factor-A, PDGF-A, platelet-derived growth factor-B (PDGF-B), transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF), MnSOD, and iNOS mRNA from lung and lipopolysaccharide (LPS)-stimulated alveolar macrophages (AM) were assessed by RT–PCR. Among these factors, IL-1α, TNF-α, IL-6, bFGF, and iNOS would be the possible parameters for the risk assessment of fibers. In a follow-up study, we investigated the time course (3 days, 1 week, 1 month, and 3 months) of expression of IL-1α and TNF-α by LPS-stimulated AM exposed to mineral fibers in vivo. Male Wistar rats were instilled intratracheally with saline or fibers (2 mg of Union Internationale Contre le Cancer (UICC) crocidolite or potassium octatitanate whisker [TW]). The expression of IL-1α mRNA by fibers was greatest in TW, crocidolite, and rhyolite, and RCF-instilled rat AM, in that order. The increase of IL-1α and TNF-α mRNA in AM peaked at 1 month and 3 days after exposure to crocidolite or TW, respectively. The expression of IL-1α by fibers (crocidolite, chrysotile, TW, and RCF) may be a good indicator of the pathologic potential of fibers. — Environ Health Perspect 105(Suppl 5):1173–1178 (1997)

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Introduction

Recently, various types of man-made fibers (MMF) have been developed as substitutes for asbestos; the demand for these products is increasing. Some of these fibers are thought to possess the same adverse biological effects as asbestos because of their similar physiochemical properties (1).

Asbestos fibers and man-made fibers deposited in the lung lead to an activation of alveolar macrophages (AM). AM can release factors such as tumor necrosis factor α (TNF-α), interleukin-1α (IL-1α), interleukin-6 (IL-6), and basic fibroblast growth factor (bFGF) that augment cellular inflammation (2). Release of oxidants by these cells may lead to lung injury (3). Manganese superoxide dismutase (MnSOD) and inducible nitric oxide synthase (iNOS) are biomarkers for superoxide (4). Growth factors like platelet-derived growth factor A (PDGF-A), platelet-derived growth factor B (PDGF-B), and transforming growth factor β1 (TGF-β1) signal interstitial fibroblasts to replicate and modulate their production of connective tissue proteins (5). Fibroblast growth factors modulate potent growth of cells and neovascularization (6).

These internal tissue cells and cytokine cascades could account for the chronic nature of the inflammation. The accumulation of inflammatory cells, fibroblasts, and connective tissue matrices leads to lung remodeling such as thickening of alveolar and bronchiolar walls.

Even though the mechanism is not completely understood, evidence suggests that various factors are related to each fibrotic process in the lung (Figure 1). It is important to investigate among these factors parameters useful for the risk assessment of MMF and the kinetics of their expression in the process of the lung remodeling.

Materials and Methods

Fiber Preparation

The fibers used in this study were Union Internationale Contre le Cancer (UICC) crocidolite asbestos (crocidolite), UICC chrysotile asbestos (chrysotile); potassium octatitanate whisker (TW), and alumina silicate refractory ceramic fibers (RCF) (7). The crocidolite preparation, measured using scanning electron microscopy, had a geometric mean diameter of 0.20 μm (SD 1.5) and a geometric mean length of 1.3 μm (SD 2.3). For chrysotile, geometric mean diameter and geometric mean length were 0.085 μm (SD 1.4) and 0.7 μm (SD 1.9), respectively. For TW, they were 0.41 μm (SD 1.5) and 2.8 μm (SD 2.0), respectively. For RCF, they were 1.2 μm (SD 1.7) and 9.6 μm (SD 1.9), respectively.

Intratracheal Instillation Study 1

Ten-week-old male Wistar rats, in groups of five per treatment, were intratracheally instilled with saline or fiber (2 mg chrysotile or RCF). One month after tracheal instillation, bronchoalveolar lavage (BAL) was performed using the left lung. The cells recovered from BAL were plated in tissue culture plates and allowed to attach for 1 hr at...
37°C with RPMI-1640 medium containing 10% fetal bovine serum (7). Adhered AM were adjusted to a concentration of 1 x 10^5/ml, and were stimulated with 10 μg/ml lipopolysaccharide [LPS]; Sigma Chemical Co., St. Louis, MO) (7). Cells were cultured for 2 and 6 hr on cell culture plates at 37°C in a CO₂ incubator. After incubation, mRNA was extracted using a Quick Prep kit (Pharmacia Biotech, Uppsala, Sweden). RNA was also extracted from the right lung using the guanidinium thiocyanate–phenol–chloroform method (8).

**cDNA Synthesis, Polymerase, and Chain Reaction**

RNA was used for the synthesis of single-strand cDNA using Moloney murine leukemia virus-derived reverse transcriptase (Perkin Elmer, Norwalk, CT). Equal amounts of cDNA from each sample were then used for amplification by specific primers for rat IL-1α, IL-6, TNF-α, PDGF-A, PDGF-B, TGF-β1, bFGF, MnSOD, and iNOS. The amplification was performed with a Thermocycler (Astech, Fukuoka, Japan) under the following conditions: 94°C for denaturation for 45 sec, 60°C for 45 sec for annealing, and 72°C for 2 min for extension. β-Actin was co-amplified as an internal standard to quantitate polymerase chain reaction (PCR) amplification of mRNA. The number of thermocycles used allowed quantitation without saturation (9) (Figure 2). Four to five cDNAs per treatment group that expressed β-actin uniformly were entered into the study.

Detection of the fragments amplified by the PCR was made by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The gel was photographed with Polaroid Type 665 positive/negative film (Polaroid, Cambridge, MA) over ultraviolet (UV) light at the same exposure and developing time. The bands of the positive film were scanned and the density of each PCR product was measured using National Institutes of Health (NIH) image 1.56 software (written by W. Rasband, NIH, Bethesda, MD). The ratio of specific gene product to β-actin product was used for further analysis.

**Statistical Analysis**

Data are expressed as mean ± SEM. Comparisons were performed using the Student's t test with a paired test used for paired data. Correlation coefficients in IL-1α expression by fibers were obtained by Spearman's rank-order method; p values less than 0.05 were considered significant.

**Intratracheal Instillation Study 2**

Wistar rats in groups of five animals per treatment were intratracheally instilled with saline or fibers (2 mg of crocidolite or TW). After exposure for 3 days, 1 week, 1 month, and 3 months, AM were recovered from the left lung and stimulated with 10 μg/ml LPS for 2 hr. Expression of IL-1α and TNF-α by LPS-stimulated AM was assessed by reverse transcription (RT)–PCR as in intratracheal instillation study 1.

**Results**

**Gene Expression One Month after Intratracheal Instillation**

We examined rat IL1-α, IL-6, TNF-α, PDGF-A, PDGF-B, TGF-β1, bFGF,
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MnSOD, and iNOS from LPS-stimulated AM and right lung. When compared with the control (saline-instilled) group, rats exposed to chrysotile and RCF had significantly increased levels of IL-1α and TNF-α mRNA in AM (Figure 3). TNF-α mRNA in the lung increased only in chrysotile-exposed rats. TNF-α, iNOS, and bFGF mRNA in the lung increased significantly in chrysotile-exposed rats. IL-6 mRNA in AM was significantly increased in chrysotile-exposed rats.

Time-Course Expression of IL-1α and TNF-α

Total cell counts in the saline-instilled group were approximately $5 \times 10^5$ cells (Figure 2). In the crocidolite- and TW-instilled groups, total cell counts increased approximately 3-fold in 3 days compared with those in the control group. Subsequently, the total cell counts from these groups decreased at 1 month, then increased again 3 months after instillation. Most of the bronchoalveolar lavage fluid (BALF) consisted of AM. Contents of neutrophils in BALF were around 25% at 3 days and at 1 week after crocidolite and TW instillation (Figure 4).

Figure 5A shows the time course of gene expression of IL-1α by RT–PCR in LPS-stimulated AM after 3 days, 1 week, 1 month, and 3 months of exposure to saline, crocidolite, or TW. From the densitometric analysis, the level of IL-1α mRNA in AM peaked at 1 month after instillation of crocidolite or TW (Figure 5B). Levels of TNF-α mRNA in AM peaked at 3 days after the instillation of crocidolite or TW (Figure 6).

IL-1α mRNA Expression by Fibers

As the level of IL-1α mRNA peaked at 1 month after instillation, we compared the expression of IL-1α by fibers (crocidolite, chrysotile, TW, and RCF) 1 month after instillation. The expression of IL-1α mRNA by fibers was greatest in TW-, crocidolite-, chrysotile-, and RCF-instilled rat AM in that order (Figure 7), which was significant ($p < 0.05$) by Spearman's rank correlation coefficient.

Discussion

It has been suggested that MMF with widths and lengths similar to asbestos are most likely associated with the induction of pulmonary fibrosis and lung cancer (10). To assess molecular factors that may be useful for risk assessment of MMF, we examined the gene expression of proinflammatory cytokines, growth factors, MnSOD, and nitric oxide synthase (NOS) in mineral fiber-exposed rats using RT–PCR. RT–PCR has multiple advantages (9): a) its exquisite sensitivity allows the detection of extremely rare mRNA in small numbers of cells in a semiquantitative manner; b) it differs from the assay system of proteins, and the same methodology can be applied in analyzing the expression of many genes; and c) cDNA can be used for future studies. However, RT–PCR has several limitations. It can give useful information about transcription but does not provide information about translation, posttranscriptional processes, or cytokine exportation. In addition, because of exponential amplification over repeated cycles and differences in reaction efficiencies, RT–PCR is useful for detecting relative differences in the amount of a given mRNA. A number of studies describe semiquantitative RT–PCR techniques whereby relative mRNA production was estimated to be between samples; comparison of amplified target molecules co-amplified constitutively expressed mRNA (11). Housekeeping genes such as β-actin or D-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) often are selected as endogenous internal standard in such studies (11). This housekeeping gene may be increased under some conditions. In our experiment, β-actin expression was not altered significantly in the samples from different groups, based on AM cell numbers (Figure 2). Since the endogenous internal standard is usually a housekeeping gene, it frequently is found in higher concentration than the target message. This limitation was avoided by using different PCR cycles for both products. Because limitations of the endogenous internal standard, many investigators have used exogenous internal standards such as...
To find more sensitive and specific parameters of lung remodeling by fibers, many factors that may contribute to the process must be considered. RT–PCR may be a suitable method for surveying possible parameters of fiber-induced lung remodeling.

We chose chrysotile and RCF for intratracheal instillation study 1. Though chrysotile can induce lung cancer (fibrosis and mesothelioma), it is one of the least toxic asbestos fibers. There is considerable evidence for the fibrogenicity and carcinogenicity of RCF in laboratory animals, compared to that for other MMFs (13). For intratracheal study 2, we chose more fibrogenic fibers, UICC chrysotile and TW, to detect time-course change of gene expression. TW produces marked pulmonary fibrosis in rats with long-term exposure (14).

In the previous study, unstimulated AM obtained from rats treated with chrysotile did not significantly enhance steady-state levels of IL-1α mRNA (data not shown). AM harvested from rat lung did not express IL-1 protein, and LPS treatment of quiescent cells (after 24-hr in vitro culture) induced low-level expression of IL-1α and IL-1β (15). Short-term inhalation of RCF resulted in markedly increased IL-1β protein expression after stimulation with LPS (15). In vivo exposure of AM to LPS increased proinflammatory cytokine mRNA, although the kinetics of upregulation varied (16). For these reasons, we examined mRNA expression in 2 and 6 hr LPS-stimulated AM. As a cautionary note, results from ex vivo LPS-stimulated AM may not necessarily indicate a role for a stimulated cytokine in the pathogenesis of inflammation associated with exposure to fibers in vivo.

In intratracheal study 1, AM exposed to chrysotile or RCF were found to have upregulated IL-1α, TNF-α and IL-6 mRNA transcripts in response to LPS. These are proinflammatory cytokines with both inflammatory and fibrogenic activities such as attraction of inflammatory cells, production of superoxide and collagenases, and proliferation of fibroblasts (17). TNF-α mRNA and protein have been detected in the lung from patients with idiopathic pulmonary fibrosis (18) and in lungs from mice with pulmonary fibrosis elicited by exposure to bleomycin or silica (19). Increased release of IL-1 from AM has been reported after asbestos exposure by inhalation or intratracheal instillation (20). In inflammatory reactions, IL-6 could act as not only a proinflammatory cytokine because of its ability to induce the expression of cellular adhesion molecules on monocytes and the facilitation of their infiltration into the lung, but also as an antiinflammatory cytokine that inhibits the production of TNF and IL-1 (21). An increase has been reported in IL-6 released by bronchoalveolar cells from rats treated with asbestos or coal mineral dust (22).

Oxidants produced by inflammatory cells are thought to lead to lung injury in pulmonary fibrosis. Nitric oxide synthase (NOS) produces reactive species such as nitric oxide (NO) and peroxynitrite anion. NO' and peroxynitrite are also cytotoxic to host parenchymal cells (4). In our model, levels of iNOS mRNA increased in lungs exposed to chrysotile.
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Among the FGFs, bFGF stimulated the replication of endothelial cells in vitro and new microvesSEL growth in vivo (23). Asbestos exposure induced lavaged cells to secrete a fibroblast growth factor from 1 to 24 weeks after exposure in rats (24). In lungs exposed to chrysotile, we found increased levels of bFGF mRNA. Based on these results, IL-1α in the AM, TNF-α in the AM and the lung, IL-6 in the AM, and iNOS and bFGF in the lung would be the possible parameters of risk assessment of man-made fibers in this model. Accordingly, we set out to further investigate the time course of expression of IL-1α and TNF-α mRNA from exposed to crocidolite and TW.

As previously reported for rats (24), total cell count of BALF at 1 month after chrysotile or TW instillation was almost the same as the control group and increased at 3 months in the present study. Crocidolite or TW instillation resulted in pulmonary inflammation as evidenced by increased numbers of BALF neutrophils and macrophages at 3 days and 1 week after the exposure. Consistent with the acute inflammation, levels of TNF-α mRNA were greatest at 3 days after the exposure and decreased thereafter. In contrast to TNF-α, levels of IL-1α mRNA peaked at 1 month after crocidolite or TW exposure.

There have been many attempts to predict the toxicity of mineral fibers based on cytotoxic potentials of fibers using a variety of cell types in vitro. In an in vitro study using AM, TW caused the highest level of TNF-α production among fibers (1). This is consistent with the present study on TNF-α mRNA expression 3 days after instillation. TNF-α mRNA expression in TW-instilled animals was higher than that in crocidolite-instilled animals. Lee et al. (25) reported that crocidolite was the most potent fibrogenic agent and was 10 times more fibrogenic than potassium octatitanate (Fybex) in terms of expression concentration. In our study, the expression of IL-1α by fiber challenge (TW > crocidolite > chrysotile > RCF) may correlate with the reported pathologic potential of fibers (25,26).

Accordingly, our approach may be useful for evaluating the potential toxicity of newly developed man-made fiber. Further investigations using other fibers are necessary to confirm the general applicability of the method.

Both TNF-α and IL-1α are proinflammatory cytokines. It is necessary to demonstrate that acute inflammation completely predicts the chronic change induced by fibers. As TNF-α plays a key role in lung remodeling (27), further investigations on the correlation between proinflammatory cytokines and the order of the toxicity of fibrous materials are also required. Along this line, correlations between gene expression and pathologic changes induced by fibrous materials may prove to be a powerful approach for assessing health risks due to fiber exposure.

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