Combined Effect of Cigarette Smoke and Mineral Fibers on the Gene Expression of Cytokine mRNA

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Occupational and environmental exposure to inhaled asbestos dust causes pulmonary fibrosis, bronchogenic carcinoma, and pleural mesothelioma (2). Recently, various types of man-made mineral fibers 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 (2). Furthermore, epidemiological and clinical studies have indicated that asbestos workers who smoked cigarettes had a higher incidence of asbestos-induced disease than those who did not (3-4).

In asbestos-induced disease, many genes are thought to be involved in lung remodeling, such as fibrosis and carcinoma (5-6). Asbestos fibers deposited in the lung lead to an activation of alveolar macrophages (AMs) (5). AMs and parenchymal cells release tumor necrosis factor-α (TNFα), interleukin-1α (IL-1α), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα) mRNA from lipopolysaccharide-stimulated AMs and lungs of rats exposed to mineral fibers and/or cigarette smoke were assessed using semiquantitative reverse-transcriptase polymerase chain reaction. Exposure only to cigarette smoke increased in IL-10 mRNA levels in AMs. Chrysotile stimulated the expression of IL-10, TNFα, and IL-6 in AMs, and the expression of bFGF in lungs. RF1 resulted in increased expression of IL-10 and TNFα in AMs. Cigarette smoke stimulated the expression of inos in AMs and IL-6 and bFGF in lungs treated with chrysotile; IL-1α and IL-6 in AMs and bFGF in lungs did the same in lungs with RF1. Among these cytokines, message levels of IL-10, IL-6, and bFGF were increased in rats stimulated with mineral fibers, and the stimulating effects of mineral fibers were enhanced by cigarette smoke. Therefore, IL-1α, IL-6, and bFGF would be the possible parameters of the lung remodeling induced by mineral fibers. Key words: alveolar macrophage, bFGF, ceramic fiber, chrysotile, IL-6, IL-10, inos, lung, Mn-SOD, TNF, Environ Health Perspect 107:495-500 (1999). [Online 7 May 1999] http://ehpnet1.niehs.nih.gov/docs/1999/107/p495-500morimoto/abstract.html

To investigate which parameters are stimulated by mineral fibers and whether cigarette smoke enhanced a fiber-induced response, we examined the level of cytokine mRNA from alveolar macrophages (AMs) and lungs of rats exposed to mineral fibers and cigarette smoke in vivo. Male Wistar rats were given a single intratracheal instillation of 2 mg of Union Internationale Contre le Cancer chrysotile or refractory ceramic fiber (RF1). The animals then inhaled a side stream of smoke 5 days per week for 4 weeks. The expression of manganese superoxide dismutase, inducible nitric oxide synthase (iNOS), basic fibroblast growth factor (bFGF), interleukin-1α (IL-1α), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα) mRNA from lipopolysaccharide-stimulated AMs and lungs of rats exposed to mineral fibers and/or cigarette smoke were assessed using semiquantitative reverse-transcriptase polymerase chain reaction. Exposure only to cigarette smoke increased in IL-10 mRNA levels in AMs. Chrysotile stimulated the expression of IL-10, TNFα, and IL-6 in AMs, and the expression of bFGF in lungs. RF1 resulted in increased expression of IL-10 and TNFα in AMs. Cigarette smoke stimulated the expression of inos in AMs and IL-6 and bFGF in lungs treated with chrysotile; IL-1α in AMs and bFGF in lungs did the same in lungs with RF1. Among these cytokines, message levels of IL-10, IL-6, and bFGF were increased in rats stimulated with mineral fibers, and the stimulating effects of mineral fibers were enhanced by cigarette smoke. Therefore, IL-1α, IL-6, and bFGF would be the possible parameters of the lung remodeling induced by mineral fibers. Key words: alveolar macrophage, bFGF, ceramic fiber, chrysotile, IL-6, IL-10, inos, lung, Mn-SOD, TNF, Environ Health Perspect 107:495-500 (1999). [Online 7 May 1999] http://ehpnet1.niehs.nih.gov/docs/1999/107/p495-500morimoto/abstract.html

For chrysotile, the geometric mean diameter [standard deviation (SD)] and the geometric mean length were 0.085 (1.4) μm and 0.7 (0.19) μm, respectively (2). For RF1, the geometric mean diameter (SD) and the geometric mean length were 0.77 (2.53) μm and 12.0 (2.36) μm, respectively. RF1 was provided by the Japan Fibrous Material Research Association (Tokyo, Japan).

Experimental design. Male Wistar rats (10 weeks old) were used in this study, and 30 rats were divided into six groups (saline, chrysotile, RF1, cigarette smoke, chrysotile + cigarette smoke, and RF1 + cigarette smoke). Either saline, chrysotile suspension, or RF1 suspension (2 mg/0.2 mL) was administered to the rats intratracheally. The rats were housed in an exposure chamber and were exposed to the smoke of 20 cigarettes (Japan Tobacco, Tokyo, Japan) for 4 hr/day, 5 days/week, for 4 weeks. The mass concentration of cigarette smoke was 10 mg/m3 and was measured gravimetrically at daily intervals by the suction of air through a glass filter. The CO concentration was 79 ppm, which is below the concentration that induces specific biological effects. The chamber volume was 100 L, and the flow rate in the chamber was 50 L/min.

Bronchoalveolar lavage and preparation. After exposure, each animal was anesthetized by an intraperitoneal injection of pentobarbital. Bronchoalveolar lavage (BAL) was performed on the left lung. Cells recovered by BAL were suspended in RPMI-1640 medium, then placed on tissue culture plates and allowed to attach for 1 hr at 37°C (8). Nonadherent cells were removed by one cycle of washing with RPMI, and adherent cells were supplied with an RPMI-1640 medium.
containing 10% fetal bovine serum. They were subjected to a final concentration of $1 \times 10^{5}$ cells/mL and stimulated with 10 mg/mL lipopolysaccharide (Sigma, St. Louis, MO). The AMs were cultured for 2 and 6 hr on cell culture plates at 37°C in the CO2 incubator. After incubation, mRNA in AMs was extracted using a Quick Prep kit (Pharmacia Biotech, Uppsala, Sweden). Total RNA from the lungs was prepared in the presence of guanidium thiocyanate (9).

Preparation of RNA, cDNA synthesis, and PCR. Total RNA (0.5 µg) was used for the synthesis of single-strand cDNA using Moloney murine leukemia virus-derived reverse transcriptase (Perkin Elmer, Norwalk, CT). An equal amount of cDNA from each sample, standardized to give identical signals on gel following amplification with β-actin primer, was amplified by specific primers for each gene (Table 1) (10–16). The amplification was performed using 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 cytokines and β-actin genes.

Detection of the fragments amplified by PCR was made by electrophoresis on a 2% agarose gel. PCR products were resolved using gel electrophoresis and visualized by ethidium bromide staining. The gel was photographed with Polaroid Type 665 positive/negative film (Polaroid Corp., Cambridge, MA) 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 using National Institute of Health image 1.55 software (provided by Wane Rasband, National Institutes of Health, Bethesda, MD). The ratio of each specific gene product to the β-actin product was used for the analysis.

**Quantitative analysis.** To quantify the transcriptional level of cytokine mRNA, the following analysis was used: IL-1α samples were coamplified with 1.10β-actin-specific primers as an internal standard (Figure 1). Aliquots containing cDNA were subjected to 26, 29, 32, 35, or 38 cycles of amplification under identical conditions as in protocols. The amounts of density recovered from the excised bands were plotted as a function of the number of cycles. The rates of amplification were exponential between 29 and 35 cycles for both templates. The number of thermocycles used allowed quantitation without saturation. The data were normalized to represent equivalent RNA loading based on the density of β-actin at the appropriate cycle of both genes (7). We compared the data of 2- and 6-hr incubation of cytokines in AMs and presented the one that showed more gene expression.

**Statistical analysis.** Values were expressed as the mean ± 1 SD. The difference between values was assessed using a Mann-Whitney U-test.

**Histopathology.** Lungs were fixed in 10% buffered formalin. Embedded samples were then sectioned and stained with hematoxylin and eosin.

**Results**

**Bronchoalveolar lavage.** An increased number of total cells was recovered from rats exposed to mineral fibers and/or cigarette smoke than from saline-exposed rats (cigarette 6.4 ± 3.4 × 10³ cells; chrysotile 5.6 ± 1.9 × 10³ cells; RF1 5.5 ± 1.9 × 10³ cells; chrysotile + cigarette smoke 5.7 ± 1.5 × 10³ cells; RF1 + cigarette smoke 9.1 ± 1.6 × 10³ cells; control 4.4 ± 1.2 × 10³ cells; Table 2). AMs accounted for >95% of the cells in all groups.

**Expression of IL-1a, TNFα, and IL-6 mRNA.** In comparison with the control (saline) group, levels of IL-1α mRNA in AMs increased significantly in groups exposed to cigarette smoke, chrysotile, chrysotile + cigarette smoke, RF1, and RF1 + cigarette smoke (Figure 2A). IL-1α mRNA in the group exposed to RF1 + cigarette smoke was maximally expressed in all groups, but it was not significant compared to cigarette smoke- or RF1-exposed group. Elevation in the gene expression of IL-1α mRNA was not observed in rat lungs exposed to mineral fibers and/or cigarette smoke (Figure 2B).

A significant increase in TNFα mRNA in AMs was obtained in chrysotile- or

**Table 1. Oligonucleotides of primers of seven target genes.**

| mRNA species | mRNA | Cycle of PCR product, bp |
|--------------|------|-------------------------|
|              |      | Lung | AMs | bp  |
| IL-1α        | sense | 30   | 32  | 295 |
|              | antisense | 38   | 32  | 623 |
| IL-6         | sense | 40   | 40  | 614 |
|              | antisense | 37   | 40  | 696 |
| Mn-SOD       | sense | 28   | 40  | 383 |
|              | antisense | 36   | 40  | 260 |
| bFGF         | sense | 26-28 | 32-34 | 357 |

**Abbreviations:** AMs, alveolar macrophages; bFGF, basic fibroblast growth factor; IL-1α, interleukin-1α; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; Mn-SOD, manganese superoxide dismutase; PCR, polymerase chain reaction; TNFα, tumor necrosis factor-α.

**Figure 1.** Gene expression of interleukin-1α (IL-1α) mRNA in alveolar macrophages of rats exposed to chrysotile or saline in a cycle-dependent fashion. (A) Exposure to saline. (B) Exposure to chrysotile.
RF1-exposed rats as compared to control rats, but not in cigarette smoke-exposed rats (Figure 3A). Mineral fibers and cigarette smoke did not have combined effects on message levels of TNFα in AMs. TNFα mRNA in the lung was increased in chrysotile-exposed rats. Similar to chrysotile, RF1 tended to increase in the gene expression of TNFα mRNA; however, there were no significant differences in the levels of TNFα mRNA when compared to control rats (Figure 3B).

Chrysotile-only exposure induced increases in IL-6 mRNA levels in AMs (Figure 4A). Elevation of IL-6 mRNA was not increased in rat AMs exposed to RF1 and/or cigarette smoke. Exposure to cigarette smoke or mineral fibers did not induce an increase in IL-6 levels in the lung; however, exposure to chrysotile and cigarette smoke had a synergistic effect on the expression of IL-6 mRNA (Figure 4B).

Expression of iNOS and Mn-SOD mRNA. In rats exposed to cigarette smoke or mineral fibers, mRNA levels for iNOS in AMs were not increased (Figure 5A). On the other hand, mRNA levels for iNOS among AMs from rats treated with mineral fibers and cigarette smoke increased significantly when compared with control rats. Levels of iNOS mRNA declined markedly in cigarette smoke-exposed rat lungs (Figure 5B). Exposure to mineral fibers tended to induce an increase in iNOS mRNA; however, differences between mineral fibers and control were not significant.

Table 2. Total cell counts of bronchoalveolar lavage fluid in rats exposed to mineral fibers and/or cigarette smoke in vivo.

| Total cell counts (x 10^5) |
|---------------------------|
| Control          | 4.4 (1.2) |
| Cigarette        | 6.4 (3.4) |
| Chrysotile       | 5.6 (1.9) |
| RF1             | 5.5 (1.9) |
| Chrysotile + cigarette smoke | 5.7 (1.5) |
| RF1 + cigarette smoke | 9.1 (1.6) |

RF1, refractory ceramic fiber.

Figure 3. Effects of mineral fibers and cigarette smoke on the expression of tumor necrosis factor α (TNFα) mRNA. (A) Alveolar macrophages incubated for 2 hr. (B) Lung. Abbreviations: NS, not significant; RF1, refractory ceramic fiber. Results are expressed in ratio of TNFα to β-actin (mean ± standard error). *p<0.05 and **p<0.01 significantly different from a control (saline-exposed) group.

Figure 4. Effects of mineral fibers and cigarette smoke on the expression of interleukin-6 (IL-6) mRNA. (A) Alveolar macrophages incubated for 6 hr. (B) Lung. Abbreviations: NS, not significant; RF1, refractory ceramic fiber. Results are expressed in ratio of IL-6 to β-actin (mean ± standard error). *p<0.05 significantly different from a control (saline-exposed) group.

Figure 5. Effects of mineral fibers and cigarette smoke on the expression of inducible nitric oxide synthase (iNOS) mRNA. (A) Alveolar macrophages incubated for 2 hr. (B) Lung. Abbreviations: NS, not significant; RF1, refractory ceramic fiber. Results are expressed in ratio of iNOS to β-actin (mean ± standard error). *p<0.05 and **p<0.01 significantly different from a control (saline-exposed) group.
Figure 6. Effects of mineral fibers and cigarette smoke on the expression of manganese superoxide dismutase (Mn-SOD) mRNA. (A) Alveolar macrophages incubated for 6 hr. (B) Lung. Abbreviations: NS, not significant; RF1, refractory ceramic fiber. Results are expressed in ratio of Mn-SOD to β-actin (mean ± standard error).

Figure 7. Effects of mineral fibers and cigarette smoke on the expression of basic fibroblast growth factor (bFGF) mRNA. (A) Alveolar macrophages incubated for 2 and 6 hr. (B) Lung. Abbreviations: NS, not significant; RF1, refractory ceramic fiber. Results are expressed in ratio of bFGF to β-actin (mean ± standard error).

* p<0.05 and ** p<0.01 significantly different from a control (saline-exposed) group.

No significant changes in levels of Mn-SOD mRNA in AMs and lungs exposed to mineral fibers and/or cigarette smoke was observed (Figure 6).

Expression of bFGF mRNA. The message of bFGF in AMs was not observed in all groups, although the cycle of PCR was up to 40 (Figure 7A). Chrysotile induced an increase in bFGF mRNA in the lungs, but cigarette smoke or RF1 did not. The combined effect of mineral fibers and cigarette smoke on the message level of bFGF was significant (Figure 7B).

Pathological findings. Foreign body giant cells and mononuclear cells around bronchioles were observed in groups exposed to chrysotile and chrysotile + cigarette smoke (Figure 8). In the other four groups, definite pathological changes were not observed.

Discussion

We set the recovery time after the intratracheal instillation of mineral fibers at 4 weeks. Unlike the inhalation findings, some reports showed that nonspecific responses at the acute phase were seen after the intratracheal injection. Some investigators (17,18)
showed that saline injection in BAL caused a neutrophil recruitment by the lower respiratory tract in 72 hr, and that an increase in neutrophils and AMs recovered from bronchoalveolar lavage fluid were seen in the first week after injecting water alone. We previously reported that inhalation for one day of cigarette smoke transiently enhanced the ability of mineral fibers to stimulate AMs to produce TNF in rats (19), and later reported that no specific changes were found for AMs in rats with cigarette smoke inhalation for 4 weeks (20). To avoid the initial influence of acute response, we exposed rats to cigarette smoke for 4 weeks in this experiment. The time line for gene expression of cytokines in AMs and lungs following initial asbestos exposure is now under investigation.

Many previous reports examined protein level or gene expression of proinflammatory cytokines and growth factors that were stimulated by mineral fibers in lungs or in AMs (5,6,19–21). However, the mineral fibers used for experimental studies are not always the same among laboratories. The physiochemical properties of mineral fibers affect biological response in cells and lungs. For instance, the size of mineral fibers causes different responses even if they are the same fibers. We previously reported a difference in the production of TNFα in AMs treated with long and short mineral fibers (22). Numerous in vitro studies have also shown that long fibers induced greater cytotoxicities than short fibers in vitro (2,23,24). In this experiment each cytokine was examined from the same samples using RT-PCR under the identical experimental process, and it is useful to estimate the sensitivity and selectivity of parameters in lung remodeling induced by mineral fibers. In this experiment, these parameters were examined on the following points: whether the gene expression of cytokine mRNA is induced by mineral fibers and whether mRNA levels for cytokines are additively or synergistically increased by combined exposure to cigarette smoke and mineral fibers.

Mineral fibers increased in gene expression of TNFα, IL-1, IL-6, and iNOS mRNA in AMs and bFGF mRNA in lungs. These proinflammatory cytokine mRNA or proteins have been detected in the AMs or lungs of patients with idiopathic pulmonary fibrosis (25) and in animal lungs with pulmonary fibrosis (26). Blackford et al. (27) reported that intratracheal instillation of silica up regulated the gene expression of iNOS mRNA in AMs. These data were accordance with our data. However, mRNA expression of Mn-SOD was not increased in AMs and lung tissue from rats exposed to mineral fibers. Quinlan et al. (28) reported that the message level of Mn-SOD in rat lungs inhaling crocidolite peaked at 3–9 days and decreased toward control level thereafter. In our experiments, levels of Mn–SOD mRNA might return from peak to normal because recovery time was 4 weeks.

Cigarette smoke is thought to increase the incidence of asbestos-induced disease (3,4). It is generally accepted that smoking is associated with a higher risk of carcinoma of the lung (3); however, its association with asbestosis is controversial, and a majority of studies showed a positive interaction between smoking and asbestosis (4). Studies suggest that smoking and asbestos act in combination in the development of fibrosis. In our experiments, cigarette smoke and mineral fibers have combined effects of gene expression of IL-1α, iNOS, bFGF, and IL-6 mRNA (Figure 9).

Figure 9. Ethidium bromide staining of PCR products separated in 2% agarose gel. Abbreviations: bFGF, basic fibroblast growth factor; IL-1α, interleukin-1α; IL-6, interleukin-6; M, DNA marker (λX174/HaeIII); Mn-SOD, manganese superoxide dismutase; iNOS, inducible nitric oxide synthase; RCF, refractory ceramic fiber; TNFα, tumor necrosis factor α.
have been few experimental studies to investigate the combined effects of cigarette smoke and mineral fibers on the gene expression of proinflammatory cytokines. We previously reported that the cigarette smoke and mineral fibers had synergistic effects on matrix metalloproteinases (MMPs) in rat lungs (29). IL-1 upregulates the production of MMPs (30), and the increase in gene expression of MMPs in rats lungs exposed by two agents may be affected by IL-1. Jackson et al. (31) reported that cigarette smoke and asbestos increased DNA damage synergistically and suggested that this synergism might involve hydroxyl radical production. Free radical formation including nitric oxide radical and peroxynitrite anion through iNOS (27) might be related to lung injury induced by asbestos and cigarette smoke. The gene expression of proinflammatory cytokines, iNOS, and bFGF might be representative cytokines of inflammation, pulmonary damage by oxidants, and a neovascularization, respectively (4,27,32). Therefore, a change in gene expression of these factors, which play a key role in the inflammatory process, suggested that these factors might contribute to lung remodeling induced by cigarette smoke and mineral fibers. The lungs in rats exposed to chrysotile or crocidolite + cigarette smoke showed inflammatory cells around the bronchioles without a fibrotic form. These pathological findings may support that two agents have a combined effect on cytokines which are related to inflammation or lung injury.

Taken together, IL-1α, iNOS, and bFGF mRNA were up regulated by exposure to mineral fibers, and were affected by exposure to mineral fibers and cigarette smoke in combination. Therefore, these factors were thought to be possible parameters for a risk assessment of mineral fibers.

Summary
We examined cytokine mRNA levels from AMs and the lungs of rats exposed to mineral fibers and/or cigarette smoke in vivo. Message levels of IL-1α, iNOS, and bFGF were increased by exposure to mineral fibers and enhanced by combined exposure to mineral fibers and cigarette smoke. These data suggested that IL-1α, iNOS, and bFGF would be the possible parameters of the lung remodeling induced by mineral fibers.

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