Early Mesothelial Cell Proliferation after Asbestos Exposure: In Vivo and In Vitro Studies

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There is some evidence that proliferation of pleural mesothelial cells (MC) occurs soon after deposition of asbestos fibers. To study this effect, we instilled a single dose of 0.1 mg crocidolite into the lungs of mice for 1 and 6 weeks and counted labeled nuclei after 3H-thymidine (3HT) injection. Short fibers (<1 μm) induced little change in the lung; they were mostly phagocytized and had a minimal effect on MC labeling. Long fibers up to 20 μm damaged the bronchiolar epithelium and were incorporated into connective tissue. Increased 3HT uptake was seen in fibroblasts and epithelial cells and also in MC, which peaked at 2% labeled at 1 week compared to near 0% labeling in controls. No fibers were found in or near labeled MC, which suggested that a cytokine generated in the lung during the early response phase might induce MC proliferation. To look for a cytokine effect in vitro, we instilled asbestos into rat lungs and, after 1 and 6 weeks, bronchoalveolar and pleural lavage fluids as well as macrophages were collected. Alveolar macrophages contained fibers, but pleural macrophages (PM) did not. After short-term culture, macrophage supernatants and the lavage fluids were tested on rat lung MC in culture. At 1 week, PM secreted growth factors for MC, and the mitogenic effect was more pronounced with lavage fluids. No effects on MC were found using material prepared 6 weeks after asbestos.

The early MC growth increase was not blocked by antibodies to cytokines, such as platelet-derived growth factor, fibroblast growth factors, or tumor necrosis factor, but was inhibited by anti-keratinocyte growth factor (anti-KGF). The results show that an early growth phase of MC after asbestos exposure appears unrelated to particle translocation to the pleura but is associated with cytokine release, most likely KGF, by lung cells. — Environ Health Perspect 105(Suppl 5):1205-1208 (1997)

Key words: lung, mesothelial cells, asbestos, pleural disease

Introduction

Deposition of asbestos fibers in the lung is associated with pathologic changes in the small airways and the production of pulmonary fibrosis (1,2). To a lesser extent, cells at the pleural surface are also affected and pleural fibrosis, plaques, or mesotheliomas may result (3). The precise mechanism for inducing mesothelial cell (MC) proliferation after fiber deposition in the airways is not known but is widely thought to involve fiber penetration of lung tissue and translocation, probably by lymphatics, to the pleura (4). Although it seems likely that considerable time would be required to accumulate enough fibers at the pleural surface to induce cell injury and mesothelial cell growth, evidence indicates that these cells divide soon after one inhalation exposure to chrysotile asbestos (5). Similar results have been reported after a single instillation of amosite or crocidolite asbestos (6,7).

In this study we examine differential responses of lung MC after long- or short-fiber deposition in the lung, to determine if fiber translocation to the pleura is a requirement for MC injury and/or growth. In this system we compare an exposure that results in fibrosis (long fibers) with a comparable exposure to short fibers that does not induce fibrosis (8,9). In a second stage we instilled a single dose of mixed-length crocidolite asbestos to rats for 1 and 6 weeks. Pleural and alveolar macrophages (PLM, AM), as well as lavage fluids, were collected from these lungs to test in vitro if growth factors for MC are present and to relate results to the localization of asbestos fibers.

Materials and Methods

In Vivo Experiments

A standard UICC sample of crocidolite asbestos was separated into long- and short-fiber preparations by a sedimentation method (8). Long fibers had a mean length of 24.4 μm ± 0.5, with 88% > 2.5 μm. Short fibers measured 0.6 μm ± 0.1 (8,9). Swiss Webster mice were instilled intratracheally with 0.1 mg of either sample in 0.1 ml water and killed in groups of four up to 6 weeks later. Each animal received 2 μCi/g tritiated thymidine (3HT) 1 hr before death. Controls received only water. The lungs were inflated with fixative and processed for methacrylate embedding and sectioning at 0.75 μm. Sections from three random blocks were prepared for autoradiography (6). The overall percentage of 3HT-labeled lung parenchymal cells was found after counting labeled cells in 3000 per animal. Then the labeling index for MC was calculated after identifying and counting 1000 MC per animal. Samples of lung were taken to measure hydroxyproline (HYP) content as a biochemical index of fibrosis (6,9).

To obtain enough cells for in vitro studies, 0.5 mg of the UICC crocidolite asbestos was injected intratracheally in 0.5 ml water to 150-g male rats. They were killed in groups of six at 1 week (the time of maximal MC proliferation) and 6 weeks (when MC proliferation is over); each received 1 μCi/g 3HT. At sacrifice the pleural cavity was lavaged 3 times with 5 ml saline each time and the pleural lavage fluids (PLLF) were pooled per rat. Similarly, a tracheotomy was performed and lungs lavaged 3 times with 5 ml saline. The bronchoalveolar lavage fluids (BALF) were also pooled per rat. For each fluid a sample was used to count cells by hemocytometer. A cytospin preparation was made to obtain

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Abbreviations used: AM, alveolar macrophage(s); BALF, bronchoalveolar lavage fluid(s); DMEM, Dulbecco's modified essential medium; EGF, epidermal growth factor; FGFa, FGFb, fibroblast growth factor(s); 3HT, tritiated thymidine; KGF, FGF-7, keratinocyte growth factor; MC, mesothelial cell(s); PDGF, platelet-derived growth factor; PLLF, pleural lavage fluid(s); PLM, pleural macrophage(s); PM, pleural macrophage(s); TGF-α, TGF-β, transforming growth factors; TNF-α, tumor necrosis factor.
differential counts of macrophages, polymorphonuclear leukocytes, and other cells before calculating the total number of each cell type. On lightly stained cytospin preparations, phagocytized fibers could be identified by oil immersion microscopy. The percentages of PLM and AM that contained any particles were determined after counting 1000 of each cell type per animal at each time. A sample of pleural cells was also centrifuged to a pellet that was then processed and embedded for electron microscopy.

After lavage, the bronchus leading to the right lung was clamped; this lung was used to measure HYP. The left lung was processed for light microscopy and as described above, the percentage of 3HT-labeled MC was determined.

**In Vitro Experiments**

Each lavage fluid was spun down and the liquid phase frozen for later testing, as described below. The cells were resuspended in culture medium and added to a plastic dish for 2 hr. The supernatant with nonadherent cells was poured off and macrophages were scraped off and counted. The AM and PLM were resuspended in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS) and added to 10^6 cells/ml to 35-mm plastic dishes for 2 hr to allow adherence. The media was switched to serum-free DMEM and the cells were cultured for 1 day. Supernatants from each macrophage preparation were then collected and frozen.

The capacity for AM and PLM secretions and for BALF or PLLF to stimulate MC proliferation was then assessed on rat lung MC obtained from the American Type Culture Collection; these were subcultured for use between passages 15 and 22. Cells were prepared fresh for each experimental run using macrophage and lavage fluids from one exposed and one control rat. The MC were seeded in 24-well plates at 2 x 10^4 cells per well in DMEM containing 0.5% FBS. In sets of four wells, AM and PLM supernatants from exposed and control rats, and BALF and PLLF from the same rats (see above) were added at 10 and 25% by volume. The lavage fluids were concentrated 4-fold by centrifugation using a microconcentrating membrane with a 30-kD cutoff. The cells were maintained 2 days, with 3HT at 0.1 µCi/ml added for the final 24 hr. Each well was rinsed twice, then the cells removed by Triton X-100 before scintillation counting. The experiment was repeated with each of six sets of samples from rats exposed to asbestos for 1 and 6 weeks. The control scintillation counts for MC in each experiment were taken as 100%, and the thymidine uptakes using the various test samples were calculated as percentages of the control value.

**Cytokine Studies**

In attempts to identify a specific growth factor when a proliferative response was observed, we tested monoclonal or neutralizing antibodies (R and D Systems, Minneapolis, MN) previously shown to block the following rat cytokines: tumor necrosis factor (TNF-α), transforming growth factors (TGF-α and TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factors (FGFa and FGFb) and keratinocyte growth factors (KGF or FGF-7). Cultures were set up as described above with different doses of antibodies added. Effects on growth were quantitated by counting 3HT uptake in 24 hr in these cultures and comparing the results with those obtained from cultures without antibodies added.

**Results**

**In Vivo**

Lung sections, after instillation of short fibers of asbestos, showed an increase in AM, and most fibers appeared to be engulfed and cleared. There was little change in lung structure and no fibrosis was found morphologically or biochemically. Long fibers appeared to induce a small increase in AM, but these fibers injured the bronchiolar epithelium. Later, fibers were incorporated into the connective tissue and granulomas formed, with giant cells containing fibers. The peribronchiolar areas became more fibrotic with time and fibrosis was confirmed biochemically.

From autoradiographs, it was determined that the overall labeling of lung cells was increased after long-fiber exposure and the percentage of labeled cells reached 3% of all cells at 1 week (Figure 1). Although most lung cell types including epithelial cells showed increased DNA synthesis at this time, the later increase in labeling was due largely to fibroblasts. Little change in lung cell labeling was found in response to short fibers. MC only were then counted (Figure 2). There was a brief but small increase in label 3 days after exposure to short fibers but at other times values were at the control level of near zero. After exposure to long fibers, MC label reached a peak greater than 2% of cells at 1 week and the value did not return to normal until 4 weeks (Figures 2,3). Both AM and PLM were significantly higher in the lavage fluids of rats at 1 and 6 weeks postasbestos instillation (Figure 4). In the cytospin preparations, fibers frequently were found in AM (58.3% of cells at 1 week, 28.5% at 6 weeks) but light microscopy revealed that none of the PLM contained asbestos. Electron microscopy also revealed no fibers in cell pellets of PLM. Autoradiographs of lung sections from these rats were examined and MC labeling counted. The results were similar to those found in mice (Figure 2); increased labeling of MC was found at 1 week but not at 6 weeks postinstillation (data not shown).

**In Vitro**

Samples of macrophage supernatants and lavage fluids were tested for growth of quiescent rat lung mesothelial cells. Samples from control animals did not affect 3HT uptake by MC. When samples that were prepared 1 week after asbestos instillation were tested, the two concentrations of AM supernatant had little effect, whereas at 25% of volume, PLM supernatant stimulated MC growth by
about 40% (Figure 5). Greater increases in MC incorporation were induced when lavage fluids were tested; in particular, PLLF caused almost a doubling of \(^{3}HT\) uptake by MC (Figure 5). In contrast to these results, samples of lavage fluids and macrophage supernatants prepared from rats 6 weeks after asbestos instillation had no effect on the \(^{3}HT\) uptake by MC (data not shown).

Antibodies to various cytokines were used in attempts to block the above increase in MC growth found when cells were cultured with pleural lavage fluid from rats exposed for 1 week. No reduction in the enhanced MC DNA synthesis was found with various dose levels of anti-PDGF, anti-FGFα or anti-FGFβ, anti-TGF-β, or anti-TNF-α and only a small inhibition (15%) was produced by anti-EGF and TGFα (data not shown). The greatest inhibition was found when anti-KGF was added to mesothelial cultures (Figure 6). This antibody blocked the observed growth promotion effect of PLLF. To confirm the role of KGF, we added pure recombinant cytokine to normal resting rat MC and found increased \(^{3}HT\) uptake at 1 day (Figure 6).

**Discussion**

Fibrotic reactions at the pleura are associated with occupational exposure to asbestos and a low incidence of mesothelioma can also be found (1, 3). These changes have been attributed largely to long fibers of asbestos, and it has been suggested that a substantial number of these fibers must reach the pleura to induce such responses (4). However, a proliferative response of mesothelial cells has been observed a few days after a single inhalation of chrysotile asbestos (5), and in the present study, we show similar results after instilling long-fiber crocidolite. Short fibers of the same material were deposited in alveoli and had easier access to peripheral lung, but phagocytosis and clearance occurred with little or no lung injury and no subsequent cell proliferation or fibrosis. Long fibers elicit very little macrophagic response (8), but such fibers do injure the epithelium in the bronchoalveolar duct regions and reach the interstitium. At this time we observed that lung cell proliferation was under way and included a 10-fold increase in MC labeling; however, no fibers were seen by light or electron microscopy in the PLM population. Because of the early timing of this response (1 week) with no fibers observed, it is postulated that MC are reacting to a cytokine generated in the early response phase after lung cell injury induced by the long fibers. In support of this hypothesis is the finding that in other models of acute lung injury such as that induced by silica or bleomycin there also are early phases of MC proliferation (10). More recently, when examining the effects of amosite instillation on rat mesothelial cells, Sekhon et al. (7) found that this type of asbestos induced sustained MC proliferation in the absence of local accumulation of asbestos fibers.
We then examined cytokine production and secretion in the lung after asbestos exposure. Since macrophages are key elements in handling deposited particles, supernatants of cultured AM and PLM were tested for stimulatory effects on MC growth. Although some increase in 3HT uptake was found, the growth effect was not very pronounced for material prepared 1 week after asbestos exposure when MC growth is maximal in vivo. No effect was seen using supernatants prepared at 6 weeks when the number of lavaged AM and PLM numbers remained high, but MC labeling was normal in vivo. These supernatants stimulated lung fibroblast growth at both times (data not shown), so it appears that the macrophage-derived cytokines responsible for fibroblast growth after asbestos (11,12) had little effect on MC cells in this system.

When lavage fluids were tested, BALF, and to a greater extent PLLF, stimulated 3HT uptake by MC in vitro, again only when using samples prepared at 1 week after asbestos treatment. The magnitude of this effect suggests that any macrophage-derived cytokine may be supplemented by growth factor(s) from another source that is present in alveolar and pleural spaces, and that acts on the mesothelial cell population. In attempts to identify the factor responsible, we used blocking antibodies to well-known cytokines. MC are known to have receptors for PDGF, TNF-α, TGF-α and -β (13–15), and when antibodies to these molecules were added to the rat MC in the presence of PLFF, no inhibition of the growth effect was observed. This is further evidence that the cytokine effect on MC cells is different from that which stimulates fibroblast growth.

Mesothelial cells have properties of both fibroblastic and epithelial cells, as seen by the expression of both vimentin and cytokeratin. Since lung epithelial cells are known to respond to KGF (16), we tested this cytokine on the rat MC in culture and found evidence of growth stimulation. In addition, anti-KGF blocked the PLFF-induced increase in MC growth. Since KGF is a fibroblast-derived cytokine that acts exclusively on epithelial cells (16), its production could be upregulated at an early stage of the reaction to asbestos when there is a phase of epithelial injury and particle translocation to the interstitium (8). In vivo, epithelial injury is followed rapidly by a reparative burst of proliferation that coincides with the increase in MC proliferation seen here. It is possible that after initial epithelial cell injury a cytokine such as KGF, secreted to stimulate repair, may diffuse sufficiently to stimulate the MC population. The MC proliferative response is transient, as is the phase of epithelial injury and the regenerative response after a single exposure, but repeated exposures may induce prolonged epithelial injury, cytokine production, and mesothelial proliferation without necessarily involving direct action of asbestos fibers on these cells at the pleura.

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