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Evidence Supporting a Role for Active Oxygen Species in Asbestos-Induced Toxicity and Lung Disease

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Asbestos is an important occupational and environmental toxicant that affects several cell types in the respiratory tract. In an effort to understand how asbestos causes cell injury and/or altered proliferation and differentiation of cells, this laboratory has focused on reactive oxygen species as mediators of asbestos-induced biological effects. A compendium of experimental results reported by this laboratory and others supports this hypothesis. For example, scavengers of reactive oxygen metabolites and iron chelators (i.e., desferroxamine) prevent cytotoxicity after addition of asbestos to a variety of cell lines and macrophages *in vitro*. DNA strand breakage associated with toxicity of crocidolite asbestos in C3H10T½ cells also is ameliorated with use of desferroxamine. All types of asbestos cause lipid peroxidation in mammalian cells and artificial membranes, a phenomenon that can be prevented by removal of catalytic iron. Last, asbestos causes generation of active oxygen species after interaction with leukocytes or by reduction of oxygen on the surface of the fibers.

Introduction

Occupational exposure to asbestos is associated with the developmment of brochogenic carcinoma, mesothelioma, and pulmonary fibrosis (*1*). To elucidate mechanisms contributing to the pathogenesis of asbestos-induced diseases, we and others have focused on determining how asbestos fibers damage or alter the metabolism of cells of the respiratory tract *in vitro* (*2*).

"Asbestos" is a collective term for a family of hydrated silicates of a >3:1 aspect (length:diameter) ratio. Chrysotile ($3MgO \cdot 2SiO_2 \cdot 2H_2O$), a pliable serpentine fiber that accounts for most of the world's production of asbestos, is mined extensively in the northern hemisphere, whereas crocidolite ($Na_2O \cdot Fe_2O_3 \cdot FeO \cdot 8SiO_2 \cdot H_2O$), a more rodlike and less biodegradable fiber in the lung, is of less industrial importance. In comparison to chrysotile, crocidolite is generally more pathogenic in man (*1*). Other amphibole fibers include tremolite, anthophyllite, and amosite asbestos.

Although various types of asbestos differ from each other both chemically and physically, they are in general more toxic to mammalian cells than a variety of inert particulates such as titanium oxides or carbon particles. Factors such as size (*3*), surface charge (*4*), and surface area (*5*) have been cited as important to asbestos-induced cytotoxicity, although how asbestos damages cells precisely has remained a mystery until quite recently. In 1983, we observed that antioxidants including superoxide dismutase (SOD) and dimethylthiourea, a scavenger of the hydroxyl ($OH^\cdot$) radical, could prevent cell death upon addition of crocidolite and long (> 10 μm) chrysotile fibers to cultures of tracheobronchial epithelial cells (*6*). This observation led to the hypothesis that active oxygen metabolites are involved in asbestos-induced cell damage. Because reactive oxygen can cause lipid peroxidation and damage to DNA and other macromolecules, it is conceivable that they are important intermediates in asbestos-associated cell injury and disease. This presentation summarizes the evidence from our laboratory and others that supports these concepts.

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Results and Discussions

Prevention of Asbestos-Induced Cytotoxicity by Antioxidants

Cells derived from a number of sources can be protected from asbestos-associated cytotoxicity by simultaneous addition of scavengers of active oxygen species to cultures. For example, release of ^{75}selenium from tracheal epithelial cells prelabeled with ^{75}selenomethionine and exposed subsequently to crocidolite asbestos or long (> 10 μm length) fibers of chrysotile asbestos (Manville Corp.) is inhibited by addition of SOD or scavengers (mannitol, dimethylthiourea, sodium benzoate) of the $OH^\cdot$ radical, but not by 1,4-diazo-

bicyclo(2.2.2)octane, a scavenger of singlet oxygen (O_2^1) (*8*). Crocidolite-induced cell death, as measured by uptake in vital dyes, also can be prevented in a dosage-dependent fashion by introduction of SOD, catalase, or dimethylthiourea to rat lung fibroblasts or alveolar macrophages *in vitro* (*9*). If fibers are pretreated with desferroxamine, an iron chelator, crocidolite is not as toxic, an observation supporting the concept that iron on the surface of fibers drives the modified Haber-Weiss (Fenton) reaction and results in the production of OH$^\cdot$ from H_2O_2 and O_2^- (*9–11*).

Another piece of evidence supporting the importance of iron in catalyzing reactions that generate active oxygen species comes from the work of Goodglick and Kane (*9*), who showed that in elicited peritoneal macrophages, addition of $FeCl_3$ to titanium dioxide (TiO_2), a nontoxic compound when added alone, increases the toxicity of this particulate. SOD, catalase, and desferroxamine protect against TiO_2 and $FeCl_3$-induced cell death. These investigators also demonstrate that toxicity of crocidolite asbestos is less pronounced in a hypoxic environment.

In contrast to results published by others (*6–10*), studies by Gabrielson and colleagues show no amelioration of cytotoxicity after addition of antioxidants to human mesothelial cells exposed to amosite, a primarily iron-containing asbestos structurally similar to crocidolite (*11*). Although production of oxygen free radicals is detected by electron paramagnetic resonance in cells exposed to menadione, an uncoupler of oxidation-reduction, no increase in production is indicated after exposure of cells to a single cytotoxic amount of amosite. Several factors may account for these negative results. For example, each of the four antioxidants (SOD, reduced glutathione, *N*-acetylcystene and D-α-tocopherol) is examined at only a single concentration. Moreover, in contrast to relatively short-term (< 24 hr) assays used by others (*6–10*), cytotoxicity is evaluated by clonal growth, a test requiring culturing of cells for 8 days. In these experiments, asbestos with and without antioxidants is added 24 hr after initial plating of cells. Because the half-life of scavengers such as SOD is very short, it is conceivable that mesothelial cells are not exposed continuously to these agents.

Lipid Peroxidation by Asbestos

Asbestos, an insoluble particulate, initially interacts with the plasma membrane of mammalian cells before phagocytosis (*12*). At high concentrations, asbestos is cytolytic to red blood cells (RBCs) and mammalian cells in culture, whereas low concentrations of fibers can stimulate cell growth (*2*). One plausible mechanism by which asbestos might damage cells is lipid peroxidation, a process involving oxidation of unsaturated fatty acids. In 1975, Gabor and Anca reported that four types of asbestos result in lipid peroxidation, as documented by an increase of thiobarbituric acid substances, in RBCs (*13*). At identical concentrations, chrysotile, the most hemolytic asbestos, causes the greatest production of lipid peroxides, followed by anthophyllite, amosite, and crocidolite, in decreasing order. Crocidolite also induces a rapid, dose-related increase in lipid peroxidation in isolated rat liver and lung microsomes, a phenomenon attributed to binding of the fibers to microsomes (*14*). Results indicate that as the ratio of fibers to microsomes increases, more phospholipid per milligram of microsomal protein is exposed for oxidation.

The importance of iron as a catalyst for lipid peroxidation has been shown by both Weitzman and Weitberg (*15*) and Turver and Brown (*16*) using phospholipid emulsions and a mammalian cell line (C3H10T½), respectively. In brief, if fibers are either pretreated or added to bioassays simultaneously with desferroxamine, an iron chelator, lipid peroxidation is inhibited. The fact that DNA strand breaks caused by crocidolite in C3H10T½ cells can be ameliorated by similar approaches suggests that iron is important in DNA damage by crocidolite as well (*16*).

In a recent report, Fontecave and colleagues (*17*) describe a dramatic increase in lipid peroxidation by crocidolite or chrysotile when NADPH is added to rat liver microsomes. This synergistic effect is not inhibited by addition of SOD, catalase, mannitol, or desferroxamine, an indication that active oxygen species and iron ions released into solution from the fibers are not involved. However, lipid peroxidation in NADPH-supplemented microsomes is greatly enhanced upon addition of magnetite (Fe_3O_4), a solid iron oxide composed of ferrous and ferric irons. In contract, ferric oxides such as hematite and goethite are unable to either initiate lipid peroxidation or stimulate NADPH-dependent lipid peroxidation. Based on these results, these investigators conclude that a simple iron complex such as Fe(ADP) initiates lipid peroxidation by asbestos. This active complex probably occurs within the fiber, thus it is not readily accessible to chelation by desferroxamine.

Mechanisms of Generation of Active Oxygen Species by Asbestos

Work-to-date shows that asbestos generates reactive metabolites of oxygen by several different mechanisms. One pathway appears to involve the physical interaction of fibers with leukocytes or effector cells of disease. In this regard, opsonized chrysotile or anthophyllite asbestos stimulates chemiluminescence, an indication of increased metabolic activity, in human polymorphonuclear leukocytes *in vitro* (*18*). In contrast, both types of fibers at obviously cytotoxic concentrations suppress chemiluminescence in human peripheral blood monocytes and inhibit subsequent phagocytosis of latex beads by these cell types (*19*). Rodent alveolar and peritoneal macrophages generate O_2^- and H_2O_2 after exposure to crocidolite asbestos *in vitro* (*9,20*), although these responses are minimal in comparison to stimulation with either opsonized zymosan or 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

After IP injection of chrysotile asbestos or *C. parvum* into mice, isolated peritoneal macrophages exhibit increased chemiluminescence (*21*) and release significantly more H_2O_2 and O_2^- after incubation with TPA (*22*). Al-

though addition of exogenous H_2O_2 or xanthine and xanthine oxidase, a reaction mixture generating O_2^-, at concentrations comparable to those produced by asbestos-primed macrophages does not inhibit proliferation of lymphocytes (*22*), H_2O_2 impairs the functional activity of an α_1-protease inhibitor (*23*). Thus, release of oxidants by asbestos-exposed leukocytes might result in proteolytic tissue damage *in vivo*.

We recently examined the physical and chemical properties of asbestos important in generation of O_2^- from both rat and hamster alveolar macrophages by performing a series of experiments using crocidolite asbestos and chemically dissimilar fibers of similar dimension (*20*). Each fiber type and its nonfibrous, chemically similar analog was evaluated over a range of nontoxic concentrations. In comparison to rat macrophages, which generated O_2^- in response to both fibers and particles, hamster macrophages required exposure to opsonized zymosan to demonstrate measurable O_2^- production. Results showed that fibrous dusts (i.e., crocidolite, erionite, Code 100 fiberglass, sepiolite) caused a significant increase in release of O_2^- from rat macrophages as well as enhancement of zymosan-triggered O_2^- from hamster macrophages. In contrast, nonfibrous particles (riebeckite, mordenite, glass) were less active than respective fibers in both species. These observations suggest that the fibrous geometry of minerals is an important factor in eliciting O_2^- release from macrophages.

Studies by Weitzman and Graceffa (*24*), Eberhardt (*25*), and Zalma et al. (*26*) suggest that cell interaction is not required for generation of active oxygen species by asbestos. As shown by spin trapping, all types of asbestos catalyze the generation of $OH^\cdot$ in the presence of H_2O_2 (*24,25*) or an aqueous buffer solution (pH 7.4) (*26*). The redox activity of individual fibers appears to be a function of their Fe^{+2} content, the density of surface active sites accessible to oxygen, the configuration of these sites, and the surface area of the mineral (*26*).

Relevance to Disease

Taken in context, the results of studies described above show that asbestos causes production of active oxygen species from cells of the immune system. Since a variety of fibers (but not their nonfibrous analogs) can induce macrophages to release O_2^-, one might speculate that this mechanism of production of oxygen free radicals involves physical perturbation of the macrophage membrane by fibers, a process that could occur during phagocytosis of fibers (Fig. 1). In comparison to fibers, particles are more easily encompassed by the cell. Fibers also are phagocytized by resident cells of the lung, including epithelial cells and fibroblasts (*7,12*). Although production of oxygen metabolites from these cell types after exposure to asbestos *in vitro* has not been examined in detail, the fact that asbestos-induced cytotoxicity can be prevented by addition of antioxidants to epithelial cells and fibroblasts (*6–8*) in the absence of cocultivation with phagocytes suggests that they also generate active oxygen species.

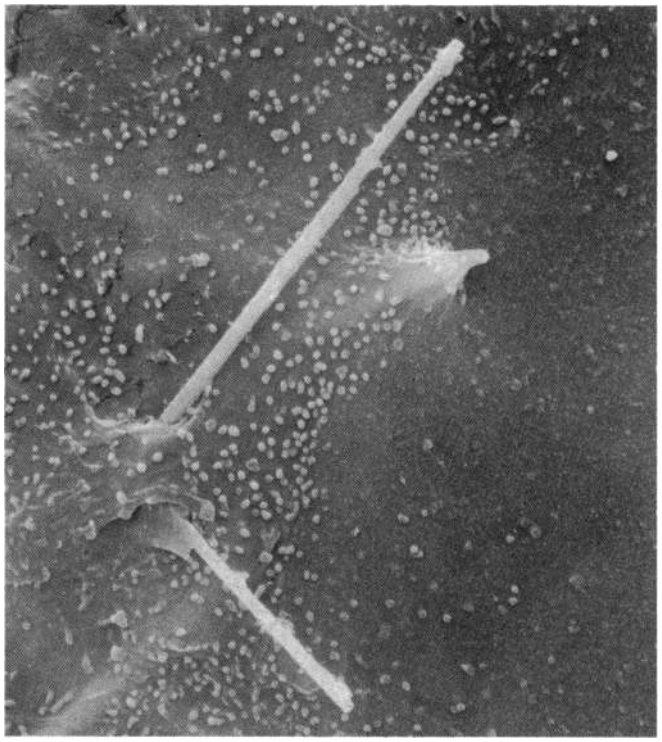

FIGURE 1. Scanning electron micrograph of unsuccessful phagocytosis of a long (> 10 μm) fiber of asbestos by a tracheal epithelial cell in organ culture. This process might result in perturbation of the membrane and production of active oxygen species.

Extracellular fibers and asbestos (ferruginous) bodies in the lung may be other sources of release of oxygen free radicals. Although the formation of the ferruginous body has been viewed in general as a mechanism whereby the reactive surfaces of asbestos are masked, the iron coating these fibers might serve as a catalyst for generation of $OH^\cdot$ and O_2^-. Clearly, the inhibition of asbestos-induced generation of $OH^\cdot$ and O_2^- from H_2O_2 (*24*), cytotoxicity (*6–11*), lipid peroxidation (*15,16*), and DNA breakage (*16*) by desferroxamine indicates that the iron content of asbestos fibers is critical to reactions favoring production of active oxygen species.

Several experiments are necessary to establish a cause-and-effect relationship between active oxygen species and asbestos-associated diseases. First, it is important to demonstrate that active oxygen species produce the same types of biological effects associated with exposure to asbestos both *in vitro* and *in vivo*. Secondly, if oxygen free radicals mediate asbestos-induced diseases, administration of antioxidants should ameliorate asbestosis, bronchogenic carcinoma, and/or mesothelioma in animals. Our current work is proceeding in this direction (*27*).

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