FINAL PERFORMANCE REPORT

Oxygen Free Radicals in Pulmonary Fibrosis

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Marie A. Shatos, Ph.D.

Principal Investigator

Brooke T. Mossman, Ph.D.

(Director of Laboratory)

Department of Pathology

Medical Alumni Building

University of Vermont

Burlington, VT 05405

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SUMMARY OF STUDIES WITH LUNG CELLS, ASBESTOS, AND OXYGEN-FREE RADICALS

My post-graduate training has been as a post-doctoral fellow in the Department of Pathology, University of Vermont and as a co-investigator with Dr. Brooke Mossman in the University of Vermont Specialized Center of Research (SCOR) in Pulmonary Fibrosis. During this time, I have gained considerable experience in lung cell culture, developed antisera to the antioxidant enzymes, superoxide dismutase and glutathione peroxidase and conducted studies which showed that asbestos-induced injury to lung cells in vitro and in vivo may be mediated by oxygen-free radicals. Much of the work completed in the following studies has been done in conjunction with my Pulmonary Fibrosis SERCA Award. Summaries of some of this work are outlined below with further detailed descriptions given in the publications attached.

1. Development of Antiserum to SOD

It has been demonstrated by Fridovich and McCord that the enzyme superoxide dismutase (SOD) which is present in all aerobic cells, plays an important role in the detoxification of the superoxide anion radical (O_2^-). SOD catalyzes the dismutation of O_2^- and hydrogen to hydrogen peroxide and oxygen. We developed and verified an antibody to SOD (Cu-Zn) in New Zealand white rabbits following conventional methods for the preparation of polyclonal antibodies. This antiserum was used to confirm the presence of SOD in tracheal epithelial cells, lung fibroblasts and alveolar macrophages in vitro. Various cells from lung sections of rats exposed to asbestos in vivo stained intensely for SOD. The work entitled "Alteration of Superoxide Dismutase Activity in Tracheal Epithelial Cells by Asbestos and Inhibition of Cytotoxicity by Antioxidants", by B.T. Mossman, J.P. Marsh, and M.A. Shatos was published in Laboratory Investigation, Vol 54:(2)204-212, 1986.

We report here the inhibition of asbestos-induced cytotoxicity in a hamster tracheal epithelial cell line by superoxide dismutase, a scavenger of superoxide (O_2^-), and by mannitol and dimethylthiourea, scavengers of the hydroxyl radical (OH•). By using these agents, cell damage was ameliorated in cultures exposed to long ($>10\mu m$ in length)fibers of chrysotile and crocidolite asbestos. In contrast, injury to epithelial cells by short ($\leq 2\mu m$) chrysotile or glass fibers was not prevented by scavengers of O_2^-, OH•, H_2O_2 or $^1O_2$ (singlet oxygen). These results implicate active oxygen species as mediators of injury by long asbestos fibers to cells of the respiratory tract.

By using immunocytochemical and biochemical techniques, we detected appreciable amounts of copper-zinc superoxide dismutase in hamster tracheobronchial epithelial cells and alveolar macrophages in vitro and in histologic sections of rat and human respiratory tract. Activity of total endogenous superoxide dismutase (copper-zinc and manganese forms) increased in tracheal epithelial cells exposed for several days in vitro to either crocidolite or chrysotile asbestos but was unchanged in untreated cells and those exposed to comparable amounts of glass fibers. After inhalation of asbestos by rats, or exposure of cells in culture to asbestos, long fibers were observed protruding from both epithelial cells and alveolar macrophages. The unsuccessful phagocytosis of long fibers of asbestos coupled with generation of oxygen-free radicals might explain the increased pathogenic potential of long fibers in asbestos-associated diseases of the respiratory tract.

Further immunocytochemical studies showed the presence of SOD in epithelial cells and submucosal glands of both rat and human airways (Shatos, et al., Am. Rev. Respir. Dis. 131:(A)187, 1985).

2. Scavengers of Active Oxygen Species Ameliorate Asbestos-Associated Injury in Rat Lung Fibroblasts and Alveolar Macrophages (Shatos, et al., Env. Res. 44:103-116, 1987)

Mechanisms of cell injury to asbestos are relevant to the pathogenesis of occupational diseases including bronchogenic carcinoma, mesothelioma and pulmonary fibrosis (asbestosis). The goal in studies here was to investigate the possible prevention of asbestos-related cell injury by antioxidants in both target and effector cells of asbestosis. The viability of a normal rat lung fibroblast line and freshly isolated rat alveolar macrophages was assessed by trypan blue and nigrosin dye exclusion after exposure of cells to a range of concentrations 2.5 - 25$\mu g/cm^2$/dish of crocidolite asbestos. In comparison to fibroblasts, macrophages were more resistant to the cytotoxic effects of crocidolite. In these studies, cytotoxic concentrations of asbestos were added to both cell types in combination with the following antioxidants: superoxide dismutase, (SOD), a scavenger of superoxide (O_2^-), (100, 200μg/ml), catalase, (25, 50μg/ml), an enzyme scavenging H_2O_2, dimethylthiourea, (20, 40mM), a scavenger of the hydroxyl radical (OH•) and deferoxamine, (1, 5, 10mM) an iron chelator. Results showed significant, dosage-dependent reduction ($p < .001$) of asbestos-associated cell death with all of the above. In contrast, asbestos-induced toxicity was not prevented after the addition of chemically inactivated SOD (100, 200μg/ml) and catalase (25, 50μg/ml) or bovine serum albumin (100μg/ml). These data suggest that asbestos-induced cell damage is mediated by active oxygen species. Moreover, the chemical composition of the fibers, presumably the iron associated with the fiber and/or its interaction with cell membranes may be critical in driving a modified Haber-Weiss reaction producing OH•.

Similar studies were performed with cristobalite (crystalline form of silica) which induced cytotoxicity in these same cell types. The antioxidants listed above did not prevent cristobalite-associated cell death, implying that silica injury to fibroblasts and macrophages may not be mediated by active oxygen species.

3. Manganese (Mn) Form of Superoxide Dismutase (SOD) is Increased in Rat Lung Fibroblasts Exposed to Asbestos (Shatos, et al. In Vitro Cellular and Developmental Biology 22:48(a), 1986.)

Studies below focus on the possible role of oxygen-free radicals on asbestos-associated injury in normal lung fibroblasts, a "target cell" in pulmonary fibrosis.

Objectives of studies here were: a) to assess baseline levels of total SOD and to determine the ratio of Cu-Zn:Mn SOD in normal Fisher-344 rat lung fibroblasts; b) to determine the activity of total SOD (Cu-Zn and Mn forms) before and after exposure *in vitro* to non-toxic concentrations of crocidolite asbestos; c) to determine whether or not exposure to asbestos results in an alteration of either Cu-Zn or Mn SOD in these same cells.

In control fibroblasts, baseline concentrations of SOD range from 23-90 U SOD/mg protein and vary with the age of the culture. Fibroblasts were found to have a 60:40 ratio of Cu-Zn:Mn SOD under these circumstances. Exposure of fibroblasts to non-toxic amounts of crocidolite asbestos (2.5 and 5 $\mu g/cm^2$/dish) results in significant dose-related elevations in SOD levels at 24, 48, 72, and 96 hours after addition of fibers ($p < .05$). Further biochemical analyses indicate the the asbestos-associated elevation in SOD levels is due to a significant increase in Mn SOD ($p = .026$), implying a possible preventive role of this SOD in asbestos-associated injury of normal lung fibroblasts. (Manuscript in preparation)

4. Characterization of a Rapid-Onset of Inhalation Model of Asbestosis in Rats (Mossman, et al., Manuscript submitted for publication/In Review)

A short-term inhalation model of asbestosis was developed in rodents to examine possible preventive approaches to lung disease. Fischer 344 rats were exposed for 10 and 20 days to N.I.E.H.S. crocidolite asbestos while sham controls were exposed to air only. To determine quantitative biochemical indicators of asbestos-induced lung disease, bronchoalveolar lavage (BAL) fluids were analyzed for lactic dehydrogenase (LDH), alkaline phosphatase, angiotensin-converting enzyme (ACE), and protein. Total and differential cell counts were performed on cell pellets form BAL. Lungs from additional rats were processed for histopathology, measurement of hydroxyproline, and autoradiography after injection of rats with ^{3}H-thymidine. Exposure to asbestos for 10 and 20 days caused increases in LDH, alkaline phosphatase and protein in BAL. In contrast, ACE was undetectable in BAL fluids from sham or asbestos-exposed rats. At both time periods, the percentage of polymorphonuclear leukocytes (PMNs) and lymphocytes in BAL were increased in asbestos-exposed rats. Total cell numbers in BAL were increased significantly at 20 days in animals inhaling asbestos. Exposure to asbestos for 10 to 20 days caused elevated amounts of hydroxyproline in lung and the development of fibrotic lesions. Asbestos-exposed rats exhibited increased numbers of interstitial cells and airspace epithelial cells incorporating ^{3}H-thymidine, whereas labeled bronchiolar epithelial cells were not elevated significantly. The quantitative changes in asbestos-associated enzyme levels, cell types and protein in BAL, as well as increases in hydroxyproline and morphologic evidence of fibrosis, are useful indices of asbestos-related lung injury.

5. Increased Synthesis of Procollagen in Rat Lung Fibroblasts Exposed to Asbestos (Mossman, et al., J. Cell Biol. 101:96a, 1985; Mossman, et al., Chest 89:(3)160-161S, 1986)

Occupational exposure to asbestos in man is associated with the development of pulmonary interstitial fibrosis (PIF). Whether the disease process reflects an increase in proliferation of fibroblasts in the lung, and/or an enhanced ability of individual fibroblasts to produce more collagen is uncertain. To determine if asbestos causes increased synthesis of procollagen from individual fibroblasts, a cell line of Fischer 344 rat lung fibroblasts was exposed to nontoxic amounts of crocidolite ($\leq 5 \mu g/cm^2$ dish) *in vitro* and the total cellular collagen and noncollagen protein measured at 24, 48 and 72 hours. Autoradiography after labelling of control and asbestos-exposed cell cultures with ^{3}H-thymidine indicated that labeling indices of asbestos-exposed fibroblasts were not increased. In comparison to controls, an increase (1.5-fold) of collagen per ng DNA was observed at 48 and 72 hours after addition of asbestos to cells. These increases were not observed after exposure of cells to latex beads. Moreover, no consistent elevations in noncollagen protein were observed with use of asbestos or latex. Using the quick-blot technique, the steady-state levels of procollagen type I mRNAs were determined using three recombinant cDNA probes (obtained from Dr. Roe, University of Connecticut). Quick blot analysis failed to show a difference between the amount of procollagen type I mRNAs in control and asbestos-exposed cells. Subcellular fractionation studies now are being initiated to determine whether there is a partitioning of procollagen mRNAs into polysomes, a phenomenon occurring in bleomycin-induced PIF.

6. Inhibition of Lung Injury, Inflammation and Interstitial Pulmonary Fibrosis by Polyethylene Glycol Conjugated Catalase in Rats Exposed by Inhalation to Asbestos (Mossman, et al., Manuscripts submitted for publication/In review.)

Several in vitro studies suggest the involvement of active oxygen metabolites in cell damage caused by asbestos. To determine if lung injury, inflammation and asbestosis could be inhibited in vivo in a rapid-onset, inhalation model of disease, a novel method of chronic administration of antioxidant enzymes was developed. In brief, Fischer 344 rats were treated with polyethylene glycol-conjugated (PEG-) superoxide dismutase (SOD) or catalase in osmotic pumps over a 10 (5 days/week for 2 weeks) or 20 day (5 days/week for 2 weeks) period of exposure to crocidolite asbestos. Controls included sham-exposed animals and those exposed to asbestos but receiving chemically inactivated enzymes. After 10 days of exposure to asbestos, lactic dehydrogenase (LDH), alkaline phosphatase and total protein in bronchoalveolar lavage (BAL) were measured in one group of rats. Total and differential cell counts in BAL also were assessed. After 20 days of exposure, lungs of an additional group of rats were evaluated by histopathology and by measurement of hydroxyproline. Asbestos-associated elevations in LDH, protein and total cell numbers in BAL were reduced in rats receiving PEG-catalase. Decreases in numbers of alveolar macrophages (AMs), polymorphonuclear leukocytes (PMNs), and lymphocytes occurred in these animals. Exposure to asbestos for 20 days caused significant increases in both the amount of hydroxyproline in lung and the severity and extent of fibrotic lesions as determined by histopathology. These indicators of asbestosis were inhibited in a dosage-dependent fashion in rats receiving PEG-catalase. Use of inactivated PEG-catalase failed to boost serum levels of catalase and did not inhibit asbestos-induced elevation of hydroxyproline in lung. These results confirm the importance of active oxygen species in asbestos-associated lung injury and suggest the possible, future use of a novel therapeutic approach to clinical asbestosis.

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16. Abstract (Limit: 200 words) Studies conducted by the authors in the areas of lung cells, asbestos (1332214) and oxygen free radicals were summarized. The research resulted in considerable experience in lung cell culture development, the processing of an antisera to the antioxidant enzymes superoxide-dismutase (SOD) and glutathione-peroxidase, and the completion of several studies which indicate that asbestos induced injury to lung cells in-vitro and in-vivo may be mediated by oxygen free radicals. Studies related to the development of the antiserum to SOD demonstrated the involvement of active oxygen species as mediators of injury by long asbestos fibers to the cells of the respiratory tract. Other studies demonstrated the unsuccessful phagocytosis of long fibers of asbestos coupled with the generation of oxygen free radicals which might explain the increased pathogenic potential of long fibers in asbestos associated diseases of the respiratory tract. Studies were also reviewed which focused on the possible role of oxygen free radicals on asbestos associated injury in normal lung fibroblasts, a target cell in pulmonary fibrosis. A short term inhalation model of asbestosis was developed in rodents to examine possible preventive approaches to lung disease. Occupational exposure to asbestos in man was associated with the development of pulmonary interstitial fibrosis. Several in-vitro studies suggested the involvement of active oxygen metabolites in cell damage caused by asbestosis. Studies were conducted which resulted in the confirmation of the importance of active oxygen species in asbestos related lung injury and suggested the possible future use of a novel therapeutic approach to clinical asbestosis.

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