Oxygen Radicals and Asbestos-mediated Disease

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Asbestos fibers are potent elaborators of active oxygen species whether by reactions involving iron on the surface of the fiber, or by attempted phagocytosis of fibers by cell types resident in the lung. The link between production of active oxygen species and the pathogenesis of asbestos-mediated disease has been highlighted by studies outlined here exploring the use of antioxidant scavengers which inhibit the cytotoxic effects of asbestos both in vitro and in vivo. The use of antioxidant enzymes ameliorates the induction of certain genes necessary for cell proliferation, such as ornithine decarboxylase, implicating oxidants as causative factors in some abnormal cell replicative events. Based on these observations, antioxidant enzymes likely represent an important lung defense mechanism in response to oxidative stress. In addition, their gene expression in lung or in cells from bronchoalveolar lavage might be a valuable biomarker of chronic inflammation and pulmonary disease after inhalation of oxidants. — Environ Health Perspect 102(10):107–110 (1994)

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Introduction

Asbestos refers to a diverse family of crystalline hydrated fibrous silicates typically exhibiting a greater than 3:1 length to diameter ratio. Asbestos is subdivided into two major classes: serpentine (of which chrysotile is the only type), and amphibole. Included in the amphibole class of asbestos are crocidolite, amosite, anthophyllite, tremolite, and actinolite. Chrysotile consists of curly, pliable fibers that tend to bundle together, while members of the amphibole class are characterized by straight, needlelike fibers (1,2). Chrysotile accounts for over 90% of the world’s asbestos production, with some amphiboles, particularly crocidolite, accounting for the remainder (2).

Asbestos exposure has been implicated in the pathogenesis of three distinct types of lung disease: asbestosis, lung cancer, and malignant mesothelioma (1,2). Asbestosis is a clinical condition marked by abnormal proliferation and increased collagen production by lung fibroblasts, leading to pulmonary interstitial fibrosis with resulting decreased lung compliance, inhibited gas exchange in the alveoli, disability, and in some cases, death. Lung cancer (bronchogenic carcinoma) arises predominantly in the tracheobronchial epithelial cells or alveolar epithelial cells, typically has a 20-year latency period after first exposure to asbestos, and arises predominantly in smokers (1,2). Malignant mesothelioma is a rare cancer of the mesothelial cells lining the pleural or peritoneal cavity, often exhibiting a 30-year or greater lag time between initial exposure to asbestos and time of diagnosis, and has a very high mortality rate. As opposed to lung cancer, mesothelioma is not linked causally with smoking (1,2). In the case of mesothelioma, asbestos acts as a complete carcinogen, being capable of both initiation and promotion. In the pathogenesis of lung cancer, asbestos acts primarily as a promoter, inducing cell proliferation, induction of ornithine decarboxylase activity, phospholipase activation, and protein kinase C activation (3–6). However, asbestos fibers may also be cocarcinogens in the respiratory tract as they can increase delivery of chemical carcinogens to tracheobronchial epithelial cells and impair their clearance from these cells (2).

Mechanisms of Formation of Active Oxygen Species by Asbestos

A causative factor strongly implicated in asbestos-related lung disease is the formation of oxygen radicals, frequently termed active oxygen species (AOS). One mechanism of generation of AOS is by redox reactions catalyzed by metals on the surface of the fibers. (7,8). For example, in studies using cell free systems, iron present on the surface of asbestos fibers can drive a modified Haber-Weiss (Fenton) reaction in which superoxide anion (O2−) and hydrogen peroxide (H2O2) are converted to the highly toxic hydroxyl (·OH) radical (9–11). In general, the greater the iron content of the fiber, the more AOS generated by this process (12). Asbestos-mediated AOS generation also occurs during the phagocytosis of inhaled fibers by alveolar macrophages or other cell types resident in the lung (13,14). If the fiber is too long the cell cannot completely ingest it, a phenomenon known as “frustrated phagocytosis.” The resultant respiratory burst generates AOS that can leak out into the external environment of the lung, damaging surrounding cells (14). Alveolar macrophages and neutrophils produce significant quantities of oxygen radicals when exposed to asbestos (7,14), as determined by the cytochrome c reduction technique.

Another pathway leading to the generation of oxygen radicals during mineral dust exposure may be the elaboration of tumor necrosis factor alpha (TNFα) by alveolar macrophages and other inflammatory cells in the lung. TNFα is believed to manifest its cytotoxic effects partly by the production of AOS, including H2O2 and O2−, when TNFα interacts with the target cells.
membranes or internal structures such as mitochondria (15–17).

**Inhibition of Cytotoxicity by Asbestos in Vitro and in Vivo**

Studies using scavengers of AOS have been particularly important in implicating AOS in the pathogenesis of asbestos-mediated lung disease. A host of antioxidant defenses exists in the lung including endogenous stores of the antioxidant enzymes, manganese superoxide dismutase (Mn-SOD), copper-zinc superoxide dismutase (Cu,Zn-SOD), catalase (CAT), and glutathione peroxidase (GPX). Mn-SOD and Cu,Zn-SOD are involved in the detoxification of O$_2^-$ and catalase and GPX cataolize H$_2$O$_2$ to other, less harmful byproducts (18). In addition to the antioxidant enzymes, there are many nonenzymatic scavengers of AOS such as mannitol, dimethylthiourea (DMTU), vitamins A, E, and C, and glutathione (19).

In our laboratory we have studied the effects of both chrysotile and crocidolite asbestos on various lung-derived cell lines, and what effect, if any, the addition of AOS scavengers to culture media would have on asbestos-induced cytotoxicity. Hamster tracheal epithelial cells (HTE) exposed both to chrysotile and chrysotile asbestos suffered substantially less cytotoxicity when either DMTU or mannitol, both scavengers of hydroxyl radical (•OH), or superoxide dismutase (SOD) added to the culture medium (20). An interesting result of this experiment was the impact of asbestos fiber length on the ability of the AOS scavengers to protect cultured cells from oxidant damage. Cell damage was ameliorated by addition of the scavengers in HTE cultures exposed to long asbestos fibers (>10 μ in length) but not in cultures exposed to short (<2 μ) chrysotile or glass fibers. This study highlights the importance of fiber geometry on AOS generation by asbestos, with longer fibers being more potent laborators of AOS than short ones (20). Studies using cultures of rat lung fibroblasts and freshly isolated alveolar macrophages (13) also revealed a diminution of the cytotoxic effects of crocidolite in these cell types by addition of the AOS scavengers used in the aforementioned experiment, and also by catalase and deferoxamine, an iron chelator. In comparison, asbestos-mediated toxicity was not reduced by addition of chemically inactivated SOD, catalase, or bovine serum albumin (13).

To determine if AOS-induced lung injury and inflammation can be inhibited in vivo by oxidant scavengers, our labora-

tory developed a rapid-onset inhalation model of disease. In one study (21) rats were exposed to crocidolite for 20 days, while being administered polyethylene glycol-conjugated SOD or catalase by an osmotic pump, and parameters of lung injury such as infiltration of neutrophils into alveolar spaces, lactate dehydrogenase (LDH), and protein levels in bronchoalveolar lavage (BAL), hydroxyproline content, and the appearance of fibrotic lesions in the lung were measured. Asbestos-induced elevations in these indicators of disease were reduced in the PEG-catalase infused rats as compared to control rats (no pump) or those infused with inactivated PEG-catalase. In contrast, the administration of PEG-SOD did not result in inhibition of the pathologic effects of asbestos inhalation, implicating H$_2$O$_2$ as a mediator of lung damage and development of asbestosis (21).

**Induction of Antioxidant Enzymes in Cells of the Respiratory Tract by Asbestos and Oxidants in Vitro**

As mentioned previously, cells of the lung contain an endogenous oxidant defense system of antioxidant enzymes (18). Studies in our laboratory have focused on the regulation of the enzymes in response to oxidant stress and have elucidated that these enzymes are differentially regulated in vitro when lung cells are exposed to a variety of oxidant generators (20,22,23). In other words, which antioxidant enzymes are induced is dependent upon both the cell type exposed and which oxidant stress is used in the experiment. Activity of total SOD increased in cultures of HTEs exposed for several days to either crocidolite or chrysotile asbestos (20), with no increases found in cells exposed to glass fibers. Exposure of HTEs to H$_2$O$_2$ (22) resulted in a dose-responsive induction of catalase mRNA in both confluent cells and during log-phase growth, with increases in mRNA reaching 3-fold as compared to control cells. GPX and Mn-SOD steady-state mRNA levels were also increased after exposure to H$_2$O$_2$; however, the increases were less striking than catalase. Confluent cells revealed the highest expression of catalase, GPX, and Mn-SOD mRNA. Cu,Zn-SOD mRNA expression revealed a different pattern than the other antioxidant enzymes in this study, with constitutive expression being greatest in actively dividing cells, and no induction of mRNA expression when log-phase or confluent HTEs were exposed to H$_2$O$_2$ (22). Mn-SOD mRNA was also selectively induced in confluent HTEs exposed to the chemical oxidant generation system xanthine/xanthine oxidase (X/XO), with steady-state mRNA levels of catalase, GPX, and Cu,Zn-SOD exhibiting no induction. Elevations in Mn-SOD mRNA were evident as early as 2 hr, peaked at 24 hr of exposure to X/XO, and correlated with increases in Mn-SOD immunoreactive protein (22). These experiments indicate cell- and cycle-specific and differential expression of various antioxidant enzymes in response to certain oxidant stresses.

Human pleural mesothelial cells (HMCs), the progenitor cells of mesothelioma, exposed to either chrysotile or crocidolite asbestos, or X/XO, also show dose-dependent increases in Mn-SOD and heme oxygenase (HO) mRNA expression (23). No induction of gene expression was seen in HMCs exposed to polystyrene beads or riebeckite, a nonfibrous analog of crocidolite. Related experiments using human adult lung fibroblasts (HAL) resulted in less striking elevations of Mn-SOD and HO mRNA levels with asbestos than that seen with HMCs; however, 5-fold increases in HO were observed in HALs after exposure to xanthine/xanthine oxidase (23). HMCs evidenced slight increases in Mn-SOD immunoreactive protein in response to both asbestos and X/XO, while HALs exhibited striking increases in Mn-SOD protein after exposure to X/XO. Thus, various cell types in the lung may vary in their responses to oxidant stresses as measured by the gene expression of antioxidant enzymes.

**Induction of Antioxidant Enzymes in Lungs of Rats after Inhalation of Asbestos or Silica**

To determine whether the results of in vitro assays on induction of antioxidant enzymes by oxidants in lung cell cultures correlate with oxidant defenses in animals after exposure to asbestos or silica, we conducted inhalation experiments using rats to examine induction of antioxidant enzyme gene response and activity in whole lung homogenates. Rats exposed to crocidolite (7–10 mg/m$^3$ air) showed increases in the activity of all measured antioxidant enzymes in lung tissue, with minor variations in the extent and time frame of increases (24,25). These elevations in enzyme activity correlate with induction of gene expression for enzymes, with Mn-SOD exhibiting the
most dramatic increases in steady-state mRNA levels, peaking at 6 to 9 days. Increases ranged from 1.4-fold above sham control values at 1 day of exposure to approximately 5-fold increases which peaked at 3 days. Elevations in Mn-SOD gene expression were observed in rats maintained in clean air even after cessation of exposures, i.e., 10 and 14 days, observations indicating that elevations in antioxi-
dant enzymes may persist for extended periods of time (24,25).

Message levels of Cu,Zn-SOD also peaked at 3 days (approximately 2-fold above sham control values), but were also elevated significantly after 9 days in the high exposure group (25). GPX and catalase exhibited increases in mRNA levels at various times, but not as high or as consis-
tently elevated as Mn-SOD. Elevations in Mn-SOD mRNA were found to correlate with increases in immunoreactive protein after 20 days of exposure (24,25).

Experiments using the fibrogenic dust, cristobalite silica, at similar doses have eluci-
dicated somewhat different patterns of antioxidative enzyme expression (24). The greatest increase in mRNA induction occurred with Mn-SOD, with higher levels found than those previously observed after exposure to similar concentrations of asbestos. In silica-exposed groups, Mn-
SOD mRNA was increased at all time points examined and remained elevated at 14 days postexposure of silica. In general, the other antioxidative enzymes (catalase, GPX) exhibited no striking changes in mRNA levels, and Cu,Zn-SOD was unaffected by exposure to silica. Mn-SOD immunoreactive protein levels also were elevated; however, no increases in enzyme activities of antioxidative enzymes were observed (24).

Experimental evidence suggests that not all lung cell types express comparable increases in antioxidative enzymes after stress. This may be a factor in the selective focal cell damage seen after certain types of oxidant stress. Recent experiments have focused on immunocytochemical localization of Mn-SOD content in various lung cells (26). In rats exposed to cristobalite or cristobalite fiber, Mn-SOD protein is primarily expressed in the mitochondria of type II alveolar cells, with other lung cell types such as fibroblasts, alveolar macrophages, or endothelial cells containing little to no detectable immunoreactive protein. The elevations in Mn-SOD in type II epithelial cells in response to mineral dust correlated with the 2-fold or greater increases in immunoreactive protein found in whole lung homogenates. Thus, this finding is in agreement with the hypothesis that type II alveolar cells are more resistant to certain types of oxidant stress because of induction of Mn-SOD or other antioxidative enzymes.

Induction of Cell Signaling Pathways, Protooncogenes, and Other Proliferation-related Genes in Vitro and in Vivo by Asbestos

A prominent feature of asbestos-related diseases is abnormal and unregulated cell prolifera-
tion of target cells of the lung or pleura giving rise to disease (1,2). The mechanisms involved in asbestos-induced cell proliferation are unclear, but may reflect alterations in expression of genes encoding transcription factors or other proteins important in the stimulation of cell division. An important regulator of cell proliferation in mammalian cells is ornithine decarboxylase (ODC), an enzyme critical in the intracellular production of polyamines, growth regulatory mol-
ecules necessary for cellular replication and differentiation (27). The specific function of polyamines in cell growth and proliferation appears to be complex, but the hyper-
plastic potential of phorbol ester compounds, tumor promoters capable of triggering pro-
liferation in the lung and other tissues, is causally linked to their ability to induce ODC activity (28).

Experiments in our laboratory have implicated AOS as mediators of induction of ODC mRNA and enzyme activity by asbestos in vitro. For example, HTEs exposed to crocidolite and chrysotile asbestos or X/XO exhibited elevations in both ODC mRNA and activity that were ameliorated by addition of antioxi-
dant enzymes and scavengers such as mannitol to the culture media (29). Related studies using calcium channel blockers and inhibitors of protein kinase C provide evidence that asbestos-mediated ODC induc-
tion is generated by a protein kinase C-dependent pathway (30). Recent in vivo rat inhalation experiments in our laboratory revealed increases in ODC mRNA levels in whole lung homogenates of rats after 9 and 20 days of exposure to crocidolite asbestos. The increases at the 20-day time period correlated with the appearance of focal fibrotic lesions in the lung and increased hydrosorptive levels, indicative of fibroblastic proliferation and increased collagen deposition (25).

The tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), which interacts directly with protein kinase C, elevates expression of the AP-1 group of transcription factors which encompasses the protein products of the c-fos and c-jun family of protooncogenes. These factors form homo- or heterodimers that bind to specific regulatory DNA sequences designated the TPA response element (TRE) (31,32). c-fos and c-jun induction is required for cells to progress from the G1 to S phases of the cell cycle. In addition, the fos and jun proteins are early response gene products involved in regulation and transcription of other genes essential for cell cycling (31,32). We have recently shown that c-jun and AP-1 binding to DNA are induced in a dose-responsive fashion in rat mesothelial and HTEs exposed to crocidolite or chrysotile asbestos in vitro (33). An interesting result in this experiment was the differential induction of c-jun and c-fos in the two cell types. Both transcription factors were induced in mesothelial cells, but only c-jun was induced in HTEs. A recent rat inhalation study revealed increases in c-jun mRNA in whole lung after 20 days of crocidolite exposure, with no increases found at earlier time points and c-fos gene expression in this model is currently under investigation (25). A model indicating the pathways of generation of AOS by asbestos and the cell signaling events possibly leading to changes in gene expression of antioxidative enzymes and proliferation-specific genes is presented in Figure 1.
developments and implications for public policy. Science 247:294–301 (1990).
3. Landesman JM, Mossman BT. Induction of ornithine decarboxylase in hamster tracheal epithelial cells exposed to asbestos and 12-O-tetradecanoylphorbol-13-acetate. Cancer Res 42:3669–3675 (1982).
4. Marsh JP, Mossman BT. Mechanisms of induction of ornithine decarboxylase activity in tracheal epithelial cells by asbestos form minerals. Cancer Res 48:709–714 (1988).
5. Sesko A, Cabot M, Mossman BT. Hydrolysis of phosphoinositides precedes cellular proliferation in asbestos-stimulated tracheobronchial epithelial cells. Proc Natl Acad Sci USA 87:7385–7389 (1990).
6. Perderiset M, Marsh JP, Mossman BT. Effects of asbestos on specific binding of phorbol ester tumor promoters and protein kinase activity in hamster tracheal epithelial cells. In: Effects of Mineral Dusts on Cells (Mossman BT, Begin RO, eds). Berlin:Springer-Verlag, 1989:433–437.
7. Kamp DW, Graceff A, Pryor W, Weitzman S. The role of free radicals in asbestos-induced diseases. Free Radic Biol Med 12:293–315 (1992).
8. Mossman BT, Marsh JP, Shatos MA, Doherty J, Gilbert R, Hill S. Implication of active oxygen species as second messengers of asbestos toxicity. Drug Chem Toxicol 10:157–165 (1987).
9. Weitzman SA, Graceff A. Asbestos catalyzes hydroxyl and superoxide radical generation from hydrogen peroxide. Arch Biochem Biophys 228:373–376 (1984).
10. Gulumian M, Van Wyk JA. Hydroxyl radical production in the presence of fibres by a Fenton-type reaction. Chem Biol Interact 62:89–97 (1987).
11. Kennedy TP, Dodson R, Rao NV, Ky H, Hopkins C, Baser M, Tolley E, Hoidal J. Dusts causing pneumoconiosis generate •OH and produce hemolysis by acting as Fenton catalysts. Arch Biochem Biophys 269:359–364 (1989).
12. Vallathyan V, Mega J, Shi X, Dalal N. Enhanced generation of free radicals from phagocytes induced by mineral dusts. Am J Respir Cell Mol Biol 6:404–413 (1992).
13. Shatos MA, Doherty JM, Marsh JP, Mossman BT. Prevention of asbestos-induced cell death in rat lung fibroblasts and alveolar macrophages by scavengers of active oxygen species. Environ Res 44:103–116 (1987).
14. Hansen K, Mossman BT. Generation of superoxide (O2•−) from alveolar macrophages exposed to asbestos and nonfibrous particles. Cancer Res 47:1681–1686 (1987).
15. Wong G, Goeddel D. Induction of Mn-SOD by TNF: possible protective mechanism. Science 242:941–944 (1988).
16. Matthew N, Watkins JF. Tumour-necrosis factor from the rabbit. I. Mode of action, specificity and physicochemical properties. Br J Cancer 38:302–309 (1978).
17. Reid TR, Torti FM, Ringold GM. Evidence for two mechanisms by which tumor necrosis factor kills cells. J Biol Chem 264:4583–4589 (1989).
18. Heffner J, Repine J. Pulmonary strategies of anti-oxidant defense. Am Rev Respir Dis 140:531–554 (1989).
19. Janssen YMW, Van Houten B, Borm PJ, Mossman BT. Cell and tissue responses to oxidative damage. Lab Invest 69:261–274 (1993).
20. Mossman BT, Marsh JP, Shatos MA. Alteration of superoxide dismutase activity in tracheal epithelial cells by asbestos and inhibition of cytotoxicity by antioxidants. Lab Invest 54:204–212 (1986).
21. Mossman BT, Marsh JP, Sesko A, Hill S, Shatos MA, Doherty J, Persuika JP, Adler KA, Hemenway D, Mickey R, Vacek P, Kagan E. Inhibition of lung injury, inflammation, and interstitial pulmonary fibrosis by polyethylene glycol-conjugated catalase in a rapid inhalation model of asbestosis. Am Rev Respir Dis 141:1266–1271 (1990).
22. Shull S, Heintz NH, Periasamy M, Manohar M, Janssen YMW, Marsh JP, Mossman BT. Differential regulation of antioxidant enzymes in response to oxidants. J Biol Chem 266:24398–24403 (1991).
23. Janssen YMW, Marsh JP, Absher MP, Gabrielson E, Borm PJA, Driscoll K, Mossman BT. Oxidant stress responses in human pleural mesothelial cells exposed to asbestos and xanthine/xanthine oxidase. Am Rev Respir Dis (in press).
24. Janssen YMW, Marsh JP, Absher MP, Hemenway D, Vacek PM, Leslie KO, Borm PJA, and Mossman BT. Expression of antioxidant enzymes in rat lungs after inhalation of asbestos or silica. J Biol Chem 267:10625–10630 (1992).
25. Quinlan TR, Marsh JP, Janssen YMW, Leslie KO, Hemenway D, Vacek P, Mossman BT. Dose responsive increases in pulmonary fibrosis after inhalation of asbestos. Am Rev Respir Dis (in press).
26. Holley J, Janssen Y, Mossman BT, Taatjes D. Increased Mn-SOD protein in type II epithelial cells of rat lungs after inhalation of crocidolite asbestos or cristobalite silica. Am J Path 141:475–485 (1992).
27. Pegg AE. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotheraphy. Cancer Res 48:759–774 (1988).
28. O’Brien TG, Lewis MA, Diamond L. Ornithine decarboxylase activity and DNA synthesis after treatment of cells in culture with 12-O-tetradecanoylphorbol-13-acetate. Cancer Res 39:4477–4480 (1979).
29. Marsh JP, Mossman BT. Role of asbestos and active oxygen species in activation and expression of ornithine decarboxylase in hamster tracheal epithelial cells. Cancer Res 51:167–173 (1991).
30. Marsh JP, Mossman BT. Mechanisms of induction of ornithine decarboxylase activity in tracheal epithelial cells by asbestos form minerals. Cancer Res 48:709–714 (1988).
31. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. Biochem Biophys Acta 1072:129–157 (1991).
32. Ransone LJ, Verma IM. Nuclear protooncogenes fos and jun. Annual Rev Cell Biol 6:539–557 (1990).
33. Heintz NH, Janssen YMW, Mossman BT. Persistent induction of c-fos and c-jun protooncogene expression by asbestos. Proc Natl Acad Sci USA 90:3299–3303 (1993).
34. Mossman BT, Marsh JP. Role of active oxygen species in asbestos-induced cytotoxicity, cell proliferation, and carcinogenesis. In: Cellular and Molecular Aspects of Fiber Carcinogenesis. Cold Spring Harbor, NY:Cold Spring Harbor Press, 1991:159–168.