Oxygen Radicals and Asbestos Carcinogenesis

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Asbestos fibers have been shown to generate reactive oxygen species using a variety of *in vitro* assays. It is hypothesized that these highly reactive metabolites mediate the development of malignant mesothelioma induced by asbestos fibers. DNA is a potential target of oxidant attack. Adaptive responses to oxidant injury have been described during exposure of mesothelial cells to asbestos fibers *in vitro*. Failure of these adaptive responses may lead to genetic instability and alterations in oncogenes and tumor suppressor genes that confer a proliferative advantage to emerging neoplastic mesothelial cells. — Environ Health Perspect 102(Suppl 10):131–136 (1994)

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Introduction

Exposure to asbestos fibers is associated with the development of diffuse malignant mesothelioma arising from the pleural, peritoneal or pericardial surfaces, and an increased risk of bronchogenic carcinoma. In humans and animal models, asbestos is a complete carcinogen at the mesothelial target tissue. The incidence of mesothelioma is not increased by cigarette smoking. In contrast, exposure to cigarette smoke and asbestos fibers greatly increases the risk of developing bronchogenic carcinoma. In the bronchial epithelium, asbestos fibers appear to function as a tumor promoter or a co-carcinogen (*1*). Physical dimensions, chemical composition, and surface reactivity contribute to the biologic effects of asbestos fibers. The iron content of asbestos is important in catalyzing formation of reactive oxygen metabolites, especially hydroxyl radicals (*2*). Reactive oxygen metabolites are postulated to play a role during the initiation and the promotion stages of carcinogenesis (*3*). This article will focus on the role of reactive oxygen metabolites during the development of diffuse malignant mesothelioma induced by asbestos fibers.

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Generation of Reactive Oxygen Metabolites by Asbestos Fibers

Asbestos fibers may generate hydroxyl radicals from molecular oxygen and hydrogen peroxide by the following reactions (*4–6*):

$$\text{reductant} + Fe^{+3} \rightarrow \text{oxidant} + Fe^{+2}$$
$$Fe^{+2} + O_2 \rightarrow F^{+3} + O_2^{\bullet -}$$
$$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2$$
$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH^\bullet$$

Hydroxyl radicals are highly reactive and have a short half-life. They can be detected in cell-free systems by molecules designed as spin traps that produce a distinctive signal using electron spin resonance (*4*). Detection of hydroxyl radicals in cells and tissues is more complex. In fact, Gabrielson et al. (*7*) have been unable to detect generation of hydroxyl radicals by asbestos fibers added to cultures of human mesothelial cells. However, generation of free radicals by phagocytic cells exposed to asbestos fibers has been detected by this technique (*8*). Phagocytic cells such as neutrophils and macrophages release much higher quantities of oxidants than mesothelial cells (*9*). The involvement of reactive oxygen species in mediating various effects of mineral fibers *in vitro* has been inferred indirectly using two approaches. First, antioxidants, including specific hydroxyl radical scavengers, decrease many of the *in vitro* activities of asbestos fibers (*10,11*). Second, potential target cell populations exposed to asbestos fibers *in vitro* show evidence of oxidant-induced injury. This oxidative damage can be measured directly using biochemical techniques or indirectly by measuring responses to oxidative damage. Lipid peroxidation has been measured in macrophages exposed to asbestos fibers *in vitro* (*12*). Peroxidized membrane lipids are unstable and decompose to form additional oxidizing species, including malonaldehyde and lipid hydroperoxides. These oxidized lipid derivatives are more stable than hydroxyl radicals and may diffuse throughout cells and tissues and produce additional damage, including additional lipid peroxidation, oxidative stress, and oxidative attack on DNA (*13–15*). Another source of reactive oxygen species and reactive lipid intermediates is the arachidonic acid pathway that is triggered in macrophages and other inflammatory cells by particulate stimuli, including asbestos fibers (*16*). Finally, phagocytic cells are capable of producing reactive oxygen and nitrogen metabolites (*17*). Exposure to asbestos fibers activates this biochemical pathway, generating extracellular release of superoxide anion and hydrogen peroxide (*10*). In the presence of iron-containing asbestos fibers, hydroxyl radicals are formed (*10,11*). Under these circumstances, an important defense reaction against invading microorganisms is converted to a deleterious reaction leading to tissue injury (*13,18*).

Cell and Tissue Responses to Asbestos

Cytotoxicity

A single ip injection of crocidolite asbestos fibers produces a rapid inflammatory response, followed by injury to mesothelial cells (*19*). Asbestos fibers cluster around lymphatic openings or stomata on the inferior surface of the diaphragm. An influx of nonspecifically activated macrophages is triggered in response to asbestos fibers, but not in response to particulate minerals such as titanium dioxide or silica (*20*). These newly recruited macrophages form aggregates around fiber clusters and attempt to

phagocytize long asbestos fibers that are not cleared through lymphatic stomata. Clearance of long fibers from the peritoneal space is limited by the diameter of lymphatic stomata (8–10 μm) (*21*). We have demonstrated that macrophages located at these fiber clusters produce $O_2^{\cdot-}$ as demonstrated by reduction of nitroblue tetrazolium *in situ* (*22*). Using scanning electron microscopy, macrophages and mesothelial cells adjacent to fiber clusters show morphologic evidence of injury: cell swelling, surface blebbing, loss of surface microvilli, and detachment from the surface (*21*). The role of oxidants as mediators of acute cytotoxicity produced by crocidolite asbestos fibers in this model system is supported by the following observation: coating fibers with deferoxamine or injection of catalase conjugated to polyethylene glycol decreases asbestos toxicity.

Repair of Mesothelial Cell Injury

Injury to the mesothelial lining is repaired by proliferation of adjacent, uninjured cells within the first week after a single ip injection of crocidolite asbestos fibers. Mineral particles that are successfully cleared by lymphatics do not elicit a proliferative mesothelial cell response. Mesothelial cell proliferation as demonstrated by incorporation of ^{3}H-thymidine or 5-bromodeoxyuridine peaks at 3 to 7 days, then declines by 21 days. However, at sites of fiber clusters, cell proliferation persists for several weeks (*21*). These proliferating mesothelial cells are potential targets of oxidant-induced genetic damage.

DNA Damage

A critical target molecule for reactive oxygen species is DNA. Direct measurement of oxidized bases or other types of DNA damage generated by free radicals in cells or tissues exposed to asbestos fibers is difficult, although oxidized bases can be detected in cell-free systems (*23*). Oxidative damage generated by asbestos fibers can be demonstrated in cell-free systems by direct measurement of the oxidized nucleoside, 8-hydroxydeoxyguanosine, using the sensitive technique of high performance liquid chromatography coupled with electrochemical detection (*24*). An alternative method to detect oxidative DNA damage is to measure unscheduled DNA synthesis. Two laboratories have demonstrated evidence of unscheduled DNA synthesis in cells exposed to asbestos and other mineral fibers using autoradiographic assays (*25,26*).

DNA repair is an error-prone process and may not be completely successful. The excision and repair of oxidized DNA bases may introduce base substitutions or mispairing that produce point mutations. Simple bacterial assays such as the Ames' test have been developed to screen for the ability of agents to cause point mutations and possibly cancer. Asbestos and other mineral fibers are negative in these assays. When examined for their ability to produce point mutations at specific loci in mammalian cells, mineral fibers were also found to be negative or marginally positive (*27*).

Chromosomal alterations, including breaks and translocations, may be produced by oxidants; these changes can be detected by cytogenetic analysis of cells arrested in metaphase. Asbestos and other mineral fibers have been shown to cause multiple types of chromosomal alterations using cytogenetic assays. The range and severity of this damage vary depending on the target cell type; however, changes in chromosome number, aneuploidy or polyploidy, as well as structural chromosome changes have been reported (*27*). Several mechanisms may produce these chromosomal alterations. It has been proposed that long, thin mineral fibers physically interfere with the mitotic spindle apparatus and perturb chromosome segregation leading to changes in chromosome number (*27*). In addition, asbestos fibers have been shown to adhere to DNA and chromosomes. This interaction may lead to fragmentation of chromosomes (*28*). Indirect generation of reactive oxygen species by asbestos fibers in the vicinity of DNA may cause single- or double-strand breaks; if these are not repaired, chromosomal deletions or translocations may result (*29*).

A new technique using fluorescence *in situ* hybridization to detect the presence or absence of centromeres in micronuclei has been developed. This technique is sensitive and reproducible, and can detect simultaneously the aneuploidogenic effect of agents such as colchicine and the clastogenic effect of agents such as ionizing radiation (*30*). We have optimized this technique for murine mesothelial cells lines *in vitro*. UICC crocidolite asbestos fibers are added to subconfluent cultures at doses of 1 to 20μg/cm^2 for 24 to 48 hr. Cytochalasin B (3 μg/ml) is included during the final 25 hr to prevent cytokinesis leading to accumulation of micronuclei. The cells are fixed in methanol:acetic acid, then prepared for *in situ* hybridization using a biotin-labeled probe for mouse gamma or major satellite DNA localized in the centromeric region. As described by Weier et al. (*31*), the cells are then stained with fluorescein-conjugated avidin, then counterstained with propidium iodide that binds to DNA. When viewed under a fluorescence microscope equipped with a dual wavelength filter, centromeres fluoresce yellow against a background of red DNA fluorescence. Exposure to ionizing radiation induces formation of micronuclei without centromeres because this treatment causes random DNA breaks. In murine mesothelial cells, there is a dose-dependent induction of micronuclei between 200 and 800 rads. The clastogenic effects of ionizing radiation are mediated by hydroxyl radicals generated from $O_2^{\cdot-}$ and H_2O_2 near the target DNA. Scavengers of $O_2^{\cdot-}$ should decrease formation of micronuclei. We successfully reduced the frequency of micronuclei from 20% to less than 5% at a dose of 600 rads using a cell-permeable mimic of superoxide dismutase, Tempol (*32*). In contrast, exposure to colchicine, a known aneuploidogenic agent that prevents polymerization of microtubules, induces micronuclei with centromeres. We discovered that crocidolite asbestos fibers induce both types of damage in murine mesothelial cells.

Our observations are consistent with recent results using the A_L hamster–human hybrid cell line. This system can be used to test for point mutations at the *hgprt* locus as well as deletions in the human marker chromosome 11. In this assay, both chrysotile and crocidolite asbestos fibers produce deletions but very few point mutations, similar to the effects of ionizing radiation (*29*).

Cellular Responses to DNA Damage

Cells adapt to chemical and physical stresses by inducing expression of a variety of genes involved in growth arrest, DNA repair, and defense against injury (*33*). One category of these genes is called DNA damage-inducible (DDI). DDI genes are induced particularly in response to ultraviolet and ionizing radiation (*33,34*). One group of DDI genes, *gadd* genes, mediate growth arrest during the late G1 phase of the cell cycle (Figure 1). This G1 arrest theoretically enables the cell to undergo

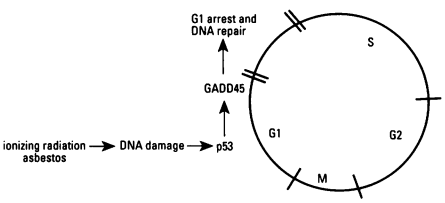

Figure 1. Mechanism of G1 arrest induced by DNA-damaging agents.

DNA repair before entering S phase. The level of induction of *gadd* genes correlates with the extent of DNA breaks or base damage. One example of a *gadd* gene is *gadd 45*. It is highly conserved among vertebrates, although its specific function is unknown (*34*). Increased expression of *gadd 45* depends on wild-type p53 protein, a nuclear phosphoprotein that is transiently expressed during the mid-G1 phase of the cell cycle. Exposure to ionizing radiation and other agents that cause DNA damage increases expression of p53 protein by a posttranslational mechanism. It is hypothesized that p53 acts as a transcription factor to induce expression of genes that arrest growth, such as *gadd 45*. Cells with a deletion in the *p53* gene or a point mutation in this gene are unable to arrest in G1 and show decreased resistance to ionizing radiation (*35*). The importance of *p53* in carcinogenesis will be discussed below (*36*).

Other DDI genes include early response genes (c-*jun*, ERG-1, and c-*fos*), cytokines such as tumor necrosis factor (TNFα), and enzymes such as collagenase (*33*). Early response genes encode for transcription factors. For example, EGR-1 is a nuclear phosphoprotein that is induced by tissue injury and functions in signal transduction during cell proliferation and differentiation. FOS/JUN heterodimers regulate transcription by binding to AP-1 sites in the promoter regions of responsive genes. Thus, primary response gene products regulate transcription of secondary response genes to produce a tissue- and stimulus-specific response to injury (*37*). In contrast to *gadd* genes, these genes are induced by membrane damage resulting in activation of protein kinase C or Src tyrosine kinase. These genes are also induced by tumor promoters such as phorbol esters, while *gadd* genes are not (*34,35*).

Asbestos fibers have been shown to induce DDI genes in mesothelial cells *in vitro*. Heinz et al. discovered increased transcription of c-*fos* and c-*jun* in rat pleural mesothelial cells exposed to crocidolite asbestos (*38*). We have preliminary evidence for increased expression of p53 protein in response to crocidolite asbestos fibers.

How do fibers trigger changes in gene expression? As illustrated in Figure 2, several pathways may be involved. First, asbestos fibers may bind to specific membrane receptors; for example, crocidolite has been shown to bind to the macrophage scavenger receptor (*39*). Physical perturbation of the membrane during phagocytosis or receptor binding may trigger second messenger pathways that transduce a signal from the cell membrane to the nucleus. Cyclic nucleotides, phosphatases or phosphorylases, or increased calcium may be involved in these intracellular signal transduction pathways (*40,41*). Second, oxidants may mediate the intracellular effects of fibers. Macrophages exposed to asbestos fibers *in vitro* release oxidants via the respiratory burst pathway triggered by an NADPH oxidase located in the phagolysosome membrane (*10*). After engulfment, asbestos fibers may catalyze formation of OH• and other reactive oxygen species from $O_2^{\bullet-}$ and H_2O_2 generated by intracellular oxidative metabolic pathways (*2*). We have demonstrated increased intracellular generation of H_2O_2 in murine mesothelial cells exposed to crocidolite asbestos *in vitro* using the fluorescent probe dichlorofluorescein diacetate. Secondary radicals may also be generated intracellularly from the products of lipid peroxidation (*43*). Reactive oxygen metabolites are proposed to be the second messengers leading to activation of the NF-*K*B family of transcription factors (*44*). Reactive oxygen metabolites are hypothesized to mediate gene expression in response to multiple other stresses, including tumor promoters and TNFα. TNFα induces expression of Mn-SOD (superoxide dismutase) as a specific defense against $O_2^{\bullet-}$. Brown et al. (*45*) demonstrated release of the inflammatory cytokines interleukin-1 and TNFα after intratracheal instillation of asbestos fibers in rats and increased Mn-SOD expression has been described in rat lungs after inhalation of asbestos fibers (*46*).

Oxidants and Asbestos Carcinogenesis

The experimental evidence described above provides support for oxidants as mediators of acute mesothelial cell injury and potential inducers of DNA damage. Most of this evidence is based on *in vitro* studies or short-term effects of asbestos fibers after inhalation or direct ip injection. Mesotheliomas develop after a long latent period of several decades in humans or 1 to 2 years in rodents. Few investigators have looked for sequential morphologic and molecular events leading to the development of this malignant neoplasm. A few clues have been provided by studying chromosomal and molecular alterations in cell lines derived from human or rodent mesotheliomas.

Multiple cytogenetic abnormalities have been described in human and rodent mesothelioma cell lines. Losses or gains of chromosomes, especially polysomy of chromosomes 5, 7, and 11 are common. Partial deletions or monosomy involving human chromosomes 1–7, 9, 11–13, 17, 18, 21, and 22 have also been reported. Tumor suppressor genes are known to be located at some of these deleted sites. Additional, more complex rearrangements and structural changes are also common. This wide spectrum of cytogenetic alterations is consistent with the aneuploidogenic and clastogenic effects of asbestos fibers observed in *in vitro* assays (*27*). Unfortunately, most of these cell lines have been derived from advanced tumors. Therefore, the appearance of these alterations at specific stages in the development of mesothelioma is unknown.

Another approach to understanding the molecular changes leading to the development of malignant mesothelioma is to study specific changes in oncogenes and tumor suppressor genes that have been observed in other human tumors. Oncogenes are activated in carcinogenesis by point mutation, amplification, or translocation. No point mutations have been detected in oncogenes in human or rat mesothelioma cell lines (*46,47*). In murine mesothelioma cell lines, expression of c-*myc* is increased, but the gene is not amplified. This gene is deregulated and expressed throughout the cell cycle. Deregulated c-*myc* expression accompanies constitutive activa-

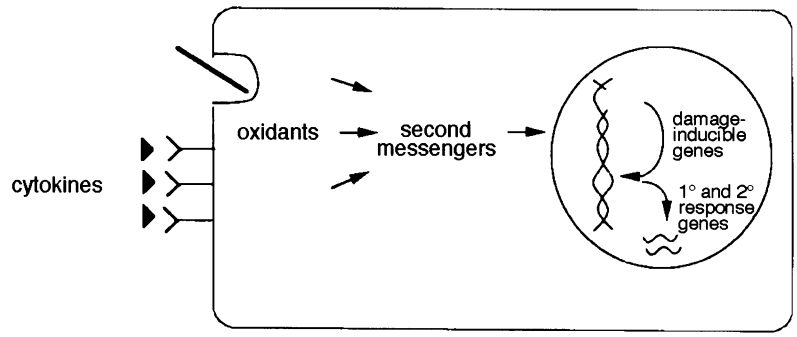

Figure 2. Effects of asbestos fibers on signal transduction and gene expression.

tion of the TGF-α growth stimulatory pathway in other neoplastic cell lines (*48*). Murine and human mesothelioma cell lines also show expression of the c-*sis* oncogene that encodes the B chain of platelet-derived growth factor. Multiple lines of evidence support constitutive activation of this growth stimulatory pathway in human, but not rodent, mesothelioma cell lines. Human mesothelioma cell lines also express additional, as yet unidentified, oncogenes as detected by transfection of DNA into NIH 3T3 cells (*27,47*).

The most frequent alterations in tumor suppressor genes in a variety of human neoplasms are point mutations in the highly-conserved exons of the *p53* tumor suppressor gene (*49*). The evidence for common point mutations in the *p53* tumor suppressor gene in human malignant mesotheliomas is conflicting (*46,50*). Point mutations in the *p53* gene are rare in murine plasmacytomas induced by a chronic inflammatory response to pristane (*51*) and murine thymic lymphomas (*52*) or osteogenic sarcomas induced by ionizing radiation (*53*). In the latter model, structural alterations in the *p53* gene were identified by altered patterns of restriction enzyme digestion on Southern blots. We searched for changes in this gene in 22 murine mesothelial cell lines. Expression of *p53* mRNA was determined by Northern blot analysis. One third of tumorigenic cell lines showed reduced or absent expression of the *p53* gene that correlates with partial deletions in exon 1. All of these cells lines were derived from mice with locally invasive tumors. DNA was digested with the restriction enzymes EcoR1 or BamH1 and analyzed by Southern blotting. Multiple structural alterations were observed in the *p53* gene including loss of the 20-kb fragment (this corresponds to the 5′ end of the gene), loss of the 6.6-kb fragment, or presence of a single large band (*54*).

We examined expression of the mutant p53 protein in these cell lines by immunohistochemistry. Only 2 of 17 tumorigenic cell lines showed any evidence of expression of mutant *p53*; less than 2% of the population expressed mutant *p53* as detected by the monoclonal antibody Pab 240. This observation was corroborated by A. Pellicer, who examined these cell lines for point mutations in exons 5 through 9 of the *p53* gene using SSCP analysis and direct sequencing after PCR amplification. He detected no point mutations in the nontumorigenic or tumorigenic cell lines (A. Pellicer, unpublished observations).

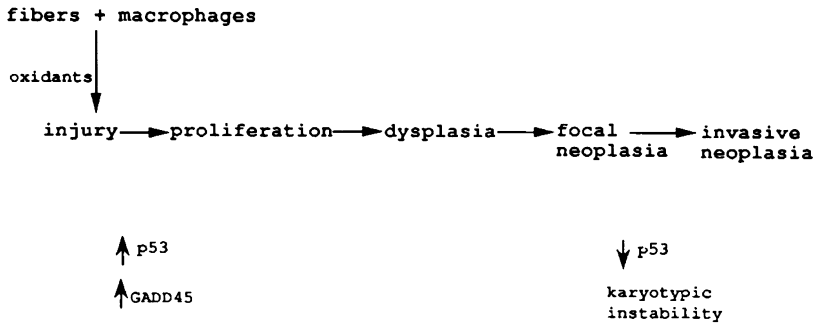

Figure 3. Proposed mechanism of malignant mesothelioma Induced by asbestos fibers.

Although these experimental studies are limited, the evidence based on the changes observed in oncogenes and tumor suppressor genes in human, rodent, and murine mesothelioma cell lines is consistent with the *in vitro* effects of asbestos fibers. As in bacterial and mammalian mutagenesis assays *in vitro*, mesothelioma cell lines do not show point mutations in oncogenes and tumor suppressor genes that are commonly altered in a variety of neoplasms. The structural alterations and deletions described in the *p53* tumor suppressor gene in murine mesothelioma cell lines are consistent with the clastogenic effects of fibers observed in *in vitro* assays. Direct physical interference of fibers with the mitotic apparatus may be responsible for changes in chromosome number or aneuploidy that is commonly observed in human and rodent mesothelial cell lines. An additional mechanism may contribute to the multiple karyotypic changes observed in mesotheliomas: inactivation of *p53* that prevents G1 arrest induced by DNA damage (*36*). As discussed earlier, DNA damage induced by ionizing radiation and base-damaging agents induces expression of wild-type p53 protein and arrests proliferating cells in G1 (*35*). We hypothesize that oxidants cause DNA breaks and induce this G1 arrest pathway in mesothelial cells after acute exposure to asbestos fibers. After repeated exposures (weekly ip injections in our murine model system), DNA damage, primarily deletions or structural changes, is induced in key genes such as *p53*. Damage or deletion in the *p53* gene allows cells to proliferate and divide despite unrepaired DNA damage (*36*). Thus, once a cell population suffers damage in the *p53* gene, additional karyotypic changes will accumulate rapidly, especially in an environment with chronic generation of oxidants catalyzed by amphibole asbestos fibers that persist in target tissues for many years.

This working hypothesis is summarized in Figure 3. The reaction of the mesothelial lining to asbestos fibers is injury that is repaired by proliferation. Experimental evidence suggests that reactive oxygen species released from macrophages during phagocytosis of asbestos fibers trapped at the mesothelial surface mediate this injury. The iron-catalyzed generation of oxidants by asbestos fibers also induces DNA damage. Initially, mesothelial cells attempt to adapt and repair this damage by induction of p53 protein, *GADD45* and other DNA damage-inducible genes. Repeated episodes of injury, DNA damage, and proliferation lead to activation of cellular oncogenes or inactivation of tumor suppressor genes that confers a proliferative advantage. Later stages in progression from focal to invasive growth of malignant mesotheliomas are characterized by reduced expression of *p53* and increased genetic instability.

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