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Serial Review: Role of Reactive Oxygen and Nitrogen Species (ROS/RNS) in Lung Injury and Diseases
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MULTIPLE ROLES OF OXIDANTS IN THE PATHOGENESIS OF ASBESTOS-INDUCED DISEASES

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Abstract—Exposure to asbestos causes cellular damage, leading to asbestosis, bronchogenic carcinoma, and mesothelioma in humans. The pathogenesis of asbestos-related diseases is complicated and still poorly understood. Studies on animal models and cell cultures have indicated that asbestos fibers generate reactive oxygen and nitrogen species (ROS/RNS) and cause oxidation and/or nitrosylation of proteins and DNA. The ionic state of iron and its ability to be mobilized determine the oxidant-inducing potential of pathogenic iron-containing asbestos types. In addition to their capacity to damage macromolecules, oxidants play important roles in the initiation of numerous signal transduction pathways that are linked to apoptosis, inflammation, and proliferation. There is strong evidence supporting the premise that oxidants contribute to asbestos-induced lung injury; thus, strategies for reducing oxidant stress to pulmonary cells may attenuate the deleterious effects of asbestos. © 2003 Elsevier Inc.

Keywords—Asbestos fibers, Cell injury, Mesothelioma, ROS, RNS, Fibrosis, Lung cancers, Free radicals

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INTRODUCTION

Asbestos is a group of naturally occurring mineral fibers (defined as having a ≥3:1 length to diameter ratio) that are associated with the development of both malignant (lung cancers, mesothelioma) and nonmalignant (asbestosis) diseases in the lung and pleura [1–3]. The role of surface activity on the toxicity and carcinogenicity of asbestos fibers has been well documented [4]. A main factor in determining the surface and biological reactivity of asbestos fibers is their ability to participate in redox reactions that generate free radicals. Free radicals generated from asbestos fibers and/or damage by fibers are linked to cell signaling, inflammation, and a plethora of other responses (mutagenesis, proliferation, etc.) associated with the pathogenesis of asbestos-associated diseases. In this review, we first discuss the types and features of asbestos fibers, determining their surface reactivity. We then review the role of reactive oxygen and nitrogen species (ROS/RNS) in asbestos-associated cell responses and their relationship to the induction of disease.

SURFACE ACTIVITY AND TYPES OF ASBESTOS

Properties such as chemical composition, atomic structure, microtopography, surface charge, and dissolution and adsorption of ions and macromolecules have been associated with the biological effects of asbestos fibers. These surface characteristics are affected by the mechanical and thermal history of the fibers, by their chemical treatment, and also by the presence of impurities in the mineral. Due to this changeability in surface characteristics, the surface activity of asbestos fibers may not only vary from one asbestos type to another, but also may change at different points on the same type of asbestos fiber. These variations may lead to the production of different levels and perhaps various species of radicals by individual asbestos types.

There are six recognized types of asbestos with different chemical compositions and morphologies.

Chrysotile

Chrysotile is a 1:1 layer silicate and is classified as the only type of the serpentine family. The chemical formula of a unit cell of chrysotile is represented as $Mg_3[Si_2O_5](OH)_4$, where layers of silicate sheet are connected to octahedral sheets of ($MgO_2(OH)_4$). Due to misfit between these two layers, the 1:1 layers are curled with the silicate sheet directed inward, towards the axis of the fibril, and the Mg-hydroxide surface on the outside. This is not the case for all the chrysotile fibrils, as some of the fibrils exist in cylindrical layers and others in spiral configurations, making the surface groups exposed to the environment different in each configuration [5].

Amosite

Amosite is a brown amphibole asbestos and its name is an acronym for the company "Asbestos Mines of South Africa." Amphiboles are double-chain silicates and, like serpentine asbestos, are characterized by units. The unit structure of amosite is represented as $Fe^{2+}{}_7[Si_8O_{22}](OH)_2$, where iron exists in the ferrous ionic state [5].

Crocidolite

Crocidolite or Cape blue asbestos is another type of amphibole asbestos. The unit cell in crocidolite is represented as $Na_2Fe^{3+}{}_2Fe^{2+}{}_3$ $[Si_8O_{22}](OH)_2$, where a double-chain is tetrahedrally coordinated SiO_4, which separates ribbons of octahedrally coordinated cations [5]. The cations occurring in the octahedral ribbons occupy different crystallographic sites known as M_1, M_2, and M_3 sites. The large asymmetric M_4 site accommodates alkali ions such as Na, K, and Ca, and the other three cationic sites are occupied by iron. From the crystal structure of crocidolite asbestos, it is known that iron in this mineral is distributed among three octahedral sites, namely M_1, M_2, and M_3.

According to Mössbauer spectra of the International Agency for Research on Cancer reference sample of UICC crocidolite, the M_1 sites are populated by both ferric (14%) and ferrous (29%) ions, while the M_2 and M_3 sites are exclusively occupied by ferric (40%) and ferrous (17%) ions, respectively [6,7]. Commercial South African crocidolite fibers have shown similar distributions of ferrous and ferric ions with a different percentage distribution of these ions at each site [8]. This is a common occurrence in mineralogy where the source of crocidolite from which the samples are obtained affects the percentage content of each ionic form of iron occupying these crystallographic sites [9]. Irrespective of these variations in crocidolite fibers obtained from different sources, the availability of the chemical groups on the surfaces will vary depending on the direction of the cleavage of the layers, as is the case with chrysotile.

The other asbestos fiber types include tremolite $[Ca_2Mg_2[SiO_{22}](OH)_2]$, anthophyllite $[(Mg, Fe^{2+})_7 [Si_8O_{22}](OH)_2]$, and actinolite $[Ca^2(Mg,Fe)_5Si_8O_{22}$ $(OH)_2]$. These have been mined and used industrially less frequently than chrysotile, amosite, and crocidolite asbestos.

SURFACE ADSORPTIVE AND ACTIVE SITES AND THE ABILITY OF ASBESTOS TO GENERATE OH$^{\bullet}$ BY REDUCING OXYGEN AND BY PARTICIPATING IN A FENTON-TYPE REACTION

Similar adsorptive sites occur on both chrysotile and crocidolite asbestos fibers [10,11]. On chrysotile, abundant basic hydroxyl sites exist on the outer layer of the octahedral sheet with minor concentrations of acceptor acidic sites. On crocidolite, surface adsorptive sites are of similar Lewis base character. Surface active sites reducing O_2 and catalyzing the decomposition of H_2O_2 are more related to the presence and ionic state of iron [12–14].

The ability of asbestos fibers to generate OH$^{\bullet}$ through their ability to reduce O_2 or to participate in a Fenton-type reaction has been studied by numerous investigators [12,13,15–17].

Reduction of oxygen

The generation of hydroxyl radical was investigated by exploring the ability of asbestos fibers to reduce O_2 to $O_2^{\bullet -}$ radical:

$$O_2 \xrightarrow{+\,e^-} O_2^{\bullet -}$$

which is then dismutated to produce H_2O_2.

$$O_2^{\bullet -} \xrightarrow{+\,e^- + 2H^+} H_2O_2$$

H_2O_2 thus produced is then decomposed to generate $^{\bullet}OH$ (Fenton-like reaction)

$$H_2O_2 \xrightarrow{+\,e^-} {}^{\bullet}OH + HO^-$$

where it can either react in a manner to produce water,

$$^{\bullet}OH \xrightarrow{+\,e^- + H^+} H_2O$$

or react with a molecule such as formate to give rise to the R$^{\bullet}$ ($CO_2^{\bullet -}$) radical or lipid hydroperoxides to produce the lipid radical as follows:

$$^{\bullet}OH + RH \longrightarrow H_2O + R^{\bullet}$$

THE ROLE OF IRON IN ASBESTOS FIBERS IN THE GENERATION OF $^{\bullet}$OH

The nature of the aforementioned free radical-generating surface sites on asbestos fibers is not yet clear. However, iron present in asbestos fibers is thought to be an important factor in the generation of $^{\bullet}OH$ produced from the reduction of O_2 or from the decomposition of H_2O_2. Studies have shown that the ability of asbestos fibers to elicit these effects is not related to total iron content, suggesting the presence of specific iron active sites that exist at the surface and become active in free radical generation only when present at specific crystallographic sites in a definite valency and coordination state.

Total bulk iron vs. oxidation state of iron

Iron present in the bulk of iron-containing minerals is important in reducing O_2 via participation in a Fenton-type reaction [12]. When crocidolite asbestos fibers and other iron-containing particles, including goethite (FeOOH α), akaganeite (FeOOH β), hematite ($Fe_2O_3\alpha$), magnetite (Fe_3O_4), and siderite ($FeCO_3$) were tested for their ability to reduce O_2, those containing Fe^{3+} iron (goethite, akaganeite, and hematite) were not active. Magnetite was also inactive despite its 2% Fe^{2+} content. Those materials having Fe^{2+} ions (siderite and crocidolite) were active, with crocidolite being more active than siderite. These results indicate, firstly, that not all iron-containing minerals share an oxygen-reducing ability to the same level. Secondly, those containing ferrous iron were more active; and, thirdly, not all ferrous-containing minerals had the same level of activity. When, on the other hand, the ability of these particulates to participate in a Fenton-type reaction was tested, although this ability depended on the ferrous content of the mineral, those particles containing ferric ions also were able to participate in this reaction.

The involvement of iron content in the bulk and the oxidation state of this transition metal ion in different asbestos fibers have been investigated further [18]. The ability of asbestos fibers to decompose lipid hydroperoxide is ranked as follows: amosite > crocidolite > tremolite > anthophyllite > chrysotile. The ability of asbestos fibers to generate $^{\bullet}OH$ is ranked as follows: crocidolite > amosite > tremolite > anthophyllite > chrysotile. Thus, once again, not only the content of iron but also the oxidation state of iron are important determinants of these reactions.

Surface-oxidized iron vs. surface-reduced iron

To further elucidate the effect of oxidized and reduced iron on the ability of asbestos fibers to induce free radical reactions, a number of investigators have used various methodologies to change the oxidation state of surface iron.

Reduction of iron

Some iron in crocidolite asbestos was reduced by H_2 at 350°C [19]. Mössbauer spectroscopy results at room

temperatures have shown that this process introduces a number of changes in the ionic distribution of different oxidation states of iron at different crystallographic sites. The total concentration of ferrous ions in all cationic sites was calculated to be 87% compared to 46% in the native crocidolite fibers, thus increasing the Fe^{2+}/Fe^{3+} ratio to 6.69. An increase in the ferrous ion concentration might change the surface activity of these fibers by rendering them more active in the reduction of O_2 and in the decomposition of lipid hydroperoxides. In addition, this process could make the iron more available to chelators for mobilization [20,21].

Oxidation of iron (detoxified crocidolite)

Some iron in commercial crocidolite fibers was converted into ferric by microwave radiation at 300°C [8]. In addition, treatment with microwave radiation abolished the hydroxyl group bending vibration assessed by infrared, an observation indicating the breakage of this bond in addition to the conversion of ferrous into ferric ions. During the latter conversion, it was proposed that a transfer of electrons to the surface of the fiber may have taken place. The changed surface charge and surface sites may then lead to reduced surface activity of these fibers [22]. These changes in surface properties could also decrease the ability of the treated fibers to decompose lipid hydroperoxides.

Treatment with ferric iron salts

Ironically, the detoxification of crocidolite is achieved by coating the fiber with extra iron [23]. Detoxified crocidolite had substantially less ability to generate $^{\bullet}OH$ and to reduce O_2. In addition, less iron was mobilized from the detoxified crocidolite as compared to native crocidolite [24–26].

Thermal treatment

Thermal treatment is achieved by heating crocidolite samples at 400°C under a vacuum or in air for 2 h. During the heating process, any surface adsorbed water is desorbed and hydroxyl groups condense via the elimination of H_2O_2 and the formation of O_2 bridges. In the presence of O_2, ferrous will be oxidized to ferric and some reduction of iron will take place, thus producing two samples with altered ferrous to ferric ratios. The ability of these two samples to reduce O_2 is dependent on the presence of ferrous ions on their surfaces, as the intensity of this reaction is 3-fold higher using samples heated in a vacuum compared to normal unheated samples. The ability of these fibers to decompose H_2O_2, on the other hand, is not much affected. In those samples heated in air, this reaction was completely inhibited [27].

Surface-bound iron vs. mobilized iron

After incubation of crocidolite asbestos with desferrioxamine, decreasing amounts of $^{\bullet}OH$ were observed and proportional to increasing concentrations of the chelating agent [28]. Since desferrioxamine depletes surface irons by mobilization, oxidant generation by crocidolite asbestos appears to be related to surface-complexed iron rather than to its total concentration within the crystal lattice of crocidolite.

To further elucidate the importance of surface-bound vs. mobilized iron in their ability to generate free radicals, supernatants and resuspended fibers of native UICC crocidolite, UICC crocidolite with increased concentrations of ferrous, and crocidolite with increased concentrations of ferric iron in the presence and absence of ferrozine were evaluated for their ability to generate $^{\bullet}OH$ from the decomposition of H_2O_2 [29]. When these fibers were incubated in buffered saline solutions, they released iron into the supernatant. In this case, samples containing high levels of ferrous iron generated increased amounts of free radicals. However, the resuspended fibers had a greater ability to generate $^{\bullet}OH$ in comparison to their corresponding supernatants. Thus, although some iron was released into the supernatant, much more was still bound on the surface of the fibers to catalyze the generation of $^{\bullet}OH$. Studies also indicate that both surface-bound and mobilized iron contribute to the generation of free radicals, but the level of radicals produced is dependent on the oxidation state of iron—whether it is surface-bound or mobilized from the asbestos fiber.

Mobilization of surface iron by ferrozine and ascorbic acid also have been reported in crocidolite asbestos-exposed cells [20,21] and in cell-free systems [30]. The cytotoxicity of crocidolite fibers is directly related to iron mobilization [21] and is followed by increased ferritin synthesis [31].

Taken together, the referenced studies show that the ionic state of iron in asbestos determines its ability to generate ROS. The change from ferric to ferrous ions increases the fiber activity and vice versa. Ferrous ions on the surface of fibers are most active. However, the amount of ferrous ion in a mineral may not be a prerequisite for this activity, as the coordination of ferrous ion to other neighboring ions may determine its availability not only to reduce O_2 or lipid hydroperoxides but also to be mobilized by chelation.

ROS/RNS IN CELL RESPONSES TO ASBESTOS

Asbestos fibers have been studied for decades in numerous in vitro assays to determine their mechanisms of cytotoxicity, DNA damage, and mutagenesis. The prevention of asbestos-induced cytotoxicity and protoonco-

gene induction by antioxidants [3,32,33] and the ability of longer, more pathogenic asbestos fibers to cause production of oxidants by frustrated phagocytosis [34,35] provides compelling evidence that oxidants contribute to asbestos-induced cell injury.

Role of oxyradicals in mutagenesis and DNA damage

Crocidolite asbestos caused the formation of 8-hydroxydeoxyguanosine (8-OHdG) and mutagenicity in Salmonella typhimurium (TA100 and TA104) in an iron-dependent fashion, phenomena linked to fiber-induced lipid peroxidation and depletion of glutathione [36–38]. Earlier attempts at defining the mutagenic potential of asbestos fibers at either the *hprt* or *oua* loci in a variety of mammalian cells have yielded largely negative results. The negative gene mutation data suggest either that asbestos is a nongenotoxic carcinogen or that mutants induced at these loci are nonviable. Using the human-hamster hybrid (A_L) cells, in which mutations are scored at a marker gene (CD59) located on human chromosome 11 (11p13), both crocidolite and chrysotile fibers were mutagenic, inducing mostly deletions involving millions of basepairs [39,40]. In recent years, several other mutagenic assays that are proficient in detecting either large deletions, homologous recombinations, or score mutants located on nonessential genes have been used to demonstrate the mutagenic potential of various fiber types [41,42]. In $hgprt^-$, gpt^+ Chinese hamster V79 cells, nitric oxide increased the iron-dependent mutagenicity of crocidolite in a synergistic fashion [42].

In crocidolite asbestos-exposed A_L cells, HRP-catalyzed oxidation of fluorescent Amplex Red reagent, a sensitive technique for release of H_2O_2 [43], occured in a dose-dependent manner, reaching a peak of 0.32 ± 0.055 μM at a 6 $\mu g/cm^2$ dish dose of fibers (Fig. 1A). Concurrent treatment of A_L cells with catalase (1000 U/ml) suppressed H_2O_2 induction by 7- to 8-fold ($p < .05$), whereas heat-inactivated catalase (boiling for 10 min) had no effect. To show that ROS induced by asbestos fibers actually mediate mutagenic events, cells were exposed to either graded doses of crocidolite fibers for 24 h or H_2O_2 in serum-free medium for 15 min, which resulted in a dose-dependent increase in toxicity of A_L cells (Fig. 1B). The normal plating efficiency of A_L cells was $80 \pm 5\%$. The surviving fraction of A_L cells treated with a 2 $\mu g/cm^2$ dose of crocidolite fibers was $62 \pm 4\%$, and the value decreased to $26 \pm 5\%$ after exposure to 4 $\mu g/cm^2$ dose of crocidolite fibers. By comparison, survival of A_L cells after the addition of 4.4 or 13.2 mM H_2O_2 was $56 \pm 10\%$ and $24 \pm 5\%$, respectively. The background mutant fraction of A_L cells used in these experiments averaged 52 ± 15 mutants per 10^5 survivors. In contrast, the negative control titanium dioxide (TiO_2), at doses up to 12 $\mu g/cm^2$, was neither cytotoxic nor mutagenic to A_L cells when tested under similar conditions (data not shown). Data show that crocidolite fibers and H_2O_2 induced dose-dependent induction of $CD59^-$ mutants in A_L cells. The mutant fraction increases with the concentration of fibers, thus correlating with cytotoxic effects.

Role of asbestos and oxidants in cell injury and apoptosis

Although lytic cell death by asbestos has been characterized in many in vitro and in vivo models, several recent studies have established that asbestos also induces apoptosis, an ATP-dependent process, typified by membrane blebbing, cell shrinkage, nuclear chromatin condensation, and DNA fragmentation [44–48]. The pathophysiologic significance of these findings as well as the mechanisms involved are not firmly established.

Accumulating evidence suggests that apoptosis of alveolar epithelial cells (AEC) has an important role in the pathogenesis of lung disorders [49]. Apoptosis is the major pathway responsible for resolving alveolar type 2 cell hyperplasia that occurs in acute lung injury; but, if exuberant, apoptosis may promote epithelial barrier dysfunction, lung injury, and fibrosis [49–51]. Acute lung injury that resolved completely demonstrated apoptotic lung epithelial cells that fully regressed, whereas those animals whose lungs progressed to the fibroproliferative phase showed persistent epithelial cell apoptosis [51]. Stimuli that induced acute lung injury, such as bleomycin and hyperoxia, triggered apoptosis in the bronchiolar and alveolar epithelium as assessed by DNA laddering and terminal deoxynucleotidyl transferase- (TdT-) mediated dUTP-biotin nick end labeling (TUNEL) [52,53]. DNA strand breaks and apoptosis also occured in the bronchiolar and alveolar epithelium of patients with idiopathic pulmonary fibrosis, a disease that shares many histopathologic features with asbestosis [54]. Consistent with these findings in humans, rats exposed to asbestos demonstrated unscheduled DNA synthesis, as detected by increased numbers of alveolar type 2 cells incorporating tritiated thymidine and TUNEL staining at the bronchiolar-alveolar duct junctions [48,55]. However, mice exhibiting increased TUNEL staining in bronchiolar and pulmonary epithelial cells after intratracheal injection of asbestos showed primarily lytic cell injury when lungs were examined by transmission electron microscopy [56]. Collectively, these data suggest an important pathophysiologic role of DNA damage, lytic cell death, and apoptosis in the response of the lung epithelium during injury and repair.

Although the mechanisms underlying asbestos-induced apoptosis are not fully understood, free radicals

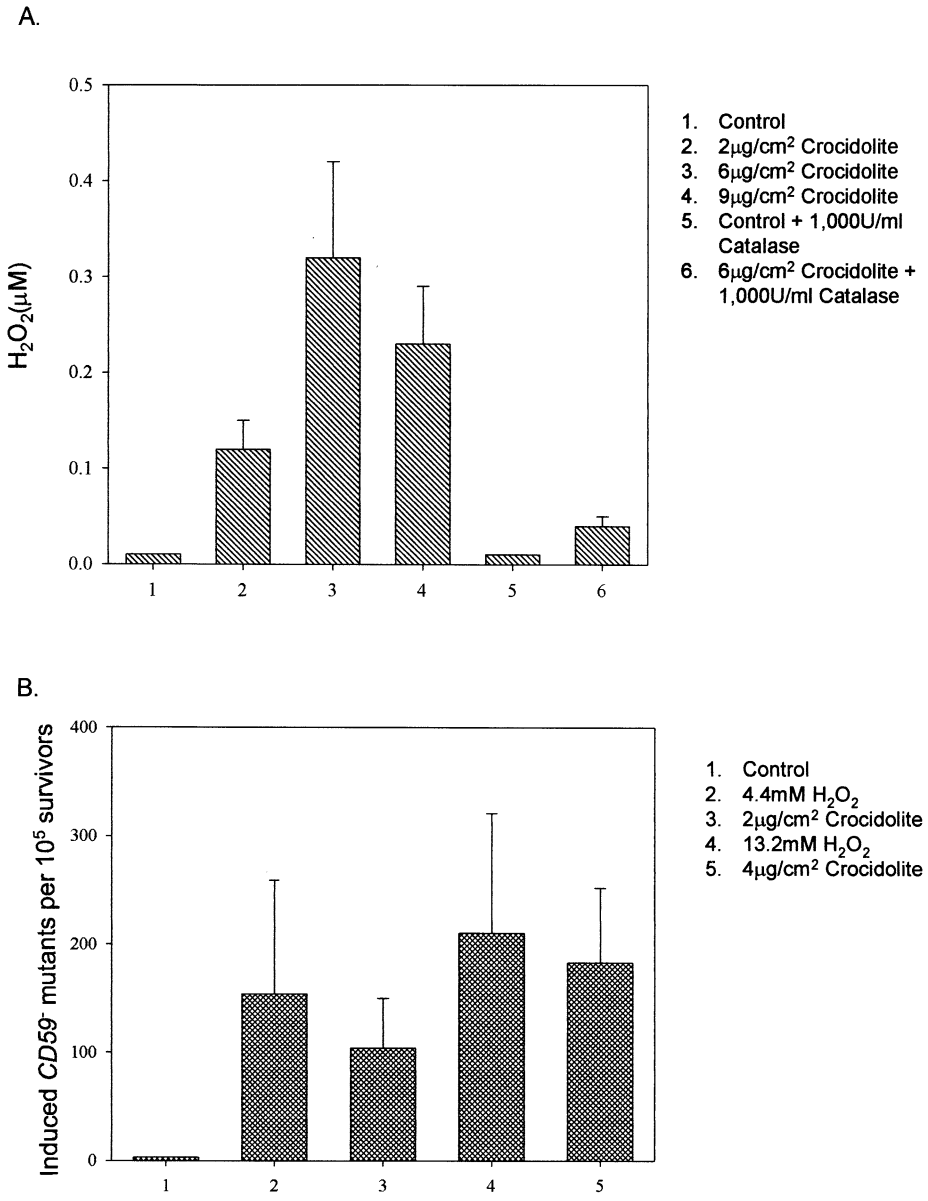

Fig. 1. (A) Production of H_2O_2 in A_L cells exposed to graded concentrations of crocidolite fibers in the presence or absence of catalase (1000 U/ml). Exponentially growing A_L cells were plated in 96-microwell dishes, and the release of H_2O_2 was examined by Amplex Red reagent in the presence of HRP. Data were pooled from three independent experiments. Error bars indicate mean ± SD. (B) Induction of *CD59*$^-$ mutant fractions per 10^5 survivors in A_L cells exposed to either crocidolite fibers or H_2O_2. Results are expressed as the number of induced mutants (total mutant yield minus background) per 10^5 survivors. The average number of pre-existing *CD59*$^-$ mutants per 10^5 survivors in A_L cells used for these experiments was 52 ± 15. Data were pooled from three independent experiments. Error bars indicate mean ± SEM.

have been implicated. For example, catalase and deferoxamine reduced mesothelial cell apoptosis, suggesting a role for iron-derived ROS [45]. Uptake of asbestos by an integrin-dependent mechanism involving vitronectin augmented oxidative mesothelial cell DNA damage [57]. Moreover, iron-derived ROS were involved since free radical scavengers and iron chelators ameliorated apoptosis in AEC [48]. In comparison to primary cultured mesothelial cells, mesothelioma cell lines were highly resistant to apoptosis, in part due to increased antioxidant defenses [e.g., manganese superoxide dismutase (Mn-SOD) and catalase] as opposed to differences in the expression of Bcl-2 apoptosis-regulating proteins [58]. In addition, antioxidants, as well as MnSOD overexpression, prevented apoptosis and cell injury caused by tumor necrosis factor-α (TNF-α), H_2O_2, and asbestos [59–61].

These data suggest that asbestos-induced ROS have a critical role in triggering cell injury and apoptosis.

There are two major pathways that regulate apoptosis: (i) the intrinsic or mitochondria-regulated pathway; and, (ii) the extrinsic pathway induced by death-receptor ligands such as TNF-α or FasL and, subsequently, caspase-8 activation [62]. Mitochondria also are emerging as an organelle that is critically important in regulating complex survival signals that determine whether cells live or die after asbestos exposure. Mammalian cells typically contain thousands of mitochondria, each with nearly 10 copies of double-stranded genomic DNA-encoding 13 polypeptides that are primarily involved with the electron transport chain for oxidative phosphorylation [63]. Mitochondrial DNA is more susceptible to oxidative damage than nuclear DNA, as evidenced by a 10- to 220-fold greater mutation rate, and mitochondrial DNA may be a particularly important target of asbestos [64].

As recently reviewed elsewhere [65], some evidence demonstrates that asbestos causes AEC apoptosis by inducing mitochondrial dysfunction. Firstly, amosite asbestos, but not inert particulates such as glass beads or TiO_2, reduced AEC cell $\Delta\Psi_m$ in a dose- and time-dependent manner. Secondly, asbestos triggered the release of cytochrome *c* and activation of caspase-9, a mitochondria-dependent caspase. In contrast, caspase-8 activation, which is an important component of the death receptor pathway, was not detected. Finally, A549 cells that overexpress Bcl-X_L were protected against asbestos-induced $\Delta\Psi_m$ and DNA fragmentation. A role for iron-derived ROS in mediating mitochondrial dysfunction is suggested by these studies, since an iron chelator (phytic acid and deferoxamine) or a free radical scavenger (sodium benzoate) completely blocked the $\Delta\Psi_m$ as well as the caspase-9 activation. These data are consistent with studies showing that iron chelators and ROS scavengers attenuated asbestos-induced AEC and mesothelial cell apoptosis [45,48]. Taken together, the evidence implicates mitochondria in regulating asbestos-induced apoptosis by mechanisms involving the generation of iron-derived free radicals.

The mechanisms by which iron-derived ROS stimulate apoptosis are not fully established. As reviewed elsewhere [66], there are multiple sources of ROS after cells are exposed to apoptogenic stimuli such as asbestos. These include mitochondria, NAD(P)H oxidoreductases (e.g., cytochrome P450 and nitric oxide synthase), molybdenum hydroxylases (e.g., xanthine oxidoreductase and aldehyde reductase), and arachidonic acid-metabolizing enzymes (e.g., cyclooxygenase and lipoxygenase). Moreover, iron associated with asbestos fibers can promote the Fenton reaction resulting in the formation of the highly toxic $^{\bullet}OH$ (see above). Recent studies also suggest that asbestos-induced DNA damage is dependent on the intracellular levels of redox-active iron as well as glutathione (GSH) depletion [67]. These findings with asbestos are consistent with accumulating evidence demonstrating that mitochondrial glutathione (GSH) is important for preventing apoptosis caused by various agents such as TNF-α, H_2O_2, and ceramide [66,68]. Also, cytochrome *c* is an iron-containing, redox-cycling protein whose redox state is largely controlled by intracellular GSH levels [69]. Future investigations should provide further insight into the origin of iron-derived ROS and the molecular mechanisms involved.

The protective effects of Bcl-X_L overexpression against the asbestos-induced AEC $\Delta\Psi_m$ and DNA fragmentation mentioned above suggests that the permeability of the outer mitochondrial membrane is a crucial regulating target for asbestos-exposed AEC [65]. These findings concur with a substantial body of evidence from other models demonstrating that antiapoptotic Bcl-2 family members prevent apoptosis by inhibiting the $\Delta\Psi_m$ and release of cytochrome *c* [70].

There is some information suggesting that the apoptotic death receptor pathway also has an important role in mediating asbestos-induced lung injury. As reviewed elsewhere [33,71], TNF-α is a key cytokine involved in the pathogenesis of asbestos-associated pulmonary toxicity. The cellular effects of TNF-α are mediated by two TNF-α receptors (TNFR) that have a similar extracellular domain but vary in their intracellular domains and size (55 kDa vs. 75 kDa). Asbestos caused inflammation and fibrosis in wild-type and single-TNFR knockout mice, but no discernible damage or fibrosis was evident in the double-TNFR knockout mice [72]. Iron-derived free radicals are important in stimulating TNF-α release from rat alveolar macrophages, since iron chelators or free radical scavengers blocked TNF-α release while ferrous sulfate or inhibition of intracellular catalase augmented TNF-α release [73]. These data provide compelling evidence that TNF-α and iron-derived ROS are key proximal mediators of asbestos pulmonary toxicity, functioning in part by activating apoptotic death pathways.

EVIDENCE FOR OXIDANTS IN ASBESTOS-INDUCED INFLAMMATORY RESPONSES AND DISEASE

Although lung fluids have a battery of antioxidants including ceruloplasmin and extracellular SOD, the lung environment may also promote formation of ROS from asbestos. For example, surfactant-coated crocidolite fibers released more iron than native fibers at both pH 4.5 and 7.2, an observation indicating that in vivo lung lining fluid coats native fibers and, therefore, affects the fiber surface chemistry and reactivity [74]. Also, it has been demonstrated that intratracheal instillation of crocidolite

asbestos causes free radical generation in rodent lungs [75]. Use of phytic acid, an iron chelator, attenuated crocidolite-induced pulmonary inflammation and fibrosis in rats [76].

The relevance of ROS to asbestos-induced inflammation and pulmonary fibrosis after inhalation of crocidolite asbestos was confirmed in rats with polyethylene glycol-conjugated catalase administered chronically in Alzet minipumps [77]. These mice, but not those receiving solvent or inactivated catalase, showed significantly decreased lung injury, inflammation, and pulmonary fibrosis.

RNS AND PULMONARY TOXICITY

In addition to ROS-induced effects, pulmonary toxicity by asbestos may be modulated by nitric oxide ($NO^{\bullet}$) or peroxynitrite ($ONOO^{-}$) [78]. Use of a nitric oxide synthase (NOS) inhibitor prevented asbestos-induced formation of 8-OHdG, supporting a role for $NO^{\bullet}$ in asbestos-induced oxidation [79]. NOS activity in rat lung and $NO^{\bullet}$ synthesis in alveolar macrophages increased after instillation of asbestos into the trachea [80]. It has been suggested that small numbers of crocidolite fibers translocate to the pleural space, where they provide a stimulus for persistent pleural space inflammation, cytokine production, and generation of toxic oxygen and nitrogen radicals [81].

The induction in iNOS protein expression and the formation of nitrotyrosine in mesothelial cells has been demonstrated after exposure to different types of asbestos fibers [82]. Using a promoter-reporter assay, asbestos-induced transactivation of iNOS also has been shown in murine macrophage-like cells in vitro [83]. Increased steady state levels of iNOS mRNA and production of nitrites/nitrates by alveolar macrophages isolated from rats were seen after inhalation of asbestos [83]. Additionally, strong immunoreactivity for nitrotyrosine, a marker of $ONOO^{-}$ formation, was detected in the lungs (at alveolar duct bifurcations and within the bronchiolar epithelium) from chrysotile- and crocidolite-exposed rats [84]. Recent studies in murine macrophages suggest that crocidolite stimulates NOS expression by decreasing the iron bioavailability and activating an iron-sensitive transcription factor [85]. Thus, asbestos-induced upregulation of iNOS or $NO^{\bullet}$ in the lungs, as well as the induction of inflammation by fibers, may contribute to the pathogenesis of lung injury [86].

ROLE OF ROS AND RNS IN ASBESTOS-INDUCED CELL SIGNALING

After interaction with cells, asbestos fibers triggered numerous signaling cascades involving mitogen-activated protein kinases (MAPK) and nuclear factor κB (NF-κB) [87,88]. Activation of transcription factors such as NF-κB and activator protein-1 (AP-1) may be linked to increases in early response genes (i.e., *jun* and *fos*), which govern proliferation, apoptosis, and inflammatory changes [89]. Recent evidence indicates that asbestos can stimulate gene expression in a variety of cell types via intracellular signaling pathways upstream of gene transactivation. As discussed above, the generation of oxidants during the phagocytic burst and/or during frustrated phagocytosis of long fibers by alveolar macrophages and other cell types may be responsible for the initiation of cell signaling and inflammatory events. Alternatively, asbestos fibers may interact with receptors triggering these pathways via metals that cause aggregation and phosphorylation of these proteins, i.e., the epidermal growth factor receptor (EGFR) [90,91].

A survey of different industrial fibers indicates the role of iron in oxidative stress and activation of transcription factors [92]. These authors concluded that intrinsic free radical activity is the major determinant of transcription factor activation and gene expression in alveolar macrophages. In pleural mesothelial cells, apoptotic concentrations of crocidolite asbestos caused phosphorylation and activation of extracellular signal-regulated kinases (ERK 1/2) in an oxidant-dependent manner [93]. Asbestos-induced ERK activation and apoptosis were blocked by catalase, N-acetyl-L-cysteine, and by chelation of surface iron from crocidolite fibers, indicating an oxidative mechanism in the activation of this signaling cascade [93]. Asbestos fibers, as opposed to nonpathogenic minerals, caused a persistent induction of the redox-sensitive transcription factors NF-κB and AP-1 in mesothelial and lung epithelial cells that was accompanied by alterations in gene expression [94,95]. Treatment of the rat lung fibroblast cell line (RFL-6) with crocidolite asbestos in the presence and absence of the membrane antioxidant vitamin E decreased levels of crocidolite-induced AP-1 and NF-κB to background levels, indicating the involvement of lipid peroxidation [96]. Exposure of tracheal epithelial and mesothelial cells or alveolar macrophages to asbestos caused persistent increases in redox factor-1 (Ref-1 or APE) and AP-1 to DNA-binding activity [94,97], an effect mediated by oxidative stress [98]. In a rat tracheal explant model, exposure to iron-loaded amosite asbestos resulted in increased procollagen gene expression, a process that is supposedly driven in part through oxidant-induced NF-κB activation [99]. Iron loading also increased gene expression of platelet-derived growth factor- (PDGF-) A and transforming growth

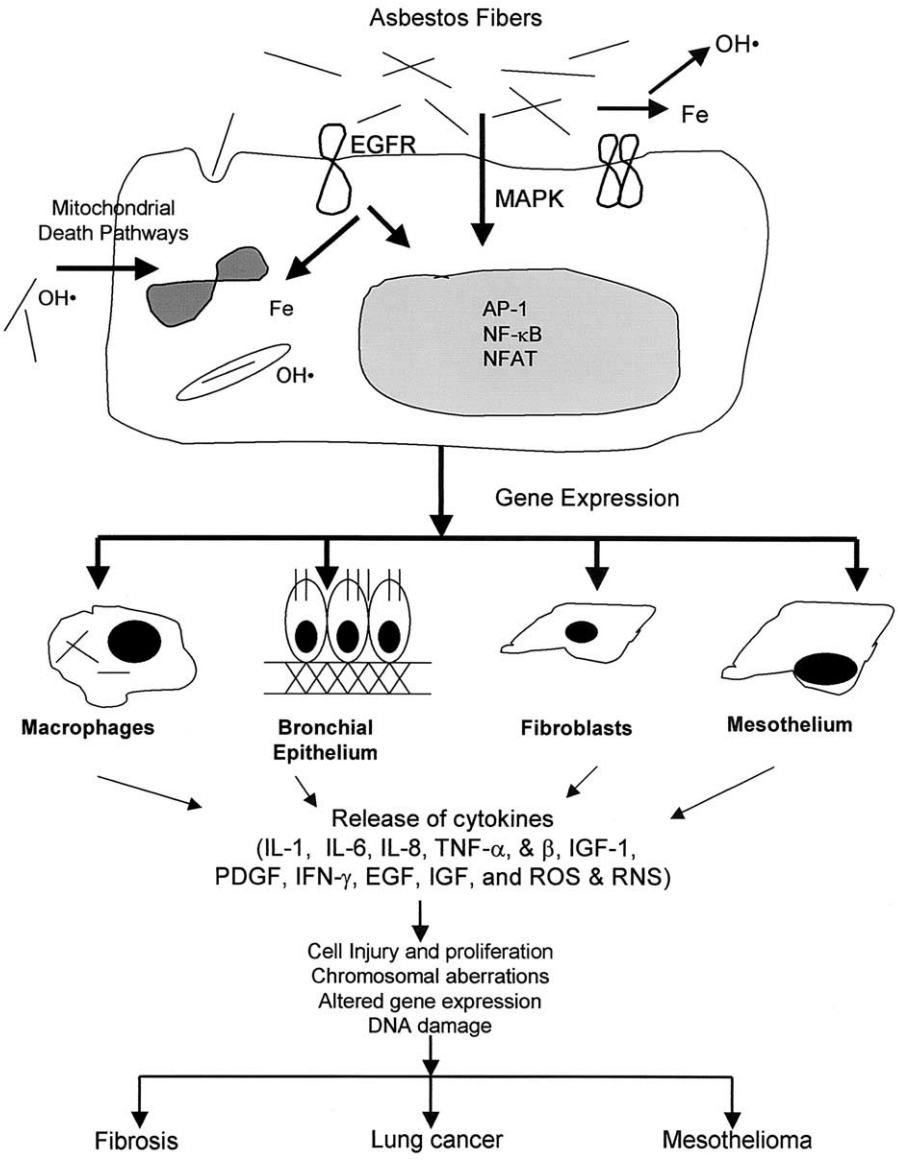

Fig. 2. Hypothetical schema of the role of oxidants in asbestos-related cell signaling, injury, and disease.

factor-β (TGF-β) through an ERK-dependent mechanism [99].

These growth factors, proinflammatory cytokines, and chemotactic peptides are strongly implicated as mediators of the pathophysiologic responses leading to asbestosis. For example, asbestos-induced redox changes and phosphorylation events activated nuclear proteins that recognized the NF-κB/NF-IL-6 binding sites of the interleukin-8 (IL-8) and interleukin-6 (IL-6) promoter and contributed to the regulation of IL-8 and IL-6 gene expression [100–102]. Asbestos-induced TNF-α gene expression and secretion were mediated by iron-catalyzed product of ROS [73] and were mediated through a process that involves NF-κB activation [103]. Crocidolite-induced activation of nuclear translocation of NF-κB as well as MIP-2 gene expression in rat alveolar type 2 cells also have been shown to be dependent on mitochondrial-derived oxidative stress [104]. Among various carcinogenic and noncarcinogenic fibers studied for their effect on translocation of NF-κB to the nucleus in A549 cells, only carcinogenic fibers were found to cause a dose-dependent nuclear translocation of NF-κB, and this effect was found to be oxidative stress-dependent [105]. Recently, another transcription factor, the nuclear factor of activated T cells (NFAT), has been shown to be induced by asbestos-induced oxidative stress in mouse embryo fibroblasts [106].

CONCLUSIONS AND CLINICAL RELEVANCE

Asbestos is a physiologically relevant oxidative stress in lung that has resulted in thousands of deaths from lung cancers, mesotheliomas, and pulmonary fibrosis. The potency of various types of asbestos in human disease was first suggested in the classical epidemiological study by Wagner and colleagues [107], which described a high incidence of mesotheliomas in South African crocidolite miners, some tumors in workers mining amosite, and no tumors in workers mining chrysotile asbestos. Over the years, the observations and reports cited herein have given rise to a free radical theory of asbestos-induced diseases. This hypothesis is supported by the direct demonstration that iron-containing asbestos participates in a Fenton-like reaction and that long asbestos fibers, which are more carcinogenic and fibrogenic than short (≤ 5 μm) fibers, cause a prolonged release of oxidants from cells due to frustrated phagocytosis. In addition, indirect evidence of oxidant stress, indicated by the upregulation of antioxidant enzymes, occurs in lungs of rodents after inhalation of asbestos [108]. The ratio of oxidative stress to antioxidant defense presumably determines whether lung or pleural disease will occur.

Chelation of iron and other approaches to modify the surface chemistry and/or mobilization of iron appear to be protective in terms of asbestos toxicity and fibrosis in rodents; but, the real issue in heavily exposed individuals with an appreciable lung content of asbestos is how to curb the constant oxidant elaboration due to fibers or recycling of fibers by cells in the lung. The results of the recent retinoid trials in individuals at high risk for lung cancer have been controversial [109–111]; dietary supplementation may not directly affect antioxidant levels in lung epithelial cells. Targeted delivery of antioxidants by nebulization or other methods of administration may prove more beneficial.

Research over the last decade has been instrumental in revealing the properties of asbestos important in toxicity, inflammation, and disease, and also has provided mechanistic information on cell signaling and other responses that may be exploited for prevention and therapy of various asbestos-induced cancers and fibroproliferative diseases. A summary of these pathways is provided in Fig. 2. Briefly, oxidant-induced activation of specific receptor-dependent (i.e., EGFR, independent signaling pathways such as MAPKs) may be critical in the activation of transcription factors including AP-1 and NF-κB. In addition, mitochondrial death pathways and other protein kinases (e.g., PKCs) or DNA-damaging effects of asbestos may be critical to cell injury and proliferation. Knowledge of these pathways, their cross-talk, and modulation is essential to the control of cell injury, proliferation, and transformation by asbestos.

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ABBREVIATIONS

AEC—alveolar epithelial cells
8-OHdG—8-hydroxydeoxyguanosine
GSH—glutathione
MAPK—mitogen-activated protein kinase
NF-κB—nuclear factor κB
NFAT—nuclear factor of activated T cells (NFAT)
$NO^{\bullet}$—nitric oxide
NOS—nitric oxide synthase
$ONOO^{-}$—peroxynitrite
RNS—reactive nitrogen species
ROS—reactive oxygen species
TdT—terminal deoxynucleotidyl transferase
TiO_2—titanium dioxide
TNF-α—tumor necrosis factor-α