Mitochondrial-derived free radicals mediate asbestos-induced alveolar epithelial cell apoptosis

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Asbestos fibers cause asbestosis and malignant bronchogenic carcinoma and mesothelioma by mechanisms that have not been fully established (26, 27, 31). Alveolar epithelial cell (AEC) injury is one important event implicated in the pathogenesis of pulmonary toxicity from a variety of agents, including asbestos (7, 27, 41). Asbestos fibers are internalized by AEC soon after exposure, resulting in cellular injury, increased permeability, and proliferation (7, 27, 31). Reactive oxygen species (ROS) such as hydrogen peroxide (H2O2), superoxide anion (O2−), and hydroxyl radical (•OH) are implicated in mediating asbestos-induced AEC injury in part by causing DNA damage, apoptosis, lipid peroxidation, and activation of signal transduction pathways (1, 25–27, 31, 39). Asbestos induces ROS production by multiple mechanisms that in part involve activation of inflammatory cells, the ionic state of the iron in asbestos fibers, the mitochondria, and other intracellular sources (24, 39). Notably, electron spin resonance trapping methods revealed that iron derived from asbestos promotes the formation of the highly reactive •OH in a cell-free system that contains H2O2 and a reducing agent (43). Thus the precise source of ROS involved in mediating asbestos-induced AEC apoptosis is unclear.

Apoptosis is an important mediator of AEC injury induced by asbestos (5, 16, 20, 35). This leads to the reduction of oxygen to O2• which is dismutated to H2O2. It is unknown whether the iron derived from the asbestos fibers or the mitochondria are the primary source of ROS production that causes AEC apoptosis. In the present study, we hypothesized that asbestos-induced AEC apoptosis is primarily caused by mitochondrial-derived ROS. We hypothesized that a functional electron transport chain is necessary for generating ROS that mediate asbestos-induced AEC apoptosis. To address this hypothesis, we utilized A549 cells that lack mitochondrial DNA (mtDNA) and a functional electron transport chain (A549-p0 cells) that were prepared by long-term growth in ethidium bromide (12).
cells lack critical respiratory chain catalytic subunits that are encoded by the mitochondrial genome, so the mitochondria cannot support normal oxidative phosphorylation or generate ROS via mitochondrial electron transport chain members that cannot support normal oxidative phosphorylation or generate ROS encoded by the mitochondrial genome, so the mitochondria

**METHODS**

Asbestos and reagents. Amosite asbestos fibers used in these experiments were Union International Centere le Cancer reference standard samples kindly supplied by Dr. V. Timbrell (40). These amphibole fibers are 70% respirable (length between 2 and 5 μm), whereas the remainder are >5 μm in length. Stock solutions (5 mg/ml) were prepared in HBSS with calcium, magnesium, and 15 mM HEPES. All suspensions were autoclaved and stored at 4°C. Samples were warmed to 37°C and vigorously vortexed before being used to ensure a uniform suspension. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

**RESULTS**

Apoptosis assays. Asbestos-induced A549 cell apoptosis was assessed by a combination of TdT-mediated dUTP nick end labeling (TUNEL)-stained nuclear morphology and histone-associated DNA fragmentation assays as previously described (1, 32). Briefly, A549 cells were exposed to amosite (0–50 μg/cm²) for 24 h, washed, permeabilized, treated for 60 min with a mixture of fluorescent-12-dUTP and TdT enzyme for 60 min, and then counterstained with propidium iodide (PI; 15 μg/ml). Cells were assessed under a fluorescence microscope by an investigator who was blinded to the experimental protocol. The apoptotic index was defined as the ratio of TUNEL-labeled green apoptotic cells:total number of red PI-labeled cells from a total of 200 cells assessed. Histone-associated DNA fragmentation (mono- and oligonucleosomes) was assessed from cell lysates using an ELISA assay (Roche Diagnostics) that was performed according to the manufacturer’s specifications (spectrophotometric wavelength: 405 nm) as previously described (1).

**FREE RADICALS MEDIATE ASBESTOS-INDUCED APOPTOSIS**

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Asbestos, unlike antimycin A, induces reactive oxygen species production in A549-\(\rho^0\) cells but significantly less than the levels noted in A549 cells. A549 (solid bars) and A549-\(\rho^0\) (open bars) cells were exposed to either control medium, amosite asbestos (50 \(\mu\)g/cm\(^2\)), or antimycin A (1 \(\mu\)g/ml) for 24 h, and then dichlorofluorescein (DCF) fluorescence was measured as described in methods. Data were normalized to fold increase over control value per microgram of protein and expressed as means ± SE (n = 6). *\(P < 0.05\) vs. control, †\(P < 0.05\) vs. A549 cells.

Asbestos-induced caspase-9 activity is decreased in \(\rho^0\) cells. We recently showed that the mitochondrial death pathway mediates asbestos-induced AEC apoptosis, as assessed by \(\Delta \Psi_m\), cytochrome \(c\) release from the mitochondria to the cytoplasm, and caspase-9 activation (32). To determine whether mitochondrial functional electron transport is necessary for mediating asbestos-induced caspase-9 activation, we compared caspase-9 activation in A549 and A549-\(\rho^0\) cells after exposure to various doses of asbestos (0, 5, 25, 50 \(\mu\)g/cm\(^2\)). As shown in Fig. 3, asbestos increased A549 caspase-9 activation in a dose-dependent manner similar to our previous report (32). Compared with A549 cells, asbestos-induced caspase-9 activation was decreased by nearly 50% in the \(\rho^0\) cells. Asbestos significantly increased caspase-9 activation in the \(\rho^0\) cells only after exposure to the highest dose tested. These data support the role of mitochondrial-derived ROS in mediating asbestos-induced caspase-9 activation, but nonmitochondrial sources of ROS may contribute to caspase-9 activation after exposure to high levels of asbestos.

A549-\(\rho^0\) cells are resistant to asbestos-induced apoptosis. To determine whether mitochondrial functional electron transport is important for mediating asbestos-induced AEC apoptosis, we compared apoptosis in A549 and A549-\(\rho^0\) cells exposed to various doses of amosite asbestos (0, 5, 25, 50 \(\mu\)g/cm\(^2\)) for 24 h. We also assessed apoptosis caused by staurosporine (1 \(\mu\)M) or etoposide (100 \(\mu\)M), two agents known to induce apoptosis in \(\rho^0\) cells. As shown in Fig. 4, asbestos increased apoptosis by nearly twofold in A549 cells as assessed by TUNEL staining. In contrast, asbestos induced negligible apoptosis in \(\rho^0\) cells after exposure to all doses of asbestos tested. Notably, A549-\(\rho^0\) cells were capable of undergoing apoptosis when exposed to either staurosporine or etoposide (Fig. 4). Therefore, despite complete loss of mtDNA and a deficient respiratory chain function, A549-\(\rho^0\) cells maintained sufficient apoptotic machinery that could be activated by staurosporine or etoposide but not by asbestos.
We also assessed asbestos-induced apoptosis using a highly sensitive assay of DNA nucleosomal fragmentation that we previously showed directly correlates with nuclear morphology and caspase-3 activation (1). As shown in Fig. 5, asbestos increased A549 cell DNA fragmentation similar to what we previously reported (1). In contrast, asbestos caused significantly less DNA fragmentation in \( \rho^0 \) cells after exposure to all doses of asbestos tested. Notably, the lowest dose of asbestos tested \( (5 \mu \text{g/cm}^2) \) caused a 2.8-fold increase of DNA fragmentation in A549 cells but a negligible increase in \( \rho^0 \) cells. Similar to our findings of DCF fluorescence, total GSH levels, and caspase-9 activation in asbestos-exposed \( \rho^0 \) cells, only the highest dose of asbestos tested increased DNA fragmentation but at levels that were nearly 50% less than seen in A549 cells.

Asbestos-induced reductions in A549 cell \( \Delta \Psi_m \), caspase-9 activation, and apoptosis are blocked by DIDS. Mitochondria-generated ROS, such as \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \), are transported to the cytoplasm by voltage-dependent anion channels (VDAC) (22, 23). The mitochondrial anion channel inhibitor DIDS protects cells by blocking the egress of ROS from the mitochondria (11). To determine whether DIDS prevents asbestos-induced A549 cell mitochondrial dysfunction, A549 cells were treated with DIDS \( (10 \mu \text{M}) \) for 1 h, and then asbestos-induced \( \Delta \Psi_m \) and caspase-9 activation were assessed. As shown in Fig. 6, DIDS completely blocked asbestos \( (25 \mu \text{g/cm}^2) \)-induced A549 cell \( \Delta \Psi_m \) and caspase-9 activation and provided partial protection \( (\sim 70\%) \) against the highest dose of asbestos tested, 50 \( \mu \text{g/cm}^2 \). To determine whether DIDS could block downstream apoptosis, we treated A549 cells with DIDS as before and then assessed asbestos-induced apoptosis after a 24-h exposure period by TUNEL staining. As shown in Fig. 7, DIDS completely blocked asbestos-induced A549 cell TUNEL staining. Collectively, these data demonstrate that DIDS inhibits asbestos-induced A549 cell mitochondrial dysfunction and apoptosis.

Role of nonmitochondrial ROS in mediating asbestos-induced caspase-9 activation and apoptosis. A role for nonmitochondrial-derived ROS in mediating asbestos-induced AEC apoptosis was suggested by the incomplete protective effects and persistent oxidative stress noted in \( \rho^0 \) cells exposed to high-dose asbestos \( (50 \mu \text{g/cm}^2) \) as described above. Although the mitochondria are a major source of ROS generation in cells, there are other sources as well (24). Also, redox-active ferrous iron in the core of the asbestos fiber or as a surface contaminant can generate ROS (39, 43). To further examine the role of nonmitochondrial-derived ROS, we determined whether an iron chelator [phytic acid \( (0.05 \mu \text{M}) \)] or a free radical scavenger \([\text{N-acetyl cysteine (1 mM)}\)] provides additional protection against asbestos \( (50 \mu \text{g/cm}^2) \)-induced caspase-9 activation and DNA fragmentation in \( \rho^0 \) cells. As shown in Fig. 8, each inhibitor significantly attenuated A549 cell caspase-9 activation and DNA fragmentation similar to what we have previously reported (32). Consistent with our earlier findings in this study, asbestos-induced caspase-9 activation and DNA fragmentation were reduced by \( >50\% \) in A549-\( \rho^0 \) cells compared with A549 cells. Notably, compared with \( \rho^0 \) cells, the addition of either an iron chelator or a free radical scavenger each significantly reduced the toxic effects of asbestos in \( \rho^0 \) cells back to levels approaching unexposed A549 cells. These data support the role of nonmitochondrial-derived ROS in mediating AEC apoptosis after exposure to high-dose asbestos.

**DISCUSSION**

Altered apoptotic mechanisms are implicated in the pathogenesis of various pathological conditions, including pulmonary fibrosis and malignancies associated with asbestos exposure. However, the molecular mechanisms, and, more importantly, the precise source of ROS are not firmly established (27, 31, 39). The novel finding of this study is that mitochondria, rather than the fiber or other intracellular sources of ROS production, are the critical site of asbestos-induced ROS generation that leads to caspase-9 activation and apoptosis. We also show that nonmitochondrial-derived ROS may be impor-
tant for causing caspase-9 activation and apoptosis, but only after exposure to high-dose asbestos (50 μg/cm²). These findings provide insight into the precise source of ROS formation in asbestos-exposed AEC that are important in mediating apoptosis to an important target, the alveolar epithelium.

The mitochondria have a pivotal role in regulating apoptosis after exposure to a wide variety of apoptogenic agents, including asbestos (5, 16, 32, 35). This organelle, which is critically involved in ATP synthesis, is also a major source of ROS production in cells and the initial subcellular compartment damaged by these ROS (20, 35). Consistent with work by others and ourselves (27, 31, 39), we showed that asbestos causes an oxidative stress on the A549 cells as assessed by both an increase in DCF fluorescence (Fig. 1) and a reduction in total GSH levels (Fig. 2). To determine the role of mitochondrial-derived ROS in mediating asbestos-induced AEC apoptosis, we utilized A549-ρ^0 cells that are incapable of generating mitochondrial-derived ROS (12). These cells were prepared by long-term ethidium bromide exposure that renders them incapable of normal oxidative phosphorylation because they are depleted of critical respiratory chain subunits that are encoded by mtDNA (11, 12, 28). The A549-ρ^0 cells are dependent on ATP derived from anaerobic glycolysis and possess a functional F1-ATPase as well as an adenine nucleotide transporter that maintains ΔΨ_m (8, 12). We show that, compared with A549 cells, the A549-ρ^0 cells were significantly protected against asbestos-induced caspase-9 activation (Fig. 3) and apoptosis as assessed by TUNEL staining (Fig. 4) and DNA fragmentation (Fig. 5). The protective effects observed were nearly complete except after high-dose asbestos exposure (50 μg/cm²). As expected in cells lacking a functional electron transport chain, the A549-ρ^0 cells, unlike A549 cells, are incapable of antimycin-induced ROS production (Fig. 1). Antimycin inhibits center “i” of complex III in the mitochondria, resulting in potent ROS production in normal cells (6, 11, 23). Notably, the A549-ρ^0 cells used in these studies had an intact apoptotic machinery because these cells were capable of undergoing apoptosis on exposure to etoposide or staurosporine, two agents known to induce apoptosis in other ρ^0 cell types (Fig. 4) (12). We utilized the well-characterized A549 cell line to prepare ρ^0 cells because it has some features of ATII cells and because we previously showed that asbestos induces DNA damage, mitochondrial dysfunction, and apoptosis to a similar degree in both cell types (1, 25, 32). Collectively, these data support our hypothesis implicating mitochondrial-derived ROS in mediating asbestos-induced AEC apoptosis.

Because mitochondria are a major source of ROS production in cells, they are particularly sensitive to oxidant-induced damage (20, 35). Mitochondrial-derived ROS utilize anion channels, such as VDAC, to exit the mitochondria into the cytoplasm (22, 23, 35). We used DIDS, a mitochondrial anion channel inhibitor known to block ROS transport from the mitochondria to the cytoplasm (11), to show that it significantly blocks asbestos-induced ΔΨ_m, caspase-9 activation, and
apoptosis as assessed by TUNEL staining (Figs. 6 and 7).

Similar to our studies with A549 p0 cells, the protective effects observed with DIDS were nearly complete except after high-dose asbestos exposure (50 μg/cm²). Our results are also consistent with our prior study showing that overexpression of Bel-XL, an antiapoptotic protein that localizes to the mitochondrial membrane, prevents asbestos-induced AEC mitochondrial dysfunction and apoptosis (32). Although the precise molecular mechanisms underlying the protective effect of Bel-XL are not firmly established, the current evidence suggests that it prevents ROS production and subsequent apoptosis by inhibiting the mitochondrial outer membrane permeabilization rather than by acting as a free radical scavenger (35). Together, our results suggest an important role for mitochondrial anion channels regulating mitochondrial-derived ROS toxicity by asbestos.

In this study, we showed that asbestos reduced total GSH levels in A549 cells in a dose-dependent manner and in close proximity to mitochondrial dysfunction and apoptosis. Moreover, our data implicate an important role for mitochondrial-derived ROS in reducing the levels of total GSH since A549-p0 cells were less affected than A549 cells. GSH, which is one of the major intracellular antioxidants important for preventing lung epithelial cell apoptosis (24, 29), is found in the lung epithelial lining fluid in concentrations that are 100-fold greater than in plasma (34, 42). Although the effects of asbestos on GSH levels in the lung epithelial lining fluid is unknown, other pulmonary diseases, such as idiopathic pulmonary fibrosis (10) and acute respiratory distress syndrome (9), are associated with markedly reduced levels. Recent studies have established that depletion of GSH and, in particular, mitochondrial GSH, occurs early after exposure to genotoxic agents that cause apoptosis (3, 15, 36). Our findings also concur with the observation that asbestos-induced DNA damage is due in part to GSH depletion by iron-derived ROS (4). Although we did not examine mitochondrial GSH levels in our model, our data parallel observations from the literature that support its important role. The protective effects of A549-p0 cells were not due to increased total GSH levels since we found reduced levels compared with A549 cells. Future studies determining whether other antioxidant defenses are altered, as occurs in other cell types, should be of interest in more fully understanding the mechanisms underlying the survival advantage of p0 cells (33).

Findings in this study suggest that high-dose asbestos (50 μg/cm²) causes apoptosis by an oxidative stress on A549 cells that is partly due to nonmitochondrial-derived ROS. The increase in DCF fluorescence (Fig. 1) and decrease in total GSH (Fig. 2) in A549-p0 cells that are incapable of generating mitochondrial-derived ROS support this conclusion. Although A549-p0 cells were partially protected (~50%) against high-dose asbestos, we observed significant levels of caspase-9 activation (Fig. 3) and apoptosis as assessed by a highly sensitive DNA fragmentation assay (Fig. 5). We also noted that an iron chelator or free radical scavenger provided additional protection to A549-p0 cells exposed to high-dose asbestos (Fig. 8). There are other sources of ROS besides mitochondria that include NAD(P)H oxidoreductase (e.g., cytochrome P-450 and nitric oxide synthase), molybdenum hydroxylase (e.g., xanthine oxidoreductase and aldehyde reductase), and arachidonic acid metabolizing enzymes (e.g., cyclooxygenase and lipoygenase) (24). Also, previous studies have established that the ferrous iron in asbestos can participate in a Fenton reaction, resulting in the formation of the highly reactive ·OH (39, 43). Another possibility is that redox active iron released from mitochondrial storage sites may contribute to oxidative injury and apoptosis. The primary focus of this study was to determine the role of mitochondrial-derived ROS given the importance of low-level asbestos exposure in causing malignancies and the questionable relevance of high-level asbestos exposure except in the occupationally exposed patients of the past. However, our findings suggest that future investigations exploring the source of nonmitochondrial ROS will be of interest.

Data presented in this study suggest that mtDNA damage is an important target of asbestos since A549-p0 cells that lack mtDNA underwent significantly less asbestos-induced caspase-9 activation and apoptosis. The protective effects of iron chelators and free radical scavengers noted in this study parallel the observations of Grishko and colleagues (21) who showed that these agents blocked oxidant-induced pulmonary vascular endothelial cell mtDNA damage as assessed using quantitative Southern and ligation-mediated PCR analyses. Compared with nuclear DNA, mtDNA is more susceptible to
oxidative DNA damage and acquires mutations at a 10-fold higher rate (17, 30, 35, 38). The explanation for this enhanced sensitivity of mtDNA to oxidative stress includes proximity to the respiratory chain, relative lack of protective histones, and limited DNA repair capacity. Human mtDNA is a 16,569-base pair closed-circular molecule that is present in high numbers per cell but varies per cell type (30). mtDNA encodes for 13 proteins, some of which are critically involved in the electron transport chain, including complex I (NADH dehydrogenase), complex III (bcl complex), complex IV (cytochrome c oxidase), and F1F0-ATP synthase (2). With the use of real-time quantitative PCR, Mambo and colleagues (30) recently showed that the D-loop in mtDNA is particularly susceptible to oxidative damage that is in part attributed to inefficient DNA repair capacity. Accumulating evidence shows that strategies aimed at augmenting mtDNA repair can limit oxidant-induced mtDNA damage and apoptosis in vascular endothelial cells (13, 14, 21). Both H2O2 and asbestos augment mitochondrial translocation of the DNA repair enzyme apurinic/apyrimidinic endonuclease (APE/Ref1), which is involved in repair of oxidative DNA damage (18, 19). Santos and associates (37) recently demonstrated, using fibroblast cell sorting experiments, that persistent H2O2-induced mtDNA damage, compared with nuclear DNA damage, caused reductions in ΔΨm and subsequent apoptotic cell death. There is also some recent direct evidence linking asbestos-induced mtDNA damage to subsequent apoptosis (39). Collectively, these data suggest that mtDNA is an important target of asbestos-induced ROS. Further studies are necessary to determine the significance of mtDNA damage in the pathogenesis of asbestos-associated pulmonary toxicity as well as the molecular mechanisms regulating the cellular response to mtDNA damage.

In summary, we have shown that mitochondrial-derived ROS are important for mediating asbestos-induced AEC apoptosis. A role for ROS from nonmitochondrial sources is also suggested, but the significance of this observation is limited to exposure to high-dose asbestos. The relevance of our in vitro findings requires further study. However, the importance of asbestos-induced mtDNA damage is supported by the observations that mitochondrial mutations are noted to occur in a wide array of degenerative diseases as well as cancer (30) and by the increasing evidence of a direct relationship between mtDNA damage and the onset apoptosis (37, 39). Our data add to the accumulating evidence that convincingly show that ROS are an important regulator of apoptosis and extend these observations by implicating mitochondrial-derived free radicals (35). We reason that strategies aimed at reducing the burden of asbestos-induced mitochondrial-derived ROS production as well as mtDNA damage should preserve the barrier function of the alveolar epithelium and thereby prevent diseases such as asbestososis and malignancies.

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