Asbestos-induced lung epithelial permeability: potential role of nonoxidant pathways

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Peterson, Michael W, and Jennifer Kirschbaum. Asbestos-induced lung epithelial permeability: potential role of nonoxidant pathways. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L262–L268, 1998.—Asbestos fibers are an important cause of lung fibrosis; however, the biological mechanisms are incompletely understood. The lung epithelium serves an important barrier function in the lung, and disrupting the epithelial barrier can contribute to lung fibrosis. Lung epithelial permeability is increased in patients with asbestosis, and asbestos fibers increase permeability across cultured human lung epithelium. However, the mechanism of this increased permeability is not known. Many of the biological effects of asbestos are postulated to be due to its ability to generate oxidants, and oxidants are known to increase epithelial permeability. However, we previously reported that altering the iron content of asbestos (important in oxidant generation) had no effect on its ability to increase permeability. For that reason, we undertook these studies to determine whether asbestos increases epithelial permeability through nonoxidant pathways. Both extracellular (H2O2) and intracellular (menadione) oxidants increase paracellular permeability across human lung epithelial monolayers. Extracellular catalase but not superoxide dismutase prevented increased permeability after both oxidant exposures. However, catalase offered no protection from asbestos-induced permeability. We next depleted the cells of glutathione or catalase to determine whether depleting normal cellular antioxidants would increase the sensitivity to asbestos. Permeability was the same in control cells and in cells depleted of these antioxidants. In addition to generating oxidants, asbestos also activates signal transduction pathways. Blocking protein kinase C activation did not prevent asbestos-induced permeability; however, blocking tyrosine kinase with tyrophostin A25 did prevent asbestos-induced permeability, and blocking tyrosine phosphatase with sodium vanadate enhanced the effect of asbestos. These data demonstrate that asbestos may increase epithelial permeability through nonoxidant pathways that involve tyrosine kinase activation. This model offers an important system for studying pathways involved in regulating lung epithelial permeability.

Asbestos. Asbestos is a collection of naturally occurring silicates that form fibers. These fibers are composed of hydrated magnesium silicates containing varying amounts of iron (6). In amosite and crocidolite asbestos, iron is incorporated into the crystal structure, and iron represents a significant proportion of the molecule (up to 27% by weight; see Ref. 29). By contrast, chrysotile asbestos does not contain iron in the crystal structure; however, iron is adsorbed to the crystal surface (29). In chrysotile asbestos, iron only represents ~2–6% of the mineral by weight. Iron is potentially important to the biological effects of asbestos because iron can catalyze the Haber-Weiss reaction, generating the reactive oxygen species -OH. Many of the biological effects of asbestos are postulated to derive from its ability to generate reactive oxygen species (18, 22).

The ability of asbestos to generate reactive oxidants is particularly relevant to epithelial permeability because reactive oxygen species increase both endothelial and epithelial permeability (19, 35). However, we previously found that altering the iron content of chrysotile asbestos had no effect on its ability to increase lung epithelial permeability (28). Based on this observation, we postulated that asbestos increases epithelial permeability through iron- and oxidant-independent pathways. We undertook the current study to test the hypothesis.

MATERIALS AND METHODS

Chemicals and reagents. Catalase (C3155), superoxide dismutase (SOD S2515), menadione, 3-amino-1,2,4-triazole, buthionine sulfoximine (BSO), and phorbol 12,13-dibutyrate (PDBu) were all purchased from Sigma Chemical, St. Louis, MO. All tissue culture media and supplements were purchased from the University of Iowa Tissue Culture Facility. [3H]mannitol (22.5 Ci/mmol) was purchased from New England Nuclear. Tyrophostin A25 and bisindolylmaleimide (GF 109203X) were purchased from Calbiochem.

Asbestos. Asbestos used in these studies was kindly provided by Dr. J. G. N. Garcia, Indiana University. The chrysotile asbestos used in these studies is a chryogeni-
cally ground Calidria asbestos previously described (32). We have demonstrated that this chrysotile asbestos increases cultured human lung epithelial permeability (25). Both asbestos samples were maintained in suspension and evenly dispersed by sonicated the sample before it was diluted and added to the cultured cells.

Cell culture and permeability assay. We used HBE16 cells in these studies. These cells were obtained from a segmental human airway and immortalized using an origin-defective SV40 (14). The cells were cultured at 37°C in 5% CO2-95% air and in Eagle's MEM containing 10% heat-inactivated fetal bovine serum, penicillin and streptomycin, and 2 mM glutamine. They were passaged weekly after brief exposure to trypsin.

RESULTS

Cell permeability. Monolayers were exposed to asbestos and the observation that catalase did not prevent asbestos-induced paracellular permeability (P\text{mann}) in both a concentration- and time-dependent pattern. Having established that extracellular oxidants could increase P\text{mann}, we next asked whether extracellular antioxidants would prevent asbestos-induced P\text{mann}. Neither extracellular SOD nor extracellular catalase prevented asbestos-induced permeability (Fig. 2). These results could be due to asbestos acting through nonoxidant mechanisms to increase permeability. However, an alternative explanation is that asbestos acts by generating intracellular oxidants, and the extracellular antioxidant enzymes cannot protect from intracellular oxidants. Our observation that HBE cells internalize the asbestos particles supports this hypothesis (11). To test this hypothesis, we exposed the cells to menadione, a vitamin K analog that is metabolized in the mitochondria. Superoxide is generated as a product of menadione metabolism (31). Menadione thus offers us a model for testing intracellular oxidant production. As shown in Fig. 3, menadione did increase P\text{mann} across HBE monolayers, and, interestingly, the prolonged time course was similar to the time course we saw after adding asbestos. However, unlike asbestos, extracellular catalase completely prevented menadione-induced permeability (Fig. 4). SOD, though, offered no protection from asbestos. These data demonstrate that extracellular antioxidants can protect epithelial cells from intracellular oxidants and further suggest that the active oxidant species is not superoxide. Coupled with the observation that catalase did not prevent asbestos-induced permeability across cultured human lung epithelial (HBE) monolayers in a time- and concentration-dependent pattern. Each bar represents 6 monolayers.

Determination of permeability. To confirm that extracellular oxidants would increase paracellular permeability across HBE monolayers, we exposed the monolayers to increasing concentrations of H2O2. As shown in Fig. 1, H2O2 increased mannitol permeability (P\text{mann}) both in a concentration- and time-

\begin{align*}
U/g = 3Ln(A_i / A_f) / v_p t
\end{align*}

where A_i is initial absorbance sample – control, A_f is final absorbance sample – control, v is volume of the sample, p is protein concentration in the sample, and t is time (1).

Statistics. All groups were compared using one-way analysis of variance with post hoc testing by Newman-Keuls using the Systat statistical package. P < 0.05 is considered significant.

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induced permeability, these data suggest that asbestos is acting through nonoxidant pathways.

We previously reported that artificially manipulating the iron content on the chrysotile asbestos had no effect on its ability to increase $P_{\text{mann}}$ across HBE monolayers. Because asbestos is thought to increase oxidants through iron-dependent catalysis of the Fenton reaction, this suggested that asbestos may be acting through nonoxidant pathways. To further confirm this observation, we exposed HBE monolayers to chrysotile or amosite asbestos. These two forms of asbestos differ in their iron content. Despite the difference in iron content, amosite and chrysotile asbestos equally increased $P_{\text{mann}}$ (Fig. 5). These data further support the conclusion that asbestos increases $P_{\text{mann}}$ through nonoxidant pathways.

We next asked whether we could accentuate the effect of asbestos by depleting the cells of their normal antioxidant defenses. We first depleted the cells of glutathione using BSO. As shown in Table 1, we could reduce cellular glutathione by 90% using BSO. However, asbestos caused no more permeability increase in cells depleted of glutathione than it did in native cells (Fig. 6). We next asked whether depleting cellular catalase would increase the effect of asbestos. We used 3-amino-1,2,4-triazole (ATZ) to deplete the cellular catalase. ATZ decreased cell catalase by 60% in the presence of either $H_2O_2$ or asbestos (Table 2). Depleting cell catalase similarly had no effect on asbestos-induced permeability (Fig. 7). These data further support the conclusion that asbestos increases lung paracellular permeability through nonoxidant pathways.

Having determined that asbestos increases paracellular permeability through nonoxidant pathways, we next asked whether asbestos acts through second messengers. Asbestos is known to increase protein kinase C (PKC) activity in cultured hamster lung epithelial cells, and PKC activation increases paracellular permeability across cultured epithelial monolayers (25, 36). To test the hypothesis that asbestos acts through PKC, we blocked PKC activation using GF 109203X (23, 43). Blocking PKC had no effect on asbestos-induced permeability (Fig. 8). As previously reported, however, GF 109203X did prevent increased permeability after PKC activation with PDBu. We next investigated the role of tyrosine kinase in asbestos-induced permeability by inhibiting tyrosine kinase using tyrophostin A25 (7). As shown in Fig. 9A, tyrophostin A25 completely prevented asbestos-induced permeability at 24 h and significantly blocked asbestos-induced permeability after 48 h of asbestos exposure. In addition, blocking tyrosine phosphatase with sodium vanadate enhanced the effect of asbestos on mannitol permeability (Fig. 9B).

**DISCUSSION**

Human epidemiological studies and animal studies provide strong support linking asbestos exposure and fibrotic lung disease. However, the biological basis for asbestos-induced lung fibrosis is incompletely understood. Recent investigation into the pathophysiology of interstitial lung disease suggests that the lung epithelium occupies a central role in subsequent lung fibrosis.

**Table 1. Effect of BSO on glutathione levels in HBE cells**

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Glutathione Levels, nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49.5 ± 4.2</td>
</tr>
<tr>
<td>Asbestos (100 µg/ml)</td>
<td>42.5 ± 5.3</td>
</tr>
<tr>
<td>BSO (50 µM)</td>
<td>5.2 ± 0.5*</td>
</tr>
<tr>
<td>Asbestos + BSO</td>
<td>4.6 ± 0.7*</td>
</tr>
</tbody>
</table>

Values are means ± SE. BSO, buthionine sulfoximine; HBE, human bronchial epithelial. *P < 0.01 compared with control cells.
Interstitial lung disease is difficult to study because most cases are idiopathic. Because of the known relationship between asbestos and lung fibrosis, asbestos provides us with an important experimental model for studying early events in lung fibrosis. Inhaled asbestos fibers preferentially deposit at bifurcations in the alveolar ducts, and the alveolar duct bifurcations are the anatomic areas where changes are first seen in animal inhalation models (3). We have previously shown that asbestos increases paracellular permeability across cultured human lung epithelial monolayers (28). Plasma proteins, including fibrinogen, will cross the altered lung epithelium and form fibrin in the distal airways (16). Gross and colleagues (13) have shown that the lung epithelium expresses significant amounts of urokinase-type plasminogen activator (uPA) that activates plasmin and leads to increased fibrin degradation. We have also shown that asbestos increases uPA expression by lung epithelial cells and that the fibrin degradation products cross asbestos-exposed epithelial monolayers more readily (11). Finally, fibrin degradation products are biologically active compounds that act as chemoattractants for polymorphonuclear neutrophils, inactivate surfactant, and increase cytokine production (20, 26). These observations have led us to propose the hypothetical pathway shown in Fig. 10 as one arm through which asbestos exposure can lead to lung fibrosis. Altered lung epithelial permeability is central in both initiating and perpetuating this pathophysiological sequence.

Both human studies and animal studies confirm that asbestos exposure increases lung epithelial permeability. With 99mTc-diethylenetriaminepentaacetate as a marker, patients with asbestosis have increased lung epithelial permeability (8, 30). Similarly, in animals exposed to asbestos, the airways contain increased protein, consistent with increased epithelial permeability (24).

Having established that asbestos increases lung epithelial permeability in our model, we designed the current study to ask whether asbestos increases epithelial permeability through oxidant-dependent mechanisms. Many of the biological effects of asbestos are thought to be due to its ability to induce or amplify oxidant injury, and extracellular oxidants increase paracellular permeability across both Madin-Darby canine kidney monolayers and rat type II epithelial monolayers (19, 35). Four experimental observations support the hypothesis that asbestos contributes to lung injury through oxidant mechanisms. First, asbestos generates \( \cdot \text{OH} \) when it is incubated with \( \text{H}_2\text{O}_2 \), and the iron chelator deferoxamine blocks \( \cdot \text{OH} \) production (33, 34). Second, \( \cdot \text{OH} \) is produced in the lungs of animals exposed to asbestos (28). Third, the antioxidant enzyme SOD is increased in the lungs of animals exposed to asbestos (16, 17). These observations, however, do not provide evidence directly linking oxidant production to biological events. A fourth study does...
suggest a more causal link. In a rapid animal model of asbestosis, antioxidants partially prevented lung fibrosis (24). Even in this model, however, protection was incomplete, and, interestingly, catalase did not prevent increased bronchoalveolar lavage protein, a marker of lung permeability. These data demonstrate that asbestosis generates oxidants in the lung; however, they do not causally link increased lung permeability with oxidant generation. Furthermore, some of the biological effects of asbestosis occur independent of oxidants. In mouse macrophages, vitamin E blocked asbestosis-induced lipid peroxidation but did not prevent asbestosis toxicity (10).

Asbestos can increase oxidant stress in the lung in two ways: by amplifying oxidant injury by catalyzing \( \bullet \)OH production from \( \text{H}_2\text{O}_2 \) or by increasing oxidant production from inflammatory cells. Both of these mechanisms can explain the experimental observations using electron spin resonance (ESR) and the experimental findings in whole animals (16, 17, 19, 28, 33–35). They do not, however, explain increased permeability in our cultured cell system. Unlike the studies using ESR, we did not provide an exogenous source of \( \text{H}_2\text{O}_2 \), and inflammatory cells were not present in our system. These data, together with the observation that altering iron content had no effect on asbestos-induced permeability (28), suggest that asbestos increases epithelial permeability through nonoxidant mechanisms.

Our current study provides strong evidence further supporting this hypothesis. First, we found that extracellular catalase but not SOD protects HBE monolayers from both extracellular and intracellular oxidants (menadione). The finding that catalase but not SOD protects from menadione suggests that the important oxidant species is not superoxide because superoxide is initially produced after menadione exposure. Superoxide is further metabolized to \( \text{H}_2\text{O}_2 \), \( \bullet \)OH, and \( \text{H}_2\text{O} \).

![Fig. 9. A: blocking tyrosine kinase with tyrophostin A25 completely prevented asbestos-induced permeability across HBE monolayers after either 24 or 48 h of exposure. Tyrophostin A25 itself had no effect on permeability. Each bar represents 6 monolayers.](image)

**Fig. 9.** A: blocking tyrosine kinase with tyrophostin A25 completely prevented asbestos-induced permeability across HBE monolayers after either 24 or 48 h of exposure. Tyrophostin A25 itself had no effect on permeability. Each bar represents 6 monolayers. B: blocking tyrosine phosphatase with 100 µM sodium vanadate enhanced the effect of asbestos on mannitol permeability. Epithelial monolayers were incubated with sodium vanadate for 5 h before asbestos was added. Sodium vanadate was maintained at 100 µM throughout the experiment. Each bar represents 6 monolayers.

![Fig. 10. Diagrammatic representation of the hypothesis by which altered lung epithelial permeability can contribute to lung fibrosis.](image)

**Fig. 10.** Diagrammatic representation of the hypothesis by which altered lung epithelial permeability can contribute to lung fibrosis. Increased lung epithelial permeability is central to initiating and perpetuating the loss of distal lung units in this model. uPA, urokinase-type plasminogen activator; FDP, fibrin degradation product.
suggests that the important oxidant in altering epithelial permeability is either H₂O₂ or -OH. Unlike menadione, extracellular catalase provided no protection from asbestos-induced permeability, consistent with asbestos acting through a nonoxidant mechanism. Furthermore, depleting the cells of either catalase or glutathione, steps that should increase sensitivity to oxidant injury, had no effect on asbestos-induced permeability. We did, however, find evidence for oxidant generation in asbestos-exposed cells because ATZ only depletes catalase in an oxidant-containing environment. In our experiments, ATZ had no effect on catalase in control cells but depleted catalase from cells exposed to asbestos.

In addition to inducing and amplifying oxidant production, asbestos is also reported to activate signal transduction pathways in cells. Asbestos is reported to activate phospholipase C and PKC in airway epithelial cells, and a related mineral, silica, activates tyrosine kinase in macrophages (15, 27). Both of these pathways are potentially important in epithelial permeability. Several investigators have reported that activating PKC increases epithelial permeability, and, recently, the PKC inhibitor GF 109203X was reported to block this permeability without inducing any injury itself (23). Our studies demonstrate that asbestos does not act through PKC because GF X109203 had no effect on asbestos-induced permeability. The role of tyrosine kinase in epithelial permeability is less firmly established. However, increasing tyrosine phosphorylation of the adherence junction protein β-catenin alters epithelial cell shape and opens gaps between cells (2). Our studies with tyrophostin A25 and sodium vanadate suggest a role for asbestos-induced tyrosine kinase activation without specifically identifying the target protein(s).

These data suggest that asbestos has nonoxidant biological effects. This is important for at least three reasons. First, it may not be adequate to screen mineral fibers for their ability to generate oxidants in an attempt to develop "safe" mineral fibers. Second, altering asbestos or other mineral fibers to decrease their oxidant-generating capacity may not completely prevent disease after exposure. Finally, increasing lung antioxidant mechanisms may not prevent mineral fiber-induced lung disease. However, using asbestos as a model, we can further our understanding of the early pathways involved in lung fibrosis and study the cellular pathways regulating lung epithelial permeability.

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