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Increased sensitivity to asbestos-induced lung injury in mice lacking extracellular superoxide dismutase

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Abstract

Asbestosis is a chronic form of interstitial lung disease characterized by inflammation and fibrosis that results from the inhalation of asbestos fibers. Although the pathogenesis of asbestosis is poorly understood, reactive oxygen species may mediate the progression of this disease. The antioxidant enzyme extracellular superoxide dismutase (EC-SOD) can protect the lung against a variety of insults; however, its role in asbestosis is unknown. To determine if EC-SOD plays a direct role in protecting the lung from asbestos-induced injury, intratracheal injections of crocidolite were given to wild-type and *ec-sod*-null mice. Bronchoalveolar lavage fluid (BALF) from asbestos-treated *ec-sod*-null mice at 24 h, 14 days, or 28 days posttreatment showed increased inflammation and total BALF protein content compared to that of wild-type mice. In addition, lungs from *ec-sod*-null mice showed increased hydroxyproline content compared to those of wild-type mice, indicating a greater fibrotic response. Finally, lungs from *ec-sod*-null mice showed greater oxidative damage, as assessed by nitrotyrosine content compared to those of their wild-type counterparts. These results indicate that depletion of EC-SOD from the lung increases oxidative stress and injury in response to asbestos.

Keywords

Asbestos; Extracellular superoxide dismutase; Pulmonary fibrosis; Oxidative stress; Nitrotyrosine; Free radical

Introduction

Asbestosis, a form of interstitial lung disease characterized by inflammation and lung fibrosis, is a chronic, debilitating disease that causes significant morbidity and mortality in affected patients [1]. Although exposure to asbestos fibers may have occurred 20 to 40 years earlier, the long latency period of the disease implies that many workers in the United States are still at risk of developing asbestosis in the upcoming years. Thus, this disease is currently and will remain a significant health problem [2,3].

In order to better investigate the pathogenesis of this disease, animal models have been used extensively to help elucidate the underlying biochemical mechanisms responsible for the development of asbestosis (reviewed in [4]). Studies conducted in these model systems have confirmed the fibrogenic and carcinogenic properties of asbestos fibers that have been observed in cases of human exposure. Even brief exposures to asbestos fibers in animal models have resulted in the persistent presence of these fibers in the lung parenchyma where they stimulate the recruitment and activation of inflammatory cells and the proliferation of fibroblasts and type II epithelial cells [5–8] (see below).

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Although the molecular mechanisms underlying asbestosis are largely unknown, current evidence suggests a role for reactive oxygen species (ROS) in the pathogenesis of this disease [4,9]. It has been previously shown that asbestos, particularly the highly fibrogenic amphibole fibers, can cause oxidative damage to the lung both directly, through hydroxyl radical formation via the Haber-Weiss reaction with fiber surface iron [10,11], and indirectly through recruitment and activation of ROS-producing inflammatory cells [12–14]. A variety of antioxidants including manganese superoxide dismutase, catalase, and iron chelators such as deferoxamine have shown protective effects in a variety of in vitro and in vivo models of asbestos-mediated lung disease [15–18].

The antioxidant enzyme extracellular superoxide dismutase (EC-SOD) is a 135-kDa tetrameric enzyme that scavenges superoxide radicals in the extracellular space (reviewed in [19]). EC-SOD is expressed in especially high levels in mammalian lungs where it is bound to the extracellular matrix through a positively charged heparin/matrix-binding domain. Proteolytic cleavage of the heparin/matrix-binding domain has been associated with loss of the enzyme from the extracellular matrix under experimental conditions that mimic human interstitial lung disease, such as bleomycin treatment or hyperoxia [20,21]. In addition, it has been shown that both bleomycin treatment and hyperoxia cause greater lung tissue damage in mice that carry a targeted disruption of the *ecsod* gene (*ecsod*-null mice) when compared to wild-type mice [22,23]. Recent evidence from our laboratory indicates that asbestos exposure in mice results in decreased lung EC-SOD protein levels and activity. The loss of this enzyme may enhance oxidative stress and injury in this model [24].

To elucidate the role of EC-SOD in oxidative stress-associated lung disease, we have examined the type and extent of asbestos-induced pulmonary damage in *ecsod*-null mice. Using a model in which wild-type and *ecsod*-null mice are exposed intratracheally to crocidolite asbestos, we have determined that lack of EC-SOD leads to increased lung injury and exacerbates asbestos-induced lung inflammation and fibrosis. These results support the hypothesis that the loss of EC-SOD from the lung results in increased oxidative lung injury and leads to increased inflammation and fibrosis in a mouse model of asbestosis.

Materials and methods

Materials

Eosin Y, phloxine B, chloramine T, methyl cellulose, titanium dioxide, and anti- β -actin antibody were purchased from Sigma (St. Louis, MO). Anti-nitrotyrosine antibody was purchased from Molecular Probes (Eugene, OR). Mayer's hematoxylin, 10% buffered formalin, and *p*-dimethylamino-benzaldehyde were purchased from Fisher Scientific (Pittsburgh, PA). Clear Rite was obtained from Richard-Allan Scientific (Kalamazoo, MI). Enhanced chemiluminescence detection reagents were purchased from Amersham Biosciences (Buckinghamshire, UK).

Animals

All animal experimental protocols were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male C57BL/6 (Taconic, Germantown, NY) and *ecsod*-null mice (congenic with the C57BL/6 strain of mice), 8–10 weeks old, were treated with 0.1 mg NIEHS crocidolite asbestos (>10 μ m in length), 0.1 mg titanium dioxide (24 h, 14 days; Sigma), or 0.9% saline vehicle (28 days) by intratracheal instillation as previously described [24,25]. Mice were euthanized 24 h, 14 days, or 28 days posttreatment. Bronchoalveolar lavage fluid (BALF) was obtained by the intratracheal instillation and recovery of 0.8 ml of 0.9% saline. Lungs were removed and either flash-frozen in liquid

nitrogen and stored at -70°C until used for biochemical analysis or inflation-fixed with 10% buffered formalin and paraffin-embedded for histologic analysis.

Histologic analysis

Standard hematoxylin and eosin staining was performed on 5- μm -thick lung sections as previously described [20]. Slides were scored by a pathologist blinded to sample group (T.D.O.) as previously described [24].

Analysis of bronchoalveolar lavage fluid

Total protein was determined by Coomassie blue protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. Total white blood cell counts were obtained with a Beckman Z1 Coulter particle counter (Beckman Coulter, Fullerton, CA). To obtain differential counts, cytopsin slides of BALF fluid were stained with a modified Giemsa stain (Dif Quik, Dade Behring Inc., Newark, DE) and the numbers of macrophages, lymphocytes, and neutrophils were counted under a light microscope. A total of 400 cells were counted for each slide.

Western blot analysis

Analysis was performed as previously described [20]. EC-SOD was detected with antibody (1:10,000 dilution) against mouse EC-SOD as previously described [20,26]. After visualization by enhanced chemiluminescence, densitometry was performed and standardized to mouse β -actin (for lung homogenates) using Kodak 1D software (Rochester, NY).

Dot blot analysis

Protein samples (10 μg) were dotted onto nitrocellulose membranes. After blocking overnight in 5% nonfat milk, the presence of nitrotyrosine in each sample was detected using an anti-nitrotyrosine antibody (1:1000 dilution) and visualized by enhanced chemiluminescence. Densitometry was performed using Kodak 1D software.

Hydroxyproline assay

Whole lungs were dried and acid hydrolyzed in sealed, oxygen-purged glass ampoules containing 2 ml of 6 N HCl for 24 h at 110°C . Samples were centrifuged at 13,000 rpm, and the supernatant was taken for hydroxyproline analysis by using chloramine-T as previously described [25].

Results

Asbestos treatment results in the loss of EC-SOD from lung parenchyma

Previous studies have demonstrated a protective role for EC-SOD in a variety of lung pathologies that are associated with high levels of oxidative stress [27–29]. Since asbestosis has been associated with both increased oxidant production and decreased levels of antioxidant enzymes (reviewed in [9]), this study was conducted to determine if loss of EC-SOD from lung tissue exacerbates asbestos-induced damage. We have established a mouse model of asbestosis in which the hallmarks of fibrotic lung disease, including patches of histologically identified interstitial fibrosis, increased collagen content as assessed by hydroxyproline levels, and increased numbers of lung neutrophils as assessed by bronchoalveolar lavage (BAL), are present 28 days after intratracheal instillation of 0.1 mg crocidolite asbestos ($>10\text{ }\mu\text{m}$ in length) (not illustrated). Although no mortality due to treatment was observed during our experiments, some weight loss in individual animals was observed but did not exceed the 15% maximum allowed by the experimental design (data not illustrated).

When lung EC-SOD protein levels were compared between asbestos-treated mice and mice treated with an inert particle control (titanium dioxide, TiO₂) by Western blotting, lung homogenates from mice treated with asbestos had significantly decreased EC-SOD compared with TiO₂-treated controls (Figs. 1A and B). In addition, EC-SOD activity in the lung tissue was also significantly decreased in asbestos-treated mice (Fig. 1C). These results suggest that loss of EC-SOD from the lung matrix is associated with the development of asbestosis in mice.

Asbestos-induced lung injury is exacerbated in *ecsod*-null mice

To further elucidate the role of EC-SOD in the progression of asbestos-induced lung disease, we assessed the effects of asbestos exposure on mice null for the *ec-sod* gene. Analysis of BAL fluid from asbestos-treated wild-type and *ecsod*-null mice showed increased total protein levels in *ecsod*-null mice at 24 h (WT vs. KO, 0.546 ± 0.019 vs. 0.851 ± 0.191) and 28 days posttreatment (WT vs. KO, 0.124 ± 0.011 vs. 0.209 ± 0.017 ; Student's *t* test, $p < 0.05$) (Fig. 2A), compared to wild-type controls. In addition, cytologic analysis of BALF fluid from both strains of mice revealed a greater number of inflammatory cells, primarily neutrophils (WT vs. KO; 0.785 ± 0.87 vs. 1.187 ± 0.124 at 24 h; 0.396 ± 0.010 vs. 0.650 ± 0.084 at 28 days; Student's *t* test, $p < 0.05$) (Fig. 2B) and lymphocytes (WT vs. KO; 0.0181 ± 0.003 vs. 0.0484 ± 0.005 at 28 days; Student's *t* test, $p < 0.05$) (Fig. 2C), in BAL fluid from asbestos-treated *ecsod*-null mice, indicating a greater inflammatory response in mice lacking EC-SOD.

Histologic analysis of lung tissue from *ecsod*-null mice examined at 28 days postasbestos treatment indicated a more extensive fibrotic response compared to that of wild-type mice (Fig. 3B vs. A). It is interesting to note the persistent presence of asbestos fibers in the lesion (Fig. 3A, inset). Assessment of the pathology index of lung tissue samples from these mice (Fig. 3C) also indicates that fibrosis was more severe in the *ecsod*-null mice (PI = 1.25 ± 0.12) compared to their wild-type counterparts (PI = 0.79 ± 0.04) (Student's *t* test, $p < 0.05$). To further assess the severity of the fibrosis present in asbestos-treated *ecsod*-null and wild-type animals, the hydroxyproline content of the lungs was determined (Fig. 3D). At 28 days posttreatment, hydroxyproline levels were significantly elevated in the *ecsod*-null animals (331.3 ± 14.3 mg) compared to those of their wild-type counterparts (274.4 ± 20.5 mg) (Student's *t* test, $p < 0.05$), indicating that *ecsod*-null mice are more sensitive to asbestos-induced lung fibrosis.

Lungs of mice lacking EC-SOD show increased nitrotyrosine levels

To determine if *ec-sod* null mice also have increased oxidative tissue damage, we assessed the levels of nitrotyrosine-modified proteins present in the lungs of asbestos-treated wild-type and *ec-sod* null mice. Fig. 4 demonstrates that mice lacking EC-SOD have increased levels of nitrotyrosine at both 24 h (Fig. 4A) and 14 days (Fig. 4B) postasbestos treatment compared to their wild-type counterparts (Student's *t* test, $p < 0.05$).

Discussion

Particle and fiber-induced lung diseases as a result of both occupational and nonoccupational exposures are a significant health concern in the United States and elsewhere. Although a detailed mechanism of action for asbestos-induced pathogenesis has not yet been elucidated, oxidant/antioxidant imbalances in the lung as a result of exposure are believed to play a key role [30]. Several studies have shown that asbestos exposure both in vitro and in vivo can lead to ROS formation and oxidative tissue damage [31,32]. In addition, other studies have shown compensatory increases in lung antioxidant levels in response to fibrogenic materials [33].

Previous studies from our laboratory and others have suggested a protective role for EC-SOD in animal models of lung injury [19,23,27–29,34,35]. We have also reported a decrease in EC-

SOD protein levels in the lungs of bleomycin-treated mice, a common model of pulmonary fibrosis [20], in a hyperoxic model of acute lung injury [21], and during the acute phase of asbestos-mediated lung disease [24]. These studies also showed that in treated animals, EC-SOD was being proteolyzed at the heparin/matrix-binding domain, resulting in the loss of EC-SOD affinity for the extracellular matrix. These data suggest that increased proteolysis of EC-SOD leads to its removal from the lung parenchyma, thus allowing for greater or more prolonged exposure to oxidative stress in the affected tissue. Furthermore, transgenic over-expression of EC-SOD [29] or treatment with compounds that mimic superoxide dismutases [36] protects against oxidative stress-induced lung injury [27,36,37]. In contrast, mice null for *ec-sod* have been shown to be more susceptible to both bleomycin and hyperoxia [22,23]. Our current study examines the direct effect of a lack of EC-SOD in the lung on asbestos-mediated injury.

Induction of asbestosis through intratracheal instillation of crocidolite asbestos fibers in wild-type mice resulted in increased levels of both BAL fluid protein and lung neutrophils in asbestos-treated animals compared to titanium dioxide-treated control mice at 28 days posttreatment (Fig. 2). Asbestos treatment also resulted in the excessive production of extracellular matrix proteins in the lung of exposed animals as demonstrated histologically and by increased hydroxyproline levels (Fig. 3). The appearance of these indicators of lung injury was also accompanied by a sustained loss of EC-SOD protein and activity from the extracellular matrix of asbestos-exposed mice (Figs. 1A–C). Previous results from our laboratory demonstrated that proteolyzed EC-SOD accumulates in the BALF of asbestos-treated mice at 24 h postinstillation [24]. In the present study, analysis of BALF EC-SOD at the later time point of 28 days showed equal amounts of EC-SOD protein in both the TiO₂ and the asbestos-treated mice (data not illustrated), suggesting that the EC-SOD present in the airway at 24 h posttreatment is being effectively cleared from the lung. The loss of lung EC-SOD is consistent with our previous observation of a similar but more pronounced loss of EC-SOD at more acute time points postasbestos treatment, which was also accompanied by indicators of lung inflammation and injury [24].

Notably, the severity and extent of both the inflammatory response (Figs. 2A and B) and the fibrotic lesions (Figs. 3B–D) were increased in mice lacking EC-SOD compared to wild-type mice in response to asbestos. These results were accompanied by increased total nitrotyrosine content of lung proteins, a marker of oxidative stress, in *ec-sod* null mice (Figs. 4A and B) at both 24 h and 14 days postasbestos exposure. These results suggest that removal of EC-SOD from the lung matrix can exacerbate oxidative lung injury and result in increased inflammation and fibrosis.

In addition, these results demonstrate that lack of EC-SOD leads to increased peroxynitrite formation as is evidenced by elevated nitrotyrosine levels in the EC-SOD knockout mice. We had previously hypothesized that EC-SOD could protect against the formation of nitrotyrosine in the lung by reducing superoxide levels [38]. Conversely, observed increases in peroxynitrite could be a result of the increased neutrophilia present in the *ec-sod* null mice. However, it is clear that the absence of EC-SOD in the lung can lead to increases in nitrotyrosine protein modifications, although the exact mechanism has yet to be elucidated. These results also indicate that the presence of extracellular superoxide and perhaps peroxynitrite plays a significant role in the pathogenesis of asbestos-induced injury.

The increase in protein nitration in response to loss of EC-SOD may have significant ramifications for the overall health of the asbestos-exposed lung. Peroxynitrite, formed upon the combination of superoxide and nitric oxide, has long been recognized as a potent oxidant, capable of inducing nitration of key proteins involved in maintaining cellular and tissue homeostasis (reviewed in [34]). Targets of peroxynitrite include apoptotic regulatory proteins

(reviewed in [39]), tyrosine kinases involved in a number of signaling cascades (reviewed in [40]), and proteins that affect mitochondrial respiration (reviewed in [41]). In the asbestos-exposed rat, nitrotyrosine was detected in conjunction with increased ERK 1/2 phosphorylation, suggesting asbestos fibers may activate the ERK signaling pathway by generating ONOO- or other nitrating species [42]. In humans, Saleh et al. showed that increased amounts of nitrotyrosine protein modification were present in patients with idiopathic pulmonary fibrosis [43]. In addition, our laboratory has observed decreased EC-SOD staining in fibrotic areas of lung tissue from IPF patients [44]. It is therefore possible that the loss of the superoxide scavenger EC-SOD from the matrix of the lung upon exposure to fibrotic stimuli may tip the oxidant/antioxidant balance in the lung in the direction of increased peroxynitrite formation and protein modification.

It is interesting to note that the role EC-SOD (or lack of) plays in our model of asbestosis shares many characteristics with other models of oxidative lung injury. It is evident that in many of the animal models that have been examined so far, the presence or absence of EC-SOD directly contributes to the extent and severity of lung injury. Studies in bleomycin, hyperoxia, asbestos (this study), and LPS-treated mice have determined that EC-SOD mediates lung injury, in part, by affecting inflammatory responses, suggesting a common effect of EC-SOD in all of these various forms of lung injury [20,21,24,28]. Available data suggest that lack of EC-SOD enhances the recruitment and activation of inflammatory cells, although we have yet to determine if EC-SOD depletion from the lung is a stimulus for the inflammatory response or is a consequence of the release of proteolytic factors associated with increased inflammation. It may be that EC-SOD is acting as a first line of defense against oxidative stress produced in these various models and that loss of this protection early on may have dire consequences for the continuing health of the lung.

In summary, the results of this study show that loss of EC-SOD exacerbates conditions that lead to fibrotic lung disease. These results support our hypothesis that EC-SOD protects against the development of asbestosis. The loss of antioxidant protection conferred by EC-SOD may increase the sensitivity of the lung to oxidative stress by enhancing inflammatory responses and nitrotyrosine formation, thus further contributing to the oxidant-antioxidant imbalance present in the lung during the progression of asbestos-mediated lung injury.

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Abbreviations

ROS	reactive oxygen species
EC-SOD	extracellular superoxide dismutase
BALF	bronchoalveolar lavage fluid
BAL	bronchoalveolar lavage

Fig. 1.

Asbestos treatment leads to decreased EC-SOD in the lung of wild-type mice at 28 days postexposure. Mice were treated with 0.1 mg TiO_2 ($n = 5$) or crocidolite asbestos ($n = 4$) and Western blots (A) performed on lung homogenates, loading 20 μg total protein for each sample. (B) Densitometric analysis of Western blots normalized to β -actin loading control. (C) EC-SOD activity in lungs from asbestos-treated and control mice ($n = 5$ for each group). Activity was assessed by inhibition of cytochrome *c* reduction by superoxide at pH 10. Error bars represent SE. Asterisks indicate significant results ($p < 0.05$, Student's *t* test).

Fig. 2.

Increased lung injury and inflammation in asbestos-exposed *ec-sod* null mice. (A) Total protein in BALF isolated from control and asbestos-treated wild-type (WT, solid bars) and *ec-sod* null mice (KO, stippled bars) at 24 h, 14 days, or 28 days posttreatment (3–5 mice per group). (B) Neutrophils in BALF isolated from control and asbestos-treated wild-type (WT, solid bars) and *ec-sod* null mice (KO, stippled bars) at 24 h, 14 days, or 28 days posttreatment (3–5 mice per group). (C) BALF lymphocytes from control and asbestos-treated wild-type (WT, solid bars) and *ec-sod* null mice (KO, stippled bars) at 24 h, 14 days, or 28 days posttreatment (3–5 mice per group). (D) BALF macrophages from control and asbestos-treated wild-type (WT, solid bars) and *ec-sod* null mice (KO, stippled bars) at 24 h, 14 days, or 28 days posttreatment (3–5 mice per group). Error bars represent SE. Asterisks indicate significant results ($p < 0.05$, Student's *t* test).

Fig. 3. Increased fibrosis in mice lacking EC-SOD after asbestos exposure. Lung sections from saline and crocidolite-treated (0.1 mg) wild-type and *ec-sod* null mice were stained with hematoxylin/eosin. (A) Wild-type mouse at 28 days postasbestos treatment. Inset is boxed area at higher magnification to show asbestos fibers. Bar equals 25 µm. (B) *ec-sod* null mouse at 28 days postasbestos treatment. Bar equals 25 µm. (C) Pathology index score of lung tissue sections from control and asbestos-treated mice at 28 days posttreatment. Mean pathology index scores for wild-type (dark bar, $n = 4$) and *ec-sod* null mice (stippled bar, $n = 4$) was determined as described under Materials and methods. (D) Analysis of hydroxyproline levels as a measure of collagen deposition/fibrosis in control and asbestos-exposed wild-type and *ec-sod* null mice ($n = 6$ each group) at 28 days posttreatment. Error bars represent SE. Asterisks indicate significant results ($p < 0.05$, Student's t test).

Fig. 4.

Increased nitrotyrosine content in lungs of asbestos-treated *ec-sod* null mice compared to wild-type mice. Mice were treated with 0.1 mg TiO₂ or crocidolite asbestos and dot blots performed on lung homogenates, loading 10 µg total protein for each sample ($n = 4-6$ mice per group). Shown are densitometric analyses of dot blots expressed as percentage control for lungs at (A) 24 h postasbestos exposure and (B) 14 days postasbestos exposure. Error bars represent SE. Asterisks indicate significant results ($p < 0.05$, Student's t test).