Leukocyte-derived extracellular superoxide dismutase does not contribute to airspace EC-SOD after interstitial pulmonary injury

Michelle L. Manni,1 Michael W. Epperly,2 Wei Han,5 Timothy S. Blackwell,5 Steven R. Duncan,3 Jon D. Piganelli,4 and Tim D. Oury1

Departments of 1Pathology, 2Radiation Oncology, 3Medicine, and 4Pediatrics, University of Pittsburgh, Pittsburgh, Pennsylvania; and 5Division of Allergy, Department of Medicine, Pulmonary and Critical Care Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee

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Manni ML, Epperly MW, Han W, Blackwell TS, Duncan SR, Piganelli JD, Oury TD. Leukocyte-derived extracellular superoxide dismutase does not contribute to airspace EC-SOD after interstitial pulmonary injury. Am J Physiol Lung Cell Mol Physiol 302: L160–L166, 2012. First published October 14, 2011; doi:10.1152/ajplung.00360.2010. —The antioxidant enzyme extracellular superoxide dismutase (EC-SOD) is abundant in the lung and is known to limit inflammation and fibrosis following numerous pulmonary insults. Previous studies have reported a loss of full-length EC-SOD from the pulmonary parenchyma with accumulation of proteolyzed EC-SOD in the airspace after an interstitial lung injury. However, following airspace only inflammation, EC-SOD accumulates in the airspace without a loss from the interstitium, suggesting this antioxidant may be released from an extrapulmonary source. Because leukocytes are known to express EC-SOD and are prevalent in the bronchoalveolar lavage fluid (BALF) after injury, it was hypothesized that these cells may transport and release EC-SOD into airspaces. To test this hypothesis, C57BL/6 wild-type and EC-SOD knockout mice were irradiated and transplanted with bone marrow from either wild-type mice or EC-SOD knockout mice. Bone marrow chimeric mice were then intratracheally treated with asbestos and killed 3 and 7 days later. At both 3 and 7 days following asbestos injury, mice without pulmonary EC-SOD expression but with EC-SOD in infiltrating and resident leukocytes did not have detectable levels of EC-SOD in the airspace. In addition, leukocyte-derived EC-SOD did not significantly lessen inflammation or early stage fibrosis that resulted from asbestos injury in the lungs. Although it is not influential in the asbestos-induced interstitial lung injury model, EC-SOD is still known to be present in leukocytes and may play an influential role in attenuating pneumonias and other inflammatory diseases.

antioxidant; asbestos-induced pulmonary fibrosis; extracellular superoxide dismutase

EXTRACELLULAR SUPEROXIDE DISMUTASE (EC-SOD) is an antioxidant enzyme that is highly expressed in the lung and localizes primarily to the extracellular matrix of vessels, airways, and alveolar septa due to its positively charged heparin/matrix-binding domain (11, 14, 22). EC-SOD is abundant in lung tissue and is expressed by bronchial epithelial cells, vascular endothelial cells, alveolar type II cells, and alveolar macrophages (14, 26).

Notably, EC-SOD is known to protect the lung against pulmonary injury from bleomycin (5, 9, 10), asbestos (12, 27), radiation (17, 25), hyperoxia (6, 13, 23), hemorrhage (2, 3), endotoxin (4, 29), and bacteria (28). Proteolysis of the heparin-binding domain of EC-SOD has also been noted in a number of interstitial lung disease models and results in the depletion of EC-SOD from the lung parenchyma and accumulation of proteolyzed EC-SOD in the bronchoalveolar lavage fluid (BALF) (10, 27). One mechanism by which EC-SOD is known to exert its protective effect in the lung is by directly binding to and preventing the oxidative fragmentation of type I collagen (9, 24), hyaluronan (15), and heparan sulfate proteoglycans (18, 19). Therefore, loss of EC-SOD from the matrix to the airspace may result in increased oxidative fragmentation of matrix components and amplification of inflammation and fibrosis.

In addition to its presence in the extracellular matrix of the lung, EC-SOD is also present in membrane-bound vesicles within phagocytic inflammatory cells (21). This intracellular localization of EC-SOD was recently shown to be important in regulating bacterial phagocytosis (21). Therefore, EC-SOD inside of inflammatory cells may also contribute to the accumulation of EC-SOD in the BALF in response to interstitial lung injuries. Indeed, a prior study reported an accumulation of proteolyzed EC-SOD in the airspaces without a loss of EC-SOD from the lung parenchyma using an airspace-only model of inflammation (bacterial pneumonia) (28). In addition, using EC-SOD transgenic mice that expressed both mouse and human EC-SOD in the lung, but only mouse EC-SOD in inflammatory cells, only mouse EC-SOD was detected in the BALF. These results suggested that inflammatory cells, which are known to contain EC-SOD (5, 20, 28), could transport and release this potent antioxidant into the airspaces after pulmonary injury. However, the contribution of EC-SOD from inflammatory cells in response to interstitial lung injury is still unknown.

In this study, chimeric mice were generated to directly test the hypothesis that leukocytes contribute to EC-SOD accumulation in the alveolar lining fluid following asbestos-induced lung injury. This study also examines the impact of leukocyte-derived EC-SOD on inflammation and early stage fibrosis in response to asbestos injury.

MATERIALS AND METHODS

Animals. Seven-week-old male C57BL/6 (Taconic, Rockville, MD), C57BL/6 (B6.PL-Thy1a/CyJ; The Jackson Laboratory, Bar Harbor, ME), and EC-SOD null (congenic with C57BL/6 background; see Ref. 6) mice were used as donors with 7-wk-old male C57BL/6 (B6.PL-Thy1a/CyJ; The Jackson Laboratory) wild-type (WT) mice and EC-SOD null mice as recipients. All animals were housed in pathogen-free conditions in microisolator cages, and all animal ex-
chimerism of these mice was verified using flow cytometry. The presence of donor cells (bold) in recipient mice after transplantation was evaluated by assessing the expression of Thy 1.1 or Thy 1.2 on total circulating T lymphocytes (Thy 1-expressing cells) from the blood of the BMC mice.

Experiments were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Generation of bone marrow chimeric mice. Donor mice were killed, and bone marrow was extracted from the long bones of the hind legs. Recipient mice received 1,000 R dose of whole body irradiation and then were injected intravenously via the tail vein with 100 μl of 1 × Hank’s Buffered Salt Solution (pH 7.1) containing 5 × 10^6 bone marrow cells. Recipient mice received 0.032 mg/ml of Baytril (Enrofloxacin; Bayer Healthcare, Shawnee Mission, KS) in their drinking water starting 2 days before and continuing until 7 days after irradiation and transplantation.

Verification of chimerism. Engraftment of donor cells was determined by flow cytometry using a BD FACS Vantage with DiVa and CellQuest analytical software (Becton Dickinson, Franklin Lakes, NJ). Peripheral blood was collected from the lateral saphenous vein in EDTA-coated anticoagulant tubes (Brinkmann Instruments, Westbury, NY) from all bone marrow chimeric (BMC) mice 33–35 days after transplantation (16). All procedures were done in fluorescence-activated cell sorter (FACS) Buffer (1× PBS with 1% BSA). To verify chimerism, whole blood cells were stained with the following antibodies for specific cell surface antigens: fluorescein isothiocyanate-conjugated mouse anti-mouse CD 90.1 (Thy 1.1), PE-Cy5-conjugated rat anti-mouse CD8a, PE-conjugated rat anti-mouse CD 90.2 (Thy 1.2), and APC-conjugated rat anti-mouse CD4. Cells were then fixed in 2% paraformaldehyde for analyses the following day. BMC mice were allowed to recover for 15 days following this blood collection before intratracheal clodronate treatment.

Preparation and administration of liposomal clodronate. Liposomal encapsulation of dichloromethylene diphosphonate (clodronate) was performed as previously described (8). In brief, 8 mg of cholesterol and 86 mg of dioleoyl-phosphatidylcholine (Avanti, Alabaster, AL) were mixed and dissolved in chloroform. The chloroform was then evaporated under nitrogen and further removed from the liposome preparation using a low-vacuum speedvac. The clodronate solution was then prepared by dissolving 1.2 g dichloromethylene diphosphonic acid (Sigma-Aldrich, St. Louis, MO) in 5 ml of sterile PBS and was added to the liposome preparation. The resulting solution was mixed, sonicated, and then ultracentrifuged at 10,000 g for 1 h at 4°C. The resulting pellet of liposomes was then resuspended in PBS and ultracentrifuged again using the same conditions. The liposomes were removed and resuspended in PBS, resulting in a final concentration of liposomal clodronate of 5 mg/ml. Seventy-five microliters of liposomal clodronate solution were administered by intratracheal instillation to the BMC mice.

Asbestos treatment. Four weeks following clodronate administration, BMC mice were treated intratracheally with 0.14 mg of crocidolite asbestos (>10 μm in length; gift from Dr. Andrew Ghio, Environmental Protection Agency, Research Triangle Park, NC; 0.1 mg/50 μl in 0.9% saline) as previously described (1, 27). BMC mice were killed at 3 and 7 days posttreatment to assess inflammation and fibrosis. Nontransplanted WT mice were treated with asbestos and titanium dioxide, an inert particulate control, as additional controls. BALF was obtained via intratracheal instillation and recovery of 0.8 ml of 0.9% saline, and lungs were inflation fixed with 10% buffered formalin and paraffin embedded for histological analyses as previously described (10).

BALF analyses. To determine the number of white blood cells present in the BALF, triplicate counts of BALF samples were made using a Z1 Coulter Counter (Beckman Coulter, Fullerton, CA). To obtain a differential count, BALF samples were adhered to glass slides and stained using DiffQuik. The number of macrophages, neutrophils, lymphocytes, and eosinophils was counted using a light microscope (200 cells/slide). The remaining BALF was centrifuged to remove the cellular content and stored at −80°C for later use.

Table 1. Chimerism of BMC mice

<table>
<thead>
<tr>
<th>Control Mice</th>
<th>BMC Mice (Recipient/Donor)</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>CS7BL/6 wild-type</td>
<td>EC-SOD KO</td>
</tr>
<tr>
<td>Thy 1.1</td>
<td>Thy 1.2</td>
</tr>
<tr>
<td>99.22 ± 0.18</td>
<td>99.04 ± 0.21</td>
</tr>
<tr>
<td>WT (Thy 1.1)/WT</td>
<td>WT (Thy 1.1)/KO</td>
</tr>
<tr>
<td>Thy 1.2</td>
<td>Thy 1.2</td>
</tr>
<tr>
<td>74.62 ± 6.91</td>
<td>80.33 ± 3.28</td>
</tr>
<tr>
<td>KO (Thy 1.2)/WT</td>
<td>KO (Thy 1.2)</td>
</tr>
<tr>
<td>Thy 1.1</td>
<td>Thy 1.2</td>
</tr>
<tr>
<td>58.87 ± 6.93</td>
<td>99.08 ± 0.60</td>
</tr>
</tbody>
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Values shown are the mean percentage of donor cells in total T lymphocytes ± SD; n = 8-12 mice/chimeric group. EC-SOD, extracellular superoxide dismutase; KO, knockout; WT, wild type. Bone marrow chimeric (BMC) mice shown above were generated through bone marrow transplantation, and the chimerism of these mice was verified using flow cytometry. The presence of donor cells (bold) in recipient mice after transplantation was evaluated by assessing the expression of Thy 1.1 or Thy 1.2 on total circulating T lymphocytes (Thy 1-expressing cells) from the blood of the BMC mice.
Immunohistochemical analyses for EC-SOD. Five-micrometer-thick sections of paraffin-embedded lung tissue from BMC mice 7 days following asbestos exposure were cut, deparaffinized, and labeled for EC-SOD. Briefly, sections were incubated in 6% hydrogen peroxide in methanol to inactivate endogenous peroxidases, and antigen retrieval was performed in 10 mM Tris Base, 1 mM EDTA, and 0.05% Tween 20, pH 9.0 at 95°C for 20 min. Sections were blocked with Superblock Blocking Buffer (Thermo Scientific, Rockford, IL) and then incubated for 2 h with an affinity-purified polyclonal antibody for mouse EC-SOD or rabbit IgG as a control. Sections were then incubated with biotin-SP donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and then with avidin-biotin complex reagent (Vector Laboratories, Burlingame, CA). All sections were developed using DAB (Vector Laboratories) and counterstained with Gill’s Hematoxylin.

Detection of leukocyte-derived EC-SOD. Total protein of the BALF was determined using Comassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Equal concentrations of BALF protein (8 μg/sample) were then subjected to SDS-PAGE and blotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA) as previously described (23). As a loading control, the polyvinylidene difluoride membranes were then stained with Ponceau Red Stain (0.2% Ponceau S, 3% trichloroacetic acid) and destained to visually verify equal loading of protein in all samples. Full-length and proteolyzed EC-SOD in the BALF was detected using a rabbit anti-mouse EC-SOD polyclonal antibody as previously described (10, 27).

Histology and fibrosis scoring. Standard hematoxylin and eosin staining was performed on 5-μm-thick lung sections and was scored by a pathologist (Oury) who was blinded to the sample groups as previously described (7, 12, 27). In brief, every field in the entire lung was observed with a light microscope (×200 magnification) and scored if at least 50% of the field was alveolar tissue. Scoring was based on the percentage of alveolar tissue with interstitial fibrosis according to the following scale: 0 = no fibrosis, 1 = up to 25%, 2 = 25–50%, 3 = 50–75%, and 4 = 75–100%. The pathological index score was then reported as a ratio of the sum of all the scores divided by the total number of fields counted for each sample.

Statistical analyses. All comparisons between three or more groups were made using a one-way ANOVA with Tukey’s posttest using GraphPad Prism 5 (GraphPad Software, San Diego, CA). All values shown are means ± SE. A *P* value of less than 0.05 was considered statistically significant.
The location of EC-SOD in the lungs of the BMC mice was determined by immunohistochemical analyses (Fig. 2). EC-SOD was localized to the lung parenchyma, resident alveolar macrophages, and infiltrating inflammatory cells seen in the lungs of WT/WT mice. In WT/KO mice, EC-SOD is present in the lung parenchyma but not localized to the resident alveolar macrophages or infiltrating inflammatory cells. In the lungs of KO/WT mice, EC-SOD is only detected in resident alveolar macrophages and infiltrating inflammatory cells. EC-SOD was not detected in the lungs of the KO/KO mice.

**Infiltrating inflammatory cells and resident macrophages are not the source of airspace EC-SOD.** To determine whether leukocytes release EC-SOD into the airspaces following asbestos-induced interstitial lung injury, Western blotting was performed on BALF from BMC mice at 3 and 7 days after instillation of asbestos. From this analysis, an accumulation of EC-SOD was detected in the airspaces of WT/KO mice at both 3 and 7 days postasbestos exposure even though the leukocytes in these BMC mice did not express EC-SOD (Fig. 3A). Furthermore, there was a lack of EC-SOD observed in the BALF of KO/WT mice following asbestos injury (Fig. 3A). Together, these data demonstrate that leukocytes do not release detectable levels of EC-SOD into the airspace 3 or 7 days following asbestos injury. Additionally, BMC mice that did not receive clodronate treatment before asbestos exposure had similar levels of EC-SOD in their BALF compared with BMC mice that received clodronate treatment (Fig. 3B). These results show that clodronate treatment does not affect EC-SOD accumulation in the BALF. However, when comparing the EC-SOD levels in the BALF of WT mice with BMC mice 3 and 7 days after asbestos exposure, WT mice had higher levels of EC-SOD in the BALF compared with BMC mice (Fig. 3C). This difference between WT mice and WT/WT BMC mice could be due to the effect of radiation treatment on the BMC macrophages.
mice, but the absolute levels of EC-SOD in the lungs of mice have not been directly measured.

Before the current study, it was thought that the EC-SOD accumulating in the airspace following an interstitial lung injury was due to a combination of EC-SOD release from the interstitium as well as direct release of EC-SOD from the infiltrating inflammatory cells. The data in Fig. 3 clearly show that the EC-SOD, which accumulates in the airspace after pulmonary injury, originates solely from the lung parenchyma as infiltrating inflammatory cells and resident alveolar macrophages do not contribute detectable levels of EC-SOD.

**Leukocyte-derived EC-SOD did not affect inflammation after asbestos exposure.** Although significant amounts of EC-SOD from leukocytes could not be detected in the BALF after asbestos-induced pulmonary injury, it is still possible that these cells may release EC-SOD at levels below detection that lessen the magnitude of pulmonary injury. To assess the amount of inflammation following asbestos instillation, the total cells in BALF of the BMC mice were counted at both 3 and 7 days following injury. These measurements revealed that leukocyte-derived EC-SOD had no effect on inflammation resulting from asbestos treatment at either time point (Fig. 4, A and B). In addition, cell differential counting of BALF cells revealed that there were no significant differences in the cellular composition of the BALF after injury (Fig. 4, A and B). Similar to previous studies (27), asbestos-treated WT mice had significantly more inflammatory cells, predominantly neutrophils, present in their BALF compared with control-treated WT mice.

Fig. 4. Leukocyte-derived EC-SOD does not alter leukocyte accumulation in response to asbestos. Total white blood cells and cell differentials in the BALF of the BMC mice were measured 3 (n = 5–6 mice/group, A) and 7 (n = 3–5 mice/group, B) days after asbestos treatment. Cellular infiltrates in the BALF of nontransplanted WT mice were also analyzed 3 (n = 3/group, C) and 7 (n = 3/group, D) days following asbestos or inert particulate control treatment. Total cells were calculated by multiplying the cell concentration (cells/ml) by the total volume of BALF recovered. Data were analyzed using a one-way ANOVA with Tukey’s posttest and are means ± SE. *P < 0.05.

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**Figures:**

A. 3 days after asbestos exposure.

B. 7 days after asbestos exposure.

C. 3 days after intratracheal treatment.

D. 7 days after intratracheal treatment.
at both 3 (Fig. 4C) and 7 (Fig. 4D) days postexposure. Previous work has shown that EC-SOD KO mice have more inflammation than WT mice 24 h and 28 days following asbestos treatment, whereas no significant difference was noted 14 days after exposure (12). Therefore, the current observations add to and are consistent with these previous data. Even though EC-SOD is known to be a potent antioxidant and anti-inflammatory agent, the EC-SOD produced by leukocytes is not abundant enough to lessen the inflammatory response induced by asbestos.

Leukocyte-derived EC-SOD does not lessen the early fibrotic response to asbestos-induced interstitial lung injury. Because EC-SOD is also known to inhibit pulmonary fibrosis, analyses were conducted to determine if leukocyte-derived EC-SOD could limit early stage fibrosis 7 days after asbestos treatment. To test this hypothesis, hematoxylin- and eosin-stained lung sections were scored by a pathologist who was blinded to sample groups and experimental treatment. Individual fields were examined with a light microscope at ×200 magnification, and scoring in each field was based on the percentage of alveolar tissue with interstitial fibrosis. Histological analyses of the lungs revealed there were fibrotic areas present in the lungs of all mice; however, there were no significant differences between any of the BMC mice (Fig. 5A). Prior studies noted that there was significantly more fibrosis in the lungs of EC-SOD KO mice compared with WT mice as early as 14 days following asbestos exposure. Seven days is known to be the beginning of the fibrotic phase in this injury model, and indeed fibrosis was noted in the lung of WT mice 7 days following asbestos exposure (Fig. 5B). Although quantifiable amounts of fibrosis are observed in murine lungs 7 days after asbestos exposure, this early fibrotic time point may explain why there is only a trend and not a significant difference in fibrosis between WT/WT and KO/KO mice (Fig. 5A). Overall, these results suggest that leukocyte-derived EC-SOD has no effect on the early fibrotic response to asbestos exposure.

In conclusion, BMC mice were generated to allow for the determination of the source of airspace EC-SOD during inflammation following interstitial lung injury. From this study, it can be concluded that leukocytes do not release detectable levels of EC-SOD into the airspaces following asbestos-induced interstitial pulmonary injury. Furthermore, leukocyte-derived EC-SOD does not appear to protect against asbestos-
induced injury in the lung, since it was found to not lessen inflammation or early stage fibrosis in vivo.

Although leukocytes are not the source of airspace EC-SOD in this lung injury model, previous work suggests that inflammatory cells may release EC-SOD into the airspaces after pulmonary bacterial infection (28). In addition, EC-SOD KO mice have greater inflammation (predominantly neutrophils) in their BALF following infection (28). Therefore, it is still possible that leukocyte-derived EC-SOD may be released into the BALF and may be effective at limiting inflammation following pulmonary infection.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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