

ALTERED EXPRESSION OF EXTRACELLULAR SUPEROXIDE DISMUTASE IN MOUSE LUNG AFTER BLEOMYCIN TREATMENT

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Abstract—The antioxidant enzyme extracellular superoxide dismutase (EC-SOD) is highly expressed in the extracellular matrix of lung tissue and is believed to protect the lung from oxidative damage that results in diseases such as pulmonary fibrosis. This study tests the hypothesis that proteolytic removal of the heparin-binding domain of EC-SOD results in clearance of the enzyme from the extracellular matrix of pulmonary tissues and leads to a loss of antioxidant protection. Using a polyclonal antibody to mouse EC-SOD, the immunodistribution of EC-SOD in normal and bleomycin-injured lungs was examined. EC-SOD labeling was strong in the matrix of vessels, airways, and alveolar surfaces and septa in control lungs. At 2 d post-treatment, a slight increase in EC-SOD staining was evident. In contrast, lungs examined 4 or 7 d post-treatment, showed an apparent loss of EC-SOD from the matrix and surface of alveolar septa. Notably, at 7 d post-treatment, the truncated form of EC-SOD was found in the bronchoalveolar lavage fluid of bleomycin-treated mice, suggesting that EC-SOD is being removed from the extracellular matrix through proteolysis. However, loss of EC-SOD through proteolysis did not correlate with a decrease in overall pulmonary EC-SOD activity. The negligible effect on EC-SOD activity may reflect the large influx of intensely staining inflammatory cells at day 7. These results indicate that injuries leading to pulmonary fibrosis have a significant effect on EC-SOD distribution due to proteolytic removal of the heparin-binding domain and may be important in enhancing pulmonary injuries by altering the oxidant/antioxidant balance in alveolar interstitial spaces. © 2001 Elsevier Science Inc.

Keywords—Extracellular superoxide dismutase, Pulmonary fibrosis, Antioxidants, Oxidative stress, Proteolysis, Free radicals

INTRODUCTION

Reactive oxygen species such as superoxide and hydrogen peroxide are important pathogenic mediators of many diseases including pulmonary diseases [1–3]. Under normal conditions, the tissue damage caused by reactive oxygen species can be prevented by endogenous antioxidant mechanisms. However, when these mechanisms no longer compensate for large amounts of damaging oxidants, irreversible tissue damage can occur.

Extracellular superoxide dismutase (EC-SOD) is an antioxidant enzyme that catalyzes the dismutation of superoxide into hydrogen peroxide and oxygen [4]. The most recently characterized of the three superoxide dis-

mutases, it shares this ability with two other SOD isozymes: the cytoplasmic and nuclear CuZn-SOD [5] and the mitochondrial Mn-SOD [6]. EC-SOD is a 135 kDa protein that exists primarily as a tetramer of four identical subunits [4]. This tetramer consists of two dimers, each containing a disulfide bond that links the heparin-binding domains in the C-terminus of two subunits together [7–9]. EC-SOD is found predominantly in the extracellular matrix of tissues and to a lesser extent in extracellular fluids [10–13]. The heparin-binding domain of EC-SOD interacts with heparin sulfate in the extracellular matrix of tissues and cell surfaces. This affinity to heparin sulfate is believed to be important in determining the specific location of EC-SOD in the extracellular matrix and allowing it to act as an efficient antioxidant. Heparin-binding affinity can be modulated through proteolysis of the heparin-binding domain and is believed to be important in regulating the distribution of

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EC-SOD in various tissues [7,14]. Notably, the activity of many proteolytic enzymes is increased during inflammation. This increase in proteolytic activity may further exacerbate the oxidative stress associated with inflammation by removing EC-SOD from the affected area.

While EC-SOD is ubiquitously expressed in mammalian tissues, both species and tissue heterogeneity exist. Mice contain high levels of EC-SOD in their lungs compared to other mammals [10]. Increased levels of this antioxidant enzyme in the mouse lung suggests an important role for extracellular superoxide scavenging in this organ, possibly as a protection against the relatively high levels of oxygen to which the lung is continually exposed. The bleomycin-treated mouse is a well-described model system for oxidative stress-induced pulmonary fibrosis, and the abundance of EC-SOD in the mouse lung makes this a good system to study the role of EC-SOD in the development of pulmonary fibrosis. Treatment of mice with bleomycin results in a well characterized fibrotic response that occurs in two distinct phases. First, there is an acute phase characterized by an influx of inflammatory cells, in particular macrophages and polymorphonuclear leukocytes (PMN). This is followed by a chronic stage characterized by extracellular matrix remodeling and collagen deposition [15,16]. EC-SOD is known to colocalize with type I collagen in the lung [13]. This relationship of EC-SOD and collagen may be particularly important since collagen is sensitive to degradation by superoxide. Collagen fragments can function as chemoattractants and activators of macrophages and neutrophils [17–20]. Thus, proteolytic removal of EC-SOD from matrices rich in type I collagen may potentiate inflammation by increasing oxidative stresses leading to collagen fragmentation.

This study tests the hypothesis that insults leading to pulmonary inflammation and fibrosis may alter the distribution of EC-SOD in the extracellular matrix of the lung via enhanced proteolysis of the heparin-binding domain of EC-SOD and the subsequent clearance of this enzyme from the extracellular space. This will result in a change in the oxidant/antioxidant balance in the pulmonary extracellular matrix and would potentially contribute to a proinflammatory environment. The effect of bleomycin treatment on EC-SOD activity and immunodistribution in mouse lung is examined.

EXPERIMENTAL PROCEDURES

Materials

Xanthine oxidase was purchased from Boehringer Mannheim (Indianapolis, IN, USA). Eosin Y, Phloxine B, Concanavalin A-sepharose, xanthine, and equine partially acetylated cytochrome *c* were purchased from

Sigma Chemical Co. (St. Louis, MO, USA). Mayer's Hematoxylin and Clear Rite were purchased from Fisher Scientific (Pittsburgh, PA, USA). Diaminobenzidine (DAB) staining kit was purchased from Vector Laboratories (Burlingame, CA, USA). Immunon Immunohistochemistry kit was purchased from Shandon (Pittsburgh, PA, USA).

Bleomycin treatment of mice

Six to 8 week old B6C3F1 mice (Taconic, Germantown, NY, USA) were given one intratracheal injection of 0.075 U of bleomycin in 50 μ l 0.9% saline (VWR, West Chester, PA, USA) or an equivalent volume of 0.9% saline and then sacrificed 2 d, 4 d, or 7 d post-treatment (4–7 mice per group). Where specified, mouse lungs were subjected to bronchoalveolar lavage by intratracheally instilling and then removing 1 ml of 0.9% saline. Lungs were removed and either processed for paraffin embedding, dried at 110° for acid hydrolysis and hydroxyproline determination, or stored at –80°C for enzyme activity assays and Western blot analysis (see below).

Hydroxyproline determination

Dried lungs were subjected to acid hydrolysis prior to the determination of hydroxyproline content. Briefly, lungs were dried at 110°C overnight in a Fisher Isotemp (Pittsburgh, PA, USA) oven. 6 N HCl was added to the vials containing dried lung and oxygen was removed from the vial by alternating vacuum with nitrogen gas for 1 min. Vials were sealed under vacuum and samples were incubated at 110°C overnight. Samples were then lyophilized and hydroxyproline content was determined as previously described [21,22].

Analysis of EC-SOD activity

Prior to their removal, lungs from treated and control mice were perfused with 5 ml of phosphate-buffered saline through the right ventricle of the heart. The lungs were homogenized in 3 ml of Buffer 1 (50 mM potassium phosphate, pH 7.4, and 0.3 M potassium bromide). After separation from CuZn-SOD and Mn-SOD by concanavalin A-sepharose chromatography [23], EC-SOD activity was measured by inhibition of partially acetylated cytochrome *c* reduction at pH 10.0 as previously described [24]. The total protein concentration in the homogenates was determined by the Coomassie Plus protein assay (Pierce, Rockford, IL, USA).

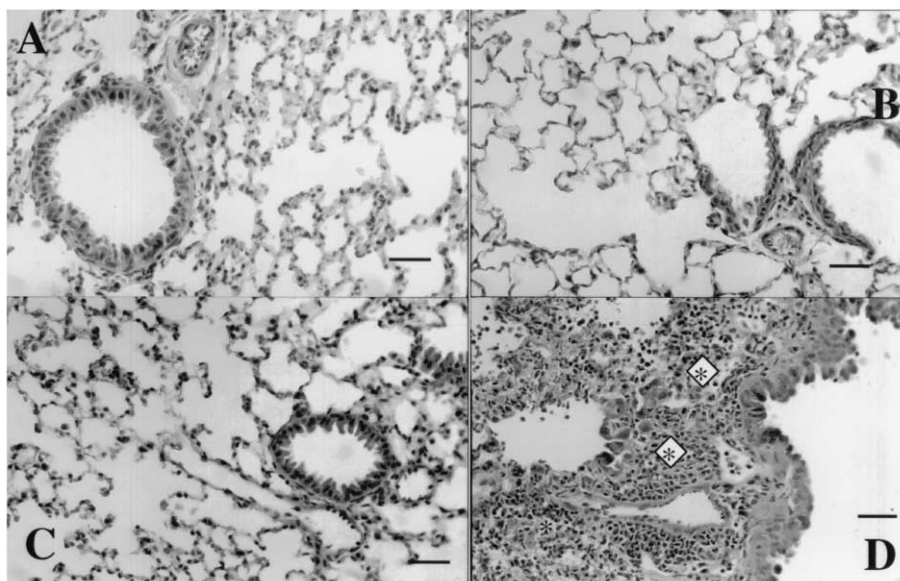


Fig. 1. Histological analysis of lung tissue from control and bleomycin-treated mice. Mice were treated with 0.075 U bleomycin or an equivalent volume of saline. Lungs were sectioned and subjected to hematoxylin/eosin staining as described in Materials and Methods. (A) Saline control; (B) 2 d post-bleomycin treatment; (C) 4 d post-bleomycin treatment; (D) 7 d post-bleomycin treatment. In D, note the significant interstitial thickening and intense inflammation (asterisk). Bar equals 50 μm .

Western blot analysis

Proteins (20 μg) from lung homogenates were subjected to SDS-PAGE on 10% polyacrylamide gels under reducing conditions and electrophoretically transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA). The membranes were blocked overnight at 4°C with 5% nonfat milk in PBS (20 mM potassium phosphate (pH 7.4), 0.15 M potassium chloride). Membranes were then incubated with rabbit antimouse EC-SOD antibody [8,25,26] or nonimmune IgG in PBS with 0.3% Tween-20, followed by incubation with HRP-conjugated goat antirabbit IgG. Antibody detection was carried out using an ECL detection system (Amersham, Braunschweig, Germany). Densitometry was performed on the resulting autoradiograph using a Kodak DC120 zoom digital camera and the Kodak Digital Science 1D analysis software (version 3.0) (Eastman Kodak, Rochester, NY, USA).

Hematoxylin and eosin staining

Four to six microns thick paraffin-embedded lung sections were deparaffinized by heating the slides at 60°C for 30 min, followed by successive incubations in Clear-Rite (Rite Scientific, Pittsburgh, PA, USA), 100% EtOH, 95% EtOH, and distilled water. Slides were stained in Mayer's hematoxylin (Fisher Scientific) for 10 min followed by a brief wash in 1% acid alcohol (1% HCl in 70% EtOH). Slides were then immersed briefly in

a 1% lithium carbonate solution and stained with eosin Y-phloxine B solution (0.1% aqueous eosin Y, 0.01% aqueous phloxine B, 75% EtOH, 0.004% glacial acetic acid), followed by dehydration in 100% EtOH and then xylene.

Immunohistochemistry

Mouse lungs were fixed with buffered neutral formalin 10% (VWR) for 4 h followed by overnight fixation in 70% EtOH at room temperature. After fixation the tissues were processed for paraffin embedding. Serial 4 μm thick sections of the paraffin-embedded tissues were cut (2 sections/slide). Sections were then deparaffinized and labeled for EC-SOD using an indirect immunoperoxidase method using an Immunon immunohistochemistry kit (Shandon, Pittsburgh, PA, USA). Briefly, sections were first incubated in 1% H_2O_2 in methanol to inactivate endogenous peroxidases, followed by antigen retrieval with 0.1% pepsin in 0.01N HCl. To reduce background staining and nonspecific binding, sections were incubated for 1 h in Immunon Maxitags protein-blocking solution (Shandon). Sections were then incubated with a biotinylated rabbit antimouse IgG and streptavidin-horseradish peroxidase. Primary and secondary antibody dilutions were determined empirically and made in Immunon dilution buffer (Shandon). As a control, one serial section on each slide was labeled with preimmune rabbit serum. In order to enhance the strength of the staining, sections were incubated in ABC reagent (Vector Labo-

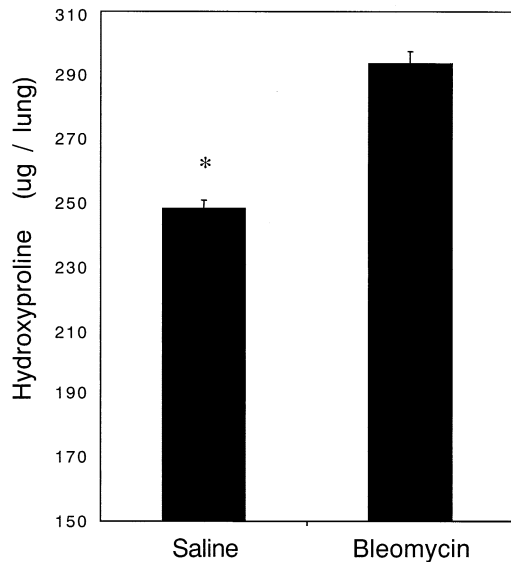


Fig. 2. Hydroxyproline levels in lungs of control and bleomycin-treated mice at 7 d post-treatment. Data shown is representative of three experiments (3–5 mice per group). The mean hydroxyproline levels ($\mu\text{g}/\text{lung}$) and standard error of the mean is depicted. Asterisk denotes significance by Student's *t*-test, $p < .01$.

ratories). The slides were developed using diaminobenzidine (DAB, Vector Laboratories) and counterstained with Mayer's hematoxylin.

Immunohistochemistry slides were scored for intensity of staining on a scale of 0–3 by three independent pathologists who were blinded to treatment and duration (T. O., C. C., S. K.). Scores were then averaged per time

point and compared to control using a Student's *t*-test. Results were considered significant if $p = .05$ or less.

Messenger RNA analysis

Bronchial epithelial cells were isolated from $0.4 \mu\text{m}$ sections of paraffin-embedded lung tissue by laser capture microscopy (Arcturus Engineering, Mountain View, CA, USA) as previously described [27]. Messenger RNA was obtained from the isolated cells or whole sections of paraffin-embedded lung tissue by acid phenol chloroform extraction followed by ethanol precipitation as previously described [28]. Purified RNA was then incubated with $1 \mu\text{g}/\text{ml}$ RNase free DNase followed by acid phenol chloroform extraction and ethanol precipitation to remove contaminating DNA. Purified RNA was subjected to RT-PCR using a GeneAmp EZ Tth RNA PCR kit from Perkin Elmer (Roche Molecular Systems, Branchburg, NJ, USA) with primers specific for either mouse EC-SOD or mouse glyceraldehyde-6-phosphate dehydrogenase (G6PD). The following primers were used: mEC-SOD forward: TTCTTGTTCTACGGCTTGCTAC; mEC-SOD reverse: CTCCATCCAGATCTCCAGCACT; mG6PD forward: TTCTTACTCCT-TGGAGGCCATG; mG6PD reverse: CATCTTGGGCTACTGA-GGAC. Amplified cDNA was analyzed by electrophoresis on a 2% agarose gel. Bands corresponding to amplified cDNA were visualized by ethidium bromide staining. To control for DNA contamination, samples were also subjected to PCR amplification with the omission of the RT step.

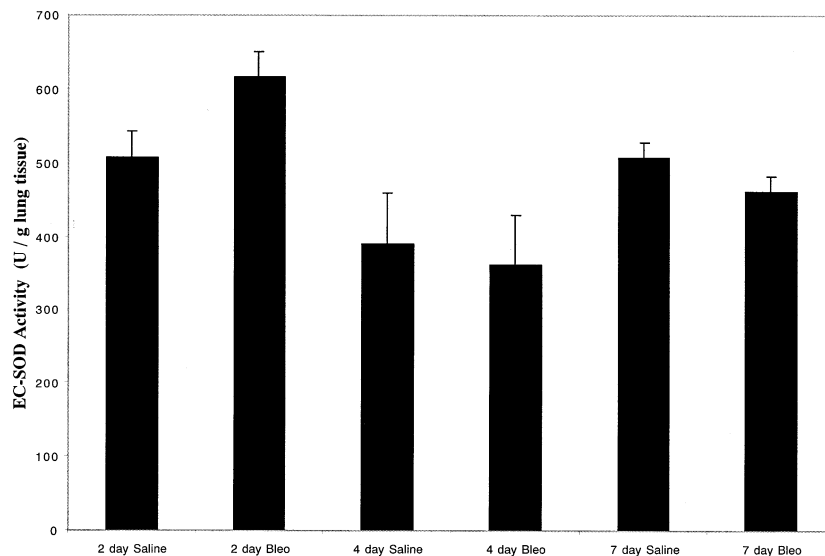


Fig. 3. EC-SOD activity in bleomycin-treated mouse lungs. EC-SOD activity was assessed by inhibition of cytochrome *c* reduction. Data shown is representative of three experiments (4–7 mice per group). The mean EC-SOD activity (units/g lung tissue) is depicted and the error bars indicate the standard error of the mean.

RESULTS

Histological analysis of lungs from bleomycin-treated mice

Histological analysis revealed no change overall in lung tissue at 2 d postbleomycin treatment as compared to the saline-treated controls (Fig. 1B vs. 1A). At 4 days post-treatment, we observed a slight thickening of the interstitial walls (Fig. 1C), but the airways and alveolar spaces remained open with little inflammation. However, at 7 d postbleomycin treatment, two significant changes in lung histology were observed (Fig. 1D). First, there was a significant thickening of the interstitium, indicative of the early stages of matrix remodeling and fibrosis. Second, there was a large infiltration of inflammatory cells, primarily macrophages and neutrophils, into the alveolar spaces and alveolar septa. These changes in lung histology are consistent with previously described characteristics of bleomycin toxicity [15].

To further assess the changes in the amount of fibrosis present in the bleomycin vs. control lungs, hydroxyproline content of the lungs was determined. It is well established that unregulated collagen deposition is characteristic of pulmonary fibrosis. Increases in collagen deposition can be analyzed by assessing the amount of hydroxyproline in a given sample. At 7 d postbleomycin treatment, the hydroxyproline content of the mouse lungs was increased significantly ($p < .05$) over the lungs from the saline-treated control mice (Fig. 2), indicating that collagen deposition was increased in bleomycin-treated mice.

EC-SOD activity in bleomycin-treated lungs

The extent and severity of oxidant injury to the lung are directly dependent upon antioxidant levels present in the tissue. Notably, we have previously shown that augmentation of SOD activity can protect against bleomycin-induced pulmonary fibrosis [22]. Therefore, to determine if bleomycin-treatment altered the level of EC-SOD, the activity of this antioxidant in control and bleomycin-treated lungs was measured. Figure 3 illustrates there was no significant change in the level of EC-SOD activity between control and bleomycin-treated lungs at any of the time points examined. In addition, there was little effect on intracellular SOD activity levels (data not illustrated). Consistent with these findings, Western blot analysis revealed no significant change in total EC-SOD protein levels (Figs. 4A and 4B). However, the ratio of proteolysed (lacking heparin-binding domain) to nonproteolysed EC-SOD was increased significantly ($p \leq .05$) in the bleomycin-treated lungs (Fig. 4C).

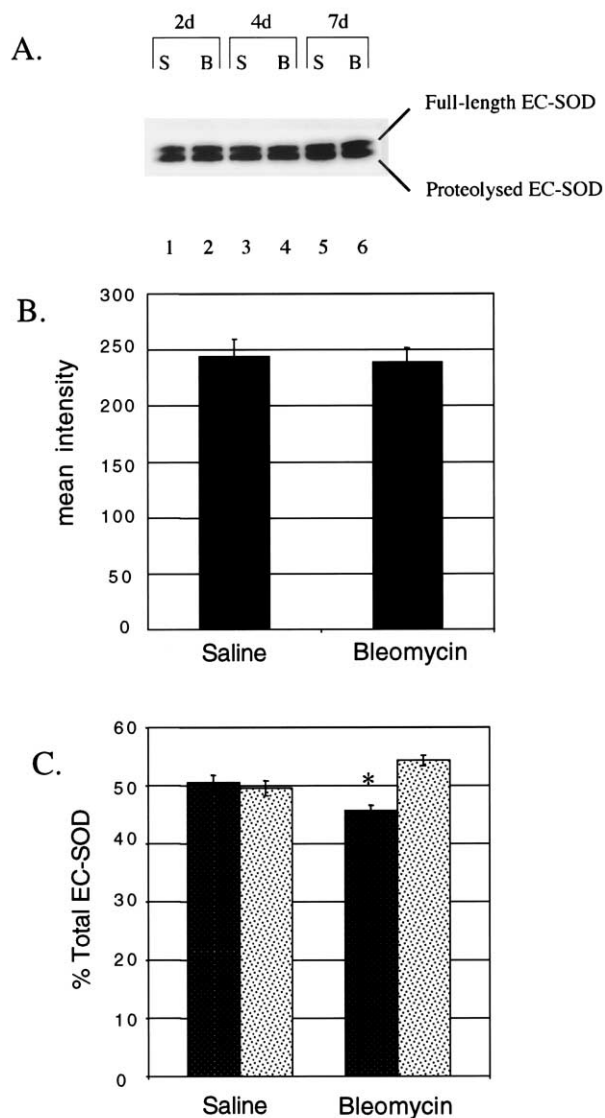


Fig. 4. (A) Western blot analysis of total protein samples from bleomycin-treated and control lungs (representative blots of a total of 3 to 5 samples analyzed per group). Full-length EC-SOD as well as proteolysed EC-SOD (lacking the heparin-binding domain) is indicated; S = saline; B = bleomycin. (B) Graphical representation of densitometric analysis of Western blot in A showing total EC-SOD protein levels at 7 d post-bleomycin treatment. Units listed are arbitrary units of intensity assigned by the Kodak Digital Science 1D analysis software. (C) Graphical representation of densitometric analysis of Western blot in A showing relative levels of full-length (dark stippled bars) and proteolysed EC-SOD (light stippled bars) in saline- or bleomycin-treated mice at 7 d post-treatment. Values expressed as percentage of total EC-SOD. Asterisk denotes significance by Student's *t*-test, $p < .01$.

Immunolocalization of EC-SOD

Using a polyclonal antibody to mouse EC-SOD [8], immunohistochemistry was performed on sections of lung tissue from control and bleomycin-treated mice. This antibody is highly specific for mouse EC-SOD and has no crossreactivity with other proteins in the mouse

Table 1. Immunohistochemical Analysis of EC-SOD Localization in Bleomycin-Treated Mice

	Day 2		Day 4		Day 7	
	Saline	Bleomycin	Saline	Bleomycin	Saline	Bleomycin
Bronchial epithelial cells						
Surface	2.24 +/- 0.20	2.38 +/- 0.05	1.96 +/- 0.16	1.68 +/- 0.13	1.70 +/- 0.21	0.95 +/- 0.17*
Intracellular	0.60 +/- 0.10	0.85 +/- 0.19	0.95 +/- 0.23	0.69 +/- 0.19	0.50 +/- 0.00	1.19 +/- 0.06*
Alveolar septa	1.95 +/- 0.22	2.15 +/- 0.23	1.78 +/- 0.08	1.75 +/- 0.10	2.31 +/- 0.19	1.59 +/- 0.08*
Inflammatory cells	1.35 +/- 0.13	1.45 +/- 0.17	1.73 +/- 0.19	1.31 +/- 0.06	1.25 +/- 0.25	1.75 +/- 0.27

Immunohistochemical staining was quantified on a scale of 0 (no staining) to 3 (intense staining). Average staining for lung sections for 3–5 mice in each group at the indicated time points are shown. Bleomycin groups were compared to the saline control at each timepoint. * Significant difference using Student's *t*-test ($p < .05$).

lung [8]. Immunohistochemical staining was quantified and the results are summarized in Table 1.

In control mice, a strong uniform extracellular staining for EC-SOD is present on the surface and matrix of alveolar septa (Fig. 5A) and also on the apical surface of bronchial epithelial cells (arrows, Fig. 5C). A similar pattern of staining was present at both 2 and 4 d post-bleomycin treatment (data not illustrated). At 4 d post-bleomycin treatment, there was a marginal decrease in EC-SOD staining on the surface of bronchial epithelial cells (Table 1). At 7 d post-bleomycin treatment, there was an extensive inflammatory response comprised mostly of macrophages and neutrophils, which displayed heavy intracellular staining for EC-SOD (Fig. 4E). Also at 7 d post-treatment, there was a significant loss of EC-SOD staining from the alveolar septa (Fig. 5A vs. 5B; Table 1). A significant loss of EC-SOD staining also occurred from the apical surface of the bronchial epithelial cells and was concurrent with an increase in intracellular staining in these cells (Fig. 5C vs. 5D; Table 1). Intracellular localization of EC-SOD has previously been seen in rabbit bronchial epithelial cells during lung development [34] and in placental trophoblasts early in gestation [30]. However, the level of intracellular staining in the saline controls fluctuated between time points (Table 1), and therefore the significance of this finding is uncertain.

To determine if the increase in intracellular staining in bronchial epithelial cells is a result of de novo synthesis or possible internalization of EC-SOD from the extracellular matrix, we isolated these cells by laser capture microdissection and determined if these cells contained EC-SOD mRNA. We were unable to detect EC-SOD message in the isolated cells, although mRNA for glyceraldehydes-6-phosphate was detected (results not illustrated). We were able to demonstrate EC-SOD mRNA from paraffin-embedded lung sections, indicating the tissue processing did not affect the ability to detect message. These results suggest that the increase in intracellular EC-SOD staining maybe due to internalization of EC-SOD protein from the extracellular matrix, although

low levels of EC-SOD mRNA below the detection level can not be completely excluded, especially since these cells have been shown to produce EC-SOD during pulmonary development [34].

Analysis of BALF from control and bleomycin-treated mice

As mentioned above, there is a significant loss of EC-SOD staining from the alveolar septa of bleomycin-treated mice. It has been previously shown that proteolysis of EC-SOD at the heparin-binding domain results in a loss of affinity to heparin [14]. It has been speculated that the loss of heparin affinity will result in the release of EC-SOD from the extracellular matrix of vessels and its subsequent accumulation in the plasma [7]. This speculation is supported by findings of increased full-length EC-SOD in the plasma after heparin treatment [31,32]. We therefore wished to determine if proteolysis of the heparin-binding domain of EC-SOD in the lungs of bleomycin-treated mice was leading to its loss from the alveolar septa. EC-SOD content of bronchoalveolar lavage fluid (BALF) from which the inflammatory cells were removed was analyzed by Western blot. There was 4 times as much EC-SOD in BALF samples from bleomycin-treated mice as compared to samples from control mice (Figs. 6A and 6B). Furthermore, the majority of EC-SOD was found in the proteolysed form (Fig. 6C), which remains active (Fig. 6D) [7]. These results suggest that the EC-SOD is being cleaved and released from the septa and leads to loss of matrix associated antioxidant protection in the lungs of bleomycin-treated mice.

DISCUSSION

In this study, the effect of bleomycin-induced lung injury on EC-SOD expression and localization was evaluated to test the hypothesis that injuries leading to pulmonary fibrosis will alter the distribution of EC-SOD in extracellular matrices and, consequently, alter the oxi-

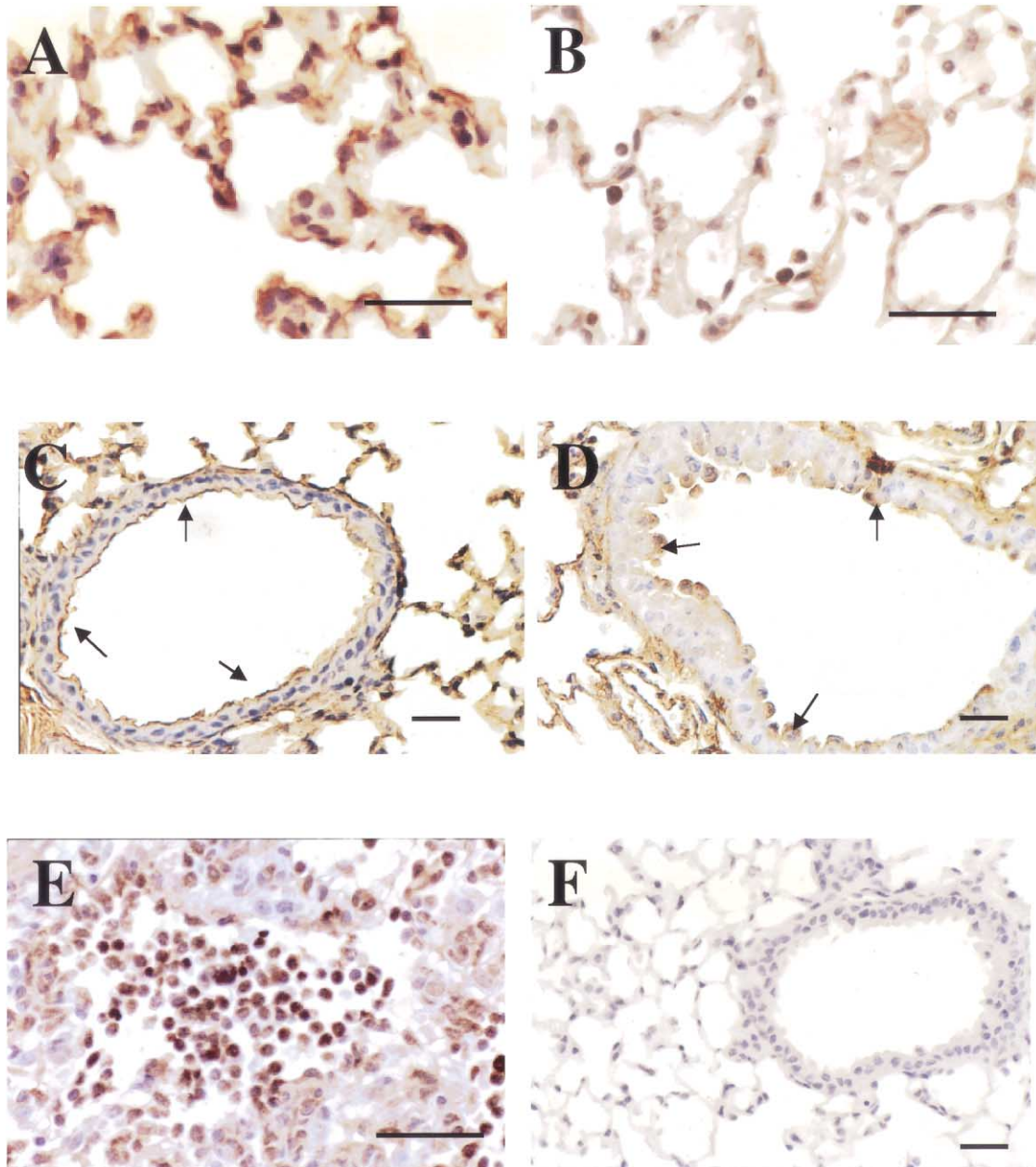


Fig. 5. Immunohistochemical analysis of lung tissue from control and bleomycin-treated mice. Mice were treated with 0.075 U bleomycin or an equivalent volume of saline. Lungs were sectioned and subjected to immunochemistry staining with a mouse polyclonal antibody to EC-SOD as described in Materials and Methods. (A) Alveolar area from a saline-treated control mouse. (B) Alveolar area from a bleomycin-treated mouse 7 d post-treatment. (C) Bronchial epithelial cells from a saline-treated control mouse. Arrows indicate intense surface staining for EC-SOD. (D) Bronchial epithelial cells from a mouse at 7 d post-bleomycin treatment. Arrows indicate intracellular staining for EC-SOD. (E) Bleomycin-treated mouse at 7 d post-treatment. Note that the inflammatory cells stain heavily for EC-SOD. (F) Immunostaining control: 7 d post-treatment incubated with preimmune rabbit sera. Bar is equivalent to 50 μm .

dant/antioxidant balance in the lung. Using a mouse model in which bleomycin was administered by intratracheal injection, several significant changes in the subcellular localization of EC-SOD protein were observed. First, with regard to the localization of EC-SOD, we show that EC-SOD in normal lung tissue is located

primarily in the extracellular matrix, with additional labeling on membrane surfaces of bronchial epithelial cells and alveoli. Within the alveoli, labeling was especially strong in the septal tips, presumably in association with type I collagen as was noted in human alveolar septal tips [13]. The localization in the alveoli is consistent with the

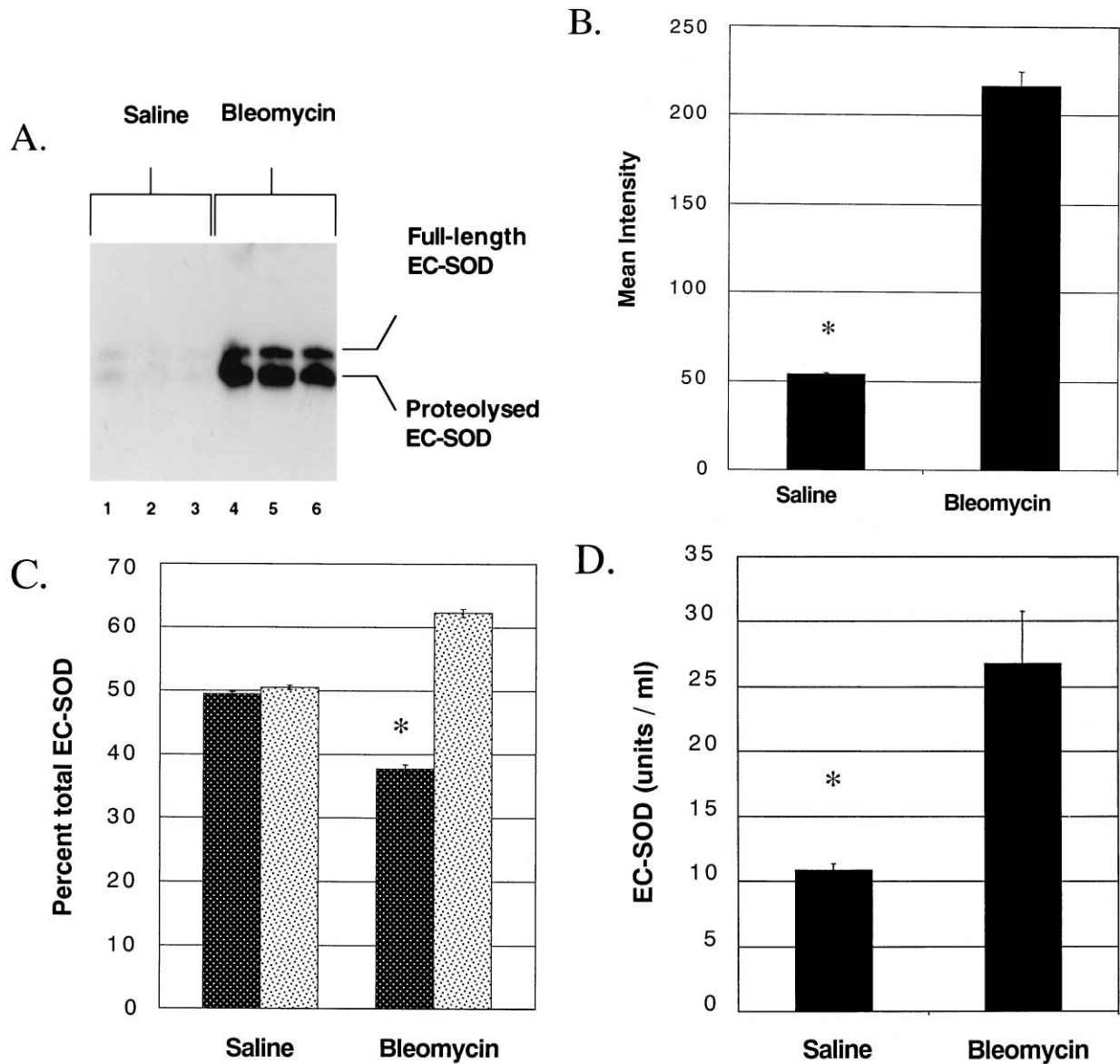


Fig. 6. (A) Western blot analysis of total protein samples from the BALF of control and bleomycin-treated mouse lungs 7 d after treatment ($n = 3$, for each group). Full-length EC-SOD as well as proteolysed EC-SOD (lacking the heparin-binding domain) is indicated. (B) Graphical representation of densitometric analysis of Western blot in A showing BALF total EC-SOD protein levels. Units listed are arbitrary units of intensity assigned by the Kodak Digital Science 1D analysis software. Asterisk indicates results are significant by Student's t -test, $p < .01$. (C) Graphical representation of densitometric analysis of Western blot in A showing relative levels of full-length (dark stippled bars) and proteolysed EC-SOD (light stippled bars) in BALF of saline- or bleomycin-treated mice. Values expressed as a percentage of total EC-SOD. Asterisk denotes significance by Student's t -test, $p < .01$. (D) EC-SOD activity in BALF from saline- and bleomycin-treated mouse lungs 7 d after treatment. The mean EC-SOD activity (units/ml) is depicted and the error bars indicate the standard error of the mean. Asterisk indicates results are significant by Student's t -test, $p < .01$.

finding that both alveolar macrophages and alveolar type II cells synthesize EC-SOD [33]. This pattern of labeling is similar to our previous findings and that of others in mouse lung [12,29]. In addition, previous immunohistochemical studies in rat lung demonstrate EC-SOD staining on the luminal surface of bronchial epithelial cells [35].

Upon treatment with bleomycin, the localization of

EC-SOD changes significantly. There is a significant loss of EC-SOD from alveolar septa as well as a significant loss of EC-SOD from the surface of bronchial epithelial cells. This is significant since the association of EC-SOD with type I collagen in the alveoli has been speculated to be important in preventing superoxide-mediated destruction of collagen (see below). The loss of EC-SOD from the surface of bronchial epithelial cells, as well as the

noted loss of EC-SOD from the extracellular space in alveoli, may compromise the lung's ability to provide adequate antioxidant defense, and thus contribute to bleomycin-induced lung injury.

Notably, the significant changes in the immunodistribution of EC-SOD in the lung did not correlate with a change in enzyme activity in the lung. In addition to the possible internalization of EC-SOD, administration of bleomycin results in a florid inflammatory response at 7 d post-treatment, characterized by the influx of large numbers of neutrophils and macrophages into the alveolar spaces. These inflammatory cells stained heavily for EC-SOD. Previous studies have shown that both macrophages and neutrophils can synthesize EC-SOD, and it has been speculated that EC-SOD may be carried to sites of inflammation by these cells to protect against the oxidants they produce [35]. It is possible that the EC-SOD inside the large number of inflammatory cells that have migrated into the airways and the intracellular EC-SOD in the bronchial epithelial cells may have masked any decrease in matrix-associated EC-SOD activity in the bleomycin-treated lungs.

Proteolysis of the heparin-binding domain of EC-SOD may also contribute to the loss of the enzyme from the extracellular matrix. It has been previously speculated that a similar proteolytic event results in the loss of EC-SOD from the matrix of blood vessels and its subsequent accumulation in plasma. Here we conclusively demonstrate for the first time the accumulation of proteolysed EC-SOD in the BALF of bleomycin-treated mice and that accumulation coincides with the loss of this enzyme from the extracellular matrix of alveolar tissue. Notably, proteolytic cleavage of the heparin-binding domain of EC-SOD does not affect enzymatic activity. This is clearly demonstrated by the increase in EC-SOD activity in the BALF of bleomycin-treated mice (Fig. 6D). Thus, the proteolysis and redistribution of active EC-SOD may serve to explain the lack of an overall decrease in EC-SOD activity observed in whole lung homogenates after bleomycin treatment.

The loss of EC-SOD from the extracellular matrix of lung tissue into the alveolar lining fluid may serve to promote collagen degradation and perhaps increase oxidative injury to alveolar epithelial cells as the enzyme is no longer present in the proper protective location. This may result in an increased inflammatory response in these areas and thus increased injury. It has been shown that type I collagen is sensitive to degradation by the superoxide anion both directly [18,19] and indirectly through the activation of latent collagenases in neutrophils [36,37]. In addition, collagen fragments are known to be both chemoattractants and activators of neutrophils [20]. Therefore, increased production or decreased scavenging of superoxide, which results in collagen degrada-

tion, may accelerate inflammatory responses and tissue destruction through neutrophil recruitment and activation.

In conclusion, this study demonstrates that injuries leading to pulmonary fibrosis can alter the oxidant/antioxidant balance in the extracellular matrix of the lung by proteolysis of EC-SOD. Loss of EC-SOD from the extracellular matrix of the alveolar septa may contribute to superoxide-mediated damage in this area. In addition, lower EC-SOD levels may contribute to inflammatory reactions by allowing increased degradation of collagen by superoxide, resulting in the recruitment of inflammatory cells into the alveolar spaces. This redistribution of EC-SOD may contribute to an increase in oxidative damage and a profibrotic environment in the lung.

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