Mice Lacking Neutrophil Elastase Are Resistant to Bleomycin-Induced Pulmonary Fibrosis

Felix Chua,* Sarah E. Dunsmore,*† Peter H. Clingen,‡ Steven E. Mutsaers,* Steven D. Shapiro,† Anthony W. Segal,* Jürgen Roes,§ and Geoffrey J. Laurent*

From the Departments of Medicine,* Oncology,‡ and Immunology and Molecular Pathology,* Royal Free and University College Medical School, London, United Kingdom; and the Department of Medicine (Pulmonary),† Brigham and Women’s Hospital, Boston, Massachusetts

Neutrophil elastase is a serine protease stored in the azurophilic granules of leukocytes. It has been implicated in the pathology of several lung diseases and is generally presumed to contribute to the tissue destruction and extracellular matrix damage associated with these conditions. To delineate the role of neutrophil elastase in pulmonary inflammation and fibrosis, neutrophil elastase-null mice were intratracheally instilled with bleomycin. In neutrophil elastase-null mice, biochemical and morphological characteristics of pulmonary fibrosis were attenuated for at least 60 days after bleomycin administration despite a typical response to bleomycin as evidenced by assessment of indices of DNA and cell damage. Neutrophil burden of bleomycin-treated wild-type and neutrophil elastase-null mice was comparable, and marked neutrophilic alveolitis was manifest in bleomycin-treated neutrophil elastase-null mice. An absence of immunostaining for active transforming growth factor (TGF)-β in lung tissue from bleomycin-treated neutrophil elastase-null mice suggested a defect in TGF-β activation, which was confirmed by biochemical assessment of TGF-β levels in bronchoalveolar lavage fluid and lung tissue. These data point to novel and unexpected fibrogenic consequences of neutrophil elastase activity in the inflamed lung. (Am J Pathol 2007, 170:65–74; DOI: 10.2353/ajpath.2007.060352)

Pulmonary fibrosis is the most severe adverse effect associated with the clinical use of bleomycin, a cytotoxic chemotherapeutic agent, and intratracheal instillation of bleomycin is a common experimental model of lung fibrosis. In this article, we describe the phenotype of bleomycin-treated neutrophil elastase-null mice. It has long been recognized that neutrophil elastase is present in fibrotic interstitial lung disease of rheumatoid and idiopathic origin but whether this protease plays a specific role in disease pathogenesis or is merely an indication of neutrophilic inflammation is unclear. The paradoxical mechanism by which neutrophil elastase, a protease that breaks down the extracellular matrix, can promote the excess matrix deposition that is characteristic of pulmonary fibrosis has not been defined.

Traditionally, much of the increase in lung extracellular matrix in the bleomycin model and in human pulmonary fibrosis has been attributed to the overproduction of interstitial collagens by cytokine-activated fibroblasts. Although several cytokines have been implicated in fibroblast activation, evidence for eminence of transforming growth factor (TGF)-β in fibrotic disorders is substantial. TGF-β is most efficiently secreted as a large latent complex in which a latent TGF-β binding protein (LTBP) is disulfide bonded to the latent portion of the TGF-β dimer. LTBP is incorporated into the extracellular matrix by transglutamination, and most TGF-β is stored in the extracellular matrix before activation.

In our experiments, we evaluated indices of fibrosis, bleomycin damage, and inflammation. We also assessed active and latent TGF-β levels in bronchoalveolar lavage fluid and lung tissue. Our results indicate that the development of fibrosis and activation of TGF-β activation are impaired in bleomycin-treated neutrophil elastase-null mice. Supported by the Medical Research Council (project grant 9715800 to S.E.M, J.R., A.W.S., and G.J.L.), the Wellcome Trust (training fellowship for Medical and Dental Graduates grant 061554 to F.C., S.E.D., and G.J.L.; and Senior Research Fellowship in Basic Biomedical Sciences grant 047608 to J.R.), the University of London Central Research Fund (to S.E.D.), the British Lung Foundation (research scientist grant P00/4 to S.E.D.), and the American Lung Association (grant RG-029-N to S.E.D.). F.C. and S.E.D. contributed equally to this study; J.R. and G.J.L. share senior authorship.

Accepted for publication October 10, 2006.

Supplemental material for this article can be found on http://ajp.amjpathol.org.

Address reprint requests to Sarah E. Dunsmore, NIGMS, 45 Center Dr., Bethesda, MD 20892-6200. E-mail: sdunsmore_uk@yahoo.com.
mice. These data support the concept that neutrophil elastase activity in the lung may have fibrotic as well as matrix-destructive consequences.

Materials and Methods

Animal Procedures

All animal procedures were approved by the appropriate regulatory body and performed in accordance with Home Office (UK) guidelines.

Generation of Neutrophil Elastase-Null Mice

Neutrophil elastase-null (NE−/−) mice were established on the 129Sv background as previously described.19 Gene inactivation was achieved by deletion of restriction enzyme sites in exon one (ATG start codon disruption) and exon two (frameshift mutation upstream of catalytic active site). Correctly targeted loci were identified by Southern blot analysis.19

Breeding, Housing, and Genotyping

Chimeric mice were mated to obtain NE+/+ and NE−/− breeding colonies. Mouse colonies were housed under conventional conditions in a nonbarrier facility. Signs of opportunistic infection were not manifest. Male and female mice between 12 and 16 weeks of age (20 to 30 g body weight) were used in experimental procedures. To genotype mice, genomic DNA was amplified by polymerase chain reaction with the following primers: common forward, 5'-CATGACACCCCCACTGTCGTGC-3'; wild-type reverse, 5'-CAATGCCAGTAGCATGGCGAG-3'; and null reverse, 5'-GGACTCCTACCTCTCTAATGGAC-3'.

Bleomycin Instillation

Mice were intratracheally injected with 50 μl sterile isotonic saline or with bleomycin sulfate (Kyowa Hakko UK Ltd., Berks, UK) in 50 μl of saline. Unless otherwise indicated, bleomycin was instilled at a concentration of 0.05 U (1 U = 1000 IU = 1 mg).

Fibrosis

Collagen Quantitation

Lung collagen was assessed in acid-hydrolyzed lung tissue by measuring hydroxyproline with a high-pressure liquid chromatography method.20

Histology

Extracellular matrix deposition was visualized by Masson’s trichrome staining of formalin-fixed, paraffin-embedded lung tissue. Histology procedures (tissue processing, sectioning, and Masson’s trichrome staining) for samples used in Figure 2 were performed in the Pulmonary and Critical Care Medicine Morphology Core at Washington University in St. Louis, MO.

Bleomycin Damage

Comet Assay

Saline-perfused lung tissue from untreated and bleomycin-instilled animals was digested with dispase (Calbiochem, Nottingham, UK) and minced into small pieces. DNA strand breaks were detected by the standard alkaline comet assay.21

Cytotoxicity

Release of lactate dehydrogenase into bronchoalveolar lavage fluid (BALF) was measured as an index of bleomycin cytotoxicity using a CytoTox 96 nonradioactive cytotoxicity assay (Promega, Southampton, UK). Absorbance at 490 nm of formazan products resulting from tetrazolium salt conversion22 was quantified.

Alveolar Leak

Alveolar leak was evaluated by measuring the amount of Evans blue dye23 in BALF or lung tissue. Evans blue dye (Sigma-Aldrich Co. Ltd., Poole, UK) was injected into the tail vein and allowed to circulate for 1 hour before procurement of blood, BALF, and saline-perfused lung tissue. Dye was eluted from powdered lung tissue by overnight incubation in formamide. Absorbances at 620 nm of plasma (diluted 1:30 with saline), BALF (undiluted), and lung tissue (20 mg) were determined.

Inflammation

Bronchoalveolar Lavage

Eight 300-μl lavages of sterile saline were performed. The amount of BALF recovered from each mouse averaged 2 ml.

Flow Cytometry

Approximately 1 × 10⁶ cells from the BALF of each animal were incubated with a phycoerythrin-conjugated antibody to Ly-6G and Ly-6C (Gr-1) (BD Pharmingen, San Diego, CA). Stained cell populations were analyzed with a BD FACScan flow cytometer (BD Biosciences, San Jose, CA).

BALF Cell Enumeration

Total leukocyte counts were attained from unstained cell preparations after red blood cell lysis. Differential leukocyte counts were acquired from cytospin preparations. Approximately 1 × 10⁵ cells were cytocentrifuged onto coated glass slides using a Shandon Cytospin 3 cytocentrifuge (Thermo Electron Corp., Pittsburgh, PA). Cytocentrifugation was for 3 minutes at 1000 rpm. Slides were stained with Diff-Quik (Baxter Dade AG, Dudingen, Switzerland), and at least 500 cells per slide were
counted. Leukocytes were classified based on morphological characteristics.

**TGF-β**

**Active TGF-β Immunohistochemistry**

LC (1-30) an antibody that recognizes active TGF-β was acquired from Kathleen C. Flanders, National Cancer Institute, Bethesda, MD. This antibody was applied to formalin-fixed, paraffin-embedded lung tissue. Secondary antibodies were horseradish peroxidase conjugated.

**Active TGF-β Quantitation**

Active TGF-β was quantified with a highly sensitive and specific bioassay based on the ability of TGF-β to stimulate transcription of plasminogen activator inhibitor-1 (PAI-1). Mink lung epithelial cells (MLECs) stably transfected with an 800-bp fragment of the 5′ end of the human PAI-1 gene fused with the firefly luciferase reporter gene were obtained from Daniel B. Rifkin, New York University Medical Center, New York, NY. The addition of active TGF-β, -β2, or -β3 at concentrations ranging from 1 to 10 pmol/L to these cells produces a linear increase in luciferase activity that can be blocked with isoform-specific antibodies. Luciferase activity is minimally increased when other inducers of PAI-1 expression are added to MLECs. Treatment of BALF from bleomycin-treated wild-type and NE−/− mice with a pan-specific TGF-β-blocking antibody (R&D Systems, Abingdon, UK) reduced luciferase activity to background levels (Supplemental Figure 1B at http://ajp.amjpathol.org) indicating that stimulation of the PAI-1 promoter by other substances present in BALF was minimal.

MLECs were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics (penicillin, streptomycin, amphotericin B, and genitin) and used between passage 30 and passage 36. MLECs were plated at a concentration of 1.6 × 10^4 cells/well and allowed to adhere for 3 hours before the addition of samples and standards. After overnight incubation in the presence of samples and standards, MLECs were treated with luciferase lysis reagent (Promega), and relative light units were read using a microplate luminometer (Applied Biosystems, Sunnyhill, CA). Concentrations of active TGF-β were determined from the linear portion of the standard curve (Supplemental Figure 1A at http://ajp.amjpathol.org).

**Collection and Activation of BALF**

Eight 300-μl lavages of sterile saline were performed. Immediately after acquisition, lavage samples were centrifuged at 2000 rpm for 10 minutes. The BALF supernatant was removed and aliquoted for storage at −80°C. The amount of BALF recovered from each mouse averaged 2 ml.

Aliquots of cell-free BALF were thawed once immediately before assay of active TGF-β. Latent TGF-β in cell-free BALF was activated by heating at 80°C in the presence of 0.5 mmol/L phenylmethyl sulfonyl fluoride and 5 μmol/L ilomastat. Exogenously added active TGF-β (0.6 ng/ml) was stable under these conditions (data not shown). In quantitative analyses (see Figure 6A), cell-free BALF from individual animals was used. BALF from several animals was combined for control experiments (Supplemental Figure 1, B and C, at http://ajp.amjpathol.org).

**Extraction of TGF-β from Lung Tissue for MLEC Bioassay**

Lung tissue (50 mg) was homogenized in Dulbecco’s modified Eagle’s medium containing 0.5 mmol/L phenylmethyl sulfonyl fluoride and 5 μmol/L ilomastat (Chemicon, Harrow, UK), heated at 80°C for 5 minutes and centrifuged for 15 minutes at 1800 rpm. Supernatants were assayed for active TGF-β, and residual material was washed in Dulbecco’s modified Eagle’s medium, homogenized, heated, and centrifuged. Supernatants obtained from the residual material were assayed for active TGF-β. This process was repeated three times for a total of five separate TGF-β extractions from a single lung tissue aliquot (Supplemental Table 1 at http://ajp.amjpathol.org). The amount of active TGF-β in lung tissue after the fifth extraction was <2 ng.

**RNase Protection**

RNase protection assays were performed with multiprobe templates (Pharmingen, Cowley, UK) on total lung RNA (3 μg). Trizol reagent (Life Technologies, Paisley, UK) was used to isolate RNA from unperfused lung tissue. Protected fragments were visualized on a FLA-3000 image analyzer (Fujifilm Medical Systems, Stamford, CT). Band intensities were quantitated with Advanced Image Data Analysis (AIDA Version 2.0) software (Raytest Italia S.R.L., Cinisello Balsamo, Italy).

**Proteolytic Treatment of Lung Tissue**

Lungs from bleomycin-treated animals were inflated with ~150 μl of a 1:1 mixture of Tissue-Tek O.C.T. compound (Sakura Finetek U.S.A., Inc., Torrance, CA) and 0.9% saline and frozen in a cryomold surrounded by O.C.T. compound. Serial 10-μm sections were cut in a cryostat maintained at −22°C. Lung sections (200 sections/well) were placed into 12-well plates and kept at −22°C until commencement of protease treatment. Every 10th serial section was stained with hematoxylin and eosin for morphometric analysis of the total lung area subjected to protease treatment.

Dulbecco’s modified Eagle’s medium containing 0.25% bovine serum albumin (1.5 ml) was added to each well of lung tissue. Protease (neutrophil elastase or plasmin) was added to some wells at a concentration of 30 nmol/L. Plates were incubated for 2 hours at 37°C. Enzymatic reactions were stopped by the addition of 0.5 mmol/L phenylmethyl sulfonyl fluoride and 5 μmol/L ilomastat. Samples were concentrated to 800 μl by centrif-
ugal filtration. Half of each sample (400 μl) was assayed for active TGF-β. The residual 400 μl was heated at 80°C for 10 minutes before measurement of active TGF-β.

**Statistical Analysis**

On graphs, data shown are the mean, and error bars depict SEM. In some instances, error bars do not extend beyond the symbols on the graph. Statistical comparison was performed using the appropriate two-tailed Student's t-test with n representing the number of mice. Values were considered to be statistically different at $P < 0.05$.

**Results**

**Neutrophil Elastase-Null Mice Are Resistant to Bleomycin-Induced Fibrosis**

In bleomycin-treated wild-type (WT) mice, the maximal increase in lung collagen deposition occurred at a dose of 0.05 U (Figure 1A) 30 days after instillation (Figure 1B). Similar results were previously obtained with 129Sv mice. Based on these data, fibrosis in NE/mice was evaluated at doses of 0.05 and 0.10 U 30 and 60 days after bleomycin instillation. No significant increase in collagen deposition was observed in bleomycin-treated NE/mice at either time point (Figure 1C).

Fibrous interstitial matrix deposition (Figure 2A) was prominent in WT mice 30 (Figure 2C) and 60 (Figure 2E) days after bleomycin instillation. In bleomycin-treated NE/mice, alveoli were more likely to be occluded by cells than by matrix (Figure 2B), and signs of acute lung injury such as alveolar collapse and inflammation were prevalent (Figure 2, B, D, and F). Histological evidence of pulmonary fibrosis in bleomycin-treated NE/mice was minimal (Figure 2, B, D, and F). Inflammation seemed to persist in the lungs of bleomycin-treated NE/mice, and even though alveolar collapse (Figure 2B) was evident in NE/lungs 60 days after bleomycin instillation, areas of intra-alveolar fibrosis were nominal (Figure 2F). The histological appearance of saline-treated controls was similar (Figure 2, G and H).

**Alveolar Destruction Is Manifest in Neutrophil Elastase-Null Mice**

Bleomycin-induced lung injury is initiated by DNA scission. In bleomycin-treated WT and NE/mice, DNA damage in the form of strand breaks was evident 4 hours after bleomycin instillation (Figure 3A). Differential repair of strand breaks in WT and NE/mice was not apparent, and the majority of single-strand DNA damage was repaired by 1 day after bleomycin instillation (Figure 3A). Cells in which DNA damage cannot be repaired may undergo necrosis and release intracellular contents into the extracellular space. The amount of lactate dehydrogenase in BALF was assessed as an index of bleomycin cytotoxicity. Bleomycin/saline ratios of lactate dehydrogenase measurements were slightly less in BALF ob-

---

**Figure 1.** Lung collagen does not increase in bleomycin-treated NE/mice. A: Bleomycin dose response. Lung collagen was assessed by quantitation of hydroxyproline 50 days after instillation of saline ($n = 9$), 0.025 ($n = 9$), 0.05 ($n = 7$), or 0.10 ($n = 7$) U of bleomycin. B: Time course of collagen deposition in WT mice. Lung collagen was assessed by quantitation of hydroxyproline 0, 15, 30, or 60 days after instillation of saline (open circles) or 0.05 U of bleomycin (filled circles). When not shown, error bars do not extend beyond the symbols. n = 7 for all time points. C: Time course of collagen deposition in NE/mice. Lung collagen was assessed by quantitation of hydroxyproline 0, 30, or 60 days after instillation of saline (open circles) or 0.05 U of bleomycin (filled circles). When not shown, error bars do not extend beyond the symbols. At each time point (n = 7 for all time points), lung collagen in bleomycin-treated NE/mice was not significantly different from the corresponding WT or NE/saline controls, which did not statistically differ from each other or from untreated mice.
tained from NE−/− mice (day 3: WT, 3.6 ± 0.48, and NE−/−, 2.5 ± 0.45; day 7: WT, 5.2 ± 0.92, and NE−/−, 4.7 ± 1.1). Statistically, however, the amount of lactate dehydrogenase released into BALF did not differ between WT and NE−/− mice at the 7-day time point.

Edema and alveolitis were the predominant histological features in the lungs of WT and NE−/− mice 3 days after bleomycin instillation. At the 7-day time point, an intense alveolitis and multifocal parenchymal sites of inflammation were obvious. Histological evidence of alveolar destruction was similar in bleomycin-treated WT and NE−/− mice at the 7-day time point. Alveolar leak (Figure 3C), which was measured as a quantitative index of alveolar destruction, was similar in bleomycin-treated WT and NE−/− mice 3 and 7 days after instillation.

**Neutrophil Burden Is Comparable in Bleomycin-Treated Wild-Type and Neutrophil Elastase-Null Mice**

Equivalent numbers of neutrophils were recovered in BALF obtained from WT and NE−/− mice 1 day after bleomycin instillation (Figure 4A). Neutrophils were also present in histological sections of the alveoli of bleomycin-treated NE−/− mice (data not shown), and myeloperoxidase activity in NE−/− lung tissue was increased after bleomycin instillation (Supplementary Table 2 at http://ajp.amjpathol.org). In cytospin preparations, the typical morphology of macrophages and neutrophils was obvious (Figure 4, C–F). No statistically significant differences in the total number of leukocytes recovered in BALF (Figure 4B) or in the percentages of macrophages, neutrophils, and lymphocytes in BALF (Table 1) were observed at the 7-day time point.

**TGF-β Is Not Activated in Bleomycin-Treated Neutrophil Elastase-Null Mice**

Staining for active TGF-β in the lungs of bleomycin-treated NE−/− mice was minimal 7 days after bleomycin instillation (Figure 5A). In contrast, staining for active TGF-β in the lungs of bleomycin-treated WT mice was widespread at this time point (Figure 5B) and particularly evident in damaged alveoli (Figure 5B, inset). In many areas active TGF-β appeared to be associated with the extracellular matrix (Figure 5B, inset). Macrophages and epithelial cells also stained positively for active TGF-β in the lungs of bleomycin-treated WT mice (Figure 5, B and inset).

The total pool of TGF-β recovered in BALF from NE−/− mice (saline, 0.4 ± 0.02 ng/ml; bleomycin, 0.6 ± 0.05 ng/ml) 7 days after bleomycin instillation was significantly less than that of WT mice (saline, 0.6 ± 0.03 ng/ml; bleomycin, 1.1 ± 0.03 ng/ml) despite similar proportions of active (30%) and latent (70%) TGF-β (Figure 6A). The diminished level of TGF-β in NE−/− BALF was not attributable to decreased expression of TGF-β1 or -β2 (Figure 7) or the absence of TGF-β in lung tissue (Figure 6B) from bleomycin-treated NE−/− animals (saline, 101 ± 8.2 ng/lung; bleomycin, 168 ± 9.8 ng/lung).

**Neutrophil Elastase Can Release TGF-β from Lung Tissue**

TGF-β was released from lung tissue by neutrophil elastase and by plasmin (Figure 6C). The relative proportions of active (46%) and latent (54%) TGF-β released by plasmin were identical to that detected in control (no protease) samples. The proportion of latent TGF-β released by neutrophil elastase (58%) was slightly greater. Activation of recombinant small latent TGF-β (299-LT-005; R&D Systems) by neutrophil elastase was similar to that of heat treatment and of plasmin (Supplemental Figure 2A at http://ajp.amjpathol.org), but neutrophil elastase had negligible effects on activation of latent TGF-β complexes recovered in BALF (Supplemental Figure 2B at http://ajp.amjpathol.org). BALF complexes were readily activated by heat treatment (Figure 6A) and by plasmin (Supplemental Figure 2B at http://ajp.amjpathol.org). Because neutrophil elastase activity was demonstrable in BALF from bleomycin-treated animals (Supplemental Figure 2C at http://ajp.amjpathol.org), these results may indicate that proteolysis of small latent TGF-β complexes by neutrophil elastase is not favored in vivo.

**Discussion**

In the present report, we demonstrate that in bleomycin-treated neutrophil elastase-null mice, collagen deposition does not increase despite a similar response to bleomycin as wild-type mice. The diminished fibrosis in bleomycin-treated neutrophil elastase-null mice may be attributable to insufficient TGF-β activation. These data imply that neutrophil elastase activity may be essential for the initiation of regenerative pathways that lead to matrix deposition in the lung.

Historically, pulmonary fibrosis was supposed to emanate from neutrophilic or lymphocytic alveolitis,27–32 and neutrophilic alveolitis is most often detected in patients diagnosed with fibrosis of unknown cause or idiopathic pulmonary fibrosis.27–34 Neutrophilic alveolitis was identified in bleomycin-treated neutrophil elastase-null mice. Thus, it is likely that neutrophils are in the appropriate pathophysiological location in the lungs of bleomycin-treated neutrophil elastase-null mice and that the phenotype we describe in this article reflects the specific lack of cleavage of a fibrogenic neutrophil elastase substrate rather than nonspecific absence of oxidative and/or proteolytic activity of other neutrophil enzymes in areas of bleomycin-induced inflammation.

Although the possibility that attenuated lung injury contributes to the diminished fibrosis in neutrophil elastase-null mice cannot be discounted, another explanation for the inability of neutrophil elastase-null mice to increase collagen deposition after bleomycin instillation is impaired TGF-β activation. TGF-β is a potent stimulator of collagen production35–37 that has distinct and cell-type-
specific functions in each phase of acute wound healing.\textsuperscript{38,39} Tissue fibrosis may result from excessive or prolonged TGF-β activity during wound remodeling.\textsuperscript{40} Although reagents that block TGF-β activity or signaling have been shown to limit fibrosis in animal models,\textsuperscript{41–44} application of TGF-β antagonists to human fibrotic diseases continues to be a challenge.

Impaired TGF-β activation has been observed in other mouse models. Significant pathological abnormalities attributed to a lack of TGF-β activation are apparent in unchallenged thrombospondin-1-null mice.\textsuperscript{45} In β6 integrin-null,\textsuperscript{26} CD44 antigen-deficient,\textsuperscript{46} and interleukin-13-null\textsuperscript{47} mice, levels of active TGF-β in bleomycin-treated animals are decreased compared to the appropriate wild-type controls. Thrombospondin-1 and the 6 integrin subunit participate in nonproteolytic mechanisms of TGF-β activation. Proteolysis does appear to be involved in the TGF-β activation pathways mediated by CD44 and interleukin-13. The advantage of multiple and redundant TGF-β activation mechanisms is underscored by the lethal phenotype of TGF-β,β6-null mice.\textsuperscript{48}

We have explored in vivo rescue and reconstitution strategies that would experimentally link the lack of active

---

**Figure 2.** NE<sup>−/−</sup> mice are resistant to bleomycin-induced fibrosis. Histology shown is representative of observations from three to six mice of each treatment group. A: Fibrous areas of matrix deposition in WT lung. Lung tissue was obtained 60 days after bleomycin installation, fixed, and stained with Masson's trichrome. Fibroblasts (arrowhead) were present in areas of excess matrix accumulation. B: Focal areas of cell accumulation in NE<sup>−/−</sup> mice. Lung tissue was obtained 60 days after bleomycin installation, fixed, and stained with Masson's trichrome. Fibroblasts were not evident in collapsed alveoli (arrowhead). C: Masson's trichrome staining of bleomycin-treated WT lung. Lung tissue was obtained 30 days after bleomycin installation. D: Masson's trichrome staining of bleomycin-treated NE<sup>−/−</sup> lung. Lung tissue was obtained 30 days after bleomycin installation.

**Figure 3.** The response of the lung to bleomycin is typical in NE<sup>−/−</sup> mice. A: DNA damage. DNA damage was visualized by propidium iodide staining of lung cells after single-cell electrophoresis. In this procedure, damaged DNA assumes a comet-like appearance with a brightly fluorescent head and a tail with length proportional to the amount of DNA strand breakage. In untreated cells, nuclei are intact. DNA strand breakage is evident in cells isolated 4 hours after bleomycin instillation and is repaired by 1 day after instillation. Samples from at least two WT and two NE<sup>−/−</sup> mice were analyzed at each time point. B: Cytotoxicity. BALF was collected 3 (n = 3, WT; n = 4, NE<sup>−/−</sup>) and 7 (n = 6) days after instillation of bleomycin (bleo) or saline. Lactate dehydrogenase (LDH) was measured with a colorimetric assay. Data are expressed as a ratio of the absorbance in BALF from bleomycin-treated animals to that of saline controls. C: Alveolar leak. Alveolar leak was assessed by the extravasation of Evans blue dye into BALF at 3 (n = 5, WT; n = 4, NE<sup>−/−</sup>) and 7 days (n = 5, WT; n = 4, NE<sup>−/−</sup>) after bleomycin instillation. Data are expressed as a ratio of the absorbance of BALF to that of plasma from the same mouse. D: Cytospin preparation of BALF collected from a NE<sup>−/−</sup> mouse 1 day after bleomycin instillation. E: Cytospin preparation of BALF collected from a WT mouse 7 days after bleomycin instillation. F: Cytospin preparation of BALF collected from a NE<sup>−/−</sup> mouse 7 days after bleomycin instillation. G–F: Large arrows indicate macrophages. Neutrophils are identified by arrowheads. Scale bars = 20 μm (G–F).

**Figure 4.** Bleomycin-induced inflammation is similar in WT and NE<sup>−/−</sup> mice. A: Neutrophils. The number of neutrophils in BALF obtained 1 day after bleomycin (bleo) instillation was determined by flow cytometry. B: Leukocytes. Leukocytes in cytospin preparations from BALF obtained 7 days after bleomycin (bleo) instillation were identified based on morphological characteristics and enumerated. Bleo: n = 6, saline: n = 4. C: Cytospin preparation of BALF collected from a WT mouse 1 day after bleomycin instillation. D: Cytospin preparation of BALF collected from a NE<sup>−/−</sup> mouse 1 day after bleomycin instillation. E: Cytospin preparation of BALF collected from a WT mouse 7 days after bleomycin instillation. F: Cytospin preparation of BALF collected from a NE<sup>−/−</sup> mouse 7 days after bleomycin instillation. C–F: Large arrows indicate macrophages. Neutrophils are identified by arrowheads. Scale bars = 20 μm (C–F).
TGF-β in bleomycin-treated neutrophil elastase-null mice to the diminished fibrosis in these animals. Because technical limitations obviated direct intratracheal instillation of TGF-β or neutrophil elastase, a bone marrow transplant protocol was established so that a TGF-β soluble receptor could be dispensed 1 week after bleomycin instillation to neutrophil elastase-null mice reconstituted with wild-type bone marrow. Neutrophil elastase-null mice transplanted with wild-type bone marrow did develop fibrosis when instilled with bleomycin. In pilot experiments, however, excessive mortality resulted from the administration of a TGF-β soluble receptor to bleomycin-treated reconstituted animals, and the extent of lung injury in surviving animals precluded any biochemical assessments of TGF-β levels or fibrosis.

In summary, we have demonstrated that in bleomycin-treated neutrophil elastase-null mice, collagen deposition...
McAnulty, Caroline Owen, Anastasia Papafili, and Louise Reynolds for helpful discussions; Daniel B. Rifkin and Anna Ludlow for mink lung epithelial cells; and Kathleen C. Flanders for LC (1-30) antibody.

Acknowledgments

We thank Manlio Barbarisi, Steve Bottoms, and Robert Thomas for technical assistance; Jeff Packman, Mick Keegan, and Jamie Evans for animal husbandry; Luigi Atzori, Geoffrey Bellingan, Rachel Chambers, Sarah Herrick, Michael Hill, Gisli Jenkins, Patricia Leoni, Robin

---

**Figure 7.** TGF-β expression is similar in bleomycin-treated WT and NE−/− mice. A: RNase protection assay. RNA was isolated from unperfused lung tissue collected 7 days after instillation of WT or NE−/− mice with bleomycin. Relative positions of undigested probes for TGF-β1, TGF-β3, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and RNase-protected fragments of TGF-β1, TGF-β3, and GAPDH are shown. B: Gel densitometry. Data were acquired in pixels and are expressed as a ratio relative to GAPDH. For data shown in the graph, n = 8 for bleomycin-treated WT and NE−/− mice.

---

Neutrophil Elastase Has Profibrotic Activity

**References**

absence of neutrophil elastase and cathepsin G. Immunity 2000, 12:201–210