Elastin protein levels are a vital modifier affecting normal lung development and susceptibility to emphysema

Adrian Shifren, Anthony G. Durmowicz, Russell H. Knutsen, Eiichi Hirano, and Robert P. Mecham

Departments of 1Internal Medicine, 2Pediatrics, and 3Cell Biology and Physiology,
Washington University School of Medicine, Saint Louis, Missouri

Submitted 9 September 2006; accepted in final form 26 November 2006

Shifren A, Durmowicz AG, Knutsen RH, Hirano E, Mecham RP. Elastin protein levels are a vital modifier affecting normal lung development and susceptibility to emphysema. Am J Physiol Lung Cell Mol Physiol 292: L778–L787, 2007. First published December 1, 2006; doi:10.1152/ajplung.00352.2006.—Cigarette smoking is the strongest risk factor for emphysema. However, sensitivity to cigarette smoke-induced emphysema is highly variable, and numerous genetic and environmental factors are thought to mitigate lung response to injury. We report that the quantity of functional elastin in the lung is an important modifier of both lung development and response to injury. In mice with low levels of elastin, lung development is adversely affected, and mice manifest with congenital emphysema. Animals with intermediate elastin levels exhibit normal alveolar structure but develop worse emphysema than normal mice following cigarette smoke exposure. Mechanical testing demonstrates that lungs with low levels of elastin experience greater tissue strains for any given tissue stress compared with wild-type lungs, implying that force-mediated propagation of lung injury through alveolar wall failure may worsen the emphysema after an initial enzymatic insult. Our findings suggest that quantitative deficiencies in elastin predispose to smoke-induce emphysema in animal models and suggest that humans with altered levels of functional elastin could have relatively normal lung function while being more susceptible to smoke-induced lung injury.

smoking

PULMONARY EMPHYSEMA IS CHARACTERIZED by destruction of alveolar septae resulting in pathological alveolar air space enlargement (50). The etiology of emphysema is most frequently related to cigarette smoking, although not all smokers develop clinical disease (28). Current hypotheses of the pathogenesis of emphysema center on imbalances in protease/antiprotease or oxidant/antioxidant levels, among others. In most cases, pathways of tissue damage in cigarette smoke-induced injury involve the destruction of elastin-rich structures in the lung.

The mechanical properties of the lung are determined principally by the connective tissue networks laid down during development. These extracellular matrices are composed chiefly of elastin, collagen, and proteoglycans, and it is elastin that is primarily responsible for providing reversible distensibility during respiratory cycling. Within the parenchyma, elastin is distributed extensively within the alveoli, including the alveolar septae, septal junctions, and along the septal-free edges. Organization of this elastin architecture begins during the pseudoglandular stage of lung development, increases throughout the canalicular and saccular stages, and peaks during alveolation, an event that in mice begins a few days after birth. Alveogenesis requires the participation of alveolar myofibroblasts at the tips of the developing alveolar septae, and active elastin expression by these myofibroblasts suggests that elastic fiber deposition and proper elastic fiber formation are critical for alveolar septation and normal alveolar development (5, 11, 31, 38). Consistent with this hypothesis is the observation that mice deficient for elastin (Eln–/–) demonstrate arrested perinatal development of terminal airway branches, resulting in dilated distal air sacs with attenuated tissue septae, a condition characteristic of emphysema (55).

Elastic fibers are composite structures containing at least two morphologically distinguishable components: amorphous elastin and microfibrils. To build a functional fiber, the production of these components must occur in a well-defined temporal sequence during tissue development. Many proteins have been studied over the years. Intrapulmonary instillation of elastolytic enzymes, for example, confirmed that destruction of elastic fibers is difficult to repair when damaged. Fiber reconstruction requires the coordinated reexpression of all of the molecules that constitute the elastic fiber as well as the cross-linking enzymes. This is an inefficient process in adult tissue, most often resulting in production of elastin that either does not polymerize or does not organize into a functional three-dimensional fiber.

The central role of elastin in the pathology of emphysema is apparent from the numerous disease models that have been studied over the years. Intrapulmonary instillation of elastolytic enzymes, for example, confirmed that destruction of existing elastic fibers leads to emphysema-like lesions (24, 27, 46). There is also evidence that functional variants of elastin may increase lung susceptibility to cigarette smoke-induced damage by altering elastic fiber assembly and integrity (25). More recently, experiments in mice have shown that deficiency in elastin or in the elastic fiber proteins fibrillin-1, LTBP-3 and -4, fibrulin-4 and -5, and EMILIN (elastin microfibril interface located protein) all result in emphysema-like lung morphology at birth (6, 35, 36, 49, 51, 56, 58). This congenital or developmentally acquired emphysema results largely from abnormal elastin deposition or altered elastic fiber-mediated growth factor signaling during development (36, 58) and is fundamentally different from the destruction of...
normal, mature fibers seen in acquired emphysema. Genetic disorders affecting many of these same elastic fiber proteins have also been linked to emphysema-like disease in humans. Marfan syndrome, for example, caused by dominant mutations in the fibrillin-1 gene, results in emphysema in a subset of adult Marfan syndrome patients. Similarly, mutations in fibrillin-4 and -5 have recently been linked to severe emphysema-like lung disease in some individuals (17, 22, 53). Mutations in elastin that result in the synthesis and incorporation of mutant tropoelastin into the extracellular matrix have been linked to emphysema in individuals with cutis laxa (39, 53).

Whereas the reports cited above describe emphysematous changes in the lung as a result of alterations in elastin quality, in this report we show that elastin quantity is a more important modifier of susceptibility to disease than has been heretofore appreciated. Lower than normal levels of elastin resulting from genetic or environmental abnormalities, or elastic fibers that are functionally compromised because of mutations within elastin or elastic fiber genes, may convey a predisposition to emphysema when the lung is challenged by insults such as cigarette smoke or oxidant injury. Furthermore, genetic disorders that compromise elastic fiber deposition and function during lung maturation may contribute to neonatal alveolar seaption defects that manifest as developmental emphysema. The identification of elastin and elastic fiber proteins as modifiers of predisposition to emphysema is important in identifying underlying mechanisms leading to human disease and in the discovery of markers that can be used as predictors of disease susceptibility.

MATERIALS AND METHODS

**Animals.** C57BL/6J mice bearing a heterozygous deletion of exon 1 in the elastin gene (Eln+/−) (30) backcrossed for more than five generations in the C57BL/6J background were used for all studies. Eln−/− littersmates from Eln−/− crosses were used as wild-type (WT) controls. hBAC−/− mice (human Eln transgene positive, mouse Eln gene null) were generated using a human bacterial artificial chromosome (BAC) clone (CTB-51122) containing the complete human elastin gene. BAC circular DNA was injected into fertilized mouse oocytes from C57BI/6xCBA mice, which were implanted into the uterus of pseudopregnant foster mothers. After birth, founders expressing the transgene were identified using RT-PCR. Of the six founder lines, line 3 was found to have the highest transgene expression level and was used throughout this study. Eln gene expression levels, based on RT-PCR, were approximately one-third of those found in WT mice (manuscript in preparation). Transgene-positive animals were mated to WT (C57BL/6) mice for more than five generations to stabilize the line. All housing and surgical procedures were performed in accordance with institutional guidelines. All experimental procedures were approved by the Washington University institutional Animal Studies Committee.

**Lung volume estimation.** Three-month-old WT, Eln+/−, and hBAC−/− mice were euthanized with a lethal dose of intraperitoneal pentobarbital. Lungs were dissected from the thoracic cavity after ligating the trachea prior to transection to prevent fluid entering the lungs. Lungs were rinsed with saline to remove any blood, and excess fluid was removed by blotting. Lungs were then weighed. The trachea was cannulated, and, without deaeration, the lungs were inflated with formalin at 25 cm of water pressure. Lungs were then reweighed. Calculated differences in weight are directly proportional to the volume of infused formalin. Lung volume was calculated by multiplying the difference in lung weight by the specific gravity of formalin.

**Lung elastin, hydroxyproline, and total protein content measurements.** Three-month-old mice from all three genotypes were euthanized with pentobarbital. Lungs were removed, dissected free of the extra-pulmonary airways and connective tissue, washed, weighed (wet weight), and snap-frozen on dry ice. Frozen lungs were lyophilized and reweighed (dry weight). Ratios of dry lung weight-to-body weight were then analyzed. Dried left lungs were hydrolyzed with 6 N HCl at 110°C for 72 h. Total protein, hydroxyproline, and desmosine and isodesmosine levels in protein hydrolysates were determined using a Beckman 6300 amino acid analyzer as previously described (4). Each group analyzed contained a minimum of three animals.

**Static lung compliance assessment.** Three-month-old mice from all three genotypes were euthanized with pentobarbital, and the tracheas were cannulated through a neck incision. The lungs were not flushed with solution to avoid altering surfactant and thereby the alveolar air-liquid interface. Lungs were removed en bloc with the cannula in situ and placed in a humidity chamber to avoid desiccation. The cannula was connected via a three-way stopcock system to a graduated syringe and a modified pressure arteriograph (Pressure/Myograph System P-100; Danish Myotechnology, Copenhagen, Denmark) used previously in our laboratory (12, 54). The equal volume method of determining lung history was used. Briefly, all lungs were preconditioned with 3 inflation-deflation cycles using 0.5 ml of air for each cycle. Starting at the residual volume (no inflation, trachea open to atmospheric pressure) the lungs were inflated in 0.1-ml increments (range: 0.1–1.2 ml) every 30 s to a maximum inflation volume of 1.2 ml [total lung capacity (TLC) in C57Bl/6J mice: ~1.0 ml; Ref. 23]. Thereafter, lungs were allowed to passively deflate in 0.1-ml decrements every 30 s. Pressure and volume on inflation and deflation were recorded using Myoview Acquisition software (Danish Myotechnology) and used to produce a static pressure-volume curve. Expiratory curves are represented. Specific compliance was determined by calculating the slope (∆V/∆P) of the expiratory curve at functional residual capacity (FRC) (~0.25 ml) (29, 41), whereas chord compliance was calculated over 100-µl increments at inflation volumes ranging from 200 µl to 1,100 µl. Each group analyzed contained a minimum of five animals.

**Respiratory system compliance assessment.** Mice from all three genotypes were anesthetized using sodium pentobarbital, and the tracheas were cannulated through a neck incision. Regular quasi-sinusoidal mechanical ventilation was achieved using a volume-controlled model 687 ventilator (Harvard Apparatus, Holliston, MA) delivering a respiratory rate of 160 breaths/min and a tidal volume of 5 ml/kg. PEEP was kept at 0 throughout. Briefly, the whole-body pressure plethysmography chamber (PLY3111 for mice; Buxco Electronics, Sharon, CT), was calibrated for both pressure and flow. Pressure was calibrated with the use of a manual manometer after excluding leaks. The preamplifier signal was adjusted to 0 V and 20 cmH2O pressure was applied to the system. Peak steady voltage was defined as 20 cmH2O. Flow was calibrated by excluding leaks, zeroing the preamplifier voltage, and steadily injecting 1 ml of air into the sealed chamber. Peak flow was determined by the software and accepted if the flow met the effective range for the chamber (10–20 ml/s). Each ventilated mouse was placed in the plethysmography chamber, and an esophageal catheter was placed for monitoring changes in pleural pressure. The chamber-pressure-time wave was continuously measured via a transducer connected to a computer data acquisition system running Biosystem AX software (Buxco Electronics) (15, 29). Real-time readouts of all measured variables (respiratory system compliance, airway resistance, peak airway pressures, and peak expiratory flows) were provided by the data acquisition system, and all animals were carefully monitored over a period of 3 min during which all readings were recorded. The pressure equation for the lung states that total measured pulmonary pressure (P_{eq}) is the sum of the elastic (P_{elas}) and resistive (P_{res}) pressures of the
respiratory system ($P_{tot} = P_{al} + P_{aw}$). This equation can be expanded to: $C = V/(P - F \times R)$, where $C$ = compliance, $V$ = volume, $P$ = pressure, $F$ = flow, and $R$ = resistance. From this formula, we understand that compliance is directly related to volume and resistance but inversely related to pressure. Each group analyzed contained a minimum of four animals.

**Stress-strain measurements.** Three-month-old mice from all three genotypes were euthanized with sodium pentobarbital. The lungs were removed from the thoracic cavity, placed into balanced physiological saline (130 mM NaCl, 15 mM NaHCO3, 5.5 mM dextrose, 4.7 mM KCl, 1.2 mM MgSO4?7H2O, 1.2 mM KH2PO4, 0.026 mM EDTA, and 1.6 mM CaCl2, pH 7.2) on ice, and separated into individual lobes. Lung parenchymal strips were obtained by cutting tissue strips from the long axis of each lobe. Tissue strips were fixed to adjustable metal posts mounted on a force transducer (Danish Myotechnology). A central region along the attached tissue strip length was chosen for diameter and length measurements. Tissue strip length was adjusted manually using a micrometer attached to one of the metal posts. The starting length (inter-post distance: $l_0$) and cross-sectional area ($A_0$) for each strip were determined after 3 preconditioning cycles from 0 to 0.1 mm. At the starting length, the force was set to 0 and the strips were stretched from 0 to 0.4 mm (or until yield strength was exceeded) in steps of 0.02 mm for 45 s to allow for force equilibration at each new length. At 45 s the force was recorded. All measurements were performed in a heated tissue bath containing balanced physiological saline at 37°C within 1 h of death. The length and force measurements were normalized to obtain stress ($\sigma$) and stretch ratio ($\lambda$) as $\sigma = F/A_0$; $\lambda = (l_0 + l_i)/l_0$, where $l_i$ is stretched length, $l_0$ is starting length of the strip, $F$ is measured force, and $A_0$ is cross-sectional area of the strip corresponding to $l_0$. The elastic modulus ($Y$) of each tissue was calculated by determining the slope of the stress-strain curves ($\Delta\sigma/\Delta\lambda$) over known changes in measured length ($l$). Each group analyzed contained a minimum of four animals and utilized at least one strip per lobe from each animal.

**Smoke exposure.** Three-month-old WT and Eln+/– mice were exposed to cigarette smoke from two filtered 2R4F University of Kentucky research cigarettes (cigarette weight 1,060 mg, filter vented during smoking) for 6 days per week, for a total of 6 mo with the use of a nose-only KCl, 1.2 mM MgSO4, 0.026 mM EDTA, and 1.6 mM CaCl2, pH 7.2) on ice, and separated into individual lobes. Lung parenchymal strips were obtained by cutting tissue strips from the long axis of each lobe. Tissue strips were fixed to adjustable metal posts mounted on a force transducer (Danish Myotechnology). A central region along the attached tissue strip length was chosen for diameter and length measurements. Tissue strip length was adjusted manually using a micrometer attached to one of the metal posts. The starting length (inter-post distance: $l_0$) and cross-sectional area ($A_0$) for each strip were determined after 3 preconditioning cycles from 0 to 0.1 mm. At the starting length, the force was set to 0 and the strips were stretched from 0 to 0.4 mm (or until yield strength was exceeded) in steps of 0.02 mm for 45 s to allow for force equilibration at each new length. At 45 s the force was recorded. All measurements were performed in a heated tissue bath containing balanced physiological saline at 37°C within 1 h of death. The length and force measurements were normalized to obtain stress ($\sigma$) and stretch ratio ($\lambda$) as $\sigma = F/A_0$; $\lambda = (l_0 + l_i)/l_0$, where $l_i$ is stretched length, $l_0$ is starting length of the strip, $F$ is measured force, and $A_0$ is cross-sectional area of the strip corresponding to $l_0$. The elastic modulus ($Y$) of each tissue was calculated by determining the slope of the stress-strain curves ($\Delta\sigma/\Delta\lambda$) over known changes in measured length ($l$). Each group analyzed contained a minimum of four animals and utilized at least one strip per lobe from each animal.

**Morphometric analysis of alveoli.** Mice from all three genotypes were euthanized, and the lungs were inflated (without deaeration) through the trachea to 25 cm water pressure with 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA). Lungs were removed en bloc, formalin-fixed for 24 h, and embedded in paraffin (59). Serial 5-μm-thick parasagittal sections were stained with hematoxylin and eosin (H&E), Hart stain, and periodic acid-Schiff stain. For chord length measurements, H&E sections were utilized exclusively. Lung images were sampled in a blinded fashion from all lobes at ×200 magnification using a random number generator and random starting points. Only sections showing obviously gross preparation artifact or distortion were excluded from analysis. Images were digitally captured in eight-bit grayscale using a Zeiss Axioskop microscope with an inline Zeiss Axioscam camera and Axiosvision software (all Carl Zeiss, Thornwood, NY). Chord length analysis was performed using NIH Image software (version 1.63; National Institutes of Health, Bethesda, MD) with a chord length macro (available at http://rsb.info.nih.gov/nih-image). Binarized, inverted lung micrographs were manually thresholded and subjected to a logical “AND” operation with horizontal and vertical grids of parallel lines. The line lengths overlying alveolar spaces were measured and averaged to yield mean chord length. Chord lines touching the edges of the image fields were excluded from analysis to ensure that mean chord length included only line segments subtending alveolar walls at both ends (9, 16, 37). Chord length measurement was chosen over mean linear intercept as it has the advantage of being independent of alveolar septal thickness. A minimum of 10 fields per slide, from each of 4 slides per animal (total = 40 fields per animal) were measured. Each group analyzed contained a minimum of six animals.

**Macrophage immunohistochemistry.** Macrophage staining was performed with rat anti-mouse Mac-3 antibody (BD Pharmingen, San Diego, CA). Primary antibody (1 μg/ml) or diluting buffer (negative control) was applied to the sections and incubated overnight in a humidity chamber at 4°C. The sections were developed with the Vector ABC Kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine applied for 30–60 s (16, 47). The slides were counterstained with 2% Gill’s hematoxylin (Fisher Scientific). Lung fields for analysis were selected using a random number generator and random starting points. The number of Mac-3-positive cells in each field was counted manually in a blinded fashion. Counts included both tissue and alveolar macrophages. A minimum of 6 fields per slide, and 4 slides per animal (total = 24 fields per animal) were counted. Each group analyzed contained a minimum of six animals. All counts were normalized to alveolar wall length to account for tissue loss in emphysematous lungs.

**Statistical analyses.** Data analysis was performed using one-way analysis of variance (ANOVA), to determine the differences between >2 groups of data, and Student’s t-tests to determine differences between pairs of data. All results are presented as mean values ± SE. P values <0.05 were chosen as the threshold for statistically significant differences.

**RESULTS**

**Elastin content in Eln+/– and hBAC–/– lungs is reduced.** Three groups of mice with differing levels of lung elastin (Table 1) were used in this study. All animals were on the C57BL/6 background and were maintained under identical conditions. Mice bearing a heterozygous deletion of exon 1 of the elastin gene (Eln+/–) (30) have lung elastin levels that are 45% lower than WT mice ($P = 0.01$). The third group of mice was generated using a BAC containing the complete human elastin gene. The BAC transgene was expressed in founder animals, which were then bred with Eln+/– mice to produce mouse elastin null/human elastin transgene-positive animals (hBAC+/–). These mice are unique in that the human elastin transgene rescues the perinatal lethal elastin null (Eln–/–).

**Table 1. Pulmonary elastin content is reduced in Eln+/– and hBAC+/– mice**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Eln+/–</th>
<th>hBAC+/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastin content, pmol (desmosine + isodesmosine)/g lung weight</td>
<td>118±5</td>
<td>65±2*</td>
<td>40±3**</td>
</tr>
<tr>
<td>% Elastin</td>
<td>100</td>
<td>55</td>
<td>37</td>
</tr>
<tr>
<td>Collagen content, nmol hydroxyproline/g lung weight</td>
<td>65±2</td>
<td>65±3</td>
<td>58±2***</td>
</tr>
<tr>
<td>% Collagen</td>
<td>100</td>
<td>100</td>
<td>90</td>
</tr>
</tbody>
</table>

Values are means ± SE. Left lungs from all 3 genotypes were weighed, hydrolyzed, and analyzed for total elastin (desmosine and isodesmosine) and collagen (hydroxyproline) content. Elastin content is expressed as picomole of desmosine and isodesmosine per gram of wet lung weight, and collagen content as nanomole of hydroxyproline per gram of wet lung weight; $n = 3$ mice per group. *$P = 0.01$ vs. wild-type (WT) controls; **$P = 0.006$ vs. WT controls; ***$P = 0.03$ vs. WT controls. Eln−/−, mice bearing a heterozygous deletion of exon 1 in the elastin gene; hBAC+/–, mice that are human Eln transgene-positive, mouse Eln gene null.
animals (manuscript in preparation). *hBAC*−/− animals have lung elastin levels that are ~65% lower than those found in WT animals (*P* = 0.006). Lung collagen levels (Table 1) expressed as a measure of wet lung weight demonstrated no differences between WT and *Eln*+/− animals, but *hBAC*−/− lungs showed significantly decreased collagen levels (*P* = 0.03).

**Pulmonary structure is similar in WT and *Eln*+/− mice.** Lungs from WT and *Eln*+/− mice were inspected macroscopically for evidence of gross anomalies. Ex vivo appearance of WT and *Eln*+/− lungs was identical (Fig. 1A), and no differences in lobe number or major airway branching pattern were noted between the genotypes (data not shown). Lung weights normalized to body weights showed a trend towards decreased

---

**Fig. 1.** Lung development is dependent on pulmonary elastin content. Lungs from 3-mo-old wild-type (WT; labeled as *Eln*+/+), *Eln*+/−, and *hBAC*−/− mice were inflation-fixed with 10% neutral buffered formalin at 25 cmH2O and removed en bloc (A). Ruler scale = centimeters. Representative hematoxylin and eosin (H&E)-stained sections (B) are shown for all 3 groups of animals. Scale bar = 200 μm. Chord length measurements (C) from WT (white bar), *Eln*+/− (black bar), and *hBAC*−/− (gray bar) mice were used to quantify alveolar size. *N* = 6 mice per group. Error bars represent SE. *P* < 0.0001 vs. WT controls. *Eln*+/−, mice bearing a heterozygous deletion of exon 1 in the elastin gene; *hBAC*−/−, mice that are human *Eln* transgene positive, mouse *Eln* gene null, generated using a human bacterial artificial chromosome (BAC) clone (CTB-51J22) containing the complete human elastin gene and the human elastin gene promoter.
lung weight in \(Eln^{+/−}\) mice that did not reach statistical significance \((P = 0.17)\). Analysis of lung volume data showed evidence of increased lung volume in \(Eln^{+/−}\) animals \((0.94 ± 0.02\) ml\) compared with WT littermate controls \((0.90 ± 0.02\) ml\), but the difference was not statistically significant \((P = 0.29)\). There was no obvious difference in large airway morphology between \(Eln^{+/−}\) and WT controls as assessed by histochemical staining. Alveolar architecture \(Fig. 1B\) appeared identical in WT and \(Eln^{+/−}\) mice, and there was no difference in chord length between 3-mo-old WT and \(Eln^{+/−}\) animals \((31.9 ± 1.0\) vs. \(31.9 ± 1.4\) \(\mu m\); Fig. 1C).

\(Eln^{+/−}\) mouse lungs are more susceptible to cigarette smoke-induced lung injury. To determine how elastin insufficiency impacts the ability of the lung to tolerate injury, WT and \(Eln^{+/−}\) animals were exposed to cigarette smoke twice per day for 6 mo. Cigarette smoke exposure was well tolerated by both genotypes over the entire 6-mo exposure. One WT animal developed a pulmonary carcinoma and was excluded from all analyses. Figure 2 shows representative lung fields from smoked and nonsmoked WT and \(Eln^{+/−}\) mice with corresponding chord length measurements. Age-matched nonsmoking controls from both strains showed no significant difference in their chord lengths \((WT = 31.4 ± 0.7\) \(\mu m\), and \(Eln^{+/−} = 31.5 ± 0.9\) \(\mu m\); \(P = 0.37)\). These values were designated as baseline chord length. The lack of change in chord length between 3 and 9 mo in either group of nonsmoking animals indicates that degenerative change is not a factor in this model over the time course studied. Emphysema was readily apparent in both strains of smoked mice as evidenced by destruction of both alveolar sac and alveolar ductal walls \(Fig. 2A\). However, air space enlargement secondary to alveolar septal destruction in \(Eln^{+/−}\)
mice (43.3 ± 1.2 μm) was more extensive than that in WT controls (38.1 ± 0.9 μm) (P < 0.0001; Fig. 2B).

Eln<sup>+/−</sup> mouse lungs demonstrate an augmented inflammatory response to cigarette smoke. Macrophages play an important role in the pathogenesis of emphysema and account for many known features of the disease (13, 14, 16, 44). Macrophages are known to localize to the sites of alveolar wall destruction, and correlation between macrophage numbers and disease severity is well documented (10). The pulmonary macrophage response to 6 mo of cigarette smoke exposure in both genotypes of mice is shown in Fig. 3. Comparison of macrophage number normalized to alveolar surface length revealed no significant difference between Eln<sup>+/−</sup> and WT nonsmoking controls (3.2 ± 0.31 vs. 3.2 ± 0.34 cells/mm alveolar wall). However, in smokers, normalized macrophage number was significantly increased in both Eln<sup>+/−</sup> (12.4 ± 0.44 cells/mm alveolar wall) and WT lungs (10.5 ± 0.48 cells/mm alveolar wall) (P < 0.01). Whereas both genotypes showed a marked influx of macrophages into the lung, the response in Eln<sup>+/−</sup> mice was 1.25× greater than smoked WT controls (P = 0.02).

Low levels of lung elastin result in congenital emphysema. In contrast to the normal size and lung morphology of WT and Eln<sup>+/−</sup> mice, 3-mo-old hBAC<sup>−/−</sup> mice demonstrated grossly enlarged thoraces and lungs with evidence of marked volume increase (Fig. 1A). Analysis of lung volume data showed that lung volume in the hBAC<sup>−/−</sup> mice (1.41 ± 0.01 ml) was significantly greater than both the WT and Eln<sup>+/−</sup> animals (P < 0.0001). Microscopic examination revealed obvious air space enlargement (Fig. 1B), and chord length analysis yielded a mean chord length of 57.4 ± 1.9 μm, an increase of 165% compared with WT control mice (P < 0.0001) (Fig. 1C). Closer inspection showed that enlargement of alveoli subtending alveolar ducts occurred in addition to that occurring in the alveolar sacs. Larger airways, as evaluated by histochemical staining techniques, were identical to those in both Eln<sup>+/−</sup> and WT animals.

Lung compliance is increased in mice deficient in elastin. Figure 4A shows the expiratory static pressure-volume curves of isolated WT, Eln<sup>+/−</sup>, and hBAC<sup>−/−</sup> mouse lungs. At all given volumes, the pressure-volume curve in Eln<sup>+/−</sup> lungs is shifted to the left compared with WT controls, indicating that the lung elastic recoil pressure in Eln<sup>+/−</sup> lungs is reduced. The hBAC<sup>−/−</sup> mouse shows the lowest elastic recoil pressures of all three genotypes and similarly has the greatest lung compliance, irrespective of lung volume. Calculation of specific lung com-
Compliance at FRC showed that lung compliance for WT = 0.03 ml/cmH2O, Eln\(^{+/−}\) = 0.05 ml/cmH2O, and hBAC\(^{+/−}\) = 0.09 ml/cmH2O (P < 0.01).

Chord compliance (Fig. 4B), calculated from the slopes of the curves (ΔV/ΔP) over 100-μl increments, indicate that at low lung volumes, Eln\(^{+/−}\) lungs have much greater compliance than WT lungs. However, at high volumes, this difference is no longer evident. Similarly, at both low and high lung volumes the compliance of Eln\(^{+/−}\) lungs tends to remain constant, whereas compliance of the WT lungs increases at higher volumes. hBAC\(^{+/−}\) lungs demonstrate the greatest chord compliance at any given lung volume. In addition, chord compliance in these mice tends to remain constant at all measured lung volumes.

Whole body plethysmography was used to measure respiratory system compliance (C\(_{\text{resp}}\)) in mechanically ventilated animals (Fig. 4C). The chest wall and thoracic structures in mice are extremely compliant, with almost all of the elastic recoil of the respiratory system attributable specifically to the lung (23, 29). Since chest wall compliance is not a significant factor in our model, respiratory system compliance measurements in WT and Eln\(^{+/−}\) animals (0.01 ± 0.008 and 0.02 ± 0.001 ml/cmH2O; P < 0.001) support our static compliance findings that lung compliance at FRC in Eln\(^{+/−}\) animals is approximately double that found in WT controls. Similarly, hBAC\(^{+/−}\) animals had the greatest C\(_{\text{resp}}\) (0.03 ± 0.001 ml/cmH2O; P < 0.0001) as would be expected in animals with grossly emphysematous lungs.

Tidal volumes in WT (0.15 ± 0.006 ml) and Eln\(^{+/−}\) (0.15 ± 0.006 ml) mice were identical (P = 0.45) as would be expected with volume-controlled ventilation. The pressure equation predicts the maximum pressure change during tidal volume ventilation in WT lungs (1.59 ± 0.010 cmH2O) to be greater than that in Eln\(^{+/−}\) lungs (1.35 ± 0.007 cmH2O), as was indeed the case (P < 0.0001). Pulmonary resistance contributes significantly to airway pressure and may account for observed pressure differences during ventilation. Measurement of pulmonary resistance demonstrated no difference between WT (0.95 ± 0.012 cmH2O·s\(^{-1}\)·ml\(^{-1}\)) and Eln\(^{+/−}\) (0.95 ± 0.002 cmH2O·s\(^{-1}\)·ml\(^{-1}\)) lungs (P = 0.48), eliminating pulmonary resistance as a contributing factor to the different lung pressures. These dynamic measurements suggest, therefore, that measured pressure differences are a function of different lung compliances. WT mice (0.08 ± 0.0008 cmH2O·ml\(^{-1}\)·s\(^{-1}\)) perform less work of breathing during tidal volume ventilation than Eln\(^{+/−}\) mice (0.07 ± 0.0004 cmH2O·ml\(^{-1}\)·s\(^{-1}\)) (P < 0.001). Since it requires less energy to ventilate more compliant lungs at similar volumes, the data suggest that WT and Eln\(^{+/−}\) lungs operate at similar lung volumes, with the higher compliance of Eln\(^{+/−}\) lungs leading to lower physiological ventilation pressures (as opposed to similar pressures at different lung volumes).

Eln deficiency markedly alters the tissue properties of isolated lung strips. Figure 5 shows the stress-strain curves of isolated lung tissue strips from WT, Eln\(^{+/−}\), and hBAC\(^{+/−}\) lungs at stress and strain amplitudes approximating the range experienced during tidal volume breathing at FRC. The net effect of decreasing total lung elastin content is a shifting of the stress-strain curve to the right.

Calculation of incremental elastic modulus (Y) at intermediate strains indicated that around FRC there is a direct relationship between elastin dosage and mechanical stiffness with WT tissue stiffness (100% elastin dosage; Y = 9.25 N/cm\(^2\)) being ~1.7× greater than Eln\(^{+/−}\) tissue stiffness (~55% elastin dosage; Y = 5.16 N/cm\(^2\)), which is ~1.4× greater than hBAC\(^{+/−}\) tissue stiffness (~35% elastin dosage; Y = 3.35 N/cm\(^2\)) (P = 0.004). Thus the incremental elastic modulus (Y) of lung tissue is decreased as elastin content is lowered, indicating that the lung tissue stiffness is reduced when lung elastin is lacking.

DISCUSSION

A critical elastin protein level is required for normal lung development. Elastin is known to have a pivotal role in lung development. In mice deficient for elastin (Eln\(^{−/−}\)), or in mice deficient in key growth factors such as platelet-derived growth factor that have an effect on the onset of elastin expression, perinatal development of terminal airway branches is arrested, resulting in distal air sacs that are dilated with attenuated tissue septae (3, 55). Our data show that mice with elastin levels about half those found in WT animals (Eln\(^{+/−}\)) have lungs that are macroscopically normal, with normal lobe numbers and major airway branching patterns (see also Ref. 55). On microscopic examination, neither the pulmonary parenchyma nor the resident inflammatory cells offer an obvious difference from the WT controls, and chord length measurements show no difference between the two genotypes. This is in contrast to what was found for the hBAC\(^{−/−}\) animals where lung morphology was grossly abnormal. These animals, with approximately one-third normal elastin expression levels, manifest with severe congenital emphysema that is present from birth and is not progressive. In many respects, the lungs in these animals resemble the dilated and thinner-walled lungs found in elastin null (Eln\(^{−/−}\)) animals (55) even though the hBAC\(^{−/−}\) animals are rescued from the perinatal lethality observed with the Eln\(^{−/−}\) phenotype. The finding that development up to the terminal bronchiole level is microscopically normal and that alveoli at both the alveolar duct and alveolar sac levels are perturbed suggests a defect in alveolar septal development and alveolar growth, processes that are known to be driven by Eln expression (31). Based on this data, we conclude that low levels of elastin become a limiting factor in normal lung development. It appears that a minimum amount of cross-
linked elastin is required to sustain the scaffolding needed for functional alveolar formation and growth. Abnormalities in lung signaling related to elastin and extracellular matrix composition may also play a role.

Altered Eln gene dosage affects pulmonary responses to injury. Mice heterozygous for the elastin gene develop worse emphysema when exposed to cigarette smoke than do WT mice (Fig. 2), suggesting that levels of elastin are an important determinant of susceptibility to lung injury. By histological analysis, Eln+/− animals showed an almost twofold increase in air space enlargement after smoking compared with WT controls. Our data also indicate that Eln+/− mice have half the amount of cross-linked elastin as WT mice, suggesting that there is less enzyme substrate present for smoke-induced degradative proteases, resulting in more significant fiber destruction given the same degree of injury. Lower than normal amounts of elastin may also make the lung more susceptible to injury by mechanisms other than proteolysis, such as oxidant/antioxidant imbalance (8, 18, 19, 42). It is interesting that Eln+/− mice develop more severe macrophage inflammation during smoking than do WT mice. Monocyte influx into the lung is mediated to a large extent by soluble elastin peptides generated through degradation of insoluble elastin fibers by macrophage-derived proteases (20). Thus the higher number of macrophages and the more severe emphysema observed in the smoking Eln+/− animals is consistent with enhanced monocyte recruitment by elevated levels of elastin peptides.

Another likely factor in the propagation of lung damage in Eln+/− mice is force-induced alveolar destruction resulting from the altered mechanical properties of the elastin-deficient lung. This is consistent with the model by Suki et al. (52) wherein protease-induced degradation of elastic fibers leads to their mechanical failure and, ultimately, to collapse of the alveolar wall. Alveolar wall collapse, in turn, results in the progression of emphysema. It seems likely, given the material properties of the Eln+/− mouse lung (that experiences greater tissue strain for any given degree of tissue stress) that the alveolar elastic fibers in Eln+/− mice experience more stress under progressive loading than WT animals, predisposing them to greater damage following proteolysis as a consequence of mechanical forces.

Altered elastin gene dosage affects pulmonary mechanical function. Static pressure-volume analysis showed important mechanical differences when lungs from Eln+/− animals were compared with WT lungs. The preserved collagen content and reduced elastin content in the Eln+/− lungs results in an altered collagen-elastin ratio of the lung parenchyma, an alteration that would be expected to lead to a change in mechanical properties of the lung. Because elastic fibers account for lung compliance changes occurring around FRC, with collagen acting to limit TLC, an Eln+/− mouse lung would be expected to show a decrease in elasticity and therefore an increase in lung compliance, predominantly at lower lung volumes. This is indeed the case in our animals (Fig. 4B), where inflation of Eln+/− mouse lungs generates lower pressures around FRC than the WT controls. Conversely, around TLC the compliance is similar to that in WT mice, suggesting that the preserved collagen content of the Eln+/− phenotype has the predicted effect on limiting lung expansion. The data also suggest that the leftward shift of the entire pressure volume curve in the Eln+/− lungs (Fig. 4A) is primarily due to changes in the curve at low lung volumes (different slopes) with little or no contribution occurring at higher lung volumes since the curves are in fact parallel (equal slopes). Furthermore, the data in the Eln+/− animals (Fig. 4B) supports the finding that collagen is an important determinant of lung compliance even at low lung volumes (45, 57), especially when elastin is deficient. AβAc+/− lungs show the greatest chord compliance at all lung volumes (Fig. 4B), with chord compliance tending to remain constant irrespective of inflation volume. This suggests that the low levels of elastin in these animals may have little effect on the mechanical properties of the lung at any given volume and that collagen may be the primary matrix determinant of compliance in these animals.

Studies of lung deflation from TLC have shown that elastic recoil pressure decreases notably in fluid-filled lungs compared with air-filled controls, implying that the surface film contributes significantly to lung elasticity (1, 2). More recent work, however, has shown that for small pressure-volume changes like those occurring with tidal breathing, the hysteresis of the surface film is small and that surface active forces are less important determinants of mechanics than lung tissue viscoelasticity (43). Similar findings were observed in experiments comparing isolated lungs with lung tissue strips lacking an air-liquid interface (40). By measuring dynamic respiratory system compliance at tidal-breathing volumes, the effect of surfactant is effectively negated, suggesting that the observed differences in our animal model result almost exclusively from the viscoelastic properties of the lung and are therefore due to the decreased elastin content of the Eln+/− mouse lung tissue. The absolute values for dynamic compliance differ from the static measurements due to differences in experimental technique (dynamic measurement within an intact chest static vs. static measurements on isolated lung). However, the data demonstrate that the relationship between decreased elastin content and increased lung compliance persist despite differences in measurement technique.

Eliminating the effects of the air-liquid interface critical for surfactant function using isolated lung tissue strips in a tissue bath allowed us to focus primarily on the role of the extracellular matrix in lung mechanics. Previous studies have confirmed that the elastin-collagen fiber network dominates the macroscopic elastic properties of lung tissue strips (57), making this model relevant to investigating the role of elastin content in lung mechanics. To ensure the physiological relevance of our results, we utilized stress and strain amplitudes approximating the range experienced during tidal volume breathing around FRC. Our data strongly suggest that pulmonary elastin content contributes to lung tissue elasticity across the range of applied strains corresponding to FRC. Since incremental loss of lung tissue elastin leads to progressive decreases in tissue stiffness, one would hypothesize that lungs with decreased elastin content would be progressively more compliant if tissue properties are an important determinant of lung mechanical behavior. This hypothesis is confirmed by both the static and respiratory system compliance measurements.

Altered elastin gene dosage in human disease. Our studies in mice relate directly to humans where elastin insufficiency can result directly from genetic factors such as loss of function mutations in the elastin gene and indirectly through elastin insuf-
ficiency resulting from environmental factors. Loss of function mutations leading to elastin haploinsufficiency result in the autosomal dominant disease supravalvular aortic stenosis (SVAS). Emphysema does not appear to be a component of SVAS, which is in agreement with our animal studies showing that Eln<sup>−/−</sup> mice have normal lung anatomy until challenged. If the animal studies are accurate predictors, however, emphysema will only develop in the presence of other risk factors, such as smoking, with such individuals being more susceptible to smoke-induced damage because of lower than normal elastin levels.

Nongenetic causes may also result in elastin insufficiency in the lung and other elastin-rich tissues. A relationship between nutrition and elastin insufficiency has been shown for the developing cardiovascular system where infants with low birth weights have higher blood pressures in later life due to effects of nutritional impairment on vascular elastin production (32, 33). A similar situation could occur in the lung. If nutritional deficits existed during the critical period of elastin expression in the lung, elastin deposition could be compromised, leading to insufficiency and enhanced susceptibility to lung damage. In fact, nutritional status is known to be a contributory/causative factor in the pathogenesis of emphysematous lesions in animal models, with alterations in pulmonary protein levels resulting in rapid loss of alveoli (34). Data from human studies support the animal work, suggesting that the starvation phenotype predisposes to emphysema (7).

Studies of human diseases arising from gain of function mutations in the elastin gene suggest another mechanism whereby qualitative, as opposed to quantitative, differences in elastic fibers might result in a predisposition to emphysema. In a previous study, we described a rare, missense variant of elastin (G773D) in a pedigree with severe early onset emphysema (25). This single amino acid change compromises the ability of the mutant protein to undergo normal elastin assembly (29). Other functional consequences include altered proteolytic susceptibility of the COOH-terminal region of elastin and reduced ability of a cell recognition sequence in elastin to interact with matrix receptors on cells. These results suggest that the G773D variant confers structural and functional consequences relevant to the pathogenesis of emphysema. Similarly, individuals with elastin mutations linked to autosomal dominant cutis laxa have severe, very early onset emphysema (53). Like the G773D polymorphism, the cutis laxa mutations alter the functionality and perhaps stability of the elastic fiber (21). The result is a fiber that can support normal lung development but is more easily damaged and degraded when exposed to degradative enzymes or oxidative stress.

**Critical thresholds for elastin exist in lung development and response to injury.** In summary, our findings suggest that qualitative deficiency in elastin can predispose to smoke-induced emphysema. We have presented data showing that lungs from mice containing approximately half the amount of normal elastin (Eln<sup>−/−</sup>) are morphologically similar to, but develop worse emphysema on cigarette smoke exposure than, WT mice. The data presented also show that mice expressing elastin at around one-third of normal levels have congenital emphysema. In addition, clinical and animal studies suggest that mutations that alter the integrity of the elastic fiber predispose to emphysema in both mice and human populations. Together, these findings imply that an initial threshold level of functional elastin is critical for normal lung development and that a second higher threshold is, in turn, necessary for timely and efficient response to lung injury. When elastin levels are below this second critical threshold, the response is not productive and the lungs are more prone to undergo accelerated alveolar destruction leading to emphysema.

**ACKNOWLEDGMENTS**

We thank Terese Hall for administrative assistance and Chris Ciliberto for expertise with animal care and technical support. We also gratefully acknowledge helpful discussion from Jack Pierce, Robert Senior, and Jessica Wagenseil. Current address: Anthony G. Durmowicz, Federal Drug Administration, Division of Pulmonary and Allergy Products, 1098 New Hampshire Ave., Building 22, Room 3375, Silver Spring, MD 20993-0002.

**REFERENCES**

15. Hamelmann E, Schwarz J, Takeda K, Oshiba A, Larsen GL, Irvin CG, Gelfand EW. Noninvasive measurement of airway responsiveness in...


