Progressive adult-onset emphysema in transgenic mice expressing human MMP-1 in the lung

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Progressive adult-onset emphysema in transgenic mice expressing human MMP-1 in the lung. Am J Physiol Lung Cell Mol Physiol 284: L727–L737, 2003. First published January 10, 2003; 10.1152/ajplung.00349.2002.—Mice with lung-specific expression of human matrix metalloproteinase-1 (MMP-1) develop emphysematous changes similar to those seen in smoking-induced emphysema in humans. Morphometric analyses of three transgenic lines [homozygous colony (Col) 34, Col 50, and Col 64] with varying temporal expression of MMP-1 were undertaken to determine the validity of this animal as a model of adult-onset emphysema. Line 50 mice, which have early expression of MMP-1 (14 days postconception), exhibited morphometric changes by 5 days of age. In contrast, homozygous line 34 and 64 with delayed expression (birth and 2 wk of age) were normal up until 4 wk of age when progressive changes in their mean linear intercept were first noted. In contrast, heterozygous mice from line 34 with lower transgene expression did not develop emphysema until 1 yr of age. The changes in mean linear intercept coincided with an increase in lung compliance. Emphysema in these mice was associated with decreased immunostaining for type III collagen within the alveolar septa. This study provides evidence that MMP-1 induces progressive adult-onset emphysema by the selective degradation of type III collagen within the alveolar wall.

Collagen; compliance; morphometry; elastin; matrix metalloproteinase

Emphysema is a devastating pulmonary condition characterized by the development of dilated respiratory air spaces and destruction of alveolar septa (1). These structural changes lead to increased lung compliance that, in many cases, can cause severe airway obstruction and hyperinflation (22). The exact mechanisms that lead to the structural changes in the lung are a matter of continued debate. Significant evidence has implicated elastin in the pathogenesis of this disease (14). Two principal findings support the role of elastase and elastin in emphysema. For one, smokers with a hereditary deficiency of α1-antitrypsin (α1-AT, the main inhibitor of neutrophil elastase) were predisposed to the development of early-onset emphysema (30), and, secondly, the intratracheal administration of elastase, but not other proteases, led to the development of emphysematous changes within the lungs of experimental animals (44). More recently, it was shown that mice deficient in matrix metalloproteinase (MMP)-12, a major elastolytic protease, were resistant to the development of smoke-induced emphysema (20). These studies provide significant affirmative evidence for the importance of elastin and elastolytic enzymes in this disease process.

In the past decade, studies have emerged suggesting that collagen and collagen-degrading enzymes may be key elements in the pathogenesis of emphysema. In a transgenic mouse line with lung-specific expression of MMP-1, morphometric evidence of emphysema developed indistinguishable from that seen in the human disease (9). Given that MMP-1 has activity against principally fibrillar collagens (collagens types I, II, and III) and not elastin, this demonstrated that emphysema could develop via an elastin-independent mechanism. Subsequently, studies in guinea pigs showed that cigarette smoke exposure increased expression of collagenase and generated morphometric evidence of collagen breakdown and repair within the lung (39, 50). Correlative studies (24, 28) in humans have now also shown increased expression of MMP-1 within the alveolar epithelial cells and alveolar macrophages of emphysematous patients compared with matched controls. Thus both animal and human studies demonstrate the importance of collagenase and collagen in the formation of emphysema.

Although the MMP-1 transgenic mouse provided insight into the role of collagen in emphysema, it has been postulated in the literature that the MMP-1 mice develop emphysema because of impaired lung development (40, 41). Development of the mouse lung is not complete until at least 4 wk after birth. Numerous transgenic mouse models (PDGF, transforming growth factor-α, and TNF-α) (16, 19, 23) interfere with normal lung development leading to the formation of emphysematous-appearing alveoli. They do so by interfering with the process of alveolar septation, thereby increasing the size and decreasing the number of alveoli.
within the lung. This, indeed, is a mechanism quite distinct from smoking-induced emphysema seen in humans. The current study was undertaken to carefully characterize the development of emphysema in the MMP-1 using temporal morphometric measurements in several transgenic lines with varying levels of transgene expression. Morphometric, biochemical, and physiological data were obtained to define the time course of emphysema development and associated changes in the lung matrix in these mice.

MATERIALS AND METHODS

Transgenic mice. The generation of the lung-MMP-1 transgenic mice has been formerly described (9). Transgenic lines 34, 50, and 64, harboring a single integration of the transgene, were established. Homozygous mice from these lines were produced through intercrossing of progeny into the CBA × C57BL/6 background. Heterozygote line 34 transgenic mice were compared with wild-type littermate controls. The animals were genotyped by PCR analysis as described previously (32).

Temporal expression analysis of lung-collagenase transgenic mice. Matings were set up between wild-type females and representative mice from the three lung-transgenic lines. Pregnant females were killed at 14, 16, and 18 days postconception (dpc), and the fetuses were studied. Newborn and 2-wk-old mice were also analyzed. Genotyping was performed by PCR amplification and confirmed by Southern blot analysis. RNase protection assays were then performed using mRNA isolated from the lungs of the transgenic mice as described (9).

Histology and immunohistochemistry. At the time of death, a 20-gauge catheter was placed into the trachea and then secured with a silk suture. After mediastinal dissection, the lungs were removed en bloc and then pressure perfused with 25 cmH2O with 10% buffered formalin for 20 min. Paraffin-embedded tissues were prepared, and sections (3–4 μm) mounted onto slides were stained with hematoxylin and eosin (H&E) for histological analysis. Serial sections were stained by Elastica Van Gieson stain for elastic fibers as previously described (9). For immunohistochemical analyses of type I and III collagens, lung sections were deparaffinized with xylene and rehydrated. Protease type XXIV (Sigma, St. Louis, MO), which is commonly used for immunohistochemistry of collagen (26), was utilized for antigen retrieval in this experiment. After blocking endogenous peroxidase with 0.3% H2O2 in methanol for 30 min, we treated the sections with 0.05% protease type XXIV in 0.05 M Tris-HCl buffer, pH 7.6, for 30 min at 37°C and rinsed them in phosphate-buffered saline (PBS). They were incubated for 30 min with normal goat serum and then reacted for 12 h at 4°C with rabbit polyclonal antibodies against type I (25 μg/ml) or type III collagen (12.5 μg/ml; Rockland, Gilbertsville, PA) or rabbit nonimmune immunoglobulin (25 μg/ml) as a control. They were incubated with EnVision+, rabbit/horseradish peroxidase (DAKO, Carpinteria, CA), for 30 min and afterwards rinsed with PBS. Color was developed with 0.05% 3,3'-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl buffer, pH 7.6, and counterstaining was performed with hematoxylin. The monospecificity of the antibodies to mouse type I and III collagens has been established by the company (Rockland).

Morphometric analysis of transgenic mice. On the basis of the above temporal analysis, we examined the lungs of these animals at birth for any obvious histological changes in lung architecture. However, no changes were apparent. We therefore proceeded with a detailed morphometric analysis of the lungs of the mice to detect any subtle changes that would not be seen by gross examination. Eight transgenic and eight control mice were analyzed at each time point. For the newborn and 5-day time point, it was necessary to fix the lung as follows. Animals were asphyxiated with CO2. The thorax was isolated by a cut at the level of the neck and another below the diaphragm and fixed in toto by immersion in formalin for 24 h. In the fixed condition, the lung was then dissected away from the thorax. Randomly sampled blocks of lung tissue were taken for histology. Sections (26 μm) were taken at 24-mm intervals. Sections were then stained with H&E. We calculated the mean linear intercept (MLI) and internal surface area according to established methods (13, 46) using the light microscope linked to a Macintosh computer and Adobe Photoshop imaging software. Two sections were chosen in a random stratified manner for analysis. A rectangular grid of dots at ~1-mm intervals was applied to all lobes in each section. From a random starting position on the grid, sequential and spaced images were digitally recorded using ImagePro 4.5 software. We performed a test system was randomly superimposed upon each image. Horizontal lines were used to count alveolar surface intersections. End points were used to calculate alveolar volumes. Results were analyzed with Students t-test to compare the differences between mean values and were considered significant at <0.05.

Morphometric analysis for the adult mice was conducted on H&E-stained slides of 10 wild-type and transgenic mice. Morphometric assessment consists of the determination of the average interalveolar distance (MLI) (46). For each pair of lungs, we evaluated 40 histological fields both vertically and horizontally. Examination of this number of fields means that practically the entire lung area of each section was evaluated. The lung sections from the mice were coded, and the individual performing the analysis was blinded to the identity of the mice during the performance of this test. The MLI was calculated by the Thurlbeck method (46).

Quantification of type III collagen staining. Blinded analyses of type III collagen staining were performed on transgenic and control line 34 heterozygous mice at 2, 6, and 12 mo of age (n = 5 in each group). We quantified staining for type III collagen in the alveolar region using ImagePro 4.5 software. Five ×40 images from each mouse were quantified and then averaged. The values from five mice in each group were averaged and compared against other groups. Paired t-test analyses were conducted to determine whether significant differences existed between the studied groups.

Determination of lung compliance. To determine the pulmonary compliance of the lung, we utilized an open-chest model. The mice were killed by an overdose of forane, and, after careful neck dissection, a 20-gauge catheter was inserted into the trachea and secured with a silk suture. A mediastinal dissection was performed, and the anterior chest wall was completely resected away. A water manometer was connected in circuit with plastic tubing that was inserted into a three-way stopcock attached to the tracheal cannula. Using a syringe attached to the three-way stopcock, we performed 0.2-ml installations of air until the lungs were inflated to total lung capacity (30 cmH2O transpulmonary pressure). The lungs were then subsequently deflated following the same procedure. After each 0.2-ml installation or deflation, the three-way stopcock was adjusted so that the pressure in the system could be measured. A 30-s pause was observed between each volume change to ensure that no leaks were present in the system. If the pressure decreased during the
30-s pause, then a leak was considered to be present, and measurements in that mouse were discarded. In this manner, both inspiratory and expiratory curves for the mouse were obtained. These pressure-volume measurements were repeated a total of three times for each mouse to ensure that we obtained reproducible curves. Pressure-volume "history" was not observed (i.e., curves 1, 2, and 3 were superimposable). Static expiratory compliance was then determined as the slope of the linear regression of the data over the initial volume range plus 0.5 ml) as established in the literature (16).

Bronchoalveolar lavage. After the mice were euthanized, the trachea was isolated by careful dissection, and a 20-gauge catheter was inserted into the airway as described. The lungs were then instilled with 1 ml of PBS, and the fluid was gently aspirated back. This procedure was repeated twice so that ~2 ml of lung lavage fluid were obtained from each mouse. The lung fluid was then centrifuged at 200 g at 4°C for 10 min. Lavage studies were then performed on cell-free supernatants.

Lung lavage MMP-1 activity. The lavage fluid from three transgenic and wild-type mice was pooled and then concentrated 20-fold with Centricon YM-10 filters (Millipore, Bedford, MA). Two groups of transgenic mouse fluid were compared with two wild-type groups, so that a total of six mice in each group were analyzed. The levels of MMP-1 activity were determined immunologically using a commercially available ELISA (Amersham Biotech, Piscataway, NJ).

Lung tissue MMP-1 levels. A whole lung is homogenized in 1 ml of an SDS buffer [0.67 M Tris-HCl, 10% SDS, and 44% glycerol (vol/vol), pH 8.8]. The homogenate is centrifuged at 3,000 g for 20 min at 4°C, and the supernatant is collected. The supernatant is then dialyzed against 1× PBS, 12 h × 2. The supernatant (100 μl) is tested in duplicate for total MMP-1 (free and MMP-1/tissue inhibitor of metalloproteinases complexed) using Amersham's Biotrak MMP-1 ELISA following the manufacturer's instructions. The value of MMP-1 is standardized per milligram of lung tissue protein.

Zymography. Fivefold concentrated, pooled lung lavage fluid from two groups of transgenic mice was compared with identically treated lung lavage from wild-type mice. Gelatin zymography was performed on this fluid as previously described (21).

Lung lavage elastase activity. Lavage samples from three mice were pooled and then concentrated fivefold as described above. Twelve transgenic and twelve wild-type mice were used for this experiment. An assay buffer of 0.2 M Tris-HCl, pH 8.0, was prepared. Lavage samples were assayed in 96-well flat-bottom plates. To each well were added 100 μl of assay buffer, 50 μl of substrate (0.5 mg/ml of N-succinyl-Ala-Ala-Ala-p-nitroanilide), and 50 μl of sample. All samples were assayed in duplicate. EDTA (10 mM) was added to samples to inhibit metalloelastase, respectively. These assays were also carried out in duplicate. Negative control wells of 150 μl of assay buffer and 50 μl of substrate were used. We assessed background absorbance of each lavage sample by incubating 150 μl of assay buffer with 50 μl of lavage fluid. This value was then subtracted from the absorbance of the test wells. The absorbance of the wells was measured at 405-nm wavelength (11).

Determination of lung elastin levels. Lung samples from 1-yr-old transgenic and wild-type mice were lyophilized for 12 h to dry weight, measured, minced, and then placed into 1 ml of 0.25 M oxalic acid. The suspension was then heated at 100°C for 1 h. The specimen was centrifuged, and the supernatant was collected. The above procedure was repeated for a total of five times until all the insoluble elastin had been converted into a soluble product (α-elastin) (7). One milliliter of the collected supernatants for each mouse was then dialyzed against water using 10,000 molecular-weight cutoff Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, Illinois). We then determined levels of α-elastin using the Fastin elastin assay (Bio-color, Belfast, N. Ireland) and following the specific protocol outlined in the kit (36). To ensure that all of the α-elastin had been measured, we performed a sixth treatment of oxalic acid on sample mouse lung tissue, and determinations of this aliquot revealed that no further α-elastin was being released after the fifth treatment of the tissue.

Hydroxyproline assay. Hydroxyproline levels were determined following the protocol of Voessner (49). The lung tissue was lyophilized for 12 h. The lung was then minced and weighed. The mince was hydrolyzed with 4 ml of 6N HCl at 125°C at 200 psi of pressure in an autoclave for 12 h. One milliliter of the hydrolysate was then taken and evaporated. The powder was then reconstituted with 1 ml of distilled H2O (dH2O) and then reevaporated. The powder was then reconstituted with 5 ml of dH2O. Hydroxyproline standard solutions of 0, 1, 2, 4, 6, 8, and 10 μg/ml were made. Sample with 5 ml of oxalic acid (Bio-color, Belfast, N. Ireland) and following the specific protocol outlined in the kit (36). To ensure that all of the α-elastin had been measured, we performed a sixth treatment of oxalic acid on sample mouse lung tissue, and determinations of this aliquot revealed that no further α-elastin was being released after the fifth treatment of the tissue.

Pyridinoline levels. One hundred microliters of the above hydrolysate solution were taken and neutralized with 230 μl of 4 M Tris base. The levels of pyridinoline cross-links were measured with a Metra Serum pyridinoline enzyme immunoassay kit (Quidel, San Diego, CA). All samples were diluted 1:20 in assay buffer and then measured following the company's protocol (48).

Determination of collagen III/I ratio and content. Lung samples were homogenized in an SDS buffer [100 mM Tris-HCl, 4% SDS (wt/vol), and 20% glycerol (vol/vol), pH 6.8] and then centrifuged at 10,000 g for 30 min to pellet the collagen and elastin. The supernatant was discarded, 10 ml of dH2O were added, and the sample was again centrifuged at 10,000 g for 30 min. This washing procedure was performed a total of three times. The samples were then lyophilized for 6 h. Lyophilized tissue samples (15 mg) were added to 1 ml of 70% formic acid. Two milligrams of purified lyophilized type I and III standards were added to separate 1-ml aliquots of 70% formic acid. The sample mixtures were heated for 1 h at 60°C. Cation bromide (1 g; Sigma) was added to 2 ml of 70% formic acid under a hood. The cation bromide mixture was added to make its final concentration in the samples 50 mg/ml (~100 μl of mixture were added). We deoxygenated the samples by bubbling nitrogen gas through them for 1 min. The samples were then incubated with constant stirring (on an agitator) for 24 h. The samples were then centrifuged at 10,000 g for 30 min, and the supernatant was collected. The 1-ml samples were then placed individually into using 10,000 molecular-weight cutoff Slide-A-Lyzer dialysis cassettes (Pierce). The supernatants were then dialyzed twice against PBS for 12 h each time. The supernatant samples were concentrated 10-fold with YM-10 Centricon filters (Millipore). Samples mixed 1:10 with 2× SDS loading buffer were loaded onto a 15% acrylamide gel (40 μl/well) and run for 2 h at 130 V. The gel was then stained for 45 min with Coomassie blue and then destained with 7% acetic acid for 16 h. The peptides chosen for quantification were α(I)III-CB5 for type III collagen and α(I)IV.
α1-(I) CB8 for type I collagen (31, 45). Densitometric analysis was conducted with Bio-Rad software (Hercules, CA), and the data are reported as the ratio of type III to type I collagen. We then determined the content of type III collagen present in the lung by multiplying the ratio of collagen III/I obtained during cyanogen bromide analysis by the absolute amount of collagen present in the sample as determined by hydroxyproline analysis.

**Statistical analysis.** Data are expressed as the means ± SE. Paired t-test analyses were performed using commercially available software (Microsoft Excel, Seattle, WA). A difference was considered statistically significant at *P* = 0.05.

**RESULTS**

**Temporal expression analysis of lung-collagenase transgenic mice.** The RNase protection assay demonstrated expression of the transgene in line 50 as early as 14 dpc. Homozygous and heterozygous line 34 did not express the transgene until the newborn time point (Fig. 1), and line 64 expressed the transgene as late as 2 wk postnatally (data not shown). Heterozygotes, as expected, had decreased expression compared with homozygotes and the expression of the transgene was lung specific (Fig. 1B).

**Lung morphometry.** Measurements on the newborn (Table 1) and 5-day-old mice (Table 2) demonstrate a difference in line 50 in surface area beginning at day 5. This coincided with the mice in this line expressing the transgene early in embryonic development. As there was no difference found in the homozygous line 34 at the newborn and 5-day time points, we proceeded to analyze these animals throughout adulthood to identify any changes in the MLI. Differences in emphysema formation were noted between homozygous and heterozygous line 34 mice. Homozygous line 34 mice developed changes in MLI beginning at 5 wk of age (Fig. 2A). Heterozygous line 34 mice did not differ statistically from the wild-type mice until the 1-yr time point (Fig. 2B, *P* = 0.005). At the 12-mo time interval, the difference between wild-type and heterozygous mice was apparent even on gross examination, as the transgenic mice demonstrated large bullae and dilated cystic air spaces. These changes were not seen in the wild-type mice.

**Lung compliance.** Compliance measurements were obtained in the heterozygous line 34 mice at 2 mo (*n* = 10 transgenic and *n* = 12 wild-type) and 12 mo of age (*n* = 6 transgenic and *n* = 9 wild-type). No difference in lung compliance was detected at the 2-mo time point. However, coincident with the development of morphometric changes, a significant increase in lung compliance was seen after 1 yr of age (Fig. 3A, *P* = 0.037). Typical curves for transgenic and wild-type lungs at this time point are shown in Fig. 3B. Of note, for several of the 1-yr-old transgenic mice, the expiratory pressure did not return to zero after the complete removal of the instilled volume. This is suggestive of air trapping in these mice.

### Table 1. Lung volumes and lung morphometry in newborn wild-type and MMP-1 transgenic mice

<table>
<thead>
<tr>
<th>Line</th>
<th>WT/TG</th>
<th>Parenchymal Volume</th>
<th>Surface Area</th>
<th><em>μm</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>50 WT</td>
<td>0.33 ± 0.03</td>
<td>31.2 ± 2.7</td>
<td>21 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>50 TG</td>
<td>0.37 ± 0.03</td>
<td>34.1 ± 2.9</td>
<td>22 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>34 WT</td>
<td>0.39 ± 0.03</td>
<td>32.9 ± 2.8</td>
<td>24 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>64 WT</td>
<td>0.37 ± 0.02</td>
<td>31.4 ± 2.7</td>
<td>21 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

Values between wild-type (WT) and transgenic (TG) mice, given as means ± SE, are not statistically different at the newborn time point. Parenchymal volume is the fractional volume of parenchyma tissue per lung; Surface area refers to the alveolar surface area of parenchyma tissue per lung per millimeter; *μm* is mean alveolar wall thickness.

### Table 2. Lung volumes and lung morphometry in wild-type and MMP-1 transgenic mice at 5-day time point

<table>
<thead>
<tr>
<th>Line</th>
<th>WT/TG</th>
<th>Parenchymal Volume</th>
<th>Surface Area</th>
<th><em>μm</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>50 WT</td>
<td>0.40 ± 0.02</td>
<td>35.2 ± 3.1*</td>
<td>23 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>50 TG</td>
<td>0.36 ± 0.02</td>
<td>29.1 ± 2.5*</td>
<td>22 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>34 TG</td>
<td>0.40 ± 0.03</td>
<td>33.9 ± 2.9</td>
<td>24 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>64 WT</td>
<td>0.37 ± 0.04</td>
<td>32.4 ± 2.8</td>
<td>23 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>64 TG</td>
<td>0.35 ± 0.01</td>
<td>31.6 ± 2.7</td>
<td>22 ± 2.2</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. *Demonstrates statistically significant difference in alveolar surface area between wild-type and transgenic mice, *P* < 0.03.
Both of these performed at 2, 6, and 12 mo (\(7/H_{11005}\)). Immunostaining procedures for collagen I and III were performed at 2, 6, and 12 mo (\(7/H_{11005}\)). The Van Gieson elastin staining was conducted on both groups of mice at 12 mo of age (\(n = 7\) for each group). No significant difference in staining for elastin was noted between the groups (data not shown).

Lavage cell count and differential. There was no significant difference in the lavage cell count between heterozygous line 34 mice and controls (16.3 \(\pm\) 3.5 \(\times\) \(10^4\) vs. 13.4 \(\pm\) 3.6 \(\times\) \(10^4\), \(P = 0.31\); \(n = 5\) in each group).

Protease activity in lavage. MMP-1 has been shown to inactivate protease inhibitors such as \(\alpha_1\)-AT. Therefore, lavage studies were conducted to see whether the presence of the transgene within the lung altered the activity of other proteases in the lung. The only detectable difference of protease activity within the lavage of these mice was for MMP-1, as expected. Levels of active MMP-1 were 3.25 \(\pm\) 0.21 ng/ml in the transgenic mice (\(n = 3\)). No active MMP-1 was detected in the control mice as anticipated. No difference in elastase-like activity nor gelatin zymography could be detected between these groups of mice (data not shown).

Lung tissue MMP-1 levels. Heterozygous line 34 mice were tested at 2, 6, and 12 mo for MMP-1 protein levels in the tissue homogenate (\(n = 3\) at each time point). The values obtained ranged were 189.1 \(\pm\) 20.5, 171 \(\pm\) 14.7, to 192 \(\pm\) 16.3 ng/mg lung protein in the 2-, 6-, and 12-mo groups, respectively. No significant age-related difference was detected.

Lung hydroxyproline, pyridinoline, and elastin levels and collagen III/I subtype ratio. The levels of hydroxyproline and elastin expressed as microgram per milligram dry wt of tissue were not significantly different at the 1-yr time point (\(n = 7\) for both transgenic and wild-type) despite the appearance of emphysematous changes in these mice (Table 3). The levels of collagen tended to be higher in the transgenic mice, but this did not reach statistical significance. The levels of pyridinoline cross-links expressed as nmol/mg were also similar between littermates and transgenic mice at the 1-yr time interval (Table 3). A significant change in the ratio of collagen subtypes at the 1-yr time point was detected. The ratio decreased from 51% in the control mice to 43% in the transgenic mice (\(P < 0.005\); \(n = 8\) in each group). Calculated type III collagen lung content was lower in the transgenic mice (362 \(\pm\) 64 \(\mu\)g/lung) compared with wild-type mice (430 \(\pm\) 82 \(\mu\)g/lung), although this did not reach statistical significance (\(P = 0.11\)). Coupled with the immunohistochemistry data, these results suggest that type III collagen loss is occurring within the alveolar region of the transgenic mice over time (Table 3). A decrease in intensity of \(\alpha\)(III) CB5 peptides is noted in the transgenic ani-

**Immunostaining for collagen I and III and elastin.** Immunostaining procedures for collagen I and III were performed at 2, 6, and 12 mo (\(n = 7\) for each group). Both of these fibrillar collagens stained prominently in bronchovascular structures and the pleura in both wild-type and transgenics at 2 and 6 mo. The patterns of staining for these fibrillar collagens did not change over the period of 1 yr in the wild-type mice. However, the transgenic mice demonstrated a clear decrease in staining for type III collagen from the alveolar septa at the 1-yr time interval (Fig. 4). The 2-mo wild-type and transgenic mice (Fig. 4, A and B) show prominent staining for type III collagen in the alveolar region. At the 1-yr time point, there is a marked decrease in staining in the transgenic mice (Fig. 4D) compared with the wild-type mice (Fig. 4C). This data suggest that a selective loss of type III collagen has occurred in this region. The decrease in type III collagen was further confirmed by video microscopy quantitative analysis of the stained type III collagen. One-year-old line 34 heterozygote mice had significantly decreased staining (654 \(\pm\) 180 \(\mu\)m\(^2\)/field) compared with 1-yr-old control, 6-mo-old transgenic, 6-mo control, 2-mo-old transgenic, and 2-mo-old control (993 \(\pm\) 187, 1,040 \(\pm\) 45, 1,206 \(\pm\) 300, 1,259 \(\pm\) 218, and 951 \(\pm\) 151 \(\mu\)m\(^2\)/field respectively; \(P < 0.05\) in every group). No difference in staining for type I collagen was noted in any of the groups tested (Fig. 5).
Fig. 3. A: compliance of the 2-mo- and 1-yr-old transgenic and wild-type mice. At baseline, compliance measurements were comparable between the groups. However, the transgenic mice show a significant increase in compliance ($P = 0.037$) at the 1-yr time point. B: representative compliance curve of 1-yr-old wild-type and transgenic mice.
DISCUSSION

The results of this study affirm that the MMP-1 transgenic mouse is a valid model of adult-onset emphysema and demonstrates that MMP-1 causes both morphological and physiological emphysematous changes without affecting normal lung development. As importantly, the study reveals that the development of emphysema in these mice is associated with a loss of type III collagen within the alveolar wall. The lungs of heterozygote line 34 mice at 2 mo of age are identical in all measurements available to us compared with wild-type littermate controls. No differences were found in morphometry, compliance, or matrix staining at this time point. Actual emphysematous changes were not seen in these mice until they were 1 yr of age, suggesting that this animal is a true adult-onset model of emphysema.

The timing of expression of MMP-1 was shown to be a critical determinant in the formation of emphysema in this animal model. Unlike line 50 mice, the line 34 mice in this study did not express the transgene during early fetal development. The premature emphysematolike changes seen in the line 50 mice are in contrast to the delayed-onset emphysema seen in line 34 and 64 mice. Line 50 studies suggest that MMP-1 expression in an early period of lung development may interfere with events such as terminal airway branching (18, 35) that are occurring during this stage of lung formation and may account for the abnormal development seen in the line 50 mice. During the immediate postnatal period, when lines 34 and 64 express the transgene, MMPs are normally upregulated (17). At this time, alveolarization and exuberant collagen remodeling (2) are occurring. The transgene is probably better tolerated during this phase of development since collagenases are normally expressed, and this may explain why MMP-1 expression at the newborn time period had no effect on the normal development of the lung.

Additionally, the present study demonstrates that the level of transgene expression is linked to the development of emphysema. Homozygous line 34 mice develop emphysema by 10 wk of age. The heterozygous mice used in this study did not show a change in lung morphology until the 1-yr time point. The finding underscores the importance of human studies demonstrating increased expression of MMP-1 within inflammatory cells and the lung epithelium of emphysema patients (24). The level of MMP-1 expression seen in these studies is not merely a factor associated with emphysema but, as judged by the present data, clearly has the potential over time of causing proteolytic damage that contributes to the formation of emphysema.

Previously, we had shown a decrease in type III immunostaining in the alveolar regions of homozygote...
line 34 mice (42). However, in those animals the abnormalities were apparent in the first month of life. The mice we used in the present experiment had a lower expression of MMP-1 and initially demonstrated normal immunostaining for type III collagen in the alveolar septa, therefore indicating that formation of this structural element occurred normally in the transgenic mice. At the 1-yr time point, the development of emphysematous changes was associated with a significant decrease in staining for type III collagen in the septa of the transgenic mice. These data were complemented by biochemical studies demonstrating that the ratio of type III/I collagen was decreased in the lungs of these mice at the 1-yr time point. These findings suggest that MMP-1-mediated breakdown of type III collagen from this region may have been a significant contributory factor in the formation of emphysema. This finding is in accordance with the fact that MMP-1 has 20-fold higher activity against type III collagen compared with type I collagen (25).

The present findings suggest that changes in the distribution of collagen subtypes within the lung may have significant structural and physiological effects on the lung even in the presence of unchanged levels of total collagen and elastin. Collagen comprises 60% of total lung protein with ~60% being type I collagen and 20–30% type III collagen (4). Despite the fact that collagen constitutes a significant portion of the extra-

Fig. 5. Immunostaining for type I collagen in the lungs of wild-type and transgenic mice (×40 views). Similar staining patterns are noted in the 1-yr-old wild-type (A) and transgenic mice (B).
yet fully understood (29, 43). The lag to the biomechanical properties of the lung is not cellular matrix, the exact contribution of type III collagen to the biomechanical properties of the lung is expressed as μg/mg of dry weight of lung tissue. Pyridinoline is expressed as μmol of pyridinoline/mg of dry weight of lung tissue. No difference is seen between lung concentration of α-elastin, hydroxyproline, and pyridinoline. The III/I collagen ratio is expressed as the ratio of band α(III)-CB8 over α(1)-CB8. This ratio was significantly decreased in the transgenics (§). ‡P = 0.32, data are expressed as μg/mg dry lung tissue (n = 7 in each group); ‡P = 0.17 data are expressed as μg/mg dry lung tissue (n = 7 in each group); §P < 0.005 data are expressed as the ratio of type III to type I collagen (n = 7 in each group).

Data are expressed as means ± SD. α-Elastin and hydroxyproline content of lung is expressed as μg/mg of dry weight of lung tissue. Pyridinoline is expressed as μmol of pyridinoline/mg of dry weight of lung tissue. No difference is seen between lung concentration of α-elastin, hydroxyproline, and pyridinoline. The III/I collagen ratio is expressed as the ratio of band α(III)-CB8 over α(1)-CB8. This ratio was significantly decreased in the transgenics (§). ‡P = 0.32, data are expressed as μg/mg dry lung tissue (n = 7 in each group); ‡P = 0.17 data are expressed as μg/mg dry lung tissue (n = 7 in each group); §P < 0.005 data are expressed as the ratio of type III to type I collagen (n = 7 in each group).

Table 3. Lung matrix content (1 yr)

<table>
<thead>
<tr>
<th></th>
<th>α-Elastin</th>
<th>Hydroxyproline</th>
<th>Pyridinoline</th>
<th>III/I Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic</td>
<td>6.45 ± 1.06a</td>
<td>9.19 ± 1.62a</td>
<td>530 ± 20‡</td>
<td>0.43 ± 0.02§</td>
</tr>
<tr>
<td>Littermate</td>
<td>4.65 ± 1.19</td>
<td>7.88 ± 1.65</td>
<td>530 ± 10</td>
<td>0.51 ± 0.02</td>
</tr>
</tbody>
</table>

It is interesting to note that, aside from the differences in type III collagen at the 1-yr time point, the lung matrices of the transgenic and wild-type mice were remarkably similar. No difference could be detected in type I collagen distribution nor in hydroxyproline levels between the two groups of mice. In addition, staining for elastin and biochemical determination of elastin concentration did not reveal any changes over the time period studied. These data suggest that neither type I collagen nor elastin were significant contributory factors to the development of emphysema in the MMP-1+ transgenic mice. Studies also failed to detect any difference in the levels of pyridinoline cross-links measured within the lung. These cross-links are formed between collagen fibrils over time and are believed by some investigators to be reflective of the age of collagen (47). This lack of difference may not be surprising due to the fact that overall levels of collagen were not changed between groups of mice. Also, collagen remodeling is occurring throughout the life of the lung, which may explain why several groups have failed to detect a correlation between pyridinoline cross-links and tissue age (6, 10).

Changes in lung collagen have been demonstrated in several experimental models of emphysema. Increased hydroxyproline levels are seen in the lung of mice exposed to cigarette smoke (37). Intratracheal elastase causes an increase in collagen deposition and aberrant collagen remodeling (15) in rats and, in hamsters, leads to an increase in the transcription of type I collagen coinciding with the development of emphysema (33). Human studies also demonstrate that the overall collagen content is increased in the lung tissue from patients with emphysema (5, 28). How then is the finding of increased collagen consistent with the fact that collagenolysis leads to emphysema? One possible mechanism is that MMP-1 alters the normal turnover of collagen in the lung, leading to a decrease in the content of type III collagen. If the total collagen content is unchanged and the ratio of type III/I collagen is decreased, there must be a net increase in type I collagen. Transgenic mouse studies in our laboratory have shown that expression of MMP-1 directly stimulates the transcription of collagen (32). This suggests that the lung in emphysema comprises a remodeling tissue with loss of type III collagen and deposition of type I collagen. The alteration of the collagen subtypes and the overall remodeling that occurs may change the mechanical properties of the lung and lead to the pathology observed. Further studies are warranted to evaluate the role of these collagen subtypes in the pathogenesis of this disease.

In summary, moderate expression of MMP-1 in the lungs of these transgenic mice leads to the development of adult-onset emphysema. The formation of emphysema in this animal model is associated with increased lung compliance and with the selective loss of staining for type III collagen in the alveolar region. The present study not only affirms the causative link between MMP-1 and adult-onset emphysema but also provides evidence to suggest that type III collagen located in the alveolar wall may be the principal target of MMP-1 in this disease model. Further studies will need to be conducted to explore the role of this collagen subtype in human cigarette smoking-induced emphysema. These results suggest that MMP-1 may be a pivotal factor in disease formation and inhibition of MMP-1 activity within the lungs of emphysema pa-

![Collagen Subtype Analysis](image)

Fig. 6. 15% acrylamide gel of cyanogen bromide peptides from collagen controls and the lungs of 1-yr-old wild-type and transgenic mice. Lane 1, the molecular wt marker; lane 2, the type I collagen standard; lane 3, the type III collagen standard; lanes 4–6, wild-type mice; lanes 7–9, the transgenic mice. The top arrow indicates α(1) CB-8, and the bottom arrow indicates α(III) CB-5.
tients may interfere with the progressive changes seen in this disease.

The authors thank Tina Zelenina and Victor Nieves for technical assistance in the conduct of these studies.

Jeanine D’Armiento is a recipient of the Burroughs Wellcome Career Award.

REFERENCES


