Transgenic expression of matrix metalloproteinase-9 causes adult-onset emphysema in mice associated with the loss of alveolar elastin

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Foronjy R, Nkyimbeng T, Wallace A, Thankachen J, Okada Y, Lemaître V, D’Armiento J. Transgenic expression of matrix metalloproteinase-9 causes adult-onset emphysema in mice associated with the loss of alveolar elastin. Am J Physiol Lung Cell Mol Physiol 294: L1149–L1157, 2008. First published April 11, 2008; doi:10.1152/ajplung.00481.2007.—Matrix metalloproteinase (MMP)-9 has been consistently identified in the lungs of patients with chronic obstructive pulmonary disease (COPD). However, its role in the development of the disease remains undefined. Mice that specifically express human MMP-9 in their macrophages were generated, and morphometric, biochemical, and histological analyses were conducted on the transgenic and littermate control mice over 1 yr to determine the effect of macrophage MMP-9 expression on emphysema formation and lung matrix content. Lung morphometry was normal in transgenic mice at 2 mo of age (mean linear intercept = 50 ± 3 littermate mice vs. 51 ± 2 transgenic mice). However, after 12 mo of age, the MMP-9 transgenic mice developed significant air space enlargement (mean linear intercept = 53 ± 3 littermate mice vs. 61 ± 2 MMP-9 transgenic mice; P < 0.04). Lung hydroxyproline content was not significantly different between wild-type and transgenic mice, but MMP-9 did significantly decrease alveolar wall elastin at 1 yr of age (4.9 ± 0.3% area of alveolar wall in the littermate mice vs. 3.3 ± 0.3% area of alveolar wall in the MMP-9 mice; P < 0.004). Thus these results establish a central role for MMP-9 in the pathogenesis of this disease by demonstrating that expression of this protease in macrophages can alter the extracellular matrix and induce progressive air space enlargement in mice.

SEVERAL STUDIES HAVE IMPLICATED matrix metalloproteinase (MMP)-9 in the pathogenesis of emphysema. Increased MMP-9 levels were detected in emphysema lung samples (36), and enhanced expression of this protease was noted in alveolar macrophages from chronic obstructive pulmonary disease (COPD) patients (11, 43). Similarly, elevated MMP-9 expression and activity were identified in animal models of the disease (58, 68) and were thought to play a role in the destructive process. While these studies associate MMP-9 activity with emphysema, they do not establish that MMP-9 is directly capable of generating structural changes in this disease. Indeed, MMP-9 could be functioning primarily to mediate postinjury repair response in the lungs of COPD patients (29, 30).

Alveolar macrophages are a major site of production of matrix-degrading enzymes in the lung (11), and these cells exert a key role on the alveolar destruction that occurs in this disease (65, 66). Macrophages secrete several proteases that have been implicated in emphysema formation, including the elastolytic cysteine proteinases (cathepsin L and cathepsin S) and several MMPs (MMP-1, -9, and -12; Ref. 49). MMP-9, however, is the protease that has been most consistently identified from the alveolar macrophages of COPD patients (32, 44). Macrophages from COPD patients have increased MMP-9 expression (12) and release greater amounts of MMP-9 upon stimulation with cigarette smoke compared with macrophages from smoking and nonsmoking control subjects (43). These findings are relevant, as MMP-9 is capable of degrading key components of the extracellular matrix (24). Thus its expression by macrophages could significantly affect the matrix remodeling and ultra structural changes that are noted in emphysema (10).

While clinical studies (11, 43) have documented an increase in the expression of MMP-9 in alveolar macrophages from COPD patients, the causative role that this production plays in the disease remains unclear. To address this question, we generated mice that specifically expressed human MMP-9 in their macrophages. Time-course experiments were performed over 1 yr to determine how the chronic expression of MMP-9 in this cell type affected matrix content and emphysema formation in these mice.

MATERIALS AND METHODS

Generation of human MMP-9 transgenic mice. The macrophage was used as a sensitization-dependent delivery system for MMP-9 in the lung. This system has been successfully utilized to examine the role of MMP-1 in atherosclerosis (31) and MMP-9 in fibrosis (4). The macrophage delivery system provides specific directed expression of the transgene (21, 31). The generation of these mice was described previously (4). To screen the mice, the primers for PCR were left (5' to 3'), CAGAACAGAATACCAGTTTGTATC, and right (5' to 3'), GCCAGGAGCCTTCTACTGGCGCGT. For the experiments described below, we utilized mice that exhibited macrophage MMP-9 activity levels in culture comparable with human macrophages (4).

Generation of the study population. The transgenic mice were crossed into a pure C57BL6/J background for six generations. Heterozygous mice were used for these studies, and the controls were littermates that were determined not to carry the transgene. Mice were housed in specific pathogen-free conditions and provided with food and water ad libitum. All studies and procedures were approved by the Columbia University Institutional Animal Care and Use Committee. For the morphometric analyses, 2-mo-old, 6-mo-old, and 1-yr-old mice were used. All other analyses were conducting using 2-mo-old and 1-yr-old mice.

Histology. At the time of euthanization, a 20-gauge catheter was placed into the trachea and then secured with a silk suture. The lungs were inflated with \begin{enumerate}
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\end{enumerate}
were pressure perfused at 25 cm H₂O with 10% formalin for 24 h. Paraffin-embedded tissues were prepared, and sections (3–4 μm) mounted onto slides were stained with hematoxylin and eosin for histological analysis. Serial sections were stained with Verhoef's solution for elastic fibers and picro-Sirius red for collagen as described previously (8).

**Morphometric analysis.** Morphometric analyses were conducted on hematoxylin and eosin stained slides of 10 mice in each group in a standard fashion as previously reported by our laboratory (14, 15, 51). Morphometric assessment consists of the determination of the average interalveolar distance (mean linear intercept; Ref. 55). The left lung was used for these experiments. The fixed lungs were serially sectioned in the coronal plane into 3-mm blocks. Two blocks were randomly chosen to be processed together for paraffin embedding. All of the 3-mm blocks of tissue from each mouse were processed in this manner for embedding. After embedding, serial 4-μm sections were made by cutting parallel to the face of the blocks from top to bottom and then random slides were chosen for analysis. This ensures that there is no regional sampling bias. Forty histologic fields (×40 magnification) were then evaluated from at least two separate sections from each mouse to calculate the fractional volume of parenchyma tissue per lung, the alveolar surface area per unit volume, and the mean linear intercept as per standard protocol (9, 55, 61, 62). Analyses were conducted with the examiner blinded to the genotype of the mouse.

**Bronchoalveolar lavage.** Lavage was obtained following established protocols of the laboratory (15). Ten mice in each group were used. The lungs of the mice were instilled with 1 ml of PBS, and the fluid was gently aspirated back. This procedure was repeated twice. The lung fluid was then centrifuged at 200 g at 4°C for 10 min. The cellular pellet was resuspended in 200 μl of PBS. Total cell number was counted with a hemocytometer, and a differential analysis was conducted after cytopsin preparation and Quik Diff staining of the cellular pellet prepared on a glass slide.

**Quantification of alveolar wall collagen staining.** Fifteen ×100 images were analyzed from 1-yr-old transgenic and littermate mice (n = 12 in each group). These images were chosen at random and contained only alveoli. The intensity of red staining from each image (expressed in pixels) was measured using ImagePro software and then corrected for alveolar wall area (mm²). Alveolar wall area was calculated using ImagePro software by subtracting the air space area of an image from the total area of the same image.

**Determination of the elastin content in the alveolar wall.** To measure alveolar wall elastin content, Verhoef-stained lung tissue sections were quantified using ImagePro software. Fifteen ×100 images were analyzed from 1-yr-old transgenic and littermate mice (n = 12 in each group). At this magnification, elastin fibrils were easily discernable within the alveolar walls of the mice. For each image, the area occupied by elastin fibrils was measured by outlining each fibril using ImagePro software. To determine the percentage of the alveolar wall that was occupied by elastin, the area occupied by elastin fibrils in a particular image was divided by the total alveolar wall area of that same image. Fifteen such measurements were taken in each mouse and then averaged. The averages from each mouse were used to calculate the average elastin percent area for each group of mice.

**Lung volume measurements.** After the lungs were pressure fixed for 24 h, the total lung volume was determined by immersing the lungs into a beaker of water. With the assumption that the density was 1, the volume of the lungs was calculated by determining the change in weight of the beaker from baseline.

**Subculture of alveolar macrophages.** The cellular pellet obtained from one mouse during lung lavage was resuspended in 1 ml of DMEM (Invitrogen, Carlsbad, CA) media. The cells were pelleted by centrifugation at 200 g and then were resuspended in 1 ml of DMEM media. A proportion of this media was added to ensure that the well received ~250,000 cells. Then, fresh DMEM was added to each well to bring the final volume of that well up to 1 ml. The cells were allowed to adhere for 1 h, and then nonattached cells were aspirated away and 1 ml of fresh media was added to each well. Visual confirmation ensured that each well was ≈80% confluent. The cells were incubated at 37°C with 5% CO₂ for 24 h. The media were collected and stored at −80°C until analysis. RNA from the macrophages was collected using the TriZol (Invitrogen, Carlsbad, CA) method as per the manufacturer’s instructions.

**Zymography.** The media from alveolar macrophages cultured for 24 h were used for this assay. The protein concentration of the media was determined using the Bradford assay (Pierce, Rockford, IL). Lanes were loaded with 20 μg of protein per well, and casein and gelatin zymography were performed as described previously (19). As a positive control for human MMP-9, 15 μl of media cultured from human macrophages (U937 cells) were used. As a positive control for mouse MMP-9, 20 μg of lung tissue protein from littermate mice were utilized.

**Lung myeloperoxidase assay.** Lung myeloperoxidase activity was measured in the lung tissue protein homogenates of littermate and MMP-9 transgenic mice using a lung myeloperoxidase assay kit (EMB Biosciences, Gibbstown, NJ). Seven-hundred seventy-five micrograms of protein were utilized per mouse, and data are expressed as nanograms of myeloperoxidase per micrograms of lung tissue protein (n = 5 in each group). Error bars represent SE.

**Expression of transgene in lung macrophages.** mRNA was isolated from cultured alveolar macrophages of wild-type and MMP-9 transgenic mice as noted in Subculture of alveolar macrophages and converted into cDNA using the Superscript cDNA kit (Invitrogen). Quantitative PCR was performed using an Applied Biosystems 7500 Real Time PCR system. Each reaction (a total volume of 25 μl) contained 5 μl of the diluted cDNA, Taq Man PCR Master Mix (Applied Biosystems), and 0.4 μM each of forward and reverse probe labeled specific primers (human MMP-9, part number 112640, and mouse GAPDH, part number 4352932E; Applied Biosystems). PCR conditions included heating for 2 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 56°C, and then 30 s at 72°C. A melt curve analysis was performed to ensure specific amplification. For each target gene, relative levels of expression were normalized using the GAPDH signal [ΔCt = (Ct_GAPDH − Ct_Target)]. Differences in the expression levels between the two groups was determined according to the following formula: 2^{ΔΔCt}, where ΔΔCt = (ΔCt_transgenic − ΔCt_littermate). The differences from the qPCR of individual RNA preparations were statistically analyzed to get means ± SE (n = 3 in each group).

**Determination of lung elastin levels.** Lungs 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; Ref. 7). The acid was cleared by centrifuging the samples in Centricon filters with a 10,000 molecular-weight cutoff (Millipore, Billerica, MA). The proteins were reconstituted with 1 ml of distilled water. Levels of elastin were determined following the protocol of Woessner (64). The lung tissue was lyophilized for 12 h. The lung was then minced and weighed. The mince was hydrolyzed with 4 ml of 6 N HCl at 125°C at 200 pounds per square inch 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 and then reevaporated. The
powder was then reconstituted with 5 ml of distilled H2O. Hydroxyproline standard solutions of 0, 1, 2, 4, 6, 8, and 10 μg/ml were made. Sample solution (2 ml) was taken and oxidized with 1 ml of chloramine-T (Sigma) for 20 min. The reaction was then stopped with 1 ml of 3.15 M perchloric acid. After 5 min, 1 ml of p-dimethylaminobenzaldehyde solution was added. The sample was vortexed, incubated in a 60°C bath, and then cooled under tap water for 5 min. The absorbency of the solutions was determined at 557 nm. The hydroxyproline concentration was determined from the standard curve.

Electron microscopy of lung. Lungs of 1-yr-old littermate and transgenic mice were processed for electron microscopy as per standard laboratory protocol (25). For transmission electron microscopy, lungs of 1-yr-old transgenic and littermate mice were fixed in 2.5% glutaraldehyde and 2.5% formaldehyde in 0.1 M sodium cacodylate buffer, postfixed with aqueous 1.25% osmium tetroxide, stained with 4% aqueous uranyl acetate, dehydrated through an ethanol series, embedded in Polybed, sectioned on a Reichert-Jung Ultra Cut, poststained in 4% uranyl acetate and lead citrate, and viewed on a Zeiss 902 electron microscope. All reagents for electron microscopy were purchased from Electron Microscopy Sciences (Ft. Washington, PA). Quantification of fibril density in the alveolar wall was determined by performing morphometric analyses on the electron microscopy images of the wild-type and MMP-9 transgenic mice. Three images from three mice in each group were utilized for this purpose. The fractional volume area (%) that was occupied by fibrils was calculated using standard morphometric technique (55).

Immunoblot for α1-anti-trypsin. The inferior vena cava was severed, and the right heart was then injected with 3 ml of sterile saline to clear blood from the lungs. The tissue was homogenized in lysis buffer (10 mM Tris pH 7.4, 2 mM EGTA pH 8.0, 150 mM NaCl, 2 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged. One-hundred micrograms of tissue protein were electrophoresed on a 10% acrylamide gel under reducing conditions. Gel proteins were transferred to a nitrocellulose membrane that was subsequently probed with a 1:1,000 dilution of IgG against α1-anti-trypsin (GenWay, San Diego, CA). This was subsequently probed with a 1:5,000 dilution of biotinylated anti-chicken IgY antibody (R&D Systems; Minneapolis, MN). The membranes were then treated with streptavidin-peroxidase conjugated (Vector Labs).

In situ zymography. One gram of low gelling temperature agarose (Bio-Rad, Hercules, CA) was dissolved in 100 ml PBS, pH 7.45, under continuous stirring and heating in a water bath (80°C) until a clear solution was obtained. This was then stored at 4°C in airtight vials. When ready for use, the solution was heated to 60°C before incubation. DQ-gelatin (Molecular Probes) was dissolved in a concentration of 1 mg/ml in distilled water and then diluted 1:10 (100 μg/ml final concentration) in the agarose-containing solution. The DQ-gelatin agarose mixture (150 μl) was put on top of the cryosections (8–10 μM) of lung tissue and covered with a coverslip of 24 mm, and the agarose was gelled at 4°C. The slides were then incubated for 12 h at 37°C. Fluorescence of FITC was detected with excitation at 460–500 nm and emission at 512–542 nm. Control incubations were carried out on serial cryostat sections by addition of 20 mM EDTA or a selective MMP inhibitor (30 mM captopril; Sigma) to the incubation medium. The presence of autofluorescence in the agarose-containing medium that lacks DQ-gelatin. Nuclei were counterstained by adding DAPI (1 μg/ml; Invitrogen) or propidium iodide (0.5 μg/ml) to the incubation medium. To colocalize fluorescence to the macrophage, immunofluorescence for macrophages was simultaneously performed on the cryostat sections. Slides were treated with a 1:10 dilution of specific rat anti-mouse monoclonal antibodies for MAC-3 (BD Pharmingen, San Diego, CA) for 1 h at room temperature. Secondary antibody was applied as a 1:100 dilution of goat anti-rat IgG-TRITC (AbD Serotec, Oxford, UK) for 1 h at room temperature.

Statistical analyses. Data are means ± SE of measurement. Two-way ANOVAs were conducted using commercially available software (Microsoft Excel, Seattle, WA). A difference was considered statistically significant at P = 0.05.
RESULTS

MMP-9 expression in the alveolar macrophages of the transgenic mice. Quantitative PCR for human MMP-9 demonstrated that this gene was, as expected, only expressed in the transgenic mice (Fig. 1A). Expression levels were similar in 2-mo-old and 1-yr-old transgenic mice (data not shown).

Increased gelatinolytic activity in the alveolar macrophages of MMP-9 transgenic mice. A gelatinolytic band corresponding to human MMP-9 (92 kDa; Fig. 1B, top band, lanes 7–8) was detected in the macrophages of the MMP-9 transgenic mice and was absent in the macrophages of the littermate mice (lanes 4–6). The band detected in the transgenic mice migrated at a level consistent with human MMP-9 (lane 2) and was slightly lower than mouse MMP-9 (lane 3). Consistent with the published literature (28, 39), we did not detect the presence of other proteases such as MMP-3, -7, and -12 under these conditions. Gelatin zymography did demonstrate a band corresponding to mouse MMP-2 (72 kDa) in both the littermate and transgenic macrophages (bottom band, lanes 4–8).

Effect of MMP-9 expression on inflammation in transgenic mice. MMP-9 expression had no affect on airway inflammation as determined by lung lavage cell count. Total cell counts in the 1-yr-old MMP-9 transgenic mice were comparable with age-matched littermates (17.8 ± 2.7 × 10^3 in littermate mice vs. 17.7 ± 2.6 × 10^3 in MMP-9 transgenics; P = NS). In addition, MMP-9 expression had no affect on the cell profiles obtained from the lung lavage. In both groups, >95% of the cells obtained in the lavage were macrophages (n = 8 in each of the groups tested). Of note, similar findings were obtained with 2-mo-old transgenic and littermate mice. Thus the age of the mice had no affect on the inflammatory status of the lung. In addition, lung myeloperoxidase assays were conducted to assess for differences in lung neutrophilia between the transgenic and littermate mice. Myeloperoxidase is a peroxidase enzyme that is present in the azurophic granules of neutrophils. Myeloperoxidase has been utilized as a reliable marker of lung neutrophilia (14). As demonstrated in Fig. 1C, comparable levels of myeloperoxidase activity were noted in the wild-type and transgenic mice. Thus the expression of the transgene did not affect overall lung neutrophil levels.

Increased gelatinolytic activity in the macrophages in the lungs of the MMP-9 transgenic mice. To evaluate gelatinolytic activity within the lung macrophages in vivo, in situ zymography was performed on lung tissue sections from littermate and MMP-9 transgenic mice. DAPI nuclear staining was performed for both the littermate (Fig. 2A) and transgenic mice (Fig. 2E). To determine if gelatinolytic activity co-localized to the macrophage, MAC-3 staining was performed on these same sections. As can be seen in Fig. 2, macrophages were similarly distributed in both the littermate (Fig. 2B, see arrows) and the MMP-9 transgenic mice (Fig. 2F, see arrows). However, gelatinolytic activity as determined by in situ zymography was significantly lower in the littermate mice (Fig. 2C) compared with MMP-9 transgenic mice (Fig. 2G). DAPI, MAC-3, and in situ zymography images were merged for the littermate (Fig. 2D) and MMP-9 transgenic mice (Fig. 2H). The littermate mice demonstrated primarily a red signal, indicating that these macrophages had low levels of gelatinolytic activity (Fig. 2D, white arrows). In contrast, the macrophages from the transgenic mice exhibited an orange-yellow color, which demonstrated that gelatinolytic activity was correlating with MAC-3 staining (Fig. 2H, white arrows). Thus these results indicate that the macrophages from the transgenic mice possess greater gelatinolytic activity than control macrophages. Captopril treatment completely eliminated FITC fluorescence of the lung tissue sections (data not shown).

Effect of MMP-9 expression on the lung extracellular matrix. A nonsignificant decrease in lung collagen content, as reflected by hydroxyproline levels (Fig. 3A), was detected in the 1-yr-old transgenic mice compared with age-matched littermate mice (21.9 ± 3.5 μg/mg lung protein littermate mice vs. 15.6 ± 2.5 μg/mg lung protein MMP-9 transgenic mice; n = 8 in each group; P = 0.10). Likewise, picro-Sirius red staining for alveolar wall collagen (Fig. 3, B and C) demonstrated a nonsignificant decrease in alveolar collagen in the...
1-yr-old MMP-9 transgenic mice (11.9 pixels/mm²) in the littermate mice vs. 9.5 pixels/mm² in the MMP-9 mice; \( P = 0.15; n = 12 \). Overall elastin levels (Fig. 4A) were unaltered in the 1-yr-old transgenic mice (α-elastin 26 ± 2 µg/mg lung tissue in littermates vs. 26 ± 3 µg/mg in the MMP-9 transgenics; \( n = 5 \) in each group). However, a marked decrease in elastin content was noted in the alveolar regions (Fig. 4, B and C) of the 1-yr-old transgenic mice (4.9 ± 0.3% area of alveolar wall in the littermate mice vs. 3.3 ± 0.3% area of alveolar wall in the MMP-9 mice; \( P < 0.004; n = 12 \)). Consistent with these results, electron microscopy (Fig. 4D) demonstrated a decrease in the fractional volume area of fibrils within the alveolar walls of the 1-yr-old transgenic mice (9.0 ± 1.7% wild type vs. 3.9 ± 1.3% transgenics; \( P < 0.04 \)). These changes in alveolar elastin could not be ascribed to alterations in lung α₁-antitrypsin levels, as immunoblot analysis performed on lung homogenates from 1-yr-old transgenic and littermate mice showed equivalent levels of α₁-antitrypsin in both groups of mice (data not shown).

**Effect of MMP-9 expression in the macrophage on emphysema formation.** Baseline lung morphometry was normal in both groups of mice at 2 mo of age (50.0 ± 3 age-matched littermate mice vs. 50.0 ± 2 in the MMP-9 transgenic mice; \( n = 6 \) in each group; \( P = \text{NS} \)). However, after 2 mo of age, the MMP-9 transgenic mice developed a progressive increase in the mean linear intercept (Fig. 5A). By 1 yr of age, the transgenic mice had air space enlargement (Fig. 5, B and C) and a significant increase in the mean linear intercept (52 ± 3 age-matched littermate mice vs. 61 ± 2 1-yr-old MMP-9 transgenic mice; \( P < 0.04; n = 10 \) in each group; units = \( \mu \)). This increase in mean linear intercept in the MMP-9 transgenic mice is equal to or greater than that which has been reported in the cigarette smoke-exposure model of emphysema (3, 5). Surface area per unit volume was also significantly decreased in 1-yr-old transgenic mice (39 ± 3 age-matched littermate mice vs. 33 ± 1 1-yr-old MMP-9 transgenic mice; \( P < 0.04; n = 10 \) in each group; units = mm⁻¹).

**DISCUSSION**

This study establishes that the expression of MMP-9 in macrophages generates progressive air space enlargement in mice that is associated with the loss of alveolar elastin without an increase in inflammation. Importantly, the increase in mean linear intercept that occurred in the 1-yr-old MMP-9 transgenic mice was comparable with that seen in the smoke exposure model of emphysema (5, 13, 54). Thus this animal model demonstrates that increases in MMP-9 that have been reported in emphysema are likely capable of inducing alterations in the lung matrix that contribute to the initiation and progression of this disease.

MMP-9 is a potent elastase that on a molar ratio is 30% as effective as human leukocyte elastase at solubilizing elastin (48). Elastin is a key component of the lung extracellular matrix that provides the tissue the resiliency to tolerate repeated mechanical stress (17, 50). The degradation of elastin exerts a key role in disease development (23) as has been demonstrated in experimental animal models (46). Alveolar...
macrophages from smokers release greater amounts of elastases compared with control macrophages (41), and this activity is largely due to MMPs that are expressed in the cell (47). In this study, the expression of human MMP-9 within the macrophages of the transgenic mice led to progressive air space enlargement in the lung coupled with a marked decrease in elastin within the alveolar walls of the transgenic mice. The 1-yr-old littermate mice (left) had abundant elastin fibrils present within their alveolar walls (see arrows). These fibrils were markedly decreased in age-matched MMP-9 transgenic mice (right). Images are at ×100 magnification. Each mouse had 15 images analyzed, and there were 12 mice in each group. C: quantification of alveolar wall elastin. Elastin fibrils appeared as distinct black lines coursing through the alveolar walls. To quantify elastin content within this alveolar region, the area of all the fibrils were calculated using ImagePro software. The area from each elastin fibril in an image was combined and then expressed as a percentage of the total alveolar wall area from that same image. Total alveolar wall area for each image was calculated using ImagePro software. The 1-yr-old littermate mice (open bar) had significantly higher levels of elastin detected within their alveolar walls compared with 1-yr-old MMP-9 transgenic mice (black bar; P < 0.004). This was a consistent finding amongst all the mice that were analyzed (n = 12 in each group). D: electron microscopy of alveolar wall. To further characterize changes in the alveolar wall, electron microscopy was performed on the lungs of 1-yr-old transgenic and control littermate mice (n = 3 in each group). Eight images were prepared for each mouse. As demonstrated by these representative images, littermate mice (left) had a higher content of fibrils (see arrow) within the alveolar wall compared with MMP-9 transgenic mice (right).

Fig. 4. Changes in lung elastin in the MMP-9 transgenic mice. A: lung α-elastin levels. Whole lungs from 1-yr-old littermate and transgenic mice were subjected to heat treatment according to methods. Biochemical analyses were then conducted to measure the α-elastin levels obtained from the lungs of 1-yr-old transgenic and littermate mice (n = 5 in each group). At 1 yr of age, α-elastin levels were nearly identical in littermate (open bar) and MMP-9 transgenic mice (black bar). B: Verhoeff staining of the alveolar wall. Verhoeff staining for elastin was conducted on from 1-yr-old transgenic and control littermate mice. The 1-yr-old littermate mice (left) had abundant elastin fibrils present within their alveolar walls (see arrows). These fibrils were markedly decreased in age-matched MMP-9 transgenic mice (right). Images are at ×100 magnification. Each mouse had 15 images analyzed, and there were 12 mice in each group. C: quantification of alveolar wall elastin. Elastin fibrils appeared as distinct black lines coursing through the alveolar walls. To quantify elastin content within this alveolar region, the area of all the fibrils were calculated using ImagePro software. The area from each elastin fibril in an image was combined and then expressed as a percentage of the total alveolar wall area from that same image. Total alveolar wall area for each image was calculated using ImagePro software. The 1-yr-old littermate mice (open bar) had significantly higher levels of elastin detected within their alveolar walls compared with 1-yr-old MMP-9 transgenic mice (black bar; P < 0.004). This was a consistent finding amongst all the mice that were analyzed (n = 12 in each group). D: electron microscopy of alveolar wall. To further characterize changes in the alveolar wall, electron microscopy was performed on the lungs of 1-yr-old transgenic and control littermate mice (n = 3 in each group). Eight images were prepared for each mouse. As demonstrated by these representative images, littermate mice (left) had a higher content of fibrils (see arrow) within the alveolar wall compared with MMP-9 transgenic mice (right).

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fibrils are in close proximity within the alveolar wall (15, 52). Thus elastin loss can shift mechanical forces causing excessive strain that results in the fatigue and breakdown of neighboring collagen fibrils (53).

In addition to directly cleaving the lung matrix, there are several possible mechanisms whereby MMP-9 could influence the structural changes observed in the 1-yr-old transgenic mice. MMP-9 has the potential to enhance lung proteolytic activity by inactivating inhibitors such as α₁-anti-trypsin and α₁-antichymotrypsin (33). This is an unlikely mechanism in the transgenic mice, since immunoblot analyses exhibited comparable levels of α₁-anti-trypsin in the littermate and transgenic mice. Second, MMP-9 could increase protease activity by enhancing the influx of inflammatory cells into the lung. MMP-9 cleaves CXCL8 (IL-8) increasing its chemotactic activity for neutrophils 10-fold (56), and proteolytic processing by MMP-9 can activate proinflammatory cytokines such as IL-1β (45), transforming growth factor-β (67), and TNF-α (16). Despite these known effects, baseline inflammatory cell number and profile and lung myeloperoxidase activity were nearly identical for the transgenic and littermate mice. Consistent with our data, the loss of MMP-9 in mice had no affect on the inflammatory response to the intratracheal administration of bleomycin (2). Thus the matrix changes that we detected cannot be attributed to alterations in lung inflammation. Therefore, direct cleavage of alveolar elastin by MMP-9 was the most likely mechanism responsible for the phenotype in this mouse model.

Given that collagen breakdown and elastin peptides have been shown to exert strong chemotactic potential in the lung (22, 60), it is somewhat surprising that inflammation in the MMP-9 transgenic mice was comparable with littermate control mice. However, turnover of these elements in the MMP-9 transgenic mice occurred over a 1-yr span. This gradual matrix turnover under baseline conditions is unlikely to generate peptide levels that would significantly affect lung inflammation. Furthermore, the interrelationship among air space enlargement, inflammation, and MMP-9 expression is complex. Elias and colleagues demonstrated that the loss of MMP-9 expression in IL-13 transgenic mice actually increased lung inflammation while decreasing air space enlargement (27). These prior findings coupled with our data indicate that MMP-9 mediates air space enlargement in the lung by directly targeting elements of the lung extracellular matrix.

This study emphasizes the importance of macrophage-derived MMP-9 in the formation of emphysema. Animal models have shown that macrophages are critical to the development of smoke-induced emphysema (18, 35, 40), and changes in macrophage biology have been well documented in COPD patients (6, 20, 38). However, direct evidence linking macrophage-mediated MMP-9 expression to the destruction of emphysema has been lacking. This had raised questions as to whether the expression of MMP-9 by macrophages was an initiator of smoke-induced air space enlargement or merely a part of the postinjury repair mechanisms of the lung. Our results provide evidence that macrophage MMP-9 activity over time can directly induce the loss of alveolar elastin, lead to a decrease in collagen content, and generate emphysematous changes in the lungs of mice. Thus these results support the assertion that
MMP-9 production by macrophages contributes to the development of this disease.

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