Cathepsin S Deficiency Confers Protection from Neonatal Hyperoxia-induced Lung Injury

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Rationale: Bronchopulmonary dysplasia (BPD) is a chronic lung disease that adversely affects long-term pulmonary function as well as neurodevelopmental outcomes of preterm infants. Elastolytic proteases have been implicated in the pathogenesis of BPD. Cathepsin S (cat S) is a cysteine protease with potent elastolytic activity. Increased levels and activity of cat S have been detected in a baboon model of BPD.

Objectives: To investigate whether deficiency of cat S alters the course of hyperoxia-induced neonatal lung injury in mice.

Methods: Newborn wild-type and cat S-deficient mice were exposed to 80% oxygen for 14 days. Histologic and morphometric analysis were performed and bronchoalveolar lavage protein and cells were analyzed. Lung elastin was assessed by real-time polymerase chain reaction, *in situ* hybridization, desmosine analysis, and Hart's stain. Distribution of myofibroblasts was analyzed by immunofluorescence. Hydroxyproline content of lung tissues was measured.

Measurements and Main Results: Hyperoxia-exposed cat S-deficient mice were protected from growth restriction and had improved alveolarization, decreased septal wall thickness, lower number of macrophages, and lower protein concentration in bronchoalveolar lavage fluid. α -Smooth muscle actin-expressing myofibroblasts accounted for at least some of the increased interstitial cellularity in hyperoxia-exposed mouse lungs and were significantly less in cat S-deficient lungs. Lung hydroxyproline content was increased in hyperoxia-exposed wild-type, but not in cat S-deficient lungs. Desmosine content was significantly reduced in both genotypes with hyperoxia.

Conclusions: Cathepsin S deficiency improves alveolarization, and attenuates macrophage influx and fibroproliferative changes in hyperoxia-induced neonatal mouse lung injury.

Keywords: cathepsin; bronchopulmonary dysplasia; hyperoxia; myofibroblast

Advanced perinatal care over the past decade has resulted in improved survival of very-low-birth-weight (VLBW) (birth weight < 1,500 g) infants (1). However, surviving VLBW infants

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Cathepsin S is a lysosomal papain-like cysteine protease with elastolytic activity. Limited information is available on the *in vivo* role of cathepsin S in normal or diseased lung, particularly in relationship to the alterations in extracellular matrix.

What This Study Adds to the Field

Deficiency of cathepsin S protected newborn mice from hyperoxia-induced growth restriction, impaired alveolarization, fibroproliferation, and macrophage influx. These improvements were not accompanied by any differences in lung elastin content.

have increased risk of major morbidities, particularly bronchopulmonary dysplasia (BPD). BPD has a negative impact on not only short-term and long-term pulmonary function but also on overall growth and neurodevelopment of VLBW infants (2–4). The major pathologic findings associated with BPD in surfactant-treated lungs are inflammation, disrupted alveolar development, and variable amounts of fibrosis (5). Exposure of the immature lung to hyperoxia, prolonged mechanical ventilation, and antenatal or postnatal infections are primary risk factors for BPD (6, 7). However, the molecular mechanisms that link these factors to disruption of alveolarization are not completely understood and there are currently no evidence-based strategies to prevent or treat BPD.

Several studies have shown that alveolar elastin expressed by myofibroblasts is a critical component of alveolar development (8, 9). The impaired alveolar development in BPD is also accompanied by changes in deposition of elastin (10). Bruce and coworkers demonstrated that urinary excretion of elastin degradation products is increased in neonates who later developed severe BPD (11). Alterations in elastin synthesis can be influenced by several factors, including the degree of lung expansion during mechanical ventilation (12), oxygen exposure (13), and retinoic acid (14, 15). Furthermore, although elastin is a remarkably stable extracellular matrix (ECM) component, it is subject to degradation by elastases released by inflammatory cells. Historically, neutrophil elastase has been linked to elastin degradation in BPD (16). However, in surfactant-treated human and baboon infants with otherwise uncomplicated BPD, free neutrophil elastase levels in the airways are very low or undetectable (17, 18). Recently, we have shown up-regulation of several papain-like lysosomal cysteine proteases in a baboon model of BPD (19). Among these proteases, cathepsin (cat) S is a potent elastolytic cysteine protease expressed by macrophages (20).

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Unlike other lysosomal enzymes, cat S maintains significant elastolytic activity at neutral pH and can be secreted from cells under certain conditions both as a proenzyme and a mature enzyme (21, 22). Cat S has an important role in promotion of atherosclerosis (23, 24) and angiogenesis (25, 26) via its effect on the ECM. Although several lung diseases, including BPD and emphysema, are characterized by altered regulation of ECM, relatively few studies have addressed the potential role of cat S in lung injury. Zheng and colleagues reported that pharmacologic inhibition as well as a null mutation of cat S significantly decreased apoptosis, inflammation, and lung volumes in a mouse model of emphysema induced by overexpression of IFN- γ (27).

Although there are no ideal rodent models of BPD, hyperoxia exposure of newborn mice results in decreased number of alveoli, enlarged terminal airspaces, and interstitial fibrosis, similar to the pathology observed in human infants with BPD (28, 29). In mouse models, these alterations occur by exposure to higher concentrations of inspired oxygen compared with that required for the treatment of the average surfactant-treated premature infant with respiratory distress syndrome and risk of BPD. In this study, we tested whether deficiency of cat S had an impact on hyperoxia-induced pathologic changes in newborn mouse lungs.

METHODS

Detailed methods are provided in the online supplement.

Animals and Hyperoxia Exposure

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Children's Hospital and Harvard Medical School, Boston. Wild-type (Jackson Laboratories, Bar Harbor, ME) and cat S^{-/-} mice of C57BL/6 background (30) were bred, and homozygote pups were used in the hyperoxia experiments. Within 12 to 24 hours of delivery, newborn pups and mothers were placed in a hyperoxic chamber (Biospherix, Redfield, NY) and exposed to 80% O₂ for 14 days. Mothers were rotated between oxygen-exposed and room-air-exposed litters every 24 hours to avoid oxygen toxicity.

Tissue Harvesting and Bronchoalveolar Lavage

Mice were killed by CO₂ narcosis and cervical dislocation. The right lung was immediately frozen in liquid nitrogen for RNA and protein isolation. The left lung was inflated to a fixed pressure of 25 cm H₂O with 10% buffered formalin. Bronchoalveolar lavage (BAL) was performed using a total volume of 1.5 ml phosphate-buffered saline. BAL fluid (BALF) was centrifuged and protein concentration of the supernatant, and total and differential cell counts were analyzed.

Histologic and Morphometric Analysis

To evaluate the general lung architecture, sections were stained with hematoxylin and eosin. The distribution of lung elastin was assessed on sections stained by Hart's method with tartrazine as a counterstain as previously described (10). To characterize airspace size, morphometry was performed on midsagittal sections stained with modified Gill's stain as previously described (31). Alveolar wall thickness was measured on images captured at $\times 400$ magnification.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry for cat S was performed as previously described (19). For immunofluorescence, the sections were incubated with a mouse monoclonal anti- α -smooth muscle actin (anti- α -SMA) antibody (clone 1A4; Sigma-Aldrich Co., St. Louis, MO) at a dilution of 1:800 as the primary antibody. A goat anti-mouse IgG conjugated with Alexa 488 (Invitrogen, Carlsbad, CA) was used as the secondary antibody at a dilution of 1:500.

Desmosine and Hydroxyproline Analysis

A portion of the fresh-frozen right lung tissue was hydrolyzed in 400 μ l of 6 N HCl for 24 hours at 100°C. The hydrolysate was evaporated to

dryness and analyzed for desmosine by radioimmunoassay as described (32). Analysis of hydroxyproline as an index of collagen content was done using an amino acid analyzer as described previously (33).

Isolation of Total RNA and Reverse Transcription

Total RNA was isolated from frozen mouse lung tissues using Trizol reagent (Invitrogen) and was treated with DNase I (Invitrogen). First-strand cDNA was synthesized from 0.5 μ g of RNA using the Super-script II First-Strand Synthesis System (Invitrogen) with 0.5 μ g oligo-deoxythymidine (oligo-dT).

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) analysis was performed using the Mx4000 Multiplex Quantitative PCR System (Stratagene, Inc., La Jolla, CA) and the brilliant SYBR Green QPCR Master Mix (Stratagene, Inc.). β -Actin was used as an internal reference to normalize the target transcripts.

In Situ Hybridization for Tropoelastin mRNA

The expression of elastin mRNA was localized by *in situ* hybridization using biotinylated antisense and sense riboprobes derived from a rat elastin cDNA. Sections were counterstained with nuclear fast red.

Statistical Analysis

Group means were compared using the nonparametric Wilcoxon Mann-Whitney U test. Values are expressed as mean $\pm$ SEM and $P < 0.05$ is considered to be significant.

RESULTS

Effect of Hyperoxia on Cat S Expression in the Lung

To determine if hyperoxia exposure would be a suitable model to study the role of cat S in neonatal lung injury, we analyzed the expression of cat S after 14 days of hyperoxia exposure in mouse lung tissues by real-time PCR and immunohistochemistry (Figure 1). Relative steady-state mRNA levels of cat S were approximately 2.5-fold higher in whole lung homogenates of wild-type mice after 14 days of neonatal hyperoxia exposure (Figure 1A; $P < 0.05$). By immunohistochemistry, cat S immunoreactivity was observed in macrophages in hyperoxia-exposed mouse lungs (Figure 1B). Hyperoxia-exposed cat S^{-/-} mouse lung tissues did not demonstrate any immunoreactivity for cat S and served as a negative control (Figure 1B).

Effect of Cat S Deficiency on Growth of Newborn Mice during Hyperoxia Exposure

Exposure to hyperoxia impairs overall body growth as well as lung growth in newborn rodents (28, 34, 35). Consistent with previous observations, hyperoxia exposure resulted in significant growth restriction in wild-type hyperoxia-exposed animals in comparison to normoxia control animals ($P < 0.001$; Figure 2). In contrast, the body weights of hyperoxia-exposed cat S^{-/-} mice were similar to those of the normoxia-exposed cat S^{-/-} mice. Of note, cat S^{-/-} mice also were heavier than wild-type mice under baseline normoxic conditions ($P < 0.001$). However, despite the difference in body weights, cat S^{-/-} and wild-type mice had comparable degree of alveolarization on Postnatal Day 14 under baseline normoxic conditions (see below). Thus, the larger size of cat S^{-/-} newborn mice did not appear to be associated with accelerated lung maturation. To our knowledge, the underlying cause for the mild obesity in cat S^{-/-} mice has not been reported.

Effect of Neonatal Hyperoxia on BAL Protein and Cell Counts

BAL samples were obtained immediately after animals were killed. Hyperoxia exposure resulted in a fivefold increase in BAL total protein concentration in wild-type mice (Figure 3A;

Figure 1. Cathepsin S (cat S) expression is induced in hyperoxia-exposed newborn lungs. (A) Real-time reverse transcriptase–polymerase chain reaction for cat S was performed on total lung RNA obtained from 14-day-old wild-type mice exposed to normoxia (WN) ($n = 8$) or hyperoxia (WH) ($n = 8$). Results are representative of two separate assays and normalized to β -actin. (B) Representative paraffin-embedded lung tissue samples showing cat S immunoreactivity in

macrophages in a WH group animal and no immunoreactivity in a cat S-deficient, hyperoxia-exposed (SH) group animal (cat S^{-/-}, exposed to 14 d of hyperoxia), thus confirming the specificity of the cat S antibody.

$P < 0.001$ vs. wild-type normoxia group). Surprisingly, hyperoxia-exposed cat S^{-/-} mice were almost completely protected from this effect ($P < 0.001$ vs. wild-type hyperoxia group). Total cell, macrophage, and neutrophil numbers were all significantly higher in wild-type hyperoxia group compared with normoxia group animals (Figure 3B). In contrast, total and differential cell counts of hyperoxia-exposed cat S^{-/-} animals were similar to cat S^{-/-} normoxia control animals. Remarkably, the number of macrophages in hyperoxia-exposed cat S^{-/-} mice was significantly lower compared with hyperoxia-exposed wild-type mice (Figure 3B), whereas the total number of neutrophils was similar in both groups.

Effect of Neonatal Hyperoxia on Lung Histology, Morphometry, and Collagen Content in Cat S-deficient Mice

Histologic analysis of hyperoxia-exposed lungs demonstrated larger and fewer terminal airspaces in both wild-type and cat S^{-/-} mice in comparison to normoxia control animals. Notably, interstitial hypercellularity was less pronounced and terminal airspace structures were more complex in hyperoxia-exposed cat S^{-/-} mice in comparison to hyperoxia-exposed wild-type mice (Figure 4A). These observations were verified by quantitative morphometry, which revealed a significant increase in mean chord length in hyperoxia-exposed lungs compared with normoxia-exposed control lungs in both genotypes ($P < 0.001$; Figure 4B). Cat S^{-/-} mice were significantly protected from airspace enlargement with lower mean chord lengths in comparison to wild-type animals ($P < 0.01$; Figure 4B).

Initial observations of decreased interstitial cellularity in hyperoxia-exposed cat S^{-/-} mice on histologic examination were further investigated by measurements of terminal airspace septal wall thickness (Figure 4C). Hyperoxia exposure resulted in a significant increase in septal wall thickness in both wild-type and cat S^{-/-} mice compared with their respective normoxia controls ($P < 0.001$). Consistent with the histologic analysis, sep-

tal wall thickness of hyperoxia-exposed cat S^{-/-} mice was less compared with wild-type control animals ($P < 0.01$). Increased collagen deposition accompanies the increased interstitial thickness in hyperoxia-exposed newborn lungs (28). We determined hydroxyproline content of the lung tissues as a measure of total lung collagen (Figure 4D). Hyperoxia exposure resulted in a significant increase in total lung hydroxyproline content in wild-type mice compared with the normoxia control animals ($P < 0.05$). Consistent with morphometric analysis, hydroxyproline levels were significantly lower in hyperoxia-exposed cat S^{-/-} mice compared with hyperoxia-exposed wild-type mice ($P < 0.05$). Furthermore, hydroxyproline levels were similar in cat S^{-/-} mice in normoxic and hyperoxic conditions.

Effect of Neonatal Hyperoxia on Elastin Expression in Cat S-deficient Mice

Cat S has potent elastolytic activity. To determine the *in vivo* effect of cat S on lung elastin expression, we first assessed steady-state levels of tropoelastin mRNA levels in whole lung homogenates by real-time reverse transcriptase–PCR (Figure 5A). As previously reported by Bruce and colleagues (36), hyperoxia significantly repressed tropoelastin mRNA expression in wild-type mice ($P < 0.05$; Figure 5A). Similarly, tropoelastin mRNA levels were lower in hyperoxia-exposed cat S^{-/-} mice compared with normoxia-exposed control animals ($P < 0.05$). Surprisingly, tropoelastin mRNA levels were further repressed in hyperoxia-exposed cat S^{-/-} mice compared with hyperoxia-exposed wild-type mice ($P < 0.05$). A similar trend was observed between normoxia-exposed wild-type and cat S^{-/-} mice, but these differences did not reach statistical significance.

Because elastin is expressed by pleural mesothelial cells, airway and vascular smooth muscle, vascular endothelium, and alveolar myofibroblasts, quantitative changes in elastin mRNA in whole lung could result from a variety of sources. To localize elastin mRNA expression, we performed *in situ* hybridizations (Figure 5B). In room-air-exposed mice, a subset of alveolar myofibroblasts were positive for elastin mRNA, and this was more abundant in peripheral sites adjacent to the pleural surface. Hyperoxia markedly reduced alveolar elastin mRNA expression, essentially eliminating positive tropoelastin mRNA signal from alveolar cells in cat S^{-/-} mice. Examination of conducting airways and adjacent arteries in the lung showed that elastin mRNA was localized to artery walls. In addition, occasional positive cells in airway smooth muscle cells were observed in normoxia-exposed animals and were repressed in all animals exposed to hyperoxia.

Next, we investigated the effect of cat S on lung elastin content and distribution at the protein level in hyperoxia-exposed lungs. Lysine residues in tropoelastin monomers are extensively oxidized by the lysyl oxidase family of enzymes, resulting in desmosine cross-links within and between monomers. Desmosine in whole lung homogenates was analyzed as a measure of

Figure 2. Cathepsin S (cat S)-deficient mice are protected from hyperoxia-induced weight loss. Body weights of mice on Postnatal Day 14. $n = 22$ –27/group. SH = cat S-deficient mice exposed to hyperoxia; SN = cat S-deficient mice exposed to normoxia; WH = wild-type mice exposed to hyperoxia; WN = wild-type mice exposed to normoxia.

Figure 3. Cathepsin S (cat S)-deficient mice have decreased bronchoalveolar lavage (BAL) protein concentration and macrophages. BAL was performed after 14 days exposure to normoxia (N) or hyperoxia (H) in wild-type or cat S^{-/-} pups (n = 7–10 for each group). (A) Total protein concentration was measured and (B) total and differential cell counts were performed. * $P < 0.001$ versus WN; ** $P < 0.01$ versus WN, * $P < 0.05$ versus WH. SH = cat S-deficient mice exposed to hyperoxia; SN = cat S-deficient mice exposed to normoxia; WH = wild-type mice exposed to hyperoxia; WN = wild-type mice exposed to normoxia.

insoluble elastin. Desmosine content was significantly reduced after hyperoxia exposure in both genotypes ($P < 0.01$, wild-type normoxia vs. wild-type hypoxia, and $P < 0.05$, cat S normoxia vs. cat S hyperoxia; Figure 5C). In contrast to the mRNA data, desmosine levels were similar in cat S^{-/-} mice in comparison to wild-type mice after both normoxia and hyperoxia exposures.

Elastic fibers localize to the rims of shallow saccules in newborn mice and encircle alveolar openings as the lung progresses

to the alveolar stage of development. In room-air-exposed mice, these elastic fibers appear as densely staining cables at tips of emerging alveolar septae in wild-type and cat S^{-/-} mice (Figure 5D). After hyperoxia exposure, alveolar elastic fibers in wild-type mice are of smaller diameter, stain less intensely, and often appear as collections of individual strands rather than as a dense cable. In the cat S^{-/-} mice, an intermediate appearance is notable, with elastic fibers at tips of alveolar septae appreciably

Figure 4. Cathepsin S (cat S)-deficient mice have improved alveolarization and less fibrosis after hyperoxia. C57BL/6 wild-type (W) and cat S^{-/-} (S) mice were exposed to normoxia (N) or hyperoxia (H) between Postnatal Days 1 and 14 (n = 8 for each group). Lungs were inflated under fixed pressure. (A) Representative hematoxylin-and-eosin-stained sections; scale bar, 50 μ m. (B) Morphometric analysis of lung sections expressed as mean chord length and (C) airspace wall thickness in micrometers. (D) Hydroxyproline content was determined as a measure of total lung collagen (n = 5–10 for each group). SN = cat S-deficient mice exposed to normoxia; WH = wild-type mice exposed to hyperoxia; WN = wild-type mice exposed to normoxia.

Figure 5. Elastin expression in cathepsin S (cat S)-deficient mice. (A) RNA was isolated from whole lung homogenates and relative steady-state mRNA levels of tropoelastin in mouse lung tissues were determined by real-time reverse transcriptase-polymerase chain reaction; $n = 8$ animals per group. (B) Representative tropoelastin mRNA *in situ* hybridizations of a minimum of four similar evaluations per group. (C) Desmosine content in mouse lung tissues at 14 days was quantified using radioimmunoassay; $n = 5$ –10 mice per group. (D) Distribution of elastin was assessed on paraffin-embedded lung sections using Hart's stain. Representative images of a minimum of four similar evaluations per group are shown. Scale bar, 50 μ m. SN = cat S-deficient mice exposed to normoxia; WH = wild-type mice exposed to hyperoxia; WN = wild-type mice exposed to normoxia.

denser and more tightly packed than in wild-type mice exposed to hyperoxia.

Effect of Cat S on Myofibroblast Density and Distribution

Myofibroblasts that are located at the tips of secondary crests synthesize alveolar elastin and are critical in normal alveolar development. In contrast, interstitial myofibroblasts contribute to the remodeling process as the key mediators of ECM deposition during and after lung injury. Although recruitment and activation of interstitial myofibroblasts may represent normal response to injury, persistence of these cells in injured areas has been implicated in the development of lung fibrosis (37). Both alveolar and interstitial myofibroblasts express α -SMA. In our initial experiments, we characterized the time course of α -SMA expression in the perinatal mouse lung as well as the effect of hyperoxia on α -SMA expression (Figure E1 of the online supplement). Consistent with observations reported by Rishikof and colleagues (38), the density of α -SMA-expressing myofibro-

blasts at the alveolar septal tips were increased between Days 4 and 7, and were significantly diminished by Day 14. Hyperoxia-exposed lungs with arrested alveolar development had α -SMA-positive myofibroblasts in the interstitium and around alveolar ducts instead of septal tips. To determine whether decreased terminal airspace wall thickness in cat S^{-/-} mice was associated with an alteration in distribution or total number of myofibroblasts, we analyzed α -SMA expression in mouse lung tissues, excluding areas with α -SMA-positive smooth muscle cells, such as large airways and blood vessels (Figure 6). α -SMA-positive cells were irregularly distributed along the walls of terminal airspaces and in the interstitium in the wild-type hyperoxia-exposed lungs (Figure 6A). In cat S^{-/-} mouse lungs, α -SMA cells were preferentially located at the rudimentary septal tips (red arrows). The overall number of α -SMA-expressing cells corrected for the number of terminal airspaces was significantly lower in cat S^{-/-} mice in comparison to wild-type mice after 14 days of hyperoxia exposure ($P < 0.01$; Figure 6B).

Figure 6. Cathepsin S (cat S)-deficient mice have decreased number of interstitial myofibroblasts. (A) Immunofluorescence staining for α -smooth muscle actin (α -SMA) was performed on paraffin-embedded lung tissue sections of hyperoxia-exposed wild-type (WH) and cat S^{-/-} (SH) mice ($n = 6$ –8 for each group; v, vessel; scale bar, 25 μ m). Red arrows indicate α -SMA-positive myofibroblasts at the rudimentary septal tips. (B) Number of α -SMA-positive cells normalized to the number of terminal airspaces.

DISCUSSION

The goal of this study was to determine whether cat S played a role in hyperoxia-induced neonatal lung injury in mice. Based on the elastolytic activity of cat S, our initial hypothesis was that deficiency of cat S would confer protection from neonatal lung injury by decreasing damage to elastin that provides a scaffold for the newly developing alveoli. Although proving our basic hypothesis that cat S is involved in the pathogenesis of hyperoxia-induced lung injury, our studies revealed a novel role for cat S. Deficiency of cat S decreased fibroproliferative changes induced by hyperoxia and this effect, at least in part, was mediated via modulation of the response of myofibroblasts to hyperoxia. Furthermore, deficiency of cat S resulted in decreased number of macrophages and protein concentration in BAL, and improved alveolarization. Importantly, these alterations were not accompanied by any significant quantitative differences in lung elastin content between hyperoxia-exposed wild-type and cat S^{-/-} mice.

Cat S is a papain-like lysosomal cysteine protease that was originally cloned from human alveolar macrophages (20). Unlike other lysosomal proteases, cat S is active and stable at neutral pH (reviewed in References 39 and 40). Both proenzyme and mature forms of cat S can be released from several different cell types, including activated macrophages and smooth muscle cells (23), and pro-cat S can be autoactivated at neutral pH in the presence of glycosaminoglycans (41). Taken together, these features suggest that cat S can have extracellular functions in addition to its well-established role in antigen presentation as a lysosomal enzyme (42).

Cat S is constitutively expressed in macrophages and IFN- γ is a potent inducer of cat S expression in several cell types, including macrophages (43), smooth muscle cells (23), endothelial cells (44), and the A549 cell line (45). In transgenic mice overexpressing IFN- γ , cat S expression is significantly up-regulated, and cat S is detected in alveolar epithelial cells (27). We have not detected cat S immunoreactivity in any cell type other than macrophages in baboon (19) or mouse lungs (Figure 1). This discrepancy may be due to different sensitivities of the antibodies or the differences in the models used. In our mouse model of neonatal hyperoxia as well as in the baboon model of BPD, IFN- γ levels are increased, but are likely much lower than those in transgenic mice overexpressing IFN- γ (S.C., unpublished data; and Reference 46). Cat S activity can be detected in the supernatant of a mouse macrophage cell line (RAW 264.7, American Type Culture Collection) after 2 hours of hyperoxia exposure (M.W. and S.C., unpublished data). On the basis of these data, activated macrophages are likely the major source of cat S in our model. However, in our more recent experiments, we have also detected cat S expression in primary mouse lung fibroblast cultures (M.W. and S.C., unpublished data) and studies are underway to investigate the biological significance of this preliminary observation.

The current paradigm regarding the elastolytic and, although not as potent, collagenolytic activities of cat S involves facilitating the migration of cells that are destined for certain functions via degradation of ECM proteins. This paradigm has evolved on the basis of several studies (*see* Table E1). For example, in an *in vitro* model of angiogenesis, cat S expression is induced in stimulated endothelial cells and inhibition of cat S activity reduces microtubule formation by impairing cell invasion (25). Endothelial cells from cat S^{-/-} mice have reduced collagenolytic activity and cat S^{-/-} mice display defective microvascular development during wound repair. By impairing angiogenesis-dependent tumor cell proliferation, deficiency of cat S impairs the growth of solid tumors (26). The results of our

study do not appear to support the current paradigm, but this may be due to the cell type that was identified as one of the targets for cat S activity in our model—the myofibroblast. Deficiency of cat S resulted in decreased numbers of interstitial myofibroblasts in association with decreased terminal airspace wall thickness and lower hydroxyproline levels in hyperoxia-exposed newborn mouse lungs. These findings are remarkable given that the fibroproliferative changes induced by hyperoxia exposure are modest compared with other experimental models of fibrosis such as bleomycin treatment. Although cat S may have a unique role in the response of the developing lung to hyperoxic injury, it is likely that it could also play a role in the fibrotic response of adult lungs to injury. Indeed, increased cat S activity in bleomycin-treated rat lung tissues and alveolar macrophages (47) supports the latter possibility. Amelioration of hyperoxia-induced fibrotic changes in cat S^{-/-} mice suggests that the impact of cat S on accumulation, maintenance, or ECM-producing functions of myofibroblasts is greater than its impact on ECM protein degradation as a proteolytic enzyme in this model (*see* Figure E2).

Decreased numbers of macrophages and protein concentration in BAL of cat S^{-/-} pups indicate that cat S plays a role in regulation of the inflammatory response in neonatal hyperoxia. An attenuated macrophage response could be one of the factors that resulted in decreased fibroproliferative changes and improved alveolarization in hyperoxia-exposed cat S^{-/-} mice. Deficiency of cat S in IFN- γ -overexpressing mice also results in significantly decreased numbers of total cells in BAL, including macrophages and neutrophils (25). Although total cell number in BAL showed a similar trend in our study, this difference did not reach statistical significance due to variability. This variability may have been due to the technical challenges of obtaining BAL from 14-day-old mice. Cat S is one of the up-regulated genes during inflammatory trafficking of monocytes into alveolar spaces (48). However, the exact role of cat S in this process or its potential effect on the inflammatory activity of macrophages is not known.

There are other potential mechanisms that could account for the decreased fibroproliferation and improved alveolarization in hyperoxia-exposed cat S^{-/-} mice. Consistent with the existing paradigm, cat S could facilitate the recruitment of fibroblasts to the interstitium by degradation of ECM proteins, and this effect may not be necessarily detected by measurements of total lung elastin and collagen content. Transforming growth factor- β signaling is potentiated in lungs of pups exposed to hyperoxia (49), and molecules on this pathway could be susceptible to proteolytic activation or inactivation by cat S. Another potential mechanism could be inactivation of antifibrotic molecules, such as IFN- γ , by extracellular cat S. Such a feedback loop between IFN- γ and cat S could play a role in maintaining the balance between fibrotic and antifibrotic factors in the lung. These potential mechanisms are currently being explored in more robust models of experimental lung fibrosis.

Hyperoxia exposure caused a more marked decrease in tropoelastin mRNA levels in cat S-deficient mouse lungs compared with wild-type control lungs. However, desmosine levels, as a measure of total lung elastin content, were similar between these two groups. This uncoupling between elastin mRNA and protein levels in hyperoxia-exposed neonatal cat S^{-/-} mice may be explained by decreased elastin turnover, such that, in the absence of cat S, less elastin is degraded and less elastin mRNA is synthesized. Despite similar levels of lung desmosine in cat S^{-/-} and wild-type lungs, cat S deficiency had a positive impact on alveolarization in hyperoxia-exposed lungs. Thus although elastin is a significant ECM component for secondary septation to occur, other factors appear to also influence this process in

the injured developing lung. Improved alveolarization in hyperoxia-exposed cat $S^{-/-}$ pups despite similar levels of lung elastin could be secondary to attenuated fibroproliferation and/or inflammation, or result from a direct effect of cat S on alveolar epithelial cell survival or proliferation. Indeed, several other cathepsins play a role in apoptosis (50, 51), and Zheng and coworkers have reported that cat S has a role in IFN- γ -induced apoptosis of alveolar epithelial cells (27). We have not detected an increase in apoptosis of alveolar epithelial cells in mouse lungs after 14 days of hyperoxia exposure (G.B.-K. and S.C., unpublished observations). However, we cannot exclude this possibility without examining earlier time points because one study has reported increased apoptosis with hyperoxia exposure in the peripheral lung of neonatal mice between Days 3.5 and 5.5 (34).

Two other important observations in this study were protection of hyperoxia-exposed cat $S^{-/-}$ newborn mice from growth restriction and alveolar-capillary leak as evidenced by low BAL protein concentrations. These findings indicate the physiologic significance of the improved lung pathology in cat $S^{-/-}$ mice and underscore the need for further studies to better understand the role of this protease in inflammatory and fibroproliferative processes associated with lung diseases such as BPD.

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