SERPINB1 upregulation is associated with in vivo complex formation with neutrophil elastase and cathepsin G in a baboon model of bronchopulmonary dysplasia

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Bronchopulmonary dysplasia (BPD), a common and serious complication of prematurity, was first described in 1967 in premature infants treated with prolonged artificial ventilation and 80–100% O2 for hyaline membrane disease (29). Since the original description of BPD, major advances in neonatology, such as use of antenatal steroids, gentler ventilation techniques, and surfactant replacement therapy, have significantly improved the care of premature infants. However, many premature infants, especially those with birth weights of <1,000 g, continue to develop a different form of BPD, referred to as the “new” BPD (3, 22). These infants are at increased risk for having abnormal neurodevelopment, impaired growth, frequent respiratory infections requiring hospitalization, and reactive airway disease (17, 18, 39, 40).

The present epidemiologic and clinical features associated with new BPD are different than those originally described (“old” BPD) (3). Infants with new BPD have lower gestational ages and lower birth weights. Some of these infants initially have minimal lung disease but develop a need for increasing oxygen and ventilator support over the next few weeks of life. The new BPD also has different pathological features compared with the old BPD (9). A partial to complete arrest in alveolar and microvascular development as well as abnormal structure and distribution of elastin are the most striking features of the new BPD (9, 24, 26).

Several factors, including antenatal and postnatal infections, baro- and volutrauma, oxygen toxicity, nutritional deficiencies, proinflammatory cytokines, and an imbalance between elastolytic proteases and antiproteases, have been implicated in the pathogenesis of BPD (5, 6, 20, 36, 44). Seminal studies that identified protease-antiprotease imbalance as a risk factor in BPD focused on the increased activity of neutrophil elastase (NE) and decreased activity of its inhibitors, α1-antitrypsin [α1-AT, serine protease inhibitor (SERPIN)A1] and secretory leukocyte protease inhibitor (SLPI) in the airways (28, 30, 51). Increased activity of NE was also reported in association with other commonly encountered complications of premature infants, such as nosocomial infections and pulmonary interstitial emphysema (15, 50). However, all of these studies were conducted in the presurfactant era. In studies with relatively small numbers of surfactant-treated preterm infants, elastase activity in tracheal aspirates was detected in only a minority of patients, and there was no difference between the study controls and the BPD groups (16, 42, 49).

Another NE inhibitor that could be relevant in the pathogenesis of BPD is SERPINB1 (monocye neutrophil elastase inhibitor, MNEI). SERPINB1, a member of the clade B serpins (32–34), is a fast-acting stoichiometric inhibitor of neutrophil-derived proteinases NE, cathepsin G (cat G), and proteinase-3 (14, 32). SERPINB1 is detected at low levels in the airways of healthy individuals (13). In the airways of patients with cystic fibrosis (CF), a neutrophil-mediated lung disease, SERPINB1 is increased but mostly inactive either in complexed or cleaved forms (13). Initially purified from monocyte-like cells,
SERPINB1 is also expressed in macrophages and neutrophils. It is not known whether these cells are the only sources of SERPINB1 in the airways. Transcriptional factors that are prominent in the inflammatory response, such as nuclear factor-κB and PU.1/Spi-1, have been identified as regulatory elements for the SERPINB1 gene (54).

In a rat model of lung injury, recombinant SERPINB1 decreased hemorrhage and epithelial permeability in the lung after instillation of human NE or protease-containing sputum of CF patients (31). In two other recent studies, recombinant SERPINB1 protected surfactant proteins A and D, members of the collectin family, from degradation by neutrophil proteases (19, 35). Collectively, these studies suggest that SERPINB1 could provide protection against the destructive effects of neutrophil proteinases in inflammatory lung diseases.

In this study, we investigated the hypothesis that SERPINB1 contributes to the regulation of neutrophil proteases NE and cat G in the lung tissue and airways during the development of BPD in previously well-characterized baboon models of BPD (8, 10, 11, 24, 26, 48). We also examined whether prematurity is associated with decreased levels of SERPINB1 in the lung and airways. Furthermore, because the role of NE in new BPD has not been fully characterized, we compared the activity levels of NE in bronchoalveolar lavage (BAL) fluid (BALF) of baboons with new and old BPD.

MATERIALS AND METHODS

Animal model. Frozen and paraffin-embedded baboon lung tissue and BALF samples were provided by the Southwest Foundation for Biomedical Research (San Antonio, TX). All animal procedures were reviewed and approved by the animal care committees of the Southwest Foundation for Biomedical Research and the University of Texas Health Science Center in San Antonio. In the new BPD model, baboons that were delivered by hysterotomy at 125 days were intubated, treated with exogenous surfactant (Surfactant; donated by Ross Laboratories, Columbus, OH), and maintained on pressure-limited, time-cycled infant ventilators (donated by InfantStar; Infrasonics, San Diego, CA) for 14 days (125-day/14-day BPD group). The ventilator settings were adjusted to maintain arterial partial pressure of CO₂ between 45 and 55 Torr, and oxygen was provided on an as-needed basis to maintain arterial partial pressure of O₂PaO₂ between 55 and 70 Torr. Animals that were killed at 14 days had pathological and biochemical findings that were characteristic of the new BPD seen in human infants (11, 24–26, 48). Thus these baboons exhibited alveolar hypoplasia, variable saccular wall fibrosis, decrease in capillary vasculature, and significant elevations of several proinflammatory cytokines compared with age-matched gestational controls. Baboons that were delivered at 125 or 140 days and killed immediately served as the age-matched gestational controls (125-day or 140-day groups). A third control group consisted of baboons that were born via natural delivery at full-term gestation (~185 days) and killed 2–3 days later (full-term group). In the old BPD model (140-day/10-day BPD group), baboons were delivered at 140 days of gestation and were ventilated for a total of 10 days with 80–100% oxygen (10). The lung pathology of these animals was comparable to that originally described by Northway et al. (29) in human infants with BPD and was striking for increased interstitial cellularity, peribronchial and peribronchiolar fibrosis, and hyperplastic and metaplastic changes of the airways (8, 10). After euthanasia by intravenous infusion of pentobarbital sodium, the lungs were perfused by infusion of phosphate-buffered saline (PBS) in the right heart, and then lung tissue was snap-frozen in liquid nitrogen or inflated with 4% paraformaldehyde at 20-cmH₂O pressure for fixation.

Isolation of total RNA and reverse transcription. Total RNA was isolated from fresh-frozen baboon lung tissue, BALF cells, or primary airway epithelial cells with TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (Invitrogen), following the manufacturer’s instructions. First-strand cDNA was synthesized from 0.5 μg of RNA with the Superscript First-Strand Synthesis System (Invitrogen) with 0.5 μg of oligo(dT). The reaction mixture was incubated at 42°C for 50 min, followed by incubation at 72°C for 15 min. cDNA was stored at –20°C until use.

Quantitative polymerase chain reaction. Quantitative polymerase chain reaction (PCR) analysis was performed with the Mx4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA) and the brilliant SYBR Green QPCR Master Mix (Stratagene). The sequences of PCR primers for were as follows: human SERPINB1 forward primer 5’-AGGACAGTTGACTTGGAAA-3’, reverse primer 5’-AAAGAGATCCCTGACACCTA-3’, human GAPDH forward primer 5’-GGTGGTCTCTCTGACTC-3’, reverse primer 5’-CTTTCCTTGGTGCTCTTTG-3’.

For PCR analysis, 2 μl of cDNA was diluted 1:10, and the reactions were performed in 20 μl of reaction volume with the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min. In initial experiments, quantitative PCR products were verified by agarose gel electrophoresis. A standard curve was plotted for each primer set with the critical threshold cycle values obtained from amplification of 10-fold dilutions of cDNA from normal baboon lung tissue to calculate the PCR efficiency of the primer set. GAPDH was used as an internal reference to normalize the target transcripts, and the relative differences were calculated. Each experiment was performed in triplicate and repeated at least twice.

Preparations of baboon lung tissue homogenates and BALF. BALF samples were obtained at the time of necropsy by instillation of sterile 0.9% saline into the left lower lobe until the lobe was completely filled and withdrawal of the saline. This procedure was repeated for a total of five times. BALF samples were then concentrated with Vivaspin 500 centrifugal concentrators (Sartorius Stedim Biotech, Goettingen, Germany) and treated with DNase I (Invitrogen), following the manufacturer’s protocol. Samples that were used for immunoprecipitation experiments were dialyzed in lysis buffer [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 1 mM protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)]. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA). Whole lung tissues were homogenized in lysis buffer with a Dounce glass homogenizer. After centrifugation for 20 min at 4°C, the supernatant was removed and stored at –80°C until use.

Immunoblot analysis and densitometry. Protein samples were analyzed by immunoblotting under reducing conditions as previously described (7). Briefly, 2× Laemmli sample buffer was added to samples, and each sample was heated to 95°C for 5 min. The proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membrane was blocked in a buffer containing PBS, pH 7.4, 0.1% Tween 20, and 5% dried milk for 1 h and incubated with the primary antibody for 1 h. All incubations were performed at room temperature (RT). The primary antibodies were used at the following dilutions: rabbit anti-SERPINB1 polyclonal antibody, 1:2,000 (31); rabbit anti-human cat G, 1:500 (Dakocytomation, Carpinteria, CA); rabbit anti-human NE, 1:1,000 (Athens Research, Athens, GA); and anti-β-actin, 1:5,000 (Sigma, St. Louis, MO). Subsequently, the membranes were rinsed in wash buffer and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). After three rinses in wash buffer, the protein bands were visualized by chemiluminescence (ECL Western blotting analysis system; Amersham Biosciences, Piscataway, NJ), and quantitated by densitometry with NIH Image 1.63. All samples were normalized to β-actin.

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded baboon lung tissue sections as...
previously reported (7). Briefly, sections were deparaffinized in xylene, rehydrated in graded ethanol solutions, and rinsed in deionized water. Between the absolute and 95% ethanol washes, sections were incubated for 30 min in a 0.3% H2O2 (vol/vol) methanol solution to quench endogenous peroxidase activity. Microwave antigen retrieval was performed with antigen unmasking solution (Vector Laboratories, Burlingame, CA). After washing in PBS, the sections were blocked by incubating with 15% goat serum diluted in PBS with 2% (wt/vol) BSA (Sigma) for 1 h. Slides were rinsed in PBS and incubated with the primary antibody (rabbit anti-SERPINB1 polyclonal antibody; 1:1,000) for 1 h at RT. Next, sections were washed for 10 min in PBS and then incubated with a biotinylated horse anti-rabbit IgG (Jackson Immunoresearch Laboratories). After a 10-min rinse in PBS, the secondary antibodies were detected by incubation for 30 min with an avidin-biotin-peroxidase complex (Vectastain Elite ABC HRP kit; Vector Laboratories), followed by a 10-min PBS wash and then a 5 min incubation with a 3,3’-diaminobenzidine substrate-chromogen solution (Vector Laboratories). Sections were counterstained with methyl green.

Recombinant proteins and in vitro SERPINB1-protease complex formation. Recombinant (r)SERPINB1 was produced in Sf9 insect cells as previously described (12). rSERPINB3 and rSERPINB4 proteins were prepared as glutathione S-transferase (GST)-fusion proteins in Escherichia coli as previously described (37, 38). Bacterial expression plasmids for these serpins were provided by Dr. Gary Silverman (University of Pittsburgh, PA). In vitro complexes of rSERPINB1 and purified NE (Athens Research) or cat G (Athens Research) were formed by incubating the serpin with each protease at a 5-to-1 (inhibitor:enzyme) molar ratio in PBS, pH 7.4, for 5 min at 37°C. The samples were heated to 95°C in Laemmli sample buffer for 5 min and subjected to immunoblotting as described above.

Immunoprecipitation. Tissue lysates (total protein 500 μg/sample) or concentrated and pooled BALF samples (total protein 700 μg/sample) were immunoprecipitated with 30 μl of protein G-agarose beads (Santa Cruz Biotechnology) bound to SERPINB1 monoclonal antibody overnight at 4°C with shaking. The monoclonal mouse anti-human SERPINB1 antibody (ELA-1) was raised against rSERPINB1 by standard methods (E. Remold-O’Donnell et al., manuscript in preparation). The samples were centrifuged at 1,000 g for 5 min, and agarose-bound immune complexes were washed five times with lysis buffer and then resuspended in 50 μl of SDS sample buffer. After boiling for 3 min at 95°C, samples were subjected to immunoblotting as described above with either anti-NE or anti-cat G antibody as the primary antibody. In vitro formed complexes between rSERPINB1 and NE or cat G served as positive controls for immunoprecipitation experiments.

HPLC ion trap mass spectrometry (liquid chromatography-tandem mass spectrometry). BALF samples of the 125-day/14-day BPD group animals that contained high-molecular-mass (HMM) SERPINB1 were pooled, immunoprecipitated with an anti-SERPINB1 monoclonal antibody, and subjected to electrophoresis with a 12% Ready Tris·HCl gel (Bio-Rad). The gel was stained with colloidal Coomassie blue (Invitrogen), and a 66-kDa band was identified for sequencing. Sequence analysis was performed at the Wistar Institute Proteomics Facility (Philadelphia, PA) with microcapillary reverse-phase HPLC nanospray tandem mass spectrometry (MS/MS) on a Thermofinnigan LCQ quadrupole ion trap mass spectrometer.

Measurement of free NE levels. The level of free NE in baboon BALF samples was determined by the cleavage of a synthetic substrate, methoxyxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (MetOSuc-AAPV-pNA; Calbiochem, La Jolla, CA), as previously described (53). Briefly, BALF samples were dialyzed in a buffer containing 20 mM Tris·HCl, pH 7.4, and 500 mM NaCl and concentrated to 1 mg/ml total protein concentration. One millimolar MetOSuc-AAPV-pNA was added to a 100-μl sample, and the change in optical density at 410 nm was measured with a spectrophotometer (SpectraMaxM2 microplate reader, Molecular Devices, Sunnyvale, CA). A standard curve was obtained by cleavage of the substrate by known amounts of NE for each experiment. The level of free NE was determined by measurement of the change in optical density between the initial elastase level and the level after preincubation of samples with rSERPINB1 or the synthetic serine protease inhibitor Pefabloc SC (Sigma) at an inhibitor-to-enzyme ratio of 10:1 (based on the initial elastase activity) for 10 min at 37°C.

Statistical analysis. Data were analyzed with Kruskal-Wallis and Wilcoxon-Mann-Whitney U-test with SAS version 9.1 (SAS Institute, 2003, Cary, NC). Values are expressed as means ± SE, and P < 0.05 is considered to be significant.
**SERPINB1** protein expression during lung development and in BPD. At the protein level, SERPINB1 expression was analyzed by immunoblotting and densitometry using whole lung tissue homogenates. First, the specificity of the polyclonal SERPINB1 antibody was confirmed by immunoblotting using rSERPINB1 and two other related serpins that are expressed in the lung, SERPINB3 and SERPINB4 (Fig. 2). Equal amounts of recombinant proteins were analyzed by SDS-PAGE and Coomassie blue staining (3 μg/lane; Fig. 2A), followed by immunoblotting with the polyclonal SERPINB1 antibody at a 1:2,000 dilution (150 ng protein/lane). As expected, SERPINB1 antibody reacted only with rSERPINB1 and not with GST-SERPINB3 or GST-SERPINB4 proteins (Fig. 2B).

In baboon lung samples, SERPINB1 was detected as a 42-kDa band in all gestational controls and both the 125-day/14-day and 140-day/10-day BPD groups (Fig. 3A). Serpins inhibit their target proteinases by formation of covalent SDS- and heat-stable HMM complexes. These inhibitory complexes (66 kDa) were detected at low levels in all BPD samples (Fig. 3A). In overexposed blots, complexed SERPINB1 could also be visualized in some samples from the full-term group (not shown). All lung tissue samples also contained low levels of cleaved SERPINB1 (Fig. 3A). By densitometry, native SERPINB1 protein levels (42 kDa) in the 125-day/14-day BPD group were increased compared with 140-day gestational control samples (P < 0.05) but were comparable to the full-term samples (Fig. 3B). In contrast, native SERPINB1 protein levels were decreased in 140-day/10-day BPD samples compared with full-term and 125-day/14-day BPD samples (P < 0.05). Similar to alterations observed at the mRNA levels, SERPINB1 protein levels were significantly higher in full-term samples compared with the 125-day or 140-day controls (P < 0.05).

**Immunolocalization of SERPINB1 in the lung.** SERPINB1-expressing cells were identified by immunohistochemistry using the polyclonal anti-SERPINB1 antibody in formalin-fixed, paraffin-embedded baboon lung tissue sections (Fig. 4). At 140 days of gestation, SERPINB1 was detected in mast cells and conducting airway epithelium (Fig. 4A). In the full-term (Fig. 4B) and 125-day/14-day BPD (Fig. 4, C and D) samples, SERPINB1 immunoreactivity was localized to macrophages, neutrophils, interstitial mononuclear cells, some of which were mast cells, and bronchial and glandular epithelial cells.

**Detection of SERPINB1 protein in BALF.** SERPINB1 is detected as an inactive cleaved or complexed serpin in BALF samples of patients with CF (13). For qualitative assessment of SERPINB1 in the airways in BPD, we analyzed concentrated BALF samples by immunoblotting using the polyclonal SERPINB1 antibody (Fig. 5). BALF samples were standardized to total protein. SERPINB1 was detected as a 42-kDa protein in all BALF samples obtained from both the control and BPD groups. In addition, SERPINB1 was consistently detected as a 66-kDa band in all BPD samples. In contrast, a 66-kDa band was detected in only one sample from the full-term group.

**SERPINB1 complexes with NE and cat G in BPD.** We detected SERPINB1 as a HMM (66 kDa) band in both lung tissue lysates and BALF of BPD group baboons. To determine whether this band represented a complex between either NE or cat G and SERPINB1, coimmunoprecipitation experiments were performed (Fig. 6A). SERPINB1 was immunoprecipitated from pooled BALF or lung homogenate samples with a polyclonal anti-SERPINB1 antibody in formalin-fixed, paraffin-embedded baboon lung tissue sections. At 140 days of gestation, SERPINB1 was detected in mast cells and conducting airway epithelium (Fig. 4A). In the full-term (Fig. 4B) and 125-day/14-day BPD (Fig. 4, C and D) samples, SERPINB1 immunoreactivity was localized to macrophages, neutrophils, interstitial mononuclear cells, some of which were mast cells, and bronchial and glandular epithelial cells.
monoclonal antibody specific for SERPINB1 and then subjected to immunoblotting using either anti-NE or anti-cat G antibodies. In both BALF and tissue samples from the 125-day/14-day BPD group, the 66-kDa proteins that were immunoprecipitated with SERPINB1 antibodies immunoreacted with anti-NE and anti-cat G antibodies. Thus NE and cat G were found in complexes with SERPINB1 in BPD samples.

To confirm the results obtained by immunoprecipitation experiments, pooled BALF samples were subjected to SDS-PAGE after SERPINB1 immunoprecipitation. A 66-kDa band was identified and analyzed by tryptic in-gel digestion followed by liquid chromatography (LC)-MS/MS. SERPINB1 was one of the multiple proteins found in this band, with six unique peptides, all with high scores and good spectra (Fig. 6B). One of these peptides was reported as “predicted chimpanzee SERPINB1” and was identical to human SERPINB1 protein sequence except for one amino acid mismatch (Val in human vs. Ala at position 106 in chimpanzee). This finding suggests that the baboon SERPINB1 sequence contains an Ala at this position, similar to the chimpanzee sequence. The majority of other proteins identified were immunoglobulin-related and likely represented contamination.

Detection of free NE in new and old BPD models. NE was detected as an inactive protease complexed to SERPINB1 in 125-day/14-day BPD samples. To determine whether free NE was also present in these samples and to compare the NE activity in BALF between new and old models of BPD, NE activity level was measured by a spectrophotometric assay using the substrate MetOSuc-AAPV-pNA. NE activity was detectable only in low levels in new BPD (125-day/14-day BPD) samples and was significantly higher in old BPD (140-day/10-day BPD) samples (Fig. 7; \( P < 0.05 \)).

DISCUSSION

In this study, we have shown that SERPINB1 is expressed in bronchial and glandular epithelial cells in addition to neutrophils, macrophages, and mast cells. SERPINB1 mRNA and protein levels increased with advancing gestational age in the baboon lung tissues. SERPINB1 expression was also characterized in two different baboon models of BPD. The new BPD model was induced in 125-day surfactant-treated baboons with ventilation and as-needed oxygen administration for 14 days, whereas the old BPD model was induced in non-surfactant-treated 140-day baboons with ventilation and hyperoxia for 10 days. Thus the new BPD model differed from the old BPD model in that it was induced in more immature baboon infants who had received surfactant treatment and were exposed to lower oxygen concentrations than in the old BPD model. In the new BPD model (125-day/14-day BPD), SERPINB1 mRNA and native SERPINB1 protein levels in whole lung lysates were significantly increased compared with the gestational controls and were similar to those in the full-term group. In contrast, SERPINB1 mRNA and native SERPINB1 protein
levels were significantly decreased in the old BPD model (140-day/10-day BPD) compared with the new BPD or full-term samples. Another striking alteration in SERPINB1 protein expression in BPD was detection of SERPINB1 as a 66-kDa complex in both BALF and lung tissues. This observation suggested the presence of proteases complexed to SERPINB1 in these samples. Indeed, we pursued these findings by coimmunoprecipitation experiments and demonstrated that both cat G and NE were complexed to SERPINB1 in BALF and tissue samples of 125-day/14-day BPD group baboons. Furthermore, the presence of SERPINB1 in these inhibitory complexes was verified by LC/MS-MS. Free NE activity was detected only at low levels in BALF in the new BPD model (125-day/14-day BPD) and was significantly lower than in the old BPD model.

SERPINB1 belongs to a subgroup of serpins known as ovalbumin-related serpins (ov-serpins) or clade B serpins (33, 41). To date, at least 10 of the 13 clade B serpins have been reported to have inhibitory activity against serine or cysteine proteinases (41). However, data on the physiological roles of clade B serpins have lagged behind their biochemical characterization. Among ov-serpins, SERPINB1 has a unique inhibitory specificity for both NE and cat G (14) and has been shown to be an ancestral gene of this group (4). Human SERPINB1 was previously identified in complexes in airway fluids of patients with CF (13), a disease that is characterized by high activity levels of airway neutrophil proteases (27, 35). In the present study, we have identified similar SERPINB1 complexes in a baboon model of BPD and demonstrated NE and cat G in these complexes by coimmunoprecipitation. Furthermore, the presence of SERPINB1 in these HMM complexes was confirmed by tryptic in-gel digest followed by LC-MS/MS. The mass spectrometry data failed to show a proteinase in this complex. One possible explanation for this may be lack of Lys residues in the NE protein sequence, because trypsin preferentially hydrolyzes peptide bonds after Lys or Arg residues. For example, five of the six SERPINB1 peptides that were identified by LC-MS/MS were sequences that followed a Lys residue (data not shown).

Unlike plasma serpins such as α1-AT, clade B serpins lack a signal peptide and are not secreted via the classic secretory pathway. However, several clade B serpins, including SERPINB1, have been detected in extracellular fluids (13, 52). In this study, we detected significant amounts of native SERPINB1 in BALF of premature as well as full-term baboons. Both native and complexed SERPINB1 were detected in BPD group BALF samples. The mechanism by which clade B serpins are released into the extracellular environment is not known. One possibility is release of these molecules from necrotic cells. The serpin could then bind to and neutralize its target protease(s) extracellularly. Another possible explanation

Fig. 6. Analysis of high molecular mass SERPINB1 complex by immunoprecipitation and liquid chromatography-tandem mass spectrometry (LC-MS/MS). A: BALF and lung homogenates from 125-day gestational controls or 125-day/14-day BPD group baboons were immunoprecipitated (IP) with anti-SERPINB1 monoclonal antibody (ELA-1) and subsequently immunoblotted with anti-neutrophil elastase (NE) (top) or anti-cathepsin G (cat G) (bottom) antibodies. Protease alone (NE, top; cat G, bottom) and respective IP of in vitro-generated complex with SERPINB1 (complex, positive control) are shown. Note that the 66-kDa complex is found in both BALF and lung homogenates of 125-day/14-day BPD but not in 125-day gestational control (negative control) samples. B: amino acid position and sequence of peptides identified by LC-MS/MS analysis of the 66-kDa complex immunoprecipitated from pooled 125-day/14-day BPD group BALF samples. Five peptides were identical to human SERPINB1, and one was identified as “predicted chimpanzee SERPINB1” (position 97–110) because of the presence of Ala at position 106 vs. Val in human SERPINB1 sequence.

Fig. 7. Free NE levels in old and new BPD. Free NE protein level was measured in concentrated necropsy BALF samples obtained on day 14 from baboons with new BPD (left) and on day 10 from baboons with old BPD (right) by a spectrophotometric assay using the specific substrate methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide. A standard curve was obtained by cleavage of the substrate by known amounts of purified NE for each experiment; n = 6–8 animals/group. *P < 0.05 vs. new BPD group.
for detection of SERPINB1 and protease complexes in BALF is release of intracellularly formed complexes from necrotic cells. However, this could occur only in cells that harbor both SERPINB1 and its target proteases, such as neutrophils. We acknowledge as a study limitation that in tissue samples the process of homogenization could have artificially induced serpin and protease complexes by providing physical proximity.

Consistent with previous observations (32, 34), we detected SERPINB1 immunoreactivity in neutrophils, macrophages, and also mast cells in baboon lung tissues. All of these cell types are involved in the inflammatory response that is observed in human infants with BPD (23, 45). In the baboon model of new BPD, a dominance of macrophages was observed on differential cell counts of BALF obtained on day 14 (1), whereas in the old BPD model, neutrophils were predominant although macrophage numbers were also elevated (10). A novel finding was detection of SERPINB1 in the suprabasal conducting airway and glandular epithelial cells. Several clade B serpins are expressed in the lung (2), and at least two of them, SERPINB3 (SCCA1) and SERPINB4 (SCCA2), are expressed in the airway epithelium in a pattern similar to that of SERPINB1 (7). The polyclonal SERPINB1 antibody that was used for immunohistochemistry in this study does not cross-react with either of these serpins (Fig. 2). This result is also consistent with detection of SERPINB1 mRNA in bronchial epithelial cells of asthmatic patients (cDNA provided by Dr. Craig M. Lilly, Brigham and Women’s Hospital, Boston) (data not shown). The expression of SERPINB1 in the airway epithelium could explain why SERPINB1 is detected in comparable amounts in BPD and full-term lungs that have only a few inflammatory cells. In both groups, intracapillary inflammatory cells could have contributed to the SERPINB1 levels assessed by immunoblotting in whole lung lysates.

An association between increased activity of NE and development of BPD was reported in several studies before the widespread use of antenatal steroids and surfactant replacement therapy in neonatal care (28, 30). However, this association has been evaluated in only a limited number of surfactant-treated infants (16, 42, 49). By decreasing surface tension and improving lung compliance, surfactant treatment improves oxygenation (reviewed in Ref. 21). Decreased use of supplemental oxygen in ventilated premature infants could favorably influence the balance between NE and its inhibitors by limiting the oxidative inactivation of NE inhibitors, such as α1-AT and SERPINB1. Furthermore, our findings indicate that SERPINB1 expression is regulated at the mRNA level in both models of BPD. The finding of lower SERPINB1 mRNA and protein levels in old BPD compared with new BPD suggested that NE activity could be higher in the old BPD model. Indeed, free NE activity in necropsy BALF samples of baboons with old BPD was higher compared with those with new BPD. These results are consistent with those of two other studies that examined free total elastase (49) or NE activity (42) in tracheal aspirate fluids (TAF) of intubated preterm human infants, the majority of whom had received surfactant treatment. In these trials, free elastase activity was low or undetectable in infants who developed BPD. Furthermore, low elastase activity was associated with adequate inhibitory activity of α1-AT and SLPI in TAF (42).

Consistent with the above observations, a randomized clinical trial of α1-AT supplementation reduced the incidence of pulmonary hemorrhage but did not affect the incidence of BPD in premature infants with respiratory distress syndrome who had also received surfactant treatment (46). In this trial, the plasma concentrations of α1-AT were not significantly different between the placebo and control groups (47) and thus could account for the ineffectiveness of α1-AT. Our study as well as the previously published studies (42, 49) suggest that low levels of NE in new BPD may have contributed to the lack of a statistically significant clinical benefit from α1-AT supplementation. However, even in the surfactant era, a subgroup of premature infants continue to suffer from the classic BPD (43) and may benefit from augmentation of antielastase capacity.

In conclusion, we found increased levels of SERPINB1 and detected inhibitory complexes of SERPINB1 with NE and cat G in lungs and BALF samples of baboons with new BPD. This was associated with decreased levels of free NE in new BPD. In contrast, lower SERPINB1 levels were associated with higher activity levels of NE in old BPD. These findings suggest that SERPINB1 upregulation in new BPD is part of a protective mechanism and SERPINB1 contributes to the regulation of NE activity along with other well-known elastase inhibitors, such as α1-AT and SLPI, in new BPD.

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