Structure-function relations in an elastase-induced mouse model of emphysema

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METHODS

Animal Preparation

Six groups of male C57BL/6 mice (24.1±1.1g, Charles River Laboratories, Boston, MA) at age 10 weeks were enrolled. All procedures were approved by the Animal Care and Use Committee of Boston University. The mice were initially anesthetized with isoflurane and treated with oropharyngeal instillation of either 50 μg (0.25 units) of porcine pancreatic elastase (PPE; Sigma-Aldrich, St. Louis, MO) dissolved in 100 μl of phosphate buffered saline (PPE treated group, n=18) or the same amount of phosphate buffered saline (Saline control group, n=18). Animals in both groups were subdivided into subgroups (n=6) based on time of sacrifice at 2, 7 and 21 days after the initial treatment. Following measurement of respiratory mechanics, 12 lungs were used to evaluate structure-function relations. These lungs were fixed with 10% buffered formalin, and the remaining lungs were homogenized for Western blot analysis. Before the experiment, the animals were deeply anesthetized by intraperitoneal injection of pentobarbital sodium (80 mg/kg), tracheostomized, and cannulated in the supine position. The cannula was connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Quebec, Canada).

Measurement and Modeling of Respiratory Mechanics

Mice were mechanically ventilated with room air using a tidal volume (VT) of 8 ml/kg at a frequency of 240 breaths/min. After stabilization, dynamic respiratory mechanics were measured at five different positive end-expiratory pressure (PEEP) levels (0, 3, 6, 9 and 12 cmH_2O) in the closed chest condition using the forced oscillation technique combined with the optimal ventilator waveform (OVW) (E1). The OVW is a composite waveform consisting of 5 sine waves so that in the time domain the waveform is similar to a tidal breath while allowing a smooth estimation of the impedance. During measurement, the peak-to-peak OVW amplitude was matched to the VT delivered by the mechanical ventilator. To standardize volume history, each measurement was preceded by two consecutive inflations of the lungs to total lung capacity (TLC) defined as a tracheal pressure of 25 cmH_2O kept for 3 seconds. Measurements were performed every 3 min after changing the PEEP level. The impedance spectra were then fitted to an impedance model including a heterogeneous distribution of tissue elastance (HTE) (E2).

The model is composed of a set of parallel branches with each branch corresponding to an airway pathway. A single pathway was modeled by a linear airway compartment, characterized by the airway impedance (Z_{aw}), and a linear viscoelastic lung tissue impedance (Z_{ti}),

connected in series. The Z_{aw} was represented by an equivalent tube, having a linear flow resistance (R_{aw}) and gas inertance (I_{aw}).

$$Z_{aw}(\omega) = R_{aw} + j\omega I_{aw} \quad (1)$$

where j is the imaginary unit, and ω is the circular frequency. The Z_{ti} was modeled as the constant phase tissue impedance (E3) given as follows:

$$Z_{ti}(\omega) = (G - jH)/\omega_n^{\alpha}, \quad \text{with } \alpha = 2/\pi \arctan(G/H) \quad (2)$$

where G and H are the coefficients of tissue damping and elastance, respectively, $\omega_n = \omega/\omega_0$ is the normalized circular frequency whereas ω_0 is an arbitrary reference frequency. By choosing ω_0 to be 1 rad/s, the numerical values of G and H will be consistent with those in previous reports. The Z_{aw} was the same in each pathway whereas H was assumed to be distributed among the pathways according to a probability function p(H), which followed a hyperbolic decay, p(H) ~ 1/H, between a minimum (H_{min}) and a maximum (H_{max}) value. Since G and H in Eq. 2 have units of impedance, we define the tissue compliance (C) as $C = 1/(H\omega_0)$ which has units of compliance, i.e. volume over pressure. Using a transformation of variables, it is easy to show that the distribution of C is also hyperbolic, i.e. p(C) ~ 1/C between a minimum ($C_{min}=1/H_{max}$) and a maximum value of C ($C_{max}=1/H_{min}$). If we define F to be

$$F = \ln(C_{max}/C_{min}), \quad (3)$$

it is easy to show that the expected value of C, C_{mean}, can be obtained as

$$C_{mean} = (C_{max} - C_{min})/F \quad (4)$$

Finally, Eq. 2 can be rewritten in the following form:

$$Z_{ti}(\omega) = (\eta - j)H/\omega_n^{\alpha} \quad (5)$$

where $\eta=G/H$ is the hysteresivity (E4). The parameters R_{aw}, I_{aw}, η, H_{min}, and H_{max} were estimated by fitting the HTE model to the measured impedance data. The functional heterogeneity of tissue compliance was then characterized by the standard deviation of tissue compliance (SD_C) calculated from the estimated values of C_{min} and C_{max} as:

$$SD_C = \sqrt{\frac{(C_{max}^2 - C_{min}^2)}{2F} - \frac{(C_{max} - C_{min})^2}{F^2}} \quad (6)$$

The absolute error, the root mean square difference between the model and the measured impedance spectra, was minimized using a global optimization algorithm (E5) which provided estimates of the model parameters.

Tissue Processing

After assessing respiratory mechanics, a median abdominal incision and sternotomy were performed under deep anesthesia. Euthanasia was obtained during exsanguination by cutting the abdominal aorta and inferior vena cava to allow blood to leave the lungs while the pulmonary artery was flushed with phosphate buffered saline. To evaluate structure-function relations, lungs were inflated with 10% buffered formalin (Fisher Scientific, Pittsburgh, PA) via the tracheal cannula at a constant airway pressure of 30 cmH_2O for 20 minutes. The heart-lung was isolated en block and immersed in formalin. After two weeks, the mediastinal lobes of the lung were divided into anterior and posterior parts, and each part was separately embedded in paraffin. To assess the constituents of the soluble part of the ECM, Western blot analyses were performed. Using an electric homogenizer (PowerGen 125, Fisher Scientific, Pittsburgh, PA), the lung without the hilum was homogenized in 2 ml of PBS containing protease inhibitors (Halt Protease Inhibitor Cocktail kit,, Pierce Biotechnology, Rockford, IL). The homogenates were then centrifuged at 10,000 x g for 15 minutes, and the supernatant was stored at -20˚C until further processing.

Morphometry

Lung sections of 5μm thickness from the mediastinal lobe were stained with hematoxylin and eosin.The slides were placed under the microscope, an image was taken and the slide was moved under the objective so that the new region did not overlap with the previous one. No selection bias was applied. In each lung, at least 16 randomly selected sections were photographed under a bright-field microscope (Nikon Eclipse TS100, Nikon Instruments Inc. Melville, NY), and the microscopic fields from each cross-section were digitized by a camera (SPOT Insight 2MP Firewire Color Mosaic, Diagnostic Instruments, Inc., MI, USA). After the large blood vessels and central airways were manually masked, the images were automatically segmented and the area of each airspace unit was measured. To quantify the airspace enlargement, the equivalent diameter (D) of alveolar airspaces was determined. A minimum of 500 D values per mouse larger than 5 μm obtained from a sample were used to calculate the mean equivalent diameter (D_{eq}). This also allowed us to compare D_{eq} between mice. To characterize the heterogeneity of structure, the area weighted mean equivalent diameter (D_2) was also computed (E6). The parameter D_2 has been shown to be sensitive to both airspace enlargement and increases in the variability of airspace sizes. A schematic of the image processing and calculation of D_2 is shown in Figure E1.

Western Blot

The samples were normalized to equal volume and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10% acrylamide), then transfered to PVDF membrane (Bio-Rad, Hercules, CA). The primary antibodies used were rabbit polyclonal anti-Collagen I (1:2,000, GeneTex, Inc., San Antonio, TX or Abcam Int., Cambridge, MA), mouse monoclonal anti-Collagen III (1:2,000, GeneTex, Inc., San Antonio, TX), goat polyclonal anti-Elastin (1:5,000, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and mouse monoclonal anti-β-actin (1:10,000, Abcam Inc., Cambridge, MA). The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat (Vector Laboratories, Burlingame, CA) antibodies as the secondary antibodies for 1 hour. The immune complexes were detected with a chemiluminescence kit (SuperSignal West Pico, Pierce Biotechnology, Rockford, IL), and quantified by computerized optical densitometry. The density from each sample was then normalized with the corresponding density of the housekeeping β-actin as a loading control. The data were expressed in arbitrary units. The mean value for saline control of 21 days after treatment was set to 1.0, and the remaining values were adjusted accordingly.

Network Modeling

We considered a two dimensional network model consisting of linear elastic springs arranged in a rectangular lattice. To obtain a microscopic structure that resembled the normal mouse lung parenchyma, springs were removed from this regular lattice until the coefficient of variation (CV) of the network pore diameters matched the CV of D measured from histological sections of the saline control animals. The spring constants had unit values and were the same inside the network. To mimic the pleural stiffness associated with the high tension (E7), the springs around the boundary of the network had a spring constant 10 times higher than that of the internal springs. A uniform negative pressure (P_0) was applied around the outer boundary of the network as follows. The application of pressure on a boundary spring generates a force $\boldsymbol{f}=(P_0\, l)\, \boldsymbol{n}$ to act on this spring where l is the length of the spring and $\boldsymbol{n}$ is the corresponding unit outward normal vector. The total free energy of the system was then minimized iteratively. At the end of each step, the value of $\boldsymbol{n}$ and l were updated. The minimization process was continued until the change in $\boldsymbol{n}$ and l between two consecutive minimization steps became smaller than a certain preset tolerance. Once the network was pressurized, we simulated the progression of emphysema in 3 different ways. In the first method, springs were uniformly weakened by 2% at subsequent iterations. In the second method, at each step, springs were randomly eliminated from the network. In the third, we invoked the notion that alveolar walls experiencing high stress are more likely to break than walls under less stress (E8). Thus, the springs that carried the highest strain were removed from

the network at subsequent iterations. In all the three methods, at each step we kept track of the area weighted mean diameter of network pores (D_2). Additionally, the 2-dimensional compliance C of the network was also calculated at each step as the ratio $C=\Delta A/\Delta P$ where ΔA is the change in total area of the network that results from the application of a small decrease in pressure ΔP around the network. All three processes were then repeated until C increased by a factor of 2.

To be able to compare the structure-function relations from the experimental data to those from the various models, we used the following normalization approach. The normalized D_2 axis in Figure 7 indicates $D_2/D_{2,n}$ for each treated animal where $D_{2,n}$ is the mean D_2 of the control mice. Similarly, the normalized compliance axis shows C/C_n for each treated animal where C_n is the mean compliance of the control mice. There are two mice from each group, the day 2, day 7 and day 21 groups. On this normalized plot, the mean C and D_2 of the control mice have coordinates $(D_2,C) = (1,1)$ which is indicated by an open circle with bars indicating the group SD. The rationale behind such normalization was to enable us to plot D_2 and C from our computer simulations of the progression of emphysema, which are in arbitrary units, on the same graph as the experimentally measured values of D_2 and C from PPE treated mice. The D_2 and C of the network at different stages of destruction were normalized by the D_2 and C of the initial network and plotted along with the experimentally measured structure function data from PPE treated mice.

Statistical Analysis

All data were expressed as means ± SD. The differences between treatment conditions were compared with unpaired t-test. One-way ANOVA with Bonferroni's multiple comparison tests and two-way repeated measure ANOVA were used to evaluate differences among different time points and PEEP levels. The Kolmogrov-Smirnoff test was used to compare the distributions of D corresponding to different animals within a given treatment group at a given time point. Correlations between structural and functional parameters were evaluated by linear regression analysis. Statistical significance was accepted for $p<0.05$. Prism 5.0a (GraphPad Software, Inc. San Diego, CA) and the statistical package R (http://www.r-project.org/) were used for the statistical analyses.

Results

In order to assess whether surface tension might be involved in this emphysema model, we carried out a number of Western blots based on equal amounts of protein from lung

homogenates to measure the levels of SP-B, the most important protein component of surfactant regulating surface tension. We found that SP-B levels in the saline treated lungs at day 2 were 27% higher than at day 7 ($p<0.05$). Additionally, SP-B was also higher at day 2 in saline than in PPE treated lung (13%, $p<0.02$). We speculate that this may have been due to a compensatory mechanism following the saline treatment which is similar to a mini lavage depleting surfactant from the lung. However, at days 7 and 21, there were no differences between the saline and PPE groups. Since at day 7 in the treated lungs, the same amount of SP-B was distributed over a smaller total surface area, but in regions with larger radius of curvature, the recoil pressure due to surfactant (assuming that all other protein and lipid concentrations were also the same) would be less in the PPE treated lungs. Figure E2 shows representative blots at day 21. Figure E3 presents an enlarged view of the dotted rectangle from the collagen stained tissue in Fig. 3D in the main text. The arrow points to a thin wall under tension containing only collagen.

References

E1. Lutchen KR, Yang K, Kaczka DW, Suki B. Optimal ventilation waveforms for estimating low-frequency respiratory impedance. *J Appl Physiol* 1993;75:478-488.

E2. Ito S, Ingenito EP, Arold SP, Parameswaran H, Tgavalekos NT, Lutchen KR, Suki B. Tissue heterogeneity in the mouse lung: Effects of elastase treatment. *J Appl Physiol* 2004;97:204-212.

E3. Hantos Z, Daroczy B, Suki B, Nagy S, Fredberg JJ. Input impedance and peripheral inhomogeneity of dog lungs. *J Appl Physiol* 1992;72:168-178.

E4. Fredberg JJ, Stamenovic D. On the imperfect elasticity of lung tissue. *J Appl Physiol* 1989;67:2408-2419.

E5. Csendes T. Nonlinear parameter estimation by global optimization—efficiency and reliability. *Acta Cybernetica* 8: 361-370, 1989.

E6. Parameswaran H, Majumdar A, Ito S, Alencar AM, Suki B. Quantitative characterization of airspace enlargement in emphysema. *J Appl Physiol* 2006;100:186-193.

E7. Hajji MA, Wilson TA, Lai-Fook SJ. Improved measurements of shear modulus and pleural membrane tension of the lung. *J Appl Physiol* 1979;47:175-181.

E8. Kononov S, Brewer K, Sakai H, Cavalcante FS, Sabayanagam CR, Ingenito EP, Suki B. Roles of mechanical forces and collagen failure in the development of elastase-induced emphysema. *Am J Respir Crit Care Med* 2001;164:1920-1926.