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ORIGINAL RESEARCH

Alveolar fractal box dimension inversely correlates with mean linear intercept in mice with elastase-induced emphysema

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Rationale: A widely applicable model of emphysema that allows efficient and sensitive quantification of injury is needed to compare potential therapies.

Objectives: To establish such a model, we studied the relationship between elastase dose and the severity of emphysema in female C57BL/6J mice. We compared alveolar fractal box dimension (D_B), a new measure which is an assessment of the complexity of the tissue, with mean linear intercept (L_m), which is commonly used to estimate airspace size, for sensitivity and efficiency of measurement.

Methods: Emphysema was induced in female C57BL/6J mice by administering increasing intratracheal doses of porcine pancreatic elastase (PPE). Changes in morphology and static lung compliance (C_L) were examined 21 days later. Correlation of D_B with L_m was determined in histological sections of lungs exposed to PPE. The inverse relationship between D_B and L_m was supported by examining similar morphological sections from another experiment where the development of emphysema was studied 1 to 3 weeks after instillation of human neutrophil elastase (HNE).

Results: L_m increased with PPE dose in a sigmoidal curve. C_L increased after 80 or 120 U/kg body weight ($P < 0.05$), but not after 40 U/kg, compared with the control. D_B progressively declined from 1.66 ± 0.002 (standard error of the mean) in controls, to 1.47 ± 0.006 after 120 U PPE/kg ($P < 0.0001$). After PPE or HNE instillation, D_B was inversely related to L_m ($R = -0.95$, $P < 0.0001$ and $R = -0.84$, $P = 0.01$, respectively), with a more negative slope of the relationship using HNE ($P < 0.0001$).

Conclusion: Intratracheal instillation of increasing doses of PPE yields a scale of progression from mild to severe emphysema. D_B correlates inversely with L_m after instillation of either PPE or HNE and yields a rapid, sensitive measure of emphysema after elastase instillation.

Keywords: chronic obstructive pulmonary disease, pulmonary emphysema, lung morphometry, lung compliance

Introduction

Development of new drugs for the treatment of emphysema is hindered by the time required for the development of the disease state and by the lack of a sensitive, efficient measure to quantify the severity of injury. Furthermore, without a standardized model, it is difficult to compare results across studies. The most common methods of inducing emphysema are intratracheal instillation of either porcine pancreatic elastase (PPE), or human neutrophilic elastase (HNE).[1–3] A bolus of elastase instilled intratracheally into mice produces acute lung injury, alveolar destruction, and airspace enlargement that stabilizes within 21 days.[2] Previous studies of elastase-induced emphysema in mice

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235

have used males or females of various strains and ages, with variable susceptibility to injury.[2,4–6] In an effort to identify a standardized therapeutic model, the C57BL/6J wild-type strain was chosen for study because it possesses intrinsically low levels of alpha-1-antitrypsin, because it is the parent strain of the *pallid* mutant, which has reduced secretion of alpha-1-antitrypsin from the liver,[7] and because it is amenable to creation of transgenic derivatives.[8] Only one other group of investigators has examined the relation of multiple doses of elastase to morphometry in C57BL/6J mice.[9] However, that study did not establish a general model of elastase-induced emphysema because the mice used by them varied in age from 6 to 10 weeks, ages that span the end of very active lung growth into much slower growth,[10] where susceptibility to elastase changes considerably.[11]

The C57BL/6J strain has been characterized as "mildly susceptible" to cigarette smoke-induced emphysema.[12,13] Serum antitrypsin levels in male C57BL/6J mice are about 70% of those in other wild-type murine strains.[13] Female C57BL/6J mice have about half the serum antitrypsin levels of males,[13,14] and this probably explains why the amount of elastase required to produce emphysema is lower for females than for males.

Ideally, parameters used to evaluate the severity of emphysema should allow a high degree of discrimination. Mean linear intercept (L_m) is the most common morphometric method to assess emphysema in animal models.[15] Although the calculation of L_m provides reliable measurements of emphysema, it is not a true measure of alveolar size.[16] Measurement of L_m is labor intensive, and requires subjective distinction between alveolar structures and surrounding tissue.[17] Image analysis software is available for performing more rapid calculations of L_m, but is expensive, and still requires subjective interpretation of the tissue. Other morphometric measures of emphysema, such as internal surface area (ISA), alveolar surface area, and alveolar number, also depend on subjective interpretation, volume measurements, which can be highly variable, and either meticulous manual measurements or expensive software. Thus, a more efficient measure is desirable; one that is sensitive to subtle changes in emphysema. One possible approach is to compare fractal properties of the lungs.

Measures of fractal dimension have recently gained importance because of their discriminatory capabilities. A fractal is an object or function that has self-similarity over several orders of scale.[18] The fractal dimension is a measure of complexity, and tends to be characteristic for a normal, healthy form or process. Furthermore, the fractal dimension may change significantly with disease.[19,20] When fractal properties are measured by laying a series of boxes over an image of a structure, the measure is called a fractal box dimension, or D_B. It is the slope of the regression line for the log–log plot of box size (or scale) C and count N, where N is the number of boxes that contain a part of the image, from a box counting scan. In nature, the log–log plot normally yields a slightly concave line, so by convention D_B is the limit as scale approaches zero: $D_B = \lim_{C \to 0} [\ln N / \ln C^{-1}]$.[21] Rigaut and coworkers established that lung alveolar surfaces have fractal properties at the microscopic level.[22] Karperien has developed a user-friendly Image J software plug-in, called FracLac,[21] which makes it possible to get fast, reliable measurements of D_B from two dimensional images.

We report that, in elastase-induced emphysema, D_B is inversely correlated with L_m, regardless of the type of elastase used, and is rapidly determined using the same digital images for measurement of L_m.

Materials and methods
Reagents
High-purity PPE was obtained from Elastin Products, Inc (Owensville, MO). Each mg of protein contained 137 units PPE activity, where one unit solubilizes 1 mg of elastin-orcein substrate in 20 minutes at pH 8.8 and a temperature of 37°C. HNE was obtained from the same source, in a concentration of 850 units/mg, where 1 HNE unit hydrolyzes 1 Nmole suc-ala-ala-ala-pNA/min at pH 7.5 and a temperature of 25°C. It must be noted that the activity of these two enzymes cannot be directly compared. Despite having a 40% homology, and the same active site mechanism, they differ with regard to pH and temperature optima, hydrophobicity, and substrate preferences.[23] Ketamine HCl and ace promazine maleate were obtained from USP Fort Dodge Animal Health (Fort Dodge, IA). Xylazine was obtained from Phoenix Scientific, Inc (St Joseph, MO), and isoflurane and 10% buffered formalin acetate from Fisher Scientific (St Louis, MO).

Mice
Seven-week-old C57BL/6J female mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in the vivarium at the Harry S Truman Memorial VA Hospital (Columbia, MO) in standard hanging shoebox cages, in isolation from other mice. Food and water were supplied ad libitum. Room temperature was 72°F–76°F, relative humidity was 40%–50%, and the day/night cycle was set at 12 hours/12 hours. Mice were acclimatized for 1 week before elastase instillation. All animal treatment

was in accordance with a protocol approved by the Harry S Truman VA Subcommittee for Animal Studies.

Elastase instillation

Elastase was administered when mice were 8 weeks old, the midpoint of the transition from rapid to near stationary growth for this strain. At this age, the lungs have completed their most rapid growth[10] and retain a moderate susceptibility to elastase. In preparation for instillation, mice were anesthetized with 5% isoflurane for induction, and then maintained at 3%–5% isoflurane, with oxygen set at 0.6 L/min. Once mice were sedated, a 20 μL ketamine/xylazine cocktail containing 25 mg/mL ketamine HCl, and 0.625 mg/mL xylazine was injected intraperitoneally to prevent laryngeal spasms. A 20-gauge catheter (Beckton Dickinson, Rutherford, NJ) was inserted into the trachea via the mouth, connected to a manometer (custom made), and placement verified by briefly observing the characteristic liquid displacement of tidal breathing. After disconnecting the manometer, a bolus of elastase was then delivered into the catheter using a micropipette with a fine tip. For recovery, animals were given oxygen for 1 minute, and then placed in a warm chamber enriched with oxygen until they recovered consciousness, usually within 5 minutes.

For the study of PPE dosage effects, mice were given 40, 80, or 120 units of PPE per kg of body weight (abbreviated U/kg) in 100 μL of phosphate-buffered saline (PBS). This range of doses was found in preliminary experiments (data not shown) to be adequate to produce emphysema in our model. Each dosage group, consisting of eight to ten mice after instillation, was subdivided for morphometry and lung compliance measurements to eliminate the possibility that morphometric results might be affected by manipulations of the lungs during compliance measurements. Mice were sacrificed 21 days after instillation.

To compare the effects of HNE on the relationship between the fractal box dimension and mean linear intercept, female mice from the same source and of the same age and strain received 700 HNE U/kg body weight. We selected this dose because, in preliminary experiments, it was shown to reliably produce emphysema within 21 days, while higher doses resulted in pulmonary hemorrhage. Mice were sacrificed 7, 14, or 21 days postinstillation. Controls in each study received 100 μL PBS alone.

Static lung compliance (C$_L$)

Animals were sacrificed by overdosing with 200–300 μL ketamine/xylazine/ace promazine maleate cocktail (100:10:2 mg/mL), administered intraperitoneally.

The body cavity was opened and an incision was made in the trachea. A 20-gauge catheter was inserted into the trachea and secured with suture.

Static lung compliance was measured with a 5 cc syringe connected to the trachea via a 20-gauge catheter and to a water manometer via a three-way stopcock. For inflation curves, 0.2 cc of air was manually injected stepwise, up to a total of 3.0 cc. The resultant pressure from each incremental injection was read from the manometer approximately 1 second after the injection. Deflation was read by the same method, manually withdrawing 0.2 cc at a time, until 3.0 cc was withdrawn. Inflation and deflation curves were measured twice for each animal. Volume was plotted as a function of pressure. C$_L$ was determined by the average slope of each deflation curve at its midpoint.

Morphometry

For morphometric studies, animals were sacrificed as described above. Lungs were excised from the body cavity with heart and trachea attached and fixed in 10% neutral buffered formalin acetate at a pressure of 25 cm of H_2O for 8 hours. Whole lungs were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Three sagittal lung sections of 4 μm thickness were taken from the middle of each lung, so that a portion of each lung lobe was included in each section. Each subsequent section was spaced 200 μm from the previous section. Paraffin embedding, sectioning, slide preparation for light microscopy, and staining with hematoxylin and eosin was done by the University of Missouri Research Animal Diagnostic Laboratory.

Mean linear intercept (L$_m$)

L$_m$ was measured according to the method of Dunnill using blind scoring.[15] Identifying information on each slide was temporarily masked with a coded label, until after L$_m$ measurements were completed. Typically, there were 25 fields of lung tissue on each microscope slide. The fields were digitally photographed and saved (in jpeg format). Twelve suitable fields were randomly chosen and counted; a total of 36 fields were counted per animal. Areas with large airways were not selected for counting. L$_m$ was derived by superimposing a grid over each image and counting the number of times the alveolar walls intercepted the grid lines. The equation

$$L_m = (N)(L)/m$$

where N = number of times the transverses were placed on the tissue, L = length of the transverses and m = the sum of all intercepts, gave L$_m$. Because the 10% formalin fixation

and paraffin embedding of mouse lungs has only a small effect on linear dimension,[24] a correction factor was not applied to L_m.

Alveolar box counting fractal dimension (D_B)

D_B of the alveolar walls was measured using the FracLac plugin, v 2.5, for Image J.[21] The images used for the measurement of L_m were autocorrected for brightness and contrast using Microsoft Office Picture Manager (within Microsoft Office 2007; Microsoft, Redmond, WA), converted to grayscale, and exported to the tagged image file format. They were then imported into Image J in stacks, set to binary and unstacked, and saved in a new folder. FracLac was set for standard box count of binary images, using default parameters for grid calibers, and with added options for scanning, data processing, and graphics unchecked. Results were saved automatically. Accuracy was verified by scanning images of known fractal dimension. Six standard images scanned by this method tested within ±1% of their known values. The binary images were scanned as a group. The correlation coefficient for each image scan, a test of the linear regression, was ≥0.995 and indicates that the measured parameters are truly fractal.[25]

Statistical analysis

The mean and standard error were calculated for each outcome measure for different levels of elastase. A P value of <0.05 was considered statistically significant. One-way analysis of variance (ANOVA) was used to make comparisons between the outcomes of different groups (Table 1, Figure 2). The residual plot from the fitted ANOVA model shows no serious violation of the normal assumption for the error terms. For estimating the correlations between the alveolar fractal box dimension (D_B) and the mean linear intercept (L_m), Spearman's correlation coefficient was chosen over Pearson's correlation coefficient, due to the non-normal distribution of outcome measures. Simple regression models were used to estimate slopes in Figure 3A and B. The effects of the two different types of elastase upon the inverse relationship between D_B and

L_m were compared by an F-test of the regression coefficients. Data analysis was compiled with the Kaleidagraph graphing and analysis program (v 4.0; Synergy Software, Reading, PA) and verified using SAS (v 9.2; SAS Institute Inc, Cary, NC).

Results
Morphology and morphometry

Low-power magnification images showed that, compared with the airspaces of PBS-treated controls, the airspaces of animals that received PPE were larger (Figure 1). Mice that received 40 U/kg had slightly enlarged airspaces and there was progressively greater airspace enlargement with higher PPE doses.

L_m increased with increasing doses of PPE ($R = 0.98$, Figure 2). L_m of all PPE groups differed significantly ($P < 0.05$) from that of the PBS controls. However, L_m after 120 U/kg was not significantly greater than L_m after 80 U/kg ($P = 0.11$). Similar results were obtained when L_m measurements were compared with those from successively deeper cuts, verifying the uniformity of distribution (data not shown).

D_B decreased with increasing doses of elastase. However, D_B did not differ between the groups receiving 80 and 120 U/kg (Table 1). There was an inverse linear relationship between D_B and L_m measurements ($R = -0.94$, Figure 3A).

In this study, to determine the relation of D_B to L_m independently, we compared the effect of PPE dose on each of the two measures. D_B was linearly related to elastase dose by the following equation: $D_B = 1.68 - 0.00137$ X dose, with a correlation coefficient of −0.895. Despite the sigmoid relationship of L_m to elastase dose, there was still a correlation of 0.978 for a linear relationship, where $L_m = 51.9 + 0.563$ X dose. Solving both equations yields $D_B = 1.81 - 0.0024$ X L_m. This is remarkably close to the experimentally observed equation of the line in Figure 3A: $D_B = 1.83 - 0.0028$ X L_m.

Effect of HNE

D_B and L_m were calculated from images of lungs of mice that were sacrificed 7, 14, and 21 days after instillation of HNE (Figure 3B). An inverse relationship between

Table 1 Effect of porcine pancreatic elastase dose on alveolar fractal box dimension (D_B)

Dose (U/kg)	Mean D_B	SEM	Images (N)	*P (compared to control)
0	1.66	0.0016	293	−
40	1.58	0.0035	299	<0.0001
80	1.47	0.0052	372	<0.0001
120	1.47	0.0055	368	<0.0001

Notes: *$P < 0.0001$ for all comparisons of pairs, except that D_B values after instillation of 80 and 120 U/kg did not differ. $P = 0.75$, $R = -0.996$. The P value of the global test = 0.007 from the fitted analysis of variance model.
Abbreviation: SEM, standard error of the mean.

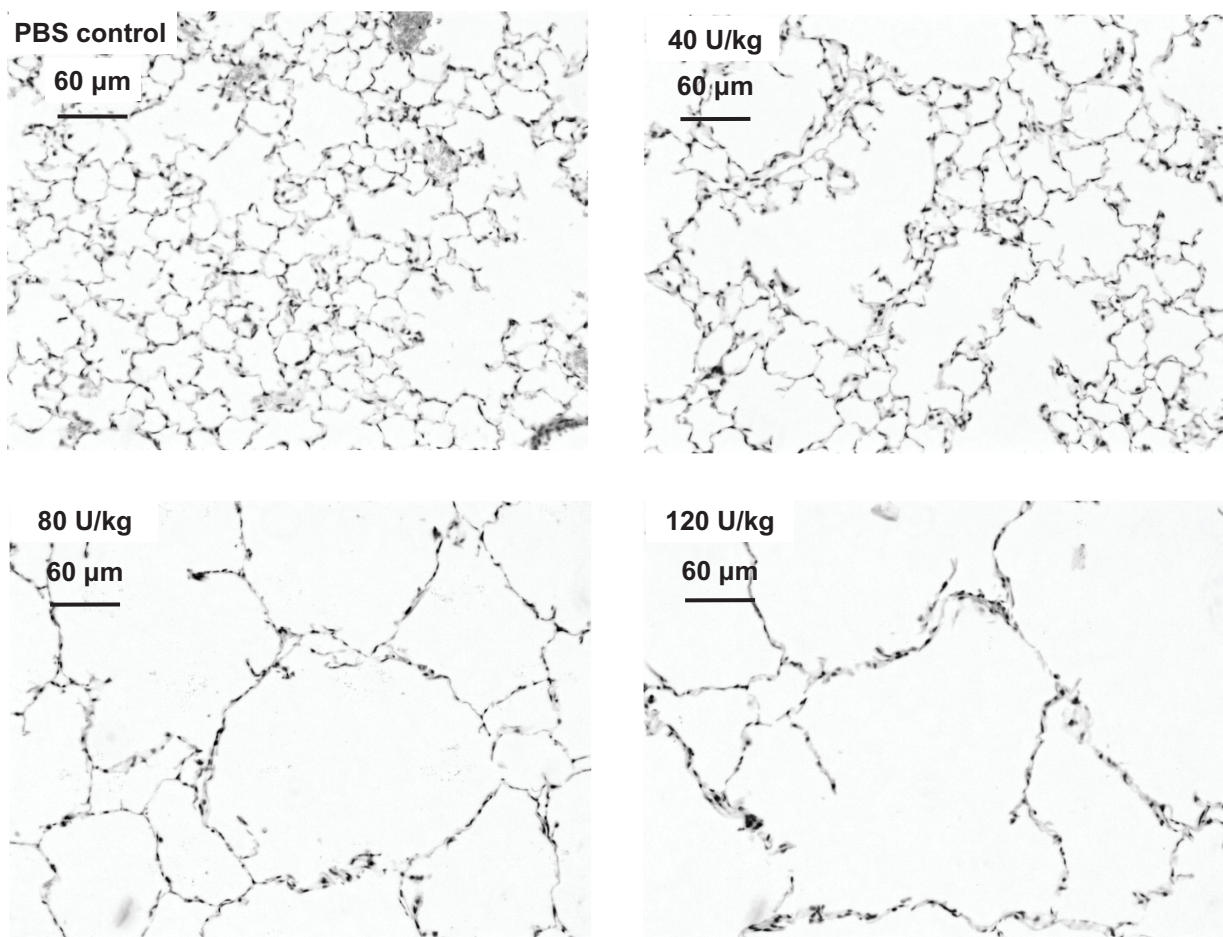**Figure 1** Photomicrographs of lungs 21 days after elastase instillation, stained with hematoxylin and eosin (magnification 200×). Representative sections after instillation of phosphate-buffered saline (PBS) control, 40 U/kg body weight, 80 U/kg, and 120 U/kg are shown.
Note: Scale bars represent 60 μm.

D_B and L_m was seen ($R = -0.83$, $P = 0.01$). The slopes of the linear relationships of D_B and L_m were shown to be different when compared using an F-test (-0.0028 for PPE and -0.0041 for HNE, respectively [$P < 0.0001$]).

While L_m increased over time after HNE was administered, D_B decreased (Table 2). By 21 days after HNE, D_B and L_m differed significantly from those of saline-instilled controls ($P < 0.0001$ for D_B, and $P = 0.05$ for L_m).

Static lung compliance

Figure 4 shows changes in static lung compliance 21 days after instillation of PPE. C_L did not increase after instillation of 40 U/kg ($P = 0.25$). However, after 80 or 120 U/kg, C_L was greater than the control values, and values obtained after 40 U/kg ($P < 0.001$ for each).

Discussion

This study addressed the need for a more standardized and efficient means of comparing results in murine studies of emphysema. It characterized the dose-response to PPE in 8-week-old female C57BL/6J mice, and compared morphometric and functional parameters of measurement of alveolar damage to find the best means of distinguishing the degree of injury. Alveolar fractal box dimension (D_B) was identified as a measure of alveolar lung damage, which is inversely related to L_m. This finding was confirmed in a separate study using HNE.

PPE affected the severity of emphysema as follows: 40 U/kg produced mild emphysema, where C_L was unaffected, but changes in L_m, and D_B were noted. 80 U/kg produced moderate emphysema, with progressively greater changes in L_m and D_B. 120 U/kg produced the most severe degree of emphysema, with marked changes in all the measured parameters, but also resulted in some fatalities immediately after instillation, in less than 20% of the mice, mostly due to acute pulmonary hemorrhage. Together, these findings provide a scale of reference for the comparison of alveolar injury in this model. The relationship between L_m and PPE
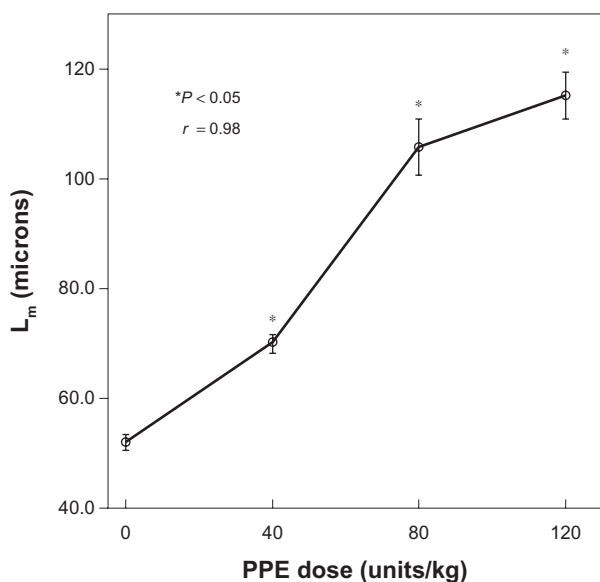
Figure 2 Relationship between the mean linear intercept (L_m) and the dose of porcine pancreatic elastase (PPE). L_m increased when the PPE dose increased.
Notes: Error bars ± standard error of the mean (N = 4 or 5 for each dose). *Significantly different from control by analysis of variance (ANOVA) ($P < 0.05$). All groups were significantly different by ANOVA, except L_m of 80 U/kg was not different from an L_m of 120 U/kg ($P = 0.11$). Pearson's correlation, $R = 0.98$. P value of the global test = 0.001 from the fitted ANOVA model.

(Figure 2A) resembles a typical dose-response curve for a toxin,[26] where the effective concentration required to achieve one half of the maximum effect (EC_{50}) is about 50 U/kg.

D_B

D_B, a measure of the complexity of the alveolar surface, progressively declined with increasing doses of elastase, as airspace size L_m increased. D_B is inversely correlated with L_m regardless of the type of elastase used, but, with HNE, each unit increase of L_m was accompanied by a correspondingly greater decline in D_B than with PPE. This may reflect differences in the action of the two enzymes upon lung structures, as well as differences in susceptibility to naturally occurring inhibitors present in the lung, such as alpha-1-antitrypsin and secretory leukocyte protease inhibitor.[23,27]

Only a few other investigators have attempted to measure lung damage by some type of fractal measure and their findings are not easily compared because each used different parameters to generate a fractal dimension. Among these, Witten et al[28] found that the perimeter length of airspaces of COPD patients had a lower fractal dimension than did those of healthy individuals. Nagao et al[29] reported increased fractal dimension of radioaerosol density probabilities in human emphysema, but these data were criticized for not measuring a fractal characteristic.[30] In contrast, Sato et al[25] examined fractal parameters of alveolar size distribution
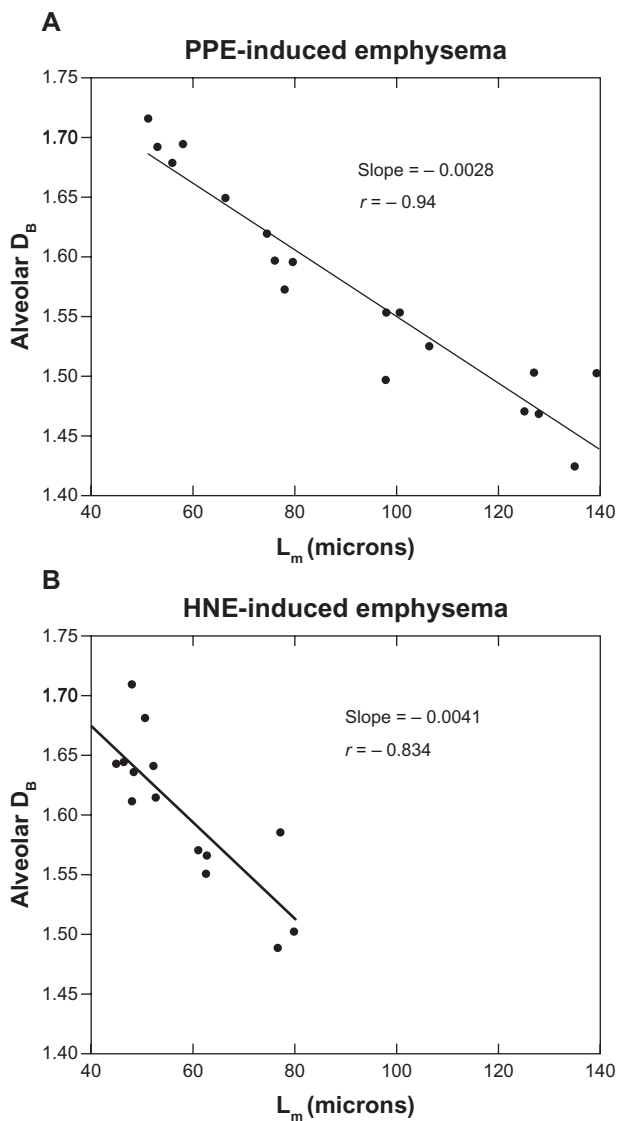

Figure 3 Relationship between alveolar fractal box dimension (D_B) and mean linear intercept (L_m). Each datum point is the mean D_B and L_m of one animal. (**A**) Porcine pancreatic elastase (PPE)-induced emphysema (N = 18). Estimated Spearman's coefficient of correlation $R = -0.94$, $P < 0.0001$. (**B**) human neutrophil elastase (HNE)-induced emphysema (N = 14). Spearman's coefficient, $R = -0.834$. $P = 0.0001$.
Notes: The slopes of the lines, −0.0028 and −0.0041, respectively, are significantly different when compared by an F-test ($P < 0.0001$). The negative correlation coefficients indicate that as airspace size L_m increases there is a corresponding decline in D_B, regardless of the type of elastase used to induce emphysema. The difference between the slopes of the lines indicates that HNE reduces D_B more for each unit of increase in airspace size L_m than does PPE.

and shape distribution in klotho mice, which spontaneously develop emphysema during aging. They found that those parameters are truly fractal and declined with the development of emphysema. Measures of fractal dimension can be applied to computed tomography (CT) scans of the human lung to examine the extent and localization of emphysema. Although such measures have been hampered by variability between scans, Keller et al[31] have recently reported a technique to address this problem. The box counting method we

Table 2 Effect of human neutrophil elastase on alveolar fractal box dimension (D_B) and mean linear intercept (L_m)

Time	Mean D_B	SEM of D_B	L_m	SEM of L_m
Control[a]	1.69	0.03	38.5	2.1
7	1.62	0.020	51.3	15.2
14	1.62	0.029	53.9	4.6
21	1.57*	0.026	60.4*	7.0

Notes: [a]Saline instilled controls were sacrificed 21 days postinstillation; *significantly different than controls.
Abbreviation: SEM, standard error of the mean.

have used on alveolar surfaces is not equivalent to any of the other methods. We have shown that it is truly a fractal measure of alveolar surfaces and that it is inversely related to the L_m in this emphysema model.

When HNE is used to induce emphysema in our model, the more negative slope of the relationship between D_B and L_m, compared to that found with PPE, is due to a greater decline in D_B for each unit of increase in L_m. The airspaces do not increase in size as much as with PPE, because the animals will die of hemorrhage at doses of HNE that increase L_m above about 80 μm. However, at the same time, a change is occurring that affects D_B relatively more than does PPE for the same change in L_m. The cause of death with either enzyme most frequently appears to be pulmonary hemorrhage, suggesting that pulmonary vascular tissues may be more readily damaged by HNE than by PPE and that the integrity of the vascular tissue may be more crucial to survival than airspace size. Again, it is important to stress that these two enzymes have less in common than suggested by calling
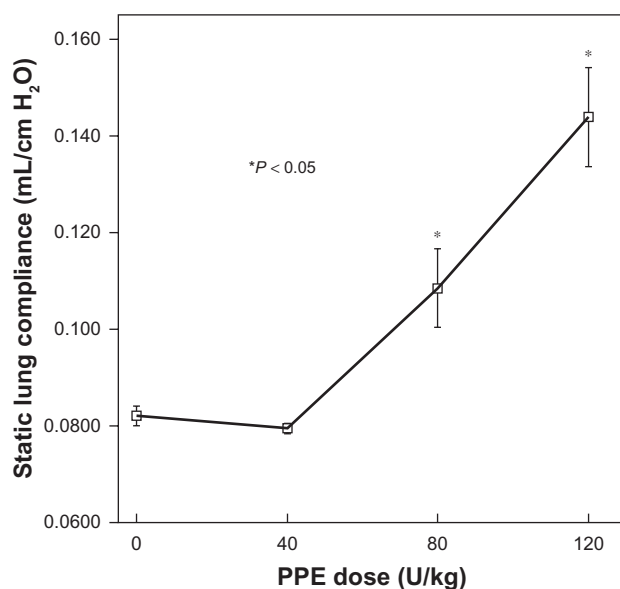
them both elastase; HNE is hydrophobic and more active against vascular tissues, whereas PPE is hydrophilic. Each acts upon other substrates besides elastin and PPE is less susceptible to elastase inhibitors normally found in the lung than is HNE.[23,27]

In our study, the D_B of controls was close to 1.7, a value that is commonly found for diffusion-limited processes, such as the growth of vasculature,[18] whereas D_B in elastase-exposed mice was reduced, regardless of the type of elastase used. The maximum tolerated dose of PPE, 120 U/kg, roughly corresponds to a minimum D_B of about 1.4 to 1.5 in this study. With HNE instillation, the maximal L_m was only about half that obtained with PPE, while D_B declined nearly as much as with PPE, resulting in a significantly steeper negative relationship between D_B and L_m.

Since D_B is measured by digital scanning, it may be calculated rapidly, and is limited primarily by available computer memory. In our study, FracLac scanned each binary image in seconds. The option to automatically save the results permitted the processing of more than 1200 images overnight without supervision. D_B may prove to be a very useful measure for comparative studies of emphysema, especially in mild emphysema where changes in other parameters may be small or difficult to measure.

Static lung compliance

Static lung compliance measurements were relatively insensitive to the dose of PPE (Figure 4), compared with L_m and D_B. C_L increased only with the highest doses of PPE. Our results are similar to those of other investigators. Takubo and colleagues[32] used a smoking model of emphysema to compare changes in the C57BL/6J strain with its pallid derivative. The wild-type strain developed mild emphysema but did not exhibit an increase in C_L, whereas the pallid derivative[5] had significantly more emphysema and an increase in C_L. Similarly, Foronjy and coinvestigators[33] found that transgenic mice that overproduce matrix metalloprotease-1 developed increased L_m, but not increased C_L after 2 months. However, after 1 year, with further increase in L_m, C_L also increased. Thus, a modest increase of airspace size, as measured by L_m, is not necessarily associated with increased C_L in mice and changes in C_L, while functionally important, are not a sensitive measure of elastase-induced lung damage.

Figure 4 Relationship of static lung compliance (C_L), 21 days after porcine pancreatic elastase (PPE) instillation, to PPE dose.
Notes: Error bars ± standard error of the mean (N = 4 or 5 for each dose). *Significant difference from control ($P < 0.05$).

Conclusion

This study characterized a dose-dependent response to PPE injury in the mouse lung. L_m showed a classical,

sigmoid dose-response curve, with an EC_{50} corresponding to approximately 50 U/kg body weight for females of strain C57BL/6J. D_B inversely corresponded to L_m regardless of the type of elastase used, whereas C_L was relatively unaffected by the lowest dose of PPE.

D_B and L_m are both sensitive measures of emphysema development after elastase instillation, able to discriminate between mild and more severe lung damage. D_B provides rapid assessment of the severity of emphysema and may prove to be especially useful in comparing therapies for emphysema.

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