Sequential and Quantitative Analysis of a Murine Model of Elastase-Induced Emphysema

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Emphysema, one of chronic obstructive pulmonary disease (COPD), is characterized as destruction of airway wall and small airway inflammation. To assess the kinetics of disease progression in murine model of elastase-induced emphysema, we used micro-computed tomography (CT) compared with morphological changes. Two week after elastase administration, a significant increase in the volume of low-density areas, recognized as —800—600 Hounsfield units by micro-CT, was observed. Coefficient of correlation between mean linear intercept (Lm) and low-density area examined by CT, was 0.79 (p<0.01). Micro-CT can quantitatively and sequentially detect murine emphysematous changes, offering a practical method to sequentially analyze the therapeutic effects of treatments in a murine model of emphysema.

Key words emphysema; computed tomography; elastase

Chronic obstructive pulmonary disease (COPD) is one of the serious causative diseases in death of aging people.1, 2 Emphysema, one of COPD, is characterized as destruction of airway wall and small airway inflammation.3–5 An imbalance between elastinolytic proteases and their natural inhibitors is thought to play a role in the pathogenesis of emphysema. One animal model of emphysema is made by administrating of elastase into the lungs.2–4 Cigarette smoke-induced emphysema is another common model of emphysema. A study of elastase-deficient mice has revealed that elastase is also critical in smoke-induced emphysema.5, 6 As several months are required to establish cigarette smoke-induced emphysema, the elastase-induced model is more convenient for assessing drug effects.

To quantitatively assess the murine model of emphysema, historical assessment using mean linear intercept (Lm) has been used.6 Because the elastase-induced destruction of airway wall is not homogenous, random several views are taken to estimate the destruction.

The non-homogenous region of the disease is also applicable for human. In human, noninvasive computed tomography (CT) is commonly used for diagnosis and disease staging by assessing areas of low attenuation.1, 7 The primary benefits of CT monitoring are the ability to assess the lungs in their entirety and the ability to follow the same mice over time.

Micro-computed tomography has been developed, resulting in low-noise CT images in a small animal. CT images are used to follow-up tumor growth in the lung.8, 9 High respiration rates of mice cause difficulty for high quality of images of the lung. Although invasive methods using tracheostomy enable high quality images,9, 10 we employed free-breathing method to take the images serially in a deeply-anesthetized condition, in which respiration rates were reduced. The purpose of present study is to clarify whether the kinetics of a murine elastase-induced emphysema model can be assessed using CT and to compare the results with morphological analysis.

MATERIALS AND METHODS

Administration of Elastase C57BL/6N mice were obtained from Charles River Japan (Kanagawa, Japan) and bred in the animal facilities of Musashino University School of Medicine under specific pathogen-free (SPF) conditions. Care and use of animals followed the guidelines of the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research.

Four cycles of sequential CT analysis were performed using 26 mice, six of which were also under histological examination at the end points. For histological analysis only, 12 mice were used for each time points, and in 8 different time points were examined. Another six mice were employed under CT and histological analysis once at the same day.

Mice were anesthetized with ether or intraperitoneal injection of ketamine (90 mg/kg) and xylazine (1 mg/kg) and given intranasal administration of 0.3 or 1.2 units of porcine pancreatic elastase (Sigma-Aldrich, St. Louis, MO, U.S.A.) in 30 μl saline solution. Control mice were intranasally administered saline alone.

CT Assessment After anesthesia was induced with ketamine (90 mg/kg) and xylazine (1 mg/kg), mice were placed in the chamber of the CT scanner for small animal (LaTheta; Aloka, Tokyo, Japan).2, 3 Anesthesia using ketamine and xylazine induces weak and reduced respiration. The calibration of the CT is conducted using the standard phantom followed by manufacturer’s instruction. The X-ray intensity and X-ray attenuation are adjusted each time to the level measured at the factory. The X-ray intensity of air is −1000 Hounsfield units (HU) and water is 0 HU. Both X-ray and detector rotated around the mice. The scanning was employed continuously, X-ray was 50 kV with 1 mA s. Scanning time was 4.5 s for one slice. Slice thickness was 0.25 mm. The size of image matrix was 480×480 and the field of view was 48 mm in resolution of 0.10 mm per pixel. Because previous reports have revealed that area of lower than −620 HU are significantly increased in parallel to the degree of emphysematous

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lungs and set the threshold level as “around −600 HU”,\textsuperscript{10,11} areas between −800 and −600 HU were recognized as areas of low density for lung field analysis. Total volume of low-density areas in the whole lung was calculated by attached software for measuring area of indicated HU in LaThea,\textsuperscript{9} The area of each slice was reconstructed using slice thickness (0.25 mm). Three dimensional analyses were undertaken using software Vgstudio Max (Nihon Visual Science Inc.), using reconstructing images possessing 0.25 mm thickness.

The absorbed radiation dose is 8 mGy per scanning one slice. All mice recovered from anesthesia after several hours. Anesthesia and CT scanning did not influence body weights.

**BALF Cell and Histological Examination** BALF was obtained from select mice by incubating and washing the lung with 1 ml of saline each time. We repeated BAL until the recovered volume was 5 ml. BALF was centrifuged at 1500 rpm for 10 min at 4 °C. Pellets were resuspended in 1 ml PBS and the number of cells was counted. Cytospin specimens were obtained by centrifugation at 640 rpm for 2 min. Cytospin slides were stained with Diff Quik (International Reagents Corporation) and the cell fractions were examined by microscope.

For histological analysis, the lungs were inflated and fixed by intratracheal administration of 10% formalin at a constant pressure of 25 cmH\textsubscript{2}O. The lung was serially divided into transverse section as CT scan and embedded by paraffin. Specimens stained with hematoxylin and eosin (HE) were assessed by analyzing Lm, as determined on random 20 photomicroscopic images per animal.\textsuperscript{6,12} Photomicroscopic images were taken using Digital sight (Nikon, Kanagawa, Japan).

To measure intercept, horizontal and vertical lines were drawn in the images. Intercepts of alveolar walls with these lines were counted and length of mean linear intercept was estimated.

**RNA Extraction and Quantification of mRNAs** Lungs were frozen in liquid nitrogen immediately after isolation and were used for RNA extraction. Lung tissue was homogenized at 4 °C and total RNA was extracted using ISOGEN, which is a modified acid guanidium–phenol–chloroform method (Nippon Gene Co., Ltd., Tokyo, Japan). RNA was treated with 10U DNase (Qiagen, Hiden, Germany) following manufacturer’s instruction. The purity of the RNA was established by spectrophotometer using a DNA purity calculating program (Hitachi Seisakusho, Tokyo, Japan). The absorbance OD260/280 ratio was 1.8. After the amount of total RNA was measured, cDNA synthesis was performed with 0.125 μM oligo-dT (Takara Biochemicals, Tokyo, Japan) as previously described.\textsuperscript{13} The levels of mRNA were examined by real-time PCR using the Light Cycler-Fast Start DNA Master Syber GreenI kit (Roche Diagnostics, Mannheim, Germany). In this system, double-stranded DNA is labelled with Syber Green I and then detected. Quantification was performed on the basis of the standard curve obtained using serial dilution of specific PCR products. To produce specific PCR products, cDNA were amplified by 35 cycles (30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C), using specific primer and taq polymerase. Initial denaturation step was 2 min at 95 °C and final extension period was 5 min at 72 °C. PCR products were then electrophoresed using 2% agarose gel with ethidium bromide, followed by purification using QIAquick Gel Extraction kit (Qiagen).

Results of real time PCR are shown as ratios of the level of mRNAs standardized by the level of β-actin mRNA.

The primers used were as follows; β-actin 5’-CCTGTAT-GGCCCTGTCGTA-3’ 5’-CCATCTCTGCGTCAACTG-3’ 260 bp, TNF-α 5’-CAGACCTCAACTCGATCA-3’ 5’-GTCCCTTGAAGAACCCTGG-3’.\textsuperscript{13,14}

**Statistics** For comparisons of multiple parameters, we used Wilson’s rank-sum tests or Mann–Whitney’s U test. Data are expressed as mean±standard deviation of the mean (S.D.). The coefficient of correlation was evaluated using Spearman’s correlation coefficient by rank. The level of statistical significance was set at $p<0.05$.

**RESULTS**

**Time Course Assessment of Inflammation Induced by Elastase** After elastase administration, significant neutrophil accumulation was observed on day 1 ($p<0.05$) (Fig. 1A). This inflammation was resolved by 1 week. The cytokine mRNA expression was also examined at the same time by real-time PCR.

![Fig. 1. BALF Cell Analysis (A) and TNF-α mRNA Expression (B)](image)

(A) BALF cells were placed on glass slides using Cytospin. Slides were then stained with Diff Quik, and cell differentiation was assessed microscopically. The ordinate exhibited the number of cells in total BALF (5 ml). Each bar indicates means±S.D. of five different mice. (B) TNF-α mRNA expression was determined by real-time PCR. RNA was extracted from whole lung. The ordinate indicated the mRNA expression compared the standardized level of β-actin mRNA. Data are mean±S.D. of four different mice.
time. TNF-α, which is ascribed one of the causative cytokines of emphysema, was significantly increased on day 1 and returned to baseline by 1 week ($p < 0.01$) (Fig. 1B).

**Time Course Assessment of Air Wall Destruction Using CT and Morphometry** Elastase-treated mice (Fig. 2B) exhibited a low-density area compared to controls (Fig. 2A). Next, we sequentially analyzed the same mice once a week after administration of elastase (0.3 units), using CT (Fig. 3). For quantitative assessments, areas from −800 to −600 HU were accumulated and showed as volume of low density lung. Volume of low density area for elastase treated mice was $0.01118 \pm 0.00968$ cm$^3$, significantly greater than that of control mice ($0.00096 \pm 0.00034$ cm$^3$, $n = 11$ each group, $p < 0.01$), but not statistically significant compared with elastase treated mice Day 1. At 2 weeks, a significant increase in total volume of low-density areas was observed compared with control mice and elastase-treated mice Day 1 ($p < 0.01$ and $p < 0.05$, respectively), and a further increase was observed at 3 weeks (Fig. 3).

To obtain whole lung images by CT scanning, 3-dimensional reconstruction was performed using software Vgstudio Max (Fig. 4). This revealed an expanding pattern of lung destruction from 2 to 3 weeks (Fig. 4). This analysis also showed that low density lesions were not homogenous and that differences existed in areas of lung destruction (Fig. 4).

To compare time-course analysis of CT with previous methods, histological examination was performed. For histological time-course analysis, mice had to be sacrificed at each time point ($n = 6$ in each time point). All mice were treated in the same manner as mice in Fig. 2. Histological analysis revealed destruction of the lung from day 1 (Fig. 5). Lung destruction continued to 3 weeks (Fig. 4). $L_m$ was calculated to estimate alveolar wall destruction, as this is the most commonly used method to assess emphysematous lungs. Significant increases in $L_m$ were observed at 1 week.

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**Fig. 2.** CT Images for Saline-Treated Mice (A) and Elastase-Treated Mice (B)
Example of low density area −800 and −600 HU is shown by arrow in (B).

**Fig. 3.** Sequential Quantification by CT
Areas of lung between −800 and −600 HU were recognized as low-density areas. Total volume of low-density areas was calculated. The trachea and artifacts were removed from calculations. Each bar represents mean±S.D. of values obtained from 6—11 mice.

**Fig. 4.** Three-Dimensional Reconstructions of CT
Yellow areas indicate −800 to −600 HU. Although the trachea and bronchi are shown as yellow areas in this figure, the trachea and bronchi were removed from calculations. The density of trachea and bronchi was about −900, which was lower than that of the lung. If these areas were included, low density areas were overestimated. Elastase 1 at 2 and 3 weeks are from the same mice. Elastase 1 and Elastase 2 are for different animals treated with elastase in exactly the same manner. Left panel is shown from the front and right panel is from the back of the same mice.

**Fig. 5.** Histological Analyses by HE Staining
Mice were sacrificed at the day indicated after administration of elastase or saline. Lungs were slowly inflated using 25 cmH$_2$O pressures to avoid further damage to the lungs. Original magnification $\times 200$. 
(p<0.05; Fig. 6), earlier than the CT evaluation. Control mice did not exhibit any changes during experiments.

After 6 months, 3 elastase-treated mice (same mice in Fig. 3) were examined by CT to obtain follow-up images. Mean volume of accumulated low-density area on CT at 6 months was 0.08±0.0127 cm³, significantly greater than that at 3 weeks (p<0.01), suggesting that destruction of alveolar walls had not recovered. Mean Lm for these mice was 52.0±5.2 μm, significantly greater than that of control mice (28.1±0.5 μm).

**Direct Comparison of CT and Morphometry** To clarify correlations between CT and Lm, we analyzed CT results and morphometry using the same mice at the same time. To obtain severe destruction, we used 1.2 units of elastase and shook the mice after elastase administration. This method allowed us to obtain the ratio of destroyed lung to whole lung, reaching 55.6±5.2% at 3 weeks. Both the percentage of the low-density area (A) and the mean CT level (B) significantly correlated with Lm (Fig. 7). Coefficients of correlation were 0.80 and 0.85, respectively (p<0.01 each).

**DISCUSSION**

In the present study, we quantified the kinetics of murine elastase-induced emphysema model by computed tomography, and compared the results with morphological analysis. The primary benefits of CT monitoring are the ability to assess the lungs in their entirety and the ability to follow the same mice over time. Our data demonstrate that CT can detect emphysematous changes in the elastase treated lung, comparable to morphological analysis by measuring Lm. CT images obtained from anesthetized mice could not detect alveolar destruction earlier than morphometric analysis, but had the advantage of allowing the same mouse to be measured sequentially and quantitatively. As intranasal administration of elastase does not damage the lung homogeneously, sequential whole-lung analyses provide more accurate quantification.

Elastase activates protease-activated receptor-2, resulting inflammatory mediator synthesis and neutrophil influx.14—16) The both neutrophil and macrophages are proposed to play a critical role in the pathogenesis of emphysema in human and animal models.1,3,17) Among inflammatory cytokines, TNF-α is known as a most causative one, which is produced by macrophages and epithelial cells.17) In elastase-induced model in our experiments, both influx of neutrophil and up-regulation of TNF-α mRNA were observed and returned to normal level by 1 week. Further destruction of airway wall is partly attributable to mechanical stress.2,11)

In humans, noninvasive CT follow-up is a common procedure to assess emphysema.7,18) In the murine model, quantitative analysis is commonly undertaken by measuring Lm on formalin-fixed specimens. This method of quantifying Lm is authorized,6) but cannot estimate the whole lung. Micro-CT has become available to assess various animal models. Micro-CT has also been applied to assess pulmonary fibrosis and metastatic lung cancer.8,19,20) The high respiration rate of mice forces using fixed lung or tracheostomized and breath fold lung for precise analysis, resulting in visualization of alveoli and estimation pulmonary function even in small animals.10,11,21,22) Recently respiratory-gating methods is developed and provide clearer vision to estimate even airway diameter.23) The micro-CT, which we used in this manuscript,
has been used for quantifying fat volume and bone mineral density. In order to assess mice sequentially, we used deep anesthetized mice, which have been previously reported to deserve for evaluation. Although artifacts due to respiratory movement could not be eliminated in our study and areas of low density may have been slightly underestimated, we found that it is applicable to the sequential estimation of detecting murine emphysematous lung. By using the software contained in CT apparatus, which has been validated for quantifying fat volume and bone mineral density, low density area was calculated and the value exhibited good correlation to the value estimated by Lm. The absorbed radiation dose is 8 mGy per scanning one slice, which is relatively lower compared to other micro-CT and not lethal level.

For human low attenuation area was determined as lower than 900 HU. However, studies reveal lowest value is around 800 HU and never reached 950 HU in murine emphysematous lung. In addition, mean density of murine normal lung is around -500 to -600 HU by ours and Plathow et al., which is quite different from that of human (-700 HU). Therefore, we used the level between -800 and -600 HU for estimating low density area in a murine model.

The estimation of low density area of our study was comparable to previous reports, although CT apparatus and method of anesthesia are different. Froese et al. used knockout mice which develop emphysema spontaneously, resulting that correlation of morphology to CT densities was $r^2$ of 0.53. Pastnov et al. have shown possibility of assessment of elastase-induced emphysema in murine model, but the lung was examined once on day 14. In this report, we showed usefulness of sequential quantitative analysis. We were able to detect sequential increases in tissue destruction in the same area of the lung using CT images. In addition, these data correlated well with morphometric analyses using Lm ($r^2$ of 0.72, Fig. 7B). As alveolar wall destruction was not heterogeneous, evaluation of the whole lung is worthwhile in this emphysema model. Micro-CT is expected to provide a practical method for sequential analysis of therapeutical effects in a murine model of emphysema.

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