Evaluation of cigarette smoke-induced emphysema in mice using quantitative micro-computed tomography

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Submitted 1 December 2014; accepted in final form 25 March 2015

Chronic obstructive pulmonary disease; mouse

THE DEFINING FEATURE OF CHRONIC obstructive pulmonary disease (COPD) is irreversible airflow obstruction caused by elevated resistance in the small airways and increased lung compliance due to emphysema and lung destruction (12). Emphysema is defined pathologically as the permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls, without obvious fibrosis (5). The severity of emphysema is clinically evaluated as low attenuation area (LAA) volume divided by total lung volume (%LAA) on high-resolution computed tomography (CT) (17). It has been reported that high %LAA of lung is related to high mortality, frequent exacerbations, and poor quality of life among COPD patients (11).

Although no current animal model recapitulates all features of human COPD (26), different mouse models of emphysema are available, including intratracheal instillation of elastase, cigarette smoke (CS) exposure, and genetic alteration (24). The elastase-induced pulmonary emphysema model is known to induce severe dose-dependent alveolar destruction with rapid onset after a single intra-tracheal instillation, and it is a preferred model to study airspace enlargement, although its direct clinical relevance as a model for human COPD, which requires decades to develop, remains questionable (27). CS exposure seems to be an appropriate model to study the process of emphysema, but it requires relatively long times to develop. Using a nose-only CS exposure system (22), we have established a mouse model exhibiting an emphysematous phenotype after 4 mo of CS exposure (23). Evaluation of emphysema severity in these models was primarily based on end-stage procedures of histopathology and physiology, precluding dynamic evaluation of disease progression in individual animals. Histological parameters have been established for quantification of emphysematous changes, although the pathophysiological differences among these models have not yet been fully elucidated.

In vivo high-resolution micro-CT allows for longitudinal image-based measurements in animal models of lung diseases, but it remains technically challenging because of respiratory and cardiac movement artifacts (3, 6). Noninvasive, serial imaging of animal models should ideally result in quantitative datasets that allow for longitudinal assessment, comparisons between different groups, including the effect of therapeutic interventions, and detailed topographic information documenting the extent of disease in individual animals. Micro-CT-based protocols for the quantification of a variety of pulmonary emphysema models in mice have been proposed, and agreement with current gold standard evaluation of histopathology has been reported (2, 8, 21). We also have recently reported the use of micro-CT to monitor the progression of emphysematous changes in an exacerbation mouse model of COPD by a single administration of elastase followed by lipopolysaccharide (14). Different parameters are used to quantify the extent of the emphysematous disease, such as the mean CT density, identification of low-density areas (i.e., LAA) using a variety of thresholds, and %LAA. However, no study that evaluated the time course of mouse lung structural changes and defined the...
appropriate parameters following chronic CS exposure using micro-CT has been reported.

In this report, a new set of protocols is presented, and the images and histogram of micro-CT for longitudinal in vivo quantitative assessment of CS-induced pulmonary emphysema in mice are shown. Moreover, our understanding of the distinct pathophysiology of CS-induced emphysema in contrast to elastase-induced emphysema in mice is extended.

MATERIALS AND METHODS

Mice. Female C57BL/6J (9–10 wk old) mice were purchased from Oriental Japan (Tokyo, Japan). The mice were housed in plastic cages under a 12:12-h light-dark cycle, fed standard chow (CE-2; Nihon CLEA, Tokyo, Japan), and given free access to food and water. All experimental protocols and procedures were approved by the Animal Use Committee at Keio University School of Medicine.

CS exposure. Mice were exposed to mainstream CS generated from commercially available filtered cigarettes (Marlboro, 12 mg tar/1.0 mg nicotine) and inhaled CS through their nose as we have previously reported (23). The SIS-CS system (Shibata Scientific Technology, Tokyo, Japan), consisting of both a CS generator (SG-300) and an inhalation chamber, to which 20 body holders were set at a time, was used. Fresh cigarettes purchased within 1 mo before use were used throughout the experiments. The following experimental settings were used to generate CS: stroke volume of 15 ml and 10 puffs/min. The CS was diluted with compressed air, in which the mass concentration of total particulate matter was 1,202 mg/m3. The mice were exposed to CS for 60 min/day, 5 days/wk for up to 4, 12, and 20 wk. Age-matched control mice were exposed to air over the same time period.

Elastase-induced emphysema mouse model. Mice were intratracheally instilled with 1.5 units of porcine pancreatic elastase (Elastin Products, Owensville, MO) dissolved in 40 μl of sterile phosphate-buffered saline via a 22-gauge intravenous catheter, as previously described (28). At 4 wk after the elastase instillation, the mice were scanned by micro-CT and then killed for morphological analysis.

Morphometric assessment. The mice were exsanguinated by severing the abdominal aorta under CO2 narcosis, and the lungs were inflated and fixed by intratracheal instillation of 4% paraformaldehyde at a constant pressure of 25 cmH2O. The lungs were then removed, fixed, and embedded in paraffin, and 3-μm-thick sections of lung were cut (along with lobar bronchi) and stained with hematoxylin and eosin. Alveolar size was evaluated by quantifying the mean linear intercept (Lm), and alveolar damage was evaluated by quantifying the destructive index (DI) in 10 randomly selected fields per lung specimen for each mouse. Lm and DI were manually counted from images taken using the Biozero BZ-8100 (Keyence, Osaka, Japan), as previously reported (19, 28).

Micro-CT imaging. The X-ray micro-CT system (R_mCT2; Rigaku, Tokyo, Japan) was operated with the following parameters: 90 kV, 160 μA, chest CT; respiratory and cardiac reconstruction mode, 24 × 24 mm field of view (50 × 50 μm pixel size). The scan time for 4.5 min yielded an average whole body exposure of 1,653 mGy/scan. Mice were scanned in the prone position with inhalation anesthesia of mixed isoflurane (Pfizer Japan, Tokyo, Japan) and oxygen through a nose cone. Respiratory and cardiac reconstruction mode captures the X-ray view to reconstruct lung images only at the diastolic phase of the heart during the end-expiratory period by simultaneously monitoring the movement of both breathing and the heart under radiographic guidance (Fig. 1). For Hounsfield unit (HU)
calibration, a water phantom (15-ml tube filled with water: 0 HU, air: −1,000 HU) was scanned.

Image analysis. Micro-CT images were converted into DICOM data format. End-expiratory lung volumes and average lung CT values were calculated using a Lexus 64 workstation (AZE, Tokyo, Japan). By visual examination of the images on micro-CT, the individual animals with atelectasis were excluded from the analysis. Lung parenchyma was arbitrarily defined as a region with X-ray attenuation values between −1,200 and −300 HU, and intrapulmonary and surrounding extrapulmonary tissues (e.g., airways, large pulmonary vessels, heart, mediastinal structures, and diaphragm) were excluded. LAA thresholds were arbitrarily established to less than −700 HU because %LAA of a normal mouse was <5% referring to a previous publication (2), and it was also set high to make the difference between the groups clearer. The %LAA was calculated using the ratio of the total LAA volume to the end-expiratory lung volume. End-expiratory lung volume, corresponding to functional residual volume, was defined as the calculated total lung volume at the phase of end expiration, and it evaluated air trapping (7).

Statistical analysis. Data are expressed as means ± SE and were analyzed by Student’s t-test and paired t-test. Linear regression was used for comparing two variable sets. P values <0.05 were considered significant. All data were analyzed using the JMP version 11.0.0 software for Windows (SAS Institute, Cary, NC).

RESULTS

Histological assessment of emphysema in the lungs. Mice were repetitively exposed to CS, whereupon the lungs of the mice were excised at 4, 12, and 20 wk and subjected to histological examination. As shown in Fig. 2A, alveolar enlargement was observed as early as 4 wk following CS exposure. The $L_m$ and DI, which are indicative of emphysematous changes, were quantified on histological sections. The $L_m$ value was significantly increased in CS-exposed mice compared with control mice at 4, 12, and 20 wk (Fig. 2B). DI was significantly increased in CS-exposed mice compared with controls at 12 and 20 wk, but not at 4 wk (Fig. 2C). Elastase-treated lungs showed significantly higher $L_m$ and DI values than CS-exposed mice for 20 wk.

Micro-CT images and histogram of lungs following CS exposure. Figure 3A presents representative color-coded images of the lungs. The early to late response (4–20 wk) of the lungs to CS exposure was detectable as HU alterations on micro-CT images. LAAs (less than −700 HU) are shown in blue in coronal slices and in three-dimensional images. The increase in LAA was not obvious in the CS-exposed lungs on micro-CT images, although it was visible in the lungs with elastase treatment. The histogram curves of the lungs are shown in Fig. 3B. The histogram robustly shifted toward the left in the elastase-treated lungs compared with control lungs, which implied an increased lung volume with low density. In the mice with CS exposure, on the other hand, the curve shifted slightly toward the right at 4 wk, and subsequently the histogram curve showed elevation of peak CT volume at 12 and 20 wk, which was a distinctly different pattern from elastase-treated mice.

Quantitative assessment of emphysema following CS exposure on micro-CT. The end-expiratory lung volume of CS-exposed mice was significantly larger than that of air controls at 12 and 20 wk (Fig. 4A). The lungs of elastase-treated mice had further larger end-expiratory lung volumes compared with the mice exposed to CS for 20 wk. In contrast to elastase-treated mice showing a decreased average lung CT value, no decrease was observed in CS-exposed mice at any time point. CS-exposed mice had rather higher average lung CT values at 4 and 12 wk (Fig. 4B). Although %LAA was significantly elevated in elastase-treated lungs compared with that of air controls at 20 wk (43.18 ± 3.42 vs. 2.97 ± 0.34%), there was no increase of %LAA in CS-exposed mice at any time point (Fig. 3), and no CS-exposed mice showed %LAA >5% even at 20 wk.
Correlations between end-expiratory lung volumes on micro-CT and Lm or DI on histological sections. The end-expiratory lung volumes on micro-CT were significantly correlated with Lm ($R^2 = 0.7466$, $P < 0.0001$) and DI ($R^2 = 0.8074$, $P < 0.0001$), implying that end-expiratory lung volume on micro-CT might be comparable to microscopic detection of emphysema on histology (Fig. 5).

Longitudinal evaluation of disease progression in individual mice on micro-CT. Serial quantitative measurements on micro-CT were performed in five CS-exposed mice and six controls. End-expiratory lung volume was significantly increased at 4, 12, and 20 wk compared with that at 0 wk only in CS-exposed mice but not in air controls (Fig. 6A). The average lung CT value was significantly higher at 4 and 12 wk compared with that at 0 wk only in CS-exposed mice (Fig. 6B). The average lung CT value was not changed over time in air controls.

DISCUSSION

In this study, the structural changes of the lungs following CS exposure were evaluated in mice using micro-CT. Mice with chronic CS exposure displayed dynamic time-dependent changes on micro-CT. At 4 wk, the average lung CT value was temporarily increased, along with hyperinflation (air trapping), which was subsequently sustained without increases of areas of low density for up to 20 wk. This implies that the parameter of end-expiratory lung volume on micro-CT might be comparable to microscopic detection of emphysema on histology. A reason why the increase in end-expiratory lung volume on micro-CT...
preceded the significant increase of DI in CS-exposed mice might be that the increase of end-expiratory lung volume on micro-CT reflects the structural narrowing and/or functional resistance of small airways that could be overlooked by artificial inflation of lungs with fixative for evaluation techniques used in histopathology. Elastase-treated mice also displayed a high expiratory total lung volume, as was previously reported (7, 14), but it is due to the increase of low-density volume, which is in sharp contrast to the CS exposure model.

Traditionally, histological analysis has been the gold standard to quantitate emphysematous changes (25). CS-induced emphysema is considered to be “mild” compared with elastase-induced emphysema according to the histological quantification, and $L_m$ and DI cannot distinguish the intrinsic differences between the two models. However, three-dimensional micro-CT images can determine the density distribution and the volume of whole lung parenchyma and reveal some phenotypic differences in the pathophysiology of emphysema between the

![Fig. 5](image-url)

Fig. 5. Correlations between end-expiratory lung volume on micro-CT and histological measurement. Correlation with $L_m$ (A) and correlation with DI (B) from 7 controls ($\triangle$), 5 CS-exposed mice (●), and 4 elastase-treated mice (x). $L_m$ and DI are correlated with the end-expiratory lung volume ($R^2 = 0.7466$ 0.8074, respectively).

![Fig. 6](image-url)

Fig. 6. Serial micro-CT evaluation of individual mice over time. CT data of all time points were available for 6 air control mice and 5 CS-exposed mice, which served for longitudinal analysis. Time-dependent changes of end-expiratory lung volume (A) and average lung CT value (B) of air control mice (left) and CS-exposed mice (right). Paired t-tests were performed between each time point indicated. ‡$P < 0.05$ compared with each time point.
two manipulations. The temporal increase of the high-density area at 4 wk (Fig. 3B) and the average lung CT value (Fig. 4B) might mirror the CS-induced lung inflammation, as reported elsewhere (8).

In vivo pulmonary imaging in small animals is technically challenging due to inevitable artifacts caused by respiratory and heart motion. The image quality has been significantly improved by respiratory gating, either via a prospective or retrospective approach. Prospective respiratory gating implies triggering of the micro-CT apparatus to acquire images, at set phases in the respiratory cycle, resulting in images acquired in identical phases of the respiratory cycle, with minimal movement artifacts. This can be achieved through intubation and connection to a mechanical ventilator (2, 4). This approach has limitations because it requires specific technical skills. Moreover, anesthesia and paralysis alter respiratory mechanics, and repetitive intubation carries significant risks in mice (3). Retrospective respiratory gating, in contrast, involves random image acquisition throughout the respiratory cycle, with post-acquisition sorting of the images into different groups, corresponding to a single respiratory phase. The respiratory cycle is reconstructed based on intrinsic or extrinsic gating techniques (7, 13). The degree of movement artifacts is largely reduced, albeit to a lesser extent compared with the prospective approach (7). In the present study, the novelty of the micro-CT method was that not only respiratory, but also cardiac, gating was performed without any external devices, such as an electrocardiogram, by which micro-CT images were automatically reconstructed by setting the region of interest on the diaphragm simultaneously monitoring the movements of both breathing and the heart (Fig. 1). This regulated image acquisition system enables minimization of the radiation dosage and time and thus results in high-quality images that can be processed for further quantitative analysis (6). In emphysematous lung diseases, enlargement of lung volumes is a hallmark feature. Quantification of the aerated lung volume thus indirectly serves as an indicator of the extent of air trapping when it is acquired during the expiratory phase. The challenge lies in correct segmentation of the air-filled lung spaces from intrapulmonary and surrounding extrapulmonary tissues. The quality of the correct lung parenchymal segmentation depends on the image quality, reflected by the image resolution.

It is well known that CS exposure provokes variable changes in the lungs, and the susceptibility for development of smoke-induced emphysema varies among mouse strains. In this study, only C57Bl/6J female mice were used for CS exposure because it has been reported that this strain shows moderate development of emphysema compared with other strains (10), and female mice developed emphysema earlier than male mice (15). A protocol of 60-min 5% CS exposure per day, in which the mass concentration of total particulate matter was tolerably high, was chosen based on our previous report (23). It remains to be examined in future investigations whether CS exposure provokes different reactions in the other strains, in males, or in response to a lower dose of CS exposure. It should also be mentioned that only a subset of animals was subjected to the longitudinal measurement because the number of animals for long-term smoking experiments was limited. Even so, the ability to monitor the time-dependent changes in individual living animals is the biggest advantage of using micro-CT.

In humans, multiple studies have shown substantial progress in using CT to quantify emphysema defined as the percentage of low-attenuation areas less than or equal to −950 HU on inspiratory CT (1, 16, 18). Recently, however, it was reported that air trapping, defined as the percentage of low-attenuation areas less than or equal to −856 HU on expiratory CT, showed a stronger relationship with severity of airflow limitation on spirometry in cigarette smokers with and without COPD (20). Although human COPD development is clinically and pathologically complicated, this CS exposure mouse model was found to radiologically mimic human cases in the early stage (20).

In conclusion, this is the first study characterizing the CS-induced emphysema model over time in mice, showing increased end-expiratory lung volume without increased low-density volume. The longitudinal analysis with micro-CT image acquisition and quantitative analysis protocol highlighted the potential of this technique to detect treatment and prevention effects on the CS-induced emphysema model in mice.

ACKNOWLEDGMENTS

We thank Takafumi Koike (Rigaku) and Tsuyoshi Sakamoto (AZE) for technical advice and support with micro-CT and image analysis; Naomi Ishikawa, Miyuki Yamamoto, and Shizuko Kagawa for exposing the mice to smoke and technical assistance; and the Collaborative Research Resources, Keio University School of Medicine, for technical support and reagents.

GRANTS

This work was supported by the Japan Society for the Promotion of Science Grant-in-Aid for Young Scientists [050454125 (M. Sasaki) and 24790820 (S. Chubachi)] and Grant-in-Aid for Scientific Research B [23390218 (T. Betsuyaku)].

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS


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