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

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Running head: Cigarette smoke-induced emphysema on micro CT

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ABSTRACT

Chronic cigarette smoke (CS) exposure provokes variable changes in the lungs, and emphysema is an important feature of chronic obstructive pulmonary disease. The usefulness of micro computed tomography (CT) to assess emphysema in different mouse models has been investigated, but few studies evaluated the dynamic structural changes in a CS-induced emphysema mouse model. A novel micro CT technique with respiratory and cardiac gating has resulted in high-quality images that enable processing for further quantitative and qualitative analyses.

Adult female C57BL/6J mice were repeatedly exposed to mainstream CS, and micro CT scans were performed at 0, 4, 12, and 20 weeks. Emphysema was also histologically quantified at each time point. Air-exposed mice and mice treated with intratracheal elastase served as controls and comparisons, respectively.

End-expiratory lung volume, corresponding to functional residual volume, was defined as the calculated volume at the phase of end-expiration, and it evaluated air trapping. The end-expiratory lung volumes of CS-exposed mice were significantly larger than those of air controls at 12 and 20 weeks, which was in line with alveolar enlargement and destruction by histological quantification. However, CS exposure neither increased low attenuation volume nor decreased the average lung CT value at any time point, unlike the elastase-instilled emphysema model. CS-exposed mice had rather higher average lung CT values at 4 and 12 weeks.

This is the first study characterizing a CS-induced emphysema model on micro CT over time in mice. Moreover, these findings extend our understanding of the distinct pathophysiology of CS-induced emphysema in mice.
INTRODUCTION:

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 intratracheal 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 months 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 due to respiratory and cardiac movement artifacts (3,6). Non-invasive, 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 weeks 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.

Cigarette smoke (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 Ltd., 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 month 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 ± 196 mg/m³. The mice were exposed to CS for 60 min/day, 5 days/week for up to 4, 12, and 20 weeks. 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 weeks after the
elastase instillation, the mice were scanned by micro CT and then euthanized 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 x 24 mm field of view (FOV) (50 x 50 µm pixel size). The scan time for 4.5
min yielded an average whole body exposure of 1,653 mGy per scan. Mice were
scanned in the prone position with inhalation anesthesia of mixed isoflurane (Pfizer
Japan Inc, 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
(Figure 1). For Hounsfield unit (HU) calibration, a water phantom (15 mL tube filled
with water: 0 HU, air: -1000 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 Ltd. 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 -1200 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 less than 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 ± standard error (SE) and were analyzed by Student’s
$t$-test and paired $t$-test. Linear regression was used for comparing two variable sets. P
values less than 0.05 were considered significant. All data were analyzed using the JMP version 11.0.0 software for Windows (SAS Institute Inc., 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 weeks and subjected to histological examination. As shown in Figure 2a, alveolar enlargement was observed as early as 4 weeks following CS exposure. The Lm and DI, which are indicative of emphysematous changes, were quantified on histological sections. The Lm value was significantly increased in CS-exposed mice compared with control mice at 4, 12, and 20 weeks (Figure 2b). DI was significantly increased in CS-exposed mice compared to controls at 12 and 20 weeks, but not at 4 weeks (Figure 2c). Elastase-treated lungs showed significantly higher Lm and DI values than CS-exposed mice for 20 weeks.

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 weeks) of the lungs to CS exposure was detectable as HU alterations on micro CT images. LAA (< -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 Figure 3b. The histogram robustly shifted toward the left in the elastase-treated lungs compared to 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 towards the right at 4 weeks, and subsequently
the histogram curve showed elevation of peak CT volume at 12 and 20 weeks, 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 weeks (Figure 4a). The lungs of elastase-treated mice had
further larger end-expiratory lung volumes compared to the mice exposed to CS for 20
weeks. 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 weeks (Figure 4b). Although LAA%
was significantly elevated in elastase-treated lungs compared to that of air controls at 20
weeks (43.18% ± 3.42% vs. 2.97% ± 0.34%), there was no increase of LAA% in
CS-exposed mice at any time point (Figure 3), and no CS-exposed mice showed LAA%
more than 5% even at 20 weeks.

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
(R² = 0.7466, p < 0.0001) and DI (R² = 0.8074, p < 0.0001), implying that
end-expiratory lung volume on micro CT might be comparable to microscopic detection
of emphysema on histology (Figure 5).
Longitudinal evaluation of disease progression in individual mice on micro CT

Serial quantitative measurements on micro CT were performed in 5 CS-exposed mice and 6 controls. End-expiratory lung volume was significantly increased at 4, 12, and 20 weeks compared to that at 0 weeks, respectively, only in CS-exposed mice, but not in air controls (Figure 6a). The average lung CT value was significantly higher at 4 and 12 weeks compared to that at 0 weeks, respectively, only in CS-exposed mice (Figure 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 weeks, 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 weeks. 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 to elastase-induced emphysema according to the histological quantification, and Lm 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 two manipulations. The temporal increase of the high-density area at 4 weeks (Figure 3b) and the average lung CT value (Figure 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 to 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 (ROI) on the diaphragm simultaneously monitoring the movements of both breathing and the heart (Figure 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 were 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 ≤ –950 HU on inspiratory CT (1,16,18). Recently, however, it was reported that air trapping, defined as the percentage of low-attenuation areas ≤ –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.

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Author contributions
M.S.: conception and design, collection and/or assembly of data, data analysis and interpretation; S.C.: conception and design, collection and/or assembly of data, data analysis and interpretation, financial support, manuscript writing; N.K., M.S., N.M. and S.T.: collection and/or assembly of data, data analysis and interpretation; T.B.: conception and design, data analysis and interpretation, financial support, administrative support, manuscript writing, final approval of manuscript.
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Figure legends

Figure 1. Respiratory and cardiac reconstruction system by monitoring movement of both the diaphragm and the heart

Radiographic images of a mouse at maximal inspiratory (a) and end-expiratory (b) phases. The region of interest (ROI) is set at the area including the heart and diaphragm. (c) A representative image showing change of brightness in ROI with time by breathing and cardiac pulsation (I: inspiratory phase, E: expiratory phase, EE: end-expiratory phase, S: systolic phase, D: diastolic phase)

Figure 2. Histological assessment of emphysema in the lungs

(a) Representative lung sections stained with hematoxylin and eosin at 4, 12, and 20 weeks of CS exposure and their air controls are shown. The elastase-treated lung is shown on the right. The scale bar of 100 μm is shown in the top left corner in each panel. (b) The mean linear intercept (Lm) values as a standard parameter of alveolar size are shown. (c) The destructive index (DI) values representing alveolar destruction are shown. Histological examination was performed for 5 mice per group. The group of elastase-treated mice (gray bars) consisted of 4 mice. The data are shown as means ± SE. Statistical analysis was performed with Student’s t-test. *P<0.05; **P<0.001 between air controls (white bars) and CS-exposed mice (black bars) at each time point. †P<0.001 as compared with CS-exposed mice for 20 weeks.

Figure 3. Micro CT images and histogram of the lungs

The panels in (a) show representative micro CT images selected from the lung fields of mice. Mouse lungs were scanned using micro CT at 0, 4, 12, and 20 weeks following
CS exposure. CT-images of the lungs at 4 weeks after the elastase treatment are shown on the right side. The top images are the lung cross-sections, and the bottom images are the three-dimensional lungs by integrating CT images, in which the LAA (below -700 HU) is colored in blue, and the whole lung field is shown as a transparent shape. A green scale bar of 10 mm is shown on the right side in each panel. (b) The CT histogram curve of the lung parenchyma is drawn based on the average CT density value of mice in each group. The volume of each HU is shown on the y-axis. Blue line: air-exposed mice (n=10 at 4 weeks, n=9 at 12 weeks, n=7 at 20 weeks), red line: CS-exposed mice (n=9 at 4 weeks, n=7 at 12 weeks, n=5 at 20 weeks), purple line: elastase-treated mice (n=4)

Figure 4. Quantitative assessment of emphysema on micro CT

End-expiratory lung volume (a) and average lung CT value (b) on micro CT at 0, 4, 12, and 20 weeks of CS exposure (black bars) and those of the air control group (white bars) are shown. The elastase-treated mice (gray bars) are shown on the right. The data are shown as means ± SE. Statistical analysis was performed with Student’s t-test. *P<0.05 between air controls and CS-exposed mice at each time point. †P<0.001 as compared with CS-exposed mice for 20 weeks.

Figure 5. Correlations between end-expiratory lung volume on micro CT and histological measurement

(a) Correlation with Lm and (b) correlation with DI from 7 controls (white triangles), 5 CS-exposed mice (black circles), and 4 elastase-treated mice (x marks). Lm and DI are correlated with the end-expiratory lung volume (R^2 = 0.7466 and R^2 = 0.8074,
respectively).

Figure 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 (a) end-expiratory lung volume and (b) average lung CT value of air control mice on the left side and CS-exposed mice on the right side. Paired t-tests were performed between each time point indicated. *P<0.05 as compared with each time point.
Figure 1

(a) Maximal Inspiratory Level
(b) End-Expiratory Phase

Region of Interest (ROI)

Image Capture Points

Maximal Inspiratory Level

Image Brightness of ROI

Time

1 sec
Figure 2

(a) Images showing the effects of Elastase and Air on tissue from different time points: 4 weeks, 12 weeks, 20 weeks, and Elastase treatment. The images compare the effects on tissue from Air and CS conditions.

(b) Bar graph showing the Mean Linear Intercept (μm) for different time points: 4 weeks, 12 weeks, 20 weeks, and Elastase treatment. The graph indicates statistically significant differences marked with asterisks (*).

(c) Bar graph showing the Destructive Index (DI) for different time points: 4 weeks, 12 weeks, 20 weeks, and Elastase treatment. The graph indicates statistically significant differences marked with asterisks (*).
Figure 3

(a) 0 weeks 4 weeks 12 weeks 20 weeks Elastase

(b) 4 weeks 12 weeks 20 weeks

Volume (mm$^3$)
Figure 4

(a) End-expiratory lung volume (mm$^3$)

(b) Average CT value (HU)
Figure 5

(a) Graph showing the relationship between end-expiratory lung volume (mm$^3$) and Lm (μm).

(b) Graph showing the relationship between DI and Lm (μm).
Figure 6

(a) End-expiratory lung volume (mm$^3$) over time (0 weeks, 4 weeks, 12 weeks, 20 weeks).

(b) Average lung CT value (HU) over time (0 weeks, 4 weeks, 12 weeks, 20 weeks).