A Experimental Study of Airway Changes on Micro-CT in a Mouse Asthma Model: Comparison With Histopathological Findings

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Purpose

Bronchial asthma: chronic inflammatory disorder of the airways

- Persistent airway hyperresponsiveness
- Intermittent reversible airway obstruction caused by hypersensitivity to various stimulants
- Airflow obstruction responses to a bronchodilator.

1. Pathology of the asthma

- Structural changes in the airways include bronchial mucosal damage, increased mucus secretion, submucosal hypertrophy, eosinophilic inflammation
- Occasionally, bronchial wall thickening caused by lymphocytes or plasma cell infiltration

2. Animal for study: mouse

- The most common species used to study physiological mechanisms and cellular components causing airway inflammation in allergic asthmatic patients.
- Pulmonary function test: necessary to diagnose and evaluate treatment responses. But, this test is difficult to perform, and its accuracy is not accepted
- For these reasons, radiological studies are performed

3. Radiological studies for asthma

- MDCT: airway remodeling, air trapping, and centrilobular nodules suggestive of small airway disease
- Lung perfusion and ventilation
- Micro-computed tomography (micro-CT)

4. Micro-CT

- Recently, many imaging techniques have been developed to examine small specimens
- Previous animal studies, conventional histological analysis: serial cutting of multiple thin slices from a tissue specimen is slow and expensive and, once sliced, the intact volume is lost, and subsequent examinations are difficult or impossible. Moreover, it does not provide 3D views of the lung.
- In contrast, micro-CT: non-invasive, allows for the generation of high spatial resolution images, provides 3D views of the lung.
- Evaluate quantitative changes in the lung as a variable; the cost, time, and number of animals are reduced.
In a recent study, micro-CT has been shown to be an innovative tool for assessing lung cancer, emphysema, and pulmonary fibrosis in a mouse model; however, studies concerning airway measurement using micro-CT in a mouse allergy model have not yet been reported.

This study was attempted to evaluate airway changes in ovalbumin-induced asthma model mice and controls using postmortem micro-CT images and pathological findings and to examine between the 2 variables.
Materials and Methods

1. Creation of a mouse asthma model

- Six-week-old male BALB/c mice were purchased from Charles River Technology Inc., Seoul, Korea.
- OVA (100 µg; Sigma, St. Louis, MO, USA), 0.2 g of emulsifying aluminium hydroxide, and 2 mL of distilled phosphate buffered saline (D-PBS) were mixed in a total volume of 200 µL, and then the mixture was injected intraperitoneally into each mouse on days 0 and 14. On days 21-23, 75 µg of OVA mixed with 1 mL of D-PBS in a total volume of 50 µL was instilled intranasally (experimental group, n=6).
- As controls, D-PBS was injected intraperitoneally on days 0 and 14, and then intranasally instilled on days 21-23 (control group, n=6). During intranasal instillation, mice were administered intraperitoneally with a mixture containing 5 mL of ketamin, 4 mL Rumpun, and 4 mL of D-PBS in a total volume of 50 µL to suppress hyperactive sneezing and reactions.
- On day 24, both the experimental and control groups inhaled methacholine (A2251 acetyl-methylcholine chloride 98%; Sigma) for 3 minutes to stimulate the airway. The flow chart of this experiment is shown in Fig. 1.

2. Micro-CT imaging acquisition

- Microfocus tube (focal spot size, 5 µm; energy range, 20-100 keV), micro-CT unit using a rotation acquisition protocol (Skyscan 1076; skyscan, Aartselaar, Belgium)
- CT was performed using the following parameters: pixel size, 35 µm; source voltage, 40 kVp, and source current, 240 µA. The X-ray detector comprised a 12-bit watercooled charge-coupled device high-resolution (4,000×2,300-pixel) camera and an X-ray scintillator.
- Metacholine inhalation on day 24, and micro-CT was conducted on day 25. Since the mouse’s diaphragm moved during respiration, anesthetics (mixture containing 5 mL of Ketamin, 1 mL of Rumpun, and 4 mL of D-PBS) were injected intraperitoneally (lethal dose, 250 µL) to eliminate motion artifacts. After respiratory arrest, 300 µL of lidocaine HCl (20 mg/mL; Huons, Seoul, Korea) was injected.
- Then, after 10 minutes of cardiac arrest, each mouse was placed in the supine position, and CT was performed.
- Micro-CT images were acquired in increments of 0.5° between projections for a total rotation angle of 360° without cardiac or respiratory synchronization.
The exposure time for each view was 0.316 seconds, and a 0.5-mm aluminum energy filter was used. Voxel (volume pixel) size was $35 \times 35 \times 35 \mu m$. The imaging acquisition time of each mouse was 30 seconds.

### 3. Image processing and analysis

- About 700 BMP (Bit MaP) modality images were obtained when the whole lung was scanned. To save scanning time and image numbers, however, scanning was performed exclusively below the main bronchus level for airway measurements, and we acquired 300 BMP images.
- Acquired BMP images were converted to the Digital Imaging and Communications in Medicine (DICOM) format to yield 3D reconstruction images, and the Lucion program was used.
- In order to measure the bronchial lumen area on Micro-CT images, we chose the most circular-shaped main bronchus lumen area after the third bronchial branch level. Three main bronchial lumen areas on both sides of each mouse were measured just at the distal main bronchus of the fourth to sixth distal branch level; thus, a total of 72 bronchial lumen areas.
- A Lucion’s smart pen (semi-automated) and a curve pen (manual) were used to measure bronchial lumen area.
- Bronchial wall thickness was obtained by measuring a diameter which was perpendicular to the longitudinal axis of the main bronchus on curved multiplanar reformation (MPR) images. Four sections (2 levels on either side) of the bronchus after the third bronchial branch from each mouse were measured, thus a total of 48 bronchial walls were evaluated.
- Measurements were made by a single radiologist with 12 years of radiological experience.

### 4. Histopathologic analysis

- After micro-CT scanning, lung tissue was immediately extracted, and 1 mL of D-PBS was injected into the bronchus to prevent air space collapse. The extracted lung tissue was immersed in 4% paraformaldehyde solution for fixation at 4°C for 1 day, and was subsequently embedded in paraffin.
- Paraffin-embedded lung tissue was sectioned at a thickness of 4 µm, and was then stained with hematoxylin and eosin (H&E).
- Periodic acid-Schiff (PAS) & smooth muscle actin (SMA) staining: To confirm mucus-producing goblet cells and hypertrophied submucosal smooth muscles in the lung tissue which were detected on H&E-stained slides.
- Histologic slides were obtained from the lesions that were matched with CT images, to know section levels.
• Bronchial wall thickness was determined-2 each, left and right-using an optical microscope at a magnification power of 100; thus a total of 48 sections.
• On pathology slides, the airway wall thickness was measured by calculating the distance from the inner side of the epithelial cell layer to the most outer side of the smooth muscle layer with the arrowhead within the microscope.

5. Statistic analysis

• Paired t-test: to compare the bronchial lumen diameter and bronchial wall thickness the experimental and control groups (P value of <0.001, considered significant)
• Pearson correlation coefficients: concordance between micro-CT and pathological results (P value of <0.05, considered significant)
• All statistical analyses were conducted using the Stata software (ver. 9.0; Stata; College Station, TX, USA).
Fig. 1: Time table of this study. Day 0 and 14: intraperitoneal injection of ovalbumin-aluminium hydroxide into BALB/c mice and distilled phosphate-buffered saline injection into controls. Day 21, 22, and 23: airway stimulation by intranasally instilled OVA. Day 24: measuring bronchial wall thickness after methacholine-challenged bronchial irritation. Day 25: performed micro-CT and tissue extraction.

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Results

Bronchial lumen area on micro-CT images

- Experimental group: ranged from 0.10 to 0.37 mm² (mean area, 0.196 ± 0.072 mm²)
- Control group: ranged from 0.07 to 0.54 mm² (mean area, 0.243±0.116 mm²)

Difference statistically significant (P=0.0077) (Table 1) (Figs. 2, 3A and B).

Bronchial wall thickness on micro-CT images

- Experimental group: ranged from 0.10 to 0.14 mm (mean thickness, 0.119±0.01 mm)
- Control group: ranged from 0.09 to 0.13 mm (mean thickness, 0.108±0.013 mm)

Difference was statistically significant (P=0.0005) (Table 2) (Figs. 4, 5).

Bronchial wall thickness, as measured in pathological specimens

- Experimental group: ranged from 0.05 to 0.08 mm (mean thickness, 0.066±0.011 mm)
- Control group: ranged from 0.03 to 0.055 mm (mean thickness, 0.041±0.009 mm)

Difference was statistically significant (P<0.0001) (Table 3) (Figs. 6, 7A and B).

Histopathological examination

- Epithelial layer was thicker in the experimental group than in the control group.
- Hyperplasia of mucus-containing goblet cells and submucosal smooth muscles was observed (Fig. 8A and B).

There was a significant correlation between the bronchial thickness measured by micro-CT and pathologic examination in the experimental group (Pearson correlation; r=0.712,
P=0.0001) (Fig. 9), while there was not in the control group (Pearson correlation; r=0.46, P=0.022) (Fig. 10).
Fig. 2: Bronchial lumen area as measured on micro-computed tomography images in the experimental and control groups. Vertical axis scale: mm². P<0.01.

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**Fig. 3:** Axial micro-CT images reveal normal airway wall thickness and lumen area in the control group (A, arrow) and diffuse bronchial wall thickening and narrow lumen area in the experimental group (B, arrow head).

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**Fig. 4:** Bronchial wall thickness as measured on micro-computed tomography images in the experimental and control groups. Vertical axis scale: mm. var 1: murine asthma models; var 2: controls. P<0.01.

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Fig. 5: Curved multiplanar reformation images show normal main bronchus wall thickness on the right side in the control group (A, arrow) and diffuse bronchial wall thickness in the experimental group (B, arrow head). A magnified picture shows measurement of the diameter perpendicular to the bronchial wall (A-1, small arrows).

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Fig. 6: Bronchial wall thickness as measured by pathological findings in the experimental and control groups. Vertical axis scale: mm. P<0.01.

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Fig. 7: Normal bronchus in the control group (A) (hematoxylin and eosin [H&E], ×200). Mucosal epithelium proliferation and submucosal smooth muscle hypertrophy in the experimental group (B) (H&E, ×200).

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Fig. 8: Airway changes in the experimental group. Goblet cell hyperplasia of the bronchial epithelium (A) (periodic acid-Schiff stain, ×200). Immunohistochemical staining for smooth muscle actin shows characteristic submucosal smooth muscle hypertrophy (B) (×200).

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Fig. 9: Correlation of micro-CT and pathologic findings in terms of bronchial wall thickness in the experimental groups (Pearson correlation; r=0.7119, P=0.0001).
**Fig. 10:** Correlation of micro-CT and pathologic findings in terms of bronchial wall thickness in the control groups (Pearson correlation; r=0.4640, P=0.0224).

**Table 1:** Bronchial lumen area as measured on micro-computed tomography (CT) images in the experimental and control groups. RLA*1: Bronchial lumen diameter of the right fourth distal branch of the bronchus level. RLA2: Bronchial lumen diameter of the right fifth distal branch of the bronchus level. RLA3: Bronchial lumen diameter of
the right sixth distal branch of the bronchus level. LLA1: Bronchial lumen diameter of the left fourth distal branch of bronchus level. LLA2: Bronchial luminal area of left fifth distal branch of bronchus level. LLA3: Bronchial luminal area of left sixth distal branch of bronchus level. O$1-6: Ovalbumin-induced asthma model mice 1-6. C%1-6: Control mice 1-6. Unit: mm2. Paired t-test. Mean of the experimental group= 0.1956±0.072 mm2. Mean of the control group= 0.243±0.116 mm2. P=0.0077. Allergy Asthma Immunol Res. 2014 Jan;6(1):75-82. http://dx.doi.org/10.4168/aair.2014.6.1.75 Copyright © 2014 The Korean Academy of Asthma, Allergy and Clinical Immunology • The Korean Academy of Pediatric Allergy and Respiratory Disease

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Table 2: Bronchial wall thickness of murine asthma models and controls on micro-CT images. RBWT*1: Bronchial wall thickness of distal main bronchus after right third branch level. RBWT2: Bronchial wall thickness of more distal main bronchus after right third branch level. LBWT#1: Bronchial wall thickness of distal main bronchus after left third branch level. LBWT2: Bronchial wall thickness of left more distal main bronchus after left third branch level. O$ 1-6: Ovalbumin-induced murine asthma model 1-6. C% 1-6: Control mouse 1-6. Unit: mm. Paired t-test. Mean of ova=0.1188±0.010 mm. Mean of control=0.1078±0.013 mm. P=0.0005.
**Table 3:** Bronchial wall thickness of murine asthma models and controls determined by pathology

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RBWT*1: Bronchial wall thickness of right fourth bronchus. RBWT2: Bronchial wall thickness of right fifth bronchus. LBWT#1: Bronchial wall thickness of left fourth bronchus. LBWT2: Bronchial wall thickness of left fifth bronchus. Unit: mm. Paired t-test. Mean of ova= 0.0665±0.011 mm. Mean of control= 0.0406±0.009 mm. P=0.0001. O$1-6$: Ovalbumin-induced murine asthma model 1-6. C%1-6: Control mouse 1-6.
Conclusions

On micro-CT images,

Bronchial lumen area was smaller and bronchial wall thickness was thicker in the experimental group than in the control group, which significantly correlated with pathological findings.

The thickened airway wall on micro-CT images was due to mucosal epithelial hyperplasia and smooth muscle hypertrophy histopathologically.

Micro-CT may be a good modality for analyzing small animal airways.
References

1. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, November 1986. Am Rev Respir Dis 1987;136:225-244.


