Airspace Enlargement With Airway Cell Apoptosis in Klotho Mice: A Model of Aging Lung

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Homozygous mutant klotho (KL−/−) mice exhibit various characteristics resembling those of human aging, including emphysema. However, age-related changes of lungs have not been fully elucidated. Here, we investigated the structural, functional, biochemical, and cell kinetic alterations of lungs in KL−/− mice at 2–12 weeks of age. Homogeneous airspace enlargement and decreased lung elastic recoil were observed in KL−/− mice with aging. The apoptotic cells in airway walls in KL−/− mice were approximately 6 times greater than those in wild-type (KL+/+) mice at 2 weeks of age. However, lipid peroxidation and elastase activity of lungs were not increased in KL−/− mice. Western blotting suggested that protein levels of epidermal growth factor (EGF) and phosphorylated extracellular signal-regulated kinase were decreased in KL−/− mice. These data suggest that significantly increased apoptosis of airway cells via inhibition of the EGF-dependent pathway may be involved in the development of the aging lungs in KL−/− mice.

Key Words: Klotho mouse—Aging lung—Airspace enlargement—Apoptosis—emphysema.

Many features of age-dependent alterations in the respiratory systems of relatively healthy people have already been elucidated (1–8). With regard to lung function, the expiratory flow rate (e.g., forced expiratory volume in 1 second, FEV1) decreases with age in relation to morphological alterations of the lung, and the age-related decline in FEV1 is further affected by noxious insults such as cigarette smoke and environmental pollution. The former age-related physiological change in lungs is defined as senile lung or aging lung, and the latter age-related pathological change in lungs is mostly described as emphysema or senile emphysema (9–15). However, in the human lung, it is difficult to distinguish a pure age effect from pathological aging because the lung is continuously exposed to air and pollution. It is difficult to study age-related lung alterations because the changes occur over a span of more than 40 years in humans. Improved mouse models are required to study the onset of age-related lung alterations and disease so that therapeutic or anti-aging interventions can be developed.

Mice deficient in a klotho gene exhibit a short life span, infertility, arteriosclerosis, skin atrophy, osteoporosis, and emphysema (16). However, in the lung, there have been conflicting reports regarding the features of airspace enlargement in klotho mice. Suga and coworkers (17) have demonstrated the histology of the lungs in homozygous mutant klotho (KL−/−) mice exhibiting heterogeneous enlargement of airspaces accompanied by obvious destruction of the alveolar walls, as indicated by the markedly increased destructive index (DI). However, Sato and coworkers (18) have reported that the morphological features of airspace enlargement in KL−/− mice were homogeneous, as indicated by the decreased fractal dimensions of airway size. Meanwhile, the functional alterations and airway cell kinetics at the different ages had not been fully elucidated in klotho mice. First, we evaluated the lung phenotypes of KL−/− mice comprehensively because the multiple approaches would be essential to differentiate the aging lung from a lung with emphysema.

Furthermore, the detailed analysis of the lung pathophysiology could be helpful to determine the underlying molecular mechanisms. Although the klotho gene is expressed mainly in the tissues important for calcium homeostasis such as distal tubule of the kidney, choroid plexus in the brain, and parathyroid gland (16), it remains to be elucidated how the lack of Klotho protein leads to the variable phenotypes of whole bodies. We evaluated the epidermal growth factor (EGF) and extracellular signal-regulated kinase (ERK) pathways as possible mediators to the pathophysiology of the lungs in KL−/− mice.

Materials and Methods

Animals

In the series of experiments reported here, all mice were generated from mating pairs of heterozygous mutant klotho mice (KL+/−) that were purchased from Nihon CLEA (Tokyo, Japan) and were bred in the Animal Research Institute of Tokyo University. Newborns were weaned at 2 weeks of age, and then polymerase chain reaction (PCR) was performed for genotyping mice by using genomic DNA, extracted from tail clips. The mice were maintained in a limited-access barrier, housed at 24 ± 2°C under an alternating 12-hour light/dark cycle, and fed a commercial diet (CE-2; Nihon CLEA) and water ad libitum. The mouse of each genotype was examined at 2, 4, 8, and 12 weeks. The experiments were in accordance with the Guidelines for...
the Care and Use of Laboratory Animals of the National Institutes of Health, and they were approved by the University of Tokyo Institutional Review Board for Laboratory Animal Use.

**Genotyping Assay for Klotho Gene**

The genotypes of mice were confirmed by PCR analysis of tail DNA by using the primers 5' - TTGTGGAGATTGGGAATGGACGAAAGAG-3' (forward), 5' - CGCCCGCA CGGAGCTGAGTA-3' (klotho mutant reverse), and 5' - CTGGACCCCTGTAAGTCAGTTAC-3' (wild-type reverse). These primers were expected to produce 815 bp (wild-type) and 419 bp (klotho mutant) amplification products (16). Reaction mixtures contained 0.2 µL of LA Taq (Takara), 2.2 µL of 25 mM MgCl2 (Takara), 2.2 µL of 10× Mg2+-free PCR buffer (Takara), 4.0 µL of dNTP mix (Takara), 1.0 µL of 10 pmol/µL each of the forward and reverse primers, and 2.0 µL of DNA template. PCR conditions were as follows: denaturation at 94°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 45 seconds, then a final extension at 72°C for 10 minutes. We subjected 10 µL of the PCR product mixed with loading buffer to electrophoresis on a 1% agarose gel, and it was further visualized after staining with ethidium bromide staining (5 µg/mL). Furthermore, the DNA/HaeIII marker (Promega, Madison, WI) was used for determination of the targeted bands.

**Evaluation of Age-Dependent Senility of the Mice Lacking a Klotho Gene**

The degree of systemic senescence of the mice was measured by the Grading Score System (GSS), which is a method for evaluating the degree of senescence in the senescence-accelerated mouse (19). The GSS has been considered to be a unique, useful, and convenient method of detecting senescence in laboratory mice. The 11 categories evaluated in this system include reactivity, passivity, glossiness, coarseness, hair loss, ulcer, periophthalmic lesions, cataract, corneal ulcer, corneal opacity, and lordokyphosis. In general, each category has 5 grades corresponding to the intensity of change (19). The total GSS score was calculated by adding grades at various weeks of age for all genotypes.

**Evaluation of Age-Related Changes in the Body Weight and Lung Weight of the Klotho Mice**

Five male mice of each genotype were weighed at 2, 4, 8, and 12 weeks of age, and their lungs were weighed using an analytical balance (Sartorius Co., Tokyo, Japan). Mean values of body weights and lung weights were compared between genotypes as the basic evaluation of the physiological age-related changes. In addition, we measured arm span instead of height because of the aging phenotype such as kyphosis (20).

**Evaluation of Morphological Changes in the Lung**

We examined the morphological changes in the lungs in genotypes of five male mice at 2, 4, 8, and 12 weeks of age. The mice were anesthetized by diethyl ether inhalation (Wako, Osaka, Japan). After killing by exsanguinations under deep anesthesia, the lungs were removed from the thorax en bloc and inflated with 4% formalin neutral buffer solution (pH 7.4) at a constant pressure of 25 cm H2O for 48 hours. After fixation, dehydration through graded alcohols and xylene, and embedding in paraffin, sections in the frontal plane at the depth of the hilum were stained with hematoxylin and eosin. The changes in airspace size were assessed by the mean linear intercepts (MLI). We used 20 randomly selected fields in each section at ×100 magnification for the calculation of MLI. MLI was calculated by the equation n × l/Si, where n equals the number of hairlines counted, l equals the length of the hairline, and Si equals the sum of the alveolar intercepts (21).

The destruction of alveolar walls was quantified by the DI (5). A grid with 25 points at the center of the hairline crosses was superimposed on the lung field. Structures lying under these points were divided into normal (N) or destroyed (D) alveolar and duct spaces. Points falling over other structures, such as duct walls or alveolar walls, were not included. The DI was calculated from the formula: DI = D/(D + N) × 100. Ten randomly selected fields in each section at ×50 magnification were used to measure DI (22).

**Bronchoalveolar Lavage Fluid**

Bronchoalveolar lavage (BAL) was performed on five male mice in each genotype group at 4 and 8 weeks of age using 1 mL of phosphate-buffered saline (PBS) five times in each genotype group. In each animal, approximately 90% (4.5 mL) of the total injected volume was consistently recovered. After BAL fluid (BALF) was centrifuged at 450 × g for 10 minutes, the total and differential cell counts of the BALF were determined from the cell fraction. The supernatant was stored at −80°C until assays were performed.

**Elastase Activity in BALF**

Elastase activity in BALF was measured by its ability to cleave p-nitroaniline from succinyl-L-alanyl-L-alanyl-p-nitroanilide (Sigma, St. Louis, MO). For the assay of enzyme activity, 250 µL of the supernatant or porcine pancreatic elastase (Sigma) for standards (10−6 U/mL) was added to 5 µL of 100 mmol/L succinyl-L-alanyl-L-alanyl-p-nitroanilide and incubated for 60 minutes at 37°C. We added 100 µL of trichloroacetic acid (Wako) to the mixture and centrifuged for 10 minutes at 4°C. For 375 µL of the supernatant, the reaction was terminated with the addition of 50 µL of 0.1% sodium nitrate (Wako), 50 µL of 0.5% ammonium amidosulfate, and 50 µL of N-1-naphthylethenediamine dihydrochloride. Elastase activity was quantified by measuring the release of p-nitroanilide (absorbance at 550 nm) by using a 96-well plate reader (Bio-Rad Laboratories, Tokyo, Japan).

**Lipid Peroxidation in BALF**

A breakdown product of lipid peroxidation, thiobarbituric acid reactive substance (TBARS) was measured. The stock solution contained equal volumes of trichloroacetic acid 15% (wt/vol) in 0.25 N hydrochloric acid and 2-thiobarbituric acid 0.37% (wt/vol) in 0.25 N hydrochloric acid. One volume of BALF and two volumes of stock reagent were
mixed in a screw-capped centrifuge tube, vortexed, and heated for 15 minutes in a boiling water bath. After cooling on ice, the flocculent precipitate was removed by centrifugation at 1000 \( \times g \) for 10 minutes and absorbance was measured at 535 nm against blank. The optical density was read at 535 nm.

Detection and Semiquantitative Assessment of Apoptosis and Cell Proliferation of Airway Cells

For the quantitative assessment of apoptosis in airway cells, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was performed on lung tissue of five male mice in each genotype group at 2, 4, 8, and 12 weeks of age (11). After being deparaffinized, the tissue sections were stained using in situ TUNEL with biotin-16-deoxyuridine 5'-triphosphate (dUTP; Roche Diagnostics, Penzberg, Germany) to identify cells demonstrating nuclear DNA fragmentation. Endogenous peroxidase activity was quenched by incubation with 2% \( \text{H}_2\text{O}_2 \) in PBS for 5 minutes. To facilitate access of the reagent to DNA fragments, sections were treated with proteinase K at 20 \( \mu \text{g/mL} \) at 37°C for 60 minutes. After washing with PBS, nick-end labeling with TdT was subsequently performed in TdT buffer containing biotin-16-dUTP at 37°C for 1 hour. The tissue sections were then washed with automation buffer for 15 minutes at 37°C and incubated with peroxidase-labeled streptavidin (Dako, Glostrup, Denmark). After washing with PBS, the sections of peroxidase-labeled tissue were visualized by incubation in diaminobenzidine for 5 minutes at room temperature. After washing with water, sections were subsequently counterstained with methyl green and dehydrated.

For the quantitative assessment of proliferation, anti-proliferating cell nuclear antigen (PCNA; Dako) was used as the monoclonal antibody for immunohistochemical staining. The tissues were deparaffinized and washed in distilled water. Endogenous peroxidase activity was reduced by incubating the samples for 20 minutes in methanol and 0.3% \( \text{H}_2\text{O}_2 \). After being rinsed with water, the slides were placed in a glass dish filled with 0.01 M sodium citrate buffer, pH 6.0. Tissue sections were boiled in a microwave oven (500 W) twice for 20 minutes each. After washing with water, the sections were immersed in 5% normal pig serum (KOHJIN BIO, Saitama, Japan) to eliminate nonspecific antibody binding. The slides were then incubated with anti-PCNA antibody overnight at 4°C, rinsed, and incubated with biotinylated antibody (Dako) for 30 minutes at room temperature. Antibody complexes were visualized by the avidin–biotin–peroxidase method. The peroxidase color reaction was developed with Tris buffer (0.05 mM, pH 7.6) containing 20 mg of 3,3′-diaminobenzidine, tetrahydrochloride, and 5 \( \mu \text{L} \) of 30% hydrogen peroxide in 100 mL of PBS and counterstained with hematoxylin.

To differentiate airway cells from endothelial cells, we also performed double staining of the lung tissues by using platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:50 as the primary antibodies. The tissue sections that underwent TUNEL and PCNA staining without nuclear staining (positive cells were colored brown) were incubated in blocking solution (2% skim milk and 0.2% Triton X-100 in PBS) at room temperature. The primary antibodies were reacted with goat anti-rabbit immunoglobulin G (IgG; Zymed Laboratories Inc., South San Francisco, CA) at a dilution of 1:50 and a secondary antibody conjugated with an alkaline phosphatase–labeled polymer. Furthermore, immunoreactants were visualized as blue color by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (Dako) and subsequently dehydrated, air dried, and mounted.

We counted the number of TUNEL- or PCNA-positive cells per 100 airway cells as indicated by the PECAM-1–negative cells at a magnification of \( \times200 \). We examined 20 randomly selected fields. The apoptotic index (AI) was determined by the percentage of TUNEL-positive cells in the total airway cells without staining with PECAM-1. The proliferation index (PI) was determined by the percentage of PCNA-positive cells in the total airway wall cells without staining with PECAM-1.

Lung Function Analysis

Lung function was measured with a whole-body plethysmograph system (Buxco BioSystem for Meuners hardware and software; Buxco Electronics, Wilmington, NC) (23). First, four mice of each genotype at 8 weeks of age and three mice of each genotype at 12 weeks of age were anesthetized with an intraperitoneal injection of ketamine and xylazine. A tracheostomy was then performed, and a 19- or 21-gauge metal tracheostomy tube was inserted and secured with a suture. The mice were attached via the tracheostomy tube to a mechanical ventilator in a sealed plethysmograph. The functional residual capacity (FRC) was determined by occluding the airway while the mice attempted to breathe. The pressure exerted by the mice and the change in their thoracic volume were measured, and these values were used for the calculation of FRC by using Boyle’s law. Next, total lung capacity (TLC), vital capacity (VC), and lung elasticity were calculated by a quasistatic pressure/volume maneuver. For this measurement, the ventilator delivered a breath to TLC (30 cm \( \text{H}_2\text{O} \)), which was calculated by adding FRC and inspiratory capacity, which was the inflated volume between 0 cm \( \text{H}_2\text{O} \) and 30 cm \( \text{H}_2\text{O} \) pressure. The pressure was then maintained for a short period of time before slow expiration to residual volume (RV). VC was calculated by subtracting RV from TLC. Pressure and volume (P-V) curves were recorded during this slow exhalation. The lung elasticity is reported as the chord compliance (Cchord), which represents the lung elastic recoil by means of the slope of compliance curve between 0 and 10 cm \( \text{H}_2\text{O} \) pressure.

We also calculated the shape constant K of the P-V curves in mice at 8 weeks of age. The P-V curve was fitted using an iterative least mean squares regression on a computer, according to an exponential equation: \( V = A - B e^{-KP} \), where \( V \) is lung volume, \( P \) is transpulmonary pressure, and \( A, B, \) and \( K \) are constants (24).

For the detection of the obstruction in lung passages, we used fast flow volume maneuvers to analyze the fast expiration induced by switching the trachea to a high negative pressure. Forced expiratory volume in 100 ms (FEV\(_{100}\)) was measured at 535 nm against blank. The optical density was read at 535 nm.
determined, and FEV100/VC was calculated for the index of the airway obstruction. Data for all maneuvers were analyzed using Biosystem XA software (Buxco Electronics). The total time of mechanical ventilation was less than 7 minutes.

**Immunochemistry for EGF**

For the assessment of the distribution of EGF in airway cells, we performed the immunochemistry using lung tissues of KL+/− mice and wild-type (KL+/+) mice at 4 weeks of age. After being deparaffinized, endogenous peroxidase activity was quenched by incubation with 0.3% H2O2 in PBS for 5 minutes. The lung tissues were primarily reacted with rabbit polyclonal antisera as cellular markers for EGF (1:50, Santa Cruz Biotechnology) for 1 hour at room temperature. These sections were then incubated with horseradish peroxidase (HRP)-conjugated anti-goat IgG (MP Biomedicals) and then washed twice in ice-cold wash buffer. The sections of peroxidase-labeled tissue were visualized by incubation in 3-3′-diaminobenzidine for 15 minutes at room temperature. After washing with water, sections were subsequently dehydrated, air dried, and mounted.

**Western Blotting for EGF and phosphorylated ERK1/2**

Membrane proteins were extracted using a Proteo Extract native membrane protein extraction kit (Calbiochem, San Diego, CA). The lung tissues of three genotypes of mice at 4 weeks of age were removed, and 50 mg of the tissues were homogenized and then washed twice in ice-cold wash buffer and incubated with extraction buffer I for 10 minutes at 4°C. Protease inhibitor cocktail (PIC; 10 μL) was added to prevent the activity of proteases released from the tissues. The tissues were then incubated with 1 mL of ice-cold extraction buffer II supplemented with 10 μL of PIC for 30 minutes at 4°C under gentle agitation. The tissue suspension was centrifuged at 16,000 × g for 15 minutes at 4°C, and the supernatant containing membrane proteins was collected. Equal protein amounts were added to sample buffer (200 mM Tris-HCl [pH 6.5], 20% glycerol, 2% sodium dodecyl sulfate [SDS], 15% 2-mercaptoethanol, 0.01% bromophenol blue) and were boiled for 5 minutes. Proteins were separated by electrophoresis on 8% SDS-polyacrylamide (PAGE) gels (Bio-Rad, Hercules, CA) and electroblotted onto polyvinylidene difluoride membranes. Nonspecific binding was blocked by incubating blots in 5% nonfat dry milk in PBS/Tween 20 at 4°C. The membrane proteins were blotted against antibodies to EGF (1:200; Santa Cruz Biotechnology), phosphorylated ERK (p-ERK1/2; 1:200, Santa Cruz Biotechnology), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:200; Santa Cruz Biotechnology). Primary antibody binding was detected with HRP-conjugated anti-goat IgG (MP Biomedicals). Specific protein bands were detected by enhanced chemiluminescence autoradiography (GE Healthcare, Little Chalfont, UK).

**Statistical Analysis**

Comparisons of data among the experimental groups were carried out using one-way analysis of variance (Scheffé test) or the nonparametric Kruskal–Wallis test. Statistical significance was determined by p values <.05, and errors were indicated by standard error (SE), unless otherwise stated. Microsoft Excel 2003 was used for data management, and all statistics were performed using Dr. SPSS II (version 14.0; SPSS Japan Inc., Tokyo).

**RESULTS**

**Senility Scores**

Senility as indicated by the GSS was increased from 4 weeks of age to 12 weeks of age in KL+/− mice, but not in other genotypes of mice. There was no difference between KL+/− mice and KL+/+ mice at any week of age (Figure 1).

**Body Weight and Lung Weight**

After 4 weeks of age, the lung weight in KL+/− mice was significantly smaller than that in the mice of other genotypes (Table 1). The ratio of lung weight to body weight was unchanged with different age in mice of each genotype (Table 1).

In contrast, the lung weight corrected with the body weight was significantly higher in KL+/− mice than in KL+/+ mice. The lung weight usually depends on not the body weight but the height (arm span), we measured arm span of mice of each genotype. The body weight ratio of KL+/− to KL+/+ or KL+/- mice at 4–12 weeks of age was approximately 40%, and the arm span ratio of KL+/− to KL+/+ or KL+/- mice was approximately 80%, indicating that the body mass index in KL+/− mice was lower than that in control groups.

**Evaluation of Morphological Changes in the Lung**

The representative histology is illustrated in Figure 2. The airspace size of the lungs as indicated by MLI was significantly greater in KL+/− mice than in mice of other genotypes. The MLI increased with age in KL+/− mice, but not
in mice of other genotypes (Figure 3A). The coefficient of variation (CV) of MLI values was <10%, suggesting the homogeneity rather than heterogeneity of airspace enlargement in KL<sup>−/−</sup> mice.

The alveolar wall destruction as indicated by DI was significantly higher in KL<sup>−/−</sup> mice than in other genotypes. However, even at 12 weeks of age, the DI in KL<sup>−/−</sup> mice was not >10%. The error bars for DI are approximately the same size regardless of the mean DI value (Figure 3B).

**Assessment of the Elastase Activity and Lipid Peroxide in BALF**

The elastase activity in BALF was almost under the detection sensitivity as compared with the standards of porcine pancreatic elastase, and there was no significant difference between the genotypes at 8 weeks of age (Table 2). Furthermore, there was no increased level of lipid peroxidation in KL<sup>−/−</sup> mice as the indication of oxidative stress in BALF, as compared with other genotypes.

**Assessments of the Cell Proportion of BALF**

More than 90% of the cells obtained were viable in every mouse at 4–8 weeks of age. The total cell count in BALF in mice of all genotypes was <2 × 10<sup>5</sup> cells; the percentage of alveolar macrophages was >95%; that of neutrophils, <1%; and that of lymphocytes, <1%. There was no differential cell proportion of BALF among the three genotypes (Table 2).

**Semiquantitative Assessment of Apoptosis and Cell Proliferation of Airway Cells**

The AI at 2 weeks of age was approximately 6 times greater in KL<sup>−/−</sup> mice than in KL<sup>+/+</sup> mice (KL<sup>−/−</sup> mice vs KL<sup>+/+</sup> mice [means ± SE]; 1.11 ± 0.35 vs 0.17 ± 0.015; *p < .05). After 4 weeks, it was consistently higher in KL<sup>−/−</sup> mice than in mice of other genotypes. Most of the apoptotic

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Table 1. Age-Related Changes of Body Weight, Lung Weight, Lung Weight/Body Weight, and Arm Span in Mice Lacking Klotho Gene (KL<sup>−/−</sup> Mice) and Mice With Klotho Gene (KL<sup>+/+</sup> and KL<sup>+/−</sup> Mice)

<table>
<thead>
<tr>
<th>Age</th>
<th>Body Weight (g)</th>
<th>Lung Weight (g)</th>
<th>Lung Weight/Body Weight</th>
<th>Arm Span (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Weeks</td>
<td>5.9 ± 0.7</td>
<td>0.26 ± 0.02</td>
<td>0.022 ± 0.002</td>
<td>59.4 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>5.8 ± 0.6</td>
<td>0.26 ± 0.03</td>
<td>0.02 ± 0.001</td>
<td>59.7 ± 2.4</td>
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<tr>
<td></td>
<td>5.6 ± 0.5</td>
<td>0.24 ± 0.03</td>
<td>0.019 ± 0.002</td>
<td>58.5 ± 2.1</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>19.5 ± 0.5</td>
<td>0.42 ± 0.03</td>
<td>0.021 ± 0.002</td>
<td>76.2 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>20.5 ± 0.7</td>
<td>0.41 ± 0.04</td>
<td>0.021 ± 0.001</td>
<td>75.9 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>7.5 ± 0.7*</td>
<td>0.31 ± 0.04*</td>
<td>0.041 ± 0.002*</td>
<td>66.5 ± 2.3*</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>20.5 ± 0.7</td>
<td>0.44 ± 0.04</td>
<td>0.021 ± 0.001</td>
<td>81.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>20.5 ± 0.5</td>
<td>0.43 ± 0.03</td>
<td>0.023 ± 0.001</td>
<td>80.8 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>7.7 ± 0.7*</td>
<td>0.32 ± 0.02*</td>
<td>0.039 ± 0.002*</td>
<td>68.2 ± 2.2*</td>
</tr>
<tr>
<td>12 Weeks</td>
<td>22.0 ± 2.0</td>
<td>0.45 ± 0.04</td>
<td>0.021 ± 0.002</td>
<td>82.5 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>22.5 ± 2.4</td>
<td>0.43 ± 0.02</td>
<td>0.022 ± 0.002</td>
<td>83.0 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>8.0 ± 1.0*</td>
<td>0.31 ± 0.02*</td>
<td>0.038 ± 0.001*</td>
<td>67.2 ± 3.0*</td>
</tr>
</tbody>
</table>

Notes: n = 5.
* *p < .05 compared with the value of KL<sup>+/+</sup> and KL<sup>+/−</sup> mice at the same age.

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![Figure 2](https://academic.oup.com/biomedgerontology/article-abstract/63/12/1289/620694/109x76 to 519x285)
cells (≥90%) were not stained with PECAM-1 antibody (Figure 4A).

The PI was also the highest in KL−/− mice at 2 weeks, and this value decreased with age. The PI in KL−/− mice was greater than in mice of other genotypes at all ages (Figure 4B). However, the ratio of PI to AI was greater in wild-type mice than in KL−/− mice. The ratio of PI to AI was >1.0 in wild-type mice. In contrast, the ratio of PI to AI was <0.5 in KL−/− mice at 2–4 weeks of age.

Functional Changes in the Lung

The lung function study demonstrated that FEV100/VC (%) and lung elastic recoil as indicated by Cchord (mL/cm H2O) were lower in KL−/− mice than in KL+/+ mice at 8 and 12 weeks of age and that RV/TLC (%) was greater in KL−/− mice than in KL+/+ mice at the same age (Table 3). There was no significant difference in lung function between KL−/− and KL+/+ mice. The P-V curves of lungs in KL−/− mice at 8 and 12 weeks of age and those in other genotypes of mice at 8 weeks of age are illustrated in Figure 5. In KL−/− mice, the P-V curve was shifted leftward and upward as compared with that in mice of other genotypes at the same age. Furthermore, the comparison of the P-V curve in KL−/− between at 8 and 12 weeks of age showed that it was shifted leftward and upward with advancing age. In addition, we calculated the shape constant K of the P-V curves in mice at 8 weeks of age. K values in KL−/− mice (0.17 ± 0.03) were significantly greater than those in KL+/+ mice (0.13 ± 0.02, p < .05) and KL+/+ (0.13 ± 0.03, p < .05).

Immunohistochemistry for EGF

We first investigated the expression of EGF by immunohistochemical staining of lung tissues. The intensity of immunostaining of EGF was greater in the lung tissues of KL+/+ mice (Figure 6A) than in the tissues of KL−/− mice (Figure 6B).

Western Blot Analysis for p-ERK1/2 and EGF

We performed western blot analysis for EGF (130 kd) and p-ERK1/2 (44/42 kd) in the lung protein of all genotypes of mice. Lower levels of EGF protein were detected in KL−/− mice than in KL+/+ mice (Figure 7). The levels of EGF protein in KL−/− mice were intermediate between those levels in KL+/− and KL+/+ mice. Furthermore, lower levels of p-ERK1/2 protein were detected in KL−/− mice than in KL+/+ mice. However, there was no obvious difference in the levels of p-ERK1/2 protein between KL+/− and KL+/+ mice (Figure 7).

DISCUSSION

The current study revealed that the airspace size of lungs enlarged with age in KL−/− mice in accordance with the systemic senile phenotype as measured using senility scores. Several investigators have demonstrated that the airspace enlargement in KL−/− mice was assessed at 4–8 weeks of age alone (17,18). Our data indicated that airspace size significantly increased at 4 weeks and progressed gradually with age up to 12 weeks, which is almost the entire life span.

Table 2. Bronchoalveolar Lavage Fluid (BALF) Examination in Mice Lacking Klotho Gene (KL−/− Mice) and Mice With Klotho Gene (KL+/− and KL+/+ Mice)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age</th>
<th>Total Cells (×103)</th>
<th>Macrophages (%)</th>
<th>Neutrophils (%)</th>
<th>Lymphocytes (%)</th>
<th>Elastase Activity (1.0 × 10−3 U/mL)</th>
<th>Lipid Peroxidation (mmol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KL−/−</td>
<td>4 wk</td>
<td>1.6 ± 0.6</td>
<td>98.0 ± 1.0</td>
<td>0.8 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KL−/−</td>
<td>8 wk</td>
<td>1.8 ± 0.7</td>
<td>97.5 ± 1.5</td>
<td>0.6 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KL+/−</td>
<td>4 wk</td>
<td>1.8 ± 0.8</td>
<td>98.0 ± 1.0</td>
<td>0.6 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>6.1 ± 1.0</td>
<td>0.1 ± 0.05</td>
</tr>
<tr>
<td>KL+/−</td>
<td>8 wk</td>
<td>1.8 ± 0.6</td>
<td>97.0 ± 1.5</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>5.6 ± 0.7</td>
<td>0.2 ± 0.10</td>
</tr>
<tr>
<td>KL+/+</td>
<td>4 wk</td>
<td>1.7 ± 0.7</td>
<td>98.0 ± 1.0</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.2</td>
<td>5.7 ± 0.7</td>
<td>0.2 ± 0.05</td>
</tr>
<tr>
<td>KL+/+</td>
<td>8 wk</td>
<td>1.5 ± 0.8</td>
<td>98.0 ± 1.0</td>
<td>0.5 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Notes: n = 5.

Diagonal lines indicate that the measurements were not performed.
of KL−/− mice. The airspace enlargement was homogeneous rather than heterogeneous in size, which was in accordance with the low value of both DI and CV of MLI values. In addition to MLI, the error bars for DI in Figure 3 were also approximately the same size, regardless of the mean DI value. This finding may imply that heterogeneity does not increase with airspace size in KL−/− mice.

Lung function was also altered in klotho mice. The quasi-static P-V curves of lungs in KL−/− mice were shifted to the left as compared with those in KL+/+ mice. The static lung compliance was increased, suggesting that the lung elastic recoil of KL−/− mice was decreased. Furthermore, the shape constant K of the P-V curves in KL−/− mice was significantly greater than those in control groups, which suggested the decreased elastic recoil (24,25). The functional results were consistent with the morphological changes of KL−/− mice.

It has been proposed that chronic obstructive pulmonary disease is characterized by an inflammatory state that induces premature aging of the lung and other organs (26). Before the development of emphysema, lungs of smokers exhibit an accumulation of macrophages, lymphocytes, and neutrophils in the walls and adjacent airspaces in the respiratory bronchioles, alveolar ducts, and alveoli (3,9,27).

In contrast, aging lung is characterized by a homogeneous enlargement of the alveolar airspaces, without major inflammation, fibrosis, or destruction of their walls (13–15). Homozygous mutant klotho (KL−/−) mice exhibit features of aging lung without exposure to tobacco smoke or environmental pollution.

In the present study, we also demonstrated that the degree of senescence in KL−/− mice was considerably greater than that in mice of other genotypes. When the alterations of the lung morphology and function in klotho mice are not accompanied by the systemic senile features including a short life span, infertility, arteriosclerosis, skin atrophy, and osteoporosis, the lung changes may be determined as pathological changes. KL−/− mice showed many of the senile features in various organs including lungs. Therefore, in KL−/− mice, age-related alterations of the lungs may not be associated with pathological aging in terms of accompanying senescence of individuals.

The morphological changes of the lung in KL−/− mice were associated with the increased apoptosis of airway cells, in comparison with endothelial cells. The AI at 2 weeks of

### Table 3. Lung Function Measurements at 8 and 12 Weeks of Age in Mice Lacking Klotho Gene (KL−/−) mice) and Mice With Klotho Gene (KL+/− and KL+/+ Mice)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>KL+/−</th>
<th>KL+/+</th>
<th>KL−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>At 8 weeks of age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLC, mL</td>
<td>1.31 ± 0.01</td>
<td>1.32 ± 0.02</td>
<td>1.15 ± 0.09</td>
</tr>
<tr>
<td>RV, mL</td>
<td>0.048 ± 0.006</td>
<td>0.050 ± 0.007</td>
<td>0.168 ± 0.018</td>
</tr>
<tr>
<td>RV/TLC, %</td>
<td>3.66 ± 0.38</td>
<td>3.79 ± 0.41</td>
<td>14.6 ± 2.0*</td>
</tr>
<tr>
<td>FEV100/VC, %</td>
<td>96.2 ± 2.8</td>
<td>94.6 ± 3.4</td>
<td>32.4 ± 2.8*</td>
</tr>
<tr>
<td>Cchord, mL/cm H2O</td>
<td>0.087 ± 0.010</td>
<td>0.095 ± 0.008</td>
<td>0.049 ± 0.009*</td>
</tr>
<tr>
<td><strong>At 12 weeks of age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLC, mL</td>
<td>1.41 ± 0.02</td>
<td>1.44 ± 0.04</td>
<td>1.25 ± 0.05</td>
</tr>
<tr>
<td>RV, mL</td>
<td>0.053 ± 0.005</td>
<td>0.056 ± 0.007</td>
<td>0.298 ± 0.022*</td>
</tr>
<tr>
<td>RV/TLC, %</td>
<td>3.76 ± 0.35</td>
<td>3.88 ± 0.53</td>
<td>23.8 ± 2.1*</td>
</tr>
<tr>
<td>FEV100/VC, %</td>
<td>95.2 ± 2.4</td>
<td>95.6 ± 3.5</td>
<td>21.4 ± 2.1*</td>
</tr>
<tr>
<td>Cchord, mL/cm H2O</td>
<td>0.085 ± 0.010</td>
<td>0.083 ± 0.009</td>
<td>0.039 ± 0.005*</td>
</tr>
</tbody>
</table>

Notes: At 8 wk of age, n = 4. At 12 wk of age, n = 3.

* p < .05 compared with the value of KL+/− or KL+/+ mice at the same age.

† p < .05 compared with the value in the same genotype at 12 weeks of age.

TLC = total lung capacity; RV = residual volume; FEV100 = forced expiratory volume in 100 ms; VC = vital capacity; Cchord = chord compliance.
age was approximately 6 times greater in KL/C0 mice than in mice of other genotypes. The apoptosis of airway cells may precede airspace enlargement. Kasahara and coworkers (28) have demonstrated apoptosis of both alveolar septal and endothelial cells in emphysematous lungs. However, most of the apoptosis was observed in airway cells, but not in endothelial cells, in the KL−/− mice, suggesting that endothelial cell apoptosis may not play a critical role in the morphological changes in lungs.

The proliferation of airway cells in KL−/− mice was also the highest at 2 weeks of age, and it decreased with age. The PI was greater in KL−/− mice than in mice of other genotypes at all ages. Thus, the KL−/− mice demonstrated increased apoptotic cell death and cell proliferation. Although the ratio of PI to AI was >1.0 in KL+/+ mice, the ratio of PI to AI was <0.5 in KL−/− mice at 2–4 weeks of age. The slightly but significantly high turnover kinetics of airway wall cells and the impaired cell repair system in the lung tissue in KL−/− mice may be involved in the homogeneous enlarged airspaces in KL−/− mice.

In humans, emphysematous lungs demonstrated lower surface areas and increased cell proliferation (29,30). The low rate of apoptosis in alveolar cells was observed in the lungs of aged nonsmokers (30). However, the kinetics of airway cells including alveolar cells in aging lung have not been fully elucidated. Because it is very difficult to prevent oxidative stress or pollution in aging humans, the klotho mouse lung is an interesting model to explore the relationship between airway cell apoptosis and aged lungs.

The apoptotic mechanism of airway cells may be important. Because inflammatory cells were not recruited into the lungs of klotho mice, inflammation and oxidative stress, which are common triggers of apoptosis pathways through the mitogen-activated protein kinases (MAPK) including c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 kinase (31,32), were not primarily relevant to this apoptosis mechanism. We considered that the suppressed growth factor-induced ERK1/2 pathway, which functioned as the MAPK survival signal (33), was important. In the development and repairing of the lungs, the signaling of EGF, which is one of the growth factors, plays an important role and it stimulates the differentiation of alveolar type II cells (34,35). In addition, a previous study reported that the immunoreactivity for EGF in the submandibular gland was decreased in KL−/− mice (36). Therefore, we focused on the expression of EGF, which induces

![Figure 5. The pressure-volume (P-V) curves of lungs in KL+/+ mice at 8 and 12 weeks of age and in other genotypes of mice at 8 weeks of age. Data at 0 and 10 cm H2O as representatives are shown as means ± standard error (n = 4 for all genotypes at 8 weeks of age, n = 3 for KL−/− at 12 weeks of age). Thick dotted line illustrates the mean value line of P-V curves of lungs in KL+/+ mice at 8 weeks of age. Thin dotted line illustrates the mean value line of P-V curves of lungs in KL−/− mice at 8 weeks of age. Solid line illustrates the mean value line of P-V curves of lungs in KL−/− mice at 8 weeks of age. Thick solid line illustrates the mean value line of P-V curves of lungs in KL−/− mice at 12 weeks of age. P-V curves of KL+/+ or KL−/− mice at 12 weeks of age were almost the same as those of each genotype at 8 weeks of age, respectively (data not shown).](https://academic.oup.com/biomedgerontology/article-abstract/63/12/1289/620694/1289)

![Figure 6. Examples of immunohistochemistry staining for epidermal growth factor in the lung tissues of KL+/+ (A) and KL−/− (B) mice.](https://academic.oup.com/biomedgerontology/article-abstract/63/12/1289/620694/1289)
the activation of ERK1/2 cascade. Because the activated ERK1/2 prevents non-inflammatory apoptosis pathways from the cells, the decreased levels of EGF caused by klotho gene deficiency may be involved in the increased levels of apoptosis of airway cells. Therefore, airway cells of the klotho gene--deficient mice are liable to commit to apoptosis, resulting in the imbalance of airway cell proliferation and apoptosis.

Moreover, the association between EGF and pulmonary emphysema may be explained by the fact that α1,6-fucosyltransferase (Fut8)-deficient mice exhibit emphysema (37). Fut8 catalyzes the transfer of a fucose residue to N-linked oligosaccharides on glycoproteins via an α1,6-linkage to form core fucosylation in mammals (38). Because the core fucosylation of N-glycans is required for the binding of the EGF to its receptor and EGF-induced phosphorylation levels of the EGF receptor were substantially blocked in Fut8−/− cells (39). These observations support some association of EGF with the pathophysiology of the lung tissues in KL−/− mice, although more detailed studies are required.

Interestingly, the major site of EGF synthesis is the distal tubule cells of the kidney (40,41); this site corresponds to the organ where the expression level of Klotho protein is predominant. Furthermore, the expression of klotho gene was indicated in the pituitary gland, and the synthesis of the growth hormone (GH) was also decreased in KL−/− mice (16). A previous study showed that the administration of GH in hypopituitary states enhances the expression of EGF (42). These mechanisms may be associated with the decreased level of EGF in KL−/− mice.

The klotho gene is expressed mainly in the kidney, brain, and parathyroid gland for calcium homeostasis (16). Suga (43) demonstrated the calcification of alveolar septa, type I collagen fiber, and basement membrane in KL−/− mice under the electron microscope. The deposition of calcium on the lung tissues of alveolar septa in KL−/− mice may contribute to the functional decline, such as decreased elastic recoil. However, we considered that the dysregulated calcium homeostasis may be not enough to fully explain the changes of lungs in KL−/− mice.

Summary

Lungs in KL−/− mice may exhibit enlarged homogeneous airspaces and increased static lung compliance, which are features of aging lung. The mechanism of the lungs in the KL−/− mice is involved in the small, but significantly increased, levels of apoptosis in airway cells without inflammation. Analysis of the pathophysiology of enlarged airspaces in klotho mice will provide a unique insight into the relationship between the aging lung and airway cell kinetics in association with premature aging and apoptosis.

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Figure 7. Characterization of epidermal growth factor (EGF; left) and phosphorylated extracellular signal-regulated kinase (p-ERK1/2; right) in all genotypes of mice. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

REFERENCES


