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Imbalance of Matrix Metalloproteinase-9 and Tissue Inhibitor of Matrix Metalloproteinase-1 is Associated with Pulmonary Emphysema in Klotho Mice

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Klotho mice, which exhibit multiple phenotypes resembling human aging, develop pulmonary emphysema. In this study, to clarify the mechanism of their emphysematous change through development, we evaluated the expression of matrix metalloproteinase (MMP)-2, 9 and the tissue inhibitors of matrix metalloproteinase (TIMP)-1, 2 in the lungs of Klotho mice and wild type mice. Klotho mice showed obvious air space enlargement at 5 weeks of age, but not at 2 weeks of age. Immunohistochemical analysis revealed that expression of MMP-9 was increased in Klotho mice compared with wild type at 5 weeks of age. Western blot analysis and gelatin zymography also revealed that the expression and the gelatinolytic activity of MMP-9 were increased in the lungs of Klotho mice. The expression of TIMP-1 decreased in the lungs of Klotho mice. MMP-2 and TIMP-2 showed no significant differences at 5 weeks of age. At 2 weeks of age, there were no significant differences in the expressions of MMP-9 and TIMP-1 between Klotho and wild type mice. These findings suggest that imbalance of MMP-9 and TIMP-1 is associated with the development of pulmonary emphysema in Klotho mice.

The pathogenesis of pulmonary emphysema has been studied based on proteinase-antiproteinase hypothesis. Recent studies reported that alveolar macrophages play an important role in the development of pulmonary emphysema (6,11,17). Among macrophage-derived proteolytic enzymes considered to be associated with pulmonary emphysema, several studies suggest that imbalance of matrix metalloproteinase (MMP)-2, 9 and their endogenous inhibitors, tissue inhibitors of matrix metalloproteinase (TIMP) -1, 2, may be essential to the pathogenesis of pulmonary emphysema (6,11,14,15,17).

Animal models of pulmonary emphysema previously reported have been established by giving some kinds of proteinases which cause acute inflammatory reaction rather than gradual destruction of the small airway. Recently, some genetic models of pulmonary emphysema were reported (4,7,16,22), although most of them showed developmental abnormalities rather than destruction of normal lung. Therefore, these animal models may be considered not to correspond to the pathogenesis of pulmonary emphysema in human.

Klotho mice which have a defect in the *klotho* gene expression, show age-related disorders, such as atherosclerosis, osteoporosis, skin atrophy, and infertility, and also develop pulmonary emphysema spontaneously (10,20). Homozygous Klotho mice grow normally up to 2 weeks of age. Emphysematous changes appear from 4 weeks of age. In addition to morphologic change,

they also show impaired respiratory function (20). While Klotho mice are unique model for pulmonary emphysema, the precise mechanism is not clear.

In this study, to investigate the balance of proteinase-antiproteinase in the mechanism of pulmonary emphysema in Klotho mice, we evaluated the expressions of MMP-2, 9, and TIMP-1, 2 in the development of Klotho mice lung.

MATERIALS AND METHODS

Animals

We used homozygous mutant Klotho mice (KL^{-/-}) and wild type mice (WT) at 2 and 5 weeks of age. All of them were a kind gift from Dr. Nabeshima of Kyoto University. KL^{-/-} were determined by southern blot analysis and polymerase chain reaction.

Tissue Preparation and Histological Analysis

KL^{-/-} and WT were anesthetized by intra peritoneal injection of pentobarbital and their lungs were perfused with heparinized physiological saline from the right ventricle. Lungs were stored at -80°C until protein extraction. For histological analysis, they were fixed by intratracheal instillation of 4% paraformaldehyde at a constant pressure of 15 cmH₂O. Then paraffin-embedded tissues were prepared, sectioned at 4 µm thickness and stained with hematoxylin and eosin. For immunohistochemical staining, the sections were incubated with phosphate buffer solution containing 1% bovine serum albumin for 10 minutes at room temperature. After washing with phosphate buffer solution, they were incubated overnight at 4°C with monoclonal anti-mouse MMP-9 antibodies (MMP-9 (Ab-2), Oncogene Research Products, Cambridge, MA, USA). The sections were then treated with 0.3% H₂O₂ in methanol for 12 minutes at room temperature to eliminate endogenous peroxidase activity. Then they were processed using the avidin-biotin-peroxidase complex method (DACO LSAB+Kit, Carpinteria, CA, USA).

Protein Extraction

Protein was extracted from the lungs for western blotting and gelatin zymography. Lungs from KL^{-/-} and WT were homogenized for 30 seconds with homogenate buffer (50 mM Tris-HCl pH 7.4, 300 mM NaCl, 1% Triton X-100, 2.5% glycerol, 5 mM CHAPS, 1 mM EGTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin, twice weight for the lung tissue of KL^{-/-} and WT). Then the homogenates were sonicated for 15 seconds and centrifuged at 40,000x g for 2 hours at 4°C. The concentration of the supernatants was analyzed by a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) according to the Bradford protein analysis method and adjusted equally to levels of 1 mg/ml in total protein concentration for each sample.

Gelatin Zymography

The gelatinolytic activity of the extracted protein solution from the lungs of KL^{-/-} and WT was analyzed by gelatin zymography. A total of 75 µg of protein extract was separated by a 10% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 for 15 minutes at room temperature. Then the gel was incubated with reaction buffer (50 mM Tris-HCl, pH 7.4, and 5 mM CaCl₂, 0.1% Brij-35) for 24 hours at 37°C. After staining with Coomassie brilliant blue and destaining in 20% methanol and 10% acetic acid, gelatinolytic activity was identified as clear band. Molecular weights of the bands were estimated through the use of prestained molecular-weight markers.

Western Blot Analysis

To investigate the expression of MMP-2, 9 and TIMP-1, 2, western blotting was performed. Each 75 µg of extracted protein was boiled for 3 minutes with SDS gel loading buffer. In the cases of MMP-2 and MMP-9, the samples were purified by gelatin-sepharose beads

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(gelatin-sepharose 4B, Bio-Rad Laboratories, Hercules, CA, USA) before boiling to remove the other proteins whose molecular weights were similar to MMPs. In brief, the samples were mixed with 20 μ l gelatin-sepharose beads and rotated for 4 hours at 4°C. After washing with phosphate buffer solution, and MMPs were eluted by 10% DMSO solution. The samples were electrophoresed through 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for MMP-2, MMP-9, and 15% SDS-PAGE gel for TIMP-1, TIMP-2 (the molecular weight of MMP-2, MMP-9 and TIMP-1, TIMP-2 were 72 kDa, 92 kDa, 25~36 kDa, and 20~24 kDa, respectively). The proteins were transferred to nitrocellulose membrane, blocked by 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with 1:1000 diluted anti-MMP-2 (MMP-2 (Ab-3), Oncogene Research Products, Cambridge, MA, USA), anti-MMP-9, anti-TIMP-1, and anti-TIMP-2 antibodies (TIMP-1 (H-150): sc-5538, TIMP-2 (H-140): sc-5539, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) in TBST for 1 hour at room temperature. After washing, the blots were incubated with 1:1000 diluted HRP-conjugated anti-mouse antibodies in TBST for 1 hour at room temperature. After washing with TBST, the membrane was incubated with detection reagents (ECL plus TM Western blotting detection reagents, Amersham Life Science, Buckinghamshire, UK) for 1 minute at room temperature and immediately exposed to X-ray film.

Bronchoalveolar Lavage (BAL)

Bronchoalveolar lavage fluid (BALF) was collected from the lungs of KL^{-/-} and WT at 5 weeks of age. The lungs were washed with 0.5 ml of phosphate buffer solution through the trachea for three times, and approximately 1 ml of lavage was consistently collected. The total cell count was measured with a hemocytometer. Smears of BALF were prepared using a Cytospin II (Shandon, Runcorn, UK) and stained with Diff-Quick solution for differential cell counts. The percentages of cell differentials were determined by counting at least 200 cells under light microscopy.

Data Analysis

Zymograms and Western blots were scanned, and the relative intensities of bands were determined by densitometry analysis. Statistical analysis was carried out by use of a commercially available program (STATVIEW, SAS Institute Inc, NC, USA), and differences were calculated by Fisher's tests and considered significant at P<0.05. The results are presented as means $\pm$ SEM.

RESULTS

Histological and Immunohistochemical Analysis

On hematoxylin and eosin staining, there were no apparent abnormalities in the lungs of WT and KL^{-/-} mice at 2 weeks of age. (Fig. 1A, B) At 5 weeks of age, KL^{-/-} mice but not WT showed obvious emphysematous changes without inflammatory cell infiltration and interstitial fibrosis (Fig. 1C, D). Immunohistochemical staining revealed that MMP-9 expression was increased in KL^{-/-} compared with WT at 5 weeks of age (Fig. 1E, F).

BAL

The total cell number of BALF in WT was more abundant than that in KL^{-/-} ($11.6 \pm 3.5 \times 10^4/\text{mm}^2$, $7.0 \pm 4.0 \times 10^4/\text{mm}^2$, respectively. P=0.02). Differential cell counts showed that neutrophil increased in KL^{-/-} compared with WT ($8.5 \pm 9.5\%$, 0% , respectively. P=0.03) (Table I). Immunohistochemical staining using non-specific IgG in the lungs of KL^{-/-} at 5 weeks of age showed no significant staining (Fig. 1G).

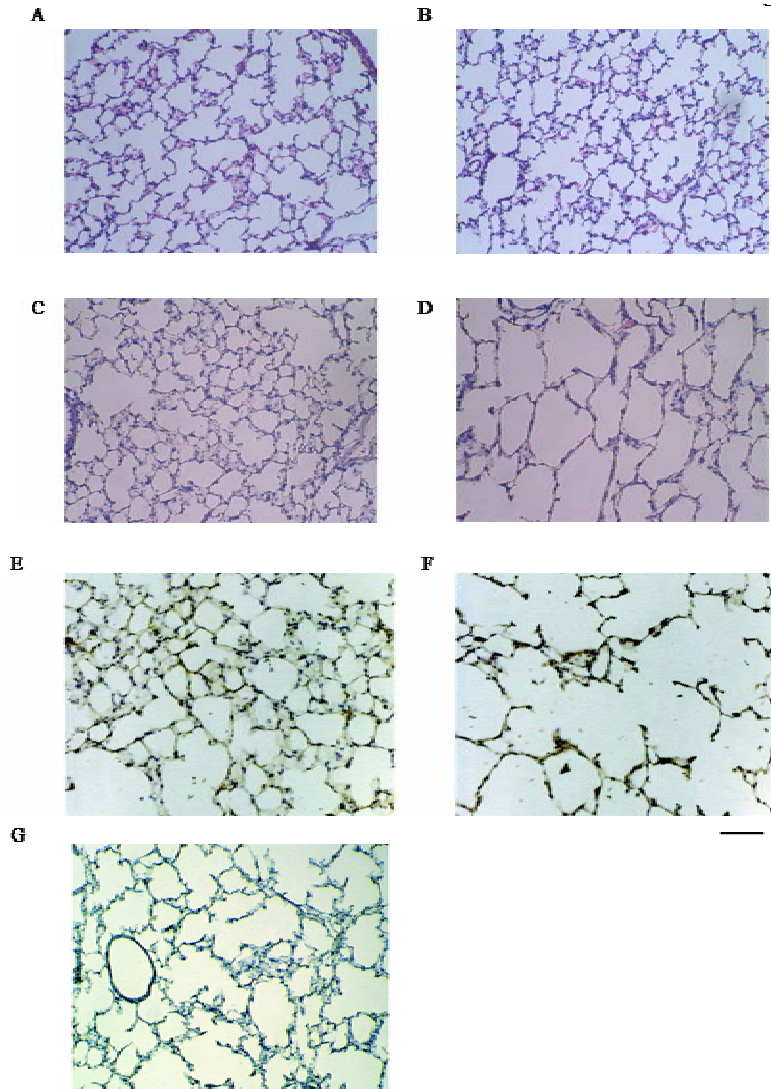


Fig. 1 Histological analysis of the lungs of WT and $KL^{-/-}$ mice. Mouse lungs of WT (A) and $KL^{-/-}$ (B) at 2 weeks of age, and WT (C) and $KL^{-/-}$ (D) at 5 weeks of age were stained with hematoxylin and eosin. Immunohistochemical staining of MMP-9 at 5 weeks of age in the lungs of WT (E), $KL^{-/-}$ (F). Immunohistochemical staining using non-specific IgG in the lungs of $KL^{-/-}$ at 5 weeks of age (G) Bar = 100 μ m

Table I. BALF analysis at 5 weeks of age.

	Total cells ($\times 10^5$)	Macrophage (%)	Neutrophil (%)	Lymphocyte (%)	Eosinophil (%)
WT	11.6 ± 3.5	99.5 ± 0.4	0	0.66 ± 0.44	0
$KL^{-/-}$	$7.0 \pm 4.0^*$	87.8 ± 8.6	$8.5 \pm 9.5^{**}$	3.7 ± 4.7	0.1 ± 0.2

Cell numbers in BALF of WT and $KL^{-/-}$ mice were counted and analyzed.

Values are means $\pm$ SD of 5 separate experiments.

* $p < 0.05$ compared with WT mice. ** $p < 0.01$ compared with WT mice.

Gelatin Zymography

To investigate the activity of MMP-2 and MMP-9 in the lungs of $KL^{-/-}$ and WT, we performed gelatin zymography of the extracted protein from their lungs. At 5 weeks of age, gelatinolytic activity of MMP-9 in the lungs of $KL^{-/-}$ increased significantly compared with WT. However there was no significant difference in MMP-2 activity between $KL^{-/-}$ and WT (Fig. 2).

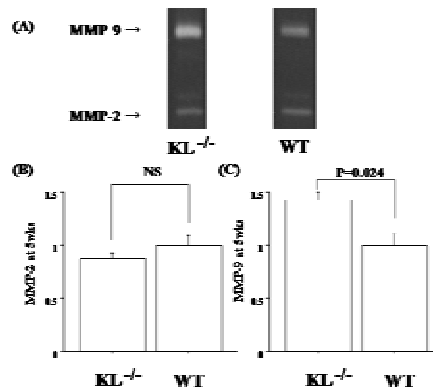


Fig. 2 (A) Gelatin zymography in lung tissue of WT and $KL^{-/-}$ mice collected at 5 weeks of age. Gelatin-sepharose purified fraction from 75 μ g of each lung extract was electrophoresed on 10% polyacrylamide gel containing 1mg/ml gelatin. Areas of MMP-2 and MMP-9 activities were seen as clear bands stained with Coomassie blue. The intensities of MMP-9 (B) and MMP-2 (C) are quantified and analyzed. Values are means $\pm$ SE of 5 separate experiments.

Western Blot

Western blotting of the extracted protein from the lungs of $KL^{-/-}$ and WT revealed that there was no significant difference of MMP-9 expression between $KL^{-/-}$ and WT at 2 weeks of age (Fig. 3A), and that MMP-9 expression was increased in $KL^{-/-}$ compared with WT at 5 weeks of

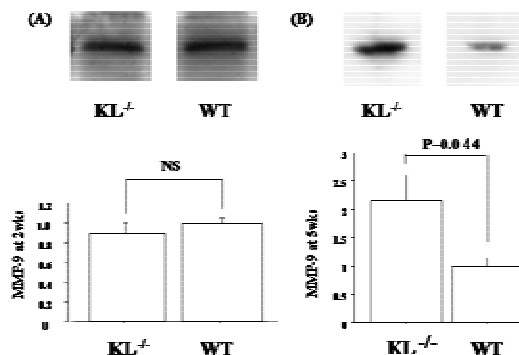


Fig. 3 Western blotting analysis for MMP-9 in the lung tissue of WT and $KL^{-/-}$ mice collected at 2 weeks (A) and 5 weeks (B) of age. Gelatin-sepharose purified fraction from 75 μ g of each lung extract was electrophoresed and blotted with anti-MMP-9 antibody. The intensity of MMP band is quantified and analyzed. Values are means $\pm$ SE of 5 separate experiments.

age (Fig. 3B). MMP-2 expression showed no significant differences at 5 weeks of age (Fig.4).

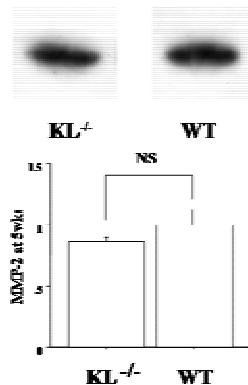


Fig. 4 Western blotting analysis for MMP-2 in the lung tissue of WT and KL^{-/-} mice collected at 5 weeks of age. Gelatin-sepharose purified fraction from 75 µg of each lung extract was electrophoresed and blotted with anti-MMP-2 antibody. The intensity of the MMP-2 band is quantified and analyzed. Values are means ±SE of 5 separate experiments.

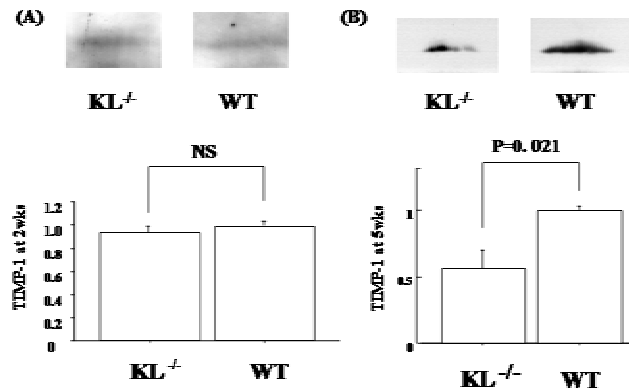


Fig. 5 Western blotting analysis for TIMP-1 in the lung tissue of WT and KL^{-/-} mice collected at 2 weeks (A) and 5 weeks (B) of age. 75 µg of each lung extract was electrophoresed and blotted with anti-TIMP-1 antibody. The intensity of the TIMP-1 band is quantified and analyzed. Values are means ±SE of 5 separate experiments.

On the other hand, while TIMP-1 expression showed no significant differences at 2 weeks of age (Fig. 5A), TIMP-1 expression decreased in KL^{-/-} compared with WT at 5 weeks of age (Fig. 5B). TIMP-2 expression showed no significant differences at 5 weeks of age between KL^{-/-} and WT (data not shown).

DISCUSSION

Klotho mice are unique animal model of pulmonary emphysema because of their association with aging, needlessness of exogenous proteinases, and lack of developmental abnormalities. Concerning with the mechanism of pulmonary emphysema of Klotho mice, Suga *et al.* reported that expression of mRNA of surfactant protein A and type IV collagen was

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increased (20). However, it may be a compensatory response to the destructive changes of the lung, and the pathogenesis of pulmonary emphysema in Klotho mice is still unclear. The imbalance of proteinase and antiproteinase is considered to be associated with the pathogenesis of pulmonary emphysema, therefore we evaluated the expression of MMP-2, 9 and TIMP1, 2 in the lungs of Klotho mice.

This study demonstrated that the expression of MMP-9 increased and TIMP-1 decreased with development of emphysema in the lungs of Klotho mice at 5 weeks of age. However, MMP-9 and TIMP-1 showed no significant difference between Klotho and wild type mice at 2 weeks of age when the emphysematous changes were not apparent. The expression of MMP-2 showed no significant difference, therefore these proteins seem not to be important for pulmonary emphysema in Klotho mice.

MMPs are family of matrix-degrading proteinases that have a zinc ion in the molecule, and TIMPs are endogenous inhibitors of MMPs. TIMPs inhibit MMPs by binding to them, and TIMP-1 and TIMP-2 inhibit MMP-9 and MMP-2 respectively (19,23). This study demonstrated that imbalance of MMP-9 and TIMP-1 existed in the lungs of Klotho mice, and suggested an imbalance between proteinases and antiproteinases play some roles in the pathogenesis of pulmonary emphysema in Klotho mice. This study also showed decrease of TIMP-1 expression. It is reported that TIMPs can modulate proliferation of various kinds of cells such as fibroblasts (3). It is possible that decreased expression of TIMP-1 may affect the proliferation of the cells that are essential to maintaining the normal structure of the small airway.

Apparent inflammatory cell infiltration was not seen in histological analysis. However, the total cell counts in the BALF of wild type was more abundant than that of Klotho mice, and the ratio of neutrophils in the BALF slightly increased in Klotho mice. As far as total cell counts, the difference of the lung size between Klotho mice and wild type may be affected the data, because Klotho mice have growth retardation (10). Compared with the BALF of human COPD patients (1,17), the ratio of neutrophils in BALF of Klotho mice may be slightly larger. MMP-9 is predominantly produced by alveolar macrophages, but is also released by neutrophils in the patients with COPD (1,15). It is possible that neutrophils play a role in the pathogenesis of pulmonary emphysema of Klotho mice. While we tried to measure the MMP activities in BALF, we could not detect clear bands (data not shown). To investigate the role of the alveolar macrophages and neutrophils, it may be necessary to culture them with some cytokines and chemical mediators.

Klotho gene expression is observed mainly in the kidney, but is not detected in the lung (10). Klotho protein is a single-pass membrane protein (13) and considered to function as a circulating hormonal factor. Since its actual function is not clear, it is difficult to understand the relationship between the lack of the Klotho protein and the changes in expression of MMP-9 and TIMP-1 expression in the lungs of Klotho mice. However, according to recent studies, there are a few possible hypotheses. Klotho mice show decreased vasodilatation response to acetylcholine and impaired nitric oxide production (18). It is reported that inhibition of VEGF receptor which is essential for the survival of endothelial cells causes pulmonary emphysema (9). Moreover, some articles showed that nitric oxide has a protective role in the lung of COPD patients (21), and that inhibition of nitric oxide causes to increase MMP-9 expression (5). One possible explanation is that endothelial dysfunction may affect the balance of MMP-9 and TIMP-1 in the small airway of Klotho mice. Another study reported overactivation of calpain in the lung of Klotho mice (12). Calpain is one of calcium-dependent cysteine proteinases present in a variety of cells and mediates proteolysis of various cellular proteins including activation NF-kappa B through degradation of I-kappa B (2). Since

activation of NF-kappa B induces expression of MMP-9 in bronchial epithelial cells (8), it may be possible that lack of Klotho protein may indirectly increase the expression of MMP-9 in the lung of Klotho mice. Further studies need to clarify this mechanism.

In conclusion, this study suggests that an imbalance of MMP-9 and TIMP-1 is associated with pulmonary emphysema in Klotho mice. Due to a unique feature of pulmonary emphysema for their spontaneous development and association with aging, Klotho mice may provide us more actual information about the mechanism of pulmonary emphysema compared with previous models.

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