論文の内容の要旨

The investigation of the pulmonary pathophysiology caused by klotho protein insufficiency

klotho 蛋白欠損に伴う肺の病態生理に関する検討

指導教員名　大内 尉義 教授

東京大学大学院医学系研究科

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石井　正紀

Background

Many features of age-dependent alterations in the respiratory systems of relatively healthy people have already been elucidated. 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. 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. However, in the lung, there have been conflicting reports regarding the features of airspace enlargement in homozygous mutant klotho (*KL–/–*) mice. Suga and coworkers have demonstrated the histology of the lungs in *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, the previous study showed 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 *KL–/–* 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, 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.

Emphysema is currently defined as a condition of an abnormal, permanent enlargement of lung airspaces distal to the terminal bronchiole, accompanied by the destruction of the alveolar walls without obvious fibrosis. Before the development of human emphysema, the lungs in smokers exhibit an accumulation of macrophages, lymphocytes and neutrophils in the respiratory bronchioles, alveolar ducts and alveoli. These chronic inflammatory responses contribute to the development of emphysema. However, not all smokers necessarily develop clinically significant emphysema, thus suggesting that other various factors affect the susceptibility to the inhaled noxious agents.

Aging is thought to be associated with these important factors. The prevalence of COPD increases with age and smoking is a potent promoter of premature aging. Recent findings also indicate the existence of alveolar cell senescence in patients with emphysema. However, these observations do not indicate whether premature aging contributes to the pathogenesis of emphysema, or whether it only occurs incidentally in association with smoking. Animal models of premature aging would thus be very useful to clarify these questions.

Several studies using *KL-/-* mice showed that increased vitamin D activity would be a major cause of features of premature aging. These phenotypes were rescued by normalizing vitamin D activity. Vitamin D has been reported to inhibit cell proliferation and induce apoptosis in normal and malignant cells. As vitamin D receptors are reported to be expressed in lung tissues, we thought that

the function of vitamin D signaling in lung tissues was very interesting. Although klotho protein functions as a transmembrane protein that binds FGF-23 predominantly in kidney and brain, the effects of secreted klotho protein in lung tissues had not been fully elucidated in association with abnormal vitamin D metabolism.

It has been reported that in *KL–/–* mice, α-2spectrin which is cytoskeletal elements is highly cleaved before the onset of age-related phenotypes. Alpha(II)-spectrin is degraded by a calcium-dependent cytosolic cysteine protease, μ-calpain. In *KL–/–* mice, μ-calpain was activated at an abnormally high level, and an endogenous inhibitor of calpain, calpastatin, was significantly decreased. Degradation of alpha(II)-spectrin increased with decreasing level of klotho protein. Interestingly, the abnormal activation of μ-calpain and decreased level of calpastatin were observed especially in lungs of klotho deficient mice. In addition, similar phenomena were observed in normal aged mice. These findings indicate that the abnormal activation of μ-calpain may be caused by decrease of klotho protein.

Although the studies of breast cancer cells have shown that exposure of cells to high levels of active vitamin D (1,25-$(OH)_2D_3$) can have apoptotic effects via activation of μ-calpain, the studies using lung cells stimulated with vitamin D have not been investigated. In the current study, we examined the effects of secreted klotho protein as a humoral factor focusing on the relationships between increased vitamin D activity and activation of μ-calpain using alveolar cells.

While the aging lung and emphysema demonstrate clearly different histologies, they could share some common factors in the pathogenesis. The investigation of the susceptibility to inhaled noxious

agents in klotho-mutant mice would be important to evaluate this linkage. To understand the pathogenesis of emphysema, elastase-inducible emphysema of mice had been established as a useful animal model. Intratracheal instillation of elastase first induces lung inflammation and subsequently resulted in alveolar wall destruction in mice. These pathological changes closely resemble those in human emphysema. This study therefore investigated the susceptibility of klotho-insufficient mice to intratracheal elastase.

Methods and materials

Animals

In the series of experiments reported here, all mice were generated from mating pairs of heterozygous mutant klotho mice (*KL* +/-). The mice were maintained at 24 ±2°C under an alternating 12-hr light and dark cycle. The procedures conformed to the guide for the care and use of laboratory animals from the National Institutes of Health, and they were approved by the University of Tokyo Institutional Review Board for Lab Animal Use.

Intratracheal elastase treatment

The mice weighing 19-21 g at 8 weeks of age were anesthetized with the intraperitoneal injection of ketamine and xylazine. A metal tracheostomy tube was inserted into the trachea. The mice were treated with a single intratracheal instillation of 0.5 IU (i.e. 25 IU/kg), 5.0 IU (i.e. 250 IU/kg) of porcine pancreatic elastase (PPE) in 100 µl of sterile saline solution, or 100 µl of saline alone. On days 7 and 21 after the treatment, the mice were sacrificed and further analyses were performed.

Lung function analysis

The lung function was measured with a whole body plethysmograph. A 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. Total lung capacity (TLC), vital capacity (VC), and pressure-volume curves indicating lung elasticity were calculated by a quasistatic pressure/volume maneuver. The lung elasticity was reported as the chord compliance (Cchord mL/cm H_2O), which represents the lung elastic recoil by means of the slope of compliance curve between 0 and 10 cm H_2O pressure. 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 (FEV100) was determined, and FEV100/VC was calculated for the index of the airway obstruction.

Bronchoalveolar lavage fluid (BALF)

BAL was performed using 1 ml of phosphate-buffered saline 5 times. After BALF was centrifuged, the total and differential cell counts of the BALF were determined. Thiobarbituric acid reactive substance (TBARS) of BALF, the biochemical marker of lipid peroxidation and elastase activity were measured. .

Histological evaluation of lungs

The removed lungs were inflated with 10% formalin neutral buffer solution (pH 7.4) at a constant pressure of 25 cmH_2O for 48 hr. The sections in the frontal plane at the depth of the hilum were

stained with hematoxylin and eosin. We measured the airspace size as indicated by the mean linear intercepts (MLI) and the destruction of alveolar walls as indicated by the destructive index (DI).

Assessment of apoptosis and proliferation in airway cells

To evaluate apoptosis, the tissue sections were stained using the in situ TUNEL method. The sections were subsequently counterstained with methyl green. The tissue sections that underwent TUNEL staining without counterstaining were also reacted with the primary antibodies against platelet endothelial cell adhesion molecule-1 (PECAM-1).

For the quantitative assessment of proliferation, immunohistochemical staining was performed using the monoclonal antibody against proliferating cell nuclear antigen (PCNA). The sections were counterstained with hematoxylin. Then, we performed double staining of the lung tissues by using PECAM-1 as mentioned above.

The number of TUNEL or PCNA positive cells per 100 alveolar epithelial cells was counted. We examined 20 randomly distributed fields. The apoptotic index (AI) and the proliferation index (PI) were determined by the percentage of the PECAM-1-negative cells, as previously described.

Immunochemistry for EGF

For the assessment of the distribution of epidermal growth factor (EGF) in airway cells, we performed the immunochemistry using lung tissues of *KL*-/- mice and wild-type (*KL*+/+) mice.

Immunoblotting

Western blotting was performed using the following antibodies recommended by the manufacturers: EGF, p-ERK1/2, μ-calpain and calpastatin.

Cell culture

We evaluated the cell-protective effects of recombinant klotho protein (1μg/μl) with or without FGF-23 (1μg/ml). We used rat normal type Ⅱ alveolar epithelial cells (L2 cells) in the presence of 1,25-$(OH)_2D_3$ (400nM for 24hrs) or hydrogen peroxide (H_2O_2) (50μM for 24hrs).

Assessment of cell death rate induced by vitamin D (1,25-$(OH)_2D_3$) or H_2O_2

The percentages of dead cells were quantified by propidium iodide and Hoechst 33258 staining. We calculated the percentages of propidium iodide positive cells in Hoechst 33258 positive cells as the evaluation of the cell death rate.

Measurement of increase of intracellular calcium ion (Ca^{2+})

We measured the increase of in intracellular Ca^{2+} using the fura-2 fluorescence method. The Ca^{2+} fura-2 fluorescence of the suspended cells was measured by a spectrofluorometer. The excitation wavelengths were 340 and 380 nm, and emission was measured at 500 nm. The intracellular increase of Ca^{2+} was evaluated by fluorescence ratio as indicated by Fex.340nm/Fex.380nm.

Results

Firstly, we investigated the structural, functional, biochemical, and cell kinetic alterations of lungs in *KL–/–* mice at 2–12 weeks of age. Our data showed that airspace size significantly increased at 4 weeks and progressed gradually with age up to 12 weeks, which is almost the entire life span of *KL–/–* mice. The airspace size of the lungs as indicated by the 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. The alveolar wall destruction as indicated by DI was significantly higher in *KL–/–* mice than in other genotypes. However, even at 12 weeks of age, the DI in *KL–/–* mice was not >10%. The error bars for DI are approximately the same size regardless of the mean DI value.

Lung function was also altered in *KL–/–* mice. The lung function study demonstrated that FEV100/VC (%) and lung elastic recoil as indicated by Cchord (mL/cm H_2O) 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. There was no significant difference in lung function between *KL+/–* and *KL+/+* mice. 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.

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 age was approximately 6 times greater in *KL–/–* mice than in mice of other genotypes. However, most of the apoptosis was observed in airway cells, but not in endothelial cells, in the *KL–/–* mice. 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, *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. However, lipid

peroxidation and elastase activity of lungs were not increased in the BALF study of *KL–/–* mice. In addition, the total cell count in BALF in mice of all genotypes was <2 x 10^5 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.

We performed immunohistochemical and Western blot analysis for EGF and p-ERK1/2 as the possible mediators to the pathophysiology of the lungs in *KL–/–* mice. The intensity of immunostaining of EGF was greater in the lung tissues of *KL+/+* mice than in the tissues of *KL–/–* mice. Lower levels of EGF protein were detected in *KL–/–* mice than in *KL+/+* mice. 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.

On the other hand, to investigate the susceptibility of klotho insufficiency to inhaled noxious insults, *KL+/-* and *KL+/+* mice were treated with elastase to develop emphysema for the functional, biochemical and cell kinetic analysis. A lung function analysis showed significantly more increased static lung compliance, significantly more decreased FEV100/VC, and Cchord, and significantly more increased RV/TLC in *KL +/-* mice in comparison to *KL+/+* mice. These findings were compatible with emphysema. Both the enlargement of airspaces and the destruction of alveolar walls after the PPE treatment were also significantly more prominent in *KL+/-* mice than in *KL+/+* mice. Thiobarbituric acid reactive substances, reflecting the lipid peroxidation, were significantly more increased in the BALF of *KL+/-* mice on day 21 after the PPE treatment, suggesting the contribution of anti-oxidative effect of klotho protein. Furthermore, both biochemical and

cell-kinetic analyses showed significantly more prominent inflammatory cell accumulations in the BALF and more prominent cellular DNA damage on day 7 after the lower- concentration elastase treatment (0.5 IU) in the lungs of the *KL* +/- mice, preceding the emphysematous changes. No significant differences were observed in the evaluation of inflammatory cell accumulation and cellular DNA damage at the higher concentrations of elastase (5 IU) between the two genotypes.

Klotho protein, regardless of the presence of FGF-23, reduced the alveolar cell death induced by 1,25-$(OH)_2D_3$ or H_2O_2. Western blotting analysis showed that the 1,25-$(OH)_2D_3$ -induced and H_2O_2-induced cell death might be characterized by the increased level of μ-calpain and decreased level of calpastatin. Although klotho protein did not prevent the activation of μ-calpain caused by H_2O_2, it reduced the activation of μ-calpain caused by 1,25-$(OH)_2D_3$. We confirmed that 1,25$(OH)_2D_3$ induced the increase of intracellular Ca^{2+} which might trigger the activation of μ-calpain and that the klotho protein reduced the increase of intracellular Ca^{2+} caused by 1,25$(OH)_2D_3$.

Conclusion

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 *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 *KL–/–* mice will provide a unique insight into the relationship between the aging lung and airway cell kinetics in association

with premature aging and apoptosis.

In addition, we concluded that *KL*+/- (klotho protein insufficiency*)* mice were very susceptible to elastase, comparing with *KL*+/+ mice. While the previous investigations had indicated that the increased vitamin D activation contribute to the premature aging in *KL*-/- mice, the serum level of phosphorus or 1,25-$(OH)_2D_3$ was not so high in *KL*+/- mice, thus suggesting that the susceptibility to elastase-induced emphysema would therefore be associated with other functions of klotho protein. Moreover, the present study suggested that klotho protein might play a role as a humoral factor in lung tissues to reduce cell death caused by increased level of vitamin D in association with aging.