

## Zinc Deficiency may Accelerate Aging by Inhibiting Klotho mRNA Expression

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### Summary

**Background**—Zinc (Zn) is critical for the functional and structural integrity of cells, in which it participates in a number of processes, including gene expression. Zn deficiency has effects that resemble the symptoms of human aging, including low immunity, dermatitis, delayed wound healing, and impaired taste. In this study, we examined the effect of Zn deficiency on the expression of the aging-related gene Klotho in rats. In mice, Klotho is involved in a murine developmental syndrome that resembles human aging.

**Methods**—Male Sprague-Dawley rats (161–162 g) were fed a diet deficient in Zn or containing 0.01 % Zn for 4 weeks. Kidneys were removed after 0, 1, 2, 3 and 4 weeks, and the expression of Klotho mRNA was determined using real-time polymerase chain reactions.

**Results**—Data were analyzed using the comparative threshold (CT) method. The mean  $\Delta\Delta\text{CT}$  values for renal Klotho mRNA expression were much lower in the Zn-deficient rats than in the control rats. In the Zn-deficient group, values of  $\Delta\Delta\text{CT}$  were  $-1.15 \pm 0.46$  at 2 weeks,  $-1.69 \pm 0.33$  at 3 weeks, and  $-2.08 \pm 0.53$  at 4 weeks.

**Conclusion**—Zn deficiency was associated with decreased Klotho gene expression, suggesting that Zn deficiency might accelerate aging by inhibiting the expression of Klotho.

### Introduction

Klotho, an anti-aging protein expressed primarily in the kidney, is named after the goddess who spins the threads of life. As reported first by Kuro-o *et al.*<sup>1)</sup>, mice lacking the Klotho gene exhibit multiple aging-like phenotypes, such as arteriosclerosis, ectopic calcification in various soft tissues, osteoporosis, growth retardation, infertility, skin atrophy, premature thymic involution, muscle atrophy, and premature death at 2 months of age. These aging-like phenotypes are similar to those observed in zinc (Zn) deficiency<sup>2–4)</sup>, which induces dysgeusia, growth retardation, thymus atrophy, alopecia, atrophy of the testis, and other symptoms<sup>5,6)</sup>. Prasad *et al.*<sup>5)</sup> first characterized human dietary Zn deficiency in 1963. Today, the worldwide prevalence of dietary Zn deficiency is believed to be more than 20 %. Indeed, it is reported 25 % in Japanese elderly people<sup>7)</sup>.

Zn is an essential trace element that is important for the

activities of many enzymes, including alkaline phosphatase, carbonic anhydrase, angiotensin I-converting enzyme<sup>8)</sup>, and Cu/Zn superoxide dismutase (SOD), an antioxidant enzyme. Cu/Zn SOD plays a critical role in the defense against reactive oxygen species, which are thought to be involved in the cellular modifications leading to senescence, but whether Zn deficiency actually induces oxidative stress resulting in accelerated aging is controversial. Zn deficiency might affect the expression of anti-aging proteins, such as Klotho. Many people are thought to be at risk of Zn deficiency in both developing and developed nations, including the United States<sup>9)</sup>, Europe<sup>10,11)</sup> and Japan<sup>7)</sup>.

### Materials and Methods

#### 1. Zn-deficient rat model

Male Sprague-Dawley rats (100 ± 5 g) were obtained from Tokyo Laboratory Animal Science Co., Ltd. (Tokyo,

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Japan). They were acclimated for 1 week before use; they were housed separately in a temperature-controlled room with a 12-h light/dark cycle and fed a standard diet. During the subsequent 4-week experiment, Zn levels were controlled by providing the rats with a isocaloric Zn-deficient diet (ZnD;  $n = 10$ ) or an isocaloric control diet containing 0.01% Zn (Cont;  $n = 10$ ), as reported previously<sup>6,7</sup>. As shown in Table 1, the Zn-deficient and control diets were otherwise identical in composition in terms of protein, carbohydrate, fat, vitamin, and mineral levels. The rats had *ad libitum* access to distilled water.

For the analysis of Klotho gene expression in kidneys, rats were placed under deep anesthesia with diethyl ether and their left kidneys were removed at time 0 (pre-experiment), at 1 week, 2 weeks, 3 weeks and 4 weeks after fed with the zinc deficient diet or the control diet. Kidney samples were stored in a freezer at  $-80^{\circ}\text{C}$  until sample preparation for analysis. Experimental protocols for the animal study were approved by the Animal Research Committee of Saitama Medical University.

## 2. Total RNA isolation and realtime polymerase chain reaction (PCR)

Total RNA from the cortex of the left kidney was extracted using a commercial kit (Isogen; Wako Pure Chemical Inc., Osaka, Japan), according to the manufacturer's instructions. Approximately 50 mg of kidney cortex was homogenized in 1 mL of Isogen reagent in a microcentrifuge tube. Then, 200  $\mu\text{L}$  of chloroform was added, and the samples were vortexed for 30 s. After centrifugation, the aqueous phase was transferred to a new microcentrifuge tube containing 0.7 vol of isopropanol, and the RNA was recovered by centrifugation at 15,000 rpm for 15 min at  $4^{\circ}\text{C}$ . The pellet was washed in cold 70 % ethanol, centrifuged, dried in a vacuum centrifugal evaporator (Tomy MV-100; Tomy Digital Biology Co., Ltd., Tokyo, Japan) for 10 min, and then dissolved in 50  $\mu\text{L}$  of diethyl pyrocarbonate-treated water. The concentration of dissolved RNA was estimated based on the optical absorbance at 260 nm.

One microgram of total RNA was subjected to first-strand cDNA synthesis in a 20- $\mu\text{L}$  reaction mixture using a commercial kit (iScript cDNA Synthesis kit; Bio-Rad Laboratories Inc., Tokyo, Japan). Next, 2  $\mu\text{L}$  of the synthesized cDNA was mixed with real-time PCR reagent (iQ SYBR Green Supermix; Bio-Rad) and specific primers for Klotho (forward, 5'-CGT GAA TGA GGC TCT GAA AGC-3'; reverse, 5'-GAG CGG TCA CTA AGC GAA TAC G-3') or glyceraldehyde phosphate dehydrogenase (GAPDH; forward, 5'-AAT GCA TCC TGC ACC ACC AA-3'; reverse, 5'-GTA GCC ATA TTC ATT GTC ATA-3') in a total volume

of 50  $\mu\text{L}$ . PCR was carried out in a thermal cycler (iCycler iQ System; Bio-Rad) for 40 cycles of  $94^{\circ}\text{C}$  (30 s),  $60^{\circ}\text{C}$  (30 s), and  $72^{\circ}\text{C}$  (60 s).

Relative quantification of the Klotho and GAPDH mRNA was performed using the comparative threshold (CT) method. The  $2^{-\Delta\Delta\text{CT}}$  method provides a relative quantification ratio according to a calibrator that allows statistical comparisons of gene expression among samples. The CT values for the reference (GAPDH) and target (Klotho) genes in each sample set were determined according to the  $2^{-\Delta\Delta\text{CT}}$  method, and changes in mRNA expression levels were calculated after normalization against GAPDH expression.

## 3. Measurement of serum Zn levels

For determination of serum zinc, blood was removed at 4 weeks after fed with zinc deficient diet or control diet. Serum Zn levels were determined using an atomic absorption spectrophotometer (Z-6100; Hitachi Co., Tokyo, Japan), as previously reported<sup>7</sup>. After 4 weeks of the experimental or control diet, rats were anesthetized under diethyl ether. Whole blood was removed from the vena cava and centrifuged for 3,500 rpm in centrifuge tubes previously washed with acid solution to remove trace metals. The resulting serum samples were transferred into acid-washed tubes, diluted with ion-exchange-purified water, and aspirated into the atomic absorption spectrophotometer. Zn levels were then determined based on the optical absorbance at 213.9 nm.

## 4. Statistical analysis

All results are expressed as the mean  $\pm$  standard error of the mean (SEM). Statistical evaluation was performed using analysis of variance (ANOVA) and Student's *t*-test with or without Bonferroni correction. Differences were considered statistically significant at  $p < 0.01$ .

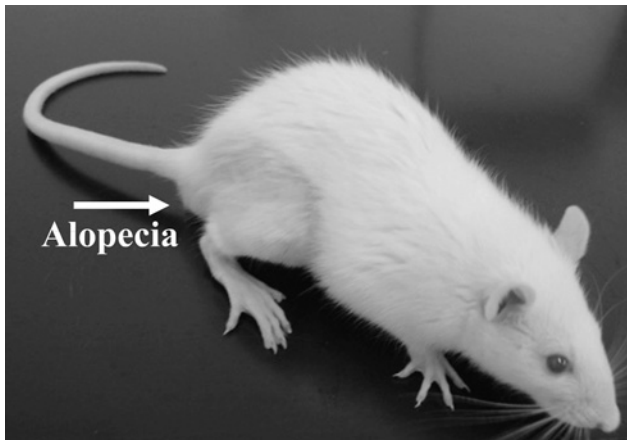
## Results

Rats fed a Zn-deficient diet exhibited mild alopecia after 4 weeks (Fig. 1). Other zinc deficient characteristic symptoms were not seen in Zn-deficient group.

The body weights of the control rats increased gradually from 0 to 4 weeks, but in the Zn-deficient group, body weights did not change over the course of the experiment (Table 2).

As shown in Fig. 2, in rats fed the Zn-deficient diet, serum Zn levels at 4 weeks decreased dramatically compared with the control rats, the difference between groups was significant at  $p < 0.0001$ .

In the mRNA expression of Klotho assessed by real-time



**Fig. 1** Characteristic alopecia in Zn-deficient rats. Rats fed a Zn-deficient diet for 4 weeks developed alopecia. The arrow indicates an area of alopecia on the right thigh.

**Table 1** Special diet for zinc deficiency

Components	%
Carbohydrate	65.7
Protein	20.0
Fat	10.0
Minerals	3.13
Vitamins	1.17

Control diet contains 0.01 % zinc

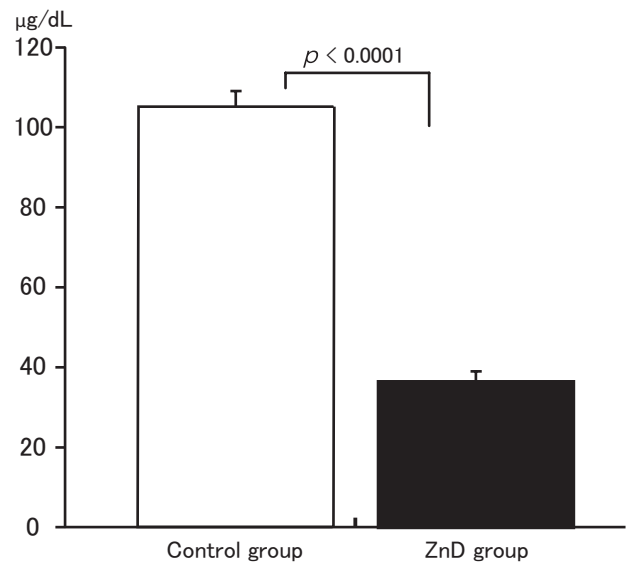
**Table 2** Changes of body weight during experiment

	Control group Means $\pm$ SD (n = 10)	Zn group Means $\pm$ SD (n = 10)	p values
Pre-experiment	155.0 $\pm$ 9.0	155.0 $\pm$ 7.8	1.0
1 week	154.8 $\pm$ 5.0	150.8 $\pm$ 4.6	0.0496
2 weeks	168.3 $\pm$ 5.7	161.0 $\pm$ 5.2	0.0007
3 weeks	174.2 $\pm$ 8.3	164.7 $\pm$ 5.7	0.0001
4 weeks	183.2 $\pm$ 8.0	163.3 $\pm$ 6.4	0.0001

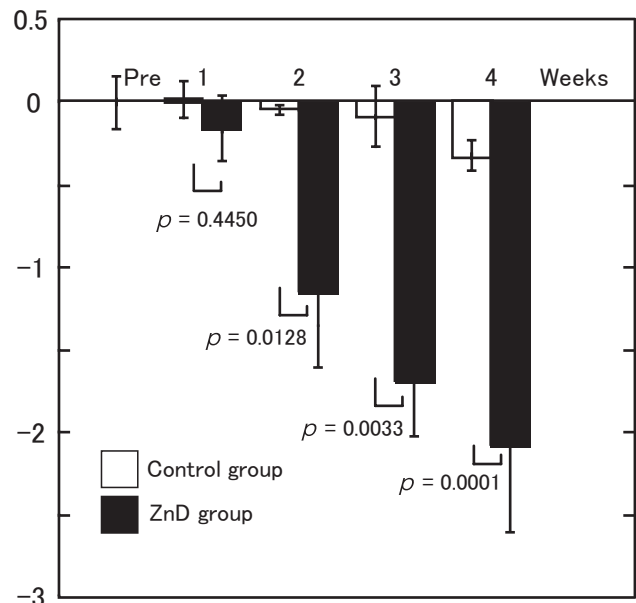
PCR, the Zn-deficient group expressed significantly less Klotho mRNA than the control group did at 2 weeks after fed a zinc deficient diet (Fig. 3). At 4 weeks after fed, expression level of Klotho mRNA was further decreased compared with the levels of control rats.

## Discussion

The kidney is the predominant site of Klotho expression, and patients with chronic renal failure exhibit dramatically decreased Klotho expression. Although Klotho is known to be expressed at particularly high levels in cells of the distal convoluted tubule<sup>1)</sup>, its function in the kidney is poorly understood. Renal Klotho mRNA expression is inhibited through an angiotensin-II (AG-II) mediated mechanism



**Fig. 2** Serum Zn levels in rats fed Zn-deficient and control diets. The data are expressed as means  $\pm$  SEM of five independent experiments.



**Fig. 3** Expression of Klotho mRNA in rat kidney as determined by real-time PCR.  $\Delta\Delta CT$  values are expressed as the mean  $\pm$  SEM of five independent experiments.

that might be a useful target for therapeutic intervention in AG-II-mediated end-stage renal disease. AG-II is critical factor in cardiac hypertrophy, in which myocytes are lost through apoptosis. AG-II-induced apoptosis is caused by an increase in  $Ca^{2+}$ /calmodulin-dependent protein kinase activity mediated by reactive oxygen species<sup>12)</sup>. The AG-II and reactive oxygen species could be involved with decreases of Klotho mRNA expression.

Zn deficiency has been reported to reduce the activity of Cu/Zn SOD<sup>6,13)</sup>. In mice, defects in Klotho gene expression result in a syndrome that resembles human aging<sup>1,14)</sup>, with symptoms including a shortened lifespan, infertility, arteriosclerosis, mitral annular calcification, skin atrophy, osteoporosis, and emphysema. Nabeshima *et al.*<sup>15)</sup> reported that Klotho is a key player in calcium and phosphate ho-

meostasis; Klotho-mutant mice suffer disorders such as hypercalcemia and hyperphosphatemia because of increased vitamin D biosynthesis and phosphate reabsorption in the kidney.

In this study using rats fed a special diet, Zn deficiency was associated with decreased expression of Klotho mRNA in the kidney. In rats fed a Zn-deficient diet, the mean serum Zn concentration decreased with time, reaching 23 % of its pre-experiment concentration after 4 weeks. After only 2 weeks of the Zn-deficient diet, the rats also exhibited decreased Klotho mRNA expression levels, suggesting that Zn may affect Klotho expression.

Skin lesions are a common manifestation of Zn deficiency in humans and animals. Cui *et al.*<sup>16)</sup> reported that dermatitis of the extremities, balanitis, stomatitis, and alopecia occur in male rats weaned on a Zn-deficient diet. However, in our experiments, characteristic alopecia occurred only after 4 weeks of the Zn-deficient diet (Fig. 1); perhaps the animals used by Cui *et al.* were more sensitive to Zn-deficiency because of their youth. In addition to characteristic alopecia, we also observed decreased serum Zn levels and growth retardation in our Zn-deficient rats; these symptoms are similar those previously described in Klotho-mutant mice<sup>1)</sup>.

When over-expressed, the Klotho gene suppresses aging and extends lifespan, and when under-expressed, it accelerates aging-like phenotypes. Recently, Yamamoto *et al.*<sup>17)</sup> reported that Klotho protein expression increases resistance to oxidative stress by inhibiting insulin/insulin-like growth factor-1 (IGF-1) signaling. Bruno *et al.*<sup>18)</sup> demonstrated that Zn deficiency increases oxidative stress, partially by increasing cytochrome C activity and partially by reducing the level of hepatic  $\alpha$ -tocopherol. In addition, McNall *et al.*<sup>19)</sup> reported a 46 % decrease in levels of serum IGF-1 and in IGF mRNA expression in Zn-deficient rats relative to control rats. Insulin contains a Zn atom, suggesting that Zn-dependent genes and proteins activate IGF-1 and insulin, hormones known to regulate healthy aging.

Lifestyle and dietary habits, including Zn consumption, affect the aging process. Cell-mediated immune dysfunction and increased oxidative stress are common in elderly people. Taken together, this study illustrates the importance of nutrition, and Zn in particular, as a determinant of healthy aging.

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