Defects in growth and bone metabolism in *klotho* mutant mice are resistant to GH treatment

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Abstract

Klotho mutant (*kl/kl*) mice exhibit growth retardation after weaning, and previous electron microscopic examination of GH-producing cells in pituitary glands revealed a reduction in GH granules. However, it has not been known whether growth retardation in *klotho* mutant mice is related to the loss of GH function. We therefore examined whether treatment with GH could rescue the retardation of growth. At the end of 3 weeks of treatment with human GH, the body weight of wild-type (WT) mice was increased. In contrast, body weight was not increased in *klotho* mutant mice even after the treatment with human GH. Another feature of *klotho* mutant mice is the presence of osteopetrosis in the epiphyses of long bones

and vertebrae. Treatment with human GH increased trabecular bone volume in the epiphyseal region of WT tibiae. Interestingly, increase in trabecular bone volume by GH treatment was also observed in *klotho* mutant mice and, therefore, the phenotype of high bone volume in the *klotho* mice was further enhanced. These findings indicate that a GH receptor system in cancellous bones could operate in mutant mice. Thus, growth retardation in the *klotho* mutant mice is resistant against GH treatment even when these mice respond to GH treatment in terms of cancellous bone volume.

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Introduction

Klotho mutant mice were discovered based on their severe growth retardation, short lives and their phenotypes including emphysema, atherosclerosis, gonadal dysplasia, ectopic calcification, ataxia, skin atrophy, defects in hearing, thymic atrophy and defects in B-cell differentiation (Kuro-o *et al.* 1997, Lyons 1997, Roush 1997). Some of these manifestations resemble aging-associated phenomena. *Klotho* gene encodes a putative membrane protein (Matsumura *et al.* 1998, Shiraki-Iida *et al.* 1998) with two β -glucosidase like domains, called KL1 and KL2. However, its precise function has not yet been identified (Aizawa *et al.* 1998, Ohyama *et al.* 1998, Saito *et al.* 1998, 1999, Kato *et al.* 2000, Shapiro 2000, Suga *et al.* 2000).

The bones in *klotho* mutant mice are normal until weaning, but due to the growth retardation after weaning, their long bones are shorter than those in age-matched wild-type (WT) mice (Kuro-o *et al.* 1997). About 4 weeks after birth, cancellous bones in the metaphyseal regions of long bones as well as vertebrae in *klotho* mutant mice become

dense, based on X-ray analysis (Yamashita *et al.* 1998). Micro CT examination revealed that bone volume fraction in these regions in *klotho* mice is higher than that in the corresponding regions in the bones of WT mice (Kawaguchi *et al.* 1999, Yamashita *et al.* 2000a). A bone marrow ablation study indicated that new bone formation after bone marrow ablation in *klotho* mutant mice was observed within one week, similar to that seen in WT mice (Yamashita *et al.* 2000b). In WT mice, newly formed trabecular bones were resorbed within two weeks after bone marrow ablation. However, no such bone resorption was observed in *klotho* mutant mice, indicating the presence of a defect in bone resorption (Yamashita *et al.* 2000b). The osteopetrotic phenotype in bone is always observed to be associated with the onset of growth retardation and other phenotypes in *klotho* mutant mice.

The homozygous *klotho* mutant mice grow normally until weaning; however, their growth starts to retard 2–3 weeks after birth. Although this growth retardation appears similar to the phenotype in mice with growth hormone (GH) deficiency (Van Buul & Van den Brande

Figure 1 Growth of mice in response to human GH treatment. (a) The body weight of mice after treatment with human GH. The body weight was measured at the start point and the end point of the experiments and the ratios of the body weight were obtained. (b) The whole body length of mice. The body length was measured at the start point and end point of the experiments on the soft X-ray films, and the ratio of the body length was obtained. (c) The body composition of mice. The ratios of fat/body weight were determined using DEXA. (d) IGF-I mRNA levels in the liver of mice. RT-PCR was conducted using liver RNA prepared 24 h after GH injection. IGF-I mRNA data were normalized against GAPDH mRNA levels. Mice were prepared as described in Materials and Methods and were injected with GH or saline (CTRL) for 3 weeks. Asterisks indicate significant difference ($P < 0.05$). Means $\pm$ S.D.

1978), it has not been known whether any defects related to GH are involved in the retardation of the growth of *klotho* mutant mice except for the report that electron microscopic examination showed reduction in the secretory granules in GH-secreting cells in the pituitary glands (Kuro-o *et al.* 1997). Therefore, we examined whether treatment with GH could rescue retardation of growth in *klotho* mutant mice. We also examined whether GH treatment would affect osteopetrotic phenotype in the *klotho* mutant mice.

Materials and Methods

Human GH was a kind gift from Eli Lilly Co. *Klotho* mutant mice and WT mice were obtained by crossing heterozygote mice (*kl/+*) with a TA20 strain background with mutation in the *klotho* gene locus (C3H/J and C57BL/6J mixed background), as described previously. WT and *klotho* mutant mice were used for this study. Four-week-old mice were s.c. injected with 3 μ g/g of recombinant human GH per day for 3 weeks (one

Figure 2 X-ray pictures of the epiphyseal regions of tibiae. At the end of 3 weeks of treatment with GH or saline (CTRL), metaphyseal regions were subjected to X-ray examination. High radiopacity was observed in *klotho* mutant mice but not in WT mice and osteopetrotic changes were observed in *klotho* mutant mice regardless of the GH treatment.

injection per day, 5 days per week). This dosage of the GH was set according to the previously published data (Van Buul & Van den Brande 1978). As control, half of the mice in each of the groups were injected with saline. Body weight was recorded every day.

Body composition

We estimated the weight of fatty tissue in whole body by using a dual-energy X-ray absorptiometry (DEXA) apparatus (Pixi-MUS; Lunar, WI, USA).

X-ray and bone mineral density (BMD) analyses

X-ray pictures of tibiae were taken at 20 kV and 0.8 mA for 2 min after killing. BMD was measured using a small animal version of the Pixi-MUS DEXA apparatus. BMD was measured in the epiphyses of tibiae at their proximal ends (one-fifth of the total length) and in the diaphyses of tibiae (three-fifths of the total length).

Three dimensional (3D) micro-computed tomography (CT) analysis

The 3D trabecular bone analysis was conducted using a desk-top 3D micro-CT analysis system (Micro-CT20; Scanco Medical, Zurich, Switzerland), as described previously (Yamashita *et al.* 2000a). In brief, the bone samples were fixed with 70% ethanol after killing and the measurements were conducted in a globular region with a diameter of 0.80 mm in the epiphyses adjacent to (0.36 mm away from) the growth plates. Thresholds for measurement were set at 275 for 3D micro-CT analysis.

RNA preparation and semi-quantitative RT-PCR

Animals (three mice per group, total of 12 mice) were killed 24 h after the last GH or saline injection, and livers were collected. Total RNA was isolated according to the acid-guanidium-phenol-chloroform (AGPC) method. The first-strand DNA synthesis was conducted as follows. Aliquots of RNA were incubated at 65 °C for 10 min using oligo dT primer (Roche), followed by incubation at 37 °C for 60 min in the presence of RNAase inhibitor (Promega) and MMLV RT polymerase (Gibco Life Technologies). One microgram of cDNA was amplified using primers as described previously (Oomizu *et al.* 1998) for 24 cycles. The cycle number of PCR amplification was set to obtain band intensities within a linear range. Ethidium bromide stained bands were quantitated using an image analyzer, Bio-1D system (Vilber Lourmat, Marne, France). As a control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used for normalization. Data were expressed as ratios of insulin-like growth factor-I (IGF-I)/GAPDH.

Histomorphometric analysis

Femora were fixed in 4% paraformaldehyde/PBS, and decalcified with 10% formic acid for 3 days. The samples were embedded in paraffin and 5 µm thickness sections were made and stained with toluidine blue.

Statistical analysis

All data are expressed as means $\pm$ S.D. Statistical significance of the difference in the values was evaluated by the Mann-Whitney U-test. Difference with *P* values less than 0.05 were regarded as statistically significant.

Results

By the end of the 3 week experimental period (mice were 7 weeks old at the end of the experiments), the body weight of WT mice injected with saline alone was

Figure 3 BMD based on DEXA. At the end of 3 weeks of treatment with GH or saline (CTRL), tibiae were subjected to DEXA as described in Materials and Methods. (a) BMD in the proximal epiphyseal regions of tibiae. (b) BMD in the diaphyses of tibiae. Asterisks indicate significant differences ($P<0.05$). Means $\pm$ S.D.

increased by $28.6 \pm 2.3\%$. As reported previously (Van Buul & Van den Brande 1978), treatment with human GH enhanced the increase in the body weight of WT mice by about 40% during the 3 weeks ($P<0.05$, Fig. 1a). In control *klotho* mutant mice, which were injected with saline alone, body weight increment was $13.9 \pm 5.3\%$ and no statistically significant difference between the body weights at the start and end point of the 3 week period was observed. Furthermore, in contrast to the WT mice, the body weight increase after the 3 week treatment with GH was only $4.0 \pm 5.9\%$ and no statistical difference was observed between the body weights of GH- and saline-treated *klotho* mice (Fig. 1a). Thus, GH did not rescue the growth retardation in *klotho* mice.

The whole body length, measured on X-ray from nose to sacrum, was increased by $10.4 \pm 3.3\%$ in the saline-treated control group and by $17.0 \pm 2.6\%$ in the GH-treated group ($P<0.05$) (Fig. 1b) in WT. The body length in *klotho* mutant mice was increased by $12.3 \pm 3.2\%$ in the control group and by $7.6 \pm 6.3\%$ in the GH-treated group (Fig. 1b). Thus, no GH effect on the whole body length was observed in the mutant mice.

We also examined whether no change in the body weight in GH-treated *klotho* mutant mice was associated with change in body composition. GH treatment did not significantly alter body composition regarding fat to tissue ratio in either *klotho* mutant mice or WT (Fig. 1c).

We further examined IGF-I gene expression levels by RT-PCR using livers of the mice. Contrary to our prediction, expression levels of IGF-I mRNA in liver in *klotho* mice were elevated compared with the WT (Fig. 1d). Furthermore, IGF-I mRNA levels were similar to the control 24 h after the last GH injection in WT mice. In contrast, GH treatment normalized the levels of IGF-I mRNA to the levels similar to the saline-injected group 24 h after the last injection in *klotho* mutant mice.

X-ray analysis of the proximal ends of the tibiae of *klotho* mutant mice taken at the end of the 3 week period indicated the presence of radiopacity in the epiphyses of long bones (Fig. 2) and vertebrae (data not shown) in the saline-injected group as reported previously (Kuro-o *et al.* 1997, Yamashita *et al.* 1998). Such high radiopacity was still observed in the *klotho* mutant mice after 3 weeks of treatment with GH (Fig. 2).

BMD in the proximal epiphyses of the tibiae (proximal one-fifth of the total length) was increased significantly by the GH treatment in WT mice (Fig. 3a, $P<0.05$). Basal levels of BMD in saline-injected *klotho* mutant mice were higher than those in saline-injected WT (Fig. 3a, $P<0.05$). GH treatment also enhanced the level of BMD in the proximal regions of the tibiae in *klotho* mutant mice (Fig. 3a, $P<0.05$). These observations indicated that the response to GH at the levels of cancellous bone tissues was not impaired in *klotho* mice. In contrast, BMD of the

Figure 4 3D micro-CT analysis of trabecular bone of tibiae. (a) 3D images of micro-CT analysis. Proximal ends of tibiae are shown. (b–h) Morphological analysis of trabecular bone. The measurements were conducted as described in Materials and Methods, and all parameters were analyzed by software based on an IPL package in the micro-CT apparatus. (b) Bone volume/tissue volume. (c) Trabecular number. (d) Trabecular thickness. (e) Bone surface. (f) Bone surface/bone volume. (g) Trabecular separation. (h) Anisotropy. Asterisks indicate significant difference ($P<0.05$). CTRL=control saline-treated mice. Means $\pm$ S.D.

(a) WT

(b)

(c)

(d)

(e)

(f)

(g)

(h)

Figure 5 Growth plate of the femora. WT and *klotho* mutant mice were treated with GH or saline (CTRL). Irregularity of chondrocyte columns in *klotho* mutant mice was not rescued by a 3 week GH treatment.

diaphyseal cortical bone (middle three-fifths of the total length) was not increased by the GH treatment in both groups, i.e. WT mice and *klotho* mutant mice. Therefore, osteoporotic changes in the cortical bone known to exist in *klotho* mutant mice were not improved by the GH treatment (Fig. 3b).

To investigate the structural aspects of the response of trabecular bone to GH treatment more precisely, we examined the trabecular bone in the epiphyses of tibiae by using a 3D micro-CT analysis system (Fig. 4a). Trabecular bone volume/tissue volume (BV/TV), trabecular thickness, trabecular bone surface and trabecular number were significantly increased by GH treatment in WT mice (Fig. 4b–e) and similar GH effects were observed in *klotho* mutant mice (Fig. 4b–e). Trabecular separation and bone surface ratio to bone volume were significantly decreased by GH treatment in both groups (Fig. 4f and g). Anisotropy, which indicates directions of the trabecular axis, was not significantly altered by GH treatment (Fig. 4h).

In WT mice, chondrocytes in the growth plate formed columns along the longitudinal axis of the femora and such chondrocyte columns appeared to be elongated after GH

treatment (Fig. 5). In *klotho* mutant mice, chondrocytes did not clearly form columns, and the size and the shape of chondrocyte columns were irregular. These phenotypes of chondrocytes in *klotho* mutant mice were not rescued by the GH treatment (Fig. 5).

Discussion

Growth retardation is one of the representative phenotypes in *klotho* mutant mice (Kuro-o *et al.* 1997). In these mice, electron microscopic examination indicated that the number of vesicles in the GH-producing cells in the pituitary gland was reduced (Kuro-o *et al.* 1997), suggesting that the growth retardation in *klotho* mutant mice could be in part due to GH deficiency (Kuro-o *et al.* 1997). We therefore examined directly the effect of exogenous GH on the growth retardation in *klotho* mutant mice and found that the mechanism responsible for the growth retardation is independent from GH function.

The increase in BMD in the epiphyses after GH treatment of *klotho* mutant mice suggests that at least in the epiphyseal cancellous bone regions, the GH pathway

appears to affect the metabolism of *klotho* mutant mice similarly to the WT. Therefore, *klotho* gene mutation is potent enough to override the growth-promoting effects of GH used in our experiment. A recent report on the abnormality in the energy homeostasis in the *klotho* mutant mice (Mori *et al.* 2000, Utsugi *et al.* 2000) may also suggest such a defect independent of the GH pathway in *klotho* mutant mice. In this report, glucose tolerance and insulin sensitivity in *klotho* mice are shown to be increased compared with those in WT mice based on i.p. glucose and insulin tolerance tests (Mori *et al.* 2000).

Lack of changes in the body weight of *klotho* mice may not mean that there is no tissue response to GH therapy. For example, GH may be increasing fat-free mass but decreasing fat mass to the same extent with no net effect on body weight. However, in our experiments the body composition was not significantly altered in either *klotho* mutant mice or WT mice by GH treatment.

In addition to body weight gain, we also examined the effect of GH on the bone phenotypes in these mice (Yamashita *et al.* 1998, 2000a,b, Kawaguchi *et al.* 1999). GH treatment significantly increased trabecular bone volume, but not cortical bone in WT mice. This site-specific effect of GH was also observed in bovine GH transgenic mice (Graichen *et al.* 1999). These site-specificities were maintained in *klotho* mutant mice. *Klotho* mutant mice show osteopetrosis in their metaphyseal regions about 4 weeks after birth (Yamashita *et al.* 1998). This has been seen in association with high levels of osteoprotegerin expression in the bone marrow (Yamashita *et al.* 2000b). The osteopetrosis in the metaphyses is one of the early phenotypic changes in *klotho* mutant mice. As mentioned above, GH treatment increased trabecular bone volume and BMD in the epiphyseal regions of tibiae in *klotho* mice indicating that the GH receptor and down-stream signaling system could be operating. However, GH did not improve the levels of osteopetrosis. Therefore, in *klotho* mutant mice, there would be a defect in body systems other than GH/IGF-I axis stimulation.

Against our prediction, IGF-I mRNA in the liver in *klotho* mice was elevated. Furthermore, GH treatment normalized the IGF-I mRNA levels in the liver in *klotho* mice. This observation cannot be simply explained based on the currently accepted understandings on the GH/IGF-I system. It appears that an as yet unidentified novel system might enhance IGF-I mRNA expression in the liver in *klotho* mice and that such a system might also normalize the IGF-I mRNA levels upon GH treatment. Even if this is the case, whether such IGF-I mRNA elevation is a compensatory response related to growth retardation in *klotho* mice or alternatively is totally independent from growth abnormality in these mice is still to be elucidated. IGF-I mRNA levels were similar to control 24 h after the last GH injection in WT mice. This may indicate that the effects of GH injection on IGF-I mRNA levels could return to base line within 24 h in WT mice.

In conclusion, growth retardation in *klotho* mutant mice is resistant to GH treatment even though this hormone can act on trabecular bone in these mice.

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