Histological evidence of the altered distribution of osteocytes and bone matrix synthesis in klotho-deficient mice*

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Summary. Mice homozygous for klotho gene deletion are well established aging models as they mimic certain aspects of human senescence e.g. osteoporosis. Induced senescence may affect cellular functions and alter the histological properties of the extracellular matrices. The present study examined the histological and ultrastructural features of osteocytes and the surrounding bone matrix in klotho-deficient mice. As expected, osteoblasts showed a flattened shape with a weak immunoreactivity for alkaline phosphatase, and the bone matrix contained many empty osteocytic lacunae. The walls of both normal and empty lacunae were intensely immunopositive for osteopontin and dentin matrix protein-1, but featured an inconsistent immunoreactivity for osteocalcin and type I collagen. Not surprisingly, TUNEL-positivity, indicative of apoptosis, was found in many osteoblasts, osteocytes, and bone marrow cells of the klotho-deficient mice. In transmission electron microscopy, an amorphous matrix containing non-collagenous organic materials was recognizable around osteoblasts and in the osteocytic lacunae. Some osteoblasts on the bone surface featured these amorphous materials in vacuoles associated with their trans-Golgi network, indicating that, under klotho-deficient conditions, they synthesize and secrete the non-collagenous structures. Some osteocytes displayed pyknosis or degenerative traits. Thus, our findings provide histological evidence that klotho gene deletion influences the spatial distribution of osteocytes and the synthesis of bone matrix proteins in addition to the accelerated aging of bone cells.

Introduction

Mice homozygous for klotho gene deletion, known as a model for accelerated aging, can grow normally until the third postnatal week but then become less active and ultimately die by 8–9 weeks of age (Kuro-o et al., 1997). They exhibit osteoporosis, skin atrophy, ectopic calcification, pulmonary emphysema, gonadal dysplasia, and defective hearing. This model also displays various metabolic abnormalities which closely resemble those found during starvation periods, e.g. a low level of blood glucose and insulin, as well as little glycogen storage in the liver and few lipid droplets in brown adipose tissue (Mori et al., 2000). The amino acid sequence of human klotho protein and the analogous mouse protein matches in 86%. The human klotho mRNA is encoded by a genomic DNA
that spans over 50 kb on chromosome 13q12 (Matsumura et al., 1998), yet no clinical premature-aging syndromes have been linked to this region: it has been linked to the chain of deteriorative events of chronic renal disease (Koh et al., 2001). Klotho gene polymorphism in humans is involved with the pathophysiology of bone loss associated with aging (Kawano et al., 2002), with spondylodiscitis (Ogata et al., 2002), with the trafficking and catalytic activity of klotho (Arking et al., 2002), and with occult coronary artery disease (Arking et al., 2003).

The klotho gene encodes secretory and membrane-bound forms of its protein, containing respectively one and two β-glucosidase-like domains in both humans (Matsumura et al., 1998) and mice (Shiraki-Iida et al., 1998). The membrane-bound isoform predominates in mice but is the lesser in humans. Even though its predicted molecular weight is 70 kDa, the klotho protein weights 130 kDa in its secretory form as it forms an oligomerized complex (Imura et al., 2004). The klotho gene is predominantly expressed in the kidney, the choroid plexus (Kuro-o et al., 1997), and parathyroid gland (Nabeshima, 2002) as well as in cortical renal tubules (Kato et al., 2000) and in the stria vascularis and spiral ligament of the inner ear (Kanemori et al., 2002). Since klotho, however, severely affects many organs that do not express this molecule, Xiao et al. (2004) suggested that the klotho protein is a circulating factor related to human aging.

Osteoporotic patients lose bone volume and show an altered skeletal architecture, forming a group with increased fracture risk. Klotho-deficient mice exhibit a low-turnover osteoporosis, with bone formation rates falling sharper than bone resorption, mimicking senile osteoporosis (Kawaguchi et al., 1999). On the other hand, Yamashita et al. (1998, 2000a) reported that klotho-deficient mice had elongated metaphyseal trabecular bone as well as an increase in the volume, number, and thickness of this bone, suggesting a unique function of klotho in bone metabolism. Serum levels of calcium, phosphorus (Kuro-o et al., 1997; Kawaguchi et al., 1999) as well as osteoprotegerin (Yamashita et al., 2000b) increase in mice, consistent with the inhibition of osteoclastic activities. In addition, a decrease in B lymphocytes relates to a reduced number of osteoclasts in klotho-deficient mice (Manabe et al., 2001). Osteoclastic activity appears to be co-related with the osteoclastic one (Nishino et al., 2001; Sakagami et al., 2005), i.e. the cellular coupling between osteoclasts and osteoblasts (Frost, 1964). Taken together, these findings indicate that the klotho deficiency may merely reduce the activity of osteoblasts or may cause abnormalities other than those seen in osteoporosis. To our knowledge, there has been no detailed histological examination of osteoblasts, osteocytes and the bone matrix of klotho-deficient mice.

This study therefore examined histological and ultrastructural alterations in the osteoblasts, osteocytes, and bone matrix of klotho-deficient mice in order to define whether klotho has a special function related to bone cells and the matrix synthesis.

Materials and Methods

Tissue preparation

Animals were handled according with the Guiding Principles for Care and Use of Animals approved by Niigata University. Under anesthesia with an intraperitoneal injection of chloral hydrate (40 mg/kg body weight), 6-week-old male SPF/VAF mice and klotho-deficient mice (Japan CLEA, Tokyo) were perfused through the left ventricle either with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4), or 4% paraformaldehyde and 0.0125% glutaraldehyde in a 0.067 M phosphate buffer for light or electron microscopic observations, respectively. The 4th and 5th lumbers were immediately removed and immersed with the same fixative for additional 8 h at 4°C. The specimens were decalcified with 10% ethylenediamine tetraacetic disodium salt (EDTA-2Na) solution for light microscopy, or 5% EDTA-2Na solution for electron microscopy. For light microscopy, after the decalcified specimens were dehydrated in an ascending series of alcohols prior to paraffin embedding, the sections were mounted onto silanized glass slides. For transmission electron microscopy (TEM), the decalcified specimens were post-fixed with 1% osmium tetroxide with 1.5% potassium ferrocyanide in a 0.1 M cacodylate buffer for 3 h at 4°C, dehydrated in ascending acetone solutions, and embedded in epoxy resin (Epon 812, Taab, Berkshire, UK). Ultrathin sections were prepared with an ultramicrotome and examined with an electron microscope (H-7000: Hitachi, Tokyo) following a brief staining with tannic acid, uranyl acetate, and lead citrate.

Immunohistochemistry for alkaline phosphatase (ALP), osteopontin, dentin matrix protein-1, type I collagen and osteocalcin, and enzyme histochemistry for tartrate resistant acid phosphatase

Most of the histochemical procedures for ALP, osteopontin, osteocalcin have been described previously (Amizuka et al., 1994, 1998). In brief, after the inhibition of endogenous peroxidase activity with methanol containing 0.3% hydrogen peroxidase for 30 min, the dewaxed paraffin sections were pretreated with 1% bovine serum
albumin (BSA; Seelodicals Proteins Inc., Kankakee, IL, USA) in PBS (1% BSA-PBS) for 30 min. They were then incubated for 2–3 h at room temperature with either rabbit polyclonal antiserum against alkaline phosphatase (ALP, Oda et al., 1999), osteopontin (LSL, Tokyo), dentin matrix protein-1 (DMP-1, Takara BIO Inc., Ohtsu), type I collagen (Cosmo BIO, Tokyo), or goat polyclonal antiserum against osteocalcin (Biomedical Technology Inc., Stoughton, USA), respectively diluted at 1:250, 1:2,500, 1:500, 1:3,500 and 1:100 with 1% BSA-PBS. For DMP-1 detection, sections were pretreated with 3% trypsin for 30 min. Sections were then incubated either with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Amersham Bui., Tokyo), or HRP-conjugated anti-goat IgG (Amersham, Bui.). The immunoreaction was visualized by an incubation with 0.04% 3,3′-diaminobenzidine and 0.003% hydrogen peroxidase. The sections were faintly counterstained with methyl green. The detection of tartrate-resistant acid phosphatase (TRAP) was performed as previously described (Amizuka et al., 1998); slides were rinsed with PBS and incubated in a mixture of 2.5 mg of naphthol AS-BI phosphate (Sigma, St. Louis, MO, USA), 18 mg of red violet LB (Sigma) salt, and 100 mM L (+) tartaric acid (0.76 g) diluted in 30 mL of a 0.1 M sodium acetate buffer (pH 5.0) for 15 min at 37°C.

Detection of apoptosis reaction

We used ‘TACS™ 2TdT-Blue Label In Situ Apoptosis Detection Kit’ (TREVIGEN Ins., Gaithersburg, MD,
Fig. 2. Legend on the opposite page.
USA) for terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL). Dewaxed sections were incubated with 1% proteinase K (TREVIGEN Ins.) diluted 1:200 at 37°C for 15 min, and the endogenous peroxidase activity was inhibited with absolute methanol containing 0.3% hydrogen peroxidase at room temperature for 5 min. After incubation with the TdT enzyme diluted 1:50 at 37°C for 1 h, the sections were then reacted with HRP-conjugated streptavidin at room temperature for 15 min. The apoptosis reaction was visible after incubation with a blue label solution. The sections were faintly counterstained with nuclear fast red.

Results

Histological and immunohistochemical observations of the vertebrae of 6-week-old klotho-deficient mice and their wild-type littermates

Corresponding areas of wild-type and klotho-deficient transverse processes of lumbers were observed under light microscopy (Fig. 1). The transverse process of the wild-type lumbar showed well-defined cortical bone, representing typical compact bone (Fig. 1a). However, klotho−/− transverse processes appeared porous with numerous osteocytes, therefore failing to have formed compact bone (Fig. 1b). Plump osteoblasts overlay the bone surfaces of the wild-type lumbar (Fig. 1c) while klotho−/− lumbar bone featured flattened osteoblasts (Fig. 1d). At a higher magnification, wild-type lumbars had several osteocytes embedded in the bone matrix (Fig. 1e), while klotho−/− mice revealed abundant osteocytes and random empty lacunae without the common traits of compact bone (Fig. 1d). Some osteocytes showed pyknosis. Intense hematoxylin-positive amorphous materials were associated with these pyknotic osteocytes and with osteocytic lacunae (Fig. 1d). The hematoxylin-positive materials were found on the endosteal bone surfaces as well. Therefore, klotho deficiency appeared to result in a bone developmental deformity.

We next examined the localization of bone matrix proteins both in wild-type and klotho−/− bones, including osteopontin, DMP-1, osteocalcin, and type I collagen. The walls of the osteocytic lacunae and canaliculi in the wild type showed DMP-1 immunoreactivity (Fig. 2a, f), which is supposed to be expressed specifically in osteocytes (Toyosawa et al., 2001) and odontoblasts (MacDougall et al., 1998). Immunoreactivity for osteopontin was faint throughout the bone matrix, being weak at the osteocytic canaliculi though relatively intense at the bone surface and walls of the osteocytic lacunae (Fig. 2b, g). Osteocalcin was evenly distributed but weak in the osteoid superficial layer (Fig. 2c, h) while type I collagen immunoreaction was uniformly found in the vertebral bone matrix (Fig. 2d, i). Osteoblasts at both the periosteum and the endosteum were strongly immunoreactive for ALP (Fig. 2e, j).

Unlike the wild-type bone, klotho-deficient vertebral bone exhibited intense positivity for DMP-1 and osteopontin immunoreaction (Fig. 3a, b). When observed at a higher magnification, osteopontin and DMP-1 associate with osteocytic lacunae and the bone surfaces (See Fig. 3f, g) paralleling the distribution of the hematoxylin-positive materials presented in Figure 1d. In contrast, type I collagen immunoreactivity was weak at the periphery of the osteocytic lacunae (Fig. 3d, i) while an inconsistent immunopositivity for osteocalcin was found in the klotho-deficient bone matrix (Fig. 3c, h). Osteoblasts were flat and weakly immunopositive for ALP (Fig. 3e, j).

Apoptosis in the vertebrae of 6-week-old klotho-deficient mice

The higher incidence of empty lacunae in klotho−/− lumbers led us to further examine the TUNEL reaction in order to verify the likeliness of apoptotic cell death. In the wild-type counterparts, few osteocytes and marrow cells showed TUNEL positivity (Fig. 4a), with osteoblasts hardly revealing any TUNEL-reaction (Fig. 4b). In contrast, many TUNEL-positive osteocytes, marrow cells, and lining osteoblasts were discernible in klotho−/− mice (Fig. 4c, d). Empty osteocytic lacunae in klotho−/− bones were not positive for TUNEL reaction (Fig. 4d).

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Fig. 2. a–e: Low powered magnifications of the transverse processes of the wild-type lumbar immunostained for DMP-1 (a), osteopontin (b), osteocalcin (c), type I collagen (d), and ALP (e). f–j: Higher magnifications of the boxed areas in panels a–e. Osteocytic lacunae and canaliculi have DMP-1 immunopositivity (brown color in f), while osteopontin accumulates chiefly on the walls of osteocytic lacunae, but faintly in canaliculi (brown color in g). Note that bone surface is also positive for osteopontin. h: Osteocalcin immunoreactivity (brown) is uniformly distributed in bone matrix except for the superficial layer, indicative of an osteoid. i: In contrast to osteocalcin, type I collagen (brown) is uniformly localized in bone. j: Periosteal osteoblasts display a strong immunoreaction for ALP. Scale bars: 125 μm (a–e), 20 μm (f–j)
Fig. 3. Legend on the opposite page.
Histological examination of klotho deleted bone

Fig. 3. a–e: Lower magnifications showing the klotho deficient transverse processes (a: DMP-1, b: osteopontin, c: osteocalcin, d: type I collagen, e: ALP). f–j: Higher magnifications of the boxed areas in panels a–e. f and g: Abundant DMP-1 (f) and osteopontin (g) accumulate osteocytic lacunae as well as on the bone surfaces. The walls of the osteocytic lacunae also show strong DMP-1 and osteopontin immunopositivities (insets in f and g). h: The bone matrix expresses a spotty immunoreaction for osteocalcin. Inset: The matrix encompassing the empty lacunae does not display immunopositivity. i: Unlike the distribution of type I collagen in the wild type counterpart, the klotho deficient bone reveals patchy immunolocalization for type I collagen. Inset: Bone matrix around the empty lacunae lacks type I collagen immunopositivity. j: Flattened periosteal osteoblasts have weak ALP-immunoreactivity (inset). Scale bars: 200 \( \mu m \) (a–e), 35 \( \mu m \) (f–j), 25 \( \mu m \) (insets)

Fig. 4. Apoptosis detection on the wild-type (a, b) and klotho deficient lumbars (c, d). In wild type mice, some bone marrow cells reveal TUNEL-positive reaction (arrows in a), whereas osteoblasts hardly show any TUNEL reaction (b). In klotho-deficient mice, however, several osteocytes in the bone lacunae have TUNEL positivity (arrows in c), and some osteoblasts also indicate TUNEL reaction (arrows in d). Scale bars: 20 \( \mu m \)
TEM observations of amorphous materials seen in klotho-deficient mice

I pondering about the origin of the amorphous structures seen in klotho-deficient bone, we assumed that these materials could be debris from degraded osteoblasts and osteocytes that had undergone apoptosis. To verify this postulation, TEM was used to assess the ultrastructures of osteoblasts, osteocytes, and amorphous materials in klotho−/− bone. In some areas, nodules containing amorphous materials without collagenous fibrils were observed over the bone surface (Fig. 5a) and often included...
osteoblasts with intact cell organelles (Fig. 5b). The amorphous material contained in these nodules seemed to mirror those structures positive for osteopontin/DMP-1 previously observed in Figures 3f and g. These intriguing structures do not seem to be derived from osteocytic debris.

At higher magnifications, the osteoblastic trans-Golgi network revealed vacuoles enclosing amorphous structures, therefore indicating that osteoblasts are responsible for their synthesis (Fig. 5c). Degraded osteocytes shared their lacunae with these unspecified structures (Fig. 5d). Thus, it would seem that, instead of being debris from dead osteoblasts and osteocytes, the amorphous structures are a product of osteoblastic cells in a condition of klotho deletion.

Discussion

The present findings have verified a higher density of osteocytic loci in klotho-deficient bone compared with that in their wild-type counterparts, but most of them were empty and contained amorphous organic materials intensely stained for osteopontin and DMP-1. TEM observations demonstrated that osteoblasts synthesize these amorphous materials, it being implausible that they could have been the debris of dead osteocytes. Thus, a klotho deficiency provokes abnormal protein synthesis in osteoblasts while making it easier for the bone matrix to entrap osteocytes.

Previous reports have stated that klotho−/− mice feature a phenotype similar to senile osteoporosis, including inhibited osteoblastic and osteoclastic activities (Kawaguchi et al., 1999; Yamashita et al., 1998, 2000a, b, 2001). In our study, klotho−/− bones were characterized by flat osteoblasts weakly positive for ALP and a lower number of TRAP-positive osteoclasts (data not shown) when compared with wild-type mice. It is well known that receptor activator of NFκB (RANK) ligand (RANKL) has been identified as a member of the membrane-associated TNF ligand family expressed on the plasma membranes of osteoblasts/stromal cells which induces osteoclastic differentiation. RANKL is a ligand for RANK, but osteoprotegerin serves as a soluble decoy receptor for RANKL. The above finding of ours is supported by reports in which the mRNA levels of receptor activator of NF-κB (RANK) and RANK ligand (RANKL) in klotho−/− mice were shown to be similar to those of heterozygous and wild-type littermates, whereas osteoprotegerin levels were higher in klotho−/− mice (Yamashita et al., 2000b, 2001). These findings may explain the low bone turnover mimicking senile osteoporosis in the klotho−/− phenotype (Kawaguchi et al., 1999).

A klotho deficiency may accelerate premature cellular differentiation without any accompanying improvement in cellular proliferation. This may lead not only to a senile osteoporotic phenotype, but also to a range of other abnormalities. The higher incidence of osteocytes in vertebral bone suggests that osteoblasts were easily trapped by the bone matrix. This finding is intriguing, as it may contradict the notion of lower bone turnover in klotho−/− mice (Kawaguchi et al., 1999; Yamashita et al., 1998, 2000a, b, 2001). Therefore, the klotho−/− mice appear to feature dubious aspects of human senile osteoporosis and its unique abnormalities. This idea is also supported by the presence of amorphous organic materials intensely positive for osteopontin and DMP-1 in the periosteal lucencies of the klotho−/− bones that were not of such a common occurrence in normal osteocytes and their lacunae (Fig. 2f, g) as previously reported (Toyosawa et al., 2001; Morinobu et al., 2003). The abundant production of osteopontin and DMP-1 was not accompanied by profuse type I collagen formation in the vicinity of the osteocytic lacunae, ratifying the ultrastructural observations that failed to show any collagen fibrils associated with amorphous organic materials (Fig. 5). The huge amount of osteopontin and DMP-1 but not collagen fibrils in the amorphous organic indicates that this amorphous material may not well-mineralized and disrupts the normal assembly of collagen fibrils and collagen-associated mineralization in bone. Taking all these finding together, a klotho deficiency may specifically regulate the transcriptional activities of some extracellular proteins, but the precise mechanism is still an on-going discussion—which includes the higher expression of osteopontin inducing osteoclasts to adhere to the bone surface, modulates the mineralization, and plays a pivotal role in bone formation and remodeling under mechanical stress (Terai et al., 1999; Morinobu et al., 2003). Interestingly, the klotho deficiency enhanced the production of DMP-1, which is not only a secretory protein essential for normal postnatal chondrogenesis and subsequent osteogenesis (Fen et al., 2002; Ye et al., 2005), but can also translocate into the nucleus (Inoue et al., 2000). Supposing that DMP-1 participates in the transcriptional activities of extracellular matrix proteins, it can be assumed that a deficiency of klotho may affect the biological functions of bone cells by indirectly mediating proteins such as DMP-1. Previous reports stated that the klotho gene was predominantly expressed in tissues other than bone, such as the kidney, parathyroid gland, choroid plexus, cortical renal tubules, stria vascularis and spiral ligament of the inner ear (Kuro-o et al., 1997; Kato et al., 2000; Kamemori et al., 2002; Nabeshima 2002). Bone cells might have a receptor and/or acceptor for klotho, a topic for further research.

Biglycan-deficient mice showed an increased apoptosis
of bone marrow stromal cells and impaired synthesis of type I collagen (Chen et al., 2002; Nielsen et al., 2003). It seems likely that an altered synthesis of bone extracellular matrix affects apoptosis and the production of other extracellular matrices through pathways that are still not clear. In our observations, osteoblasts accumulated amorphous materials in vacuoles of the trans-Golgi network and, after releasing them, were entrapped by massive amorphous structures. Although these osteoblasts seemed intact and featured normal organelles (Fig. 5b, c), the osteocytes enclosed by these amorphous structures were shown to have undergone an apoptosis-driven death. To our current knowledge, there is no simple way of defining the relations between the secretion of amorphous materials and osteocytic death. It is possible that the accumulated amorphous materials interrupt the metabolic cycle of osteocytes, or that osteocyte death has no connection at all with the production of amorphous structures. Further examinations, including a thorough search for and possible discovery of a receptor and/or acceptor for klotho in bone cells, will be indispensable for resolving these issues.

In summary, our study has provided histological evidence that klotho gene deficiency not only accelerates aging in bone cells but influences the overall number of osteocytic loci as well as the synthesis of bone matrix proteins.

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References


Frost HM: Dynamics of bone remodeling. In: Bone biodynamics (Frost HM ed), Little, Brown, Boston, 1964 (p.315-333)


Koh N, Fujimori T, Nishiguchi S, Tamori A, Shiomi S, Nakatani T, Sugimura T, Kinoshita S, Kuroki T,


