Histological and immunohistochemical changes in the submandibular gland in klotho-deficient mice

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Summary. The submandibular gland (SMG) has been regarded as an age-stable organ in spite of reports on its structural changes with aging. Although the klotho gene is involved in aging, little information is available regarding its effects on morphological changes of SMGs. The present study examined the histological and immunohistochemical features of SMGs in klotho-deficient mice — which are well-established aging models — by immunohistochemical and histochemical techniques. Five kinds of cellular markers — against NGF, EGF, Mn- and Cu/Zn-SOD, and RITC-conjugated phalloidin — were used for the identification of cell types. In klotho-deficient mice, the SMGs lost their granular ducts and each lobe diminished. The granular duct showed strong immunoreactivities for NGF and EGF in the wild-type mice, but the NGF- and EGF-immunopositive ducts decreased in number remarkably in klotho-deficient mice. Interestingly, instead of a loss of the granular duct, the striated duct located on the distal portion in the homozygous mice came to show NGF- and EGF-immunoreactions. Neither Mn- and Cu/Zn-SOD immunoreactivities in the duct system nor the phalloidin-reaction in the myoepithelial cells differed between the wild-type and klotho-deficient mice. Our findings suggest that the klotho gene inhibited the differentiation of the granular duct from the striated duct due to the repression and/or down-regulation of sexual and growth hormones.

Introduction

Klotho-deficient mice, known as a model for accelerated aging, develop normally until 3 weeks after birth and then become less active, ultimately dying by 8–9 weeks of age (Kuro-o et al., 1997). The mice exhibit osteoporosis, skin atrophy, ectopic calcification, pulmonary emphysema, gonadal dysplasia, and defective hearing — all of which appear in human aging. These animals also induce various metabolic abnormalities which closely resemble those in conditions of food deprivation: they have lower levels of blood glucose, insulin, and glycogen storage in the liver and lipid droplets in brown adipose tissue (Mori et al., 2000).

A human klotho protein shows an 86% amino acid identity with the mouse protein, and its gene is encoded by a gene that spans over 50 kb on chromosome 13q12 (Matsumura et al., 1998), though no premature-aging syndromes have been linked to this region. Koh et al. (2001) reported a relation between klotho gene expression and the process of deterioration to multiple complications in human chronic renal disease. Klotho
gene polymorphism in humans is also involved in the pathophysiology of bone loss with aging (Kawano et al., 2002), spondylosis (Ogata et al., 2002), in the trafficking and catalytic activity of klotho (Arking et al., 2002), and an independent factor for occult coronary artery disease (Arking et al., 2003), suggesting that the klotho protein is a serum factor related to human aging (Xiao et al., 2004).

The submandibular gland (SMG) has been reported to increase in proportional volume of fat and connective tissues with a reduction of that of acini with aging, though without any remarkable change in the volume of the duct system (Scott, 1975, 1977a–c, 1979, 1987, Nagler, 2004). Accordingly, many researchers believe that the salivary constituents indicate age-stability due to the absence of major medical problems and medications (Ship et al., 1995). Nevertheless, previous reports have shown an up-regulation of some molecules under pathological conditions. For instance, advanced chronic sialadenitis and Sjögren’s syndrome result in a higher expression of the transforming growth factor-β1 (TGF-β1) (Teymoortash et al., 2003) and increased contents of IL-2 and IL-6 in saliva (Streckfus et al., 2001), respectively. Here, the reserve functional capacity of the salivary glands probably compensates for the loss of functional parenchyma (Ghezzi and Ship, 2003).

Our recent study revealed a characteristic distribution of osteocytes and the synthesis of bone matrix proteins as well as the accelerated aging of bone cells in klotho-deficient mice (Suzuki et al., 2005). Throughout that study, we noticed some morphological changes in the submandibular glands. Little information is available regarding the precise changes in the SMGs caused by the loss of klotho gene except for a study by Masuda et al. (2005): they briefly described a lack of eosinophilic granules in the SMGs of klotho-deficient mice. The present study was therefore undertaken to examine the histological and immunohistochemical features of the SMGs in klotho-deficient mice as compared with those in wild-type mice.

Materials and Methods

Tissue preparation

The care and use of animals followed the Guiding Principles for the Care and Use of Animals, as approved by Niigata University in accordance with the principles of the Helsinki Declaration.

We used a total of 6 male SPF/VAF (n=3) and klotho-deficient mice (n=3) at 7 weeks of age in this study. Klotho-deficient mice were purchased from Japan CLEA (Tokyo). Both types of mice were anesthetized by an intraperitoneal injection of chloral hydrate (40 mg/100 g b.w.). They were perfused transcardially through the left ventricle with 4% paraformaldehyde in a 0.067 M phosphate buffer, pH 7.4. SMGs were immediately removed en bloc and immersed with the same fixative for additional 8 h at 4°C. Some specimens were equilibrated with a 30% sucrose solution overnight for cryoprotection, rapidly frozen in liquid nitrogen, and cut at a thickness of 10 μm in a cryostat (HM-500, Carl Zeiss, Jena, Germany). Other tissue blocks were dehydrated through ascending ethanol and embedded in paraffin. Both cryostat and paraffin sections were mounted onto silanized glass slides (Dako Japan, Tokyo).

Deparafinized sections were performed with hematoxylin and eosin or Masson-Goldner (MG) staining for histologic observations. For the demonstration of myoepithelial cells, cryostat sections were reacted with rhodamine isothiocyanate (RITC)-conjugated phalloidin (Sigma, St. Louis, MO, USA) for 1 h. They were counterstained with DAPI and examined with a fluorescent microscope.

Immunohistochemistry

Cryostat sections were first treated with 0.3% H2O2 in absolute methanol to quench any endogenous peroxidase activity. They were primarily reacted with rabbit polyclonal antisera for 2–3 h at room temperature. We used four kinds of antisera as cellular markers for duct cells: nerve growth factor (NGF: 1:2500, Chemicon International Inc., Temecula, CA, USA) and epithelial growth factor (EGF:1:250, Chemicon International Inc.) for the granular duct, and manganese- and copper/zinc-superoxide dismutase (Mn- and Cu/Zn-SOD; 1:1000, StressGen Biotechnologies Corp., Victoria, Canada) for the duct system. These sections were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Amersham Bui., Tokyo). The antigen-antibody reaction sites were made visible by incubation with 0.04% 3,3′-diaminobenzidine and 0.003% hydrogen peroxidase. After counter-staining with 0.03% methyl green, the immunostained sections were dehydrated through an ascending series of ethanol and cover-slipped with Permount (Fisher Scientific, Springfield, NJ, USA).

Immunohistochemical experiments were performed by replacing the primary antisera with normal non-immune rabbit serum or 0.01M PBS and omitting the HRP-conjugated anti-rabbit IgG. These sections did not show any specific immunoreaction.
Results

Histological differences between the wild type and klotho-deficient mice

The SMGs of both the wild-type and klotho-deficient mice consisted of acini, a duct system, and interstitial connective tissues. The nuclei of the acinal cells were restricted to the basal side, and their cytoplasm filled clear granules in both phenotypes.

In the wild-type mice, all types of duct cells — intercalated, granular, striated, and excretory cells — were clearly identified in the duct system of the SMGs (Fig. 1a–c). In klotho-deficient mice, on the other hand, the interlobular connective tissue increased in volume, resulting in its becoming smaller in size for each individual lobule (Fig. 1d). In spite of the absence of any remarkable change in striated ducts (Fig. 1f), the SMGs of klotho-deficient mice lacked eosinophilic granules in some ducts (Fig. 1d–f). The intercalated ducts were observed as consisting of cuboidal cells with a poor cytoplasms as observed in the wild-type mice. Thus, the duct system in the SMGs of klotho-deficient mice was composed of intercalated, striated, and excretory ducts. In addition, the staining ability with eosin in the cytoplasm of acinar cells decreased more in klotho-deficient mice than in the wild type mice, appearing as a clear cytoplasm. However, these cells were serous cells because their nuclei were not restricted to the basal side (Fig. 1e).

Immunohistochemical features in the wild-type and klotho-deficient mice

In the SMGs of the wild-type mice, immunostaining showed an intense immunoreactivity for NGF (Fig. 2a) and EGF (Fig. 2c), a dot-like appearance, in the granular duct cells. No cells of the intercalated, striated, or excretory ducts had any immunoreactivity for NGF or EGF (Fig. 1a, c inset). Using the Mn-SOD antibody, the duct cells of granular and striated cells in addition to some intercalated and excretory cells displayed immunoreactivities with various immuno-intensities (Fig. 3a). On the other hand, all duct cells — the intercalated, granular, striated, and excretory cells — exhibited immunoreactivities for Cu/ Zn-SOD with various immuno-intensities (Fig. 3c). However, there was a different immuno-expression pattern between Mn- and Cu/Zn-SOD in the granular duct; the Mn-SOD-immunoreaction was found both in cytosolic granules as well as the cytoplasm (Fig. 3a) while Cu/Zn-

SOD-immunoreactions were discernable in the cytoplasm of the duct cells (Fig. 3c). Any labeling of phallolidin was only recognizable in the myoepithelial cells around the acini and endothelial cells (Fig. 3e).

We next examined the immuno-localization of NGF, EGF, Mn- and Cu/Zn-SOD in SMGs of klotho-deficient mice. The striated duct cells located in the distal portion had a weak immunoreactivity for NGF and EGF (Fig. 2b, d inset). Compared with the wild type, these positive cells decreased in number (Fig. 2b, d). Neither Mn- (Fig. 3b) and Cu/Zn-SOD (Fig. 3d) immunoreactivities in the duct system nor the phallolidin-reaction in the myoepithelial cells (Fig. 3f) differed between the wild-type and klotho-deficient mice.

Table 1 summarizes immunohistochemical changes in the SMGs between the wild-type and klotho-deficient mice.

Discussion

Previous studies have pointed out quantitative alterations in volume — including an increase in fat and connective tissues — but a reduction of the acini in the SMGs with aging (Scott, 1975, 1977a–c, 1979, 1987, Liu et al., 2000, Nagler, 2004). However, since the numbers of the ducts and salivary constituents have been reported unchanged, the SMGs are considered an age-stable organ (Ship et al., 1995; Nagler, 2004). Current observations in klotho-deficient mice confirm the notion of a comparatively stable structure for the SMGs with aging except for a loss of granular ducts. One noteworthy finding is that the distal striated duct came to show NGF- and EGF-immunoreactions in klotho-deficient mice in spite of the lack of the granular duct. Histological observations failed to find any apparent granular duct in klotho-deficient mice. This finding suggests that the granular ducts could not be differentiated from the striated duct in klotho-deficient mice, resulting in a compensative synthesis of growth factors instead of the granular duct.

A similar regression of the granular ducts as observed in klotho-deficient mice has been reported in castration and hypophysectomy (Chretien, 1977), with remarkable decreases in the concentration of EGF (Hiramatsu et al., 1994) as well as the androgen receptor (Li et al., 2005) in rat SMGs. The granular ducts of the SMGs in mice have been considered to differentiate from the striated duct at about 5 weeks of age in dependency on testosterone, a representative sexual hormone (Chretien, 1977). Furthermore, a subcutaneous injection of testosterone induced a re-differentiation of the granular duct in the castrated mice (Chretien, 1977), and an administration of testosterone propionate the caused a recovery of EGF to normal levels in castrated rats (Hiramatsu et al., 1994). All these findings strongly suggest the development and
Fig. 1. Legend on the opposite page.
Fig. 1. Photomicrographs of the submandibular glands (SMGs) in the wild-type (a–c) and klotho-deficient (d–f) mice. Stained with hematoxylin and eosin (a, b, d, e) and Masson-Goldner staining (c, f). a: Low magnified view of the SMG in the wild-type mouse. b, c: The SMG has all types of duct cells including intercalated (I), granular (G), striated (S), and excretory cells. The granular ducts contain many eosinophilic granules in their cytoplasm (b). The cells of the striated ducts develop the basal striation (c). d: Compared with the wild-type mouse, the interlobular connective tissues appear to increase, resulting in a smaller size for each lobe in a klotho-deficient mouse. e, f: The SMG of a klotho-deficient mouse are devoid of granular ducts which fill eosinophilic granules. Instead of the granular duct, it possesses many striated ducts with basal striation (inset). Scale bars: 250 μm (a, d), 50 μm (b, c, e, f), 25 μm (inset)

Fig. 2. Expression of immunoreactivity for NGF (a, b), EGF (c, d) in the wild-type (a, c) and klotho-deficient (b, d) mice. The granular ducts of a wild-type mouse display strong NGF- and EGF-immunoreactions (a, c), but the cells of the striated duct — in addition to the intercalated and excretory ducts — exhibit non-immunoreactivity for NGF and EGF (a, c inset). On the other hand, NGF- and EGF-immunoreactivity appears to decrease in a klotho-deficient mouse (b, d), while the striated duct located in the distal portion shows a weak immunopositivity for them (b and d inset). G: granular duct, S: striated duct. Scale bars: 50 μm (a–d), 25 μm (insets)
Fig. 3. Expression of immunoreactivity for Mn-SOD (a, b), Cu/Zn-SOD (c, d), and fluorescent microscopic images for phalloidin (e, f) in the wild-type (a, c, e) and klotho-deficient (b, d, f) mice. The Mn-SOD immunoreaction has a granular appearance in the cytoplasm of the duct system in the wild-type (a) and klotho-deficient mice (b). On the other hand, Cu/Zn-SOD immunoreactions are discernible in the cytoplasm of the duct cells of the wild-type (c) and klotho-deficient mice (d). Endothelial cells and myoepithelial cells around the acini are labeled with RITC-conjugated phalloidin (e, f). Scale bars: 50 μm (a–d), 25 μm (e, f)
Table 1. Immunohistochemical characterization of wild (+/+) and klotho-deficient (-/-) mice.

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ID: intercalated duct, GD: granular duct, SD: striated duct, ED: excretory duct, MC: myoepithelial cell, ND: not developed.

Histological examination of klotho-deleted SMG

The maintenance of the granular ducts are under the control of testosterone. The lack of granular ducts in klotho-deficient mice is also explained by findings of the atrophy of the testis (Kuro-o et al., 1997) and experimental data showing that the exogenous klotho expression regained responsibility in the granular ducts for testosterone in the SMG of klotho-deficient mice (Masuda et al., 2005).

Many researchers agree with the notion that the development and maintenance of the granular ducts in the SMGs are under control of some other hormones — including thyroxin and adrenal corticoids — besides testosterone. A relationship between the growth-hormone and development of the SMG has also been suggested in addition to testosterone (Gresik, 1994; Lobie and Waters, 1997). The granular ducts of the mouse SMG develop and persist depending on the growth-hormone derived from the pituitary gland (Gresik, 1994, Lobie and Waters, 1997). Kuro-o et al. (1997) reported that growth hormone-producing cells in the pituitary gland become smaller with reduced numbers of secretory granules in klotho-deficient mice. Thus, it is easy to suppose that the absence of the granular duct found in klotho-deficient mice was a change incident to an abnormality in the pituitary gland.

Mn- and Cu/Zn-SODs are respective mitochondrial and cytosolic enzymes (Slot et al., 1986), both of which protect cells against oxidative injury. Tsay et al. (2000) reported the increase of oxidative potential — including the up-regulation of SODs — with aging. On the other hand, the high levels of expression of the enzymes in the brain suggest that the klotho gene is involved in the regulation of brain aging. Since Nagai et al. (2003) demonstrated that the klotho protein plays a role in the regulation of antioxidative defense oxidative stress, these enzymes may be involved in the aging process of the brain. However, the present observations demonstrated no obvious difference in the expression pattern of immunoreactions for Mn- and Cu/Zn-SOD between the wild-type and klotho-deficient mice; klotho-deficient mice showed Mn- and Cu/Zn-SOD-immunoreactivities in the intercalated, striated, and excretory ducts, but never in the acinar cells, consistent with previous reports on rats (Yamamoto et al., 1999, 2002). A close relationship between klotho and apoptosis has been suggested by findings that a deletion of klotho induced an increase in apoptotic cells in various tissues (Sugiura et al., 2005; Suzuki et al., 2005) as well as in vitro (Mitobe et al., 2005). On the other hand, a recent report showed a reduction of klotho expression by oxidant stress injury in a dose-dependent manner (Mitobe et al., 2005). Taken together, these data show it is likely that Mn- and Cu/Zn-SODs protect duct cells from apoptosis even under conditions with a deficiency of the klotho protein, which can inhibit apoptosis.

The klotho-deficient mouse exhibits a lower level of insulin and a higher sensitivity to insulin with hypoglycaemia compared with the wild-type mouse (Mori et al., 2000; Utsugi et al., 2000). Kurosawa et al. (2005) recently have reported that the klotho protein appears to
repress intracellular signals of insulin/IGF1, resulting in an extension of the life span, as indicated by the findings of IGF1 deficient and IGF1 receptor-heterozygous mice (Hsieh et al., 2002a; b; Holzenberger et al., 2003). Mammals with genetic or acquired defects in the insulin signaling pathway are at risk for an age-related decrease and increased mortality, as suggested by the loss of the IGF1 receptor ultimately leading to a short life span for mice (Holzenberger et al., 2003). Previous studies have indicated that the duct cells display the immunoreactivity for IGF1 (Kerr et al., 1995) and that IGF1 protein and mRNA levels decrease with age in the SAMPI mouse (Kobayashi et al., 2004). It is accepted that SMGs function as not only an exocrine but also an endocrine organ (Rougeot et al., 2000). Taken together, the findings indicate that IGF1 secreted from SMG may influence the blood circulation more or less with the klotho protein.

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References


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