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Regulation of angiogenesis by the aging suppressor gene *klotho*

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Abstract

Advanced age is a major risk factor of peripheral artery disease. We examined the effects of the aging-suppressor gene klotho on angiogenesis in response to ischemia by introducing ischemic hindlimb model in mice heterozygously deficient for the klotho gene and in wild type mice. Blood flow recovery as assessed by laser doppler perfusion imaging and angiogenesis as assessed by density of PECAM-1/CD31-positive positive capillaries were markedly impaired in mice heterozygously deficient for the klotho gene (both <0.05). Our findings show that the aging-suppressor gene klotho affects angiogenesis and the possibility that age-related impairment of angiogenesis might be regulated by the klotho gene. Our results present a new possibility of therapeutic angiogenesis for patients of advanced age.

Keywords: Aging; Angiogenesis; Endothelial factors; Ischemia; Klotho gene

Advanced age is a major risk factor of peripheral artery disease [1] as well as other factors, such as hypertension, hyperlipidemia, diabetes mellitus, and smoking. Patients with multiple risk factors are highly susceptible to ischemic vascular disease. However, clinical evidence suggests that elderly patients can develop ischemic diseases even in the absence of any of these risk factors suggesting aging itself to be a potent risk factor of ischemic vascular disease. It is also known that some patients fail to develop effective collateral circulation in response to either ischemia [2] or to exogenous administration of growth factors although the reason for this is unknown [3,4]. A recent study demonstrated that both age-related endothelial dysfunction and reduced expression of the angiogenic/mitogenic factor VEGF (vascular endothelial growth factor) are possible mechanisms of age-related impairment of angiogenesis [5]. These findings suggest that other factors involved in the aging process might also contribute to impaired angiogenesis in the elderly.

We have recently identified the factor Klotho as a new gene which is involved in the regulation and suppression of aging [6]. Originally identified on the basis of insertional mutagenesis in mice, disruption of the klotho gene results in a phenotype which resembles human aging including decreased activity, short life span, infertility, arteriosclerosis, osteoporosis, skin atrophy, and ectopic calcification [6]. Investigation of the effects of klotho on the vasculature showed that disruption of the klotho gene is associated with decreased vasodilatation in response to acetylcholine which reflects decreased production of nitric oxide (NO) in endothelial cells [7,8]. Attenuated levels of NO were also seen in klotho disrupted mice. Interestingly, this endothelial dysfunction was rescued by parabiosis between wild type mice and mice heterozygously deficient for the klotho gene [7,8] suggesting that the klotho gene product may be a humoral factor. Adenovirus-mediated transfer of klotho into rats with endothelial dysfunction (e.g. Ohtsuka–Long Evans–Tokushima Fatty rats) ameliorated this endothelial function as well as the reduced nitric oxide levels. These findings collectively showed that klotho, an aging-suppressor gene, is involved in the regulation of endothelial function likely through a pathway mediated by NO.

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Given that involvement of age-related endothelial dysfunction, including reduced NO production [11], impaired proliferation and migration of endothelial cells [9,10] has been implicated in impaired angiogenesis in aged animals, and importantly as these pathogenic characteristics closely parallel the actions of klotho, we reasoned that klotho is likely to play a role in angiogenesis, and if so, although further investigation is required, would be a promising potential therapeutic factor for use in therapeutic angiogenesis [11], especially in an elderly population.

As a first step, we examined the effects of the klotho gene on angiogenesis in the present study. Our results demonstrate that reduced klotho expression significantly impairs angiogenesis.

Materials and methods

Animals. Thirty-five- to forty-week-old adult male mice heterozygously deficient for the klotho gene were used. All protocols involving experimental animals were in accordance with the local institutional guideline for animal care of the University of Tokyo and complied with the 'Guide for the Care and Use of Laboratory Animals' (NIH publication No. 86-23, revised 1985).

Mouse ischemic hindlimb model. Unilateral hindlimb ischemia was induced in 35- to 40-week-old male mice heterozygously deficient for the klotho gene and in wild type mice [12] obtained as siblings of heterozygous klotho mice, so that the influence of genetically different background can be avoided. The animals were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). An incision was made on the skin overlying the middle portion of the right hindlimb. The proximal portion of the femoral artery was ligated, followed by ligation of the distal portion of the saphenous artery. The artery and all side branches were then dissected free and excised. The skin was closed with a 5-0 surgical suture.

Monitoring of hindlimb blood flow. Hindlimb blood perfusion was measured with a Laser Doppler Perfusion Imager (LDPI) system (Moor Instruments Limited, Devon, UK). Excess hair was removed from the limbs using depilatory cream before imaging, and mice were placed on a heating plate at 40 °C to minimize the influence of temperature. The results are expressed as the ratio of perfusion in the right (ischemic) versus left (normal) limb to correct for ambient light and temperature.

Immunohistochemistry. The mice were sacrificed at five weeks after surgery by intraperitoneal administration of pentobarbital. The muscle was immediately fixed in methanol. Bone was carefully removed, and 5-mm-thick tissue sections were cut and paraffin-embedded for immunohistochemical analysis. Immunohistochemical staining of platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) was performed to identify endothelial cells with a rat-monoclonal antibody against murine CD31 (clone MEC13.1, pharmingen) [12].

Measurement of capillary density. Capillaries were identified by positive staining for CD31 and morphology. The number of capillaries was counted under 20× objective and 7.5× lenses. Capillary density was expressed as the number of capillaries per square millimeter. Five different fields from each muscle were randomly selected, and the number of capillaries were counted.

Statistics. All data are expressed as mean ± SEM. Statistical comparisons among strains were performed by ANOVA followed by Student's t test. A p-value of <0.05 was considered significant.

Results

Hindlimb ischemia model of angiogenesis in klotho-deficient mice

In order to analyze native angiogenesis in response to ischemia, hindlimb ischemia was induced in mice heterozygously deficient for the klotho gene and in wild type mice. Homozygously deficient mice could not be used because they do not thrive beyond 10 weeks and do not grow to a size allowable for operation and further observation (e.g. 8 weeks span) of the results. Older heterozygous mice were selected to avoid unstable results as a consequence of performing femoral artery excision to smaller mice.

Impaired blood flow recovery in klotho-deficient mice

Blood flow as assessed by laser doppler perfusion imaging was significantly impaired after surgery in both mice heterozygously deficient for the klotho gene and in wild type mice (Fig. 1). There was no significant difference in the degree of post-operative ischemia. However, in wild type mice ($n = 5$) blood flow of the ischemic hindlimb increased significantly after 7 days, compared with that of mice heterozygously deficient for the klotho gene ($n = 9$). Mice heterozygously deficient for the klotho gene showed significantly impaired blood flow restoration up to 14 days. Significant difference was not seen afterwards although a trend of impaired recovery still remained at 35 days likely reflective of the use of aged mice (Fig. 2). These results show that klotho-deficient mice show impaired recovery of blood flow following hindlimb ischemia.

Impaired capillary growth in klotho-deficient mice

Laser Doppler perfusion imaging visualizes blood flow on the basis of the presence of erythrocytes but cannot determine whether changes in blood flow is due to capillary growth (e.g. angiogenesis). To assess whether the reduced recovery of blood flow in klotho-deficient mice was due to impaired capillary growth, capillary density was examined by histopathology. In the ischemic hindlimb harvested at five weeks after surgery, immunohistochemical staining of CD31 (PECAM-1), which is a marker of new capillary growth (Fig. 4), showed that there was reduced capillary growth in mice heterozygously deficient for the klotho gene (mice heterozygously deficient for the klotho gene 253.4 ± 114.7 mm^{-2} vs. wild type mice 367.3 ± 157.9 mm^{-2}) (Fig. 3). These findings show that lower capillary density and thus impaired angiogenesis are seen in klotho-deficient mice.

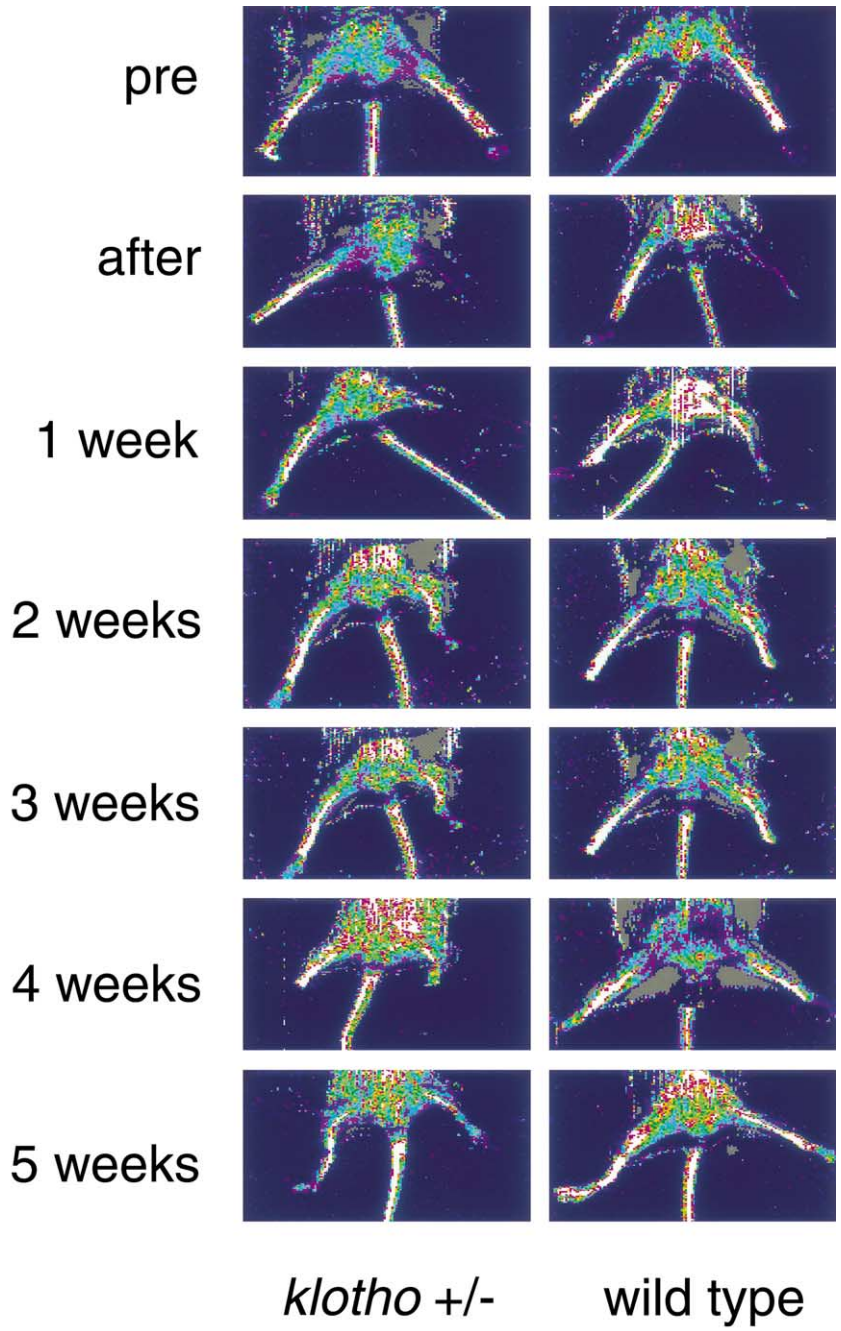

Fig. 1. Blood flow recovery is impaired in mice heterozygously deficient for the klotho gene as assessed by a hindlimb ischemia. Blood flow recovery as shown by color-coded laser doppler perfusion imager (LDPI) system images. Normal baseline (control) perfusion in both hindlimbs is shown in white. Marked reduction in blood flow in the right hindlimb immediately after unilateral femoral artery excision, is shown in dark blue. In wild type mice, blood flow began to recover from 7 days after surgery, and returned quickly to almost normal values by day 35. In contrast, recovery of hindlimb perfusion in mice heterozygously deficient for the klotho gene was significantly retarded in earlier stages compared with wild type mice.

To summarize, our results demonstrate that the klotho gene might play an important role in age-dependent impairment of angiogenesis. Klotho-deficient mice showed impaired restoration of blood flow in early stages after induction of ischemia. Significant difference with wild type mice was not seen at later stages, but impaired blood flow restoration remained in later stages in wild type mice as well. This is likely because we used aged mice in our experiments. Nonetheless, mice heterozygously deficient for the klotho gene still showed inferior recovery to the wild mice of the same age both in terms of blood flow and capillary density.

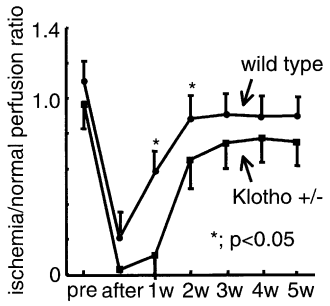

Fig. 2. Serial recovery of blood flow in response to hindlimb ischemia. Ratio of blood flow in the ischemic limb to that in the control limb of wild type mice ($n = 5$) and heterozygous mice heterozygously deficient for the klotho gene ($n = 9$) at each time point is shown. Differences in recovery between wild typemice and mice heterozygously deficient for the klotho gene were statistically significant during the first two weeks.

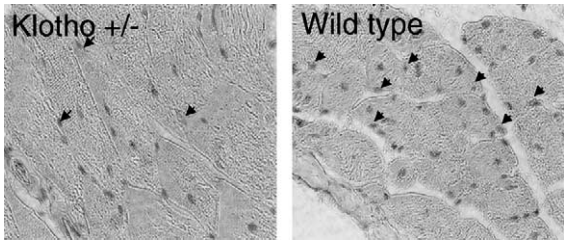

Fig. 3. Impaired angiogenesis in mice heterozygously deficient for the klotho gene. Immunohistochemical staining for CD31/PECAM-1 as an index of new capillary growth in hindlimb muscle of mice at day 35. CD31/PECAM-1-positively stained caplillaries are reddish-brown shown by arrows. Photomicrographs demonstrate decreased capillary density in mice heterozygously deficient for the klotho gene versus wild type mice.

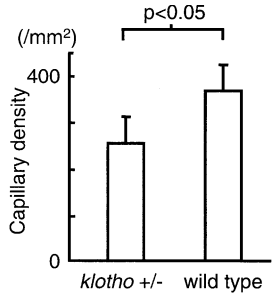

Fig. 4. Immunohistochemical examination of CD31/PECAM-1 expression in mice heterozygously deficient for the klotho gene. Quantitative analysis of capillary density. Capillary density was calculated as the number of capillaries in 1 mm^2.

Discussion

Hindlimb ischemia was induced in klotho-deficient mice to investigate the role of the aging-suppressor gene klotho in angiogenesis. Our results indicate that blood flow restoration is significantly impaired in klotho-deficient mice. Capillary density, as measured by immunohistochemical staining of CD31, was also lower in mice heterozygously deficient for the klotho gene than in wild type mice showing that angiogenesis is impaired in these mice as well. These findings suggest that klotho is involved in angiogenesis and restoration of blood flow in induced ischemia.

Angiogenesis is a process involving activation, migration, and proliferation of endothelial cells [13]. Various factors such as cytokines, growth factors, and NO contribute to endothelial cell activation which is essential for angiogenesis. In the case of tissue ischemia, endothelial cells are activated and various angiogenic growth factors, such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), or fibroblast growth factor (FGF) are released. Recently, hepatic growth factor (HGF) has also been reported to be a promising angiogenic growth factor for therapeutic angiogenesis. A common feature of these factors is that they are secreted proteins and function as humoral factors in both autocrine and paracrine manners which is important for potent therapeutic use in angiogenesis. Gene transfer of some of these factors have already been tested for therapeutic angiogenesis in humans [14]. Notably, vascular endothelial growth factor (VEGF), which has shown to play a central role in both pathological and physiological angiogenesis [15–17], has shown promising results in clinical trials [14].

Uniquely, the klotho gene product is thought to be present in at least two forms, both a secreted form and membrane-bound form. The klotho gene is expressed predominantly in the kidney, but its anti-aging phenotypes are multi-systemic. A circulating form of the protein mediating the actions of this factor would explain these multi-systemic effects. Indeed, this has been shown to be the case as a secretory product of the klotho gene has been identified. In further support of the secretory/circulatory effects of klotho is the parabiosis experiment which suggests that the klotho factor acts as a humoral factor. Therefore, one pathway by which the klotho factor affects angiogenesis is likely by a secreted form of the klotho protein which implies a direct action of the klotho gene on angiogenesis similar to the known growth factors (e.g. VEGF, FGF). An alternative theory is that the klotho protein can induce secretion of a hitherto unknown circulating factor(s) which affects angiogenesis and other phenotypes suggestive of an indirect pathway.

The membrane-bound form of klotho may also be involved in regulating angiogenesis. It has been suggested that growth factors and their actions as mediated by receptors is not sufficient to induce the biological process of angiogenesis. Interaction of endothelial cells with both the extracellular matrix and endothelial cells themselves also plays a critical role in angiogenesis. These interactions are mediated by transmembrane proteins such as integrins [18–20] and VE-cadherin [21–23]. Integrins associate directly with growth factor

receptors and induce intracellular signaling events which are unique for each growth factor [19]. Blocking agents of transmembrane proteins as exemplified by the angiogenic-suppressor factors, such as endostatin, and antagonists of and antibodies against these proteins have been shown to be clinically applicable for anti-angiogenic therapy of various diseases including cancer [20,23–25]. Some of them are currently being evaluated in clinical trials, and an antibody against integrin has successfully completed phase I trials [26]. Although the expression of klotho in vascular cells is still a controversial issue, if the klotho protein is present in its transmembrane form, indirect contribution of the transmembrane form of the klotho protein to angiogenesis as a mediator of intracellular signal is a plausible pathway.

As a mechanism of the action of klotho in angiogenesis, NO also likely plays a key role. The importance of NO in both endothelial function and in angiogenesis in response to growth factors has been extensively addressed [27–30]. For the VEGF-induced signaling pathway, VEGF induces NO production through its actions on NO synthase which lies downstream of VEGF [31]. NO has also been reported to play a permissive role in endothelial cell migration [32,33] and in angiogenesis [31,34]. Previous reports have shown mice heterozygously deficient for the klotho gene have significantly attenuated levels of endogenous NO metabolites as compared to wild type mice [8]. Another animal model of human type-II diabetes mellitus, OLETF rats, which show low levels of klotho gene expression, also demonstrated impaired endothelial function suggested to be due to decreased endogenous NO production [35,36]. Gene transfer of klotho improved endothelial function as well as NO metabolite levels in OLETF rats [37]. Taken together, these studies showed that the klotho gene might play a critical role in endogenous NO production through which it likely affects angiogenesis.

Previous studies have shown that age-dependent impairment of angiogenesis [5] and endothelial dysfunction may be induced as a function of aging [9,10,38–42]. A recent study demonstrated that VEGF can rescue age-dependent impairment of angiogenesis although the response in elderly animals was inferior to that in younger animals [5]. Age-dependent angiogenesis is therefore a diverse pathway and likely involves more hitherto unidentified factors and pathways. Given the role of klotho in endothelial cell dysfunction caused by reduced endogenous NO levels, we reasoned that klotho deficiency might contribute to impaired angiogenesis. The results of the present study provide a strong argument for the involvement of the aging-suppressor gene in regulating angiogenesis, and provides a new factor and working hypothesis to be tested in future studies.

In summary, angiogenesis was impaired in mice deficient for the klotho gene. Since the klotho gene is a regulatory factor of aging, our findings are intriguing as they implicate a role of regulation of aging as a critical factor in angiogenesis. The klotho gene may therefore play a key role in age-dependent impairment of angiogenesis. Furthermore, the two different forms of the klotho gene product being a secretory and membrane-bound form might regulate angiogenesis both as a humoral factor and as a mediator of the intracellular signaling cascade. Although further investigation is warranted, the use of klotho gene delivery as a new strategy of therapeutic angiogenesis for elderly patients can be envisioned.

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