Angiogenesis and Vasculogenesis Are Impaired in the Precocious-Aging klotho Mouse

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Background—The effects of aging on angiogenesis (vascular sprouting) and vasculogenesis (endothelial precursor cell [EPC] incorporation into vessels) are not well known. We examined whether ischemia-induced angiogenesis/vasculogenesis is altered in klotho (kl) mutant mice, an animal model of typical aging.

Methods and Results—After unilateral hindlimb ischemia, laser Doppler blood-flow (LDBF) analysis revealed a decreased ischemic-normal LDBF ratio in kl mice. Tissue capillary density was also suppressed in kl mice (+/+ > +/kl > kl/kl), Aortic-ring culture assay showed impaired angiogenesis in kl/kl mice, accompanied by reduced endothelium-derived nitric oxide release. Moreover, the rate of transplanted homologous bone marrow cells incorporated into capillaries in ischemic tissues (vasculogenesis) was lower in kl/kl mice than in wild-type (+/+ ) mice, which was associated with a decrease in the number of c-Kit⁺CD31⁺ EPC-like mononuclear cells in bone marrow and in peripheral blood. Finally, the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor cerivastatin restored the impaired neovascularization in kl/kl mice, accompanied by an increase in c-Kit⁺CD31⁺ cells in bone marrow and peripheral blood, and enhanced angiogenesis in the aortic-ring culture.

Conclusions—Angiogenesis and vasculogenesis are impaired in kl mutant mice, a model of typical aging. Moreover, the age-associated impairment of neovascularization might be a new target of statin therapy. (Circulation. 2004;110:1148-1155.)

Key Words: aging ■ angiogenesis ■ ischemia ■ statins ■ vasculature

Postnatal neovascularization comprises angiogenesis and vasculogenesis and is regulated by a complex interplay among various growth factors, circulating bone marrow (BM)-derived precursor/stem cells, and inflammatory cells. Angiogenesis is defined as the formation of new blood vessels by way of sprouting of preexisting mature endothelial cells, whereas vasculogenesis is considered the creation of blood vessels by differentiation of endothelial precursor cells (EPCs).

Aging is one of the important risk factors for arteriosclerosis. The occurrence of coronary artery disease and peripheral arterial occlusive disease increases with age, even in a population without other major risk factors. Recent studies have shown that aging impairs ischemia-induced neovascularization. However, little is known as to whether ischemia-induced neovascularization, especially vasculogenesis, becomes impaired with age, probably because of the paucity of appropriate animal models of aging.

Kuro-o et al recently developed klotho mutant (kl/kl) mice that show typical age-related phenotypes, such as arteriosclerosis, skin atrophy, osteoporosis, and infertility. The appearance of kl/kl mice is normal until 4 weeks after birth, after which they become inactive and die prematurely at 8 to 9 weeks. All of these phenotypes are caused by disruption of the single klotho gene, and interestingly, the klotho protein functions as a humoral factor, or “antiaging” hormone. In the present study, taking advantage of klotho mice, we investigated the effects of aging on ischemia-induced neovascularization, with a special focus on angiogenesis and vasculogenesis.

Methods

Animals
Heterozygous klotho mutant (+/kl) mice were generated as described previously. kl/kl and wild-type (WT; +/+ ) littermate mice were obtained by crossing +/kl mice.

Mouse Model of Angiogenesis
The Institutional Animal Care and Use Committee of Kurume University School of Medicine approved the experimental protocol. A mouse model of hindlimb ischemia was prepared as described previously. WT, +/+ , and kl/kl mice were subjected to unilateral hindlimb ischemia. Systemic blood pressure and heart rate were

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measured in the conscious state with a tail-cuff blood pressure analyzer (MK2000, Muromachi).

**Laser Doppler Blood-Flow Analysis**

At 6 time points (before and after surgery and on postoperative days 3, 7, 14, 21, and 28), we measured limb blood flow with a laser Doppler blood flow (LDBF) analyzer (MoorLDI, Moor). LDBF data were expressed as the ratio of ischemic-limb to normal-limb LDBF.9

**Tissue Capillary Density**

Additional mice (n=6 for each group) were euthanized by intracardiac injection of 30 mg pentobarbital sodium on day 28 after limb ischemia. Thigh adductor skeletal muscles of bilateral limbs were isolated, embedded in OCT compound, and snap-frozen in liquid N2. Multiple cryostat sections 5 μm thick were prepared and subjected to alkaline phosphatase staining to identify capillary endothelial cells.10

**Aortic-Ring Culture for Angiogenesis Assay**

Descending thoracic aortas were isolated from WT and kl/kl mice (n=3 from each group). Under a dissecting microscope, multiple 1-mm-thick aortic rings were prepared. Rings were then placed between 2 layers of growth factor–reduced Matrigel (Becton Dickinson) supplemented with medium 199, 20% fetal bovine serum, 10 U/mL heparin, antibiotics, and bovine pituitary extracts (Life Technologies).11 Microvascular sprouting was characterized by the incorporation of 1,1’-dioctadecyl-3,3,3’3’-tetramethylindocarbocyanine perchlorate–labeled acetylated LDL (DiI-acLDL).12 We measured the length of endothelial sprouts at 4 randomly selected points at right angles to each other and calculated the mean length.

**Analysis of EPCs**

We quantified EPC-like mononuclear cells (MNCs) in both peripheral blood (PB) and bone marrow (BM) after induction of hindlimb ischemia because EPCs mobilize from BM.13 WT and kl/kl mice
(n = 5 each) were subjected to limb ischemia and euthanized on day 14 to collect PB (0.2 mL) and BM cells. PB-MNCs were isolated by centrifugation through a Histopaque-1083 density gradient. BM cells were obtained from the bilateral femurs by flushing the BM cavity with heparinized saline. PB-MNCs and BM cells were suspended in phosphate-buffered saline containing 1% bovine serum albumin and were subjected to flow cytometry.

EPC-like MNCs were identified as c-Kit+/CD31− or CD34+/CD31− cells. PB-MNCs or BM cells were incubated with a fluorescein isothiocyanate–conjugated anti-mouse c-Kit monoclonal antibody (clone 2B8, Immunotech) or with a fluorescein isothiocyanate–conjugated anti-mouse CD34 monoclonal antibody (PharMingen), followed by phycoerythrin–conjugated anti-mouse CD31 monoclonal antibody (clone MEC13.3, PharMingen). Cells were finally fixed with 1% paraformaldehyde (pH 7.5) in phosphate-buffered saline and analyzed by flow cytometry.

In addition to WT and kl/kl mice (n = 5 each), BM cells were isolated as described earlier. BM-MNCs were then obtained by centrifugation through a Histopaque density gradient, and cells (7 × 10⁶) were subjected to culture assay in 2% gelatin–coated, 12-well plastic dishes to quantify the number of differentiated EPC-like attaching cells.

Homologous BM Cell Transplantation
Donor WT and kl/kl mice (n = 10 each) were subjected to hindlimb ischemia and were euthanized on day 14 to collect BM cells. Donor BM cells were labeled with the green fluorescent marker PKH2-GL (Sigma). Recipient WT and kl/kl mice (n = 10 each) were also subjected to unilateral hindlimb ischemia, and they received BM cell transplantation through the tail vein (1.4 to 1.8 × 10⁷ cells per mouse) on the day of surgery. Each recipient mouse received PKH2-GL–labeled BM cells isolated from 1 donor mouse. On day 14, the recipient mice were euthanized, and ischemic skeletal muscles were harvested. Tissues were embedded in OCT compound and snap-frozen in liquid N₂. Five-micron-thick sections were stained with an anti–von Willebrand factor (vWF) monoclonal antibody (clone 4F9, Immunotech) or an anti-CD31 monoclonal antibody (clone MEC13.3) followed by a tetramethylrhodamine isothiocyanate–conjugated secondary anti-IgG1 (Jackson ImmunoResearch) antibody, respectively, to detect endothelial cells by fluorescence microscopy. We calculated the percentage of PKH2-GL+/CD31− or PKH2-GL+/vWF− double-positive cells among the population of CD31+ or vWF+ endothelial cells as the BM cell incorporation (BMCI) ratio with the following formula: BMCI ratio (%) = number of PKH2-GL+/CD31+ cells (or PKH2-GL+/vWF+ cells)/total number of CD31+ cells (or vWF+ cells) × 100. Five microscopic fields (×40 magnification) were examined from samples of 6 animals in each group, and the mean BMCI ratio was calculated.

Urine NOₓ and Tissue cGMP Content
We measured urinary excretion of NOₓ (nitrite [NO₂⁻] plus nitrate [NO₃⁻]) in WT and kl/kl mice to examine whether systemic nitric oxide (NO) production was decreased in kl/kl mice. NOₓ was measured by high-performance liquid chromatography after reduction of total NO₃⁻ to NO₂⁻. Thigh skeletal muscles were harvested from ischemic hindlimbs of WT and kl/kl mice (n = 5 from each group) on day 14. Samples were weighed, snap-frozen in LN₂, and stored at −80°C until biochemical analyses of tissue cGMP were performed.

Effects of Statin Therapy on Neovascularization in kl/kl Mice
An additional 15 kl/kl mice were treated with cerivastatin (Bayer), a lipophilic 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (statin). These animals were administered cerivastatin (5 mg·kg⁻¹·d⁻¹) for 28 days by subcutaneous injection on the dorsal skin starting on the day of surgery. The dose of cerivastatin, which had been determined in a previous study, effectively enhanced vascular endothelial NO synthase activity and induced vasodilatation in mice. We measured the ischemic-normal hindlimb LDBF ratio and the capillary density. Serum total cholesterol levels were measured with a commercially available kit (Wako). The ex vivo aortic-ring culture assay was also performed to examine whether ex vivo angiogenesis was altered in cerivastatin-treated kl/kl mice. Finally, we determined the number of c-Kit+/CD31+ EPC-like MNCs in BM and PB-MNCs in the cerivastatin-treated animals.

Statistics
Data are expressed as mean±SE. Differences among the 3 groups were analyzed by 1-way ANOVA followed by Scheffe’s comparison for 2 groups. Differences between the WT and kl/kl mice were analyzed by unpaired Student’s t test. The comparative incidence of...
limb amputation was evaluated by $\chi^2$ test. Probability values <0.05 were considered statistically significant.

**Results**

**Unilateral Hindlimb Ischemia in klotho Mutant Mice**

After the left femoral artery and vein were excised, the ratio of ischemic (left) to normal (right) hindlimb blood flow decreased to comparable levels (0.09 to 0.11, $P=\text{NS}$) in all 3 groups, blood pressure and heart rate did not differ among the 3 groups during the experimental period.

**Ischemic-Normal Hindlimb Blood Flow Ratio**

The data in Figure 1A show the trends in hindlimb LDBF, which disclose a progressive recovery of LDBF in the
ischemic limb of WT mice (Figure 1B). In contrast, LDBF in \textit{kl/kl} mice remained impaired throughout the follow-up period, and the ratio was persistently lower in \textit{kl/kl} mice than in WT mice (\(P<0.001\), Figure 1B). In heterozygous +/\textit{k} mice, the LDBF ratio was lower than that of WT mice; however, it was greater than that of \textit{kl/kl} mice (\(n=10\) each). Therefore, the degree of impairment of the LDBF ratio was related to the severity of allele mutation of the \textit{klotho} gene (LDBF ratio: +/+ > +/\textit{k} > \textit{kl/kl}; Figure 1B).

### Tissue Capillary Density

The findings in Figure 1C are photomicrographs of alkaline phosphatase–stained skeletal muscles with or without ischemia. Capillary density in ischemic hindlimbs, as examined on day 28, was significantly reduced in \textit{kl/kl} mice and in +/\textit{k} mice compared with WT mice (Figure 1C and 1D). Quantitative analysis revealed that the degree of reduced capillary density was related to the severity of allele mutation of the \textit{klotho} gene (capillary density: +/+ > +/\textit{k} > \textit{kl/kl}; Figure 1D). In contrast, the capillary density in nonischemic hindlimbs examined on day 28 did not differ among the 3 groups (Figure 1D, \(n=6\) each).

### Aortic-Ring Culture Assay for Ex Vivo Angiogenesis

The findings in Figure 2A are photographs representing microvascular sprouting from cultured aortic rings. Sprouted vessels incorporated Dil–acLDL, indicating that the sprouts were endothelial cells.\(^{12}\) On day 7 of culture, quantitative analysis revealed that the mean length of the vessels sprouted from the edge of aortas was significantly shorter in \textit{kl/kl} mice than in WT mice (\(n=3\) each, Figure 2B and 2C). Thus, ex vivo angiogenesis was impaired in \textit{kl/kl} mice compared with WT mice.

### Tissue cGMP Contents and Urinary NO\(_x\) Secretion

To examine whether the \textit{klotho} mutation affected NO production in the ischemic hindlimbs, we examined the tissue contents of cGMP. The tissue cGMP level was significantly lower in \textit{kl/kl} mice than in WT mice. Also, the amount of urinary NO\(_x\) secretion, which indirectly represents endothelin-derived NO formation, was significantly lower in \textit{kl/kl} mice than in WT mice (\(n=5\) each, Figure 2D).

### EPCs in BM and in PB-MNCs

On day 14 after hindlimb ischemia, we analyzed EPC-like MNCs in BM and PB by flow cytometry. EPC-like MNCs were identified as c-Kit\(^+\)CD31\(^+\) or CD34\(^+\)CD31\(^+\) MNCs. The numbers of c-Kit\(^+\)CD31\(^+\) cells and CD34\(^+\)CD31\(^+\) cells in both BM and PB were significantly lower in \textit{kl/kl} mice than in WT mice (\(n=5\) each, Figure 3A and 3B). BM-MNC culture assay further revealed that the number of differentiated EPC-like attaching cells was significantly lower in \textit{kl/kl} mice than in WT mice (Figure 3C).

### BM Transplantation for Vasculogenesis Analysis

We examined whether intravenously transplanted homologous PKH2-GL–labeled BM cells were incorporated into vascular structures in ischemic tissue. Histological analysis on day 14 revealed that PKH2-GL\(^+\) BM cells were incorporated into microvascular structures in ischemic tissues, and most of the cells were also stained for either CD31 (Figure 4A) or vWF (Figure 4B), markers of endothelial cells. The number of PKH2-GL\(^+\) cells per microscopic field was significantly lower in \textit{kl/kl} mice (receiving \textit{kl/kl} BM cells) than in WT mice (receiving WT BM cells, Figure 4C and 4D). The number of CD31\(^+\) or vWF\(^+\) capillary endothelial cells was also significantly lower in \textit{kl/kl} mice than in WT mice. The
incorporation rate of transplanted BM cells into capillary structures as assessed by the BMCI index was significantly lower in kl/kl mice than in WT mice (Figure 4C and 4D). LDBF analysis showed a decreased ischemic-normal hindlimb blood flow ratio on day 14 in kl/kl mice compared with WT mice (data not shown).

**Effects of Cerivastatin on Neovascularization in kl/kl Mice**

After hindlimb ischemia, LDBF analysis showed that the ischemic-normal hindlimb LDBF ratio was significantly greater in cerivastatin-treated kl/kl mice than in kl/kl mice (Figure 5A and 5B). On day 28, capillary density was significantly greater in cerivastatin-treated kl/kl mice than in untreated kl/kl mice (Figure 5C and 5D). Microvascular sprouting from aortic rings was also greater in cerivastatin-treated kl/kl mice than in untreated kl/kl mice (Figure 6A and 6B). These changes were accompanied by an increased tissue cGMP content and increased number of c-Kit+/CD31+ EPCs in BM and PB after cerivastatin treatment (Figure 6C and 6D). Serum levels of total cholesterol and triglycerides did not differ among the WT, kl/kl, and kl/kl-cerivastatin groups (data not shown), indicating that cerivastatin did not change the ischemia-induced neovascularization by altering the serum lipid profile.

**Limb Amputation Ratio**

After hindlimb ischemia, spontaneous limb amputation occurred in only 3.1% of 32 WT animals. Among kl/kl mice, however, spontaneous amputation occurred in 24% of 25 animals. Cerivastatin treatment resulted in significant limb salvage in kl/kl mice, and autoamputation of the ischemic limbs occurred in only 6.3% of 48 tested animals ($P<0.05$ vs untreated kl/kl mice, Figure 7A and 7B).

**Discussion**

In the present study, in vivo ischemia-induced neovascularization was impaired in klotho mutant mice, and both ex vivo angiogenesis and in vivo vasculogenesis were also inhibited in klotho mice. Interestingly, cerivastatin treatment rescued the impaired ischemia-induced neovascularization in kl/kl mice.

Because our aortic-ring organ culture assay can avoid the contribution of EPCs to neovascularization, the data indicate that angiogenesis is impaired in kl/kl mice. There may be several mechanisms for the impaired angiogenesis in kl/kl mice. We previously showed that angiogenesis depends on endothelium-derived NO. Furthermore, endothelium-derived NO lies downstream of vascular endothelial growth factor–induced angiogenesis and plays a central role in endothelial migration, an essential process for angiogenesis. Because endothelium-derived NO production declines with age and because endothelium-dependent vasorelaxation is impaired in klotho mice, the insufficient endothelium-derived NO formation may at least partly account for the impaired angiogenesis in kl/kl mice. Consistently, the tissue cGMP
levels, a downstream molecule of NO, and urinary NO secretion were reduced in kl/kl mice in the present study.

We next examined whether vasculogenesis was impaired in kl/kl mice. Circulating EPCs mobilize from BM and are incorporated into angiogenic foci. Because BM function declines with age, it is conceivable that the number, mobilization, and/or function of EPCs would be decreased in kl/kl mice. Vasa and coworkers recently showed that the migratory property of EPCs was impaired by the presence of coronary risk factors, including aging. However, the effects of aging on the numbers of EPCs have not been well examined previously. In the present study, the numbers of EPC-like c-Kit⁺/CD31⁻ as well as CD34⁺/CD31⁻-MNCs were significantly decreased in both BM and PB in kl/kl mice. Thus, EPC differentiation and/or EPC mobilization from BM is likely impaired in kl/kl mice.

The mechanism by which the number of EPCs is decreased in kl/kl mice remains to be determined. A recent study indicated that the klotho protein regulates the BM microenvironment, including macrophages, fibroblasts, endothelial cells, and extracellular matrixes, and the 3-dimensional structure composed of these cells is an important environment for hematopoiesis and EPC differentiation. A deficiency of the klotho gene has also been reported to cause osteoporosis, which further disturbs the BM microenvironment. Taken together, the reduced number of EPCs in kl/kl mice might be caused at least partly by alterations of the BM microenvironment.

We then performed homologous BM cell transplantation studies to further clarify in vivo vasculogenesis. The incorporation rate of BM cells into capillary structures (BMCI ratio) was significantly reduced in kl/kl mice. Therefore, it is possible that ischemia-induced vasculogenesis is also inhibited in kl/kl mice. The mechanism of the reduced BM cell incorporation into capillaries is currently unknown. However, a recent study showed that vascular endothelial growth factor could mobilize EPCs from BM and induce EPC homing to ischemic tissues. Because ischemia-mediated vascular endothelial growth factor induction decreases with age, the decreased vasculogenesis may be explained in part by a decreased vascular endothelial growth factor formation in kl/kl mice. This issue should be further examined.

Because “antisenescence” is one of the major topics of recent biomedical research, we explored a potential therapeutic strategy against the impaired neovascularization in kl/kl mice. In the present study, cerivastatin accelerated recovery of the LDBF ratio and increased capillary density in kl/kl mice. Cerivastatin also increased tissue cGMP levels, indicating recovery of the tissue NO-cGMP pathway. Consistently, ex vivo angiogenesis in aortic-ring culture was restored in cerivastatin-treated kl/kl mice. Cerivastatin also increased the number of c-Kit⁺CD31⁻ MNCs within both BM and PB. The latter findings are consistent with the results of recent reports showing that statins not only enhance angiogenesis but also stimulate the mobilization of EPCs from BM. Therefore, cerivastatin likely augmented both angiogenesis and vasculogenesis in kl/kl mice, resulting in increased limb blood flow and significant limb salvage in kl/kl mice.

There is a limitation in the present study. It is still unknown whether our findings were caused by the klotho gene mutation per se or secondarily by the aging phenotype of kl/kl mice. Currently, the precise role of the klotho protein in terms of antisenescence efficacy is not well known. Abnormalities in calcium/phosphorus metabolism have been postulated in kl/kl mice, but its role in reduced angiogenesis is still unknown. Nevertheless, the present study demonstrates that the klotho gene product may function as a maintenance factor for endothelium-
would be intriguing to test whether statins could rescue impaired neovascularization in elderly patients.

In summary, our findings have several clinical implications. First, reduced neovascularization with age could contribute to the increased incidence and severity of ischemic peripheral/heart diseases in the elderly. Second, age-related impairment of neovascularization could be rescued by statins. Because statins have been reported to inhibit some age-associated disorders (e.g., osteoporosis and dementia), it would be intriguing to test whether statins could rescue impaired neovascularization in elderly patients.

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