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Role of Klotho in migration and proliferation of human dermal microvascular endothelial cells

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ABSTRACT

Purpose: To examine the possible role of Klotho (Kl) in human microvasculature.
Methods: The expression level of Kl in primary human dermal microvascular endothelial cells (HDMECs) and primary human dermal fibroblasts (HFb) was detected by real-time polymerase chain reaction amplification (qRT-PCR), Western blot analyses and immunohistochemistry. Migration of HDMECs and HFb was examined in monolayer wound healing "scratch assay" and Transwell assay. Proliferation of these cells was examined using Cell Proliferation BrdU incorporation assay.
Results: Our results have shown that downregulation of Kl abrogated HDMECs migration after 48 h. On the other hand, migration of HFb significantly increased after blocking Kl. Lack of Kl decreased expression of genes involved in the activation of endothelial cells and enhanced expression of genes involved in extracellular matrix remodeling and organization of connective tissue.
Conclusions: This study for the first time provides the evidence that Kl is expressed in HDMECs and HFb. Additionally, we have demonstrated that Kl is implicated in the process of angiogenesis of human dermal microvasculature.

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Introduction

Angiogenesis is a process through which new vessels are formed from pre-existing capillaries or venules. The whole process is divided into two phases: the activation phase and the resolution phase. During activation phase there is increased vascular permeability, detachment of periendothelial cells from endothelium, degradation and remodeling of basement membrane followed by migration and increased cell division of endothelial cells (Markiewicz et al., 2011; Carmeliet and Collen, 2000; Goumans et al., 2003; D'Amore and Thompson, 1987; Senger and GE, 2011). In the resolution phase, proliferation and migration of endothelial cells is decreased and the basement membrane is rebuilt leading to vessel maturation (Goumans et al., 2003). The molecular and cellular mechanisms that regulate these processes are under investigation. Cell migration is essential to angiogenesis. This process is directionally regulated by chemotactic, haptotactic, and mechanotactic stimuli and further involves degradation of the extracellular matrix to enable progression of the migrating cells (L1 et al., 2007).

The human Klotho gene encodes the α-Kl protein. Three α-Kl protein types with possibly different functions have been identified: a full-length transmembrane α-Kl, a secreted α-Kl and a truncated soluble α-Kl (sKl). sKl is a protein released from the cell membrane and after entering the urine and/or the blood, sKl functions as a hormone (Xu and Sun, 2015). The transmembrane Kl protein, homologous to β-glucuronidase was shown to be required for FGF23 (fibroblast growth factor 23)-mediated receptor activation. Kl binds to multiple FGFRs and increases their affinity for FGF23. Klotho-FGFR co-expression delineates the tissue specificity of FGF23 effects (Urakawa et al., 2006; Kurosu et al., 2006). Klotho which is an anti-aging gene plays an important role in angiogenesis. It has been shown in hindlimb ischemia heterozygously K1 gene deficient mice model that capillary density is decreased and Kl plays a role in restoration of blood flow in these mice (Fukino et al., 2002). Additionally, in mice which lack the Kl gene, aortic-ring culture assay demonstrated reduced angiogenesis accompanied by reduced endothelium derived nitric oxide release (Shimada et al., 2004). Studies published by Kusaba et al., reported that vascular endothelium in Klotho deficient mice is hyperpermeable because of increased apoptosis and decreased expression of VE-cadherin (vascular endothelial) (Kusaba et al., 2010). Interestingly, Kl suppresses tumor necrosis factor-α (TNF-α) induced expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells (Maekawa et al., 2009). These adhesion molecules are essential for formation of new vessels (DeLisser et al., 1997). Although previous studies have shown that Kl has anti-apoptotic and anti-senescent effects on

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Table 1
Primers for real time RT-PCR.

B_2MG F 5′- GGCATTCCTGAAGCTGACAG -3′, R 5′- TGGATGACGTGAGTAAACCTG -3′
VCAM1 F 5′- TCTACGCTGACAATGAATCCTG -3′, R 5′- AGGGCCACTCAAATGAATCTC -3′
PECAM1 F 5′- GAGTCTGGAGAGGACATTGTG -3′, R 5′- CTTCTGCTTGGTCCAAAATGC -3′
MMP9 F 5′- CAGTTTCCATTCATCTTCCAAGG -3′, R 5′- CATCACCGTCGAGTCAGC -3′
KLOTHO F 5′- GACCACCAAGAGAGATGATGC -3′, R 5′- CTGTAACCTCTGTGCCACTC -3′
LAMB3 F 5′- CTTCACTGGACTCACCTACG -3′, R 5′- GCACACTGGTCACATTTGG -3′
MMP8 F 5′- GCAACCCTATCCAACCTACTG -3′, R 5′- CGACTCTTTGTAGCTGAGGATG -3′
MMP15 F 5′- CTTCTCCAGCACTGACCTG - 3′, R 5′- TTGTCAACGTCCTTCCACTG -3′

endothelial cells (Ikushima et al., 2006), Klotho's role in migration and proliferation of endothelial cells is not well understood. Expression of Kl in human umbilical vein endothelial cells (HUVECs) decreases with cellular senescence suggesting the role of this gene in aging as well as in age related vascular diseases (Carracedo et al., 2012).

It is known that angiogenesis requires an interaction between cells and extracellular matrix (ECM). However, the contribution of the cellular and fibrillar microenvironment in angiogenesis still remains unresolved. Fibroblasts and extracellular matrix deposited by these cells are the major players involved in this process. Matrix metalloproteinases (MMPs) are proteinases which take part in ECM degradation. Moreover, these proteinases play a significant role in many biological processes, such as embryogenesis, normal tissue remodeling, wound healing, and angiogenesis (Visse and Nagase, 2003). MMP's especially MMP-2 and MMP-9 play a key role in angiogenesis by degrading basement membrane and other ECM components, allowing endothelial cells to detach and migrate into new tissue (Genersch et al., 2000a; JE, 2003). They are also involved in the release of ECM bound proangiogenic factors (bFGF, VEGF and TGF β) (JE, 2003).

This is the first report showing expression of Kl in HDMECs and HFb. In this study, we will investigate the possible role of Kl in migration and proliferation of these cells. Furthermore, we will explore the role of Kl in regulation of genes involved in the activation of endothelial cells and ECM remodeling and organization of connective tissue.

Materials and methods

Cell culture

Human dermal microvascular endothelial cells and human dermal fibroblasts were isolated from foreskins as previously described (Richard et al., 1998). The cells were cultured on collagen type I coated flasks in the presence of endothelial cell growth medium 5% EBM2-MV (endothelial basal cell growth medium) with supplements (Lonza, Inc.) and incubated at 37 °C with humidified 95% air/5% CO_2. Human dermal fibroblasts culture was established from foreskins of healthy newborns from the Medical University of South Carolina Hospital in compliance with the Institutional Review Board for Human Studies.

Immunohistochemistry

Klotho expression was identified in HDMECs and HFb. α-SMA (NeoMarkers Inc.) was detected in HFbs by IHC, according to the previously described protocol (Markiewicz et al., 2007). Isolated HDMECs were labeled with Dil-Ac-LDL (Low Density Lipoprotein acetylated and labeled with fluorescent probe Dil), according to the manufacturer's protocol (Biomedical Technology Inc.).

Quantitative Real time PCR (qRT-PCR).

Total RNA was isolated from HDMECs or HFb using RNeasy Mini Kit (Qiagen, Inc). Briefly, 5 μg of total RNA was reverse transcribed with random hexamers using Transcriptor First Strand cDNA Synthesis Kit (Roche, Inc.) according to the manufacturer's protocol. (See Table 1).

B_2MG (beta-2 microglobulin) was used as a control. Amplification of product was performed using the PTC-200 Peltier Thermal Cycler at the reaction condition described by Tang et al. (Tang et al., 2011).

Western blot analyses

Confluent untreated HDMECs, HFb or transfected with scrRNA (control), Klotho siRNA (siKl) (Origene, Inc.) or treated with recombinant human Klotho (rhKL) (0.5 μg/ml) (Prospec, Inc.) were lysed in RIPA buffer. Protein concentration was quantified using the BCA Protein Assay kit (Pierce, Rockford, IL). Forty micrograms of protein was

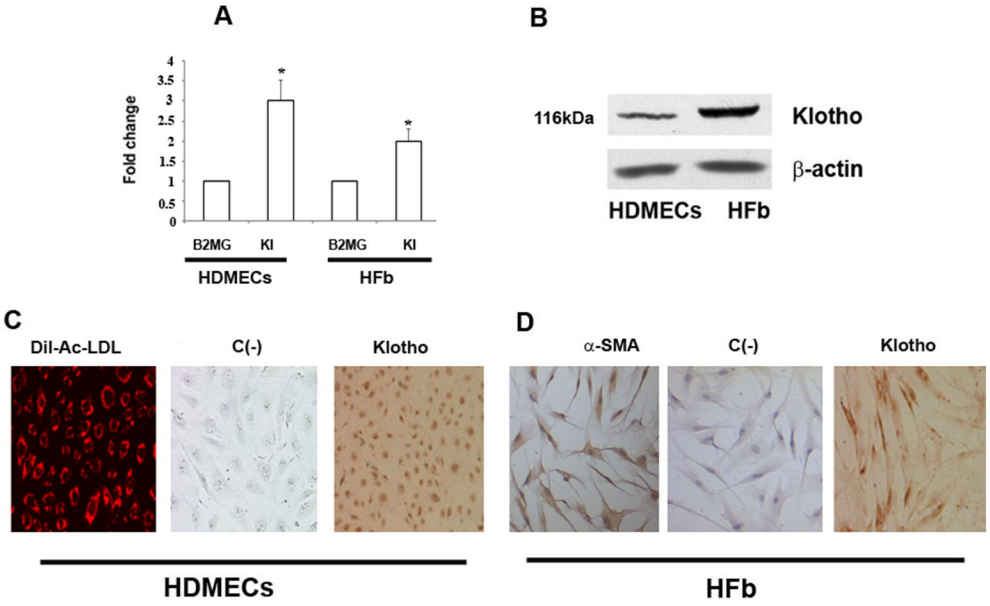

Fig. 1. Expression of Klotho in HDMECs (A, B, C), and HFb (A, B, D). (A) qRT-PCR analysis shows Kl mRNA in both HDMECs and HFb. Kl mRNA is normalized for B_2MG (beta-2 microglobulin) mRNA. (B) Klotho protein level was determined by Western blot analysis. The blot was probed O/N with primary antibody at 4 °C. β-actin was used as a control for equal loading (C, D). Cultured HDMECs and HFb were isolated from foreskins and subjected to IHC for Klotho. Immunoreactivity was detected by using DAB substrate kit. C(−) represents the negative control (no primary Ab) in HDMECs and HFb. Isolated HDMECs were labeled with Dil-Ac-LDL and visualized under fluorescence microscopy. α-SMA is the positive control for HFb. Bars represent mean ± SEM. Asterisk symbol indicates statistically significant values:*$P < 0.05$.