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Original Contribution

Senescence marker protein-30 deficiency impairs angiogenesis under ischemia

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ABSTRACT

Aging decreases collateral-dependent flow recovery following acute arterial obstruction. However, the mechanisms are partially understood, therefore critical management has been lacked in clinical setting. Senescence marker protein-30 (SMP30) is a novel aging marker, which is assumed to act as an anti-aging factor in various organs. Therefore, we studied the effect of SMP30 on ischemia-induced collateral growth in SMP30 knockout (KO) mice, young and old C57BL/6 mice. The SMP30 expression in gastrocnemius tissue was decreased in old mice compared to that of young mice. The recovery of cutaneous blood flow in hind limb after femoral artery ligation and tissue capillary density recoveries were suppressed in SMP30 KO and old mice compared to those in young mice. Nitric oxide generation induced by L-arginine and GSH/GSSG in aorta of SMP30 KO and old mice were lower than those in young mice. The levels of NADPH oxidase activity and superoxide production in the ischemic tissue were higher in SMP30 KO and old mice than in young mice. The phosphorylated eNOS and Akt levels and VEGF levels in ischemic muscle were lower in SMP30 KO and old mice than in young mice. Deficiency of SMP30 exacerbates oxidative stress related to NADPH oxidase activity enhancement and impairs eNOS activity, which leads to rarefaction of angiogenesis induced by ischemia. These results suggest that SMP30 plays a key role in disrupting collateral growth under ischemia in aging.

1. Introduction

The occurrence of coronary and peripheral artery diseases increases with age, even in a population without other major risk factors [1,2]. Additionally, aging augments the severity of tissue injury after acute and chronic ischemia [3]. The impairment of collateral growth would be a major exacerbating factor in the prognosis of ischemic disease. It was first demonstrated >10 years ago that aging decreases collateral-dependent flow recovery following acute arterial obstruction [4]. However, whether aging reduces the extent of the collateral circulation has been controversial particularly because the mechanisms of age-associated decline in vascular function are complicated. Senescence marker protein-30 (SMP30), a 34-kDa protein, is a novel molecule whose expression decreases with age in a sex-independent manner. In humans, the SMP30 gene is located in the p11.3-q11.2 segment of the X chromosome. In mice, SMP30 transcripts are detected in various organs, including the liver, kidney, cerebrum, testis, lung, and heart [5,6]. SMP30 protects cellular functions from age-associated deterioration in several organs, such as lung, brain, and heart. SMP30 knockout (KO) mice were generated by disruption of the third exon of SMP30 gene on the X chromosome [7]. Although these mice show about 20% lower in body weight and a shorter life span compared to the wild type, they are fertile and apparently healthy, and many of the phenotypic changes mimic the premature aging processes, as hepatic lipid droplets, abnormally enlarged mitochondria with indistinct cristae, and enlarged lysosomes, deposition of lipofuscin as an aging marker in renal tubular epithelial cells [8]. In addition, SMP30 KO mice have an increased susceptibility to apoptosis induced by TNF-α, Fas ligand, and calcium ionophore in hepatocytes [5,9,10]. Taken together, SMP30 is assumed to behave as an anti-aging factor. Recently, endothelial nitric oxide synthase (eNOS) and glutathione contribute the angiogenesis induced by various stimuli [11,12] and we reported that the decrease of SMP30 impairs not only eNOS activity but also anti-oxidative ability of glutathione in heart [13,14]. Moreover,

Abbreviations: SMP30 KO mice, senescence marker protein-30 knockout mice; CBF, cutaneous blood flow; NO, nitric oxide; L-NAME, L-nitro arginine methyl ester; eNOS, endothelial nitric oxide synthase; GSH, reduced glutathione; GSSG, oxidized glutathione

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SMP30 has a protective role in maintaining coronary circulation against oxidative stress as well as a cytoprotective role in a cell line [9,15]. Therefore, we speculated that decrease of SMP30 is a key factor to exacerbate collateral growth related to nitric oxide and glutathione with aging. Groleau et al. have reported that oxidative stress increases with aging and that the deficiency of superoxide dismutase impairs angiogenesis in a senescence model [16]. We hypothesized that SMP30 plays an important role in collateral growth under ischemia that is related not only to angiogenetic factors, including eNOS and GSH, but also to defense against oxidative stress in aging. Therefore, we investigated the effect of SMP30 on angiogenesis with limb ischemia and the impact of oxidative stress on collateral development in SMP30 KO mice, young and old C57BL/6 mice.

2. Material and methods

2.1. Animal

SMP30 KO mice were created from C57BL/6 mice using the gene-targeting technique previously described [7]. Young and old wild type (WT) C57BL/6 male mice [young; 8 weeks (W), body weight (B.W.) 23.6 ± 2.6 g, old; 74 weeks, B.W. 25.2 ± 2.8 g] and SMP30 KO male mice (8 weeks, B.W. 22.8 ± 2.8 g) were housed and bred in a room at 22 ± 3 °C, with relative humidity of $50 \pm 10\%$ and a 12-h light–dark cycle. The mice were given food that included vitamin C (21 mg/100 g, CLEA Japan, Tokyo, Japan) and water ad libitum. The experiments were conducted according to the Guidelines on Animal Experiments of Fukushima Medical University, the Japanese Government Animal Protection and Management Law (No. 105), and *Guide for the Care and Use of Laboratory Animals* (NIH Publication no. 85-23, revised 1996).

2.2. Hind limb ischemia

The mice were anesthetized with 1.25% isoflurane/O_2, and the hind limbs were depilated. The rectal temperature was maintained at 37.0 ± 0.5 °C. The left femoral artery was exposed aseptically and ligated distal to the inguinal ligament and proximal to the saphenous-popliteal bifurcation using 7–0 suture. The artery and all of the branches were carefully dissected free from the veins and nerves and were excised with ligation. The wound was closed and cefazolin (50 mg/kg im), furazolidone (topical) and pentazocine (10 mg/kg im) were administered. Sham operation is without femoral artery ligation but skin incision in right hind limb.

2.3. Laser Doppler perfusion imaging

Under 1.125% isoflurane/O_2 anesthesia, scanning laser Doppler perfusion imaging (Moor Instruments, Wilmington, DE, USA) was used to record the hind limb perfusion before ligation and on postoperative days 0, 1, 7, 10 and 14. Hair was removed using depilatory cream before imaging, and the mice were placed on a heating pad at 37 °C to minimize temperature variation. Average perfusion was recorded from the plantar surface, which indexes overall limb blood flow, and is expressed as the ischemic/non-ischemic ratio for assessing the variables, including ambient light and temperature [17].

2.4. Capillary density measurement

After the laser Doppler imaging on day 14 post-surgery, the mice were anesthetized (pentobarbital 60 mg/kg body weight i.p.) and sacrificed. The gastrocnemius tissue was excised from the right (non-ischemic area) and left (ischemic area) hind limb, and the snap was frozen in O. C. T. compound (Sakura Finetek, Tokyo, Japan) for cryosectioning. For mouse capillary density analysis, the hind limb sections from each mouse were stained using a mouse-specific CD31 (Santa Cruz Biotechnology, Dallas, TX, USA), followed by Alexafluor-594 secondary antibody. For quantification, the numbers of CD31-positive cells were counted in 10 randomly selected transverse sections in each animal. The results were averaged, and capillary density is expressed in terms of the number of CD31-positive cells per mm^2 field [18].

2.5. Detection of superoxide anions and NADPH oxidase activity in the ischemic muscle

Intracellular superoxide anions were quantified using a high-performance liquid chromatography/fluorescence assay that employs dihydroethidium as a probe [19]. A stable fluorescent product, 2-hydroxyethidium, is formed from the reaction between dihydroethidium and superoxide anions. After the laser Doppler imaging on day 14 post-surgery, left gastrocnemius tissue was incubated in Krebs-HEPES buffer containing 50 μmol/L of dihydroethidium (Molecular Probes, Eugene, OR) at 37 °C for 15 min. The samples were washed to remove the free probe and were incubated in Krebs-HEPES buffer for 1 additional hour at 37 °C. The tissue was homogenized in 4 °C cold methanol and centrifuged at 12,000 rpm. The supernatant was analyzed using high-performance liquid chromatography/fluorescence (Beckman Coulter, Brea, CA) in 37.0% acetonitrile in 0.1% aqueous trifluoroacetic acid solution. The data were normalized against tissue protein levels. NADPH oxidase activity was quantified using lucigenin-enhanced chemiluminescence [20]. NADPH (100 μmol/L, Sigma-Aldrich, St. Louis, MO, USA) was added to the buffer containing the tissue (30 μg protein in 500 μL), and lucigenin was injected automatically at 5 μM to avoid known artifacts when used at higher concentrations. NADPH oxidase activity was calculated by subtracting the basal values from those in the presence of NADPH.

2.6. Total thiol and tissue glutathione concentrations in aorta

Total thiols were determined in aorta homogenates by measuring the absorbance of 5-thio-2-nitrobenzoic acid, the reaction product of sulfhydryl groups with 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent). An equal volume of 10% metaphosphoric acid was added to the samples, the resulting precipitated proteins were pelleted by centrifugation, and the supernatant was neutralized with 50 μL/mL of 4 mol/L triethanolamine. Thiols were measured by adding 200 μL of Ellman's reagent from a commercially available assay to 50 μL of the neutralized supernatant. The absorbance of the Ellman's reagent adduct was measured at 405 nm [21]. Tissue concentrations of glutathione (total, reduced, and oxidized) were measured in tissue homogenates (10 wt%/vol%) after deproteinization using metaphosphoric acid in an enzymatic recycling method with glutathione reductase provided by a commercially available assay (Cayman Chemical, Ann Arbor, MI, USA) [22]. The values were normalized to protein concentration in the homogenate.

2.7. Evaluation of intracellular nitric oxide (NO) level in aorta

To examine the production of NO at 14 days after femoral artery ligation, the mice were sacrificed and the aorta was removed and rinsed with cold physiological salt solution (PSS) composed of the following (in mM): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.6 $CaCl_2$, 1.18 NaH_2PO_4, 24 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose. After removal of any adhering connective tissue, the aorta was cut into several segments. Each segment was opened with a fine scissors and was pinned onto a piece of rubber with the endothelium facing up, so