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Cathepsin S activity controls ischemia-induced neovascularization in mice

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ABSTRACT

Background: Evidence from human and animal studies has demonstrated elevated levels of the cysteine protease cathepsin S (CatS) in hypoxic atherosclerotic lesions. We hypothesized that silencing of CatS gene would suppress ischemia-induced angiogenic action.

Methods and results: Left femoral artery ligation-induced ischemia in mice showed the increased expression and activity of CatS in the ischemic muscle. The CatS-deficiency ($CatS^{-/-}$) mice showed impaired functional recovery following hindlimb ischemia and reduced levels of peroxisome proliferator-activated receptor-γ (PPAR-γ), phospho-Akt (p-Akt), p-endothelial nitric oxide synthase, p-extracellular signal-regulated kinase1/2 (Erk1/2), p-p38 mitogen-activated protein kinase, and vascular endothelial growth factor (VEGF) proteins, as well as reduced levels of matrix metalloproteinase-9 and macrophage infiltration in the ischemic muscles. *In vitro*, CatS silencing reduced the levels of these targeted essential molecules for angiogenesis and vasculogenesis. Together, the results indicated that the effects of CatS knockdown led to defective endothelial cell invasion, proliferation, and tube formation. This notion was reinforced by the finding that CatS inhibition led to a decreased PPAR-γ level and VEGF/Erk1/2 signaling activation in response to ischemia. $CatS^{-/-}$ resulted in decreased circulating EPC-like $CD31^{+}$/c-Kit^{+} cells, accompanied by the reduction of the cellular levels of PPAR-γ, p-Akt, and VEGF induced by ischemic stress. Transplantation of bone-marrow-derived mononuclear cells from $CatS^{+/+}$ mice restored neovascularization in $CatS^{-/-}$ mice.

Conclusions: CatS activity controls ischemia-induced neovascularization partially *via* the modulation of PPAR-γ and VEGF/Akt signaling activation.

1. Introduction

Postnatal angiogenesis or neovascularization occurs in response to wound healing and to ischemic stress. It is well known that the process of new blood vessel formation is associated with extracellular matrix (ECM) remodeling involving various proteolytic systems [1]. Substantial evidence supports the involvement of both matrix metalloproteinases (MMPs) and serine proteases in this process [2,3]. However, several studies show that cysteine protease also participates in the angiogenesis of pathophysiological conditions [4–6].

Cathepsins — including cathepsin S (CatS) — were originally identified as members of the cysteine protease family localized in lysosomes [7]. However, over the past decade emerging data revealed unexpected roles of cathepsins in pathological conditions such as tumor growth, metabolic disorder, and atherosclerosis-based cardiovascular disease [8,9]. CatS together with CatK were the first cathepsins found to be expressed in human atherosclerotic lesions. Human and animal atherosclerotic hypoxic plaques were shown to have increased levels of CatS and CatK proteins and vascular ECM breakdown activity [10–12].

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It was recently reported that CatK inhibition confers resistance to carotid artery injury by decreasing inflammation and adventitia vasa vasorum formation [5]. Previous studies showed that CatL was required for endothelial progenitor cell (EPC)-induced neovascularization in response to ischemic stress [4,6]. In tumor growth, CatL was shown to modulate the antiangiogenic activities of human endostatin [13]. In 2010, a genetic engineering study revealed that silencing CatL accelerates tumor angiogenesis and growth [14]. Thus, further work is necessary to determine the full spectrum of anti- or proangiogenic activity of the individual cathepsin expressed in both tumor-related angiogenesis and ischemia neovascularization.

The nuclear receptor peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a member of the nuclear superfamily of ligand-inducible transcription factors [15]. By binding to PPAR-responsive regulatory elements as obligate heterodimers with retinoid X receptor, PPAR-γ controls the expression of networks of genes involved in adipogenesis, angiogenesis, and the maintenance of metabolic homeostasis [15]. PPAR-γ activation has been implicated in the regulation of a variety of cellular biological events including cell differentiation, migration, and proliferation [15]. A study demonstrated that PPAR-γ agonist pioglitazone improved cardiovascular dysfunction in patients with rheumatoid arthritis [16]. Accumulating evidence supports a close interaction between PPAR-γ and Samd2/3 signaling pathways in cardiovascular disease [17,18]. A recent study of CatS knockout mice revealed that the deficiency confers resistance to an experimental abdominal aortic aneurysm formation by decreasing the lesion adventitia microvessel content and the inflammatory action [19]. More recently, it was demonstrated that CatS activity controls ischemia-induced cardiac repair through the modulation of Sma2/3 activation-related fibroblast trans-differentiation in mice [20]. On the basis of these findings and the pharmacological data that CatS inhibition reduced Sma2/3 activation *in vivo* and *in vitro* [20], we hypothesized that there might be an association between CatS activation and PPAR-γ activation induced by acute hypoxic stress in the neovascularization process.

In the present study, we used CatS-deficient ($CatS^{-/-}$) mice and an ischemic hindlimb model to conduct the first investigation into whether this protease contributes directly to neovascularization in response to ischemic stress.

2. Methods

2.1. Reagents

CatS inhibitor (CatS-I, Z-FL-COCHO), GM6001 (also known as an MMP inhibitor) and E-64*d* [(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucine (3-methylbutyl) amide] were purchased from Calbiochem (San Diego, CA, USA). Morpholinurea-leucine-homophenylalanine-vinylsulfone-phenyl (LHVS) was purchased from Arris Pharmaceutical (South San Francisco, CA). All powdered reagents were dissolved in sterilized dimethyl sulfoxide.

2.2. Mice

The male CatS knockout mice (KO, $CatS^{-/-}$) [11] and wild-type (WT, $CatS^{+/+}$) littermates used in this study were 8 weeks old and weighed between 21 and 25 g. All animal experiments were performed in accord with the guidelines on animal care of the Nagoya University Graduate School of Medicine.

2.3. Model of hindlimb ischemia

For the hindlimb ischemia model, mice were subjected to unilateral hindlimb ischemic surgery under sodium pentobarbital anesthesia (50 mg/kg intraperitoneally). In the specific experiments, the specific CatS inhibitor CatS-I (5 mg/kg) dissolved in DMSO (CatS-I group) or vehicle (control group) was injected into the abdomen of $CatS^{+/+}$ mice every other day from 3 days prior to surgery until 14 days after surgery. At the indicated time points after surgery, mice were euthanized by an overdose of sodium pentobarbital. Skeletal muscles were dissected out and immediately frozen using liquid nitrogen before they were stored at −80 °C until analysis.

2.4. Analysis of hindlimb blood flow

Hindlimb blood flow was determined using a laser Doppler blood flow (LDBF) analyzer (PIM II LDI, Lisca Development, Linkoping, Sweden). LDBF analyses were performed on the legs and feet of the mice before the surgery and on the indicated postoperative days. Blood flow is shown as the changes in the laser frequency using different color pixels. The results of the quantitative analysis of blood flow are expressed as the ratio of left (ischemic) to right (non-ischemic) LDBF to avoid data variations because of ambient light and temperature [21].

2.5. Measurement of capillary density

We assayed the capillary density in cross-sections of adductor muscle (6-μm cryosections) at postoperative day 14 or 28 with anti-PECAM-1 (CD-31, Santa Cruz Biotechnology, Santa Cruz, CA). Capillary endothelial cells (ECs) were quantified by measuring the number of cells positive for the CD31 per high-power (400×) field. The number of capillaries per muscle fiber was measured in 12 randomly chosen microscopic fields from three different sections in each tissue block [2].

2.6. Western blotting

Protein was extracted using a RIPA lysis buffer and Western blotted against antibodies for phospho-p38 mitogen-activated protein kinase (p-p38MAPK), total p38MAPK, phospho-endothelial nitric oxide synthase (p-eNOS), total eNOS, phospho-mammalian target of rapamycin (p-mTOR), total mTOR, phospho-Akt (p-Akt), total Akt, p-extracellular signal-regulated kinase1/2 (p-Erk1/2), total Erk1/2, phospho-glycogen synthase kinase-3β (p-GSK-3β), total GSK-3β (Cell Signaling Technology, Beverly, MA), vascular endothelial growth factor (VEGF), PPAR-γ, insulin receptor substrate-1 (IRS-1), IRS-2, CatS, CatK (Santa Cruz Biotechnology), CystC, CatB (Upstate, Charlottesville, VA), human CatS (R&D Systems, Minneapolis, MN), PPAR-α (BioVision, Milpitas, CA), CatL, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (Sigma-Aldrich, St. Louis, MO; later both as loading control) [5].

2.7. Levels of VEGF protein

At day 4, the contents of human VEGF165 in mouse plasma ($n = 6$) and muscles were detected by using a commercially available ELISA kit (R&D Systems) according to the manufacturer's instruction [2].

2.8. Gelatin zymography

For gelatin zymography, 20 μg of artery protein extract was mixed with a sodium dodecyl sulfate sample buffer without reducing agent and loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel containing 1 mg/mL gelatin as described [5].

2.9. Assay of collagenolytic activity

Total protein (100 μg) from the extracts of muscle tissues and cells was incubated with 500 μg/mL fluorescein-labeled type I collagen (Molecular Probes, Eugene, OR) for 6 h. Reactions were performed in the absence or presence of several protease inhibitors at indicated concentrations as described [22].