Cathepsin S Activity Controls Injury-Related Vascular Repair in Mice via the TLR2-mediated p38MAPK and PI3K−Akt/p-HDAC6 Signaling Pathway

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Materials and Methods

Mice

The male CatS knockout mice (KO, $CatS^{-/-}$)[1] and wild-type (WT, $CatS^{+/+}$) littermates used in this study were 8 weeks old and weighed between 22 and 25 g. We use PCR and western blotting assay to evaluate the expression of CatS gene and protein in $CatS^{+/+}$ and $CatS^{-/-}$ mice (Sup. Fig.1C). All of the animal studies were performed in accord with the animal care guidelines of the Nagoya University Graduate School of Medicine.

Models of the carotid artery injuries and tissue collection

The mouse was anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Dainippon Pharmaceutical, Osaka, Japan). For the ligation injury model, the right common carotid artery of each 10-wk-old mouse was ligated just proximal to its bifurcations as described[2]. In the specific experiments, the specific HDAC6 inhibitor tubastatin A (10 mg/kg/day; #SML0044, Sigma-Aldrich, St. Louis, MO), dissolved in saline (tubastatin A group) or vehicle alone (control group) was injected into the abdomen of each $CatS^{+/+}$ mouse every day from 2 days before the surgery to 14 days after the ligation surgery. For the negative control experiments, $CatS^{-/-}$ mice that had undergone the ligation injury were injected subcutaneously with either vehicle or tubastatin A (10 mg/kg/day) as indicated. In addition, $CatS^{-/-}$ mice that also had undergone the ligation injury were injected subcutaneously with either vehicle or CatS-I (5 mg/kg/day) as indicated. At the indicated time points after the injury, the mice were euthanized by an overdose of sodium pentobarbital. For the biological analysis, the mice were perfused with saline, and then the carotid artery was isolated and kept in RNAlater® Stabilization solution (#AM7020, Life Technologies, Carlsbad, CA) (for the genes assay) or liquid nitrogen (for the proteins assay). For the morphometry, after being immersed in fixative for 24 h (4°C), the arteries were embedded in paraffin or in

optimal cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo) and stored at −30°C.

Morphometric and immunohistological analyses

The praffin-sections (5 μm) of the mouse carotid arteries were prepared at 2 mm proximal to the ligated site. Corresponding sections were stained with hematoxylin and eosin (H&E) and Van Gieson staining. In all immunohistologic and morphometric analyses, six cross-sections (two sections each from the proximal, middle, and distal regions) of vessels in each artery were measured for internal elastin length, media, and neointima, and then the results were averaged as described. The internal elastic lamina (IEL) and the external elastic lamina (EEL) were obtained by tracing the contours on digitized images. The medial areas were calculated by subtracting the area defined by the IEL from the area defined by the EEL, and the neointimal areas were determined by subtracting the lumen area from the area defined by the IEL. In addition, carotid arterial slices on separate slides were processed for the immunohistochemical analysis of TLR2. The primary rabbit polyclonal antibody against TLR2 (1:50; sc-10739, Santa Cruz Biotechnology, Santa Cruz, CA) was applied to the sections, which were then left overnight at 4°C. Then, the sections were sequentially treated with appropriate secondary antibodies (1:200, Vector Laboratories, Burlingame, CA) for 2 h at 4°C, and were then visualized with a corresponding substrate kit (Vector Laboratories).

Detection of cell proliferation in vivo

In vivo bromodeoxyuridine (BrdU) labeling was performed to identify proliferating cells in ligation-injured arteries by the detection of DNA synthesis with the BrdU Immunohistochemistry Kit (ab125306, Abcam, Cambridge, MA). BrdU (40 μg/g mouse) was injected intraperitoneally 12 h before the preparation of the artery. We counted the BrdU-labeled and unlabeled cells in the neointima at ×400. A quantitative analysis was performed in five independent sections for each mouse. The BrdU labeling index was determined by dividing the number of BrdU-labeled cells by the number of total cells, as described[3]. We also performed proliferating cell nuclear antigen (PCNA) immunostaining by using an anti-PCNA mouse mAb (Cat NA03, Merck Millipore, Darmstadt, Germany) and the M.O.M.™ detection kit (BMK-2202, Vector Laboratories, Burlingame, CA). The quantitative analysis was conducted by dividing the number of PCNA immunopositive cells by the intima and media areas of injured arteries.

Western blot analysis

Protein samples were obtained from homogenized arteries and from cultured cells, and the protein concentration of each sample was determined. Protein samples were western blotted against antibodies for p-Akt^{s473} (4060), t-Akt (2967), p-Erk1/2$^{t1202/t1204}$ (4377), t-Erk1/2 (9107), p-p38MAPK$^{t180/t182}$ (2331), t-p38MAPK (9212), p-eEF2^{t56} (2331), t-eEF (2332), t-HDAC6 (2162) (from Cell Signaling Technology, Beverly, MA), CatS (6686-100, BioVision, Milpitas, CA), β-actin (sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA), and p-HDAC6^{s1035} (from Dr Zhang as co-author).[4] The band intensity was analyzed by densitometry using Image J software. Quantification of western blots is normalized to total protein levels.

Quantitative real-time polymerase chain reaction

Total RNA was harvested from tissues with the use of the RNeasy® Micro kit (74004, Qiagen, Valencia, CA) and subjected to reverse transcription[5]. The resulting cDNA was subjected to a quantitative real-time polymerase chain reaction (RT-PCR) analysis with the use of a Bio-Rad CFX96™ Real-Time PCR Detection System and Power SYBR® Green PCR Master Mix (4367659, Applied Biosystems, Foster City, CA). All experiments were performed in triplicate. The sequences of the primers for the targeted genes are provided in supplemental Table II. We calculated the changes in gene transcription by the $2^{-\Delta\Delta Ct}$ method and normalized the values to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Plasma platelet-derived growth factor BB detection

The plasma platelet-derived growth factor BB (PDGF-BB) concentrations were measured using the Mouse/Rat PDGF-BB Quantikine ELISA Kit (MBB00, R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

HDAC6 activity assay

Protein samples were obtained from homogenized arteries and from cultured cells, and the protein concentration of each sample was determined. 5 μl of protein samples and plasma was used to dectect HDAC6 activity by using the Fluorogenic HDAC6 Assay Kit (50076, BPS Bioscience, San Diego, CA) following the manufacturer's instructions with a small modification. Briefly, constructed a standard curve by using a series of diluted HDAC6 human recombinant enzyme. 5 μl of tubastatin A (20μM) was added to the samples as an inhibitor control.

Cell culture

We obtained VSMCs from the media of mouse aortas of both genotypes ($CatS^{+/+}$ and $CatS^{-/-}$) by the tissue explant method and cultured them in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics[2]. Briefly, the thoracic aortas of 8-wk-old mice were removed, and turned "inside out" in DMEM with the use of a unique catheter under a microscope. After the aortas were cut into 1×1-mm explants, the explants were individually plated into a 10 cm culture dish and cultured in 8 mL of DMEM containing 20% FBS for 7 days before the next change of medium. The obtained cells retained SMC characteristics (purity > 90%). we used western blotting assay to detect the protein expression of CatS (Sup. Fig.).

Cell proliferation assay

We assessed the VSMC proliferation with the Cell Titer 96®AQ Assay kit (G5430, Promega, Madison, WI) in vitro[2]. Cells were plated on plates at 5,000 cells in 100μL of DMEM per well and incubated in the presence of PDGF-BB (10 ng/mL) for 48 h. Then, 20 μL of a mixture of tetrazolium compound and phenazine methosulfate was added for an MTS assay, and the absorbance was detected at 492 nm. Experiments were performed five separate times for each group in triplicate. In the specific experiments, cells were plated on plates at 500 cells in 50μL of DMEM per well and incubated in the presence of PDGF-BB (10 ng/mL) for 48 h. Cell numbers were counted for each well.

Wound-induced migration assay

A wound-induced migration assay was performed as described[6]. Briefly, VSMCs were seeded into a 6-well plate at 8,000 cells/well and cultured in DMEM supplemented with 10% FBS and antibiotics until they reached 70% confluence. The cell monolayer was then scraped in a straight line with a p1000 pipet tip to create a ‘‘scratch’’. The debris was removed and the edge of the scratch was smoothed out by washing the cells twice with 1 ml of the growth medium. The medium was then replaced with 1.5 ml of medium specific for the in vitro scratch assay. The dish was placed in a culture incubator in DMEM containing PDGF-BB (20 ng/mL or 50 ng/mL or 2% FBS, respectively) at 37°C for 24–36 h. The images for wound healing were pictured in ×50 fields. The numbers of cells migrated into the scratched area were calculated.

Cell cycle analysis

A cell cycle analysis was conducted by using a modified propidium iodine-based flow cytometry protocol[7]. Briefly, cells were cultured in DMEM containing 10% FBS until 70% confluence. After starvation for 12 h, the cells were subjected to different

treatments in DMEM containing 10% FBS for 24–36 h in a normoxic or hypoxic box. Subconfluent cells were trypsinized and fixed in 70% ethanol at 4°C overnight. Fixed cells were washed once with phosphate-buffered saline (PBS) followed by incubation with KRISHIAN buffer (0.1% sodium citrate, 0.02 mg/mL RNAse A [Sigma-Aldrich], 0.3% NP-40 [Sigma-Aldrich], and 0.05 mg/mL propidium iodide [#P1304MP, ThermoFisher Scientific]) for 30 min at room temperature in the dark. Cell suspensions were analyzed for the propidium iodide labeling of DNA by flow cytometry. Cell-cycle progression was measured as the percent of cells in the G2/S/M stage.

HDAC6 plasmid transfection

Flag-tagged HDAC6 (pBJ-HDAC6F) and vector plasmids (kanamycin resistance) were made and amplified as described[8]. Seeded VSMCs were brought to 70%–90% confluence at transfection, and then HDAC6 and vector plasmids were transfected into the cells for 48–72 days by using Lipofectamine® LTX & PLUS™ Reagent (15338030, Life Technologies).

Preparation of explants and the migration assay

The aortic explants were prepared as described[2]. Briefly, we removed and opened out thoracic aortas of mice, and removed the endothelium gently by abrasion. After the aortas were cut into 1×1-mm explants, we plated the explants individually into collagen type 1-coated 24-well plates with the lumen side down and cultured them in 500 μL of DMEM containing 0.1% bovine serum albumin (BSA), transferrin, insulin, and PDGF-BB (50 ng/mL, #315-18, PeproTech, London). At the indicated time points, the VSMC sprouts at the edge of the explants were analyzed by Beta 4.0.3 Scion Image software. The VSMC migratory ability is expressed as sprouted total cell numbers and areas (average of seven explants for each animal). We fixed and stained the cells for smooth muscle α-actin to characterize the cell migration from explants.

Short interfering RNA (siRNA) transfection

Specific siRNAs against TLR2 (Mm_mTlr_5214-s, Mm_Tlr_5124-as; F#806) and HDAC6 (Mm_Hdac61178_s, Mm_Hdac61178_as, F#838), and non-targeting control siRNA (Mission_SIC-001_s and Mission_SIC-001_as as the negative control) were purchased from Sigma-Aldrich. SMCs were grown on 60-mm dishes until 50% confluence. The siRNA solution mixed with serum-free and antibiotic-free DMEM-2 medium containing Lipofectamine RNAiMAX reagent (Invitrogen) was supplied to each well to achieve a final siRNA concentration of 100 pM. The cells were treated at

37°C for 48 h, and the levels of targeted gene were analyzed by PCR. Transfected cells were also used for cell proliferation experiments. Silamin A/C (D0010500105, Dharmacon, Brébières, France) was used as a positive control.

Statistical analysis

All measurements were conducted by two observers. Data are expressed as means ± standard error of the mean (SEM). Student's t-test (for comparisons between two groups) and a one-way analysis of variance (ANOVA; for comparisons of three or more groups) followed by the Bonferroni post-hoc test were used for the statistical analyses. SPSS software version 17.0 (SPSS, Chicago, IL) was used. P-values <0.05 were considered significant.

References:

1. Sukhova GK, Zhang Y, Pan JH, Wada Y, Yamamoto T, Naito M, Kodama T, Tsimikas S, Witztum JL, Lu ML, Sakara Y, Chin MT, Libby P, Shi GP. Deficiency of cathepsin S reduces atherosclerosis in LDL receptor-deficient mice. *J Clin Invest.* 2003;111:897-906.
2. Hu LN, Cheng XW, Song HZ, Inoue A, Jiang HY, Li X, Shi GP, Kozawa E, Okumura K, Kuzuya M. Cathepsin K Activity Controls Injury-Related Vascular Repair in Mice. *Hypertension.* 2014;63:607-615.
3. Uemura Y, Shibata R, Ohashi K, Enomoto T, Kambara T, Yamamoto T, Ogura Y, Yuasa D, Joki Y, Matsuo K, Miyabe M, Kataoka Y, Murohara T, Ouchi N. Adipose-derived factor CTRP9 attenuates vascular smooth muscle cell proliferation and neointimal formation. *Faseb J.* 2013;27:25-33.
4. Williams KA, Zhang M, Xiang SY, et al. Extracellular Signal-regulated Kinase (ERK) Phosphorylates Histone Deacetylase 6 (HDAC6) at Serine 1035 to Stimulate Cell Migration. *J Biol Chem.* 2013;288:33156-33170.
5. Cheng XW, Kuzuya M, Kim W, Song HZ, Hu LN, Inoue A, Nakamura K, Di Q, Sasaki T, Tsuzuki M, Shi GP, Okumura K, Murohara T. Exercise Training Stimulates Ischemia-Induced Neovascularization via Phosphatidylinositol 3-Kinase/Akt-Dependent Hypoxia-Induced Factor-1 alpha Reactivation in Mice of Advanced Age. *Circulation.* 2010;122:707-716.
6. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc.* 2007;2:329-333.
7. Burger D, Kwart DG, Montezano AC, Read NC, Kennedy CRJ, Thompson CS,

Touyz RM. Microparticles Induce Cell Cycle Arrest Through Redox-Sensitive Processes in Endothelial Cells: Implications in Vascular Senescence. *J Am Heart Assoc.* 2012;1:e001842.

8. Zhang XH, Yuan ZG, Zhang YT, Yong S, Salas-Burgos A, Koomen J, Olashaw N, Parsons JT, Yang XJ, Dent SR, Yao TP, Lane WS, Seto E. HDAC6 modulates cell motility by altering the acetylation level of cortactin. *Molecular Cell.* 2007;27:197-213.