Cathepsin S contributes to macrophage migration via degradation of elastic fibre integrity to facilitate vein graft neointimal hyperplasia

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Received 19 November 2012; revised 20 November 2013; accepted 28 November 2013; online publish-ahead-of-print 6 December 2013

Aims Cathepsin S (Cat S) is a potent lysosomal protease that is secreted into the extracellular space and has been implicated in elastin and collagen degradation in diseases such as atherosclerosis. Elastin degradation plays an important role in vascular remodelling. However, the mechanism by which Cat regulates this process and its contribution to vein graft remodelling remains unclear.

Methods and results Using a murine vein graft model, we examined the expression pattern of Cat family members. Expression of cathepsin genes was induced in vein grafts, with that of Cat S being the highest. Elevated Cat S expression was confirmed in both mouse and human vein grafts. To explore the role of Cat S, vein grafts were created between wild-type (WT) littermates and Cat S knockout (Cat S KO) mice. Knockout of Cat S in the recipients (vein^CatS-KO^-artery^CatS-KO^ or vein^WT^-artery^CatS-KO^) significantly inhibited neointima formation and reduced the accumulation of macrophages and proliferation of smooth muscle cells in vein grafts. Knockout of Cat S preserved the elastic fibre integrity of vein grafts and inhibited the migration of macrophages across the elastin fibres.

Conclusion These results demonstrated that Cat S contributes to macrophage migration via degradation of elastic fibre integrity to facilitate neointima formation of vein grafts, which might provide a novel therapeutic target for preserving vein graft patency.

Keywords Vein graft • Cathepsin S • Neointima • Hyperplasia • Elastin

1. Introduction

The development of obliterative stenosis of vein grafts is primarily due to neointima formation and accelerated atherosclerosis. No successful therapeutic strategy to ameliorate vein graft failure exists, thus a deeper insight into the vein graft remodelling mechanism is needed.

When grafting a vein into an artery, the vein graft is exposed to the arterial haemodynamic pressure, then inflammatory cells are recruited followed by proliferation and migration of vascular smooth muscle cells (VSMCs) in the vein grafts. We previously identified several signalling molecules that participate in neointima formation in vein grafts, such as early growth response-1 transcriptional factor, insulin-like growth factor 1 receptor, serum–glucocorticoid-regulated kinase 1, and fibroblast-specific protein 1 (FSP-1). In addition to these cellular signalling molecules, the extracellular matrix also undergoes remodelling in vein grafts. However, the mechanism that regulates extracellular matrix remodelling and its role in neointima formation of vein grafts remains unclear.

A key component of the extracellular matrix, elastic fibres consist of cross-linked elastin cores surrounded by a mantle of fibrillin-rich microfibrils that constitute the internal elastic lamina (IEL) and external elastic lamina (EEL), which provide barriers between the intima and media and the media and adventitia. Elastin degradation is associated with numerous vascular diseases, including aneurysm and atherosclerosis. Recently, it was reported that degradation of elastic fibres occurs in vein grafts and is suppressed with inhibition of elastase. IEL and EEL disruption can facilitate pro-inflammatory cell infiltration and migration of VSMCs entering the neointima of vein grafts, and elastin...
degradation is associated with macrophage migration and VSMC proliferation and migration.\textsuperscript{21–23} However, the mechanism underlying elastin degradation in vein grafts remains unclear.

Elastin degradation is mediated by numerous proteinases derived from the infiltrating inflammatory cells and activated vascular cells.\textsuperscript{24,25} Recent studies have shown that cathepsin S (Cat S) are expressed next to the elastin degradation site in vein grafts, suggesting their involvement.\textsuperscript{14} The Cat family is a papain superfamily located in the acidic lysosome and endosome. Among its members, Cat S, L, and K have elastolytic and collagenolytic activities.\textsuperscript{26,27} The Cat family plays an important role in the pathogenesis of cardiovascular diseases.\textsuperscript{28,29} For example, Cat S and K are expressed in atherosclerotic lesions, and knockout of Cat S, K, or L inhibits the development of atherosclerosis in low-density lipoprotein receptor knockout (LDLR\textsuperscript{−/−}) mice and apo-lipoprotein E (apoE) knockout mice.\textsuperscript{27} Cat B, K, and S are highly expressed in cerebral aneurysms, and knockout of Cat K decreases aneurysm severity in mice.\textsuperscript{30,31} We recently showed that knockout of Cat S prevents aortic aneurysm development.\textsuperscript{32} However, the expression pattern of Cat family members and their role in vein graft neointima formation remain unclear.

In this study, we examined the expression pattern of Cat family members in mouse veins and vein grafts, and found that Cat S was highly expressed in vein grafts. Knockout of Cat S suppressed neointima formation and macrophage accumulation and VSMC proliferation in vein grafts. Cat S degraded the integrity of elastic fibres of vein grafts and increased the migration of macrophages across the fibres. These results demonstrated that Cat S contributes to macrophage migration via elastic fibre degradation to facilitate vein graft neointimal hyperplasia.

\section{2. Methods}

\subsection{2.1 Reagents}

Antibodies against Cat S, F4/80, and α-smooth muscle actin (α-SMA) were from Abcam (Cambridge, MA, USA). Antibody against Mac-2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Elastin was from Sigma-Aldrich (St. Louis, MO, USA).

\subsection{2.2 Animals}

Cat S knockout (Cat S KO) in C57/B6 genetic background mice were described.\textsuperscript{32} Two-month-old Cat S KO mice and littermate wild-type (WT) male mice were used. The Institutional Animal Care and Use Committee of Capital Medical University, Beijing, China, approved all studies. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Mice were anaesthetized by intraperitoneal (i.p.) injection with one dose of ketamine (200 mg/kg) and xylazine (10 mg/kg) in 50 μL of saline. Anaesthesia was monitored by pinching the toe.

Veins grafts were performed as described previously.\textsuperscript{10} In brief, the vena cava from a donor mouse was grafted between two ends of a carotid artery by ‘sleeving’ the ends of the vein over the artery cuff and secured with 8.0 silk sutures. Four types of vein grafts (N \textsuperscript{≥} 4 for each group) were created (vena cava from WT mice was grafted to the carotid artery of WT mice; vena cava from Cat S KO mice was grafted to the carotid artery of Cat S KO mice; vena cava from WT mice was grafted to the carotid artery of Cat S KO mice; vena cava from Cat S KO mice was implanted to the carotid artery of WT mice). Post-operative analgesia (buprenorphine, 0.05 mg/kg/12 h, i.p.) was administered for 48 h. After 4 weeks, mice were sacrificed with carbon dioxide narcosis.

The vein grafts were harvested, and the intima plus media were measured as the region between the lumen and the adventitia. Vessel wall thickness was measured as the cross-sectional area of the vessel minus that of the lumen using a Nikon Labophot 2 microscope equipped with a CCD-Iris/RGB colour video camera attached to a computerized imaging system and the Image-Pro-Plus 3.0 software (ECLIPSE80i/90i; Nikon, Tokyo, Japan). Five cross-sections were examined as described.\textsuperscript{10}

\subsection{2.3 Bone marrow transplantation}

Bone marrow (BM) transplantation was performed as described previously.\textsuperscript{11} After mice were sacrificed with carbon dioxide narcosis, BM of WT or Cat S KO mice were washed away from femurs and tibias with a 25-G needle, and filtered. Then cells were washed and resuspended at 2 × 10\textsuperscript{7} cells/mL. The recipient Cat S mice were anaesthetized with one dose

Table I Primers used for qRT-PCR

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<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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of ketamine (200 mg/kg, i.p) and xylazine (10 mg/kg, i.p) in 50 μL of saline and transferred with 2 × 10^7 cells per mouse by tail vein injection. Anaesthesia was monitored by pinching the toe. Post-operative analgesia (buprenorphine, 0.05 mg/kg/12 h, i.p.) was administered for 48 h. WT and Cat S KO BM chimera will be referred as BMWT and BMKO, respectively, throughout the manuscript. Five weeks after transplantation, vein grafts (N ≥ 4 mice for each group) were created in each group.

2.4 Human vein grafts tissue
Human failed primary bypass grafts were obtained by coronary artery bypass re-grafting surgery, according to protocols approved by the Medical Ethical Committee of Capital Medical University, Beijing, China, that comply with the principles outlined in the Declaration of Helsinki. All the patients gave informed consent for coronary artery bypass re-grafting before surgery.

2.5 Histopathology and immunohistochemistry
Vein grafts from WT and Cat S KO mice fixed in 10% formalin were processed and paraffin embedded. Tissue sections (5 μm) were then stained with haematoxylin and eosin (HE). Immunohistochemistry was performed on vein grafts as described with minor modifications. After removing paraffin and rehydrating, sections were incubated for 10 min in 3% H2O2 in methanol at room temperature, washed with phosphate buffered saline (PBS), and heated in a microwave to 199°F for 5 min. Sections were blocked with serum for 30 min and then incubated with primary antibodies. Sections underwent immunohistochemical staining with the antibodies against Cat S (1:150), F4/80 (1:100), α-SMA (1:200), and Mac-2 (1:400). After washing, sections were incubated with secondary antibodies. The immunostaining was visualized with a peroxidase substrate kit and counterstained with haematoxylin. For a negative control, the same protocol was used with antigen dilution reagent instead of the primary antibodies. Images were viewed and captured and analysed by an Image-Pro-Plus 3.0.

2.6 Immunofluorescent staining
The immunofluorescent staining was conducted with frozen sections of vein grafts and control veins. Sections underwent double immunofluorescence staining with primary antibodies against Cat S (1:200) and F4/80 (1:200), Mac-2 (1:400), or PCNA (1:400) at 4°C overnight and Alexa Fluor 488-conjugated or 594-conjugated secondary antibodies (Molecular Probes, Carlsbad, CA, USA). The slides were mounted in glycerol-based vectashield medium (Vector Laboratories, Burlingame, CA, USA) containing 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining.

Figure 1 Cat S was expressed in vein grafts. (A) Vein grafts were created in WT mice and harvested 3 days later. Expression of Cat family members in normal veins and vein grafts were quantified using qRT-PCR and expressed as the percentage of the internal control α-tubulin. N = 4 for each group. ND, not detected. (B) Vein grafts were created in WT mice and harvested 4 weeks later. The control veins and vein grafts were subjected to immunohistochemistry with a primary antibody against Cat S or IgG control. (C) Normal human saphenous veins and failed vein grafts were harvested, sectioned, and subjected to immunostaining with a primary antibody against Cat S. (D) Control veins and vein grafts were frozen-sectioned and subjected to immunofluorescence staining with primary antibodies against Cat S (TRITC) and F4/80 (FITC). Nuclei were stained with DAPI. (E) Control veins and vein grafts were frozen-sectioned and subjected to immunofluorescence staining with primary antibodies against Cat S (TRITC) or CD4 (FITC). Nuclei were stained with DAPI. Scale bar: 50 μm. Data represent four independent experiments.
2.7 Flow cytometry
Peripheral blood cells were washed twice with Hanks’ balanced salt solution (HBSS) buffer with 2% fetal bovine serum after erythrocytes were lysated, then underwent cell-surface antigen staining with fluorochrome-conjugated monoclonal rat anti-mouse antibodies against CD11b and CD45 (BioLegend, San Diego, CA, USA) at 4°C for 30 min. Flow cytometry was used to quantify the number of circulating monocytes in Cat SKO and WT mice after surgery by the use of Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA). Data were analysed with the Summit software (Beckman Coulter).

2.8 Western blot analysis
Western blot was performed as described.34 Cells were extracted in a lysis buffer containing 20 mM Tris–HCl pH 8.0, 150 mM NaCl, 90 mM KCl, 2 mM ethylene diamine tetraacetic acid (EDTA), 1% Triton X-100, 1 mM Na3VO4, 10 mM NaF, and protease and phosphatase inhibitor cocktails. Equal amounts of protein extracts (30 μg/lane) were loaded on a 12% SDS–PAGE gel, transferred onto the nitrocellulose membrane, and probed with goat anti-mouse Cat S polyclonal antibodies (1 : 1000). Immunoblot for housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1 : 3000, Santa Cruz) was used to assure equal protein loading.

2.9 In situ zymography
In situ zymography was performed as described with minor modification. Vein graft elastinolytic activity was determined on 6-μM frozen sections using elastin conjugated with quenched fluorescein (DQ elastin; Elastase Assay Kit, Molecular Probes) as a substrate, which requires cleavage by elastinolytic enzymes to become fluorescent. DQ elastin (1 mg/mL in H2O) was mixed 1:10 with 1% low-melting agarose (Sigma-Aldrich). This mixture (20 μL) was added on top of each section, coverslipped, and gelled at 4°C. Following incubation at 37°C for 48 h, fluorescence was examined using a fluorescent microscope. Cysteine protease activity was determined using a pH 5.5 buffer containing EDTA (a chelator of calcium to inhibit matrix metalloproteinase (MMP) activity; 10 mM). MMP activity was determined using a pH 7.4 buffer containing E64 (a non-selective inhibitor of cysteine proteases; 20 μM). Fluorescence intensity, as measured using computer-assisted image quantification, is expressed in the percentage of fluorescence area over neointimal area of each cross-section.

2.10 Quantitative real-time PCR
Total RNA was isolated from grafted veins and macrophages with Trizol Reagent (Invitrogen, Carlsbad, NY, USA), and 2 μg RNAs were reversed transcribed with moloney murine leukemia virus and oligo (dT) primer (Invitrogen). Real-time quantitative PCR involved use of the Bio-Rad iQ5 system (Bio-Rad) with SYBR Green I (Takara, Shiga, Japan). Amplification was at 95°C for 5 min, 95°C for 45 s, and 60°C for 1 min for each step for 45 cycles. The housekeeping gene tubulin was used as a control. Table 1 summarizes the primers used.

2.11 Elastin staining
Elastin fragmentation was graded based on the degree of elastin filament break, as described with modification. Elastin in the grafted veins was stained with the Gomorri’s aldehyde-fuchsin staining method, with the elastic fibre stain kit (Maixin, Bio, Fuzhou, China). In brief, after deparaffin and rehydrating, sections were incubated for 5 min in Lugol’s iodine solution, washed with PBS, and then incubated with sodium thiosulfate for 5 min. After washed with PBS and 70% ethanol, the sections were incubated with aldehyde-fuchsin for 10 min and acid Orange G for seconds. The elastic fibre is intense violet colour, and the background is pale yellow.

Figure 2 Recipient Cat S knockout inhibited vein graft neointima formation. (A) Veins from WT mice and Cat S KO mice were grafted to WT and Cat S KO mice. Vein grafts were harvested 4 weeks later, paraffin-sectioned, and subjected to HE staining. The area and ratio of the lumen and neointima were measured (B and C). (D) Cat S deficiency in BM-derived cells decreased the extent of neointima formation. The area and ratio of the lumen and neointima were measured (E and F). Green arrows show the IEL. The enlarged area is defined by the black box. Black arrows indicate the neointima. BMWT, BM cells derived from WT mice; BMKO, BM cells derived from Cat S KO mice. Data represent the mean ± SEM; n = 4 for each group; *P < 0.05; NS, not significant. Scale bar: 50 μm.
2.12 Apoptosis assays
Apoptotic VSMCs were detected using the in situ Cell Death Detection Kit (Promega), according to the manufacturer’s instructions.

2.13 Cell culture
Macrophages were isolated from BM of mice and grown in macrophage colony-stimulating factor (M-CSF; PeproTech, Rocky Hill, NJ, USA) as described. Briefly, after mice were sacrificed with carbon dioxide narcosis, BM cells were isolated from femurs and tibias of 8- to 12-week-old mice. Suspensions were cleared of adipose tissue and connective tissue by filtration and then underwent ficoll gradient centrifugation to clear residual erythrocytes and non-lymphocytes. Myeloid origin macrophage were cultured in DMEM medium (HyClone, Waltham, MA, USA) supplemented with 10% heat-inactivated FBS in the presence of 50 ng/mL of M-CSF. Mouse VSMCs were isolated from thoracic aorta of C57BL/6 WT male mice. Briefly, excised thoracic aorta was washed in ice-cold PBS solution, and then ventricular media was minced into small pieces by use of scissors in a mixture of 0.2% collagenase type I for 20 minutes at 37°C with agitation. The dispersed cells were incubated in culture dishes for 2 hours. VSMCs were grown in DMEM supplemented with 10% FBS.

2.14 Transmigration across the elastin and collagen
The experiment was conducted as previously described.38 Briefly, the upper chamber of the 24-well transwell insert (8 mm pore size; Corning Costar, New York, NY, USA) was coated with elastin (100 μg/mL) or collagen (100 μg/mL) overnight. The bottom chamber was filled with DMEM containing recombinant mouse monocyte chemoattractant protein-1 (MCP-1; 50 ng/mL; Peprotech). Macrophages (1 × 10^5) were added on the upper chamber. After incubation at 37°C in 5% CO_2 for 12 h, top macrophages were removed by gently swabbing with cotton swabs, and bottom cells that had migrated were fixed and stained with DAPI to visualize and count. Cat S inhibitor (Cbz-Phe-Leu-COCHO) (Calbiochem, Germany) was used (15 nmol/L) for 12 h to inhibit endogenous Cat S.39

2.15 Statistics
All data are expressed as the means ± SEM. The unpaired two-tailed t-test was used to compare the two groups. Comparisons between multiple groups were performed using one-way analysis of variance by the Newman–Keuls multiple comparison test using the Graphpad software (GraphPad Prism version 5.00 for Windows; GraphPad Software, Inc., San Diego, CA, USA). A P-value of < 0.05 was considered statistically significant.

3. Results
3.1 Cat S expression was increased in vein grafts
To examine the involvement of Cat family members in vein graft remodeling, the expression of Cat in normal or grafted veins was examined using quantitative real-time PCR (qRT-PCR). As shown in Figure 1A, expression of Cat S, C, D, K, L, and Z was significantly up-regulated in the vein grafts at Day 3 after surgery. Among these genes, Cat S, C, and D were highly expressed in grafted veins.

To confirm the expression of Cat S in vein grafts, immunohistochemistry was performed in mouse vein grafts and human failed vein grafts. As shown in Figure 1B and C, expression of Cat S was increased in the both mouse and human vein grafts, respectively. Immunoglobulin G (IgG) control antibody was used as a negative control (Figure 1B).

To further identify the cellular localization of Cat S in vein grafts, immunofluorescent staining was performed. Cat S was primarily expressed in macrophages (F4/80 positive) in the murine vein grafts (Figure 1D; see Supplementary material online, Figure S1). Cat S was not expressed in T cells in the vein grafts (Figure 1E).

3.2 Cat S knockout decreased the extent of vein graft neointimal hyperplasia
To assess the role of Cat S in vein graft neointimal hyperplasia, veins from WT or Cat S KO mice were grafted to the carotid arteries of WT (WT–WT or KO–WT) or Cat S KO (KO–WT or WT–KO) mice, respectively. As shown in Figure 2A–C, WT veins grafted to WT recipient showed significant neointima formation, whereas Cat S KO veins grafted to WT recipient showed similar neointima formation. However, both vein grafts from WT and Cat S KO mice implanted into Cat S recipients exhibited significantly reduced neointima formation. These results suggested that the Cat S-expressing cells in the recipient are essential for vein graft neointimal hyperplasia. Moreover, Cat S expression was quite high in both the WT–WT and KO–WT grafts and low in the WT–KO and KO–KO grafts, suggesting that circulating cells expressed Cat S. Furthermore, there were fewer changes in the expression of other Cat genes in WT–WT, WT–KO, KO–WT, and KO–KO grafts (see Supplementary material online, Figure S2). To establish the role of Cat

Figure 3 Cat S knockout decreased VSMC and macrophage accumulation in vein grafts. (A) The harvested vein grafts were subjected to immunofluorescence staining with anti-Mac-2 (FITC) antibodies. The positive area was calculated and expressed as the positive ratio of the neointima area. (B) Scatter plots were gated on circulating monocytes (CD45^−CD11b^− cells) analysed from WT and Cat S KO mouse blood 3 days after vein graft surgery. (C) The harvested vein grafts were subjected to immunofluorescence staining with antibodies against PCNA (TRITC) and α-SMA (FITC). The positive area was calculated and expressed as the positive ratio of the neointima area. (D) TUNEL and α-SMA double staining were performed to detect apoptotic VSMCs in the neointima. The positive number was calculated in each field of the neointima area. Nuclei were stained with DAPI. Data represent the mean ± SEM; n = 4 for each group; *P < 0.05 vs. the WT group. Scale bar: 50 μm.
S-expressing BM-derived cells in neointima formation, BM transplantation was performed, where WT or Cat S KO BM was used to repopulate these cells in irradiated Cat S KO mice. After BM transplantation, vein grafts were built. As shown in Figure 2D–F, WT BM transplantation in Cat S KO mice resulted in increased neointima formation.

3.3 Cat S knockout decreased macrophage infiltration and VSMC proliferation in vein grafts

To further explore the role of Cat S in vein graft neointimal hyperplasia, we examined the events needed for vein graft neointima formation, including proliferation and apoptosis of VSMCs and macrophage infiltration. Veins from WT or Cat S KO mice were grafted into the carotid arteries of WT or Cat S KO recipients, respectively. As shown in Figure 3A, macrophage infiltration into the neointima was significantly inhibited in Cat S KO vein grafts. The number of circulating monocytes (CD11b+ CD45+) at Day 3 after surgery was not significantly different between Cat S KO and WT mice (Figure 3B). This finding suggested that differences in circulating monocytes merely account for the reduced number in Cat S KO vein grafts. These results suggested that Cat S is involved in the macrophage infiltration process in vein grafts.

As inflammatory cell infiltration is followed by proliferation and migration of VSMCs in vein grafts,9,10 we next examined the proliferation of

![Figure 4](https://academic.oup.com/cardiovascres/article-abstract/101/3/454/460148)
VSMCs in the vein grafts of both WT- and Cat S-deficient mice. As shown in Figure 3C, WT vein grafts exhibited massive VSMC proliferation, which was significantly reduced in Cat S KO vein grafts. VSMC apoptosis in WT vein grafts was increased compared with that of Cat S KO vein grafts (Figure 3D). However, Cat S deficiency significantly decreased the accumulation of VSMCs in vein grafts. These results suggested that the net effect of Cat S is required for the accumulation of VSMCs, contributing to vein graft neointimal hyperplasia.

### 3.4 Cat S knockout decreased elastin degradation in vein grafts

To determine the mechanism by which Cat S promotes macrophage infiltration in vein grafts, we first examined the relationship between elastin integrity and macrophage infiltration in the vein grafts. As shown in Figure 4A and B, Gomorri’s aldehyde-fuchsin staining of elastin showed interruption in and fragmentation of elastin in the vein grafts. Meanwhile, macrophages were observed to transmigrate across the degradation site of elastin towards the neointima of the vein grafts. These results suggested that degradation of elastin facilitates macrophage infiltration in vein grafts.

To further evaluate elastin degradation in vein grafts, elastin was stained and graded according to its integrity (Figure 4C). We then compared the degree of elastin degradation in WT and Cat S KO vein grafts. As shown in Figure 4C and D, massive degradation of elastin was observed in the WT vein grafts, which was significantly abrogated in the Cat S KO vein grafts. Along with elastin preservation, Cat S KO was associated with the reduced MMP expression (Figure 4F) and collagen deposition (Figure 4E). In situ zymography showed that Cat S KO decreased elastinolytic activity of vein grafts. Since blood vessel walls emit autofluorescence, a Cat inhibitor (E64d) or MMP inhibitor (EDTA) was used to quantify changes in Cat and MMP activities in the neointima of vein grafts. Changes in these enzymatic activities in the neointima were determined by subtracting that of inhibitor-treated from untreated sections. As shown in Figure 4G, there was a significant reduction in Cat and MMP activity in Cat S KO vein grafts compared with that in WT vein grafts. These results suggested that Cat S is involved in elastin degradation in vein grafts.

### 3.5 Cat S mediated macrophage migration across elastin fibres

To confirm the role of Cat S in elastin degradation, the migration ability of WT or Cat S KO macrophages across elastin was examined with elastin-coated chambers. In the presence of the chemokine MCP-1, WT macrophages migrated across the elastin fibres, while the migration of Cat S KO macrophages was significantly inhibited (Figure 5A). Without the presence of elastin, Cat S deficiency did not significantly affect macrophage migration (Figure 5B). As previous studies showed that Cat S has collagenase activity, we also examined the effect of Cat S on the migration of macrophages across collagen fibres. We found that Cat S deficiency also significantly decreased the migration of macrophages across collagen (see Supplementary material online, Figure S3). Pharmacological inhibition of Cat S showed a similar effect as Cat S knockout; Cat S inhibition decreased macrophage migration and caused decreased macrophage-mediated elastin degradation (Figure 5A and B). Moreover, expression of tumour necrosis factor alpha (TNF-α), and interleukin (IL)-1 was higher in macrophages from Cat S KO mice than in WT mice, whereas the expression of IL-10 or...
transforming growth factor beta 1 (TGF-β1) was little changed between macrophages from WT or Cat S KO mice (Figure 5C). These results suggested that Cat S expression in macrophages mediated the digestion of extracellular matrix components such as elastin and collagen and facilitated migration across them.

3.6 Macrophages facilitated VSMC migration across elastin fibres

It is possible that a proportion of the VSMCs invaded from the recipient, and Cat S-mediated breakdown of the extracellular matrix could increase this invasive capability. To test this possibility, the migration ability of VSMCs across elastin was examined using elastin-coated chambers. In the absence of macrophages, WT and KO VSMC migration was not significantly different (Figure 6A and B). However, VSMC migration was significantly increased in the presence of WT macrophages, but not in the presence of Cat S KO macrophages (Figure 6C).

![Figure 6](https://academic.oup.com/cardiovascres/article-abstract/101/3/454/460148)

**Figure 6** Cat S knockout in macrophages decreased VSMC migration across elastin fibres. (A) VSMC migration was assessed with 8 mm pore transwell filters. VSMCs (1 × 10⁴) derived from WT and Cat S KO mice were cultured on the upper chamber. MCP-1 (50 ng/mL) was added to the lower chamber. The migrated cells were harvested 24 h later and stained with DAPI. The bar graph represents the total number of migrated VSMCs in each group; four independent experiments were performed. (B) VSMC migration was assessed with 8 mm pore transwell filters, to which the upper chamber was coated with elastin. VSMCs (1 × 10⁴) derived from WT and Cat S KO mice were cultured on the upper chamber. MCP-1 (50 ng/mL) was added to the lower chamber. The migrated cells were harvested 24 h later and stained with DAPI. The bar graph represents the total number of migrated VSMCs in each group; four independent experiments were performed. (C) VSMC migration with M₉s were assessed using 8 mm pore transwell filters. VSMCs (0.5 × 10⁴) or M₉s (0.5 × 10⁴) derived from WT and Cat S KO mice were co-cultured on the upper chamber. MCP-1 (50 ng/mL) was added to the lower chamber. The migrated cells were harvested 24 h later and stained with DAPI. The bar graph represents the total number of migrated cells in each group. Data represent the mean ± SEM; four independent experiments were performed. M₉, macrophage; *P < 0.05 vs. the WT group; NS, not significant.

4. Discussion

This study showed that Cat S expression in macrophages was involved in vein graft neointima formation, which was significantly inhibited by Cat S deficiency. We also found that Cat S mediated the degradation of elastin in vitro and in vein grafts and also the migration of macrophages and VSMCs. Our studies revealed that Cat S exerts a positive effect on neointimal hyperplasia in vein grafts.

Our study demonstrated that Cat S expression in circulating macrophages was essential for neointima formation in vein grafts. Previous studies showed that Cat S is mainly expressed in macrophages, dendritic cells, B cells, and aortic smooth muscle cells. In this study, Cat S was highly expressed in macrophages that infiltrated grafted veins. Further, vein grafts from WT mice implanted into Cat S KO mice exhibited significantly decreased neointima formation. However, veins of Cat S KO mice implanted into WT mice did not further decrease vein graft neointima formation (Figure 2A). Our results suggested that Cat S derived from the recipient, especially in macrophages, but not from cells in the grafted vein, plays an important role in promoting neointima formation in vein grafts. BM transplantation demonstrated that Cat S expressed in circulating cells is responsible for neointima formation (Figure 2D). These results also extend our recent report, showing that circulating FSP-1-positive cells derived from the BM are responsible for neointima formation in vein grafts. Moreover, our recent study showed that Cat S knockout prevents angiotensin II-induced aortic aneurysm by preserving the destruction of the extracellular matrix and inflammatory responses.

We found that Cat S mediated elastin degradation in vein grafts. Cat S knockout preserved the integrity of elastic fibres in vein grafts and inhibited macrophage migration across these fibres (Figure 4). MMPs are primarily expressed in macrophages, VSMCs, and endothelial cells. Infiltration of monocytes and macrophages into the vessel wall provides a major source of proteolytic enzymes, including MMPs and cathepsins which promote matrix degradation, thus impairing the integrity of the artery wall. Our observation corresponds with our recent study, showing that the absence of Cat S markedly suppressed both MMP-2 and -9 activity in abdominal aortic aneurysms. Although our present study did not elucidate why the MMPs are altered and in which cells are expressed, the present in vivo and in situ observations might have resulted from fewer macrophages and VSMCs residing within the Cat S KO vein grafts, and Cat S KO might be associated with MMP expression. These results were consistent with the elastinolytic activities of Cat S in vitro. In vivo studies demonstrated that monocytes derived from Cat S-/- LDLR-/- showed impaired migration across elastic fibres and aortas with a preserved elastin structure. In the present study, Cat S-expressing macrophages in vein grafts showed a similar effect on elastin integrity and migration across the elastic fibres.

This study also showed that macrophages migrated across the elastin degradation site to enter the vein graft neointima (Figure 4). Cat S knockout preserved the integrity of elastic fibres and decreased the accumulation of macrophages in the neointima of vein grafts (Figures 3 and 4). Elastin degradation likely contributes to the neointima formation of vein grafts via an elastic fibre-dependent mechanism. Recent studies have shown that progenitor cells in the adventitia differentiate to VSMCs and contribute to vein graft atherosclerosis. Inflammatory cells from the adventitia and circulation are recruited to the neointima and involved in vein graft neointima formation. The disruption of elastic fibres facilitates the migration of VSMCs and inflammatory cells into the neointima.
There are differences between animal models and human diseases.\(^{55}\) and the murine model used in this study is not identical to human vein grafts. However, we used this model to address the role of the protease Cat S in regulating extracellular matrix elastin breakdown, migration of monocytes and VSMCs, and contributing to inflammation and neointima formation, which comprise the main events of human venous bypass graft remodelling.

In summary, we observed that deletion of Cat S reduced macrophage accumulation and VSMC proliferation in vein grafts and preserved the integrity of elastic fibres and inhibited macrophage migration across elastin fibres in vitro. These results demonstrated that Cat S contributes to macrophage and VSMC migration via degradation of elastic fibre integrity to facilitate vein graft neointimal hyperplasia, and suggested an elastolytic role of Cat S in this process. This study provides a novel insight into the mechanism by which Cat S prevents obliterator vein graft stenosis.

Acknowledgements

Cat S KO mice were kindly supplied by Dr Guoping Shi (Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA, USA).

Conflict of interest: none declared.

Funding

This study was supported by grants from the Chinese Ministry of Science and Technology (2009CB522205, 2012CB945104 and 2012CB17802), the National Natural Science Foundation of China (81230006 and 31090363), Beijing collaborative innovative research center for cardiovascular diseases (PMX2013_014226_07_000088), and Program for Changjiang Scholars and Innovative Research Team in University (IRT1074).

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