Increased Expression of Elastolytic Cysteine Proteases, Cathepsins S and K, in the Neointima of Balloon-Injured Rat Carotid Arteries

Xian Wu Cheng,* Masafumi Kuzuya,*
Takeshi Sasaki,* Koji Arakawa,[†] Shigeru Kanda,*
Daigo Sumi,* Teruhiko Koike,* Keiko Maeda,*
Norika Tamaya-Mori,* Guo-Ping Shi,[‡]
Noboru Saito,[†] and Akihisa Iguchi*

From the Department of Geriatrics,* Nagoya University Graduate School of Medicine, Nagoya; the Laboratory of Animal Physiology,[†] Graduate of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, Japan; and the Department of Medicine,[‡] University of California, San Francisco, San Francisco, California

The matrix-degrading activity of several proteases are involved in the accelerated breakdown of extracellular matrix associated with vascular remodeling during the development of atherosclerosis and vascular injury-induced neointimal formation. Previous studies have shown that the potent elastolytic cysteine proteases, cathepsins S and K, are overexpressed in atherosclerotic lesions in human and animal models. However, the role of these cathepsins in vascular remodeling remains unclear. In the present study, the expressions of cathepsin S and K and their inhibitor cystatin C were examined during arterial remodeling using a rat carotid artery balloon-injury model. The increase in both cathepsin S and K mRNA levels was observed from day 1 and day 3 through day 14 following the induction of balloon injury, respectively. Western blotting analysis revealed that both cathepsin S and K protein levels also increased in the carotid arteries during neointima formation, coinciding with an increase elastolytic activity assayed using Elastin-Congo red, whereas, no significant change in the expressions of cystatin C mRNA and protein was observed during follow-up periods after injury. Immunohistochemistry, Western blot, and *in situ* hybridization showed that the increase of cathepsins S and K and the decrease of cystatin C occurred preferentially in the developing neointima. These findings suggest that cathepsin S and K may participate in the pathological arterial remodeling associated with restenosis. (Am J Pathol 2004, 164:243–251)

smooth muscle cell (SMC) migration from the tunica media to the intima is a key step in the development of neointimal lesion formation.^{2,3} During the processes of SMC migration, SMCs must degrade and breach the extracellular matrix proteins surrounding each cell and internal elastic lamina. SMCs produce a large number of proteases, such as serine, cysteine, and matrix metalloproteinases (MMPs).^{4–6} Among these proteases, MMPs and the serine protease system, plasminogen/plasmin, have been believed to contribute to matrix remodeling and to play an essential role in SMC migration.^{7–10} This is supported by findings that MMPs and plasminogen activator levels are elevated after balloon injury to rat carotid arteries.^{7,8,11} However, previous observations have suggested that the even effective inhibition of MMPs and serine proteases might not sufficiently arrest neointima formation.^{12–15}

Cathepsins, lysosomal proteases within the papain superfamily, are thought to generally reside in and function optimally within acidic lysosomes.¹⁶ Despite their lysosomal origin and optimal acidic pH, some of cathepsins including cathepsin S and K can be secreted and retain a large portion of their proteolytic activity at neutral pH.^{17–19} Among the members of the cathepsin family, cathepsin S and K express potent elastolytic as well as collagenolytic activities.^{19–21} Although it has been demonstrated that vascular SMCs have the ability to express these cathepsins,^{6,22} cathepsins have received much less consideration in the involvement in the neointima formation.

Previous studies showed that cathepsin S and K are expressed in atherosclerosis lesions in humans and mice.^{6,22,23} More interestingly, it has recently been reported that deficiency of cathepsin S reduced atherosclerosis in low-density lipoprotein receptor-deficient mice.²⁴ However, the expression of these cathepsins during neointima formation remains unknown. The expression and activity of cathepsins are tightly controlled at several levels. Cystatin C is ubiquitous in human tissues and body fluids²⁵ and efficiently inhibits endogenous cathepsins.^{26,27} Changes in the temporal expression of these

Accepted for publication September 30, 2003.

Address reprint requests to Masafumi Kuzuya, M.D., Ph.D., Department of Geriatrics, Nagoya University Graduate School of Medicine, 65 Tsuruma-Cho, Showa-Ku, Nagoya 466-8550, Japan. E-mail: kuzuya@med.nagoya-u.ac.jp.

Neointima formation plays a role in the pathogenesis of restenosis after angioplasty.¹ It has been believed that

enzymes and their inhibitors may regulate the local accumulation and degradation of elastin-rich extracellular matrix and could be involved in the vascular remodeling that results in restenosis. In the present study, we analyzed cathepsin S and K and cystatin C expression during the development of neointima in the rat carotid artery after balloon injury using quantitative real-time polymerase chain reaction (PCR), immunohistochemistry, Western blotting, and *in situ* hybridization.

Materials and Methods

Animal Model

Male Wister rats (3 to 4 months old; Japan SLC, Shizuoka, Japan) were used for the present study. All animal experiments were performed in accordance with the Guidelines for Animal Care of Nagoya University School of Medicine. The animals were anesthetized by intraperitoneal injection of ketamine and xylazine (70 mg/kg and 4.6 mg/kg body weight, respectively), and a balloon catheter injury to the left common carotid artery was performed as previously described.⁷ At various time points after injury was induced, the animals were killed by means of an overdose of ketamine and xylazine. The arteries were flushed clear of blood using normal saline at physiological pressure, removed, and stripped of the surrounding connective tissue and the fatty material. Uninjured left carotid arteries (0 day) were used as controls. For quantitative real-time PCR analysis, the vessels were put in RNeasy lysis buffer from an RNeasy Protect Mini Kit and stored at -20°C . For immunohistochemistry and *in situ* hybridization analysis, the vessels were excised and fixed for 16 hours with 4% phosphate-buffered formalin. For protein extraction, the vessels were snap-frozen in liquid nitrogen and stored at -70°C .

Quantitative Real-Time RT-PCR Analysis

The total cellular RNA from rats ($n = 25$) common carotid arteries were extracted using RNeasy Protect Mini Kit using the procedures recommended by the manufacturer. Twenty ng of RNA was reverse-transcribed using cloned murine leukemia virus reverse transcriptase (PE Biosystems, Foster City, CA) and random hexamer. cDNA was amplified by real-time PCR with 1X TaqMan Buffer, 5.5 mmol/L MgCl_2 , 200 $\mu\text{mol/L}$ of each dNTP, 100 nmol/L of each primer, 200 nmol/L of probe, 0.01 U/ μL Amp-Erase uracil N-glycosylase, and 0.025 U/ μL AmpliTaq Gold (PE Biosystems, Foster City, CA). Primers and probes are listed in Table 1. Each mRNA quantity was normalized in regard to its respective GAPDH mRNA quantity. Each sample was analyzed in duplicate using ABI Prism 7700 Sequence Detector (PE Biosystems) using the following conditions: 50°C (2 minutes) for UNG incubation, 94°C (10 minutes) for AmpliTaq Gold activation, 95°C (15 seconds), and 60°C (1 minute) for 40 cycles.

Table 1. Primers, Probes, and Antisenses Used for RT-PCR and *in Situ* Hybridization

Cathepsin S
Forward primer: 5'-CTGCCACGTGTTCAAGGTACA-3'
Reverse primer: 5'-AGACTTTCTTCGTCACCGGT-3'
Probe: 5'-TGAGCTCCCCTTCGGCGATGAA-3'
Cathepsin K
Forward primer: 5'-GGAACGAGAAAGCCCTGAAGA-3'
Reverse primer: 5'-AACTGTAGAAAGGTTAAAATGTCGT-3'
Probe: 5'-ACCCGTCTCTGTGTCCATCGATGCA-3'
Cystatin C
Forward primer: 5'-GGCACTCTGCTCCTCCAGAT-3'
Reverse primer: 5'-TTAGGTCGACGTTTTACGGA-3'
Probe: 5'-CTGGAAGGCACACACACCCTGACA-3'
Cathepsin S antisenses:
5'-ACACTGATCACGACACACTTGGGTCCTCCG-3'
5'-AAAAGAAGAAACGGTTTAGATTCTGGGTA-3'
5'-ACACTTTTCATCCATGGCTTTGTAGGGATA-3'
Cathepsin K antisenses:
5'-GGCACCCGCAGAGCTGAAAGCCCAACAGGAAC-CACACTGGCCCTG-3'
5'-TCCATTCTGCTGCACATATTGGAAGGCAGTGGTC-ATATAGCCGCC-3'
5'-TCACATCTGGGGAAGCTGGCCAGGTTGGTAAT-GCCACAGGCATT-3'

In Situ Hybridization

Three 30-mer oligo-DNAs (cathepsin S nucleic acid numbers: 631–660, 1006–1035, and 1090–1119) and 45-mer oligo-DNAs (cathepsin K nucleic acid numbers: 439–483, 577–621, and 988–1032) complementary to the cathepsin S and K mRNA sequences were designed from the mRNA of rat cathepsin S²⁸ and K²⁹. A computer-assisted search (GenBank) for these oligo-DNA sequences did not detect any significant similarities with other published sequences. The oligo-DNAs were labeled with digoxigenin (DIG) using a DIG Oligonucleotide Tailing Kit according to the procedures recommended by the manufacturer.

In situ hybridization was performed as previously described with some modifications.³⁰ After permeabilization with 10 $\mu\text{g/ml}$ proteinase K for 10 minutes, the sections were immersed in 50% formamide (FA)/5X SSC (150 mmol/L NaCl and 15 mmol/L sodium citrate, pH 7.4) for 2 hours at 39°C for pre-hybridization. DIG-labeled oligo-DNA probes were mixed with 20 mmol/L Tris-HCl (pH 7.4), 2.5 mmol/L EDTA, 300 mmol/L NaCl, Denhardt's solution, 50% FA, 5% dextran sulfate, 1 mg/ml yeast tRNA, and 1 $\mu\text{g/ml}$ DIG-labeled probe heated for 10 minutes at 95°C to linearize the probes. The sections were incubated in the hybridization mixture overnight at 39°C . For immunohistochemical detection of haptenized (DIG-labeled) antisense probes, after treatment with 1% blocking reagent (DIG Nucleic Acid Detection Kit) in buffer containing 100 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 7.4) for 1 hour, the sections were incubated with alkaline phosphatase-conjugated sheep anti-DIG IgG (1:500) dissolved in 1% blocking buffer for 1 hour. Following washing three times with buffer (100 mmol/L Tris-HCl, 100 mmol/L NaCl, 50 mmol/L MgCl_2 , pH 9.5), the sections were visualized with the color substrate NBT/BCIP (DIG Nucleic Acid Detection Kit) according to the manu-

facturer's instructions. Control experiments were used to confirm the specificity of the cathepsin S or K mRNA signals. Sense probes were hybridized with some sections as a negative control. The cathepsin S and K antisenses are listed in Table 1.

Neointima Tissue Isolation

Both 7 and 14 days after injury, the carotid artery was isolated and the adventitia stripped away from the vessel. The vessel was then incised longitudinally and the neointima was dissected away from the media with the use of fine forceps and a dissecting microscope. The medial and neointimal cell layers were then snap-frozen in liquid nitrogen. To assure thorough separation of intima, media, and adventitia, after isolation the adventitia and the intima from the media, a part of the medial was fixed and 5- μ m histological sections were prepared to examine.

Isolation of Arterial Protein and Western Analysis

Western blotting was performed as previously described.³¹ Individual carotid arteries ($n = 25$) or isolated medial and neointimal cell layers ($n = 8$) were minced using a razor blade and extracted by means of a tissue homogenizer. The protein concentration for each lysate was determined using a protein assay system according to the manufacturer (Bio-Rad Dc; Bio-Rad Laboratories, Hercules, CA). The equal amounts of total protein from each sample (150 μ g for cathepsin S and K; 300 μ g for cystatin C) were separated on 15% SDS-PAGE and blotted onto a PVDF membrane. Membranes were reacted with rabbit cathepsin S, K, or cystatin C polyclonal antibodies, respectively, and immunoreactive bands were visualized by means of chemiluminescence. Each time, we prepared two gels, one to Western blot analysis; another one to Coomassie brilliant blue staining to check the loading of protein as the same amounts. Band intensity was quantitated using an image analyzer system (NIH image 1.62).

Elastase Assay

Elastolytic activity of carotid arteries ($n = 25$) was assessed by Elastin-Congo red degradation assay as previously described^{22,32} with some modification. For acidic protease activity of cathepsins,²¹ 100 μ g of total protein from each sample was incubated with 1 mg/ml Elastin-Congo red containing 0.05% Triton X-100, 20 mmol/L sodium acetate, and 1 mmol/L EDTA, pH 5.5; for a study of MMP activity, the same amounts of total proteins were added to 1 mg/ml Elastin-Congo red and incubated in 10 mmol/L phosphate, pH 7.2, containing 150 mmol/L NaCl, 1% Triton-100, 15 mmol/L CaCl_2 , 0.1% SDS, 0.5% Na deoxycholate, and 0.2% Na azide. All assays were performed in duplicate. After incubation for 24 hours using Mixer at 37°C, the results were centrifuged to measure degraded elastin (495 nm). Parallel incubations with an inhibitor of cysteine protease, *Trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64, 20 μ mol/L). Blanks

values were determined by incubation of the elastin at each pH value in the absence of the tissue extracts and these were subtracted from counts determined in the presence of the tissue extracts.

Collagenase Assay

Collagenolytic activity was performed as previously described³³ with some modification using FITC-labeled type I collagen as a substrate. One-hundred μ g of total protein from each sample was added to 96-well plate which was pre-coated with 500 μ g/ml FITC-labeled type I collagen and incubated for 6 hours at 37°C using shaker. The solubilized collagen fragments were then measured using Fluoroskan Ascent CF (Labsystems, excitation/emission maxima at 485 nm/527 nm). To identify the specific inhibitors for the collagenolytic activity present in the arterial extracts, the samples were incubated at room temperature with 10 μ mol/L N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (GM6001, a MMP inhibitor), 2 mmol/L phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor), and 20 μ mol/L E64 for 30 minutes before adding the substrate.

Immunohistochemistry

Dissected carotid segments were embedded in OCT compound. Five- μ m serial cryostat sections were incubated with polyclonal cathepsin S, K,²² or cystatin C (each rate of dilution 1:100) antibodies overnight at 4°C. Subsequent incubation for 60 minutes in biotinylated secondary antibodies (1:200 dilution) was followed by 60 minutes of incubation with ABC-AP complex. The sections were visualized using Alkaline Phosphatase Substrate Kit I and levamisole. Sections were counterstained with Mayer's hematoxylin solution. Staining for cathepsin S required a 10-minute pretreatment with 1 N HCl at room temperature before incubation in the presence of primary antibody as previously described.²² Antibodies against human cathepsin S and K were raised from rabbits by immunizing maltose-binding protein fused cathepsin recombinant proteins as previously described.^{34,35} To control for antibody specificity, immunohistochemical studies were performed in parallel with a preparation of antibody that had been incubated overnight at 4°C with a 20-fold excess of antigen as negative controls, as described previously.²²

Materials

Anti-cystatin C was obtained from Upstate Biotechnology (Lake Placid, NY). ABC-AP complex, Alkaline Phosphatase Substrate Kit I, levamisole, and goat anti-rabbit IgG second antibody were from Vector Laboratories, Inc. (Burlingame, CA). The Rneasy Protect Mini Kit was purchased from Qiagen Labtrade Inc. (Miami, FL). OCT compounds were from Sakura Finetechnical Co. Ltd. (Tokyo, Japan). DIG Oligonucleotide Tailing Kit and DIG Nucleic Acid Detection Kit were from Roche Diagnostics

(Mannheim, Germany). Ketamine was from Sankyo Pharmaceutical Co., and xylazine was from Bayer Pharmaceutical Company (both in Tokyo, Japan). FITC-labeled type I collagen and E64 were from Molecular Probes (Eugene, Oregon). GM6001 and bovine spleen cathepsin S were from Calbiochem (San Diego, CA). Elastin-Congo red and PMSF were obtained from Sigma-Aldrich (St. Louis, MO). Human MMP-1 was from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA).

Statistical Analysis

All data included in the present text were considered to be normally distributed and are presented as mean $\pm$ SD unless otherwise indicated. Statistical analysis was performed by one-way analysis of variance (analysis of variance) followed by Scheffé's multiple comparison. A value of $P < 0.05$ was considered statistically significant.

Results

Total RNA was isolated from carotid arteries at various time points after injury was induced and was analyzed by quantitative real-time RT-PCR for the expression of cathepsin S, cathepsin K, and cystatin C. As shown in Figure 1A, the cathepsin S mRNA levels were increased significantly in balloon-injured vessels as early as 1 day after injury. At 3 days after injury, the relative mRNA levels of cathepsin S had increased 3.5-fold over uninjured control vessels. Similarly, the cathepsin K mRNA levels had significantly increased between 3 and 14 days after injury, with the highest expression occurring at 7 days (Figure 1B). In contrast, no significant changes in cystatin C mRNA expression were observed in carotid arteries during the follow-up periods (Figure 1C).

We further studied the localization of cathepsin S and K mRNA expression by *in situ* hybridization in the carotid arteries at day 7 after injury. The intense signal for the cathepsin S or K mRNA was detected in the neointima compared with the media (Figure 2, A and B). There were low levels of the cathepsin S and K mRNA detected in the uninjured carotid artery (data not shown). In control sections using the cathepsin S or K sense probe for *in situ* hybridization, no signal for the cathepsin S or K was found in the media and the neointima (Figure 2, A and B).

Western blot analysis performed on the equal amounts of total protein from different vessels extracts by means of antibody to cathepsin S showed that the cathepsin S protein levels in the carotid arteries increased significantly from 3 days up to 14 days after injury, reaching their highest levels at 7 days (Figure 3, A and B). Western blot analysis using the cathepsin K antibody revealed a significant increase in cathepsin K levels between 7 and 14 days with the highest levels occurring at 7 days after injury (Figure 3, C and D). No changes of cathepsin S and K levels were observed in uninjured right carotid arteries at any of the time points (data not shown). It should be noted that the apparent size of the proteins were 28 and 29 kd, respectively, consistent with the reported sizes of the active forms of these cathepsins.²² In contrast with

A

B

C

Figure 1. Quantitative real-time PCR analysis of cathepsin S (A), cathepsin K (B), and cystatin C (C) mRNA of rat carotid arteries after injury at the indicated time points. Each mRNA level was normalized in regard to GAPDH mRNA. Data represent results from at least five separate determinations. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ versus uninjured control levels (day 0).

the cathepsins, no change of cystatin C protein expression in the carotid arteries was observed between 1 and 14 days after balloon injury (Figure 3, E and F).

To evaluate the distribution of the cathepsin S and K proteins, the neointima and the medial layers were separated from carotid artery at day 7 and 14 after injury and protein extracts of each layer were analyzed by Western blotting. As shown in Figure 4A to C, cathepsin S and K proteins were expressed preferentially in the neointima.

Figure 2. Transcripts for cathepsin S (**A**) and K (**B**) localize in the neointima. Serial cryostat sections from the carotid arteries at day 7 after injury were analyzed for cathepsin S and K transcript expression by *in situ* hybridization using DIG-labeled antisense oligo-DNA probes (**A1** and **B1**) or DIG-labeled sense oligo-DNA probes (**A2** and **B2**) for the cathepsin S or K, respectively. L, lumen; **solid arrow**, internal elastic lamina; N, neointima; M, media.

To test whether the cathepsins expressed by balloon injury in the carotid arteries can actually degrade an extracellular matrix component, we determined total elastolytic activity of the tissue extracts from injured arteries at indicated time points. When assayed under the conditions that optimize cysteine protease activity, the elastolytic activity in the carotid arteries increased significantly from 3 days up to 14 days after injury, reaching their highest level at 7 days (Figure 5A). Incubation of the tissue extracts with the cysteine protease inhibitor, E64, significantly inhibited the increased elastolytic activity in the carotid arteries (Figure 5A). In contrast, when assayed under the conditions that optimize MMP activity, neutral, metal-dependent elastolysis of the Elastin-Congo red substrate was much lower than optimized acidic condition at all time points (data not shown). In contrast to the elastolytic activity, significant increase of collagenolytic activity of the injured carotid artery extracts compared with that of uninjured control vessel was only detected at day 7 after injury (data not shown). Furthermore, the total collagenolytic activity of the injured carotid arteries at day 7 after injury was significantly but only partially inhibited in the presence of E64, a specific cysteine protease inhibitor (Figure 5B). GM6001, a specific MMP inhibitor, strongly inhibited collagenolytic activity, whereas no significant inhibition was observed in sam-

ples incubated with the serine protease inhibitor (PMSF) (Figure 5B).

Immunohistochemical analysis revealed strong staining for cathepsin S and K in the endothelial cell layer in uninjured carotid arteries and that only a weak staining was detected in the medial region (Figure 6, A1 and B1). A neointima was visible at day 3 and was well developed by 7 days. Cathepsin S and K staining of cross-sections taken on day 3, 7, and 14 after injury produced strongly positive-stained regions in the intima of the carotid arteries (Figure 6, A2, A3, A4, B2, B3, and B4). There appeared to be no grossly visible differences in the intensity of cathepsin S and K staining in the media region between the uninjured vessel walls (0 day) and those at 3, 7, and 14 days after injury. Immunohistochemical studies using anti-cystatin C antibody revealed cystatin C in the media region in the control arteries (Figure 6C1) as well as in the arteries at day 3, 7, and 14 after the induction of balloon injury, with likely weaker staining of cystatin C being observed in the intima lesion compared with the media (Figure 6, C2, C3, and C4). The lack of the reaction with the cathepsin S or K antibody preabsorbed with excess of both recombinant cathepsin S or K established the specificity of the staining under the conditions used in these experiments (Figure 6, A5 and B5). Control preparations with non-immune serum used in place of the

Figure 3. Cathepsin S, K, and cystatin C protein levels in balloon-injured rat carotid arteries. Representative Western blots for cathepsin S (A), cathepsin K (C), or cystatin C (E), respectively, are shown as the function of time after injury. The equal amount of the total protein (300 μ g for cystatin C; 150 μ g for cathepsin S and K) extracted from a single vessel at each time point was loaded in each lane. The graph of combined with data cathepsin S (B), cathepsin K (D), or cystatin C (F) protein levels. Data represent results from at least five separate determinations. *, $P < 0.01$, **, $P < 0.001$ versus uninjured control levels.

primary antibody showed virtually no background or non-specific staining (data not shown). We also confirmed that the intima as well as the media contained mainly α -actin-positive cells (data not shown).

Figure 4. Both cathepsin S and K protein levels in the medial and the neointimal cell layers of rat carotid artery between day 7 and 14 after injury. A and C: Representative Western blot of the medial and the neointima cellular extracts analyzed with both affinity-purified antibody to cathepsin S or K. Data represent results from at three separate determinations. *, $P < 0.05$, **, $P < 0.01$ versus the media at day 7 and 14 after injury.

Discussion

Proteolytic enzymes derived from SMCs are thought to facilitate cell migration, neointimal lesion formation, and degradation of the extracellular matrix associated with vascular remodeling. Although the rat carotid artery balloon-injury model does not represent human restenosis with complete accuracy, many studies using this model have been performed to reveal the mechanisms of vascular remodeling induced by balloon injury.^{7,8,11} After balloon injury, the SMCs must modulate elastin-rich matrix to penetrate the internal elastic lamina from the media to the intima to form the neointimal lesion. This process of SMC migration is critically dependent on the action of extracellular proteases. Extensive investigations regarding vascular remodeling have been performed using two proteolytic systems, the fibrinolytic (plasminogen/plasmin) and the MMP systems.⁷⁻¹⁰ Abundant evidence now supports a potential role for these two proteolytic systems in vascular remodeling.^{7,8,11} However, recent studies using mice with targeted gene deletion and inhibitors of these proteases have suggested that other proteolytic

A

B

Figure 5. Elastin degradation by the tissue extracts from carotid arteries at indicated time points after balloon injury. Tissue extracts with or without E64 (20 μ mol/L) were incubated with Elastin-Congo red for 24 hours at 37°C (A). The relative amounts of elastin degradation are presented as the absorbance determined. Elastolytic activity of cathepsin S (100 nmol) was used as a positive control. Effects of protease inhibitors on the arterial collagenolytic activity at day 7 after injury (B). The selective inhibitors, E64 (20 μ mol/L), GM6001 (10 μ mol/L), or PMSF (2 mmol/L) were used to determine the degree of FITC-labeled type I collagen degradation. The relative amounts of type I collagen degradation are presented as the fluorescence intensity determined. Collagenolytic activity of 100 ng MMP-1 was used as a positive control. Data represent observations from at least five independent experiments. *, $P < 0.01$, **, $P < 0.001$ versus control. #, $P < 0.05$, ##, $P < 0.01$; (–) E64 versus (+) E64 at day 3, 7, and 14 after injury.

systems might also contribute to atherogenesis^{6,24} and vascular remodeling.^{12–15} We believe that our experiments represent the first characterization of the expression of elastolytic cathepsins and their inhibitor in the rat carotid artery balloon-injury model.

The present study disclosed low levels of expression of cathepsin S and K in uninjured rat carotid arteries, consistent with the observation in human normal arteries.²² Based on quantitative real time RT-PCR and immunoblotting analyses, we demonstrated that the cathepsin S levels increased in response to balloon injury as early as 1 day following it and remained elevated even at 14 days in injured carotid arteries. In contrast, overexpression of cathepsin K was detected from day 3 after injury.

Cathepsins are synthesized as zymogens and undergo maturation to their enzymatically active forms,¹⁶

although it is still unclear how procathepsins mature *in vivo*. In the present study, the increase in the amount of mature cathepsin S and K levels were primarily confined to the balloon-injured vessels, which was evident based on Western blotting analyses. There was a different time course between the mRNA and matured protein expression of these cathepsins after balloon injury. Although we do not know the exact mechanism of these differences, it is possible that there may be lag periods for these cathepsins' maturation after the transcription of these zymogens.

We clearly demonstrated that total elastolytic activity in injured carotid artery increased after injury. This increase in the elastolytic activity observed in the extracts of balloon-injured carotid arteries was strongly inhibited by cysteine proteinase inhibitor. This observation correlates with our findings of the overexpression of cathepsin S and K in the balloon-injured carotid artery. It should be noted that collagenolytic activity in carotid artery increased at day 7 after balloon injury and that its activity was only partially blocked by cysteine protease inhibitor, suggesting a limited contribution of cathepsins in collagenolytic activity in the injured arteries.

The data from *in situ* hybridization, Western blotting analysis, and immunohistochemistry clearly demonstrated that both expression of cathepsin S and K are located mainly in the intimal lesions. Taken together with the fact that the predominant cellular population of these regions are SMCs suggested that SMCs could be the main source of these enzymes. Although in the present study we did not focus on the regulation of cathepsin expression in SMCs, an earlier study has demonstrated that cultured SMCs express little or no cathepsin S and K, but that inflammatory cytokines up-regulated their expression.²² Therefore, it is possible that inflammatory cytokines induced during the course of intimal lesion formation after balloon injury could enhance the expression of these cathepsins.

In contrast with cathepsins, no significant change of cystatin C levels was demonstrated in rat carotid arteries following balloon injury based on the observations of quantitative real time RT-PCR and Western blotting, consistent with the constitutive nature of the cystatin C promoter.³⁶ However, immunohistochemical analyses revealed that lower levels of cystatin C expression were observed in neointima lesions compared with the levels in the media region. These results were inconsistent with human atherosclerotic lesion, in which significant reduction of cystatin C levels was observed in comparison with control artery.³⁷ How changes in cystatin C expression in SMCs might be affected is currently unknown. It is possible that phenotypic changes in SMCs during the course of intima lesion formation, and growth factors as well as cytokines involved in lesion formation, may be involved in changes in cystatin C expression.

Even though cathepsins function optimally at a specific acidic pH,¹⁶ studies of cathepsin S and K have shown that these enzymes are stable and express potent proteolytic activity at neutral pH.^{17–19} The time frame of the changes in cathepsin S and K expression and the local-

Figure 6. Immunohistochemistry of balloon-injured rat carotid arteries with three affinity-purified antibodies to cathepsin S (A1 to A4), cathepsin K (B1 to B4), or cystatin C (C1 to C4), respectively. The cathepsin S or K antibody pre-absorbed with both 20-fold excess of recombinant cathepsin S or K was used for negative controls. L, lumen; solid arrow, internal elastic lamina; N, neointima; M, media.

ization of these enzymes in injured vascular walls support the notion that the elastolytic activity of cathepsins play some role in the neointimal lesion formation after balloon injury. Overexpression of cathepsin S and cathepsin K and the reduction of the expression of their inhibitor,

cystatin C, which induced a shift of the proteolytic balance toward proteolytics seem to contribute to the vascular remodeling associated with the neointimal lesion formation after balloon injury. However, further studies using their specific inhibitors or animals with targeted

cathepsin gene deletion will be required to confirm their contribution to vascular remodeling.

In summary, we demonstrated that cathepsin S and K expression significantly increased, but that expression of their inhibitor, cystatin C, decreased in neointima lesions in rat carotid arteries following balloon injury. We believe that cathepsin S and K participate in pathological arterial remodeling associated with restenosis in cooperation with other proteases such as MMPs and plasminogen/plasmin systems.

References

- Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993, 362:801–809
- Reidy MA, Jackson D, Lindner V: Neointimal proliferation: control of vascular smooth muscle cell growth. *Vasc Med Res* 1992, 3:156–167
- Clowes AM, Reidy M, Clowes MM: Kinetics of cellular proliferation after arterial injury: smooth muscle growth in the absence of endothelium. *Lab Invest* 1983, 49:327–333
- Leake DS, Hornebeck W, Brechemier D, Robert L, Peters TJ: Properties and subcellular localization of elastase-like activities of arterial smooth muscle cells in culture. *Biochim Biophys Acta* 1983, 761:41–47
- Carmeliet P, Moons L, Lijnen R, Baes M, Lemaître V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, Collen D: Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet* 1997, 17:439–444
- Jörmsjö S, Wuttge DM, Sirsjo A, Whattling C, Hamsten A, Stemme S, Eriksson P: Differential expression of cysteine and aspartic proteases during progression of atherosclerosis in apolipoprotein E-deficient mice. *Am J Pathol* 2002, 161:939–945
- Bendeck MP, Zempo N, Clowes AW, Galardy RE, Reidy MA: Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. *Circ Res* 1994, 75:539–545
- Zempo N, Kenagy RD, Au YPT, Bendeck M, Clowes MM, Reidy MA, Clowes AM: Matrix metalloproteinases of vascular wall cells are increased in balloon-injured rat carotid artery. *J Vasc Surg* 1994, 20:209–217
- Kanda S, Kuzuya M, Ramos MA, Koike T, Yoshino K, Ikeda S, Iguchi A: Matrix metalloproteinase and $\alpha v \beta 3$ integrin-dependent vascular smooth muscle cell invasion through a type I collagen lattice. *Arterioscler Thromb Vasc Biol* 2000, 20:998–1005
- Kenagy RD, Hart CE, Stetler-Stevenson WG, Clowes AW: Primate smooth muscle cell migration from aortic explants is mediated by endogenous platelet-derived growth factor and basic fibroblast growth factor acting matrix metalloproteinases 2 and 9. *Circulation* 1997, 96:3555–3560
- De Young MB, Tom C, Dichek DA: Plasminogen activator inhibitor type I increases neointima formation in balloon-injured rat carotid arteries. *Circulation* 2001, 104:1972–1977
- Bendeck MP, Irvin C, Reidy MA: Inhibition of matrix metalloproteinase activity inhibits smooth muscle cells migration but not neointimal thickening after arterial injury. *Circ Res* 1996, 78:38–43
- Strauss BH, Robinson R, Batchelor WB, Chisholm RJ, Ravi G, Natarajan MK, Logan KA, Mahta SR, Levy DE, Ezrin AM, Keeley FW: In vivo turnover following experimental balloon angioplasty injury and the role of matrix metalloproteinases. *Circ Res* 1996, 79:541–550
- Cheng L, Mantile G, Pauly R, Nater C, Felici A, Monticone R, Bilato C, Gluzband YA, Crow MT, Stetler-Stevenson W, Capogrossi MC: Adenovirus-mediated gene transfer of the human tissue inhibitor of metalloproteinase-2 blocks vascular smooth muscle cell invasiveness in vitro and modulates neointimal development in vivo. *Circulation* 1998, 98:2195–2201
- Kenamasa K, Ishida N, Kato H, Sakamoto S, Nakabayashi T, Otani N, Ishikawa K, Katori R: Recombinant tissue plasminogen activator prevents intimal hyperplasia after balloon angioplasty in hypercholesterolemic rabbits. *Jpn Circ J* 1996, 60:889–894
- Chapman HA, Riese RJ, Shi GP: Emerging roles for cysteine proteases in human biology. *Annu Rev Physiol* 1997, 59:63–88
- Xin XQ, Gunesekeera B, Mason RW: The specificity and elastolytic activities of bovine cathepsin S and H. *Arch Biochem Biophys* 1992, 299:334–339
- Bromme D, Bonneau PR, Lachance P, Wiederanders B, Kirschke H, Peters C, Thomas DY, Storer AC, Verner T: Functional expression of human cathepsin S in *Saccharomyces cerevisiae*: purification and characterization of the recombinant enzyme. *J Biol Chem* 1993, 268:4832–4838
- Bossard MJ, Tomaszek TA, Thompson SK, Amegadzie BY, Hanning CR, Jones C, Kurdyla JT, McNulty DE, Drake FH, Gowen M, Levy MA: Proteolytic activity of human osteoclast cathepsin K. *J Biol Chem* 1996, 271:12517–12524
- Kafienah W, Bromme D, Buttle DJ, Croucher LJ, Hollander AP: Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix. *Biochem J* 1998, 331:727–732
- Shi GP, Munger JS, Meara JP, Rich DH, Chapman HA: Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastolytic cysteine protease. *J Biol Chem* 1992, 267:7258–7262
- Sukhova GK, Shi GP, Simon DI, Chapman HA, Libby P: Expression of the elastolytic cathepsin S and K in human atheroma and regulation of their production in smooth muscle cells. *J Clin Invest* 1998, 102:576–583
- Miller BF, Kothari HV: Increased activity of lysosomal enzymes in human atherosclerotic aortas. *Exp Mol Pathol* 1969, 10:288–294
- 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 low-density lipoprotein receptor-deficient mice. *J Clin Invest* 2003, 111:897–906
- Abrahamson M, Barrett AJ, Salvesen G, Grubb A: Isolation of six cysteine proteinase inhibitor from human urine. *J Biol Chem* 1986, 261:11282–11289
- Barrett AJ, Davies ME, Grubb A: The place of human γ -trace (cystatin C) amongst the cysteine proteinase inhibitors. *Biochem Biophys Res Commun* 1984, 120:631–636
- Brömme D, Rinne R, Kirschke H: Tight-binding inhibition of cathepsin S by cystatins. *Biomed Biochim Acta* 1991, 50:631–635
- Petanceska S, Devi L: Sequence analysis, tissue distribution, and expression of rat cathepsin S. *J Biol Chem* 1992, 267:26038–26043
- Tanaka S, Tamura K, Mizuno Y: Clinical consideration with special reference to autopsy cases of malignant tumor in the oral cavity, treated with bleomycin (author's translation). *Hiroshima Daigaku Shigaku Zasshi* 1975, 8:168–175
- Koji T, Brenner RM: Localization of estrogen receptor messenger ribonucleic acid in rhesus monkey uterus by non-radioactive in situ hybridization with digoxigenin-labeled oligodeoxynucleotides. *Endocrinology* 1993, 132:382–392
- Koike T, Kuzuya M, Asai T, Kanda S, Cheng XW, Watanabe K, Banno Y, Nozawa Y, Iguchi A: Activation of MMP-2 by *Clostridium difficile* toxin B in bovine smooth muscle cells. *Biochem Biophys Res Commun* 2000, 277:43–46
- Fujie K, Shinguh Y, Yamazaki A, Hatanaka H, Okamoto M, Okuhara M: Inhibition of elastase-induced acute inflammation and pulmonary emphysema in hamsters by a novel neutrophil elastase inhibitor FR901277. *Inflamm Res* 1999, 48:160–167
- Menter JM, Cornelison LM, Cannick L, Patta AM, Dowdy JC, Sayre RM, Abukhalaf IK, Silvestrov NS, Willis I: Effect of UV on the susceptibility of acid-soluble Skh-1 hairless mouse collagen to collagenase. *Photodermatol Photoimmunol Photomed* 2003, 19:28–34
- Shi GP, Webb AC, Foster KE, Knoll JHM, Lemere CA, Munger JS, Chapman HA: Human cathepsin S: chromosomal localization, gene structure, and tissue distribution. *J Biol Chem* 1994, 269:11530–11536
- Gelb BD, Shi GP, Chapman HA, Desnick RJ: Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science* 1996, 273:1236–1238
- Olafsson I: The human cystatin C gene promoter: functional analysis and identification of heterogeneous mRNA. *Scand J Clin Lab Invest* 1995, 55:597–607
- Shi GP, Sukhova GK, Grubb A, Ducharme A, Rhode LH, Lee RT, Ridker PM, Libby P, Chapman HA: Cystatin C deficiency in human atherosclerosis and aortic aneurysms. *J Clin Invest* 1999, 104:1191–1197