Cathepsin B, K, and S Are Expressed in Cerebral Aneurysms and Promote the Progression of Cerebral Aneurysms

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Background and Purpose—A cerebral aneurysm (CA) causes catastrophic subarachnoid hemorrhage. Degradation of extracellular matrix in arterial walls is a prominent feature of cerebral aneurysms. We investigated the expression and role of cysteine cathepsins, collagen- and elastin-degrading proteinases, in CA progression.

Methods—CAs were induced in Sprague-Dawley rats with or without cysteine cathepsin inhibitor, NC-2300. Expression of cathepsin B, K, S, and cystatin C, an endogenous inhibitor of cysteine cathepsins, in aneurysmal walls was examined in quantitative RT-PCR and immunohistochemistry. The activity of cysteine cathepsins and collagenase I and IV in aneurysmal walls was also assessed. Finally, expression of cysteine cathepsins and cystatin C in human CAs was examined.

Results—Quantitative RT-PCR and immunohistochemistry revealed upregulated expression of cathepsin B, K, and S in the late stage of aneurysm progression. In contrast, cystatin C expression was reduced with aneurysm progression. Treatment with NC-2300 resulted in the decreased incidence of advanced CAs. The activity of cysteine cathepsins and collagenase I and IV in aneurysmal walls was reduced and elastin content was increased in the NC-2300–treated group. Finally, immunohistochemistry for cysteine cathepsins and cystatin C expression in human CAs showed the same expression pattern as in the rat study.

Conclusions—Data obtained by using NC-2300 revealed an important role of cysteine cathepsins in the progression of CAs. Our findings strongly suggest that an imbalance between cysteine cathepsins and their inhibitor may cause the excessive breakdown of extracellular matrix in the arterial walls leading to the progression and rupture of CAs. (Stroke. 2008;39:2603-2610.)

Key Words: aneurysm | cathepsin | cystatin C | animal model

Cerebral aneurysm (CA) is a major cause of subarachnoid hemorrhage, one of the most severe forms of stroke. Despite the progress in microneurosurgery and endovascular surgery, the mortality of subarachnoid hemorrhage is still high because a substantial number of patients die before arriving at a hospital. Therefore, the prevention of rupture of CAs is mandatory for public health. The main pathological features of CAs are disappearance of the internal elastic lamina and thinning of the media, both of which are related to the degradation of extracellular matrix (ECM). Previously, we reported the important role of matrix metalloproteinases (MMPs) in the progression of CAs by using an experimentally induced CA model. Treatment with a selective inhibitor for MMP-2, -9, and -12 reduced the incidence of advanced aneurysms in rats. However, some advanced aneurysms developed even if MMP activity was completely blocked by the inhibitor, suggesting the involvement of other proteinases. Cathepsins consist of large family members of lysosomal proteolytic enzymes. Cathepsins localize in lysosomes and endosomes and digest unnecessary intracellular or endocytosed proteins. Recent molecular biological investigations revealed that these enzymes could function outside lysosomes and endosomes and have elastolytic and collagenolytic properties. Cathepsins play crucial roles in diverse biological and pathological status, including inflammatory or autoimmune disease. More interestingly, the expression of cathepsins was upregulated in various vascular diseases such as atherosclerosis and aortic abdominal aneurysms, although there is no immunoreactive reaction of cathepsins in healthy human aorta. Cathepsins are classified into several categories according to their catalytic site, and cathepsin B, K, and S belong to cysteine cathepsins. Previous studies indicated the active participation of cysteine cathepsins in the pathogenesis of atherosclerosis. Although the expression of cathepsin D and L was reported in human CAs, the expression and role of cysteine cathepsins in CAs have not yet been examined.

In the present study, we examined, for the first time, the expression and role of cysteine cathepsins in the initiation and...
progression of CAs by using an experimentally induced CA model and human CA samples.

Materials and Methods

Induction of Experimental Cerebral Aneurysms in Rats

CAs were induced as previously described by Nagata et al. After the induction of pentobarbital anesthesia (50 mg/kg i.p.), the left common carotid artery and posterior branches of the bilateral renal arteries were ligated at the same time with 10-0 nylon in 7-week-old male Sprague-Dawley rats (Oriental BioService). Animals were fed special food containing 8% sodium chloride and 0.12% β-aminopropionitrile (BAPN; Tokyo chemical), an inhibitor of lysyl oxidase that catalyzes the cross-linking of collagen and elastin. Blood pressure was measured by tail-cuff method twice in each rat. Animal care and experiments complied with Japanese community standards on the care and use of laboratory animals.

Immunohistochemistry

Three months after aneurysm induction (3 M), rats (n=10) were deeply anesthetized and perfused transcardially with 4% paraformaldehyde. As a control, age-matched male Sprague-Dawley rats without aneurysm induction (Control) were euthanized. The anterior cerebral artery/olfactory artery (ACA/OA) bifurcation was stripped, embedded, and cut into 5-μm sections. After blocking with 5% Donkey serum (Jackson ImmunoResearch), primary antibodies were incubated for 1 hour at room temperature followed by incubation with fluorescence labeled secondary antibodies (FITC-conjugated donkey antigoat IgG antibody, FITC-conjugated donkey antirabbit IgG antibody, Cy3-conjugated donkey antirabbit IgG antibody, Cy3-conjugated donkey antimouse IgG antibody, Jackson ImmunoResearch) for 1 hour at room temperature. Then the slides were excited for fluorescence by illumination through a fluorescence microscope system (Olympus). The primary antibodies used in the present study are listed below: goat polyclonal anticaathepsin B antibody (Santa Cruz), goat polyclonal anticaathepsin K antibody (Santa Cruz), goat polyclonal anticaathepsin S antibody (Santa Cruz), rabbit polyclonal anticatstatin C antibody (Upstate Biotechnology), mouse monoclonal anti-CD68 antibody (HyCult), mouse monoclonal antimouse α-actin antibody (Laboratory Vision), rabbit polyclonal antiendothelial nitric oxide synthase (eNOS) antibody (Laboratory Vision).

Quantitative Real-Time RT-PCR

Total RNA from the whole Cir of Willis was isolated using RNeasy Fibrous Tissue Mini Kit (QIAGEN) before aneurysm induction (0 M), 1 month (1 M), and 3 months (3 M) after aneurysm induction. Age-matched male Sprague-Dawley rats without aneurysm induction were used as controls. RNA extraction was performed according to the manufacturer’s directions. Total RNA was converted into cDNA using Sensiscript reverse transcriptase (QIAGEN). Quantitative RT-PCR was performed using QuantiTect SYBR Green PCR Kit (QIAGEN) and LightCycler quick system 330 (Roche). Constructs were produced by TOPO TA Cloning Kit (Invitrogen) from cDNA according to the manufacturer’s directions. β-actin was used as an internal control. The second derivate maximum method was used for crossing point determination, using LightCycler Software 3.3 (Roche), and 6 independent samples were examined in 1 experiment. The amount of mRNA expression was calculated as the ratio to mRNA expression of control rats. The condition for PCR was 42 cycles of 95°C twenty seconds for denaturation, 53°C twenty seconds for annealing, 72°C twenty seconds for extension. Primer sets used were listed below. forward 5'-aaacaggtgcttataaggcaag-3', reverse 5'-gactgtgatgtaggtacctga-3' for cathepsin B, forward 5'-ccagacctgcatatgc-3', reverse 5'-cgtgacatagcttgctg-3' for cathepsin K, forward 5'-acagcagctgaagcaga-3', reverse 5'-tagccaccaagagcaaccc-3' for cathepsin S, forward 5'-ggattctcgactcagagtatcc-3' for cathepsin C, forward 5'-aagactgtcaggctcctcccc-3', reverse 5'-aatgctcctacccctaaag-3' for β-actin.

Cathepsin Inhibitor

As a cathepsin inhibitor, we used NC-2300 (Monosodium (2S,3S)-3-[(1S)-1-isobutoxy methyl-3-methylbutyl] carbamoyle oxiran-2-carboxylate: MW=309 (Nippon Chemiphar, Saitama, Japan). NC-2300 is a specific inhibitor for cysteine cathepsins, which could suppress autoimmune inflammation of the joints as well as osteoclastic bone resorption in autoimmune arthritis. The plasma concentration of NC-2300 was measured by HPLC.

Immediately after aneurysm induction, rats were fed food with or without (n=10) or without (n=21) 50 mg/kg/d of NC-2300, and euthanized after 3 months. The ACA/OA bifurcation was stripped and observed under a light microscope after Elastica van Gieson staining. Early aneurysmal change refers to a lesion with the discontinuity of the internal elastic lamina without apparent outward bulging of the arterial wall. Advanced aneurysm refers to an obvious outward bulging of the arterial wall with the fragmentation or disappearance of the internal elastic lamina. After immunohistochemistry for CD68, the number of CD68-positive cells was counted in a 100 μm square around the ACA/OA bifurcation on each section. Three independent researchers assessed the histopathologic changes.

Measurement of Cathepsin B, K, and S Activities in Aneurysmal Walls

Total protein from the Cir of Willis was extracted using Complete Lysis-M (Roche) and incubated with each fluorescence-labeled substrate for 1 hour at 37°C. Cathepsin K activity was measured with Z-Gly-Pro-Arg-MCA (Peptide Institute) in 100 mmol/L sodium acetate buffer including 20 mmol/L l-cysteine (pH5.5). Cathepsin B activity was measured with Z-Arg-Arg-MCA (Peptide Institute) in 100 mmol/L phosphate buffer including 2 mmol/L l-cysteine (pH6.0). Cathepsin S activity was measured with Z-Val-Val-Arg-MCA (Peptide Institute) in 100 mmol/L phosphate buffer including 2 mmol/L l-cysteine (pH6.0). Enzyme activities were evaluated from the increase in fluorescence at 460 nm (excitation 360 nm). Enzyme activities were expressed as the relative ratio of fluorescence at 460 nm to the control (rats before aneurysm induction). One hundred μg of protein was used in 1 experiment (n=3 in each group).

Measurement of Collagenase Activity

Total protein from the Circle of Willis was purified by Bio-Plex Cell Lysis Kit (Bio-Rad; n=5 in each group). Collagenase activity was measured using Collagenase Activity Measurement Kit (Life Laboratory). Briefly, fluorescence labeled type I or IV collagen was incubated with the lysate of the whole Willis ring for 1 hour at 37°C. Undigested fluorescence labeled collagen was extracted in ethanol solution and measured by fluorospectrometer with excitation 496 nm and measurement 520 nm. Collagenase activity was calculated by the fluorescence intensity and corrected by the amount of total protein according to the formula given in the manufacturer’s direction.

Elastin Content in Aneurysmal Walls

Frozen sections at the ACA/OA bifurcation were stained with Miller elastin and van Gieson stain in a standard manner. The area of elastic fibers in van Gieson–stained sections was calculated by quantitative morphological analysis with Image Analyzer (Meesoft) and Scion Image (Scion Corporation). Elastin content was expressed as area ratio of elastic fibers to arterial walls (n=5 in each group).

Immunohistochemistry for Human Samples

Human CA samples were obtained from 7 patients who underwent neck clipping for unruptured aneurysms with informed consent. As a control, we used the middle cerebral artery (MCA) obtained at the STA-MCA bypass surgery (n=5). After deparaffinization and block-
ing of endogenous peroxidase activity with 0.3% H₂O₂, primary antibodies against cathepsin B, K, and S and cystatin C (the same as used in the rat study) were incubated for 12 hours at 4°C followed by incubation with biotin-labeled secondary antibodies for 30 minutes at room temperature. Then, slides were incubated with streptavidin-conjugated peroxidase. Finally, the signal was detected by 3,3′-diaminobenzidine system (DAKO). Nuclear staining was performed by hematoxylin solution. As a negative control, we performed immunohistochemistry without primary antibodies. For double staining, the slides were incubated with primary antibodies for smooth muscle α-actin (DAKO), CD68 (DAKO), or von Willebrand factor (DAKO) for 30 minutes at room temperature, followed by the incubation with alkaline phosphatase-labeled secondary antibodies and Fast red solution (Sigma).

Statistical Analysis
The values were expressed as means±SD. Statistical analysis was performed by using Student t test for a 2-group comparison and 1-way ANOVA followed by Fisher test for a multiple comparison. The incidence of aneurysmal changes was analyzed by the use of Fisher’s exact test. Differences were considered statistically significant at P<0.05.

Results
Expression of Cathepsins and Cystatin C in Experimentally Induced CAs in Rats
The expression of cathepsin B, K, and S could not be detected in arterial walls of rats without aneurysm induction (Figure 1C, 1E, 1G). Three months after aneurysm induction, cathepsin B, K, and S (Figure 1D, 1F, 1H) were highly expressed throughout aneurysmal walls. In contrast, cystatin C was abundantly expressed mainly in the media of arterial walls in rats without aneurysm induction (Figure 1I), and its expression was reduced in advanced aneurysms (Figure 1J). Cathepsin B, K, and S were mainly expressed in smooth muscle cells, although endothelium and macrophage also expressed cathepsin B, K, and S in aneurysmal walls (Figure 2).

Expression of Cathepsins and Cystatin C mRNA in Experimentally Induced CAs in Rats
The expression of cathepsin B, K, and S mRNA did not significantly increase 1 month after aneurysm induction and was markedly upregulated after 3 months (cathepsin B P<0.01, 1 month versus 3 months, n=6; cathepsin K P<0.01, 1 month versus 3 months, n=6; cathepsin S P<0.01, 1 month versus 3 months, n=6; Figure 3A through 3C) in quantitative real-time RT-PCR. In contrast, cystatin C mRNA expression significantly decreased with aneurysm progression (P=0.031, before aneurysm induction versus 1 month, n=6; P=0.016, 1 month versus 3 months; n=6; Figure 3D). In rats without aneurysm induction, mRNA levels of cathepsin B, K, S and cystatin C did not show any age-related changes (Figure 3A through 3D).

The Effect of a Cysteine Cathepsin Inhibitor (NC-2300) on CA Formation and Progression
The plasma concentration of NC-2300 was 531±247 nmol/L (n=10). In the control group, 19 of 21 rats, (90%) developed
advanced aneurysms and 2 (10%) showed early aneurysmal changes. In the NC-2300–treated rat, only 5 of 10 rats (50%) developed advanced aneurysms and 4 (40%) showed early aneurysmal changes. The incidence of all aneurysmal changes was not different between the 2 groups. However, the rate of advanced aneurysms was significantly lower in the NC-2300-treated group than in the control group (P=0.022; Figure 4A). In both groups, systemic blood pressure was elevated after 3 months of aneurysm induction, but there was no significant difference between the control group (160.7±21.1 mm Hg, n=21) and the NC-2300–treated group (164.6±20.7 mm Hg, n=10; Figure 4B). Macrophage infiltration into aneurysmal walls did not differ between the control group (5.3±1.5 cells/field, n=14) and the NC-2300–treated group (4.9±1.7 cells/field, n=10; Figure 4C).

The Effect of NC-2300 on Activities of Cathepsin B, K, and S in Aneurysmal Walls
Three months after aneurysm induction, the activities of cathepsin B, K, and S were increased compared with rats before aneurysm induction (cathepsin B 1.97±0.54, P=0.02; cathepsin K 2.50±0.56, P<0.01; cathepsin S 2.00±0.38, P=0.017, n=3 per group; Figure 5A through 5C). The activities of cathepsin K and S in aneurysmal walls were inhibited by the treatment with NC-2300 (cathepsin K 0.84±0.34, P<0.01; cathepsin S 1.26±0.45, P=0.05, n=3 per group; Figure 5B through 5C). Cathepsin B activity was also inhibited by NC-2300, but the difference did not reach statistical significance (1.39±0.37, P=0.082, n=3; Figure 5A).

The Effect of NC-2300 on Collagenase I and IV Activities in Aneurysmal Walls
Three months after aneurysm induction, the activities of type I and type IV collagenase were increased (type I 0.35±0.059 U/mg total protein, P=0.027; type IV 0.21±0.054 U/mg total protein, P=0.045; n=5 per group) compared with rats before aneurysm induction (type I 0.17±0.11 U/mg total protein; type IV 0.15±0.048 U/mg total protein). In the NC-2300–treated rats, both activities of type I and type IV collagenase were significantly inhibited (type I 0.21±0.12 U/mg total protein, P=0.012; type IV 0.13±0.045 U/mg total protein, P=0.022, n=5 per group; Figure 5D and 5E).

The Effect of NC-2300 on Elastin Content in Aneurysmal Walls
Elastin content was reduced in aneurysmal walls 3 months after aneurysm induction (0.062±0.054, n=5) compared with...
rats before aneurysm induction (0.25 ± 0.15, n = 5; P < 0.01; Figure 5F, 5G, 5H, 5I). The reduction of elastin content was ameliorated by the treatment with NC-2300 (0.17 ± 0.058, n = 5; P = 0.047; Figure 5F).

**Expression of Cathepsins and Cystatin C in Human Cerebral Aneurysms**

Cathepsin B, K, and S were highly expressed in endothelial cell layer and the media in aneurysmal walls (Figure 6B, 6C, 6E, 6F, 6H, 6I). In the control artery, cathepsins were only faintly expressed in the endothelial cell layer (Figure 6A, 6D, 6G). Cystatin C was highly expressed in the intima and media of cerebral arterial walls (Figure 6J), and its expression was reduced in aneurysmal walls (Figure 6K and 6L). Negative controls without the incubation with primary antibodies showed no positive signal in both control and aneurysm samples (data not shown). Double immunohistostaining demonstrated cathepsin S expression in all of smooth muscle cells, macrophages, and endothelial cells (Figure 6M through 6R). Cathepsin B and K were also expressed in all three kinds of cells (data not shown).

**Discussion**

Extensive degradation of elastin and collagen is found in the arterial wall of CAs. Revealing the molecular mechanisms that trigger and promote the degradation of extracellular matrix should aid the development of new therapeutic modalities for CA. The present study demonstrated increased cathepsin B, K, and S expression in rat and human CAs (Figures 1 and 6). The main sources of cathepsins were macrophages and smooth muscle cells, although endothelial cells also secreted cathepsin B, K, and S (Figures 2 and 6). This is the first demonstration of cysteine cathepsin expression in cerebral aneurysms. In atherosclerosis, cathepsin K and S are also mainly expressed in macrophages in the shoulder regions of atheroma, and in smooth muscle cells of the fibrous cap. Data from studies of cahepsin S deficiency mice illustrated a significant reduction of atheroma size and plaque instability, suggesting that cathepsin S may promote the progression of atherosclerosis and plaque vulnerability by altering vascular remodeling in atherosclerotic plaques.

In the present study, the role of cathepsins in the progression of CA was examined by NC-2300, a selective inhibitor for cysteine cathepsins. IC50 of NC-2300 for cathepsin B, K, and S was 284, 34.5, and 186 nmol/L, respectively. NC-2300 did not inhibit other proteinases such as matrix metalloproteinases (IC50 > 1000 nmol/L). The plasma concentration of NC-2300 reached the value sufficient for inhibition of cysteine cathepsins (531 ± 247 nmol/L). Treatment with NC-2300 dramatically reduced the incidence of advanced aneurysms, not affecting the ratio of all aneurysmal changes (Figure 4A). Systemic blood pressure and macrophage infiltration into the aneurysmal walls was not different between the control group and the NC-2300–treated group (Figure 4B and 4C). The activity of cathepsin B, K and S was decreased in the NC-2300–treatment group (Figure 5A through 5C). Collagenase activities were inhibited and elastin content was increased in the NC-2300–treatment group (Figure 5D through 5I). These data suggest that NC-2300 prevented the progression of cerebral aneurysms by inhibiting ECM degradation in aneurysmal walls caused by cystein cathepsins. NC-2300 did not have an influence on the initiation of CAs. This is in line with the time course of cathepsin expression in cerebral aneurysms. The expression of cathepsin B, K, and S mRNA did not increase 1 month after aneurysm induction, when most operated rats showed early...
aneurysmal changes at the ACA/OA bifurcation. Their expression was highly upregulated 3 months after aneurysm induction, when aneurysm changes developed to advanced forms. Macrophage accumulation into the aneurysmal walls becomes prominent 3 months after aneurysm induction,3 suggesting active participation of macrophages in cathepsin-mediated ECM degradation in aneurysmal walls. Immunohistochemistry revealed that smooth muscle cells (SMCs) also secreted a large amount of cathepsins. As shown in Figures 1 and 6, SMCs in the normal cerebral artery do not express cathepsins. However, incubation with some inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), induces expression and secretion of cathepsin K and S in cultured SMCs.7 IL-1β is a major inflammatory cytokine expressed in SMCs of aneurysmal walls and promotes the progression of cerebral aneurysms.15 Another inflammatory stimuli occurring in the aneurysmal walls may also participate in the induction of cathepsins.

Cystatin C is known as an endogenous inhibitor of cysteine proteases.16,17 SMCs in the normal arteries constitutively expressed cystatin C.18 In contrast, atherosclerotic lesions and aortic abdominal aneurysms (AAA) have also low levels of cystatin C.18 Serum cystatin C concentration correlates negatively with AAA size and annual expansion rate in patients with AAA.19 Cystatin C deficiency increases aortic dilata-

**Figure 5.** Cathepsin activity, collagenase activity, and elastin content in aneurysmal walls in NC-2300–treated rats. A through C, The activity of cathepsin B (A), K (B), and S (C) in cerebral arterial walls measured by fluorescence labeled substrates. Data were analyzed by the use of 1-way ANOVA followed by Fisher test. n=3 in each group. D and E, Collagenase I (D) and IV (E) activity measured by using fluorescence labeled collagen 3 months after aneurysm induction with or without NC-2300 treatment. Data were analyzed by the use of 1-way ANOVA followed by Fisher test. n=5 in each group. F through I, Miller’s staining in cerebral aneurysmal walls of a rat before aneurysm induction (0 M) (G), 3 months after aneurysm induction (3 M; H), and 3 months after aneurysm induction with NC-2300 treatment (3 M+NC2300; I). Bar=50 μm. Data were analyzed by the use of 2-way ANOVA followed by Fisher test. n=5 in each group.
In summary, the expression of cysteine cathepsin B, K, and S was induced in the late stage of CA progression. On the contrary, an endogenous inhibitor of cysteine cathepsins, cystatin C was downregulated with aneurysm progression. The imbalance between cysteine cathepsins and cystatin C in the arterial walls may cause excessive degradation of ECM in aneurysmal walls, finally resulting in CA progression and rupture. This notion is supported by data showing that a cysteine cathepsin inhibitor prevented the progression of CAs. Therefore, cysteine cathepsins and cystatin C can be a treatment target for preventing aneurysm growth and rupture. Some kinds of proteinase inhibitor such as NC-2300 may be a promising candidate for medical treatment of CAs.

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Disclosures

None.

References


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