Macrophage-Derived Matrix Metalloproteinase-2 and -9 Promote the Progression of Cerebral Aneurysms in Rats

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Background and Purpose—Mechanisms of initiation, progression and rupture of cerebral aneurysms have not yet been fully understood despite its clinical significance. Matrix metalloproteinases (MMPs) are a family of proteinases which are involved in the remodeling of vascular walls. In the present study, we investigated the significance of MMPs in the progression of cerebral aneurysms.

Methods—Cerebral aneurysms were experimentally induced in 7-week-old male Sprague-Dawley rats. MMP-2 and MMP-9 expression was examined by immunohistochemistry and RT-PCR. Gelatinase activity in aneurysmal walls was assessed by in situ zymography. A selective inhibitor for MMP-2, -9 and -12, tolylsam, was used to examine the effect of inhibition of MMP-2 and MMP-9.

Results—Macrophages infiltrated in arterial walls of experimentally induced rat cerebral aneurysms and expressed MMP-2 and -9. Macrophage infiltration and MMP expression was increased with the progression of aneurysms. Gelatinase activity attributable to MMP-2 and MMP-9 increased in arterial walls of rat cerebral aneurysms. Furthermore, tolylsam reduced the ratio of advanced aneurysms in our rat model.

Conclusions—These data suggest that macrophage-derived MMP-2 and -9 may play an important role in the progression of cerebral aneurysms. The findings of this study will shed a new light into the pathogenesis of cerebral aneurysms and highlight the importance of inflammatory response causing the degeneration of extracellular matrix in the process of this disease. (Stroke. 2007;38:162-169.)

Key Words: aneurysm ■ inflammation ■ macrophages ■ matrix metalloproteinases

The subarachnoid hemorrhage attributable to the rupture of cerebral aneurysms is a life-threatening disease. Despite its clinical and public importance, the detailed mechanisms of initiation, progression and rupture of cerebral aneurysms have not yet been fully understood. Pathological studies revealed that the essential changes of cerebral aneurysms are disappearance of the internal elastic lamina and thinning of the smooth muscle layer. The degeneration of extracellular matrix occurs in the aneurysmal wall, promoting the progression of aneurysms. Family members of the matrix metalloproteinases (MMPs) are thought to play a critical role in the development of atherosclerosis and aortic abdominal aneurysms by causing the vascular remodeling in arterial walls. Previous reports demonstrated the expression of MMPs in human aneurysmal walls by immunohistochemistry and western blotting. Serum gelatinase activities increased in patients with cerebral aneurysms. However, little is known about the role of MMPs for the initiation and progression of cerebral aneurysms. Furthermore, the source of MMPs in the aneurysmal wall remains to be elucidated. In human cerebral aneurysms, several kinds of inflammatory cells, which are candidates for MMP secreting cells, infiltrate in the wall of cerebral aneurysms. The contribution of inflammation to aneurysm formation and progression has not been well investigated, despite considerable circumstantial evidence linking inflammation to cerebral aneurysms.

We previously established experimental animal models of cerebral aneurysms in rats, monkeys and mice, and disclosed some parts of molecular mechanisms of aneurysm formation and progression. In the present study, we clarified, for the first time, the role of inflammatory cells and MMPs in the progression of cerebral aneurysms by using the rat experimentally induced cerebral aneurysm model.

Materials and Methods

Induction of Experimental Cerebral Aneurysms

Cerebral aneurysms were induced as previously described by Nagata et al. After the induction of pentobarbital anesthesia (50 mg/kg IP), the left 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; Osaka, Japan). Animals were fed special food containing 8% sodium chloride and 0.12% β-aminopropionitrile (Tokyo Chemical), an inhibitor of lysyl oxidase that catalyzes the cross-linking of collagen and elastin.
Animal care and experiments complied with Japanese community standards on the care and use of laboratory animals.

**Immunohistochemistry**

One (n=11) or 3 months (n=10) after aneurysm induction, all rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde. As a control, age-matched male Sprague-Dawley rats were euthanized as described above. 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; Baltimore, Md), primary antibodies were incubated for 1 hour at room temperature followed by incubation with fluorescence-labeled secondary antibodies (FITC-conjugated donkey anti-rabbit IgG antibody, FITC-conjugated donkey anti-goat IgG antibody, FITC-conjugated donkey anti-mouse IgG antibody or Cy3-conjugated donkey anti-mouse IgG antibody [Jackson ImmunoResearch]) for 1 hour at room temperature. Then the slides were covered with PERMAFLUOR (Immunotec) and excited for fluorescence by illumination through a fluorescence microscope system (BX51N-34-FL-1; Olympus). The primary antibodies used in the present study are listed as follows: rabbit polyclonal anti-MMP-2 antibody (Santa-Cruz; Santa Cruz, Calif), goat polyclonal anti-MMP-9 antibody (Santa-Cruz), mouse monoclonal anti-CD68 antibody (HyCult; Uden, Netherland), mouse monoclonal anti-smooth muscle α actin antibody (Labvision; Ferment, Calif), goat polyconal anti-MMP-2 antibody (Santa-Cruz; Santa Cruz, Calif), rabbit polyclonal anti-myeloperoxidase antibody (Santa-Cruz), goat polyclonal anti-BLCAM antibody (Santa-Cruz), and mouse monoclonal anti-smooth muscle α actin antibody (Labvision; Ferment, Calif).

**Cell Counting**

The number of CD68/MMP-2 or -9 double-positive cells was counted in 1 section under a microscope (n=5). The number of smooth muscle α actin/MMP-2 or -9 double-positive cells was also counted in the serial section. 4',6-diamino-2-phenylindole (DAPI) staining was simultaneously performed to identify each cell. The ratio of the number of double-positive cells to that of total MMP-2 (or -9) positive cells was calculated.

**RNA Isolation and RT-PCR**

One or 3 months after aneurysm induction, rats were euthanized as described above. Total RNA from the whole Willis ring was isolated using the RNasy Fibrous Tissue Mini Kit (QIAGEN). Extraction was performed according to the manufacturer’s direction. By SensiScript reverse transcriptase (QIAGEN), total RNA was converted into cDNA, which was used in each PCR reaction. PCR was performed using HotStar Taq polymerase (QIAGEN). β-actin was used as an internal control. The primer sets used were: 5’-ctgtaaactggatcatgctgct-3’ and 5’-ccagccagtccgatttga-3’ for MMP-2; 5’-tcaaggagctgggtttc-3’ and 5’-ctctgagcctagaccaactta-3’ for MMP-9; 5’-aagtccctcaccctcctcaaaag-3’ and 5’-aagtcgctgctaccc-3’ for β-actin. The condition for PCR reaction was: 95°C for 10 minutes followed by 45 cycles of 95°C for 30 seconds, 53°C (for MMP-2) or 60°C (for MMP-9) cycles of 30 seconds, and 72°C for 30 seconds. PCR products were separated by the electrophoresis in 2% agarose gels. Two samples in each group were subjected to one RT-PCR analysis, and 3 independent analyses were performed. Densitometric analysis includes data of 6 samples per group.

**In Situ Zymography**

Four months after aneurysm induction, rats were euthanized as described above. Immediately after the perfusion, the ACA/OA bifurcation was stripped, embedded and frozen. Four-micrometer sections were cut and mounted on MMP in situ...
Zymo-Film or MMP-PT in situ Zymo-Film (Wako). The films were incubated for 30 hours at 37°C in a moisture box. After the incubation, films were stained with the Biebrich Scarlet Stain Solution (Wako) for 10 minutes at room temperature. The ACA/OA bifurcation in an age-matched male Sprague-Dawley rat was served as a control.

MMP Inhibitor Treatment
As an MMP inhibitor, we used tolylsam ((R)-3-methyl-2-[4-(3-p-tolyl-1,2,4]oxadiazol-5-yl)-benzenesulfonylamino]-butyric acid; Molecular weight 415.47), a competitive inhibitor for MMP-2, -9, -12 developed by Shionogi & Co, Ltd. IC50 for MMPs is listed below: MMP-2 0.005 μmol/L; MMP-9 0.049 μmol/L; MMP-12 0.034 μmol/L; MMP-3 5.558 μmol/L; MMP-8 0.397 μmol/L; MMP-13 0.201 μmol/L. IC50 for other MMPs and other proteinases is >10 μmol/L. Immediately after aneurysm induction, rats were fed food with (n=10) or without (n=21) 50 mg/kg per day of tolylsam, 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

Figure 2. MMP-2 and MMP-9 expression in experimentally induced cerebral aneurysms in rats. A, C, E, and G, Elastica van Gieson staining of the ACA-OA bifurcation in a rat 3 months after aneurysm induction (A and E) and in a control rat (C and G). B and D, MMP-2 staining of the serial section of A (B) and C (D). F and H, MMP-9 staining of the serial section of E (F) and G (H). MMP-2 (B) and MMP-9 (F) expression is predominantly present in the endothelial cell layer of the neck portion and the adventitia surrounding the apex of dome. Bar=20 μm.
bulging of the arterial wall with the fragmentation or disappearance of the internal elastic lamina. Three independent researchers assessed the histopathological changes. In both groups, systemic blood pressure was measured by the tail cuff plethysmographic method before operation and 3 months after aneurysm induction. The number of CD68 positive cells in aneurysmal walls was counted 3 months after aneurysm induction in both groups.

**Statistical Analysis**

The values were expressed as means±SD. Statistical analysis was performed using Mann-Whitney U test. Differences were considered statistically significant at \( P<0.05 \).

**Results**

**Macrophage Infiltration in Aneurysmal Walls of Rat Cerebral Aneurysm**

Macrophages were predominantly infiltrated in aneurysmal walls 1 month after aneurysm induction (Figure 1B) and the number of infiltrating macrophages increased after 3 months (Figure 1E). Macrophages were mainly located in the luminal surface of endothelial cell layer and the media around the intimal pad in early aneurysmal change. In advanced aneurysms, macrophages infiltrate also into the adventitia. No macro-

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**Figure 3.** Characterization of MMP-2 and MMP-9 producing cells in experimentally induced cerebral aneurysms in rats. Double-staining of MMP-2 (green) and CD68 (red; A), smooth muscle \( \alpha \) actin (red; B), and DAPI staining (C) of the serial sections. Double-staining of MMP-9 (green) and CD68 (red; D), smooth muscle \( \alpha \) actin (red; E), and DAPI staining (F) of the serial sections. The ratio of double-positive cells in total MMP-2 positive cells (G) and total MMP-9 positive cells (H; \( n=5 \)), mean±SD. Both macrophages and smooth muscle cells express MMP-2 (A and B) and MMP-9 (D and E). The main source of MMP-2 and MMP-9 in aneurysmal walls is macrophage (\( P<0.01 \); G and H).
Phage infiltration could be seen in arterial walls in control rats (Figure 1H). Small amounts of T cells (Figure 1J) and neutrophils (Figure 1K) also accumulated in aneurysmal walls. But no B cell could be detected by immunohistochemistry (Figure 1L).

**MMP-2 and -9 Expression in Aneurysmal Walls of Rat Cerebral Aneurysm**

By immunohistochemistry, both MMP-2 and MMP-9 were expressed in aneurysmal walls 3 months after induction (Figure 2B and 2F). No MMP-2 or MMP-9 expression was detected in control arterial walls (Figure 2D and 2H). Double staining with CD68 and smooth muscle α-actin demonstrated that MMP-2 and MMP-9 were expressed both in macrophages and in smooth muscle cells (Figure 3A, 3B, 3D, and 3E). Both MMP-2 and MMP-9 were expressed mainly in macrophages (CD68 positive 50.9±10.9%, smooth muscle α-actin positive 18.9±13.0%, P<0.01, n=5 for MMP-2; CD68 positive 52.1±10.4%, smooth muscle α-actin positive 31.0±8.7%, P<0.01, n=5 for MMP-9), showing that the major source of MMP-2 and MMP-9 in aneurysmal walls is macrophage (Figure 3G and 3H).

**Expression of MMP-2 and -9 mRNA in Aneurysmal Walls of Rat Cerebral Aneurysm**

MMP-2 mRNA was expressed 1 month after aneurysm induction and increased at 3 months (Figure 4A and 4C; P=0.011, 1 month versus 3 months). MMP-9 mRNA was not detected at 1 month by RT-PCR and upregulated at 3 month (Figure 4B and 4D; P<0.01, control versus 3 months). Both MMP-2 and MMP-9 mRNA was not expressed in arterial walls of control animals.

**Gelatinase Activity in Aneurysmal Walls of Rat Cerebral Aneurysm**

In in situ zymography, gelatinase activity was present in aneurysmal walls four months after aneurysm induction (Figure 5A). Gelatin digestion was prominent at the neck portion of aneurysms. Pretreatment with an MMP inhibitor ameliorated the gelatinase activity, confirming that the digestion of gelatin resulted from the action of MMPs (Figure 5B). No gelatinase activity was detected in control cerebral arterial walls (Figure 5C).
Finally, we examined whether a selective MMP inhibitor for MMP-2, -9 and -12, tolylsam, could prevent the initiation and progression of cerebral aneurysms in our model. The serum concentration of tolylsam measured by HPLC (0.217±0.096 μmol/L [n=5]) reached the value sufficient for inhibition of MMP-2 and MMP-9 in vitro. In the control group, 19 of 21 rats, (90%) developed advanced aneurysms and 2 (10%) showed early aneurysmal changes. In rats given tolylsam (50 mg/kg per day), only 5 of 10 rats (50%) developed advanced aneurysms and 5 (50%) 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 tolylsam group than in the control group (P=0.013; Figure 6A). 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=20) and the tolylsam group (161.2±12.2 mm Hg, n=10; Figure 6B). The number of CD68 positive cells in aneurysmal walls was not significantly different between the 2 groups (control group 5.4±1.5, n=14; tolylsam group 5.1±1.3, n=10; Figure 6C). In situ zymography, gelatinase activity was abundantly found in aneurysmal walls of control rats (Figure 6D), whereas it was not present in aneurysmal walls of tolylsam-treated rats (Figure 6E).

Discussion

Inflammation producing enzymatic remodeling of the vascular extracellular matrix has been proven to be one of the major pathological mechanisms causing a variety of vascular diseases, including atherosclerosis and abdominal aortic aneurysm. Macrophages consist of a major cellular component of atherosclerotic plaques and secrete various inflammatory cytokines and proteinases, promoting the progression of atherosclerosis and plaque rupture. Many inflammatory cells including macrophages are also prevalent throughout the wall of abdominal aortic aneurysms. Using the experimentally induced cerebral aneurysm model of rat, we have clearly shown that inflammatory cells, especially macrophages, infiltrated into the vascular wall of rat cerebral aneurysms. Macrophages accumulated in the luminal surface of the endothelium and in the adventitia, and the number of macrophages in aneurysmal walls increased with the progression of aneurysms. This is in line with the results of the study by Chyatte et al in which macrophages and T lymphocytes were frequently present in the wall of human cerebral aneurysm tissue. Although their data imply that inflammation is involved in the process of aneurysm progression or rupture, it has been still unknown whether inflammation is primary processes causing the initiation and progression of disease or secondary reactions accompanied by disease process. Our data demonstrated the presence of inflammatory cells in arterial walls of early aneurysmal change and a link between inflammatory reactions and disease process in an experimentally induced cerebral aneurysm model, suggesting that inflammatory reactions mediated by macrophages is one of the major pathological factors of the progression of cerebral aneurysms.

The Effect of MMP Inhibitor on Aneurysm Formation and Progression

Finally, we examined whether a selective MMP inhibitor for MMP-2, -9 and -12, tolylsam, could prevent the initiation and progression of cerebral aneurysms in our model. The serum concentration of tolylsam measured by HPLC (0.217±0.096 μmol/L [n=5]) reached the value sufficient for inhibition of MMP-2 and MMP-9 in vitro. In the control group, 19 of 21 rats, (90%) developed advanced aneurysms and 2 (10%) showed early aneurysmal changes. In rats given tolylsam (50 mg/kg per day), only 5 of 10 rats (50%) developed advanced aneurysms and 5 (50%) 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 tolylsam group than in the control group (P=0.013; Figure 6A). 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=20) and the tolylsam group (161.2±12.2 mm Hg, n=10; Figure 6B). The number of CD68 positive cells in aneurysmal walls was not significantly different between the 2 groups (control group 5.4±1.5, n=14; tolylsam group 5.1±1.3, n=10; Figure 6C). In situ zymography, gelatinase activity was abundantly found in aneurysmal walls of control rats (Figure 6D), whereas it was not present in aneurysmal walls of tolylsam-treated rats (Figure 6E).

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In the present study, we have also shown that MMP-2 and MMP-9 are expressed in the arterial wall of early aneurysmal change, and that its expression increases with the progression of aneurysms by immunohistochemistry and RT-PCR. Macrophages predominantly secreted MMP-2 and MMP-9 in the wall of cerebral aneurysms. Their expression was preferentially seen in the neck portion and the adventitia tissues around the apex of cerebral aneurysm. Gelatinase activity also increased at the same area, showing that most of expressed MMPs are active forms. MMPs are reported as a family of proteinases which play an important role in the degeneration of extracellular matrix such as collagen and elastin. Within the family members of MMPs, MMP-2 and MMP-9 have both gelatinase and collagenase activity and can degenerate important extracellular matrix components in the walls of cerebral aneurysms, elastin and collagen Type IV. In atherosclerosis, MMPs participate in the development of atherosclerotic plaque and thinning of fibrous cap leading to plaque rupture. MMP expression in human cerebral aneurysms has been already reported. However, data from human specimens do not tie the release of MMPs.

Figure 6. The effect of a selective MMP inhibitor (tolylsam) on the initiation and progression of cerebral aneurysms in rats. A, Incidence of aneurysmal changes 3 months after aneurysm induction. The incidence of all aneurysmal change was not different between these 2 groups. But the rate of advanced aneurysms is significantly lower in the tolylsam group than the control group (P=0.013). B, Mean blood pressure before aneurysm induction and 3 months after aneurysm induction. The blood pressure is not different between the 2 groups. C, Macrophage infiltration in aneurysmal walls 3 months after aneurysm induction. Macrophage infiltration did not significantly differ between the 2 groups. D and E, In situ zymography in a control rat (D) and a tolylsam-treated rat (E) 3 months after aneurysm induction. Gelatinase activity (arrows) is abundant in aneurysmal walls of the control rat (D) and is not detected in the tolylsam-treated rats (E). Bar=20 μm.
to a specific stage of aneurysm progression. Spatial and temporal expression pattern of MMP-2 and MMP-9 in the present study suggests that MMP-2 and -9 produced by macrophages play an important role for the progression of cerebral aneurysms. In order to examine the extent to which MMP-2 and MMP-9 contribute to the initiation and progression of cerebral aneurysms, we used a selective inhibitor for MMP-2, -9 and -12 (tolylsam) in our rat model. Tolylsam prevented the progression of cerebral aneurysms although it did not reduce the incidence of total aneurysmal changes. These results were different from the previous report by Kaufmann et al. They used doxycycline as an MMP inhibitor, which is not a selective inhibitor for gelatinases, and the dose of doxycycline may not be enough for in vivo use as they discussed. Tolylsam did not have an influence on systemic blood pressure and macrophage infiltration into aneurysmal walls, and in the tolylsam group, gelatinase activity in aneurysmal walls was absent even in advanced aneurysms. These data suggest that tolylsam prevented aneurysm progression by inhibiting gelatinase activity. There is a possibility that tolylsam only delayed the maturation of cerebral aneurysm. Even if the effect of tolylsam is not strong enough to prevent aneurysm growth and rupture completely, our data indicate that gelatinase (MMP-2 and MMP-9) is one of the important factors that promote the progression of cerebral aneurysm. Because macrophages produce a variety of proteinases and inflammatory cytokines, another proteinase or cytokine can be also a modulator of the progression of cerebral aneurysm, which remains to be elucidated.

The findings of this study will shed new light into the pathogenesis of cerebral aneurysms and highlight the importance of inflammatory response in the progression of aneurysms. Macrophages modulate the progression of cerebral aneurysms, at least in part, by the release of MMP-2 and MMP-9. At present, treatment modalities for cerebral aneurysms are confined to surgical obliteration of aneurysms by clipping or coiling. If we could disclose more detailed mechanisms of inflammatory reactions involved in the progression of cerebral aneurysms, specific anti-inflammatory treatments would be the first choice for the patients with cerebral aneurysms in the future. Macrophages or MMP-2 and -9 may become candidates of treatment target for cerebral aneurysms.

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Disclosures
None.

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