Role of TIMP-1 and TIMP-2 in the Progression of Cerebral Aneurysms

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Background and Purpose—The degradation of extracellular matrix (ECM) is a hallmark of a cerebral aneurysm; however, little is known regarding the molecular mechanism leading to this change. Tissue inhibitor of matrix metalloproteinase (TIMP) regulates the ECM degradation in vascular walls by inhibiting the activity of matrix metalloproteinases (MMPs). We investigated the role of TIMPs in the progression of cerebral aneurysms in the present study.

Methods—TIMP-1 and TIMP-2 expression was examined by immunohistochemistry and quantitative RT-PCR in experimentally-induced cerebral aneurysms in rats. The incidence of aneurysmal changes in TIMP-1/H11002 and TIMP-2/H11002 mice was compared with that in the wild-type mice.

Results—TIMP-1 and TIMP-2 were expressed mainly by smooth muscle cells in aneurysmal walls. Quantitative PCR showed an increase of TIMP-1 and TIMP-2 mRNA in the early stage of aneurysm progression (form 0 to 1 month) but not in the late stage (form 1 to 3 months), whereas mRNA expression of MMP-2 and MMP-9 dramatically increased in the late stage. In both TIMP-1/H11002 mice and TIMP-2/H11002 mice, aneurysm progression was promoted with the increased enzyme activity of MMPs.

Conclusions—Our findings suggest that TIMP-1 and TIMP-2 have a protective role for the progression of cerebral aneurysms. There is an imbalance between MMPs and TIMPs in the late stage of cerebral aneurysm formation, which may be responsible for ECM degradation leading to the progression and rupture of cerebral aneurysms. (Stroke. 2007; 38:2337-2345.)

Key Words: animal model ■ cerebral aneurysm ■ matrix metalloproteinase ■ remodeling ■ tissue inhibitor of matrix metalloproteinase

A cerebral aneurysm can cause a catastrophic subarachnoid hemorrhage, one of the most severe forms of stroke. Despite the development of modern treatments, a large number of people die of subarachnoid hemorrhage caused by cerebral aneurysms. However, the detailed mechanisms of the initiation, progression, and rupture of cerebral aneurysms are not fully understood. The main pathological features of vascular walls in cerebral aneurysms are disappearance of the internal elastic lamina (IEL) and thinning of the smooth muscle cell layer.1 These changes are accompanied by the degradation of extracellular matrix (ECM), which is a dynamic network of proteins and proteoglycans. The structural integrity of vessel walls depends on a balance between synthesis and degradation of ECM. A family of matrix metalloproteinases (MMPs) has been found to degrade most of the arterial ECM components, including elastin and collagen.2 The role of MMPs in the pathogenesis of cerebral aneurysm was extensively investigated.3-6 Recently we also demonstrated that MMP-2 and -9 promoted the progression of cerebral aneurysms by using an experimentally induced cerebral aneurysm model.7 Tissue inhibitors of MMPs (TIMPs) regulate the proteinase activity of MMPs via forming complexes and are considered the most potent inhibitors of MMPs.8 It is believed that an imbalance between MMPs and TIMPs is responsible for the excessive elastin degradation seen in atherosclerotic plaques.9 In abdominal aortic aneurysms (AAAs), there is growing evidence that TIMP-1 prevents elastin depletion in aneurysmal walls, thus inhibiting the progression of AAAs.10-14 TIMP-1 expression was also abundantly detected in human cerebral aneurysms.4 However, the role of TIMPs in the initiation and progression of cerebral aneurysms is still unclear.

In the present study, we clarified, for the first time, the role of TIMP-1 and TIMP-2 in the progression of cerebral aneurysms by using a previously established model of experimentally induced cerebral aneurysm.15,16

Materials and Methods

Induction of Experimentally Induced Cerebral Aneurysms in Rats

Rat cerebral aneurysms were induced as previously described by Nagata et al.17 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. Animals were fed special food containing 8% sodium chloride and 0.12% β-aminoproprionitrile (BAPN; 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 month (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 frozen. 5-μm sections were cut and mounted on silanecoated slides. After blocking with 5% Donkey serum, the slides were incubated with primary antibodies for 1 hour at room temperature followed by the incubation with fluorescence labeled secondary antibodies (FITC-conjugated donkey anti-rabbit IgG antibody, FITC-conjugated donkey anti-goat IgG antibody, Cy3-conjugated donkey anti-mouse IgG antibody; Jackson ImmunoResearch) for 1 hour at room temperature. Then the slides were covered with PERMAFLUOR (Immuno Tech) 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: rabbit polyclonal anti-TIMP-1 antibody (Santa Cruz), rabbit polyclonal anti–TIMP-2 antibody (CHEMICON), rabbit polyclonal anti–MMP-2 antibody (Santa Cruz), goat polyclonal anti–MMP-9 antibody (Santa Cruz), mouse monoclonal anti-CD68 antibody (Hy-Cult), mouse monoclonal anti–smooth muscle α actin antibody (Laboratory Vison), mouse monoclonal anti–endothelial nitric oxide synthase (eNOS) antibody (Biomol Research Laboratories).

**Characterization of TIMP-1 and -2 Secreting Cells**

The number of CD68/TIMP-1 double positive cells was counted in 1 section under a microscope (n=5). The number of smooth muscle α actin/TIMP-1 double positive cells and eNOS/TIMP-1 double positive cells were also counted in the serial section. To identify each cell, 4',6-diamino-2-phenylindole (DAPI) staining was performed at the same time. The ratio of each double positive cell to total TIMP-1 positive cells was calculated. The main source of TIMP-2 secreting cells was examined in the same manner.

**RNA Isolation and Quantitative RT-PCR for Rats**

Two weeks, 1 month, or 3 months after aneurysm induction, rats were euthanized as described above. Total RNA from the whole Willis ring was isolated using RNeasy Fibrous Tissue Mini Kit (QIAGEN). RNA extraction was performed according to the manufacturer’s directions. Total RNA was converted into cDNA using Sensiscript reverse transcriptase (QIAGEN).

Constructs for TIMP-1, TIMP-2, MMP-2, MMP-9, and β-actin were produced by TOPO TA Cloning Kit (Invitrogen) from cDNA manufacturer’s directions. Total RNA was converted into cDNA using Sensiscript reverse transcriptase (QIAGEN).

The primary antibodies used were the same as in the rat study.

For the evaluation of MMP-2 and -9 activity, 3 mice in each group were subjected to gelatin zymography. Protein was extracted from the whole Willis ring and adjacent brain tissue of TIMP-1−/−, TIMP-2−/−, and the wild-type mice using Sample Grinding Kit (Amersham) and subjected to gelatin zymography with Gelatin Zymo-Electrophoresis Kit (Primary Cell) according to the manufacturer’s directions.

Six animals in each group were subjected to RT-PCR. RNA extraction and conversion to cDNA was performed as described above. PCR was performed using HotStar Taq polymerase (QIAGEN). The primer sets used were: forward 5′-actctcactgcggtctgggac-3′, reverse 5′-gtaaacgctaatctgg3′-3′ for TIMP-1; forward 5′-tcaccccagccagcctcacc-3′, reverse 5′-cctccctaccccctccttc-3′ for TIMP-2; forward 5′-tgcagaagggcctcctcctc-3′, reverse 5′-ctttgctctggcattc-3′ for MMP-2; forward 5′-gtaaggatttgtgactcgg-3′, reverse 5′-ctctctttggtcataagggctaaattcatggg-3′ for MMP-9; forward 5′-agcagcagggatacaggag-3′, reverse 5′-ccctacgctgaatgaa-3′ for β-actin. The conditions for PCR reactions were: 50 cycles of 95°C 15 seconds for the denaturation, 52°C 15 seconds for the annealing, 72°C 20 seconds for the extension. PCR products were separated by electrophoresis in 2% agarose gel.

**Statistical Analysis**

Data (mean±SD) were analyzed by use of Student t test for 2 group comparison and by 1-way ANOVA, followed by a Fisher test for multiple comparison. The incidence of aneurysmal change or advanced aneurysm was analyzed by use of Fisher exact test. Differences were considered significant at P<0.05.

**Results**

**Expression of TIMP-1 and TIMP-2 in the Arterial Walls of Rat Cerebral Aneurysms**

One month after aneurysm induction, 53% (10/19) of rats showed an early aneurysmal change at the ACA/OA bifurcation, in which IEL was disrupted (Figure 1D). Both TIMP-1 and TIMP-2 were expressed in the intima, media, and adventitia of aneurysmal walls 1 month after aneurysm induction (Figure 1E and 1F). Further, their expression pattern was the same after 3 months (Figure 1H and 1I), when 91% (19/21) of rats had an advanced aneurysm with apparent outward bulging and the disappearance of IEL.
(Figure 1G). No TIMP-1 or TIMP-2 expression was detected in the cerebral arterial wall in control rats (Figure 1B and 1C). Double staining with smooth muscle α actin (SMA) (Figure 2A and 2D), CD68 (Figure 2B and 2E), and eNOS (Figure 2C and 2F) demonstrated that TIMP-1 and TIMP-2 was expressed in all 3 kinds of cells: smooth muscle cells, macrophages, and endothelial cells. 53.5±10.5% of total TIMP-1–positive cells were SMA-positive and 63±13% of total TIMP-2–positive cells were SMA-positive, showing that the major source of TIMP-1 and TIMP-2 in aneurysmal walls was smooth muscle cells (TIMP-1: SMA versus CD68 $P<0.01$, SMA versus eNOS $P<0.01$).

**Figure 1.** TIMP-1 and TIMP-2 expression in experimentally induced cerebral aneurysms in rats. Hematoxylin and Eosin staining of the ACA/OA bifurcation in a control rat (A), a rat 1 month after aneurysm induction (D) and 3 months after aneurysm induction (G). B, E, and H, Immunohistostaining of TIMP-1 (green) of the serial section as A (B), D (E), and G (H). C, F, and I, Immunohistostaining of TIMP-2 (green) of the serial section as A (C), D (F), and G (I). Red color shows immunohistostaining of smooth muscle α actin. TIMP-1 (E) and TIMP-2 (F) was expressed in the intima, media, and adventitia surrounding the dome of aneurysms after 1 month. After 3 months, the expression pattern of TIMP-1 (H) and TIMP-2 (I) was the same as after 1 month. Bar=20 μm.

**Figure 2.** Characterization of TIMP-1 and TIMP-2 producing cells in experimentally induced cerebral aneurysms in rats. Double staining of TIMP-1 (green) and smooth muscle α actin (red) (A), TIMP-1 (green) and CD68 (red) (B), TIMP-1 (green) and eNOS (red) (C), TIMP-2 (green) and smooth muscle α actin (red) (D), TIMP-2 (green) and CD68 (red) (E), and TIMP-2 (green) and eNOS (red) (F). The ratio of double positive cells in total TIMP-1 or TIMP-2 positive cells (G) (n=6). Data (mean±SD) were analyzed by use of 1-way ANOVA followed by a Fisher test. All 3 kinds of cells (smooth muscle cells, macrophages, and endothelial cells) expressed TIMP-1 (A, B, C) and TIMP-2 (D, E, F). The main source of TIMP-1 and TIMP-2 in aneurysmal walls was smooth muscle cells (G). Bar=20 μm.
pared with the wild-type mice (\textit{P}<0.01) (Figure 2G).

**Time Course of MMPs and TIMPs mRNA in the Arterial Wall of Rat Cerebral Aneurysms**

Gene expression of TIMP-1 and -2 was significantly higher 2 weeks after aneurysm induction than before aneurysm induction by quantitative RT-PCR analysis (TIMP-1: 0 month versus 0.5 month \textit{P}<0.01; TIMP-2: 0 month versus 0.5 month \textit{P}=0.049; Figure 3C and 3D). However, the increase of TIMP-1 and -2 mRNA from 2 weeks to 3 months was not statistically significant. On the other hand, the expression level of MMP-9 and -2 mRNA was elevated especially from 1 month to 3 months (MMP-9: 1 month versus 3 months, \textit{P}=0.048; MMP-2: 1 month versus 3 months \textit{P}<0.01; Figure 3A and 3B). To assess the balance between MMPs and TIMPs, the expression ratio of each MMP to each TIMP was calculated. The ratio of MMP-9/TIMP-1 (Figure 3E) and MMP-2/TIMP-2 (Figure 3F) was significantly increased from 0.5 month to 1 month \textit{(P}=0.011) and from 1 month to 3 months \textit{(P}=0.046), respectively. The ratio of MMP-2/TIMP-3 and MMP-9/TIMP-3 did not change over time (data not shown).

**Expression of MMPs and TIMPs in the Arterial Wall of Mouse Cerebral Aneurysms**

TIMP-1 (Figure 4A) and TIMP-2 (Figure 4E) were expressed in the arterial wall of wild-type mice 5 months after aneurysm induction as in rats. Their expression was absent in the corresponding knockout mice (Figure 4B and 4F). Expression of MMP-9, MMP-2, and CD68 was seen in the aneurysmal wall of the wild-type, TIMP-1 \textit{−/−}, mice and TIMP-2 \textit{−/−} mice (Figure 4C, 4D, 4G, and 4H), and expression pattern of these molecules was not significantly different among these 3 groups (Figure 4C, 4D, 4G, and 4H).

**Aneurysm Formation and Progression of TIMP-1 \textit{−/−} and TIMP-2 \textit{−/−} Mice**

At 5 months after aneurysm induction, 9 of 16 mice (56%) developed aneurysmal changes at the ACA/OA bifurcation in the wild-type mice. Among them, 3 mice (18%) developed advanced aneurysms. In TIMP-1 \textit{−/−} mice, 9 of 10 mice (90%) developed the ACA/OA aneurysms, among which 6 (60%) were advanced aneurysms. Although the incidence of all aneurysmal changes was not significantly different between the groups \textit{(P}=0.099), the incidence of advanced aneurysms was significantly higher in TIMP-1 \textit{−/−} mice \textit{(P}=0.04; Figure 5C). In TIMP-2 \textit{−/−} mice, 7 of 10 mice (70%) developed aneurysmal changes at the ACA/OA bifurcation, among which 6 were advanced aneurysms. Although the incidence of all aneurysmal changes was not significantly different between the groups \textit{(P}=0.683), the incidence of advanced aneurysms was significantly higher in TIMP-2 \textit{−/−} mice compared with the wild-type mice \textit{(P}=0.04; Figure 5C). The vascular anatomy of the circle of Willis was compared under a light microscope, and there was no anatomical difference among the wild-type, TIMP-1 \textit{−/−}, and TIMP-2 \textit{−/−} mice (data not shown).

**Blood Pressure of TIMP-1 \textit{−/−} and TIMP-2 \textit{−/−} Mice**

Blood pressure was not significantly different among the wild-type \textit{(n}=6, 90.5±17.7 mm Hg), TIMP-1 \textit{−/−} \textit{(n}=6, 80.7±14.7 mm Hg), and TIMP-2 \textit{−/−} mice \textit{(n}=8, 95.5±17.7 mm Hg; Figure 5D). After aneurysm induction, blood pressure was significantly elevated in each group (wild-type: 133.2±12.0 mm Hg, \textit{P}<0.01; TIMP-1 \textit{−/−} mice: 125.0±14.1 mm Hg, \textit{P}<0.01; TIMP-2 \textit{−/−} mice: 134.5±19.5 mm Hg, \textit{P}<0.01; Figure 5D). Blood pressure after 5 months of aneurysm induction was not different among the groups.

**Macrophage Infiltration in TIMP-1 \textit{−/−} and TIMP-2 \textit{−/−} Mice**

Macrophage infiltration was assessed by the number of CD68-positive cells per 50 \textmu m \times 50 \textmu m field around aneurysms. The number of CD68-positive cells did not significantly differ among the wild-type \textit{(4.2±1.3)}, TIMP-1 \textit{−/−} \textit{(3.9±1.1)}, and TIMP-2 \textit{−/−} mice \textit{(4.6±1.5); Figure 5E).**

**MMP Activity in TIMP-1 \textit{−/−} and TIMP-2 \textit{−/−} Mice**

Gelatin zymography showed increased MMP-9 activity in TIMP-1 \textit{−/−} mice and increased MMP-2 activity in TIMP-2 \textit{−/−} mice compared with the wild-type mice (Figure 5F). MMP-2 activity in TIMP-1 \textit{−/−} mice and MMP-9 activity in TIMP-2 \textit{−/−} mice were the same as in the wild-type mice.

**Expression of MMPs and TIMPs mRNA in Aneurysm Walls of Mouse Cerebral Aneurysm**

TIMP-1 mRNA was expressed in the wild-type mice 5 months after aneurysm induction. In TIMP-1 \textit{−/−} mice, TIMP-1 mRNA was not detected. And the expression level of MMP-9 mRNA was lower than in the wild-type (Figure 6A and 6C). TIMP-2 mRNA was also expressed in the wild-type mice 5 months after aneurysm induction. In TIMP-2 \textit{−/−} mice, TIMP-2 mRNA was not detected. The mRNA expression of MMP-2 is lower in TIMP-2 \textit{−/−} mice than in the wild-type (Figure 6B and 6D).

**Discussion**

At present, there are 4 different types of TIMP (TIMP-1 to TIMP-4), and each of them has specific affinity for different types of MMPs.5 TIMP-1 binds with high affinity to MMP-9,18 and TIMP-2 is an important inhibitor of MMP-2.19 Recently, we reported the critical role of MMP-2 and MMP-9, which have gelatinase activity, in the progression of cerebral aneurysms.20 In the present study, we demonstrated the upregulated expression of TIMP-1 and TIMP-2 in the arterial wall of experimentally-induced cerebral aneurysms in rats and mice. Although mRNA expression of TIMP-1 and TIMP-2 was increased in the early stage of aneurysm progression, the increase in the late stage was not statistically significant. In contrast, mRNA expression of MMP-9 and -2 was raised especially in the late stage, resulting in the increased ratio of MMP-9/TIMP-1 and MMP-2/TIMP-2 in aneurysmal walls in the late stage. These data indicate an imbalance between gelatinases and their inhibitors in the late stage of cerebral aneurysm progression.
Because all members of MMP family are secreted as inactive zymogen and require extracellular activation, mRNA data should not be overestimated. To examine whether TIMP-1 and TIMP-2 affect the activity of MMPs and aneurysm progression, TIMP-1/- and TIMP-2/- mice were analyzed. A deficiency of TIMP-1 or TIMP-2 resulted in promoted aneurysm progression without influencing the systemic blood pressure and macrophage infiltration into aneurysmal walls. MMP-9 activity in TIMP-1/- mice and MMP-2 activity in TIMP-2/- mice were elevated compared to control.
with the wild-type mice, suggesting that the preventive effect of TIMP-1 and TIMP-2 on aneurysm progression was derived from their ability to inhibit the enzyme activity of MMPs. A total number of aneurysmal changes did not differ between each knockout mice and the wild-type mice. This is in line with quantitative RT-PCR data showing an imbalance of mRNA expression between MMPs and TIMPs in the late stage of aneurysm progression. Our findings suggest that an imbalance between MMP-2/9 and TIMP-1/2 may determine the extent of ECM degradation in arterial walls leading to the progression and rupture of cerebral aneurysms.

Unexpectedly, MMP-9 expression in TIMP-1−/− mice and MMP-2 expression in TIMP-2−/− mice were reduced compared with the wild-type. Although there is no evidence that TIMPs are involved in transcriptional regulation of MMPs, TIMPs may enhance the gene expression of MMPs by some positive feedback mechanisms.

TIMP-1 is also known as an important modulator in the pathogenesis of AAA. TIMP-1 is highly expressed in arterial walls of AAAs compared with control arteries. Local overexpression of TIMP-1 lead to prevention of aortic aneurysm degeneration and rupture. And TIMP-1−/− mice developed larger AAA than the wild-type mice. On the other hand, gene expression of TIMP-2 did not correlate with AAA, suggesting that its role is not important in the initiation and progression of AAAs. Most cerebral aneurysms arise at the arterial bifurcation unlike AAAs. Although there are several pathological similarities between cerebral aneurysms and AAs, the mechanisms leading to aneurysm progression and rupture are likely different.
and AAAs, some molecular mechanisms occurring in the vascular wall may be different between these two diseases.

In the present study, we used an experimentally induced cerebral aneurysm model in rats and mice. Experimentally-induced cerebral aneurysms have many aspects of human cerebral aneurysms, including a location at the arterial bifurcation, a saccular form in shape and histopathological findings. We used BAPN, an inhibitor of lysyl oxidase (LOX), to accelerate aneurysm formation. LOX initiates the cross linking of collagens and elastin by catalyzing oxidative deamination of the ε amino group in certain lysine and hydroxylysine residues and participates in ECM biosynthesis. Although no direct or indirect interaction between TIMPs and LOX is known, BAPN may affect the results in the present study. Nevertheless, it is highly possible that the altered balance between MMPs and TIMPs is also one of the major causes leading to the progression and rupture of human cerebral aneurysms. In fact, a previous study reported the TIMP-1 expression in human aneurysmal walls. Although an analysis of the

Figure 5. Effect of gene targeting of timp-1 and timp-2 on the progression of cerebral aneurysms. A and B, A representative Elastica van Gieson staining of an early aneurysmal change (A) and an advanced aneurysm in mice (B). C, The incidence of aneurysmal changes 5 months after aneurysm induction. The ratio of advanced aneurysms was significantly higher in both of TIMP-1−/− mice (P=0.04) and TIMP-2−/− mice (P=0.04) than in the wild-type. Data were analyzed by the use of Fisher exact test. D, Systemic blood pressure was not statistically different among 3 groups analyzed by the use of 1-way ANOVA followed by a Fisher test. **P<0.01. E, The number of macrophages infiltrating into the arterial wall of cerebral aneurysms was not different among the groups. Data (mean±SD) were analyzed by the use of 1-way ANOVA followed by a Fisher test. F, Gelatin Zymography showed increased MMP-9 activity in TIMP-1−/− mice and increased MMP-2 activity in TIMP-2−/− mice. Representative data of 3 independent experiments were shown.
coding region of TIMP-1 and TIMP-2 failed to show an association between genetic polymorphisms and a cerebral aneurysms,27 polymorphisms in a promoter region may have an impact on cerebral aneurysm development.

Our data suggest that both TIMP-1 and TIMP-2 have a protective effect on the progression of cerebral aneurysms. At present, cerebral aneurysms are treated by surgical clipping or coil embolization. There is no medical treatment to suppress the progression and rupture of cerebral aneurysms. In the future, it might be possible by modulating MMPs or TIMPs to inhibit aneurysm progression and thereby reduce the risk of subarachnoid hemorrhage.

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Disclosures
None.

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