Abnormal Expression of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases in Brain Arteriovenous Malformations

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Background and Purpose—Excessive degradation of the vascular matrix by matrix metalloproteinases (MMPs) can lead to structural instability of vessels. In this study we examined the expression of MMPs and tissue inhibitors of metalloproteinases (TIMPs) in brain arteriovenous malformations (BAVMs).

Methods—We performed gelatin zymography for MMPs and Western blot for MMP-9, MMP-2, TIMP-1, TIMP-2, TIMP-3, and TIMP-4. MMP-9 expression was localized by immunohistochemistry.

Results—We analyzed 37 BAVM specimens and 9 control brain specimens from epilepsy surgery. Thirty-two BAVM patients had embolization treatment before resection. Eighteen BAVM patients had a history of hemorrhage from BAVMs. Neither MMP-2 nor TIMP-2 was detected in BAVMs or control brain specimens. Compared with control brain samples, BAVM samples had higher levels of total MMP-9, active MMP-9, pro-MMP-9, TIMP-1, and TIMP-3. TIMP-4 levels were higher in the control brain than in BAVM specimens. MMP-9 was localized to the endothelial cell/peri–endothelial cell layer and infiltrating neutrophils of BAVMs. BAVMs with venous stenosis >50% had higher expression of MMP-9 than BAVMs with venous stenosis <50%. There was no apparent association between total MMP-9, pro-MMP-9, or active MMP-9 levels and (1) feeding artery pressure, (2) pattern of draining vein (exclusively deep venous drainage versus any superficial drainage), and (3) BAVM size.

Conclusions—We found increased levels of MMP-9 and TIMPs in BAVMs. Abnormal balance of MMP-9 and TIMPs may contribute to vascular instability of BAVMs. (Stroke. 2003;34:925-931.)

Key Words: angiogenesis ■ cerebral aneurysm ■ cerebral arteriovenous malformations ■ subarachnoid hemorrhage ■ vascular diseases

Human brain arteriovenous malformations (BAVMs) represent a rare but treatable cause of stroke in young adults. The variable nature of the clinical course, especially with respect to spontaneous hemorrhage, recurrence, growth, and regression, may indicate that BAVMs are structurally unstable vessels.

Matrix metalloproteinases (MMPs), a family of proteolytic enzymes, degrade extracellular matrix proteins, cell surface molecules, and other pericellular substances. Excessive degradation of the vascular matrix by MMPs may result in the destabilization of vessels, which potentially leads to weakening of the vessel wall, passive dilatation, and rupture.

We hypothesized that vascular instability in BAVMs exists that is associated with abnormal expression of MMPs, and we examined expression of MMPs and tissue inhibitors of metalloproteinases (TIMPs) in BAVMs.
centrifugation at 5000 rpm for 10 minutes. An equal amount of protein was loaded and electrophoresed on 7% sodium dodecyl sulfate discontinuous polyacrylamide gels. Subsequently, proteins were transferred onto polyvinylidene difluoride membranes. The polyvinylidene difluoride membranes were blocked at room temperature for 1 hour. The membranes were then probed with a primary antibody for 1 hour, followed by appropriate species-specific horseradish-peroxidase (HRP)--conjugated secondary antibody. Protein expression was detected with an enhanced chemiluminescence detection system (ECL Plus, Amersham Pharmacia Biotech Inc.). Bands were visualized on film, and a densitometric scanner was used to estimate the relative amount of protein. We used primary antibodies against MMP-9, MMP-2, TIMP-1, TIMP-2 (NeoMarkers, Inc), TIMP-3, TIMP-4 (Chemicon International, Inc), CD45 (NeoMarkers, Inc), and CD31 (Dakko Corporation). Secondary antibodies were HRP-conjugated donkey anti-rabbit IgG and HRP-conjugated sheep anti-mouse IgG (Bio-Rad Laboratories). To determine the sensitivity and specificity of the antibodies, the following positive controls were used: human umbilical vein endothelial cell (American Type Culture Collection) lysates for CD31, recombinant human MMP-9, recombinant human MMP-1, recombinant human TIMP-1, recombinant human TIMP-2 (R&D System, Inc), human pituitary gland for TIMP-3, rat heart tissue for TIMP-4, and tonsil Jurkat cell lysates (NeoMarkers, Inc) for CD45. In preliminary experiments, controls with increasing amounts of protein verified that quantitative band intensities fell within a linear range.

**Immunohistochemistry**

Immunohistochemistry was performed with the use of paraffin-embedded tissues. The heat-induced epitope retrieval was done in a 10% zymogram gel (Invitrogen) containing 0.1% gelatin for 1 hour. The membranes were then probed with a primary antibody (Elite ABC, Vector Laboratories) for 30 minutes at room temperature. The slides were washed again and incubated with detecting reagent (Elite ABC, Vector Laboratories). The slides were counterstained with hematoxylin. Primary antibodies were polyclonal rabbit anti-human-MMP-9 antibody (NeoMarkers Inc) and anti-CD31 antibody (Dakko Corporation). Sensitivity and specificity of anti-MMP-9 antibody were verified by means of a positive control (breast cancer tissue) and a negative control (no primary antibody).

**Gelatin Zymography**

The specimen was homogenized in a buffer containing Tris. The sample protein (40 μg) was mixed with sodium dodecyl sulfate sample buffer (Invitrogen) and separated under nonreducing conditions in a 10% zymogram gel (Invitrogen) containing 0.1% gelatin incorporated as a substrate. Recombinant MMP-2 and MMP-9 proteins (R&D systems) were used as positive controls. The gel was incubated with renaturing buffer (Invitrogen) and then incubated with developing buffer (Invitrogen) overnight at 37°C. The gel was then stained with colloidal blue stain (Invitrogen).

In some samples, we used 20 mmol/L EDTA (nonspecific MMP inhibitor), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) (serine protease inhibitor), or 1 mmol/L pepstatin A (aspartic protease inhibitor) to confirm the identity of proteolytic bands. To verify pro and active form MMPs, some samples were pretreated with 0.1 mmol/L 4-aminophenyl mercuric acetate (APMA) (pro-MMP activator) at 37°C for 1 hour. Proteolytic bands in zymography were quantified by scanning densitometry with a scanner with KODAK image analysis software (Eastman Kodak Company).

**Clinical Data and Feeding Artery Pressure Measurement**

Clinical data were collected according to “Reporting Terminology for Brain Arteriovenous Malformation Clinical and Radiographic Features for Use in Clinical Trials.” Details of feeding artery mean pressure measurement were previously described elsewhere. Briefly, during superselective angiography, feeding mean arterial pressure was measured just proximal to the nidus with the use of an intracranial microcatheter. 1.8F at its distal tip. All pressure measurements were referenced to right atrial pressure and were compared with the simultaneously recorded systemic mean arterial pressure in either the extracranial internal carotid artery, vertebral artery, or radial artery.

**Statistical Analysis**

The data for total MMP-9, pro-MMP-9, active MMP-9, and TIMPs were presented as a relative expression with control brain samples as 100%. The data for CD45 were presented as a relative expression with the positive control (tonsil Jurkat cell lysates) as 100%. We used the nonparametric Mann-Whitney test for comparison, and statistical significance was taken at \( P<0.05 \).

**Results**

We analyzed material from 37 BAVM patients (21 men and 16 women) and 9 surgical epilepsy patients (6 men and 3 women). The mean ages for BAVM patients and surgical epilepsy patients were 36±16 and 33±13 years, respectively. Clinical data are shown in the Table. Thirty-two BAVM patients had embolization treatment before resection. Among these 32 BAVM patients, the mean number of embolization treatments was 1±1 (range, 1 to 3), and the mean interval between last embolization and surgery was 2±2 days (range, 1 to 7 days). Eighteen BAVM patients had a history of hemorrhage from BAVMs.

**MMP and TIMP Expression in BAVMs**

Gelatin zymography using BAVM samples showed 2 proteolytic bands corresponding to pro-MMP-9 (~97 kDa) and active MMP-9 (~88 kDa) (Figure 1A and Figure 2, top). Treatment with APMA (pro-MMP activator) converted pro-MMP-9 to active MMP-9 (Figure 1B). Treatment with EDTA, PMSF, or pepstatin A confirmed that proteolytic bands had an MMP origin (Figure 1C to 1E). The Western blot for MMP-9 further confirmed that cleavage bands were MMP-9 (Figure 2, bottom). MMP-2 activity was not detected in BAVMs or control specimens (Figure 2, top). There was a linear relationship between total MMP-9 detected by Western blot analysis and total MMP-9 detected by zymography (\( r^2=0.72 \)).

Gelatin zymography showed that BAVM samples had higher levels of total MMP-9 (956±1109% versus 100±61%; \( P<0.05 \)) (Figure 2, top, and Figure 3), active MMP-9 (801±103% versus 100±99%; \( P<0.05 \)) (Figure 3), and pro-MMP-9 (2230±3176% versus 100±103%; \( P<0.05 \)) (Figure 3).

Western blot analysis showed that MMP-2 or TIMP-2 was not detected in BAVMs or control brain samples. BAVMs had higher levels of TIMP-1 (381±319% versus 100±101%; \( P<0.05 \)) and TIMP-3 (172±71% versus 100±41%; \( P<0.05 \)) than control brain specimens (Figure 3). TIMP-4 levels were higher in control brain samples than in BAVMs (71±100 versus 100±21; \( P<0.05 \)) (Figure 3).

Using immunohistochemistry for CD31, we identified the endothelial cell layer in BAVMs (Figure 4, left). MMP-9 was localized mainly in the endothelial cell/peri—endothelial cell layer of BAVMs (Figure 4, center and right). MMP-9 was also expressed in neutrophils infiltrating the BAVM vascular wall (data not shown). Immunohistochemistry on control...
brain samples showed no apparent MMP-9 expression (data not shown).

**MMP-9 Expression and History of BAVM Hemorrhage**

We assessed the relationship of MMP-9 and TIMP expression in BAVMs to history of intracranial hemorrhage. There was no difference in total MMP-9 (863±752% versus 1044±1382%; P = 0.903), pro-MMP-9 (1955±1976% versus 2490±4027%; P = 0.606), active MMP-9 (742±657% versus 857±1296%; P = 0.600), TIMP-1 (368±350% versus 393±296%; P = 0.543), TIMP-3 (170±75% versus 173±70%; P = 0.976), or TIMP-4 (66±29% versus 77±40%; P = 0.331) levels between BAVMs with prior hemorrhage and BAVMs without prior hemorrhage.

**MMP-9 Expression and Clinical Factors Associated With BAVM Hemorrhage**

We examined the relationship between MMP-9 expression and clinical factors reported to be associated with BAVM hemorrhage.10,11 BAVMs with venous stenosis ≥50% (n = 13) had higher expression of total MMP-9 (1519±1487% versus 692±744%; P < 0.05), pro-MMP-9 (3809±4336% versus 1493±2035%; P < 0.05), and active MMP-9 (1340±1494% versus 534±497%; P < 0.05) than BAVMs with venous stenosis <50% (n = 21). There was no apparent association between total MMP-9, pro-MMP-9, or active MMP-9 levels and (1) feeding artery pressure (n = 15), (2) pattern of draining vein (n = 35), and (3) BAVM size (n = 34).

**MMP-9 Expression and Embolization Treatment**

We assessed the relationship of MMP-9 and TIMP expression in BAVMs to embolization treatment. There was no difference in total MMP-9 (875±922% versus 1163% versus 1044±1382%; P = 0.05), pro-MMP-9 (3809±4336% versus 1493±2035%; P = 0.05), and active MMP-9 (1340±1494% versus 534±497%; P = 0.05) than BAVMs with venous stenosis <50% (n = 21). There was no apparent association between total MMP-9, pro-MMP-9, or active MMP-9 levels and (1) feeding artery pressure (n = 15), (2) pattern of draining vein (n = 35), and (3) BAVM size (n = 34).

**Figure 1.** Gelatin zymogram using BAVM specimens. There were 2 proteolytic bands corresponding to pro-MMP-9 (~97 kDa) and active-MMP-9 (~88 kDa) (A). APMA converted pro-MMP-9 to active MMP-9 (B). Bands corresponding to pro-MMP-9 and active MMP-9 were inhibited by EDTA (C) but not by PMSF (D) or pepstatin A (E).
to the endothelial cell/peri–endothelial cell layer of BAVMs, we assessed an endothelial cell mass using CD31, an endothelial cell marker, in BAVMs. There was no apparent correlation between CD31 expression and expression of total MMP-9 ($r^2=0.002$), pro-MMP-9 ($r^2=0.001$), and active MMP-9 ($r^2=0.001$).

**Discussion**

In this study we demonstrated the first evidence of the abnormal expression pattern of MMP-9 and TIMPs in BAVMs. We found markedly increased MMP-9 activity in BAVMs compared with control brain samples. MMP-9 was mainly localized to the endothelial cell/peri–endothelial cell layer of BAVMs. The increased activity of MMP-9 activity can be expected to cause degradation of the vascular matrix, impairing structural stability of BAVM vessels. This may, in part, explain vascular instability of BAVMs. Although a causal relationship needs to be carefully examined in a future study, MMP-9 may serve as a potential pharmacological target to modify clinical behavior of the unstable vascular lesions.

MMP-9, known as gelatinase B, degrades components of vascular extracellular matrices including type IV and V collagen, fibronectin, and elastin. Degradation of the vascular matrix by MMP-9 or other MMPs seems to be a critical step for angiogenesis and vascular remodeling. High levels of MMP-9 expression are detected in structurally unstable vasculature including cerebral aneurysms, abdominal aortic aneurysms, and atherosclerotic carotid artery. Excessive degradation of the vascular matrix may contribute to the destabilization of vessels, leading to the weakening of the vessel wall, and vessel rupture. Gaetani et al reported higher levels of nonspecific collagenase and elastase activities in ruptured intracranial aneurysms than in unruptured aneurysms. When we examined the relationship between MMP-9 expression and clinical factors associated with propensity to hemorrhage, we found that BAVMs with higher venous stenosis had higher expression of MMP-9. Although a larger sample size may be needed to confirm the associations between propensity to hemorrhage and MMP-9 expression, BAVMs with higher MMP-9 may have unstable vessels that are at risk for hemorrhage.

In addition to increased MMP-9, we found increased expression of TIMP-1 and TIMP-3, inhibitors of MMP-9, in BAVMs. There appears to be well-orchestrated temporal-spatial regulation of MMPs and TIMPs that precisely controls overall proteolytic activity during tissue remodeling. TIMPs are often coexpressed with MMPs in the physiological tissue remodeling process. A net balance between MMPs and TIMPs may determine a clinical course of unstable vascular lesions; more detailed analysis of MMPs and TIMPs using reverse zymography and in situ zymography may be needed. Underlying mechanisms for increased MMP-9 expression in the vascular lesions are not well understood. In addition to...
serving as a major proteolytic factor during angiogenesis, MMP-9 appears to be able to initiate and sustain angiogenesis during carcinogenesis by increasing bioavailability of vascular endothelial cell growth factor (VEGF).\(^1\) Angiopoietin-2 appears to increase MMP-9 expression in the presence of VEGF.\(^2\) Increased levels of VEGF were detected in BAVMs,\(^3\) and high levels of VEGF may be associated with the unstable nature of BAVMs, such as growth and recurrence.\(^4\) Our previous study found a marked increase of angiotropin-2 in BAVMs.\(^5\) Increased expression of angiotropin-2 and VEGF in BAVMs may contribute to increased MMP-9 activity.

Our data showed the relatively wide range for MMP-9 expression levels among BAVM samples, presumably reflecting heterogeneity of BAVMs. There are a number of physiological and pathological conditions that influence MMP-9 expression. Increased MMP-9 expression observed in this study may be due in part to pathological microenvironment induced by prior hemorrhage from BAVMs,\(^6\) ischemia,\(^7\) or shunt-induced high shear stress.\(^8\) Increased levels of MMP-9 in blood and brain tissue have been observed after ischemic stroke in humans.\(^9,10\) High MMP-9 expression appears to be associated with spontaneous or thrombolytic agent–induced hemorrhagic transformation after stroke.\(^11,12\)

In our study, however, we did not find any apparent effects of prior-hemorrhage on total MMP-9, active-MMP-9, or pro-MMP-9 levels.

Embolization treatment may increase MMP expression or activate MMP through inflammatory/ischemic processes, the formation of organized thrombi, or recanalization processes. Intraluminal mononuclear and polymorphonuclear cells were observed after embolization with polyvinyl alcohol foam, a therapeutic embolization material being used at our institution.\(^13\) It is possible, although not certain, that some of the MMP-9 expression or MMP-9 activation observed in BAVMs was induced by embolization treatment. In this study, however, there was no apparent association between active MMP-9 expression and (1) the interval between last embolization and surgery and (2) the number of embolization treatments. We also assessed a degree of inflammatory cell infiltration in BAVMs using CD45, a pan-leukocyte marker. There was no correlation between CD45 expression and MMP-9 expression, suggesting that a simple increase in the number of inflammatory cells does not account for increased MMP-9 in BAVMs.

Because of the practical difficulty in obtaining normal human brain specimens, we used structurally normal brain tissue obtained from temporal lobectomy for epilepsy treatment as our controls. In rats, seizure induced by kainic acid or bicuculline (GABA-A receptor antagonist) appears to cause a transient increase in brain MMP-9 levels that returns to normal after 24 hours. To our knowledge, increased MMP or TIMP expression has not been reported in patients with chronic seizure disorder or anticonvulsant treatment. We did not detect MMP-9 in our control brain samples, which is consistent with the previous reports showing little expression of MMP-9 in normal brain or normal vasculature.\(^14,15\)

Furthermore, our previous report showed that endothelial cells in our epilepsy brain samples were angiogenically quiescent.\(^16\)

In conclusion, there is an abnormal expression pattern of MMPs and TIMPs in BAVMs that may explain in part the structural instability of BAVM vessels.

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**Editorial Comment**

**Growth and Bleeding in BAVM: Another Role for MMPs**

Matrix metalloproteinases (MMPs) are extracellular matrix degrading enzymes that have been implicated in vascular remodeling under normal and pathological conditions. Elevated levels of the MMPs have been found in cerebral aneurysms, aortic aneurysms, and atherosclerotic plaques. Hashimoto and colleagues in an elegant study in this issue of *Stroke* add brain arteriovenous malformations (BAVMs) to the list of MMP-mediated vascular pathologies. They measured gelatinases by zymography and tissue inhibitors to metalloproteinases (TIMPs) by western blots in surgically removed BAVMs, comparing them with control tissues that had been removed from a group of patients undergoing epilepsy surgery. Gelatinase B (MMP-9), TIMP-1 and TIMP-3 were increased in the BAVMs.

BAVMs are formed congenitally with the potential for growth. Angiogenesis in the abnormal vessels has been suggested by the finding of expression of angiogenic factors, such as vascular endothelial cell growth factor (VEGF) and angiopoietin-2, in the BAVM. Studies in cancer cells, where angiogenesis is critical for tumor survival, have shown that MMP-9 is important in the action of VEGF.

Inflammation induces the production of MMPs. In many situations where MMP-9 production is observed, such as acute multiple sclerosis attacks, bacterial meningitis and stroke, there is an inflammatory response with both endogenous production of the enzyme by brain cells and its delivery to the injured tissues by white blood cells. Production of MMP-9 is driven by AP-1 and NF-κB sites in the gene promoter region that are stimulated by inflammatory factors. Hashimoto and colleagues in their study showed increased levels of MMP-9 in surgically removed BAVMs, which suggests inflammation at least around the time of resection. Other factors, yet to be discovered, such as hormonal changes or intermittent infections, may stimulate the release of growth factors that activate the proteases.

MMP-9 is expressed in a latent form that requires activation. Studies in mutant mice that lack the MMP-9 gene have shown that MMP-9 is important in blood-brain barrier disruption. Reactive oxygen species and nitric oxide have been shown to contribute to the activation process. Stromelysin-1 is another MMP, which is able to activate MMP-9. Increased expression of the activated form of MMP-9 was present in the surgically resected tissues, sug-
suggesting that the factors necessary for the activation process were present. Once activated, MMP-9 attacks the macromolecules in the extracellular matrix around the blood vessels. Excessive remodeling could weaken the vessel wall, which is thought to occur in abdominal and intracerebral aneurysms.\textsuperscript{11,12}

Several MMPs have been implicated in the degradation of blood-brain barrier proteins that form tight junctions and basal lamina, including zona occludens, occludins, laminin, and fibronectin.\textsuperscript{7,13} In BAVM, the MMP-9 co-localized with cerebral blood vessels, suggesting that they are involved in the growth and bleeding of the BAVM. Gelatinase A (MMP-2), which is constitutively expressed in brain along with its inhibitor, TIMP-2 were absent in the BAVMs. Resting astrocytes contain MMP-2, and during inflammation, both astrocytes and endothelial cells secrete MMP-9. A third cell type, the pericytes or microglial cells, also produce MMP-9 as well as other factors that appear to be important in the activation of MMP-9, such as MMP-3, reactive oxygen species, and nitric oxide. The lack of MMP-2 and TIMP-2 in the BAVMs suggests they are mainly endothelial cells without significant contribution from astrocytes.

Serum levels of MMPs are elevated in stroke patients.\textsuperscript{14} It will be interesting to see if the MMP-9 is elevated in the CSF and blood of patients with BAVM. If so, will there be levels that warn of an impending bleed, and will treatment with MMP inhibitors, during that time, reduce the rate of growth or risk of hemorrhage? Is there an increase in the MMPs in cavernous hemangiomas? This study provides an interesting insight into the biology of BAVM that will lead to new questions and novel treatments.

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