Matrix Metalloproteinase-9 Inhibition Attenuates Vascular Endothelial Growth Factor-Induced Intracerebral Hemorrhage

Chanhung Z. Lee, MD, PhD; Zheng Xue, MD; Yiqian Zhu, MD; Guo-Yuan Yang, MD, PhD; William L. Young, MD

Background and Purpose—Human brain arteriovenous malformation tissue displays increased levels of vascular endothelial growth factor (VEGF) as well as matrix metalloproteinase (MMP)-9, a tissue protease associated with various intracerebral hemorrhage (ICH). We hypothesized that increased MMP-9 was associated with ICH induced by vascular endothelial growth factor hyperstimulation and that this effect could be attenuated by nonspecific MMP inhibition.

Methods—We used a mouse model with adenoviral vector-mediated vascular endothelial growth factor transduction in the Methods fusion injury in ischemic stroke4–6 or amyloids in Alzheimer specific disease processes, eg, thrombolysis-associated reper-

Results—Adenoviral vector-mediated vascular endothelial growth factor at the higher dose and at 48 hours induced MMP-9 levels 6-fold (n=6, P=0.02) and increased brain tissue hemoglobin (43.4±11.5 versus 30.3±4.1 µg/mg, n=6, P=0.003) compared with the adenoviral vector control. Immunostaining was positive for MMP-9 around the cerebral vessels and the hemorrhagic areas. Minocycline and pyrrolidine dithiocarbamate administration suppressed vascular endothelial growth factor-induced MMP-9 activity (n=6, P=0.003 and P=0.01, respectively) and the associated increases in hemoglobin levels (n=5–6, P=0.001 and P=0.02, respectively).

Conclusions—Vascular endothelial growth factor-induced ICH is associated with increased MMP-9 expression. Suppres-

ion of MMP-9 by minocycline or pyrrolidine dithiocarbamate attenuated ICH, suggesting the therapeutic potential of MMP inhibitors in cerebral vascular rupture. (Stroke. 2007;38:2563-2568.)

Key Words: intracerebral hemorrhage ■ matrix metalloproteinase ■ minocycline ■ pyrrolidine dithiocarbamate ■ vascular endothelial growth factor

Intracerebral hemorrhage (ICH) significantly increases the mobility and mortality of various cerebral vascular diseases, including brain arteriovenous malformations (BAVMs). Thus, searching for therapeutic targets to prevent or reduce ICH is of prime clinical importance. The underlying mechanism in the pathogenesis of ICH, however, is largely unknown, which is, at least partially, attributed to lack of animal models. Most reported animal ICH models either focused on events after the incidents1–3 rather than the pathogenesis of ICH or simulated the characteristics of the specific disease processes, eg, thrombolysis-associated reper-

fusion injury in ischemic stroke4–6 or amyloids in Alzheimer disease.7

Vascular endothelial growth factor (VEGF), with the ability to increase vascular permeability and cause vasodila-
tion, has been indicated in various hemorrhagic disorders. Increased expression of VEGF has been noticed in BAVM surgical specimens8 as well as in the hemorrhagic transformation of ischemic stroke9,10 and in metastatic brain tumor-related ICH.11 An in vivo model has shown that certain isoforms of VEGF can cause tumor-related ICH.12 Overexpres-

sion of VEGF and matrix metalloproteinases (MMPs) was reported recently in clinically brain tumor-associated ICH.11 MMPs are known to degrade important structures in the vascular wall, including extracellular matrix proteins, cell surface molecules, and other pericellular substances; hence, they are implicated in the disruption of neurovascular structure leading to vascular rupture and hemorrhage.13–16

Our previous studies demonstrated that cerebral MMP-9 could be induced by VEGF focal hyperstimulation in the
mouse model. In the present study, we tested the hypothesis that MMP-9 is involved in VEGF-induced ICH and that inhibition of MMP-9 can attenuate the extent of VEGF-induced brain hemorrhage.

Materials and Methods

Animals and Treatment

Animal use was approved by the UCSF Committee on Animal Research. Male CD-1 mice, weight 30 to 35 g, were purchased from Charles River Laboratory (Wilmington, Mass). The mice were allowed free access to food and water with a 12-hour alternating light–dark cycle.

Adenoviral-Mediated Vascular Endothelial Growth Factor Gene Transfer in the Brain

After induction of anesthesia with 80 mg/kg ketamine and 6 mg/kg xylazine intraperitoneally, the mouse was placed on a heating pad to maintain the body temperature and the head was secured in a stereotactic frame (David Kopf Instruments). A Hamilton syringe was inserted through a burr hole 1 mm lateral to the sagittal suture, 1 mm posterior to bregma, and 3 mm under the cortex. Two microliters of adeno-vascular expression of either adenoviral-mediated VEGF (AdVEGF) or AdFc were injected stereotactically into the right caudate putamen.

In the present study, higher dosages of AdVEGF were chosen compared with our previous dose of $5.8 \times 10^7$ viral particles that stimulated cerebral angiogenesis and MMP-9 expression.18 A dose–response study with $1.0 \times 10^7$ and $3.0 \times 10^7$ viral particles of AdVEGF and a time course study at 24 hours and 48 hours after AdVEGF transduction (3.0 $\times 10^7$ viral particles) were performed to determine the appropriate dose and time point for assessing changes of MMP expression in association with ICH induced by VEGF overexpression. AdFc was used as the control for the viral vector (AdVEGF and AdFc were generous gifts from Regeneron Pharmaceuticals, Tarrytown, NY).

Pharmacological Treatment

To study the effect of MMP inhibition on ICH induced by AdVEGF transduction, minocycline, a nonspecific MMP inhibitor, and pyrrolidine dithiocarbamate (PDTC), the inhibitor of NFkB, an upstream regulator of MMPs, were used. Minocycline (Sigma Co) was administered starting on the day before AdVEGF injection, at 30 mg/kg per day in drinking water, a dose shown to inhibit MMP-9 induced by VEGF hyperstimulation in mice. In a separate group of mice, PDTC (Sigma Co) was administered at 2 hours before and 24 hours after AdVEGF injection, at 100 mg/kg intraperitoneally, a dose regimen reported to inhibit NFkB and MMP-9 in abdominal aortic aneurysms.

Tissue Collection

Mouse brain tissue was harvested after transcardiac perfusion using ice-cold phosphate-buffered saline. Coronal sections of brain tissues, including 1.5 mm anterior and posterior to the injection site, were quickly frozen on dry ice and stored at −80°C. The brain tissue was trimmed to contain only the caudate putamen portion and homogenized with d.d. water on ice. The homogenates were centrifuged at 80°C. The brain tissue was sectioned with a cryostat at 20-μm intervals.

Spectrophotometric Assay of Intracerebral Hemorrhage

The extent of cerebral hemorrhage was quantified using a spectrophotometric assay with Drabkin’s reagent (Sigma Co). Known quantities of mouse hemoglobin were added to lysates of untreated brain tissue to generate the standard curve. Fifteen minutes after adding Drabkin’s reagent to each aliquot at room temperature, the optical absorbance of cyanomethemoglobin was measured at 540 nm.

Gelatin Zymography

Equal amounts of sample proteins were separated by electrophoresis on 10% zymogram gels (Invitrogen). The gels were subsequently stained with colloidal blue stain (Invitrogen). Proteolytic bands in zymography were quantified by scanning densitometry using Kodak image analysis software (Eastman Kodak Co).

Immunohistochemistry

Tissue sections were fixed in 4% paraformaldehyde for 20 minutes. After preincubation with 5% goat serum, rabbit anti-mouse MMP-9 antibody (1:150) (a generous gift from Dr. Robert Senior at Washington University, St Louis, Mo) and rat anti-mouse CD31 (1:100) were applied at 4°C and left overnight. The sections were then incubated with fluorescein anti-rabbit 488 (1:100) and anti-rat 591(1:500) (Invitrogen) for 1 hour at room temperature. Immunostaining was analyzed under a fluorescence microscope.

Statistical Analysis

Data are expressed as mean±SD. Parameters between different groups in the MMP expression and hemoglobin amount were analyzed using analysis of variance followed by Fisher’s PLSD test. A probability value $<0.05$ was considered statistically significant.

Results

To determine the effects of AdVEGF transduction on the disruption of the cerebral vasculature, the amount of hemoglobin (an index of ICH) and MMP expression after AdVEGF transduction were examined (Table). In the time course study, 48 hours after the intracerebral injection, AdVEGF at the dosage of $3.0 \times 10^7$ viral particles caused cerebral hemorrhage with hemoglobin increasing to 43.4 μg/mg brain tissue compared with 30.3 μg/mg brain tissue in mice treated with AdFc controls ($P=0.003$). Meanwhile, cerebral MMP levels increased nearly 6-fold ($40.35 \pm 19.937$ versus $7085.5 \pm 5473$ arbitrary units, $P=0.02$) in the group treated with a higher dose of AdVEGF compared with the AdFc controls. Although there was a trend toward increases in the amount of hemoglobin and MMP-9 at 24 hours after VEGF stimulation, the changes did not reach statistical significance. In the dose–response study, at the lower dosage of $1.0 \times 10^7$ viral particles, there was no change in hemoglobin from AdVEGF. Although there was a trend in increased MMP levels (AdVEGF $9348 \pm 4872$ versus AdFc $19795 \pm 13880$ arbitrary units), it did not achieve statistical significance. Unlike MMP-9, there was no significant change in MMP-2 expression in response to VEGF (data not shown).

Hemorrhage was observed in the mouse brain after injection of AdVEGF, but not in the viral vector control group after AdFc injection. The pattern of ICH induced by focal VEGF hyperstimulation in the brain was illustrated using hematoxylin–eosin staining (Figure 1). Forty-eight hours after injection of $3.0 \times 10^7$ viral particles AdVEGF into the caudate putamen region, various bleeding areas formed in the mouse brain.

To identify the sources of stimulated MMP-9 expression, immunohistochemistry staining was performed to examine the distribution of MMP-9 expression at 48 hours after AdVEGF gene transfer. Positive staining of MMP-9 was distributed around cerebral microvessels that were colocalized with positive staining of CD31 (a marker for endothelial...
cells) as well as in areas with a scattering of bleeding as illustrated in Figure 2. To further define the involvement of MMP-9 activity in VEGF-induced hemorrhage, we analyzed the correlation between change in MMP-9 levels and the amount of hemoglobin in brain tissue. We found a positive trend in correlation between increased MMP-9 levels and amount of bleeding (hemoglobin/H11003\textsuperscript{30.91}/H11001\textsuperscript{2.19}/H11003\textsuperscript{[MMP-9 fold of change]}, r\textsuperscript{2}/H11005\textsuperscript{0.289}, n\textsuperscript{6}; P\textsuperscript{0.30}).

To assess the contribution of MMPs in VEGF-induced ICH, pharmacological inhibition of MMPs was administered. Both minocycline and PDTC treatments attenuated the extent of VEGF-induced cerebral hemorrhage while suppressing MMP-9 expression in the mouse brain. Minocycline completely inhibited VEGF-stimulated increases in cerebral MMP-9 activity and tissue hemoglobin amount (Figure 3), whereas PDTC suppressed nearly 80% of the induced MMP-9 and 60% of the increased hemoglobin in the brain tissue (Figure 4).

Discussion
In the present study, we demonstrated that: (1) MMP-9 was increased in VEGF-induced ICH; and (2) suppression of MMP-9 activation cascade by either minocycline or PDTC attenuated the extent of hemorrhage.

Clinical studies have shown elevated levels of VEGF receptors in surgical BAVM specimens.\textsuperscript{8,22,23} Increased VEGF levels were also noted in recurrent BAVMs.\textsuperscript{8,22,23} In an animal model of cerebral venous hypertension, which mimics the landmark phenotype of arteriovenous malformations, increased VEGF expression was found in the surrounding brain tissue.\textsuperscript{24} Research studies on cancer biology suggest that overexpression of certain VEGF isoforms can lead to ICH.\textsuperscript{12} Therefore, to identify potential therapeutic targets, it is important to elucidate the pathogenesis mechanisms involved in VEGF-related ICH, which might, at least partially, contribute to BAVM hemorrhage.

MMPs, a family of zinc-dependent extracellular proteinase, disrupt basal lamina of cerebral vessels, increasing vascular permeability and causing rupture of the vascular wall.\textsuperscript{25,26} The role of MMP-9 has been intensively studied in brain damage resulting from hemorrhage in animal models using collagenase\textsuperscript{2,3,27} or direct autologous blood\textsuperscript{28} injection into the animal brain. Those studies investigated the deleterious role of MMP-9 in acute brain injury and the therapeutic strategies in brain protection using MMP inhibitors after the ICH injury. It remains unclear, however, whether MMP contributed to the pathogenesis of ICH initiation. Several other studies examined the pathogenesis of ICH and showed that MMP-9 was involved in thrombolytic therapy with tissue plasminogen activator-induced hemorrhagic transformation of ischemic stroke, or in hemorrhage from cerebral amyloid angiopathy in Alzheimer disease. In these studies, MMP-9 was induced by activators that were geared either to the

<table>
<thead>
<tr>
<th>Time Course of ICH Development</th>
<th>24 Hours</th>
<th>48 Hours</th>
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<tr>
<td>Groups</td>
<td>AdFc</td>
<td>AdVEGF</td>
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<tr>
<td>Hemoglobin ((\mu g/mg))</td>
<td>24.5±2.7</td>
<td>28.9±5.5</td>
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<tr>
<td>MMP-9 activity (arbitrary units)</td>
<td>4505±1600</td>
<td>10 096±5388</td>
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*P<0.05, AdVEGF group versus the corresponding control group AdFc.

The amount of hemoglobin, as the index of the extent of hemorrhage, and gelatinase activities in the brain tissue were assessed at 24 and 48 hours, respectively, after 3.0×10\textsuperscript{7} viral particles of AdVEGF injection in the mouse brain. At 24 hours, there were trends in increased hemoglobin and MMP-9 activity in the VEGF-stimulated group. However, only at 48 hours after the intracerebral injection did the differences between the AdVEGF and the AdFc control groups reach statistical significance. An additional dose–response study showed that after AdVEGF injection at a lower dose of 1.0×10\textsuperscript{7} viral particles, there was no difference in hemorrhage, but there was a trend in increased MMP-9 activity in the VEGF-stimulated group.

Both minocycline and PDTC treatments attenuated the extent of VEGF-induced cerebral hemorrhage while suppressing MMP-9 expression in the mouse brain. Minocycline completely inhibited VEGF-stimulated increases in cerebral MMP-9 activity and tissue hemoglobin amount (Figure 3), whereas PDTC suppressed nearly 80% of the induced MMP-9 and 60% of the increased hemoglobin in the brain tissue (Figure 4).

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![Figure 1. ICH after focal AdVEGF hyperstimulation. Hematoxylin–eosin staining of the mouse brain section shows scattered bleeding areas mainly in the caudate putamen region after injection of AdVEGF. V, ventricle; Cc, corpus callosum. Arrowhead: part of needle track entrance. Arrows: various bleeding spots. Bar=5 mm. Inset is under larger magnification to show more details of the bleeding area.](http://stroke.ahajournals.org/content/2565)
specific therapeutic intervention (eg, tissue plasminogen activator after ischemic stroke) or to the specific diseases (eg, Alzheimer). Although these specific circumstances were limited to the particular diseases mentioned, these findings indicated that MMP-9 activation was potentially involved in the mechanisms of hemorrhage development.

VEGF is one of the most potent angiogenic factors. Not only has VEGF been shown to induce expression of MMP, but MMPs can also facilitate tissue availability of bound VEGF. Overexpression of VEGF and MMP-9 was recently reported in a clinical study of brain tumor metastatic-associated hemorrhage. Cheng et al described that specific isoforms of VEGF cause hemorrhage in experimental brain tumor studies. Here, we described a reproducible model of VEGF-induced ICH in mice. In addition, we have demonstrated for the first time that MMP-9

**Figure 2.** Immunohistochemistry showing positive signals of MMP-9 around cerebral vessels and bleeding areas using fluorescence double staining. A, The green-colored fluorescence staining positive for MMP-9. B, Red-colored fluorescence staining for CD31, a marker for endothelium in the vessels. C, The merged image of the green and red fluorescence staining showing MMP-9 positive around many blood vessels and scattered in the surrounding areas with bleeding spots. Arrow: double positive staining for MMP-9 and cerebral microvessels. Arrowhead: positive staining for MMP-9 in scattered areas. Bar=50 μm.

**Figure 3.** Minocycline treatment inhibited MMP-9 activity and suppressed ICH. Minocycline, 30 mg/kg per day in the drinking water, was started 1 day before the intracerebral injection of AdVEGF. Specimens were obtained 48 hours after AdVEGF intracerebral injection. A, Minocycline treatment completely suppressed the MMP-9 activity induced by VEGF hyperstimulation. B, Minocycline treatment completely suppressed the increase of hemoglobin in the brain tissue induced by VEGF hyperstimulation. *P<0.05, AdVEGF versus AdFc group; **P<0.05, minocycline treated versus AdVEGF-only group.

**Figure 4.** PDTC treatment suppressed MMP-9 activity and attenuated ICH. PDTC, an antioxidant inhibitor of NFκB (upstream transcriptional activator of MMP-9), 100 mg/kg intraperitoneally, was given 2 hours before and 24 hours after the intracerebral injection of AdVEGF. Specimens were obtained 48 hours after AdVEGF intracerebral injection. A, PDTC treatment partially suppressed the MMP-9 activity induced by VEGF hyperstimulation. B, PDTC treatment partially suppressed the increase of hemoglobin in the brain tissue induced by VEGF hyperstimulation. *P<0.05, AdVEGF versus AdFc group; **P<0.05, PDTC treated versus AdVEGF-only group.
is mechanistically linked to the pathogenesis of ICH caused by VEGF hyperstimulation in the brain.

Tetracyclines have recently been emerging as nonspecific inhibitors of MMPs.31 There is growing evidence that MMP inhibition may be useful in the management of vascular diseases.32–34 Potential benefits to brain protection after ICH using MMP inhibitors have also been reported.2,3,28 Studies on ischemic stroke have implied that MMP inhibition can reduce hemorrhagic transformation after tissue plasminogen activator treatment.4,6 Our previous study showed that doxycycline could inhibit MMP-9 and associated cerebral angiogenesis induced by VEGF. In the present study, suppression of VEGF-stimulated cerebral MMP-9 expression by minocycline resulted in the complete suppression of VEGF-induced ICH.

The implications of inflammation and immunomodulation in vascular disorders have recently become an area of great interest.35 Our data suggest that inflammation is a prominent feature of BAVM lesional phenotype, and neutrophils appear to be a major source of MMP-9 in these lesions.36 Reports from cancer research have also shown evidence that inflammatory cell types are major sources of MMP-9 in the angiogenic switch during the early stages of carcinogenesis.37 VEGF is known as a potent proinflammatory mediator in addition to its role in promoting proliferation and migration of endothelial cells. Oxygen-derived free radicals generated in inflammatory responses have been linked to MMP upregulation.38 Activation of MMP was also linked to free radical injury in tissue plasminogen activator-induced hemorrhage.39 The fact that PTDC, an antioxidant inhibitor of NFκB, was able to inhibit the expansion of experimental abdominal aortic aneurysm along with the suppression of MMP activity20 implies that MMP production may also be induced through the activation of NFκB pathway. In our model, PDTC treatment partially suppressed VEGF-induced MMP-9 expression and attenuated the extent of ICH. These data confirmed the involvement of inflammatory reactions and the contribution that MMP-9 derived from the subsequent free radical injury in the pathogenesis of VEGF-induced ICH.

It is interesting to note that another recent study has found no change in MMP-9 expression in VEGF-aggravated brain hemorrhage using immunohistochemistry staining.40 This result not only is in contrast to our data, but also differs from many other studies showing the important role of MMP-9 in the hemorrhagic transformation in the same ischemic stroke animal model using zymography to assess expression of MMP-9.6,41 The discrepancy shown by these findings may be attributable to the differences in sensitivity of the methodology used to detect the change in expression of MMP-9.

Inhibition of MMP has been shown to reduce endogenous VEGF in the brain tissue during stroke recovery42 in line with MMPs’ role of facilitating the bioavailability of growth factors.29,30 In our study, stimulation of MMP-9 was induced by exogenously delivered VEGF mediated by adenovirus. To the best of our knowledge, minocycline or PDTC should have no influence on the efficacy of adenovirus-mediated gene transfer unless the viral vector is specially engineered with a tetracycline sensitive promoter,43 which was not applied in our case. Acknowledging the complexity of the mutual influence between MMP and VEGF, we assume the MMP inhibitor in the current study might in turn reduce the subsequent release of VEGF to the extracellular space, but we are not certain to what degree in terms of biological significance in this case. Furthermore, there is evidence that minocycline inhibits MMP-9 activity stimulated by VEGF protein in cultured vascular smooth muscle cells,44 in which case VEGF biological activity is not dependent on the function of the viral vector or facilitation from MMP-9.

In the present study, we intended to provide evidence that MMP-9 is involved in VEGF-induced ICH, and that MMP-9 is, probably in part, generated from a VEGF-induced inflammatory response. Future studies are needed to elucidate the mechanisms and potential cellular sources of MMP-9 activity. We used minocycline as the nonspecific MMP inhibitor to demonstrate the effect of MMP inhibition on the attenuation of ICH. The mechanism of MMP inhibition by tetracyclines has not been fully understood. Additionally, the causal relationship between MMP-9 activity and hemorrhage needs to be more precisely defined in future studies. We did note a trend toward a positive correlation between the MMP-9 levels and extent of bleeding in the brain tissue. It is also critical that further studies use more specific inhibitors that target only individual MMPs, eg, MMP-9, or MMP-9 knockout mice. These studies would help define the mechanisms underlying the pathogenesis of VEGF-induced ICH and thus provide more specific therapeutic targets.

Summary
Our results demonstrated that VEGF-induced ICH is associated with increased MMP-9 expression. Suppression of MMP-9 by minocycline or PDTC attenuated ICH, suggesting that MMPs contribute to VEGF-induced vascular rupture and that MMP-9 may have been, at least partially, derived from VEGF-induced inflammatory responses in the brain.

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Disclosures
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