Nitric Oxide in Vascular Endothelial Growth Factor-Induced Focal Angiogenesis and Matrix Metalloproteinase-9 Activity in the Mouse Brain

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Background and Purpose—Vascular endothelial growth factor (VEGF) can induce matrix metalloproteinase (MMP)-9 activities and focal angiogenesis. We hypothesized that VEGF activation of cerebral MMP-9 would require nitric oxide participation.

Methods—We compared the in vivo effects of: (1) N\textsubscript{G}-monomethyl-L-arginine, a nonspecific nitric oxide synthase inhibitor; (2) L-N\textsuperscript{6}-(1-iminoethyl)lysine, an inducible nitric oxide synthase selective inhibitor; and (3) doxycycline, a known nonspecific inhibitor of MMP in the mouse brain, using in situ zymography and endothelial marker CD31. 3-nitrotyrosine was used as a surrogate for nitric oxide activity. Inflammatory cell markers CD68 and MPO were used to confirm leukocyte infiltration.

Results—VEGF-stimulated MMP-9 activity expressed primarily around cerebral microvessels. N\textsubscript{G}-monomethyl-L-arginine suppressed cerebral angiogenesis ($P<0.05$), especially those microvessels associated with MMP-9 activation ($P<0.02$) induced by VEGF, comparable to the effect of doxycycline. L-N\textsuperscript{6}-(1-iminoethyl)lysine showed similar inhibitory effects. 3-nitrotyrosine confirmed nitric oxide levels in the brain. Compared with the lacZ control, VEGF increased inflammatory cell infiltration, especially macrophages, in the induced brain angiogenic focuses.

Conclusions—Inhibition of nitric oxide production decreased MMP-9 activity and focal angiogenesis in the VEGF-stimulated brain. Both specific and nonspecific inhibition of nitric oxide synthase resulted in similar reductions, suggesting that VEGF-stimulated cerebral MMP activity and angiogenesis are predominantly mediated through inducible nitric oxide synthase, a specific nitric oxide synthase isoform mediating inflammatory responses. (Stroke. 2009;40:00-00.)

Key Words: matrix metalloproteinase • nitric oxide • vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is highly expressed in cerebral vascular malformations. Exaggerated VEGF expression can induce matrix metalloproteinase (MMP)-9-related cerebral angiogenesis contributing to excessive vascular remodeling and hemorrhage. The exact role of nitric oxide (NO) in MMP activation has been controversial. We hypothesized that NO is a critical mediator in VEGF-stimulated MMP-9 activities and angiogenesis in the brain.

Materials and Methods
Male adult CD-1 mice (Charles River, Mass) were divided into 5 groups (approved by the University of California, San Francisco Committee on Animal Research): control and VEGF and 3 VEGF groups receiving (1): N\textsubscript{G}-monomethyl-L-arginine, a nonspecific NO synthase (NOS) inhibitor, at 170 mg/kg/d (2); L-N\textsuperscript{6}-(1-iminoethyl)lysine (Alexis, Calif), an inducible NOS selective inhibitor, at 17 mg/kg/d, a dosage selected at the plateau of a dose–response curve; and (3) doxycycline (Sigma, Mo), a nonspecific MMP inhibitor, at 30 mg/kg/d, a dose shown to inhibit VEGF-induced MMP-9 activity and angiogenesis. Adenoviral-mediated VEGF gene transfer in the brain was performed. Brain specimens were harvested after 2 weeks, when the newly formed microvessels increased after adenoviral-mediated VEGF transduction and while cerebral MMP expression was still elevated.

For in situ zymography, frozen brain sections were incubated with DQ gelatin conjugate (Molecular Probes). Localization of CD31, 3-nitrotyrosine (3-NT), CD68, and MPO expression was assessed after chromogenic staining or the double-labeled fluorescent staining protocols. Capillary density was used as an index for cerebral angiogenesis. Data were collected as the total number of microvessels marked by CD31 and vessels with positive gelatinolytic activities. Vessel counts were expressed as mean±SD and analyzed using analysis of variance with protected least significant difference. A probability value $<0.05$ was considered statistically significant.

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Results

Under VEGF hyperstimulation, gelatinolytic (MMP) activities colocalized with brain microvessels (Figure 1A). Similar to the doxycycline group, gelatinolytic activity around the blood vessels substantially decreased after N\textsuperscript{G}-monomethyl-\textit{l}-arginine or L-N\textsuperscript{6}-(1-iminoethyl)lysine treatment (Figure 1B). VEGF increased capillary density in the brain in comparison with the lacZ group (326±57 versus 226±53 microvessel counts, \(P<0.05\)), whereas the number of vessels with positive MMP gelatinolytic activity also increased (199±52 versus 89±40, \(P<0.05\)). Comparable to doxycycline,\textsuperscript{1} N\textsuperscript{G}-monomethyl-\textit{l}-arginine suppressed total capillary density to 265±48 (\(P<0.05\)) and vessels positive for MMP activity to 91±56 (\(P<0.02\); Figure 1C). Inducible NOS selective inhibitor, L-N\textsuperscript{6}-(1-iminoethyl)lysine, showed similar reduction.

3-NT-positive cells intensively increased in the VEGF-stimulated brain, which was blocked by N\textsuperscript{G}-monomethyl-\textit{l}-arginine and similarly by L-N\textsuperscript{6}-(1-iminoethyl)lysine as well as after doxycycline treatment (Figure 2A). 3-NT-positive signals colocalized with cells near the cerebral microvessels and vascular walls (Figure 2B).

Neutrophils (MPO) and especially macrophages (CD68) were increased under VEGF stimulation (Figure 3A). CD68 largely colocalized with 3-NT, further illustrating macrophages as one of the major sources of VEGF-induced NO activity (Figure 3B).

Discussion

We demonstrated that inhibition of NOS suppressed VEGF-stimulated MMP activity and angiogenesis in the mouse brain. Although in vitro studies have shown that NO can either inhibit or promote MMP activation depending on cell types and stimulants,\textsuperscript{3,4} our data from this in vivo mouse model provide direct evidence linking VEGF-induced NO production to increased MMP activity and cerebral angiogenesis.

Although in situ zymography may detect both MMP-2 and -9 activity, increased MMP-9 activation (but not MMP-2 in either the pro- or activated form) has been detected by gelatin zymography under VEGF stimulation in \textit{vivo} or in \textit{vitro}\textsuperscript{7} despite MMP-2 involvement in other neurovascular degradation pathways.\textsuperscript{8} The failure of VEGF to stimulate angiogenesis in MMP-9 knockout mice\textsuperscript{6} further supports its importance in the VEGF pathway.

Inducible NOS inhibition showed similar effects as non-specific NOS inhibition, suggesting a predominant role of inducible NOS in VEGF-induced cerebral angiogenesis, although endothelial NOS has been found in vascular endothelial cells in response to VEGF. Although NO and MMP can increase VEGF release from tissues, recent evidence indicates

![Figure 1. A, VEGF-stimulated MMP gelatinolytic activities colocalized with microvessels in the angiogenic focus in brain caudate putamen. Merged images (yellow): MMP activity (in situ zymography, green) and microvessels (CD31, red). B, Inhibitors decreased mouse cerebral MMP gelatinolytic activity. Bar=20 μm. C, Quantitation of capillary density (mean±SD, \(n=5\) to \(6\); \(P<0.05\)).](http://stroke.ahajournals.org/)

![Figure 2. A, 3-NT expression in the brain angiogenic focus (\(n=3\)). Arrows point to positive stainings. Inset shows larger magnification. Bar=50 μm. B, 3-NT in cells in close vicinity to and colocalizing with cerebral microvessels (lectin). Bar=100 μm.](http://stroke.ahajournals.org/)

![Figure 3. A, Increased inflammatory cells in the VEGF-stimulated brain angiogenic focus (\(n=3\)). Arrows point to positive stainings. Insets show larger magnification. Bar=100 μm. B, Macrophage marker CD68 colocalized with 3-NT-expressing cells. Bar=100 μm.](http://stroke.ahajournals.org/)
that NOS inhibition mainly alters VEGF downstream effector capacity,\textsuperscript{10} in our case, cerebral MMP activation and angiogenic response. Given that inducible NOS is a potent source of NO in leukocytes and vascular cells, increased leukocyte infiltration is consistent with VEGF-induced inflammation in brain angiogenesis.

As an important mechanism underlying VEGF-stimulated MMP activity, further studies are needed to explore anti-inflammatory manipulation by local NO inhibition to decrease pathological angiogenesis and stabilize abnormal vasculature to decrease spontaneous hemorrhage risk. On the other hand, accumulating evidence suggests that VEGF–MMP cascade augmentation may be beneficial during stroke recovery.\textsuperscript{11} The demonstrated participation of NO in the VEGF-stimulation pathway may also open a new window for therapeutic interventions to promote functional revitalization after central nervous system insults.

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Disclosures

None.

References

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