Angiopoietin-2 Facilitates Vascular Endothelial Growth Factor-Induced Angiogenesis in the Mature Mouse Brain

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Background and Purpose—A better understanding of angiogenic factors and their effects on cerebral angiogenesis is necessary for the development of effective therapeutic strategies for ischemic brain injury. Vascular endothelial growth factor (VEGF) has been shown to induce angiogenesis in the adult mouse brain. However, the function of angiopoietin-2 (Ang-2) in cerebral angiogenesis has not been clarified. The goal of this study was to identify the combined effects of VEGF and Ang-2 on cerebral angiogenesis and the blood–brain barrier (BBB).

Methods—Six groups of 6 adult male CD-1 mice underwent AdlacZ (viral vector control), AdVEGF, AdAng2, VEGF protein, VEGF protein plus AdAng2, or saline (negative control) injection. Microvessels were counted using lectin staining on tissue sections after 2 weeks of treatment. Matrix metalloproteinase-9 (MMP-9) activity was determined by zymography. The presence of zonula occludens-1 (ZO-1) protein was determined by Western blot and immunohistochemistry.

Results—Mice treated with VEGF protein infusion plus AdAng-2 significantly increased microvessel counts relative to all other groups (P<0.05). The changes in MMP-9 activity paralleled the reduced ZO-1 expression in the VEGF plus Ang-2–treated group compared with the other 5 groups (P<0.05). Double-labeled immunostaining demonstrated that ZO-1–positive staining was significantly decreased on the microvessel wall in the VEGF plus Ang-2–treated group.

Conclusions—Our study demonstrates that the combination of VEGF and Ang-2 promotes more angiogenesis compared with VEGF alone. Furthermore, the combination of VEGF and Ang-2 may lead to BBB disruption because it increases MMP-9 activity and inhibits ZO-1 expression. (Stroke. 2005;36:1533-1537.)

Key Words: angiogenesis  ■ blood–brain barrier

Animal models offer a means of developing therapeutic strategies for reducing ischemic brain injury and restoring impaired function. In particular, the use of pro-angiogenic stimulation offers the promise of accelerating neurogenesis and the recovery of ischemic brain regions that lack sufficient perfusion from collateral vasculature. A better understanding of how pro-angiogenic signals in the brain affect physiological function is needed for the development of therapeutic strategies.

Vascular endothelial growth factor (VEGF) is the most important factor in the regulation of the development and differentiation of the vascular system. By acting as a capillary permeability-enhancing agent, VEGF also affects the integrity of the blood–brain barrier (BBB). However, the relationship between VEGF expression and BBB disruption is still unclear. As multiple partners of VEGF, angiopoietins (Angs) also play a critical role in vascular development, primarily by participating in the recruitment of peri-endothelial cell support cells (smooth muscle cells and pericytes) and development of barrier function. Angs are ligands for the Tie2 receptor and have either agonistic (Ang-1 and Ang-4) or antagonistic (Ang-2 and Ang-3) actions regulating vascular survival and expansion. Ang-2, a natural antagonist of Ang-1, promotes or inhibits angiogenesis in different environments. Ang-2 induces endothelial cell (EC) death and vascular regression in the absence of VEGF. Recent investigation showed(261x421) that concerted expression of VEGF and Ang-2 resulted in increased microvessel density in solid tumors. Matrix metalloproteinases (MMPs) are also among the most potent regulators of angiogenesis. Angiogenic factors such as VEGF and Ang-2 may also enhance MMP elaboration, which participates in the induction of microvessel sprouting in the growing vascular network. Ang-2 upregulates MMP-1 and MMP-9 in the presence of VEGF in vitro. MMP-2 and MMP-9 are able to digest the endothelial basal lamina leading to the opening of BBB.

Disruption of BBB integrity causes severe brain edema and exacerbates brain injury. Tight junctions (TJ) are essential structural components, which maintain the function of the BBB. ZO-1, one of the submembranous TJ-associated proteins specific-
Materials and Methods

Adenoviral Vector Transduction and Protein Mini-Pump Infusion

Procedures for the use of laboratory animals were approved by the UCSF Institutional Animal Care and Use Committee (IACUC). Adult male CD-1 mice (Charles River, Wilmington, Mass) weighing 30 to 35 grams were anesthetized with ketamine/Xylazine (Sigma, St Louis, Mo) at 1.5 μL/g body weight intraperitoneally. After induction of anesthesia, mice were placed on a stereotactic frame with a mouse holder (Kopf Instruments, Tujunga, Calif), and a right-sided burr hole was drilled in the skull 2 mm lateral to the sagittal suture and 0.6 mm anterior to the coronal suture. A Hamilton syringe was inserted into the lateral caudate putamen 3.0 mm below the cortical surface. The tip of the needle was implanted 1 mm lateral to bregma, to a depth of 2.5 mm, which is the location of the left lateral ventricle. After euthanizing the animals, the brain was removed and 2 mm thick sections were cut. One section was used for the immunohistochemistry, and the other section was divided into the ipsilateral and contralateral hemispheres. We used the ipsilateral hemispheres for immunoblotting and MMP-9 zymography.

Lectin Staining and Vessel Counting

Microvessel counting through lectin staining microvessel density is a simple and reproducible way to morphologically identify whether VEGF produces increased numbers of microvessels.2,13 Twenty-micrometer-thick frozen sections were cut. Two coronal sections, 1 mm anterior and 1 mm posterior to the needle track, were chosen. Three areas of microvessels (left, right, and inferior to the needle track) were chosen at low magnification (10×), and microvessel counting was performed in these areas as described previously.15

Double-Labeled Fluorescent Staining

Sections were fixed with acetone at −20°C for 10 minutes and incubated with 5% normal blocking serum for 1 hour, followed by lectin staining. Sections were then incubated with ZO-1 or PCNA antibody overnight (ZO-1, 1:200 dilution; Zymed, San Francisco, Calif; PCNA, 1:100 dilution; Santa Cruz, Calif) The secondary antibody used was Texas red anti-rabbit IgG (Vector Labs). Double-labeled immunostaining sections were evaluated using a fluorescence microscope (Nikon Microphoto-SA) with a filter cube (excitation filter, 450 to 490 nm) for fluorescent isothiocyanate labeling and a filter cube (excitation filter, 515 to 560 nm) for Texas-red. Photomicrographs for double-labeling illustrations were obtained by changing the filter cube without altering the position of the section or focus.

MMP-9 Zymography

Brain caudate samples were homogenized in lysis buffer and protein concentration was determined by colorimetric assay using bovine serum albumin as a control. Equal amounts of protein were loaded and separated by electrophoresis on zymogram gels per manufacturer’s instructions (Invitrogen, Carlsbad, Calif). After development, the gel was stained with colloidal blue stain for 30 minutes. Proteolytic bands in the zymogram were quantified by scanning densitometry using KODAK image analysis software (Eastman Kodak).

Western Blot Analysis

Brain tissue was homogenized in lysis buffer and equal amounts of total protein extracts were electrophoresed on 7% Tris-acetate gels (Invitrogen) and then transferred to a hydrophilic polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, Calif) using standard procedures. After blocking with 5% nonfat dry milk, the membrane was incubated at 4°C overnight with polyclonal rabbit anti ZO-1 antibody (1:500 dilution; Zymed). After washing, the membrane was incubated with peroxidase-conjugated goat anti-rabbit antibody (1:4000 dilution; Pierce, Rockford, Ill). Finally, the membrane was incubated in Ferno detection reagent (Pierce) for 5 minutes. Relative densities of the bands were analyzed with National Institutes of Health Image 1.63 software.

Statistical Analysis

Parametric data in the groups treated with VEGF protein infusion, AdVEGF, AdAng-2, VEGF protein/AdAng-2, and AdlacZ were compared using a 2-way ANOVA followed by the Scheffe f test. All data are presented as mean±SD. P<5% was considered to represent statistical significance.

Results

Increase of Microvessel Counts in VEGF and VEGF/Ang-2–Treated Mice

Lectin fluorescent staining is a sensitive method for detecting the number of microvessels in the mouse brain. Using this semi-quantitative method, we analyzed changes in microvessel counts in the 6 groups of mice after 2 weeks of adenoviral vector and protein transduction. We found that AdAng-2 alone did not stimulate an increase in microvessel density in the adult mouse brain. The number of microvessels in the AdVEGF and VEGF protein infusion groups was greatly increased compared with the saline treated or AdlacZ-transduced mice (P<0.05; Figure 2). The microvessel counts were not different between the AdVEGF and VEGF protein infusion groups (P>0.05). Interestingly, Ang-2 plus VEGF transduction increased microvessel counts compared with VEGF transduction alone (P<0.05).
These results demonstrated that AdVEGF transduction and VEGF protein infusion induce similar angiogenesis in the adult mouse brain. Furthermore, the combination of VEGF and Ang-2 had a pronounced effect on angiogenesis. No hemorrhages in our animal model during angiogenesis were detected. These data suggest a synergistic effect between VEGF and Ang-2. We further examined EC proliferation using PCNA immunostaining. There was little PCNA-positive staining in the AdlacZ-transduced and AdAng-2-transduced mice. Compared with these 2 groups of mice, PCNA-positive staining was greatly increased in the VEGF alone and VEGF plus AdAng-2-transduced mice. The PCNA-positive staining was often located at the newly formed sprouts around mother vessels, or very tiny growing microvessels (Figure 2D).

**Increased MMP-9 Activity in VEGF and VEGF/Ang-2-Treated Mice**

MMPs are involved in vascular remodeling and extracellular matrix signaling. To test whether Ang-2 has a synergistic effect with VEGF during brain angiogenesis, we further measured MMP-9 activity in these 6 groups of mice using zymography. We demonstrated that MMP-9 activity was increased in VEGF-treated animals compared with control animals \((P<0.05); \) Figure 3). Similarly, mice treated with Ang-2 plus VEGF had significantly increased MMP-9 activity compared with the VEGF-treated group \((P<0.05)\). Our data demonstrated that MMP-9 activity was upregulated during brain angiogenesis, especially in response to the combination of VEGF and Ang-2.

**Decrease of ZO-1 Protein in VEGF/Ang-2 Mice**

ZO-1 expression was inversely related to MMP-9 expression. The combination of VEGF and Ang-2 decreased ZO-1 expression compared with other groups \((P<0.05, \) Figure 4a). We demonstrated that a combination of increased MMP-9 and decreased ZO-1 expression provides a mechanism by which Ang-2 promotes VEGF-induced angiogenesis.
expression and decreased ZO-1 expression was consistent with BBB disruption and should be investigated in further studies of barrier functionality. To identify whether ZO-1 decreased in the vessel wall, we used double-labeled immunostaining. We found that in the saline-treated mice, ZO-1 was expressed around the vessel, demonstrating that ZO-1 existed at sites of endothelial cell–cell contact and preserved the integrity of the microvessel endothelial cell after 2 weeks of treatment (Figure 4b). However, in the mice treated with VEGF and Ang-2, ZO-1–positive staining was much less than in the control groups, suggesting that Ang-2 in combination with VEGF interrupted the integrity of the BBB.

**Discussion**

AdVEGF gene transfer and the combination of AdAng-2 and VEGF protein via infusion provide useful approaches for enhancing the effect of inducing multiple angiogenic protein overexpression in the brain in vivo. Using this method, we demonstrated that: (1) the combination of VEGF and Ang-2 increased microvessel counts, although Ang-2 alone did not stimulate microvessel increase; (2) the combination of VEGF and Ang-2 increased MMP-9 activity and decreased ZO-1 expression in the brain (Figure 5); and (3) an increase in MMP-9 was closely related to a decrease in ZO-1. These data suggest that Ang-2 may act synergistically with VEGF to promote brain angiogenesis. Because the combination of VEGF and Ang-2 increases MMP-9 activity and decreases ZO-1 expression, Ang-2 may also play a disruptive role in BBB integrity during brain angiogenesis.

Angiogenesis is a step-wise process. Necessary steps include an increase of vascular permeability, degradation of surrounding matrix, proliferation and migration of endothelial cells, and stabilization of neo-microvessels. Many angiogenic molecules are involved in this progression in which VEGF and Ang-1 and Ang-2 may play fundamental roles in the microvessel. We combined VEGF and Ang-2 because both angiogenic factor expressions are greatly increased during brain angiogenesis. Our result suggests that Ang-2 may play a crucial role in the early stage of angiogenesis and may be necessary for promoting the growth of sprouts.

VEGF is essential for angiogenesis and BBB functionality. Our previous work illustrated that local VEGF transduction promoted capillary angiogenesis. Intraventricular infusion of VEGF protein induces microvascular formation in the whole rodent brain. We showed that VEGF-induced microvessel counting is similar in the adenoviral vector and protein infusion groups, suggesting that the 2 approaches are comparable. We found Ang-2 alone did not increase the number of microvessels, suggesting that Ang-2 may destabilize the vasculature and cause subsequent remodeling. However, the combination of VEGF and Ang-2 greatly increased the microvessel count, which suggests that Ang-2 may stimulate new microvessel sprouting and capillary angiogenesis in the presence of VEGF.

MMPs are members of a multigene family of metal-dependent enzymes classically implicated in extracellular matrix...
remodeling in physiological and pathophysiological conditions. During angiogenesis, some angiogenic stimulators, such as VEGF, Ang-1, and Ang-2, can increase the expression of MMPs. MMP-1, MMP-2, and MMP-9 are produced during EC migration and endothelial tubule formation. We concentrated on MMP-9 activity because we previously demonstrated that VEGF stimulated MMP-9 activity in EC and smooth muscle cell cultures. We found that MMP-9 activity was greatly increased in the group treated with VEGF plus Ang-2, compared with the group treated with VEGF alone, suggesting that Ang-2 may induce MMP-9 activation. This function is very important for the promotion of extracellular matrix remodeling. Intracerebral MMP-9 injection causes BBB disruption in the rat brain.

We are particularly interested in the structural features of BBB in neovascularized tissue because it is the key for induction of functional angiogenesis. Tight junctions in the BBB are essential for maintaining the microenvironment. ZO-1 is a peripheral tight junction protein that is found on the epithelial and EC membranes. Loss of ZO-1 from endothelial TJs facilitates capillary leakage and hence increases BBB permeability. ZO-1 may play an important role in the BBB formation by serving as an MMP-9 substrate associated with degradation when MMP-9 increases. Therefore, as a specific molecular marker, ZO-1 protein expression parallels BBB morphofunctional maturation. In cultured murine brain ECs, VEGF reduces and dislocates ZO-1 expression and enhances ZO-1 tyrosine phosphorylation. In the adult brain, most microvessels express ZO-1. Because Ang-2 induces vascular leakage and regression, these findings suggest that Ang-2 reduces the expression of ZO-1 in the brain vascular system.

In conclusion, the present study has demonstrated that focal VEGF and angiopoietin-2 hyperstimulation in the mouse brain increases MMP-9 activity and decreases ZO-1 protein, thereby inducing angiogenesis and BBB disruption, respectively. The ability to manipulate angiogenesis may have important implications in the treatment of ischemic brain injury. The mechanism of the effects of the combination of Ang-2 and VEGF on MMP-9 activity in relation to the function of BBB TJ protein will require further investigation.

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