Mechanisms of Osteopontin-Induced Stabilization of Blood-Brain Barrier Disruption After Subarachnoid Hemorrhage in Rats

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**Background and Purpose**—Osteopontin (OPN) is an inducible, multifunctional, extracellular matrix protein that may be protective against blood-brain barrier (BBB) disruption after subarachnoid hemorrhage (SAH). However, the protective mechanisms remain unclear.

**Methods**—We produced the endovascular perforation model of SAH in rats and studied the time course of OPN induction in brains by Western blotting and immunofluorescence (n=50). Then, 34 rats were randomly assigned to sham (n=3), sham+OPN small interfering RNA (siRNA, n=3), SAH+negative control siRNA (n=14), and SAH+OPN siRNA (n=14) groups, and 109 rats were allocated to sham vehicle (n=17), sham+recombinant OPN (n=17), SAH+vehicle (n=33), SAH+recombinant OPN (n=31), and SAH+recombinant OPN+L-arginyl-glycyl-L-aspartate motif–containing hexapeptide (n=11) groups. The effects of OPN siRNA or recombinant OPN on BBB disruption and related proteins were studied.

**Results**—OPN was significantly induced in reactive astrocytes and capillary endothelial cells, peaking at 72 hours after SAH, during the recovery phase of BBB disruption. Blockage of endogenous OPN induction exacerbated BBB disruption and was associated with a reduction of angiopoietin-1 and mitogen-activated protein kinase (MAPK) phosphatase-1 (an endogenous MAPK inhibitor), activation of MAPKs, and induction of vascular endothelial growth factor-A at 72 hours after SAH, whereas recombinant OPN treatment improved it and was associated with MAPK phosphatase-1 induction, MAPK inactivation, and vascular endothelial growth factor-A reduction, which was blocked by L-arginyl-glycyl-L-aspartate–containing hexapeptide at 24 hours after SAH. Vascular endothelial growth factor-B and angiopoietin-2 levels were unchanged.

**Conclusions**—OPN may increase MAPK phosphatase-1 that inactivates MAPKs, upstream and downstream of vascular endothelial growth factor-A, by binding to L-arginyl-glycyl-L-aspartate–dependent integrin receptors, suggesting a novel mechanism of OPN-induced post-SAH BBB protection.

Key Words: osteopontin ■ blood-brain barrier ■ brain injury ■ subarachnoid hemorrhage

__Aneurysmal subarachnoid hemorrhage (SAH)–induced early brain injury is a potentially fatal condition, 1 of whose key pathologic manifestation is the breakdown of the blood-brain barrier (BBB).1 To improve outcomes of SAH patients, it may be important to develop new therapies against BBB disruption.

We recently reported that recombinant osteopontin (r-OPN) suppresses nuclear factor-κB activation and matrix metalloproteinase-9 induction, thus preventing post-SAH BBB disruption in rats.2 OPN is an inducible, pleiotropic, extracellular matrix glycoprotein that is involved in variable pathophysiologic processes in a wide range of tissues.3,4 However, it is unknown whether endogenous OPN is induced and protective against BBB disruption in the setting of SAH. Moreover, the protective mechanisms of OPN against post-SAH BBB disruption have been poorly elucidated. Accumulated evidence suggests that many molecules, for example, vascular endothelial growth factor (VEGF)-A; VEGF-B; angiopoietin (Ang)-1; Ang-2; mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK) 1/2, p38, and c-Jun N-terminal kinase (JNK); and their endogenous inhibitor, MAPK phosphatase-1 (MKP-1), are involved, while acting simultaneously or at different stages during BBB disruption,5 although no study has reported whether OPN affects these molecules to protect the BBB. The aim of this study was to clarify the role of endogenous OPN__
in post-SAH BBB disruption and to find out the mediators that are involved in the protective mechanisms of OPN against it.

Materials and Methods

Experimental Model of SAH and Study Protocol

All procedures were approved by the Loma Linda University animal care committee. The endovascular perforation model of SAH was produced in male, adult, Sprague-Dawley rats (300 to 370 g; Harlan, Indianapolis, Ind) as previously described. In brief, rats were anesthetized with 2% to 3% isoflurane in 60%/40% medical air/oxygen, followed by subcutaneous injection of atropine (0.1 mg/kg). Blood pressure and blood gases were measured via the left femoral artery. Rectal temperature was kept at ~37°C with an electric heating pad. After the left common carotid artery, external carotid artery, and internal carotid artery were exposed, a sharpened 4-0 monofilament nylon suture was advanced rostrally into the internal carotid artery, and internal carotid artery were reperfused to the bifurcation of the anterior and middle cerebral arteries. Immediately after puncture, the suture was withdrawn into the external carotid artery stump, and the internal carotid artery was reperfused to produce SAH. Sham-operated rats underwent an identical procedure, except that the suture was withdrawn once resistance was felt, without puncture. Animals had free access to food and water until euthanasia.

To study whether OPN is induced in brains after SAH, 44 rats were randomly assigned to sham + sham intracisternal injection (n = 3), sham + OPN small interfering RNA (siRNA; n = 3), SAH + negative control siRNA (n = 14), and SAH + OPN siRNAs (n = 14) groups. Surviving rats were euthanized at 72 hours after SAH, and the severity of SAH, BBB permeability ( sham, n = 3; SAH, n = 7 each), and expression changes in BBB disruption-related proteins (SAH + negative control siRNA, n = 6; SAH + OPN siRNA, n = 5) were assessed after evaluation of neurologic scores and body weights.

To study the effects of exogenous OPN on BBB disruption, 109 rats were randomly divided into sham + vehicle (n = 17), sham + r-OPN (n = 17), SAH + vehicle (n = 33), SAH + r-OPN (n = 31), and SAH + r-OPN + L-arginyl-L-glycyl-L-aspartate motif–containing hexapeptide (GRGDSP; n = 11) groups. GRGDSP blocks L-arginyl-L-glycyl-L-aspartate–dependent integrins, the primary receptors for OPN. Neurologic scores, body weight changes, the severity of SAH, brain edema (n = 5 per group), and BBB disruption (n = 6 per group) were assessed, and Western blot analyses (n = 6 per group) were performed 24 hours after SAH.

Neurologic Scoring

Neurologic scores (3 to 18) were blindly evaluated before SAH production and after SAH production at 24-hour intervals until all surviving animals had been euthanized, as previously described. The evaluation consisted of 6 tests (spontaneous activity, symmetry in the movement of all 4 limbs, forepaw outstretching, climbing, body proprioception, and response to whisker stimulation) that are scored 0 to 3 or 1 to 3. Higher scores indicate greater dysfunction.
Severity of SAH

The severity of SAH was blindly assessed in all animals after euthanasia as previously described.6 The subarachnoid cistern was divided into 6 segments, and each segment was allotted a grade from 0 to 3 as follows: grade 0 = no subarachnoid blood; grade 1 = minimal subarachnoid blood; grade 2 = moderate blood clot with recognizable arteries; and grade 3 = blood clot obliterating all arteries within the segment. Each animal received a total score ranging from 0 to 18 after the individual scores were summed.

Intracerebroventricular Infusion

Under 2% to 3% isoflurane anesthesia, the needle of a 10-μL Hamilton syringe (Microliter No. 701; Hamilton Co, Reno, Nev) was inserted through a burr hole on the skull into the left lateral ventricle according to the following coordinates relative to bregma: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm below the horizontal plane of bregma.2 OPN siRNA (sense, 5′-CUAUCAGGUGAGCAGGAG[dT][dT]-3′; and antisense, 5′-ACUUGCAGAUGACCUUGUA[dT][dT]-3′; Sigma-Aldrich, St. Louis, Mo) or an irrelevant control siRNA (Dharmacon/Thermo Fisher Scientific, Lafayette, Colo) at 500 pmol/μL was infused at a rate of 0.5 μL/min at 24 hours before SAH production or sham operation. Sterile PBS vehicle (1 μL) or mouse r-OPN (0.1 μg in 1 μL; EMD Chemicals, La Jolla, Calif) was infused at a rate of 0.5 μL/min irrespective of the animal’s body weight at 1 hour before SAH production or sham operation. GRGDSP (Sigma-Aldrich, St. Louis, Mo) dissolved in 0.1N acetic acid and further diluted in PBS (final concentration of acetic acid, <1.74%). GRGDSP (100 pmol in 1 μL) was infused together with r-OPN. In rats that received a sham intracerebroventricular infusion, a burr hole was made on the skull at the same position, but neither needle insertion nor drug infusion was performed. The needle was removed 10 minutes after an infusion was finished, and the burr hole was quickly plugged with bone wax.

Brain Water Content

After euthanasia, brains were immediately divided into right and left cerebral hemispheres, brain stem, and cerebellum. Brain specimens were dried in an oven at 105°C for 72 hours. The following formula was used to calculate the percentage of water content: ([wet weight−dry weight]/wet weight)×100%.2

BBB Permeability

Evans blue dye (2%; 5 mL/kg) was injected over 2 minutes into the left femoral vein and allowed to circulate for 60 minutes. Rats were euthanized by intracardiac perfusion with PBS, and brains were removed and divided into the same regions as in the water content study. Spectrophotometric quantification of extravasated Evans blue dye was performed as previously described.2

Western Blot Analyses

Western blot analysis was performed as previously described.2 In brief, brains were divided into the same regions as in the water content study. In the study of OPN siRNA or r-OPN treatments, the left (perforation side) cerebral hemisphere was used. Equal amounts of protein samples (50 μg) were loaded on a Tris glycine gel, electrophoresed, and transferred to a nitrocellulose membrane. Membranes were blocked with a blocking solution, followed by incubation overnight at 4°C with the following primary antibodies: mouse monoclonal anti-OPN (1:200), goat polyclonal anti–Ang-1 (1:200), goat polyclonal anti–Ang-2 (1:200), mouse monoclonal anti–phospho-JNK (1:200), mouse monoclonal anti–phospho-p38 (1:200), rabbit polyclonal anti–phospho-ERK (1:200; Santa Cruz Biotechnology, Santa Cruz, Calif), rabbit polyclonal anti–MKP-1 (1:5000; Sigma-Aldrich, St. Louis, Mo), mouse monoclonal anti–VEGF (1:1000; Millipore, Temecula, Calif), and rabbit polyclonal anti–VEGF-B (1:500; Abcam, Cambridge, Mass). Immunoblots were processed with appropriate secondary antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, Calif) for 1 hour at 21°C, and bands were detected with a chemiluminescence reagent kit (ECL Plus; Amersham Bioscience, Arlington Heights, Ill). Blot bands were quantified by densitometry with Image J software (National Institutes of Health, Bethesda, Md). ß-Actin (1:2000; Santa Cruz Biotechnology, Santa Cruz, Calif) was blotted on the same membrane to be used as a loading control.

Immunofluorescence

The brains were fixed by cardiovascular perfusion with PBS and 10% paraformaldehyde. The brains were postfixed in 10% paraformaldehyde followed by 30% sucrose (weight/volume) for 3 days. Ten-micron-thick coronal sections at the level of bregma 1 mm (caudally) were cut on a cryostat (LM3050S; Leica Microsystems, Bannockburn, Ill) and mounted on poly-L-lysine-coated slides. Double-fluorescence labeling was performed as described previously.2 Sections were incubated overnight at 4°C with the following primary antibodies: mouse monoclonal anti-OPN (1:50; Santa Cruz Biotechnology, Santa Cruz, Calif), rabbit polyclonal anti–CD34 (1:100; Abbiotec, San Diego, Calif), and rabbit polyclonal anti–glial fibrillary acidic protein (1:100; Dako, Carpinteria, Calif). Appropriate fluorescence dye–conjugated secondary antibodies (Jackson Immunoresearch, West Grove, Pa)
were incubated in the dark for 1 hour at 21°C. For negative controls, the primary antibodies were omitted and the same staining procedures were followed. The sections were visualized with a fluorescence microscope, and photomicrographs were recorded and analyzed with MagnaFire SP 2.1B software (Olympus, Melville, NY).

Statistics
SAH grade and neurologic scores were expressed as median and 25th to 75th percentiles and were analyzed by Mann–Whitney U tests or Kruskal-Wallis tests, followed by Steel-Dwass multiple comparisons. Other values were expressed as mean±SD, and unpaired t tests, χ² tests, or 1-way ANOVA with Tukey-Kramer post hoc tests were used as appropriate. P<0.05 was considered significant.

Results
Natural Course of OPN Induction
Seven SAH rats died before euthanasia. The final number of animals in the 24-, 72-, and 120-hour SAH groups therefore was 10, 9, and 10, respectively. SAH was cleared from the basal cisterns as time passed, but significant hemorrhage remained even at 120 hours after SAH (Figure 1A). SAH caused a significant loss in body weight, neurologic impairment, and BBB disruption (Figure 1, B–D). Western blot analysis demonstrated a significant elevation of OPN in all brain regions studied, with a peak at 72 hours after SAH (Figure 1, E and F). This peak corresponded to the animal’s recovery of body weight loss and neurological impairment as well as BBB disruption. Immunofluorescence showed that reactive astrocytosis and angiogenesis occurred predominantly in the basal cortex covered with thick SAH at 72 hours after SAH (Figure 2). OPN was induced in the reactive astrocytes and capillary endothelial cells, with the largest increase in the basal cortex near the densest SAH and the least at a distance away.

Specific Blockage of Endogenous OPN Induction
Comparisons of mean values for body weight, arterial blood pressure, heart rate, blood gas data, and SAH grade revealed no significant differences among the groups (data not shown). Effects of OPN siRNA treatment were studied...
at 72 hours after SAH, because the BBB was disrupted at 24 hours, when OPN was not sufficiently upregulated, but returned to normal at 72 hours, when OPN induction peaked. Two OPN siRNA-treated and 1 control siRNA-treated SAH rats died before euthanasia, whereas no sham-operated rats died. SAH rats (n=25) showed significantly worse neurologic scores compared with sham-operated rats (n=6) at 48 and 72 hours after SAH (Figure 3A). At 72 hours after SAH, neurologic scores in the OPN siRNA-treated SAH rats (n=12) were significantly worse than in the control siRNA-treated SAH rats (n=13). Evans blue dye extravasation was still present in all brain regions at 72 hours after SAH in OPN siRNA-treated SAH rats (n=7) compared with sham-operated rats (n=6), but BBB breakdown had already resolved in control siRNA-treated SAH rats (n=7; Figure 3, B–E). OPN siRNA treatment (n=7) resulted in significantly increased BBB permeability in both cerebral hemispheres compared with control siRNA treatment (n=7) in SAH rats (Figure 3, B and C). Post-SAH OPN upregulation was significantly inhibited after OPN siRNA treatment (n=5) compared with control siRNA-treated rats (n=6) and was associated with a decrease in Ang-1 and MKP-1 levels and an increase in VEGF-A, phospho-JNK, phospho-p38, and phospho-ERK1/2 levels in the left cerebral hemisphere 72 hours after SAH (Figure 4). VEGF-B and Ang-2 levels were unchanged.

**Exogenous OPN Treatment**

Comparisons of physiologic parameters revealed no significant differences among the groups (data not shown). None of the sham-operated rats died. In SAH rats, mortality in the r-OPN+GRGDSP treatment group (45.5%, 5 of 11 rats) was significantly higher than in the r-OPN treatment group (16.1%, 5 of 31 rats) but not in the vehicle treatment group (24.2%, 8 of 33 rats). Among surviving animals, 8 vehicle-treated and 9 r-OPN-treated SAH rats were not used for further analysis because their SAH grading scores were 9 or less, suggesting that the BBB disruption was mild.2

The average SAH grading score was similar among the groups in each analysis (12.4±1.9, 12.3±2.2, and 11.7±0.5 in the vehicle [n=17], r-OPN [n=17], and r-OPN+GRGDSP [n=6] treatment groups, respectively, at 24 hours after SAH). The vehicle- and r-OPN+GRGDSP–treated, but not the r-OPN–treated, SAH rats showed significant neurologic impairment and body weight loss compared with the vehicle-treated (n=17) or r-OPN-treated (n=17) sham-operated rats (Figure 5, A and B). SAH caused a significant increase in brain water content (n=5 per group) and Evans blue dye extravasation (n=6 per group) in all brain regions, which was significantly attenuated by r-OPN treatment in both cerebral hemispheres (Figure 5, C–F). Western blot analysis showed that r-OPN significantly inhibited post-SAH upregulation of VEGF-A and phospho-JNK and increased MKP-1, whereas these effects of r-OPN were blocked by GRGDSP (n=6 each; Figure 6). Ang-1, phospho-p38, and phospho-ERK1/2 levels were not significantly changed.

**Discussion**

The present study demonstrates that OPN was induced in reactive astrocytes and capillary endothelial cells during the initial recovery from early brain injury in a rat model of SAH. Blockage of endogenous OPN expression exacerbated neurologic impairment and BBB disruption and was associated with a decrease in Ang-1 and MKP-1 levels and an increase in VEGF-A, phospho-JNK, phospho-p38, and phospho-ERK1/2 levels at 72 hours after SAH. In contrast, r-OPN treatment improved these parameters and was associated with MKP-1 induction, VEGF-A reduction, and JNK inactivation at 24 hours after SAH. The protective
effects of r-OPN were blocked by GRGDSP. Our experimental evidence suggests that OPN represents a naturally occurring protective factor against BBB disruption after SAH.

We have already reported that r-OPN suppressed nuclear factor-κB activation and matrix metalloproteinase-9 induction, preventing post-SAH BBB disruption. However, many other molecules are involved, acting simultaneously or at different stages during BBB disruption. VEGF-A is a potent inducer of BBB disruption, whereas VEGF-B may maintain the BBB in a steady state. Ang-1 has a potent antipermeability property and blocks the effect of VEGF-A, whereas Ang-2 may be a naturally occurring inhibitor of Ang-1 activity. In the cold-injury and focal cerebral ischemia models, VEGF-A and Ang-2 were induced and associated with BBB breakdown, and an increase in VEGF-B and Ang-1 and decreases in VEGF-A and Ang-2 were followed by restoration of the BBB. Increased expression of VEGF-A was also reported in the cerebral cortex with BBB disruption at 24 hours and in the cerebral hemisphere with angiogenesis at 48 hours after SAH in rats. However, changes in expression of VEGF-B, Ang-1, and Ang-2 have not been investigated after SAH. This study showed that both VEGF-A levels and BBB disruption were reduced by exogenous OPN at 24 hours, whereas they were increased by blockage of endogenous OPN induction at 72 hours after SAH, suggesting that the protective effect of OPN may occur by suppression of VEGF-A. Levels of VEGF-B, Ang-1, and Ang-2 were not significantly changed after SAH and r-OPN treatment. However, endogenous OPN induction may play a role in keeping Ang-1 levels within a normal range and restoring the BBB at 72 hours after SAH, because blockage of endogenous OPN decreased Ang-1.

MAPK not only mediates the effect of VEGF-A on BBB permeability but also induces VEGF-A expression. According to reports, the expression levels of MAPK after SAH have been diverse, possibly because of limited injury with an irregular pattern in the cerebral cortex. However, inhibition of MAPK by an Src-family kinase inhibitor or a JNK inhibitor consistently suppressed VEGF-A expression and prevented BBB disruption. Ang-1 also interfered with the ability of VEGF-A to disrupt the endothelial barrier or normalized retinal VEGF-A increases in diabetic animals by blocking the
Src-MAPK pathway. Thus, MAPK may have a significant role in BBB disruption both upstream and downstream of VEGF-A. In this study, restoration of the BBB 72 hours after SAH was associated with induction of OPN and the endogenous MAPK inhibitor MKP-1, whereas blockage of endogenous OPN reduced MKP-1 and caused MAPK activation, VEGF-A induction, and increased BBB permeability. In contrast, r-OPN induced MKP-1 and inhibited VEGF-A induction, JNK activation, and BBB disruption at 24 hours after SAH. Taken together, these findings suggest that MKP-1 induction mediates the protective effects of OPN against BBB disruption by inactivating MAPK that may be both upstream and downstream of VEGF-A. This study also demonstrated that r-OPN activated the protective pathways by binding to l-arginyl-glycyl-l-aspartate-dependent integrins. Further studies will clarify the more detailed molecular pathways.

This study is somewhat limited, in that it was not designed to demonstrate that OPN siRNA and r-OPN certainly affects capillaries. This study showed that a delay in OPN induction in reactive astrocytes and capillary endothelial cells restored the normal BBB. However, the morphological demonstration of the mechanisms by which OPN restores the BBB has not yet been performed, and further studies are needed.

In conclusion, we have demonstrated for the first time that a delay in OPN upregulation restores the BBB and that OPN increases MKP-1 and decreases VEGF-A levels in the brain after SAH, suggesting a novel mechanism of OPN-induced stabilization of the BBB. Multiple independent or interconnected signaling pathways may be involved in the protective effects of OPN against BBB disruption. This study showed that r-OPN is a promising treatment after SAH BBB disruption that has complex pathogenesis.

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**Disclosures**

None.
References

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