Intravenous Administration of Bone Morphogenetic Protein-7 After Ischemia Improves Motor Function in Stroke Rats

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Background and Purpose—We and others have previously reported that bone morphogenetic protein-7 (BMP-7), given before middle cerebral artery occlusion (MCAO), reduces ischemic injury in brain. Recent studies have indicated that receptors for BMP are upregulated after brain ischemia. It is possible that this upregulation may facilitate endogenous neurorepair in the ischemic brain. The purpose of this study was to determine the neuroregenerative effects of BMP-7 given parenterally after ischemia/reperfusion injury.

Methods—Adult Sprague-Dawley rats were anesthetized with chloral hydrate. The middle cerebral artery was transiently occluded by a filament inserted through the right internal carotid artery. The filament was removed after 60-minute ischemia to allow reperfusion. Some animals were killed 24 hours after MCAO to examine BMP-7 mRNA expression. Other animals received a single dose of intravenous BMP-7 or vehicle at 24 hours after MCAO and were used for subsequent behavioral studies and BMP-7 immunostaining.

Results—BMP-7 mRNA was upregulated 24 hours after MCAO in untreated animals. BMP-7 immunoreactivity was dose-dependently increased on the ischemic side of the hippocampus/dentate on day 6 after MCAO in animals receiving intravenous injection of BMP-7. Animals receiving BMP-7 also showed a decrease in body asymmetry from day 7 to day 14 and an increase in locomotor activity on day 14 after MCAO.

Conclusions—Our data indicate that BMP-7, given parenterally after stroke, can pass through the blood-brain barrier on the ischemic side and induce behavioral recovery in stroke animals at longer testing times. (Stroke. 2003;34:558-564.)

Key Words: bone morphogenetic proteins ■ growth factors ■ nerve regeneration ■ stroke

Bone morphogenetic protein-7 (BMP-7), a protein in the transforming growth factor-β superfamily, was initially considered to be a trophic factor mainly for nonneuronal tissue. BMP-7 increases proteoglycan synthesis and initiates formation of cartilage and differentiation of bone.1 Bone morphogenetic proteins (BMPs) are highly expressed in adult kidney during ischemia, as well as in fetal kidney during development.2 Systemic application of BMP-7 reduced ischemia-induced infarction and density of apoptotic cells in kidney.3 Recent studies, however, have indicated that effects of BMPs are not limited to the skeletal or urinary system. BMP-7 is also expressed in perinatal neuronal tissues, including hippocampus, cortex, and cerebellum. Exogenous application of BMP-7 to rat mesencephalic cell cultures increases both the number of tyrosine hydroxylase–positive cells and dopamine uptake.4 Receptors for BMP are also expressed in neuronal tissue. BMP receptors can be upregulated in the granule cells of dentate gyrus after transient global cerebral ischemia5 and brain contusion.6 Moreover, intraperitoneal injection of BMP-7 before general hypoxia reduces brain infarction volume and mortality in neonatal rats.7

We have previously found that intracerebral administration of BMP-7, given before middle cerebral artery (MCA) ligation, reduces stroke injury in adult rats.8 Besides its neuroprotective effect, BMP-7 also induces neurorepair in stroke animals. Intracisternal administration of BMP-7 1 day after focal cerebral infarction induces an enhanced recovery of sensorimotor function in the impaired limbs.9,10 Taken together, these data suggest that BMP-7 and its cognate receptor are involved in neuronal protection and restoration of function during ischemia. The purpose of the present study was to determine whether a procedure more relevant to
clinical stroke, involving systemic administration of BMP after focal ischemia, induces functional improvement.

Materials and Methods

Animals and Surgery

Adult male Sprague-Dawley rats (average body weight, 269.8 ± 4.1 g) were used for this study. Animals were divided into 3 groups. Animals in group A (n=12) were killed on day 6 after MCA occlusion (MCAO) (see below) or day 5 after intravenous administration of BMP-7 for immunohistochemical study. Animals in group B (n=46) were used for behavioral and mortality studies up to 50 days after MCAO and BMP-7 administration. Group C animals (n=16) were killed 24 hours after MCAO or sham surgery for BMP-7 mRNA measurements. Animals in group C did not receive BMP-7 injection. Nonstroke controls for RNA study were anesthetized and cut open but did not receive stroke surgery.

MCA Occlusion

Animals were anesthetized with chloral hydrate (0.4 g/kg IP initially and 0.1 g/kg every hour). The use of chloral hydrate has been approved by our Animal Care and Use Committee and allowed a more rapid postoperative recovery. Microfilament (4-O Monosof monofilament nylon, USSC) was coated with rubber-base-impregnation material (Omniflex, GC American Inc) and 0.5 L of 40 g/L paraldehyde (0.1 L/min) in 0.1 mol/L phosphate buffer, pH 7.3. Brains were then removed, postfixed for 2 hours at 4°C, rinsed with phosphate buffer, and sequentially transferred to 100-, 120-, and 150-g/L sucrose solutions. Brains were then frozen on dry ice and sectioned on a cryostat to obtain coronal sections 30 × 10⁻⁵ m thick.

Sections were rinsed with phosphate buffer before processing for immunocytochemistry. Nonspecific binding sites were blocked by incubating sections for 1 hour in phosphate buffer supplemented with 40 g/L bovine serum albumin and 3 g/L Triton X-100. After blocking, sections were incubated in a 1:1000 dilution of a well-characterized anti-BMP-7 monoclonal antibody (1B12, from Curis, Inc) for 24 hours at 4°C. After they were rinsed for 30 minutes in phosphate buffer, sections were processed with an ABC kit (Vector,) and the peroxidase reaction was developed with 0.5 g/L 3,3-diaminobenzidine-4 HCl and 0.3 g/L hydrogen peroxide. All microscopic observations were made by blinded observers. The immunoreactivity of BMP-7 in hippocampus/dentate was quantified with the use of Scion Image 4.02 (PC version of NIH Image).

BMP-7 mRNA Expression

BMP-7 mRNA expression in the cortex and striatum/hippocampus was compared between ischemic and control animals by reverse transcription–polymerase chain reaction (RT-PCR) with the use of 18S rRNA as an internal control. Eight hours after MCAO, the brain tissues were dissected free and frozen on dry ice. Total RNA was extracted with the use of Trizol (Life Technology). RNA samples (5 × 10⁻⁶ g) were reverse transcribed with the use of random primers at an annealing temperature of 65°C (You-Prime-the-First-Strand kit, Amersham). The resultant cDNA was amplified by PCR (DNA Engine Tetrad Thermocycler, MJ Research) for 26 one-minute cycles at an annealing temperature of 55°C and a denaturing temperature of 94°C. As a negative control, samples were amplified by PCR without prior RT. Primers used for amplification of BMP-7 cDNA were designed to unique sequences of rat BMP-7 with the use of the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The specific primer sequences were BMP-7 sense 5'-ATTTACAGCCTGGACAAACGAG-3' and BMP-7 antisense 5'-TGAAAGATCAAACCGGAAC-3'. The primer sequences used to analyze 18S rRNA were sense 5'-GTAAACCCGTGGACCCATT-3' and antisense 5'-CCATCAATTGGTAGGCG-3'. After synthesis, PCR products were assessed by gel electrophoresis (10 g/L agarose) and quantified by densitometry (Stratagene Eagle Eye II) by means of computerized image analysis (Scion).

Statistical Analysis

Statistical analyses were performed with the χ² test and 1-way or 2-way ANOVA, and post hoc Newman-Keuls test was used for statistical comparison. Significance was inferred at P < 0.05. Data are presented as mean ± SEM.

Results

Mortality

Animals were used for behavioral studies up to 50 days after MCAO. We found that BMP-7 treatment did not alter the mortality rate (Table; P = 0.891).

Motor Behavior

Body Asymmetry

An elevated body swing test was used to evaluate body asymmetry before MCAO and on days 1, 7, 10, 14, 21, and 50 after MCAO. All animals developed significant body asymmetry 1 day after MCAO (P < 0.05, 1-way ANOVA; Figure
1A). There was a spontaneous improvement in body asymmetry in vehicle control animals after MCAO (Figure 1). Intravenous administration of a high dose (10^-2 g/kg) of BMP-7 significantly reduced body asymmetry after MCAO (Figure 1A; P<0.05, 2-way ANOVA plus Newman-Keuls test). Animals receiving a high dose (10^-2 g/kg) of BMP-7 had an earlier onset of behavioral normalization on day 7 after MCAO (Figure 1), while animals receiving lower doses (10^-3 and 10^-4 g/kg) of BMP-7 developed significant reductions in body asymmetry between days 7 and 14 after MCAO (P<0.05, 1-way ANOVA plus Newman-Keuls test).

**Locomotor Activity**

Locomotor activity was examined before MCAO and on days 7, 14, 21, and 50 after MCAO. All stroke animals developed a significant reduction in vertical movement on day 7 after stroke (Figure 1B to 1D; *P<0.05 ANOVA*). Similar to the body asymmetry test, there was a spontaneous improvement in locomotor activity in stroke animals that received vehicle. Animals receiving BMP-7 at a dose of 10^-4 g/kg had significantly enhanced locomotor activity compared with the vehicle-treated controls on day 14 after MCAO (Figure 1C and 1D; *P<0.05, 2-way ANOVA*). These behavioral parameters were not significantly enhanced in animals receiving higher doses of BMP-7 (10^-3, 10^-2 g/kg).

**BMP-7 Immunohistochemistry**

A total of 12 animals (n=3 per group), given various doses of BMP-7 (0, 10^-4, 10^-3, 10^-2 g/kg IV), were killed 6 days after MCAO. There was a dose-dependent increase in BMP-7 activity on the lesioned side of the hippocampus and dentate after exogenous application of BMP-7; the greatest increase was found in the stroke animals receiving 10^-2 g/kg BMP-7 (Figure 2A to 2D). The density of BMP-7 immunoreactivity in the lesioned side of the hippocampus/dentate was quantified in all slices and then normalized by comparison with the activity in the corresponding area in the control hemisphere. We found that there is a good linear correlation between the increase in BMP-7 density and dose of BMP-7 applied (BMP-7 activity=107.252+49.718×[dose in 10^-2 g/kg of BMP-7]; *P<0.05*, r=0.665). At higher magnification, BMP-7–labeled neurons were detected in the stratum pyramidale (Figure 3) and stratum oriens in CA3 (Figure 3) on the ischemic side. No

### Mortality Animals With Different Doses of BMP-7 Posttreatment Within 50 Days After Stroke

<table>
<thead>
<tr>
<th>Dose (g/kg)</th>
<th>Animals Used, n</th>
<th>Animals That Died Within 50 Days After Stroke, n</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>10^-4</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>10^-3</td>
<td>13</td>
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<tr>
<td>10^-2</td>
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\[P=0.891, \text{chi-square test.}\]

was significantly reduced in all stroke animals on day 7 after MCAO (P<0.05 vs nonstroke animals, 1-way ANOVA). Low doses of BMP-7 (10^-4, 10^-3 g/kg) significantly enhanced vertical activity (P<0.05 vs vehicle group, 2-way ANOVA). C, Animals receiving BMP-7 also spent significantly longer time moving vertically after MCAO. D, Vertical movement, the frequency of animal rearing up over 60 minutes, was also significantly enhanced in stroke animals receiving BMP-7 (P<0.05 vs vehicle group, 2-way ANOVA). Unlike the improvement in body asymmetry, animals receiving a low dose (10^-4 g/kg) of BMP-7 developed significant locomotor behavioral improvement (B to D) on day 14 (P<0.05, 1-way ANOVA plus Newman-Keuls test).
BMP-7–positive cells were found in the ipsilateral hippocampus/dentate in the stroke animals receiving vehicle (Figure 2E) or in the contralateral nonlesioned hippocampus/dentate in animals receiving BMP-7 (Figure 3).

Expression of BMP-7 mRNA
A total of 16 animals (8 stroke, 8 nonstroke controls) were used to assess the effects of ischemia on expression of BMP-7 transcripts and 18S rRNA in the striatum/hippocampus and

Figure 2. Dose-dependent increase in BMP-7 immunoreactivity in hippocampus/dentate in animals receiving exogenous BMP-7. Animals were injected with BMP-7 (A, vehicle; B, 10^{-4} g/kg IV; C, 10^{-3} g/kg IV; D, 10^{-2} g/kg IV) through the tail vein 24 hours after MCAO. Animals were killed on day 6 after MCAO. There was a dose-dependent increase in BMP-7 immunoreactivity in the lesioned side of the hippocampus and dentate (A to D). At higher magnification, BMP-7–positive cells can be found in the dentate in an animal receiving BMP-7 (10^{-2} g/kg). F, No BMP-7–positive cell was found in the hippocampus/dentate in the animals receiving vehicle (E). Bar=560×10^{-6} m in A to D; bar=70×10^{-6} m in E and F.

Figure 3. BMP-7 immunoreactivity in hippocampus after MCAO and intravenous injection of 10^{-2} g/kg BMP-7. A, At low magnification, BMP-7 immunoreactivity was detected in all subdivisions of the hippocampus and dentate gyrus (DG) on the ischemic side (brown label) but not on the contralateral side. B to E, At intermediate (B) and higher (D) magnifications, labeled neurons were detected in the stratum pyramidale (SP; small arrows in E) and stratum oriens (SO; large arrows in E) in CA3 on ischemic side. C and E. Lack of BMP-7 immunoreactivity in CA3 on the nonischemic side. Bar=425×10^{-6} m in A; bar=75×10^{-6} m in B and C; bar=38×10^{-6} m in D and E.
In this study we found that parenteral administration of BMP-7 1 day after MCAO reduced body asymmetry and enhanced locomotor activity in stroke animals. Previous studies have indicated that maximal cerebral infarction can be achieved 24 hours after reperfusion and that BMP-7, given intracerebroventricularly 1 day after MCAO, does not attenuate the volume of infarction. On the other hand, we found that there is a behavioral improvement in animals receiving poststroke BMP-7 injection. Such behavioral normalization is therefore probably not related to the changes in the volume of cerebral infarction. We and others previously reported that BMP-7, given centrally, did not alter blood pressure, blood gas, and electrolytes. It has also been demonstrated that intravenous administration of BMP-7 did not alter serum electrolytes. We also found that BMP-7, at doses between 2.5×10^−3 and 2.5×10^−1 g/kg, did not alter blood Na⁺, K⁺, Ca²⁺, hemoglobin, cholesterol, glucose, total protein, bicarbonate, GOT, or creatinine levels (data not shown), suggesting that improvement in behavior is not secondary to the indirect changes in liver, pancreatic, or kidney function.

We found that there is a dose-dependent decrease in body asymmetry after BMP-7 treatment starting from day 7 after ischemia. The long latency of this BMP-7-mediated behavioral recovery may be related to several factors. First, in relation to the pharmacodynamic properties of BMP-7, it has been reported that BMP-7 stimulates bromodeoxyuridine incorporation into glial cells, resulting in proliferation of immature glial cells and increasing astrocyte numbers in vitro. Inhibition of bromodeoxyuridine incorporation into the glial cells abolishes BMP-7–induced trophic effects on midbrain dopamine neurons. These data suggest that BMPs have trophic effects that are indirectly mediated through activation of glial-derived factors. Supporting this hypothesis are reports that BMPs selectively promote the differentiation of oligodendroglial-astroglial progenitor cells into astrocytes.

It is thus possible that the effects of BMP-7 are indirectly mediated through the activation of astroglia, which would delay its onset of action. Second, our preliminary data have indicated that BMP-7 can elicit new neurite outgrowth. The behavioral normalization may thus also depend on the generation of new neuronal connections, which would require several days.

We found that there was a spontaneous improvement in locomotor function that occurred 3 weeks after MCAO in vehicle controls. There was a significant increase in vertical movement in the animals that received a low dose of BMP-7. Such a response, however, was not dose dependent. A more prominent recovery was found in animals treated with 10⁻³ g/kg of BMP-7. Doses higher than 10⁻¹ g/kg did not enhance locomotor activity. Similar non–dose-dependent responses were reported with BMP–induced neuroprotection in primary cortical cultures and BMP–mediated ureteric bud development. The reason for this disparity of dose dependency between body asymmetry and locomotor behavior is not clear. Previous studies have indicated that intracisternal injection of BMP-7, given on days 1 and 3 after MCAO, reduced forelimb use asymmetry but did not induce significant improvement in the adhesive removal test, a sensory function test. It has been suggested that recovery in certain motor functions can be affected by muscle strength in the
MCA-occluded rat. It is also possible that high doses of BMP-7 may affect locomotor scores through indirect motor or sensory functions.

Previous studies have indicated that the blood-brain barrier is open transiently after ischemia. This occurs within 0 to 4 hours after the onset of reperfusion. There is a second phase of reopening starting 22 hours after MCAO. In this study we injected BMP-7 at 24 hours after stroke. We found that BMP-7 immunoreactivity was present on the ischemic side in cortex, striatum, and hippocampus. No BMP-7 immunoreactivity was found in the contralateral hemisphere. These data suggest that the increase in BMP-7 immunoreactivity is specifically related to the ischemia-induced blood-brain barrier opening. We also found that the increased BMP-7 immunoreactivity in the ischemic hippocampus was dose dependent.

There was no significant increase in BMP-7 immunoreactivity in the ipsilateral hippocampus in vehicle-treated animals 6 days after MCAO. We found that there was a much greater increase in BMP-7 immunoreactivity in stroke animals given systemic BMP-7. These data suggest that the BMP-7 immunoreactivity detected in this study was mainly derived from exogenous application. Previous studies have indicated that receptors for BMP are upregulated after ischemic brain injury and brain contusion. We found that pyramidal neurons in the ischemic hippocampal CA3 regions showed marked BMP-7 immunoreactivity. It is possible that ischemia upregulates BMP receptors and thus increases the effects of exogenous BMP-7 on these neurons.

Our RT-PCR data indicate that BMP-7 mRNA in cortex and striatum/hippocampus is upregulated 24 hours after MCAO and suggest that there is an upregulation of endogenous BMP-7 expression early after stroke. As noted above, we also found enhanced BMP-7 immunoreactivity in the ipsilateral hemisphere 6 days after stroke, indicating that there is also an accumulation of exogenously administered BMP-7 protein in the lesioned hemisphere. Since BMP receptors are upregulated after ischemia, the binding of exogenous BMP-7 protein to its receptors in the ischemic hemisphere may be enhanced in stroke animals.

We found that there is also an upregulation of BMP-7 mRNA in the contralateral hemisphere after stroke. Several reports indicate that the contralateral hemisphere can compensate for the functions of the lesioned hemisphere after stroke. For example, electrophysiological excitability was increased in the neocortex contralateral to infarction after MCAO. Intracortical injection of basic fibroblast growth factor enhanced recovery of sensorimotor function and immunoreactivity of growth-associated protein 43 (GAP-43), a molecular marker of axonal sprouting, in the intact sensorimotor cortex contralateral to cerebral infarcts. The early contralateral upregulation of BMP-7 mRNA and/or other factors suggests that endogenous neuroreparative processes can be activated from the nonischemic hemisphere after stroke via these trophic factors.

We have previously demonstrated that pretreatment with BMP-7 or BMP-6 reduces ischemia/reperfusion-induced cerebral infarction and reduces behavioral deficits, suggesting that BMPs may have neuroprotective effects against brain ischemia. In this study we found that BMP-7 has additional neurorestorative function. Parenterally administered BMP-7, given after stroke, entered into the ischemic brain areas and reduced abnormal motor behavior in stroke animals. These data may have clinical implications insofar as systemic administration of BMP-7 after stroke could improve behavioral outcomes in such patients.

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References


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