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Bone Marrow Stromal Cells Induce BMP2/4 Production in Oxygen-Glucose-Deprived Astrocytes, Which Promotes an Astrocytic Phenotype in Adult Subventricular Progenitor Cells

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Abstract

Bone morphogenetic proteins (BMPs) affect cell proliferation and differentiation. Astrocytes in ischemic brain are highly responsive to bone marrow stromal cell (BMSC) treatment. We investigated the effects of BMSCs on astrocytes cultured under oxygen- and glucose-deprived conditions, which in part simulate in vivo stroke conditions, to test the hypothesis that BMSCs alter astrocytic expression of BMPs which may contribute to neurological functional recovery of stroke. Quantitative real-time RT-PCR showed that the expression of BMP2/4 mRNAs decreased within ischemic astrocytes. In contrast, BMP2/4 mRNA was significantly increased after cocultured with BMSCs. Western blotting also confirmed this increase at the protein level in the medium of ischemic astrocytes after coculture with BMSCs. As a source of neural stem and progenitor cells, cultured subventricular zone (SVZ) neurospheres exposed to medium obtained from ischemic astrocytes cocultured with BMSCs were significantly enriched in cells expressing the astrocytic marker glial fibrillary acidic protein (GFAP), but not at the expense of β -III-tubulin-positive SVZ neuroblasts. The expression of BMP2/4 subsequently increased the phosphorylation of downstream effector Smad1 and the expression of notch signal pathway-induced protein Hes1 in cultured SVZ neurospheres. BMP antagonist Noggin blocked the elevation of phosphorylated Smad1 and the expression of Hes1 as well as reducing the percentage of astrocytic SVZ progenitor cells. Our results indicate that BMSCs increase BMP2/4 expression in ischemic astrocytes. These changes enhance subventricular progenitor cell gliogenesis by activating relevant signaling pathways. BMSC-stimulated signaling of endogenous astrocytes may alter the ischemic environment, promoting remodeling of brain and hence, improve functional recovery after stroke.

Keywords

BMSC; BMPs; astrocyte; ischemia; differentiation

Bone marrow stromal cells (BMSCs) improve neurological functional recovery of rats and human beings subjected to stroke (Chen et al., 2001; Chopp and Li, 2002; Bang et al., 2005). However, the mechanisms underlying this restoration are unclear. Although transplanted BMSCs express neural markers, in taking into account the small population of these BMSCs

in the brain, it is unlikely that BMSCs improve functional recovery by replacing lost neural cells after stroke (Li et al., 2002). To gain insight into alternate mechanisms that underlie the functional benefit of BMSCs in the treatment of stroke, we tested the effects of BMSCs on the expression of bone morphogenetic proteins (BMPs) in astrocytes subjected to oxygen and glucose deprivation (ODG) in vitro, to simulate ischemic-like conditions. BMPs regulate important processes in the developing nervous system (Mehler et al., 1997, 2000; Lein et al., 2002). BMP2, BMP4, and BMP7 are the focus of this study, because they have been implicated as key regulators of cell fate neuronal cell survival, cell shape, and transmitter phenotype formation (Karsenty et al., 1996; Jordan et al., 1997; Withers et al., 2000; Chalazonitis et al., 2004; Qi et al., 2004).

BMPs initiate intracellular signaling and effect cell differentiation via binding to two kinds serine/threonine kinase receptors (BMPR), type I and type II (Ebendal et al., 1998). Subtype A of BMPR I has a high affinity for BMP2/4 and plays an important role in signal transduction (Davis et al., 2004; Keller et al., 2004). As the immediate downstream molecules of the BMP receptors, Sma- and Mad-related proteins (Smad)-1, -5, and -8 are effectors of BMP function. BMP binding to its receptors induces phosphorylation of Smad1, which enhances the binding of Smad1 to cAMP response element-binding protein and thereby stimulates Smad1-dependent transcription (Kretzschmar et al., 1997). The Notch signaling pathway instructively induces glial differentiation (Lundkvist and Lendahl, 2001). Insofar as activated Smad1 contributes to BMP2-mediated enhancement of Notch-induced Hes5 expression, BMPs may regulate the Notch signal pathway in the process of cell determination (Takizawa et al., 2003).

Neurogenesis is present throughout adulthood (Gage, 2002), and this process is amplified after stroke (Zhang et al., 2001; Sharp et al., 2002; Kokaia and Lindvall, 2003; Parent, 2003). New cells in the subventricular zone (SVZ), a major source of stem and progenitor cells in adult brain, migrate to the ischemic boundary zone (IBZ) and integrate into the tissue (Zhang et al., 2004). Thus, the newly formed cells may remodel the brain and contribute to improved neurological function. BMSC treatment may increase and influence this neurogenesis by changing the microenvironment in the IBZ.

In this study, we investigated the effects of BMSCs on astrocytes subjected to ODG to mimic the environment of the IBZ, and these astrocytes were subsequently cocultured with BMSCs. Expression of BMPs (BMP2, BMP4, and BMP7) in ischemic astrocytes cocultured with or without BMSCs were examined. Moreover, to investigate whether expression of BMPs affected neural stem and progenitor cell differentiation, SVZ neurospheres were cultured with conditioned media collected from various treatment groups, and the relevant signal transduction pathways were investigated.

MATERIALS AND METHODS

Normal Cell Culture

ACI rat BMSCs (passage 3; provided by Cognate Inc., Baltimore, MD) were cultured in HyQ MEM alpha modification medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 100 U/ml penicillin, and 100 µg/ml streptomycin. Rat cortical astrocytes, phenotype 1 (American Type Culture Collection; ATCC), were cultured in DMEM (Gibco) containing high glucose (4.5 g/liter glucose), 10% FBS, and penicillin and streptomycin. All normal cultured cells were maintained in a humidified tissue culture incubator at 37°C with 5% carbon dioxide, using 0.25% trypsin/1 mM EDTA to detach and passage cells every 3 days.

Astrocytes and In Vitro OGD

Astrocytes (1×10^5) were seeded in each well of a six-well plate containing normal medium until they were attached. Normal medium was then replaced with nonglucose culture medium, and the astrocytes were cultured in an anaerobic chamber (model 1025; Forma Scientific) for 3 hr. The oxygen level within the anaerobic chamber was routinely measured with a BD Disposable Anaerobic Indicator (Becton, Dickinson and Company, Sparks, MD), which showed that the oxygen level remained below 0.2%. Subsequently, astrocytes were cultured in a humidified tissue culture incubator at 37°C with 5% carbon dioxide with or without BMSCs. For BMSC coculture, an upper chamber of a transwell insert dish (0.4- μ m pores; Becton Dickinson Labware; Falcon) was used to ensure separation of the BMSCs and astrocytes. The ratio of cocultured BMSCs to astrocytes was 1:100 (BMSCs:astrocytes). Astrocytes cocultured with or without BMSCs were detached and collected for RNA extraction after 6 hr.

Conditioned Medium Preparation

Modified DMEM-F12 medium made from DMEM-F12 medium (Invitrogen, La Jolla, CA) containing L-glutamine (2 mmol/liter), glucose (0.6%), putrescine (9.6 mg/ml), insulin (0.025 mg/ml), progesterone (6.3 ng/ml), apotransferrin (0.1 mg/ml), and sodium selenite (5.2 ng/ml) was used as basal culture medium. This medium was added to various culture cells to obtain the conditioned media. There were five conditioned media: 1) normal differentiation medium, i.e., basal culture medium; 2) medium from BMSC alone culture (1×10^3 BMSCs); 3) medium from normal cultured astrocytes (1×10^5 astrocytes); 4) medium from ischemic astrocytes alone (1×10^5 astrocytes); and 5) medium from ischemic astrocytes cocultured with BMSCs (1×10^5 astrocytes and 1×10^3 BMSCs). After 48 hr in culture, all the conditioned media were filtered with a 0.22- μ m-pore filter and were placed on the plated SVZ neurospheres.

Stem and Progenitor Cell Culture and Differentiation

SVZ neurosphere cells were taken from normal male Wistar rats ($n = 3$, 3–4 months old; Charles River Breeding), as previously reported (Chiasson et al., 1999), then incubated in a humidified tissue culture incubator at 37°C with 5% carbon dioxide in basal culture medium containing 20 ng/ml epidermal growth factor (EGF; R&D Systems, Minneapolis, MN) and basic fibroblast growth factor (bFGF; R&D Systems). The medium was changed and the cells were passaged every 4 days. The SVZ neurospheres were cultured with normal differentiation medium or the conditioned media described above for 4 days, and then immunofluorescent staining was performed for morphological identification. To detect the relevant signal transduction proteins in these cells, Western blotting was processed after culturing for 48 hr. Recombinant mouse Noggin (a high-affinity BMP binding protein that antagonizes BMP bioactivity, 0.3 μ g/ml; R&D Systems) was used in these experiments to reduce the effect of BMPs.

Quantitative Real-Time RT-PCR

By following the user's manual of the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA), RNA was isolated from the astrocytes after various treatments, as described above. The cDNA synthesis was carried out with Moloney murine leukemia virus (MMLV) Superscript II Reverse transcriptase (Invitrogen), following the manufacturer's instructions. Quantitative PCR was performed in an ABI Prism 7000 Sequence Detection System, using the following protocols with Quantitect Syby Green PCR Kit (Qiagen, Valencia, CA). The samples underwent PCR (45 cycles) consisting of the following steps: denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. Oligo-nucleotide primers were designed for rat BMP2 (sense primer: 5'-TGG TGT CCA ATA GTC TGG

TC-3'; antisense primer: 5'-CGA AGA AGC CAT CGA GGA AC-3'), rat BMP4 (sense primer: 5'-GTC ACA TTG TGA CGG ACT AG-3'; antisense primer: 5'-ACG AAG AAC ATC TGG AGA AC-3'), rat BMP7 (sense primer: 5'-CCA AAG AAC CAA GAG GCA CTG-3'; antisense primer: 5'-TGG AGC TGT CGT CGA AGT AGA G-3'), and rat β -actin: (sense primer: 5'-CCA TCA TGA AGT GTG ACG TTG-3'; antisense primer: 5'-CAA TGA TCT TGA TCT TCA TGG TG-3'). The gene expression ratio of astrocytes was normalized with β -actin gene expression, and the normalized gene expression ratio was compared with that of normal cultured astrocytes. Each gene analysis was performed on three different samples.

Western Blotting

To concentrate the BMP2 and BMP4 in the media secreted by astrocytes, conditioned media (10 ml), made as described above, were incubated with 0.25 g heparin sepharose CL-6B (Amersham Biosciences, Ghent, Belgium) at 4°C for 24 hr. After centrifugation (700g), the supernatant was removed, and bound proteins were eluted in the same volume of loading buffer (glycerol 10% v/v; Tris 0.05 M, pH 6.8; 2% sodium dodecyl sulfate; bromophenol blue; and 2.5% v/v mercaptoethanol) by heating at 70°C for 10 min. The same volumes of these elutions were loaded on 10% Bis-Tris Gels (Invitrogen), and Western blotting was performed. To measure the various proteins in the SVZ neurospheres treated with the conditioned media for 48 hr, as described above, the cells were harvested and rinsed with PBS, then lysed in RIPA lysis buffer containing proteinase inhibitor cocktail (Roche, Indianapolis, IN). Equal amounts (20 μ g) of protein, as determined by using the bicinchoninic acid (BCA) protocol (Pierce, Rockford, IL), were loaded on 10% Bis-Tris gels and then processed for a standard Western blotting process. Primary polyclone antibodies were used in these experiments, including BMP2/4 (R&D Systems), BMPR-I (H-45), Smad1/5/8 (N-18), p-Smad1 (Ser 463/Ser 465, which can react with Ser 463 and Ser 465 phosphorylated Smad1, -5, and -8), Hes1 (N-17), and Hes5 (M-16) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The working dilution of all the above-mentioned primary antibodies was 1:1,000. A mouse monoclonal antibody of β -actin (Abcam, Cambridge, United Kingdom) was used as the internal control (working dilution 1:2,000). Respective secondary antibodies were applied following the protocol of Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and used for Western blotting. Enhanced chemiluminescence (ECL) detection was used according to manufacturer's instructions (Boehringer, Mannheim, Germany). Semiquantitative analysis of the protein was performed by using the Scion Image Beta 4.0.3 software on the bands of the Western blots, and the protein ratios were calculated by comparison with β -actin expression.

Immunofluorescent Staining

The SVZ neurospheres were cultured for 4 days in eight-well chamber slides (Nalge Nunc International, Naperville, IL) with the experimental conditioned media, as described above. After removal of the media and rinsing with PBS, the cells were fixed with 100% methanol at -20°C for 15 min and then permeated with 0.2% Triton X-100 plus 1% normal goat serum (NGS) in PBS, pH 7.3, for 5 min. Cells were incubated with primary antibody for 24 hr at 4°C and then reacted with secondary antibodies for 2 hr at room temperature. A polyclonal rabbit anti-GFAP (Dako Inc., Carpinteria, CA) or a mouse anti-TUJ1 (Covance Inc.) antibody diluted in blocking buffer at a dilution 1:500 and a secondary antibody (Cy3-conjugated goat anti-rabbit or FITC-conjugated goat anti-mouse; Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:100 were used. Total cell number was measured by staining cell nuclei with DAPI. The fraction of positive cells was determined by counting five nonoverlapping microscopic fields (± 20 cells/field, ± 100 cells/experiment). The number of slides in each group was considered as the sample number (n).

Statistical Analysis

Data were analyzed by using a paired *t*-test. All values are presented as mean $\pm$ SD. Statistical significance was set at $P < 0.05$.

RESULTS

BMSCs Increase the Expression of BMP2 and BMP4 in Ischemic Astrocytes

Quantitative RT-PCR results showed that the mRNA level of BMP2 only slightly decreased in ischemic cultured astrocytes compared with normal cultured astrocytes (from 1.00 ± 0.24 to 0.86 ± 0.27), but BMSCs significantly increased the mRNA level of BMP2 in ischemic cultured astrocytes (from 0.86 ± 0.27 to 2.10 ± 1.39 ; $P < 0.05$). The mRNA level of BMP4 decreased in ischemic cultured astrocytes compared with normal cultured astrocytes (from 1.00 ± 0.13 to 0.32 ± 0.14 ; $P < 0.01$), and BMSCs partially restored the expression ratio of BMP4 (from 0.32 ± 0.14 to 0.58 ± 0.11 ; $P < 0.05$) in ischemic astrocytes. No significant difference was observed in BMP7 mRNA levels under different culture conditions (Fig. 1A). The plot analysis data of the Western blotting bands also showed that the total concentration of BMP2/4 decreased in the medium of ischemic astrocytes compared with that from normal culture astrocytes (from 108.61 ± 1.54 to 43.78 ± 2.27 ; $P < 0.01$); however, BMSCs significantly increased BMP2/4 to nearly normal levels (from 43.78 ± 2.27 to 107.54 ± 1.16 ; $P < 0.01$) in the medium of ischemic astrocytes. Although BMP2/4 was expressed in medium derived from BMSCs culture alone (22.56 ± 1.44), the magnitude of the increase of BMP2/4 in ischemic astrocyte medium was not due to BMP2/4 secretion by BMSCs (Fig. 1B).

BMP2 and BMP4 Promote an Astrocytic Cell Fate in SVZ Progenitor Cells In Vitro

Figure 2A,B, respectively, shows the representative GFAP-positive cells and TUJ1-positive cells differentiated from the SVZ neurospheres when cultured in the conditional media. Table I and Figure 2C show the percentage of GFAP-positive and TUJ1-positive cells differentiated from the SVZ neurospheres. Compared with culture with the normal differentiation medium, medium obtained from cultured BMSCs alone had no effect on the percentage of GFAP-positive cells, and medium obtained from normal cultured astrocytes significantly increased the percentage of GFAP-positive cells ($P < 0.01$). Medium obtained from ischemic astrocytes alone decreased the percentage of GFAP-positive cells to a level comparable to that in normal differentiation medium; however, medium obtained from ischemic astrocytes cocultured with BMSCs significantly increased the percentage of GFAP-positive cells compared with that from ischemic astrocytes alone ($P < 0.01$) or normal differentiation medium ($P < 0.01$). The percentage of TUJ1-positive cells significantly increased only when the SVZ neurospheres were cultured with medium obtained from BMSCs alone ($P < 0.05$). However, promotion of an astrocytic cell fate did not occur at the expense of TUJ1-reactive neuroblasts. In fact, in those cultures enriched for GFAP, the fraction of TUJ1-positive cells either did not change or increased as well. To verify that the BMP2 and BMP4 pathway alters differentiation of the SVZ neurospheres, we used Noggin as a BMP antagonist to reduce the affect of BMP2 and BMP4. After Noggin treatment, compared with non-Noggin-treated neurospheres, the percentage of GFAP-positive cells significantly decreased when cultured with media obtained from normal culture astrocytes and cocultured ischemic astrocytes-BMSCs (both $P < 0.001$). Noggin had no apparent effect on the percentage of TUJ1-positive cells in the SVZ neurospheres cultured with all conditioned media.

BMP2/4 Signal Pathway in the SVZ Neurospheres Is Activated by the Conditioned Media of Astrocytes Cocultured With BMSCs

To test the downstream protein expression controlled by BMP2 and BMP4, we cultured the SVZ neurospheres with various conditioned media, and Western blotting was used to measure the changes of downstream protein levels of the BMP2 and BMP4 signal transduction pathway in these cells. Figure 3 shows the Western blotting results of the protein levels. The levels of BMPR1a and total Smad1/5/8 in the SVZ neurospheres treated under all culture conditions were constant. The level of phosphorylated Smad1 significantly decreased after culture with the medium from ischemic cultured astrocytes compared with that from normal cultured astrocytes ($P < 0.01$). Medium from ischemic astrocytes cocultured with BMSCs significantly elevated this phosphorylation to a higher level than that from ischemic astrocytes ($P < 0.01$). This increase was larger than the additive effect of p-Smad induced by BMSCs alone medium (Fig. 3B). These data indicate that BMSCs may regulate the process of Smad phosphorylation in ischemic astrocytes. To explore the cross-talk of the BMP signal pathway and the Notch signal pathway, we examined the expression of Hes1 and Hes5, which are controlled by the Notch signal pathway and contribute to astrocytic differentiation (Morrison, 2001; Wu et al., 2003). We found that the expression of Hes1 was significantly decreased in the SVZ neurospheres with medium from ischemic astrocytes, and it was significantly increased with medium from cocultured astrocytes-BMSCs (both $P < 0.01$); however, the expression of Hes5 did not change (Fig. 3B). Noggin prevented changes of Smad1 phosphorylation and Hes1 expression. These data show that the BMP2/4 signal pathway influences the Notch signal pathway in stem and progenitor cell differentiation.

DISCUSSION

We and others have demonstrated that BMSCs improve functional recovery in rat stroke models (Chen et al., 2001; Li et al., 2001, 2005; Zhao et al., 2002), and this improvement may be due to the factors secreted (Chen et al., 2002) or induced in parenchymal cells by BMSCs (Li et al., 2002), in that they evoke increased expression of brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and bFGF in astrocytes (Gao et al., 2005). In the present study, we found that BMSCs change the BMP expression in astrocytes in vitro. These data suggest that the factors secreted from BMSCs can subsequently up-regulate the BMP expression of astrocytes in the ischemic rat brain.

There are two important sites in the injured brain that can play a role in restoring neurological function after stroke, the IBZ and the SVZ (Fig. 4). BMSCs systemically injected into the rat after stroke can enter the cerebral parenchyma through the blood-brain barrier. These cells migrate and are directed to sites of hypoxic-ischemic brain damage. Most BMSCs (>80%, the percentage is route, dose, and time dependent) survive and are localized to the IBZ, compared with the small number of BMSCs (<5%) localized in the SVZ of the ipsilateral hemisphere, a relatively intact region remote from the lesion (Chen et al., 2001, 2003a,b; Li et al., 2002; Guan et al., 2004). Given the minuscule ratio of BMSCs to brain neural cells, it is likely that the beneficial effects of BMSC therapy are attributable to the activation of parenchymal cells by factors secreted from BMSCs.

BMP-induced astrocyte differentiation is mediated in part through the modulation of the gap junction protein connexin43 (Cx43) expression and gap junction-mediated intercellular communication (Zhang et al., 2002). Cx43 is the primary component of intercellular channels in astrocytes. Communications through gap junction channels provide a major signaling mechanism during early brain histogenesis (Rozental et al., 2000). Gap junctions function as electrotonic synapses between neurons as pathways for the exchange of metabolites and second-messenger molecules between glial cells (Reuss and Unsicker,

1998). BMP2/4, Cx43, and synaptophysin expressions were significantly increased in the IBZ in rats treated with BMSCs compared with controls. The BMP2/4 and Cx43 expression correlated with the synaptophysin expression. Most BMP2/4- and Cx43-positive cells were colocalized with GFAP-positive cells. The astrocyte differentiation from SVZ progenitor cells may promote functional recovery by guiding the new neuron migration toward the ischemic boundary zone, as in developing brain. In addition, the newly formed astrocytes may provide trophic support for the neurons in the periinfarct area.

The Notch signaling pathway plays an instructive role in driving astrocyte differentiation (Wang and Barres, 2000; Scheer et al., 2001; Gaiano and Fishell, 2002). The Notch effector Hes1 can drive precursor cells to an astrocyte cell fate at the expense of oligodendrocyte differentiation (Wu et al., 2003). Overexpression of Notch1 has an effect on gliogenesis similar to that of Hes1 and the mRNA levels of Hes1 are up-regulated in cells overexpressing Notch1 (Wu et al., 2003). Previous studies have also shown that BMP signals are linked to both astrocyte (Gross et al., 1996; Nakashima et al., 1999) and neuron (Li et al., 1998; Mabie et al., 1999; Lopez-Coviella et al., 2000) differentiation and proliferation. BMP2 and BMP4 antagonize sonic hedgehog-mediated proliferation of cerebellar granule neurons through Smad5 signaling, and overexpression of BMPs can induce progenitor cell and stem cell differentiation (Rios et al., 2004). BMP2 activates its downstream effector Smad1 and subsequently up-regulates the expression of Hes5 (Takizawa et al., 2003), so there must be cross-talk between the BMP signal pathway and the Notch signal pathway. Our data show that BMSCs increase BMP2 and BMP4 secreted by astrocytes, and this increase activates the Notch signal pathway, promoting an astrocytic cell fate in SVZ neural progenitors via increasing the expression of Hes1.

Astrocytes can modulate the environment around neurons, regulate neuronal survival during development (Shute et al., 2005), and regulate brain homeostasis, which facilitates axonal conduction, synaptic transmission, and information processing throughout the adult brain (Fields and Stevens-Graham, 2002). Thus, astrocytes likely play an important role in functional recovery after stroke. Our previous *in vivo* study has shown that BMSC treatment increases the number of astrocytic SVZ fibrils that extend into the ischemic striatum, which apparently guide the axonal growth (Li et al., 2005). In this study, we found that the expression of BMP2 and BMP4 in ischemic astrocytes was increased by BMSCs, and this increased expression promotes a GFAP-reactive astrocytic cell type in SVZ progenitors *in vitro*. These data indicate that BMSC treatment may create a microenvironment in the IBZ that facilitates the SVZ-derived progenitor cell differentiation into astrocytes.

Coculture of SVZ progenitor cells with BMSCs increases TUJ1⁺ SVZ neuroblasts, and SVZ stroke-induced neurogenesis is enhanced by BMSC treatment *in vivo* (Chopp and Li, 2002). Our *in vitro* data, however, indicate the BMSCs direct astrocytes to induce differentiation of SVZ progenitor cells toward an astrocytic cell fate. However, in ischemic brain, the SVZ is not in direct contact with ischemic astrocytes, so stroke-induced SVZ neurogenesis might not be antagonized by BMPs produced by astrocytes within the periinfarct region. Additionally, we revealed that astrocytic release of BMPs did not reduce the number of TUJ1-reactive cells. In fact, ectopic BMP4 has been reported to promote the survival of SVZ neuroblasts (Lim et al., 2000). Thus, in the ischemic brain, BMPs produced by astrocytes in the ischemic boundary might enhance the survival of incoming migrating SVZ neuroblasts, in addition to promoting an astrocytic fate of any arriving undifferentiated progenitors. It should also be noted that, in our experiments, we did not employ SVZ cells derived from ischemic brain, and it is possible that ischemia could alter the response of these cells to BMPs.

Our finding that BMSCs increase the percentage of TUJ1-positive cells differentiated from the SVZ neurospheres is in agreement with reports that some factors secreted by BMSCs increase neurogenesis (Chen et al., 2003a,b). Although Notch signal is required for both neuron and glia formation within the cerebellar neuroepithelium (Lutolf et al., 2002), the increase in TUJ1-positive cells in our study was not inhibited by Noggin, indicating that neuronal differentiation of stem and progenitor cells is not attributed to the BMP2 and BMP4 pathways.

In summary, this study demonstrates, in vitro, that BMSCs enhance the expression of BMP2 and BMP4 in ischemic astrocytes and increase the number of GFAP-positive cultured SVZ progenitor cells via the BMP-Smad-Hes1 pathway. This BMSC-directed response in ischemic astrocytes may be a mechanism that contributes to the beneficial effect of BMSC treatment of stroke.

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Fig. 1.

A: Quantitative RT-PCR mRNA expression level of BMP2, BMP4, and BMP7 in astrocytes. The BMP2 mRNA expression in ischemic astrocytes is increased when cocultured with BMSCs. The BMP4 mRNA decreased in the ischemic astrocytes and, cocultured with BMSCs, can partially recover the mRNA-expressing level of BMP4 in ischemic astrocytes compared with ischemic astrocytes alone. There are no significant changes in BMP7 mRNA expression in normal astrocytes and ischemic astrocytes cocultured with or without BMSCs. **B:** Protein level of BMP2/4 in various conditioned media. There is no BMP2/4 detected in the normal differentiation medium and low BMP2/4 in BMSCs alone. The expression of BMP2/4 is decreased in the medium of ischemic astrocytes; BMSCs increase the expression of BMP2/4 in the medium of ischemic astrocytes to a level comparable to that in the medium of normal cultured astrocytes. N, normal differentiation medium; M, BMSCs alone cultured medium; A, normal astrocytes alone cultured medium; AD, ischemic astrocytes cultured medium; ADM, ischemic astrocytes with BMSCs cocultured medium. * $P < 0.05$, ** $P < 0.01$.

Fig. 2.

Immunofluorescence shows representative GFAP-positive cells (A) and TUJ1-positive cells (B) differentiated from the SVZ neurospheres. Among all the conditioned media, the media from BMSCs alone and ischemic astrocytes alone have GFAP-positive cells comparable to those with normal differentiation medium, the media collected from normal astrocytes, and ischemic astrocytes cocultured with BMSCs obviously increase the percentage of GFAP-positive cells compare with the normal differentiation medium. The fraction of TUJ1-positive cells either did not change or increased, and, as with the GFAP positive cells, it significantly increased only when the SVZ neurospheres were cultured with medium obtained from BMSCs alone. Noggin inhibits the effect of BMSCs and decreases the percentage of GFAP-positive cells differentiated from stem and progenitor cell cultured with normal astrocyte medium and ischemic astrocytes-BMSCs coculture medium. N, normal differentiation medium; M, BMSCs alone cultured medium; A, normal astrocytes alone cultured medium; AD, ischemic astrocytes cultured medium; ADM, ischemic astrocytes with BMSCs cocultured medium. *Compared with N, $P < 0.05$; **compared with N, $P < 0.01$; ++compared with AD, $P < 0.01$; #compared with no Noggin, $P < 0.01$; #compared with no Noggin, $P < 0.001$.

Fig. 3.

A: Representative Western blot results show the protein level in the SVZ neurospheres cultured in the various conditioned media. **B:** Semiquantitative results of various protein levels. The levels of BMPR1a and total Smad1/5/8 are constant in the SVZ neurospheres in all culture conditions. The level of p-Smad1 in the SVZ neurospheres decrease after culture with the medium from ischemic cultured astrocytes compared with that from normal cultured astrocytes, but it significantly increased when cultured in the medium of ischemic astrocytes cocultured with BMSCs, and this increase was greater than the effect of p-Smad induced by BMSC-alone medium. A similar response of Hes1 expression in the SVZ neurospheres cultured with conditioned media is found. The expression of Hes5 exhibited no

significant change. Noggin can prevent all changes, as above mentioned. N, normal differentiation medium; M, BMSCs alone cultured medium; A, normal astrocytes alone cultured medium; AD, ischemic astrocytes cultured medium; ADM, ischemic astrocytes with BMSCs cocultured medium. * $P < 0.01$.

Fig. 4. Coronal section sketch of a rat brain subjected to 2 hr of middle cerebral artery occlusion with BMSC treatment. IBZ, ischemic boundary zone, where the majority of BMSCs survive in the brain and located (Chen et al., 2001). IC, ischemic core; SVZ, subventricular zone, where cells can proliferate throughout the life of the rats (Gage, 2002) and are amplified after ischemia (Chen et al., 2001).

TABLE I
Percentage of TUJ1⁺ and GFAP-Positive Cells Differentiated From Cultured SVZ Cells (n = 3)[†]

	N	M	A	AD	ADM
Without Noggin					
TUJ1 ⁺	9.27 ± 2.47	13.85 ± 2.34 [*]	9.11 ± 1.73	8.97 ± 2.30	13.13 ± 5.25
GFAP ⁺	42.42 ± 7.14	44.07 ± 8.82	71.14 ± 6.28 ^{**}	41.75 ± 6.77	73.05 ± 4.86 ^{**++}
With Noggin					
TUJ1 ⁺	9.52 ± 4.13	13.68 ± 4.36	10.41 ± 3.99	10.05 ± 3.23	12.76 ± 4.96
GFAP ⁺	38.95 ± 1.93	40.17 ± 7.94	39.44 ± 10.06 [#]	40.51 ± 5.22	39.87 ± 4.59 ^{##}

[†] N, normal differentiation medium; M, BMSCs alone cultured medium; A, normal astrocytes alone cultured medium; AD, ischemic astrocytes cultured medium; ADM, ischemic astrocytes with BMSCs cocultured medium.

^{*} Compared with N, *P* < 0.05.

^{**} Compared with N, *P* < 0.01.

⁺⁺ Compared with AD, *P* < 0.01.

[#] Compared with no Noggin, *P* < 0.01.

^{##} Compared with no Noggin, *P* < 0.001.