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Down-regulation of neurocan expression in reactive astrocytes promotes axonal regeneration and facilitates the neurorestorative effects of bone marrow stromal cells in the ischemic rat brain

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Abstract

The glial scar, a primarily astrocytic structure bordering the infarct tissue inhibits axonal regeneration after stroke. Neurocan, an axonal extension inhibitory molecule, is up-regulated in the scar region after stroke. Bone marrow stromal cells (BMSCs) reduce the thickness of glial scar wall and facilitate axonal remodeling in the ischemic boundary zone. To further clarify the role of BMSCs in axonal regeneration and its underlying mechanism, the current study focused on the effect of BMSCs on neurocan expression in the ischemic brain. Thirty one adult male Wistar rats were subjected to 2 hrs of middle cerebral artery occlusion (MCAo) followed by an injection of 3×10^6 rat BMSCs (n=16) or phosphate-buffered saline (n=15) into the tail vein 24 hrs later. Animals were sacrificed at 8 days after stroke. Immunostaining analysis showed that reactive astrocytes were the primary source of neurocan, and BMSC treated animals had significantly lower neurocan and higher growth associated protein 43 expression in the penumbral region compared to control rats, which was confirmed by Western blot analysis of the brain tissue. To further investigate the effects of BMSCs on astrocyte neurocan expression, single reactive astrocytes were collected from the ischemic boundary zone using laser capture microdissection. Neurocan gene expression was significantly down-regulated in rats receiving BMSC transplantation (n=4/group). Primary cultured astrocytes showed similar alterations; BMSC coculture during reoxygenation abolished the up-regulation of neurocan gene in astrocytes undergoing oxygen-glucose deprivation (n=3/group). Our data suggest that BMSCs promote axonal regeneration by reducing neurocan expression in peri-infarct astrocytes.

Keywords

bone marrow stromal cells; stroke; axonal regeneration; neurocan; reactive astrocytes

INTRODUCTION

The astrocytic response to CNS injury results in the formation of a “glial scar”, which is characterized by densely populated reactive astrocytes and highly expressed growth-inhibitory molecules (Davies et al. 1999; Ishiguro et al. 1993; Katsman et al. 2003). Bordering the injury site, this structure serves as a physical as well as a biochemical barrier

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that inhibits axonal regeneration. Among the inhibitory molecules, chondroitin sulfate proteoglycans (CSPGs) reduce the ability of axons to regenerate in vivo in areas of reactive gliosis (Davies et al. 1997; Davies et al. 1999; Hoke and Silver 1996).

Neurocan is one of the major CSPGs in the nervous tissue, whose expression and proteolytic cleavage are developmentally regulated in the normal rat brain. Full-length neurocan is expressed along with its proteolytic fragments in the juvenile brain, while only neurocan fragments are detectable in the adult brain (Matsui et al. 1994; Meyer-Puttlitz et al. 1995; Rauch et al. 1991). Growing evidence shows that full-length neurocan molecules reappear and accumulate around various CNS injuries ranging from kainite-induced seizures (Matsui et al. 2002), traumatic lesion (Asher et al. 2000; McKeon et al. 1999) to focal ischemic attack (Deguchi et al. 2005). Taking into account that neurocan expression is localized to reactive astrocytes in vivo (Asher et al. 2000; Deguchi et al. 2005; Matsui et al. 2002; McKeon et al. 1999) and neurocan is avoided by growing axons in vitro (Asher et al. 2000), these experimental findings highlight a role for neurocan during tissue repair and neural network reconstitution after CNS injuries.

Bone marrow stromal cells (BMSCs) are a heterogeneous subpopulation of bone marrow cells including mesenchymal stem and progenitor cells. After a decade of extensive research, the efficacy of BMSC treatment in rodents with ischemic brain injury has been established, and its underlying mechanisms of action has been narrowed down to neuro-restoration rather than neuro-substitution (Chopp and Li 2002). Among all their brain remodeling facilitating effects, BMSCs have been shown to accelerate axonal regeneration (Liu et al. 2007; Shen et al. 2007a; Shen et al. 2006) and decrease the thickness of glial scar wall (Li et al. 2005; Shen et al. 2007a). In the present study, we directed our attention to the interaction of BMSCs with reactive astrocytes in terms of neurocan expression, to shed light on the role reactive astrocytes take in axonal remodeling after stroke and the effects of BMSCs on this process.

MATERIALS AND METHODS

Experiments were performed on 34 adult male Wistar rats (Table 1). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Animal middle cerebral artery occlusion (MCAo) model

Transient MCAo was induced using a method of intraluminal vascular occlusion modified in our laboratory (Chen et al. 1992). Briefly, rats (n=31) were initially anesthetized with 3.5% isoflurane and maintained with 1.0-2.0% isoflurane in 70% N₂O and 30% O₂ using a face mask. The rectal temperature was controlled at 37°C with a feedback regulated water heating system. The right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were exposed. A length of 4-0 monofilament nylon suture (18.5-19.5 mm), determined by the animal weight, with its tip rounded by heating near a flame, was advanced from the ECA into the lumen of the ICA until it blocked the origin of the MCA. Two hours after MCAo, animals were reanesthetized with isoflurane, and reperfusion was performed by withdrawal of the suture until the tip cleared the lumen of the ECA. The following day, the modified neurologic severity scores (mNSS) test was employed to evaluate the neurological function of the animals, and only rats showing obvious deficits were selected for the following studies.

Cell transplantation procedures

Primary cultures of bone marrow stromal cells (Theradigm Inc.) were obtained from young donor adult male rats and BMSCs were separated, as previously described (Shen et al. 2006). At 24 hrs post-ischemia, randomly selected rats received BMSCs. Animals were anesthetized with 3.5% isofurane and then maintained with 1.0-2.0% isofurane in N₂O:O₂ (2:1). Approximately 3×10^6 BMSCs in 1 ml phosphate buffered saline (PBS, n=16) or control fluid (1 ml PBS, n=15) was slowly injected over a 2-3 min period into each rat via the tail vein. All animals were euthanized at 7 days after treatment, among which, 8 brains (n=4/group) were used for laser capture, 6 for Western blot assay (n=3/group), and the other 17 (8 from the PBS group, 9 from the BMSC treatment group) were processed for immunostaining (paraffin or vibratome preparations).

Immunohistochemical assessment

For paraffin embedded brain tissues, a series of 6- μ m-thick slides were cut from a standard paraffin block obtained from the center of the lesion of the forebrain, corresponding to coronal coordinates for bregma $\sim -1 \sim 1$ mm (Paxinos and Watson 1986) for immunostaining. After deparaffinizing, brain sections were placed in boiled citrate buffer (pH 6) in a microwave oven (650 to 720 W). Following blocking in normal serum, sections were incubated with antibodies against neurocan (dilution, 1:800, Chemicon, Temecula, CA), or growth associated protein 43 (GAP43, dilution, 1:50, Abcam, Cambridge, MA) at 4°C overnight. Then, the sections were incubated with avidin-biotin-horseradish peroxidase complex and developed in 3'3' diaminobenzidine tetrahydrochloride (DAB, for neurocan); or visualized with CY3-conjugated secondary antibody (for GAP43). Double immunohistochemical staining was employed to detect the cellular co-localization of neurocan and the astrocyte marker, glial fibrillary acidic protein (GFAP, dilution, 1:10,000, Dako, Carpinteria, CA). The fluorescein isothiocyanate conjugated antibody (FITC, Jackson ImmunoResearch, West Grove, PA) and CY3 conjugated antibody (Jackson ImmunoResearch, West Grove, PA) were employed for double immunoreactivity identification. Negative control sections from each animal received identical preparations for immunohistochemical staining, except that the primary antibodies were omitted.

Coronal (100 μ m thick) sections were cut on a vibratome, and processed for double immunofluorescence labeling for neurocan and GFAP. Briefly, vibratome sections were incubated with the primary antibody cocktail (GFAP, rabbit 1:10,000 + neurocan, mouse 1:800) for 4 days at 4°C, and then with secondary antibody cocktail (FITC-conjugated anti rabbit 1:200 + CY3-conjugated anti-mouse 1:200).

Fluorescence-labeled sections were analyzed and double labeling confirmed using z-stacks acquired on a laser scanning confocal microscope (Zeiss, LSM510).

Western blot assay

Three normal rats and six animals subjected to MCAo (n=3 for MCAo alone, and n=3 for MCAo + BMSC) were decapitated under deep anesthesia. Brain tissues along the ischemic boundary zone ipsilateral to the injury were then extracted, and homogenized in ice cold RIPA buffer containing 1% protein inhibitor cocktail, 10 mg/ml PMSF, and 100 mM sodium orthovanadate. After incubating for 30 mins on ice, the mixture was centrifuged at 10,000 $\times$ g at 4°C, and the supernatant was collected as total cell lysate. Standard Western blot assay was employed for GAP 43 (1:500, Abcam) and neurocan (1:250, Chemicon).

Laser capture microdissection (LCM) of reactive astrocytes

LCM is a method for procuring homogenous cells from specific microscopic regions of tissue sections. Brain coronal sections (8 μ m) were cut on a cryostat set at -20°C and kept at

-80°C until processing. For the immunostaining, the sections localized to the territory supplied by the MCA (Fig 3A, area 1-8), were air-dried for 30 secs and fixed in freshly prepared 4°C cold acetone for 2 mins. The coronal sections were incubated with GFAP antibody at 1:50 dilution for 5 mins, and then incubated with CY3-conjugated F(ab')₂ anti-rabbit IgG secondary antibody for 5 mins. After air-drying for 5 mins, GFAP positive reactive astrocytes along the ischemic boundary were cut using Leica LMD6000 system. Combined with GFAP immunostaining, this technique allows the isolation of individual reactive astrocytes within the brain tissue (Fig 3B, C). Approximately 1000 cells were dissected and collected in Eppendorf tubes containing 100 µl of lysis buffer. The samples were stored in -80°C before RNA isolation.

Cell culture

Cortices of postnatal day 2-3 pups were dissected and digested with 0.25% (w/v) trypsin. The cells were collected in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) and maintained at 37 °C in a 5% CO₂ incubator. 3-5 days later, the confluent monolayer cells were rinsed with PBS and shaken to remove contaminating non-astroglial cells.

Primary cultures of bone marrow stromal cells were obtained from donor young male animals (Theradigm Inc.). Briefly, the bone marrow was harvested from the hind legs. Bone marrow was mechanically dissociated and the cells were washed, and suspended in culture medium. After 3 days, the cells tightly adhered to the plastic flasks were heterogeneous and considered as MSCs. BMSCs were cultured in α -modified MEM (Hyclone, South Logan, UT) with 20% FBS containing penicillin-streptomycin on 75cm² tissue culture flasks (Corning, NY) in 37 °C, 5% CO₂. There was less than 1% contamination by hematopoietic cells as assessed by flow cytometry using antibodies to CD45 (T cells), Pan T cell (T cells), CD11b/c (macrophages), and CD45RA/B (B cells).

Astrocyte and BMSC coculture

8×10⁵ astrocytes per well were cultured in 6-well plates. In the same plate, 4×10⁵ BMSCs (astrocyte to BMSC ratio, 2:1), were cocultured in the upper chamber of transwell cell inserts. Thus, astrocytes and BMSCs shared the same medium environment, yet were not in direct contact.

Oxygen-glucose deprivation (OGD) and reoxygenation

As previously described (Gao et al. 2005b), OGD was induced within an anaerobic chamber (model 1025, Forma Scientific, OH). The 10% FBS, high glucose DMEM was replaced with 1 ml of glucose and serum-free DMEM in the astrocyte culture. The astrocyte culture was transferred to the anaerobic chamber saturated with 85% N₂/10% H₂/5% CO₂, at 37°C. Astrocytes were incubated in this OGD condition for 5 hrs. Astrocyte cultures were removed from the anaerobic chamber, rinsed with PBS, and fed with 3 ml fresh half glucose and 5% FBS DMEM. BMSCs were then introduced to the astrocyte culture and were incubated together with astrocytes for an additional 18 hrs under reoxygenation conditions.

Real-time reverse transcriptase-polymerase chain reaction (real time RT-PCR)

Quantitative polymerase RT-PCR was performed using SYBR Green system. Total RNA from LCM cells or cultured astrocytes was extracted using Absolutely RNA nanoprep and miniprep kit, respectively (Stratagene, CA, USA). RNA was subsequently reverse transcribed to cDNA with SuperScript First-strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed afterwards. Primer (Invitrogen) concentrations (10nM) were optimized before use. A 1x SYBR Green PCR master kit was used with the

appropriate concentrations (10nM) of forward and reverse primers in a total volume of 20 μ l. PCR reactions contained 1 μ l cDNA. Optimization was performed of each gene-specific primer prior to the experiment to confirm that 10nM primer concentrations did not produce nonspecific primer-dimer amplification signal in no-template control wells. Quantitative RT-PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems, CA) by using 3-stage program parameters provided by the manufacturer as follows; 2 mins at 50°C, 10 mins at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during RT-PCR. RT-PCR products were run on 2% agarose gels to confirm that correct molecular sizes were present. Each sample was tested in triplicate with quantitative RT-PCR, and samples obtained from multiple animals (for LCM) or three independent experiments were used for analysis of relative gene expression data using the $2^{-\Delta\Delta CT}$ method. Primers employed were: β -actin: 5'-CCA TCA TGA AGT, GTG ACG TTG-3' (fwd), 5'-CAA TGA TCT TGA TCT TCA TGG TG-3' (rev). neurocan: 5'-TTT CAG TCC ACA GCG ATC AG-3' (fwd), 5'-AGG AGA GGG ATA CAG CAG CA-3' (rev).

Quantification and statistical analysis

Immunoreactive cells were analyzed with NIH image software (Image J) based on the evaluation of an average of three histology slides (6 μ m thick, 54 μ m interval, every 10th slide) from the standard block of each animal. For semiquantification of GAP43 or neurocan immunoreactivity, 8 fields of view along the ischemic boundary zone (Fig 3A) were digitalized under a 40x objective and the percentage of immunoreactive positive area was calculated and averaged. Signal intensity of Western blot assay was quantitated using Adobe Photoshop 6.0 after scanning. One way ANOVA was used to analyze Western blot data between normal control, MCAo alone and MCAo + BMSCs as well as gene expression fold change data between normoxic, OGD, and OGD + BMSCs. Student's t-test was used to analyze all the in vivo related immunohistochemical and LCM data between 2 groups. All data are presented as mean $\pm$ SE. A value of $P < 0.05$ was taken as statistically significant.

RESULTS

Peri-infarct protein expression changes

GAP43 is a membrane-bound protein found in the axonal growth cones of sprouting CNS axons (Benowitz et al. 1988; Goslin et al. 1988; Meiri et al. 1986), which has been widely used to quantitate sprouting axons during neuroanatomical remodeling (Gregersen et al. 2001; Kawamata et al. 1997; Shen et al. 2007a; Stroemer et al. 1995). To evaluate the status of axonal regeneration along the damaged brain tissue at 8 days after MCAo, we calculated the positive areas for GAP43 in selected fields of view in the ischemic boundary zone (IBZ). As shown in Fig 1A-C, BMSC treatment significantly increased GAP43 expression compared with control rats ($P < 0.05$). The semiquantitative immunohistological data are consistent with the results of Western blot assay, which show reduction of GAP43 expression after MCAo and a significant increase following BMSC treatment in the IBZ ($P < 0.05$, Fig 1D).

Immunohistochemistry of the contralateral brain of the MCAo rats for neurocan gave little, if any, positive signal (Fig 2A). In contrast, a substantial increase in neurocan expression was observed in the peri-infarct tissue of the cerebral cortex and striatum 8 days after ischemic attack (Fig 2B, C). Due to the overlap of the neurocan positive area and GFAP positive reactive astrocyte distribution around the injured site, double fluorescent immunostaining was employed to discern the spatial relationship of these two proteins. As shown in Fig 2E-G, neurocan positive signals concentrated around GFAP expressing

reactive astrocytes, and scattered double positive yellow spots (arrows in Fig 2E-G) are present on astrocytic cell bodies and processes. To confirm the cellular origin of neurocan expression, z-stacks of vibratome section (100 μ m) were collected by confocal microscopy. Co-localizations of neurocan and GFAP were detected around the injury sites (Fig 2H), indicating that reactive astrocytes in the peri-infarct area synthesize neurocan. Quantitative analysis shows that animals receiving BMSC administration exhibited significantly lower levels of neurocan expression in the IBZ compared to control MCAo alone rats (Fig 2D, $P < 0.05$). As expected, Western blot analysis (Fig 2D) of tissue extracted from the IBZ confirms that: 1) The expression of the full-length neurocan (260 kD) was very low in normal control rats; 2) neurocan expression markedly increased after ischemic injury ($P < 0.01$ vs normal control); 3) BMSC treatment significantly decreased the expression of the full-length neurocan in the IBZ ($P < 0.05$ vs MCAo alone).

Effects of BMSCs on neurocan expression in reactive astrocytes (in vivo & in vitro)

To confirm the origin of neurocan expression, and to investigate whether or not BMSCs conferred alteration of neurocan protein resulted from the regulation of gene expression, we dissected 1,000 reactive astrocytes along the IBZ and applied RT-PCR. Our data show (Fig 3D): 1) neurocan gene was highly expressed in reactive astrocytes (the gene expression of neurocan was approximately 5% of that of housekeeping β -actin gene); 2) neurocan gene expression was significantly down-regulated in astrocytes extracted from BMSC treated brain, compared to astrocytes from MCAo alone control brain (0.42 ± 0.01 vs 1 ± 0.13 , $n=4$, $P < 0.05$). These data are consistent with alteration of protein expression detected by immunostaining and Western blot analysis, which suggests that the change of neurocan protein expression may be partially initiated by alterations at the gene level.

To obtain further evidence for the astrocytic origin of neurocan, and to investigate the impact of hypoxia as well as BMSC coculture on neurocan gene expression in astrocytes, primary astrocytes derived from postnatal pups were subjected to 5 hrs of OGD and 18 hrs of reoxygenation, and RT-PCR was performed. In agreement with our in vivo data, neurocan gene expression was significantly up-regulated in the OGD group compared to astrocytes in the normoxic control group (1.73 ± 0.25 fold of normoxia, $n = 3$, $P < 0.01$, Fig 3E); the presence of BMSCs during reoxygenation significantly decreased neurocan gene expression to 0.82 ± 0.23 fold of normoxia ($n = 3$, $P < 0.01$, Fig 3E).

DISCUSSION

Our data demonstrate that neurocan protein level is significantly increased in the peri-infarct area at 8 days after MCAo. GFAP-positive reactive astrocytes are a major source of neurocan around the lesion site. BMSC administration at 24hrs after ischemia decreases neurocan level and increases the expression of the axonal sprouting marker GAP43 in the IBZ. In agreement with the above findings, laser capture data from ischemic brains and in vitro cell culture data of astrocytes show that neurocan gene expression in astrocytes is up-regulated after stroke and OGD, and then BMSC administration and coculture during reoxygenation, respectively, significantly reverses this change.

BMSCs have been extensively studied as a candidate for stroke therapy. Based on our previous studies (Chen et al. 2001; Li et al. 2005; Li et al. 2006; Seyfried et al. 2006; Shen et al. 2007b), a dose of 3×10^6 was employed in the present study. Our goal is to investigate the mechanisms underlying the white matter change associated with the improved functional benefit induced by BMSCs on the ischemic brain.

According to previous reports, not many intravenously (IV) administered cells were detected within the parenchyma of brain tissue (1.5-3.0% of 3 million injected BMSC at 14-35 days

after treatment (Chopp and Li 2002; Zhang et al. 2004)). These cells targeted the region of the injury; more than 80% of BMSCs were located within the ipsilateral hemisphere, with the majority of them congregating in the IBZ (Chopp and Li 2002; Eglitis et al. 1999). Despite the small number of BMSCs in the brain, animals showed significant functional improvements, which usually manifested within days after transplantation (Chen et al. 2003; Shen et al. 2006; Zhang et al. 2006). BMSCs, in response to local environment, behave as small molecular factories, producing many different cytokines and trophic factors (Caplan and Dennis 2006; Chen et al. 2002). It is our hypothesis, that the beneficial effects of the limited number of BMSCs are mediated and amplified by local brain cells (Li et al. 2005; Zhang et al. 2006). Taking into account that, 1) reactive astrocytes are abundant in the IBZ and 2) BMSCs regulate the function of reactive astrocytes and promote functional recovery after MCAo (Gao et al. 2005a; Gao et al. 2005b; Li et al. 2005; Xin et al. 2006; Zhang et al. 2006), we now focus on the role of reactive astrocytes and their reaction to BMSC treatment in terms of axonal regeneration.

In response to massive neuronal death and denervation after focal ischemia, nondamaged neurons undergo axonal sprouting and establish new synaptic connections, which may underlie the partial recovery of neurological function over time (Kawamata et al. 1997; Stroemer et al. 1995; Weiller et al. 1992). As markers for axonal growth cone and synaptogenesis, respectively, GAP43 and synaptophysin levels are often measured to reflect the neuronal remodeling and plasticity after cerebral infarction (Kawamata et al. 1997; Stroemer et al. 1995). Our previous studies demonstrate that BMSC transplantation significantly increases synaptophysin expression level in the IBZ at 30 days or later after MCAo (Shen et al. 2007a; Shen et al. 2006; Zhang et al. 2006), and not surprisingly, rats treated with BMSC show less axonal loss, and more white matter bundles in the penumbra area (Li et al. 2006; Shen et al. 2007a). As the sequence of axonal regeneration is new axon sprouting followed by new terminal formation (synaptogenesis), and changes of synaptophysin expression occur at later time points after MCAo (Stroemer et al. 1995), we chose GAP43 as the indicator of axonal regeneration in the present study (animals were sacrificed at 8 days after MCAo). Consistent with our previous findings (Li et al. 2006; Shen et al. 2007a; Shen et al. 2006), BMSC administration significantly increases the number of sprouting axons in the IBZ.

As one of the major CSPGs in the CNS, neurocan is related to axonal guidance during brain development: This molecule controls the direction of axon extension by a repulsive mechanism; for example, neurocan in the roof plate prevents dorsal root ganglion axons from crossing the midline (Kato-Semba et al. 1998), and it repels retinal axons from the hypothalamus and epithalamus (Tuttle et al. 1998). In vitro experiments have also confirmed the inhibitory properties of neurocan on axon growth (Friedlander et al. 1994; Kato-Semba et al. 1998). When neurocan is arranged as stripes on culture container, neurite extension from a cerebellar explant is guided along the neurocan-free stripes (Asher et al. 2000). Being developmentally regulated, neurocan expression in the brain increases during late embryogenesis but decreases significantly within the first month after birth (Meyer-Puttlitz et al. 1996). After CNS injury, however, this protein is up-regulated and concentrates around the lesion site (Deguchi et al. 2005; Haas et al. 1999). Considering its inhibitory properties, neurocan may contribute to axonal regenerative failure in CNS injuries. Although initially described as a neuronal proteoglycan (Engel et al. 1996; Meyer-Puttlitz et al. 1996), neurocan is synthesized by cultured astrocytes as well as reactive astrocytes in the glial scar (Asher et al. 2000; McKeon et al. 1999; Oohira et al. 1994), indicating the astrocyte is one of the major sources of neurocan, especially after brain injuries. Consistent with the above findings, we found a substantial overlap of neurocan expression and GFAP positive signals in the peri-infarct region and confocal laser microscopic images confirmed that neurocan

signals were in and around reactive astrocytes, indicating that reactive astrocytes synthesize neurocan and secrete it into the surrounding spaces.

Astrocytes are the most abundant cell type in the CNS and provide many supportive activities essential for neuronal function under physiological circumstances. In response to all CNS insults, astrocytes become “reactive”, increase in both size and number (hypertrophy and hyperplasia), and eventually form “scar tissue” around the injury sites. Reactive astrocytes may contribute to the hostile CNS environment for axonal regeneration (Bovolenta et al. 1992; Fawcett and Asher 1999). Since CNS axons fail to regenerate past a lesion site even in the absence of a recognizable glial scar, it is suggested that reactive astrocytes establish a local biochemical rather than a purely mechanical barrier that inhibits significant axonal regeneration (Davies et al. 1996). Our previous work demonstrates that BMSCs reduce ischemia-induced astrocytic activation in vitro (Gao et al. 2008) and decrease the thickness of the scar wall in vivo (Li et al. 2005; Shen et al. 2007a), implying that BMSCs modulate post stroke astrocytic response for the benefit of brain repair after ischemic attack. To strengthen the above findings, the current study demonstrated that 1) BMSC administration significantly reduced neurocan expression at both RNA and protein levels in vivo; 2) Neurocan gene was significantly upregulated in primary cultured astrocytes under hypoxic condition (OGD), and this change was reversed after BMSC coculture for 18 hrs. The fact that all RNA data were collected from pure astrocytes in vivo (LCM of GFAP positive reactive astrocytes) as well as in vitro, (primary cultured postnatal astrocytes) provides basic insight into the effects of BMSCs on local astrocytes under ischemic conditions.

SUMMARY

We demonstrate that BMSC administration significantly increases the axonal sprouting marker GAP43 and reduces the expression of the inhibitory molecule neurocan in the peri-infarct glial tissue after MCAo. By targeting reactive astrocytes, BMSCs may help produce a permissive environment for axonal regeneration. More research is warranted to investigate the expression of neurocan as a function of cell dose and the relationship between neurocan expression and axonal regeneration.

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Fig 1.

A-D: BMSC administration significantly increases GAP43 expression in the ischemic boundary zone. *P<0.05 vs. MCAo, #P<0.01 vs. normal control. Scale bars: A, B = 25 μ m.

Fig 2.

A-D: Neurocan protein is barely detectable in the contralateral hemisphere (A), is increased in the peri-infarct region after MCAo (B), and is significantly reduced after BMSC treatment (C); D: Quantitative analysis of immunostaining and Western blot assay show the similar changes in neurocan expression in the IBZ, neurocan is upregulated after ischemic attack, and BMSC treatment significantly decreases neurocan expression; E-H: Double immunostaining shows that neurocan is concentrated around reactive GFAP positive astrocytes, and confocal analysis reveals the colocalization of the two proteins. *P<0.05 vs. MCAo, #P<0.01 vs. normal control, A-C = 50 μ m, E-G = 25 μ m, H = 20 μ m.

Fig 3.

A: Hematoxylin and eosin-stained coronal section illustrates the ischemic boundary regions from which reactive astrocytes are cut by LCM (area 1-8); B,C: GFAP immunofluorescent staining before and after cell capture; D: Neurocan gene expression in reactive astrocytes significantly decreases in BMSC treated animals, compared to that of the MCAo alone rats. E: 5hrs of OGD up-regulates neurocan gene expression in primary astrocytes, and coculture with BMSC during reoxygenation markedly suppresses neurocan gene expression; * $P < 0.05$, ** $P < 0.01$ vs. ischemia alone; ## $P < 0.01$ vs. normoxia. Scale bar: B, C = 25 μ m.