BONE MARROW STROMAL CELLS REDUCE ISCHEMIA-INDUCED ASTROCYTIC ACTIVATION *IN VITRO*

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Abstract—Transplantation of bone marrow stromal cells (BMSCs) improves animal neurological functional recovery after stroke. To obtain insight into the mechanisms underlying the therapeutic benefit, we directed our attention to the interaction of BMSCs with astrocytes. Astrocytes become reactive (astrogliosis) after a brain injury, such as stroke. Astrogliosis plays both beneficial and detrimental roles in brain recovery. Previously, we have shown that administration of BMSCs to animals with stroke significantly reduces the thickness of the scar wall formed by reactive astrocytes. We tested the influence of mouse bone marrow stromal cell (mBMSC) on astrogliosis under oxygen–glucose deprivation (OGD)/reoxygenation conditions *in vitro*, employing an anaerobic chamber. Our data indicate that mBMSCs down-regulate glial fibrillary acidic protein (GFAP) expression in astrocytes after 2 h of OGD and an additional 16 h reoxygenation. mBMSCs protected astrocytes from ischemia, maintaining morphological integrity and proliferation. The IL-6/IL-6R/gp130 pathway is associated with astrogliosis in response to CNS (disorders. Therefore, we examined the effects of mBMSC on the IL-6/IL-6R/gp130 pathway as an underlying mechanism of mBMSC-altered astrogliosis. Furthermore, IL-6 siRNA was used to block IL-6 expression in astrocytes to further investigate IL-6 involvement in mBMSC-altered astrogliosis. Our results indicate that the mBMSC-conferred decline of astrogliosis post-ischemia may derive from the down-regulation of the IL-6/IL-6R/gp130 pathway. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: bone marrow stromal cell, astrocyte, stroke, IL-6, IL-6 receptor, gp130.

Astrocytes are the most abundant cell type in the CNS. They perform an essential role in support of normal neuronal activities. After brain injury, including cerebral ischemia, astrocytes undergo a vigorous response termed "as-

trogliosis" characterized by escalated expression of glial fibrillary acidic protein (GFAP), hypertrophy, and cell proliferation. Accompanying these morphological changes is a range of physiological changes, including secretion of a variety of cytokines (Wu and Schwartz, 1998; Goldshmit et al., 2004). Astrogliosis has both beneficial and detrimental effects on injured brain. It promotes neuronal repair and survival with the increased production of trophic factors (Tacconi, 1998). In addition, reactive astrocytes form a glial scar which obstructs neuronal axonal regeneration and communication. Up-regulation of astrocytic intermediate filaments (IFs) is a crucial step in astrocyte activation (Wilhelmsson et al., 2004). GFAP up-regulation is the prototypical biochemical change during astrogliosis; it is almost universally recognized as a marker of reactive astrocytes (Wu and Schwartz, 1998). In this study, we therefore examined astrogliosis and GFAP expression at both protein and gene levels in response to bone marrow stromal cells (BMSCs).

IL-6 is associated with astrogliosis both *in vitro* and *in vivo* (Marz et al., 1999; Tilgner et al., 2001). As a positive autocrine loop, reactive astrocytes induce IL-6 production, which in turn leads to further activation of astrocytes (Dietrich et al., 2003). Although various CNS cell types can produce IL-6, astrocytes likely play a dominant role as the source of this cytokine in many CNS conditions (Van Wagoner and Benveniste, 1999). Under normal physiological conditions, IL-6 levels in the CNS remain constant. However, during brain injury, inflammation and disease including stroke, IL-6 levels become elevated (Acalovschi et al., 2003).

IL-6 stimulates target cells via the IL-6 and IL-6 receptor (IL-6R) complex and transsignaling. On cells that express IL-6R, IL-6 first binds to the IL-6R. The complex of IL-6 and IL-6R associates with the signal-transducing membrane protein gp130, thereby inducing its dimerization and initiating signaling. Neither IL-6 nor IL-6R alone exhibits a measurable affinity for gp130 (Jones et al., 2005; Taga and Kishimoto, 1997; Rose-John, 2001). IL-6R is not expressed by all brain cell types (Jones et al., 2005). Cells that only express gp130 but not IL-6R are unable to respond to IL-6 itself. A naturally occurring soluble form of the IL-6 receptor (sIL-6R), which has been found in various extracellular body fluids, together with IL-6 stimulates cells that only express gp130, a process named transsignaling (Rose-John and Heinrich, 1994; Peters et al., 1998; Mullberg et al., 2000; Jones et al., 2005).

BMSCs are a heterogeneous subpopulation of bone marrow cells including mesenchymal stem or progenitor cells (Friedenstein et al., 1976). Previous *in vivo* studies

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have shown that implantation of BMSCs promotes significant functional recovery in rats subjected to stroke and traumatic brain injury (Chen et al., 2001a; Li et al., 2002; Lu et al., 2003). This recovery is at least partially due to induction of brain plasticity and brain remodeling (Li et al., 2005; Hess et al., 2002; Rempe and Kent, 2002; Song et al., 2004). BMSCs reduced apoptosis in astrocytes post-ischemia, indicating BMSC protective role on astrocytes (Gao et al., 2005b). In addition to the protective role on cell survival in the brain, administration of BMSCs to animals with stroke also significantly reduces astrogliosis and the thickness of the scar wall, and enhances neuronal generation and presence within the subventricular zone and the scar boundary, respectively (Li et al., 2005; Shen et al., 2006).

To our knowledge, there are no studies reporting the interaction of BMSC and astrogliosis under ischemic/post-ischemic conditions *in vitro*. To investigate the BMSC effect on astrogliosis, we employed an anaerobic chamber to simulate *in vivo* ischemic conditions and to create an *in vitro* astrogliosis model. GFAP expression was employed as a marker of astrogliosis.

To further understand the mechanism underlying astrogliosis and the effect of BMSC on astrogliosis after stroke, we investigated whether IL-6/IL-6R/gp130 pathway is involved in the effect of BMSCs on astrocytes.

EXPERIMENTAL PROCEDURES

Cell culture

Primary cultures of BMSCs were obtained from donor young adult C57/BL6 mice, and were isolated, as previously described (Chen et al., 2001b). Briefly, the bone marrow was harvested from mouse hind legs. Bone marrow was mechanically dissociated and the cells were washed, suspended in culture medium. After 3 days, the cells tightly adhered to the plastic flasks were heterogeneous and considered as MSCs. Mouse bone marrow stromal cells (mBMSCs) were cultured in α-modified MEM (Hyclone, Logan, UT, USA) with 20% fetal bovine serum (FBS, Gibco Laboratory, Grand Island, NY, USA) containing penicillin–streptomycin on 75 cm^2 tissue culture flasks (Corning St. Louis, MO, USA). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital, and conformed to all local and international guidelines on the ethical care of animals. We minimized the number of animals used and their suffering.

Mouse cortical astrocytes, phenotype 1, were obtained from the American Type Culture Collection (ATCC, Arlington, VA, USA). Cells were cultured in high glucose Dulbecco's modified eagle medium (DMEM) with 10% FBS (Gibco), containing penicillin–streptomycin on 75 cm^2 tissue culture flasks (Corning) in 37 °C, 5% CO_2.

mBMSC and astrocyte coculture

Astrocytes (8×10^5 per well) were cultured in six-well plates. In the same plate, 4×10^5 mBMSCs (a 1:2 mBMSC to astrocyte ratio), were cocultured in the upper chamber of transwell cell inserts. Thus, astrocytes and mBMSCs shared the same medium environment, yet were not in direct contact.

Oxygen–glucose deprivation (OGD) and reoxygenation

As previously described (Gao et al., 2005b; Li et al., 2005), OGD was induced with an anaerobic chamber (model 1025, Forma Scientific, OH, USA). The 10% FBS, high glucose DMEM was replaced with 1 mL of glucose and serum-free DMEM in the astrocyte culture. Then the astrocyte culture was transferred to the anaerobic chamber saturated with 85% N_2/10% H_2/5% CO_2, at 37 °C. Astrocytes were incubated in this OGD condition for 2 h. At the end of the ischemic incubation, astrocyte cultures were removed from the anaerobic chamber, rinsed with PBS, and fed with 3 mL fresh half glucose and 5% FBS DMEM. mBMSCs or IL-6 neutralizer were then introduced to the astrocyte culture and were incubated together with astrocytes for an additional 16 h under reoxygenation conditions. Four experimental groups were employed: normoxic control (astrocytes in high glucose and 10% FBS DMEM), OGD control (astrocytes in glucose and serum free DMEM), mBMSC coculture (astrocytes cocultured with 4×10^5 mBMSCs), and IL-6 neutralizer (astrocytes in glucose and serum free DMEM containing 1.5 μg/mL IL-6 neutralizer). Treated astrocyte cultures were returned to the 5% CO_2, humidified 37 °C incubator under reoxygenation and were incubated for an additional 16 h.

Morphology assay

After 2 h of OGD and an additional 16 h of reoxygenation, cells were gently rinsed with PBS. Cells were subsequently observed under a microscope with 20$\times$ magnification (Nikon, Eclipse, TE2000-U, Chiyoda-ku, Tokyo, Japan). Each experimental group contained six wells each time, and the experiment was repeated in triplicate.

Immunostaining

Cells were cultured in six-well plates until confluent. After treating cells under the experimental conditions as described above, medium was removed and cells were rinsed with PBS. Afterward the cells were fixed with 4% paraformaldehyde for 15 min at RT (room temperature). Blocking buffer (1% BSA, 0.1% Triton-X-100, 0.002% Tween-20 in 1% TBS) was added in the cell culture and incubated for 1 h at RT. Immunofluorescent labeling was then performed by incubating anti-GFAP (1:1000), IL-6 (1:300), Ki67 (1:300, Labvision-Neo, Fremont, CA, USA) antibodies with the cells overnight at 4 °C. Ki67 is a nuclear antigen associated with cell proliferation and is present throughout the active cell cycle (G1, S, G2 and M phases) but absent in resting cells (G0). Thereafter, the cell culture was washed with T-TBS (0.2% Tween-20 in 1$\times$ TBS) for three times. Cy3-conjugated secondary antibody (diluted 1:200, Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) was subsequently added to the cells and was incubated for 1 h at RT. The cells were washed three times with T-TBS, and mounted with anti-fluorescent mounting medium. Results were observed with a fluorescent microscope (Ziess, Thornwood, NY, USA) at 20$\times$ objective and imaged with Metamorph software. Each experimental group contained six wells each time, five fields of each well were imaged, and the experiment was repeated in triplicate. Employing the Metamorph image software analysis, a constant positive signal threshold was established and the average signal intensity for each field was quantified and compared using Student's t-test.

Short-interfering RNA (siRNA) transfection

Mouse IL-6 siRNA was purchased from Qiagen. Transfection of siRNA was carried out using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Briefly, astrocytes were grown to 50% confluence in 150 mL tissue culture plates. Lipo-