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GRANULOCYTE COLONY-STIMULATING FACTOR IMPROVES ALTERNATIVE ACTIVATION OF MICROGLIA UNDER MICROENVIRONMENT OF SPINAL CORD INJURY

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Abstract—Granulocyte colony-stimulating factor (G-CSF) was investigated in the present study to examine whether it could affect the activation status of microglia under microenvironment of spinal cord injury and provide a potential therapeutic treatment for spinal cord injury. We established mouse spinal cord hemisection model and injected recombinant human G-CSF (rhG-CSF) subcutaneously. The results demonstrated that G-CSF could recruit microglia to the injury site in the first 72 h after spinal cord injury. Moreover, G-CSF inhibits the expression of pro-inflammatory factors and promotes the expression of neurotrophic factors. Additionally, G-CSF also increases the expression of markers of M2 macrophage and inhibits the expression of markers of M1 macrophage in BV2 microglia *in vitro* model, favoring the M2 polarization of microglia under the microenvironment of spinal cord hemisection. NFκB signal pathway was involved in G-CSF-induced polarization of BV2 microglia. As a conclusion, we suggested that administration of G-CSF within the first 72 h after spinal cord injury might reduce early inflammation-induced detrimental effect and promote an anti-inflammatory response that favors repair *via* improving alternative activation of microglia. Administration of G-CSF in the acute phase of spinal cord injury may be a promising strategy in restorative therapy after spinal cord injury.

Key words: granulocyte colony-stimulating factor, spinal cord hemisection, microglia, classically activated microglia, alternatively activated microglia.

INTRODUCTION

Microglia are the primary immune effector cells in the central nervous system, one of the members of mononuclear phagocytes family. They have many functional and phenotypic characteristics in common with macrophages, involving in the phagocytosis and the innate immune response in the central nervous system (David and Kroner, 2011). Mantovani et al. proposed that macrophages are divided into two types – classically activated macrophage and alternatively activated macrophage according to the different activation status and functioning (Mantovani et al., 2002, 2005). M1 macrophages secrete pro-inflammatory cytokines and chemokines and present antigen, participating in a positive immune response and has a role in immune surveillance function. Whereas, M2 macrophages have weak antigen-presenting ability and play an important role in immune regulation by down-regulating immune response *via* secreting inhibitory cytokines, such as interleukin-10 (IL-10), transforming growth factor-beta (TGF-β) and so on (Mantovani et al., 2004, 2007). Atsushi et al. demonstrated that interleukin-1 (IL-1) participated in the classical and alternative activation of microglia/macrophages after spinal cord injury (Mukaino et al., 2010). These findings indicated that microglia located within the spinal cord were activated after spinal cord injury and also had two activating status and played a double-edged function during the CNS damage repair. How to control the activating phenotype of the microglia will be a new target for treatment of spinal cord injury.

Granulocyte colony-stimulating factor (G-CSF) is a stimulating factor promoting the proliferation of bone marrow stem cells and the inhibition of granulocytes apoptosis. Its molecular weight is 19.6 KD (Welte et al., 1985). G-CSF is able to pass through the blood–brain barrier (Zhao et al., 2007; Pitzer et al., 2008). The main function of G-CSF is to promote the maturity of neutrophils and inhibit neutrophil apoptosis (Welte et al., 1985). Due to its mild side effects and non-toxic features, G-CSF is now widely used in clinical treatment, including chemotherapy-induced leukopenia, reconstruction of bone marrow, hematopoietic stem cells recruitment (Silvestris et al., 2012; Kadia et al., 2012).

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Abbreviations: BDNF, brain-derived neurotrophic factor; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; G-CSF, granulocyte colony-stimulating factor; G-CSFR, granulocyte colony-stimulating factor receptor; H&E, Hematoxylin and Eosin; IBA1, ionized calcium bindingadaptor molecule-1; IL-1, interleukin-1; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; MAG, myelin-associated glycoprotein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OMgp, oligodendrocyte-myelin glycoprotein; PBS, phosphate-balanced solution; rhG-CSF, recombinant human granulocyte colony-stimulating factor; TGF-β, transforming growth factor-beta.

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In recent years, it has been found that G-CSF receptor is commonly present in the central nervous system (Pan et al., 2008). The neuroprotective effect of G-CSF has attracted wide attention. The latest study found that granulocyte colony-stimulating factor receptor (G-CSFR) was expressed in microglia and its expression was significantly increased after spinal cord injury (Yamasaki et al., 2010). However, little is known about whether G-CSF is able to affect the activation of microglia phenotype by binding with its specific receptor and its subsequent impact on the repair after spinal cord injury.

Here, we established mouse spinal cord hemisection model and injected recombinant human G-CSF (rhG-CSF) subcutaneously, and then examined the activation of microglia at different time-points with or without G-CSF treatment *in vivo* and the expression of chemotactic factors and markers related with classical activation and alternative activation of macrophages. Furthermore, we detected the possible pathway of neuroprotective effect of G-CSF *via* activation of microglia under the microenvironment of mouse spinal cord injury.

EXPERIMENTAL PROCEDURES

Animals and drug treatment

Eighty female Kunming mice, weighing 30 ± 5 g, were obtained from the Laboratory Animal center, Shandong University. Mice were bred and housed at 23 °C under an alternating 12-h light and dark cycle and fed a commercial diet.

Eighty mice were divided randomly into two groups, for *in vivo* and *in vitro* experiments, respectively. Forty mice were divided randomly into two subgroups, injury group and G-CSF-treated group.

Spinal cord injury

All animal experiments were approved by the Shandong University Animal Care Committee. Spinal cord hemisection was performed as described previously (Mikami et al., 2002; Arvanian et al., 2009). Briefly, mice were anesthetized with 10% chloral hydrate. Dorsal laminectomy was performed to expose segments T9–T11 of the spinal cord using a superficial vein at T5–T6 as a landmark. The dura was slit (1 mm) at the midline at T10. A complete hemisection of the right hemicord at T10 was carried out with the tip of an iridectomy scissors. After surgery, the muscles and skin were closed in layers. Antibiotic (Gentamicin, 1000 U) was administered subcutaneously. Paralysis of the right hind limb indicated that the model was successful at 1 day after spinal cord hemisection. Each mouse received manual bladder expression twice daily until the recovery of sphincter control.

Drug treatment

G-CSF-treated group were injected subcutaneously with rhG-CSF (50 μg/kg day, Chugai Pharmaceutical Co., Tokyo, Japan) for three consecutive days after spinal cord hemisection. Injury group were injected subcutaneously with an equal volume of phosphate-balanced solution (PBS).

Tissue processing

Mice were perfused transcardially with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.2) at 1 day, 3 days, 5 days, and 7 days after spinal cord hemisection. The spinal cords were removed and post-fixed in the same fixative. Fifteen-micrometer sections were prepared on a cryostat.

Supernatant from injured spinal cord

Mice were deeply anesthetized with 10% chloral hydrate at 1 day, 3 days, and 5 days after spinal cord injury. Spinal cord at T10 about 0.5 cm from top to bottom was removed under sterile conditions. Cut the spinal cord tissue into pieces and centrifuge at 1000 rpm. The supernatant from the injured spinal cord was used for BV2 culture.

BV2 cell culturing

BV2 cells are derived from primary mouse (C57BL/6) microglia cells (Blasi et al., 1990). The immortalized murine microglial cell line BV2 has been used frequently as a substitute for primary microglia cultures (Henn et al., 2009). We purchased BV2 cell line from ATCC global bioresource center.

BV2 cells were maintained in high glucose Dulbecco's modified Eagle's medium (HG DMEM; Hyclone Co., Logan, UT, USA) with 10% fetal bovine serum (Hyclone Co.), 2 mM-L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml) (Sigma–Aldrich, St. Louis, MO, USA) in a 5% CO_2 incubator. The purity of the microglia cultures was assessed using CD11b antibody and more than 97% of cells were stained positively. For all experiments, BV2 cells were used at 75–80% confluency.

Cell viability assay by MTT

Cell viability was determined by the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich, USA) assay. BV2 cells were plated into 96-well culture plates (Corning Costar, USA) at density of 1×10^4 cells/ml with 200-μl serum-free DMEM per well for 1 h. After this, the medium was replaced with serum-free DMEM containing the supernatant from the injured spinal cord (10%). For G-CSF group, 100 ng/ml G-CSF was added into the serum-free DMEM medium containing the supernatant from the injured spinal cord (10%) (Liu et al., 2009). Then, 20 μl MTT solution (5 mg/ml) was added to each well and incubated at 37 °C for 4 h. The medium was aspirated and 200-μl dimethyl sulfoxide was added. The absorbance value was measured in a multi-well spectrophotometer (Bio-Rad, USA) at 490 nm. There independent experiments were conducted.

Hematoxylin and Eosin (H&E) Staining

To examine whether the right spinal cord was transected completely or not, H&E Staining was performed following standard methods. Briefly, the sections were rinsed with distilled water and then stained with Alum Haematoxylin (CI 75290). Differentiated with 0.3% acid alcohol and then stained with Eosin Y (CI 45380). And then dehydrate, clear and mount. Images were collected using a Leica microscope.

Immunofluorescence staining and double immunofluorescence staining

Ionized calcium bindingadaptor molecule-1 (IBA1) immunofluorescence staining was performed following standard methods. Briefly, sections were prepared as described above and incubated with a primary antibody against mouse anti-IBA1 (1:500, Wako, Japan) overnight at 4 °C. Sections were incubated with TRITC-conjugated goat anti-mouse IgG (1:100, Sigma) 1 h at 37 °C. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1:1000, Invitrogen, Carlsbad,