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ANTI-APOPTOTIC EFFECT OF GRANULOCYTE-COLONY STIMULATING FACTOR AFTER FOCAL CEREBRAL ISCHEMIA IN THE RAT

I. SOLAROGLU,[a,d] T. TSUBOKAWA,[a] J. CAHILL[a] AND J. H. ZHANG[a,b,c]*

[a]*Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, Loma Linda, CA, USA*

[b]*Department of Neurosurgery, Loma Linda University, School of Medicine, Loma Linda, CA, USA*

[c]*Department of Anesthesiology, Loma Linda University, School of Medicine, Loma Linda, CA, USA*

[d]*Department of Neurosurgery, Ankara Ataturk Research and Education Hospital, Ankara, Turkey*

Abstract—We investigated the molecular mechanisms of the anti-apoptotic properties of granulocyte-colony stimulating factor (G-CSF) on neurons and whether G-CSF affects glial cell survival following focal cerebral ischemia in rats. Sprague–Dawley rats were subjected to a transient 90 min middle cerebral artery occlusion (MCAO) by the intraluminal occlusion technique. Rats were treated with either a single dose of G-CSF (50 μg/kg, s.c.) at the onset of reperfusion or G-CSF (50 μg/kg body weight, s.c.) was administered starting at the onset of reperfusion and followed by the administration of the same dose per day for an additional 2 days. Brains were harvested either 24 h, 72 h or 2 weeks after reperfusion for assays of infarct volume, immunohistological studies and Western blot analysis for phosphorylated signal transducer and activator of transcription 3 (pSTAT3), Pim-1, bcl-2, Bax, cytochrome c, cellular inhibitor of apoptosis protein 2 (cIAP2), and cleaved caspase-3 levels. G-CSF significantly reduced infarct volume and ameliorated the early neurological outcome. G-CSF treatment significantly up-regulated pSTAT3, Pim-1, bcl-2 expression, and down-regulated cytochrome c release to the cytosol, Bax translocation to the mitochondria, and cleaved caspase-3 levels in neurons. The activation of the STAT3 pathway was accompanied by increased cIAP2 expression in glial cells. After MCAO, G-CSF treatment increased both neuronal and glial survival by effecting different anti-apoptotic pathways which reflects the multifactorial actions of this drug. These changes were associated with remarkable improvement in tissue preservation and behavioral outcome.

Key words: apoptosis, Bcl-2, glia, Pim-1, STAT, stroke.

*Correspondence to: J. H. Zhang, Division of Neurosurgery, Loma Linda University Medical Center, 11234 Anderson Street, Room 2562B, Loma Linda, CA 92354, USA. Tel: +1-909-558-4723; fax: +1-909-558-0119.
E-mail address: johnzhang3910@yahoo.com (J. H. Zhang).

Abbreviations: ANOVA, analysis of variance; cIAP2, cellular inhibitor of apoptosis protein 2; DTT, dithiothreitol; ECA, external carotid artery; G-CSF, granulocyte-colony stimulating factor; G-CSFR, granulocyte-colony stimulating factor-receptor; IAPs, inhibitor of apoptosis proteins; ICA, internal carotid artery; JAK2, Janus kinase2; MCAO, middle cerebral artery occlusion; STAT3, signal transducer and activator of transcription 3; TTC, 2,3,5-triphenyltetrazolium chloride, WB, Western blot.

Granulocyte-colony stimulating factor (G-CSF) is a member of the hematopoietic growth factor family, which orchestrates the proliferation, differentiation, and survival of hematopoietic progenitor cells (Demetri and Griffin, 1991). It has been widely used in clinical practice for the treatment of such conditions as neutropenia, associated with cytotoxic therapy.

However, growing evidence has suggested that G-CSF also has important non-hematopoietic functions in other tissues including the CNS. Recent studies have shown the presence of the granulocyte-colony stimulating factor-receptor (G-CSFR) in a wide variety of cells in the brain, including neurons and glial cells (Schneider et al., 2005). G-CSF and its receptor are co-expressed in neurons and are upregulated in response to neural injury, suggesting an autocrine protective signaling mechanism (Kleinschnitz et al., 2004; Schneider et al., 2005). Moreover, experimental studies have shown that exogenous G-CSF is a promising neuroprotective agent (see review) (Solaroglu et al., 2006).

An anti-apoptotic action is one of the proposed mechanisms of G-CSF related neuroprotection after CNS injury. The Janus kinase2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway has been suggested as a mediator of the anti-apoptotic actions of G-CSF (Komine-Kobayashi et al., 2005; Schabitz et al., 2003; Schneider et al., 2005); however, the precise molecular mechanisms have not been entirely explored, especially under *in vivo* experimental settings.

Pim-1, a member of the serine/threonine kinase proto-oncogene family, is one of STAT3's target genes, which has been shown to be induced by G-CSF in hematopoietic cell lines (Lilly et al., 1992). Pim-1 increases cell survival through the regulation of bcl-2 proteins (Lilly et al., 1999). In the present study, we investigated whether the anti-apoptotic effects of G-CSF was at least partially regulated by STAT3-induced Pim-1 expression, and whether G-CSF inhibits the mitochondria-dependent apoptotic pathway in neurons after focal cerebral ischemia.

Besides neuronal apoptosis, apoptotic cell death in glial cells has also been implicated in the pathophysiology of ischemic stroke. Glial cells (especially astrocytes) provide structural, trophic and metabolic support for neurons under normal conditions as well as during post-ischemic conditions (Nedergaard and Dirnagl, 2005). It has been shown that the preservation of astrocytic metabolism and survival is essential for neuronal survival and is a predictor for recovery *in vivo* models of focal cerebral ischemia (Bambrick et al., 2004; Haberg et al., 2006; Xia et al., 2004). Hence, improvement of glial cell survival is sug-

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gested to be one crucial pathway to protect neurons against ischemic injury (Bambrick et al., 2004; Nedergaard and Dirnagl, 2005; Trendelenburg and Dirnagl, 2005). Although G-CSFR has also been detected in glial cells, whether G-CSF affects glial survival after focal cerebral ischemia remains unknown. The present study was also designed to clarify the role of G-CSF in the survival of glial cells after transient focal cerebral ischemia.

EXPERIMENTAL PROCEDURES

Every effort was made to minimize both animal suffering and the number of animals necessary to complete the study. The experimental protocol conforms to U.S. National Institutes of Health guidelines for the care and use of laboratory animals and was approved by the Loma Linda University Animal Care and Use Committee.

Transient middle cerebral artery occlusion (MCAO)

Fifty-five adult male Sprague–Dawley rats weighing between 290 and 330 g were randomly allocated in three groups: Sham, MCAO and G-CSF-treated (MCAO+G-CSF) groups. Anesthesia was induced intraperitoneally with ketamine (80 mg/kg) and xylazine hydrochloride (8 mg/kg) followed by atropine at a dose of 0.1 mg/kg (s.c.). The left femoral artery was cannulated for continuous monitoring of mean arterial blood pressure, heart rate and blood sampling for analysis of arterial blood gases and glucose levels measured before, during and after the MCAO. Rats were intubated and respiration was maintained with a small animal respirator (Harvard Apparatus, Holliston, MA, USA). Rats were subjected to a MCAO as described by Yin et al. (2003), with modifications. Briefly, under an operating microscopic field, the left common carotid artery, internal carotid artery (ICA) and external carotid artery (ECA) were surgically exposed. The ECA was coagulated and a 4-0 nylon suture with silicon (Doccol Co., NM, USA) was inserted into the ICA through the ECA stump to occlude the MCA. The core temperature was maintained at 37±0.5 °C. After 90 min of MCAO, the suture was carefully removed from the ICA. The neck incision was closed and the rats were allowed to recover. The body temperature was carefully monitored during the post-operative period until the complete recovery of the animal from the anesthetic. The animals were housed individually and had free access to food and water until killed.

Treatment schedules

Animals were divided into two different treatment schedules (Fig. 1):

1) Schedule A: Rats received a single dose of 50 μg/kg (s.c.) of G-CSF (Neupogen, Amgen Inc.) at the onset of reperfusion. The MCAO group received the same volume of saline. Rats were killed 24 h after reperfusion.

2) Schedule B: G-CSF (50 μg/kg body weight, s.c.) was administered at the onset of reperfusion and again administrated with the same dosage daily for an additional 2 days. The MCAO group received the same volume of saline. Rats were killed either 72 h or 2 weeks after reperfusion.

Neurological scores

The neurological scores were evaluated by using a scoring system reported by Garcia et al. (1995) in a blinded fashion.

Determination of infarction volume

Samples from sham (n=3), MCAO (n=6) and G-CSF-treated (n=6) groups were used to evaluate the infarction volume after 24 h. Briefly, at the end of the reperfusion period, general anesthesia was reintroduced and the rats were perfused transcardially with ice-cold PBS. The brain was removed and sectioned coronally into 2 mm thick slices starting from the frontal pole. Slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, Germany) for 30 min at 37 °C in the dark. When the stain had developed, the tissue blocks were removed into 10% formalin overnight. Five coronal sections per animal were then photographed. TTC stains both neuronal and glial cells with a deep red pigment. In areas where neuronal loss occurs TTC does not stain and tissue remains white. Hence, the areas of unstained tissue (the infarcted areas) were demarcated and were analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA), version 1.32. To compensate for the effect of brain edema, the corrected infarct volume was calculated as described previously (Schabitz et al., 2003). To evaluate the infarction volume at 72 h after reperfusion samples from the sham (n=3), MCAO (n=5), and G-CSF-treated (n=5) groups underwent the same TTC staining procedure.

Nissl staining

Samples from the MCAO (n=3) and G-CSF-treated (n=3) groups that were subjected to the Schedule B treatment protocol were used for histological assessment. Briefly, 2 weeks after the MCAO, the rats were anesthetized and transcardially perfused with ice-cold PBS followed by 10% paraformaldehyde. The brains were quickly removed and post fixed in 10% paraformaldehyde and 30% sucrose for 3 days. The brains were cryoprotected and then rapidly frozen by 2-meyhtlbutane chilled in liquid nitrogen. Coronal tissue sections 10 μm thick were cut with the aid of a cryostat (Leica LM3050S).

For Nissl staining, the slices were hydrated in 0.1% Cresyl Violet for 3 min. Then they were dehydrated in ethanol and cleaned with xylenes. The slides were then examined with light microscopy and pictures were taken with a digital camera (Olympus BX51).

Immunohistochemical staining

Samples from the three groups (n=3 for each group) were used for immunohistochemistry. At 24 h after the reperfusion, general anesthesia was reintroduced and the rats were perfused transcardially with ice-cold PBS, followed by 10% phosphate buffered formalin. Double fluorescent staining was performed as previously described (Yin et al., 2003). Briefly, frozen tissue sections (10 μm thick) were incubated with primary antibodies at 4 °C overnight. The primary antibodies used for double fluorescent staining were as follows; G-CSFR, pSTAT3, cytochrome c, Pim-1 and cellular inhibitor of apoptosis protein 2 (cIAP2) (Santa Cruz Inc., 1:50), NeuN and GFAP (Chemicon, 1:400). After rinsing with PBS, the sections were incubated for 1 h in FITC-, AMCA-, or Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch, 1:100). The sections were then visualized using a fluorescent microscope (Olympus) and digital photographs were taken. For negative controls, either the primary or the secondary antibodies were omitted and the same staining procedures were followed.

For immunohistochemistry, the brain sections were incubated overnight at 4 °C with cleaved caspase-3 antibody (Cell Signaling Technology, Inc., 1:500) and sections were treated with the ABC staining kit (Santa Cruz Inc.). Analysis of the pictures was performed using MagnaFire SP 2.1B software.

Subfractionation of cellular proteins for Western blots (WB)

Rats were deeply anesthetized with ketamine 24 h after reperfusion and then decapitated. The brains were rapidly removed and the ipsilateral brain cortexes were separated and flash frozen in