INTRATHECALLY INJECTED GRANULOCYTE COLONY-STIMULATING FACTOR PRODUCED NEUROPROTECTIVE EFFECTS IN SPINAL CORD ISCHEMIA VIA THE MITOGEN-ACTIVATED PROTEIN KINASE AND Akt PATHWAYS


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Abstract—Granulocyte colony-stimulating factor (G-CSF) is a potent hematopoietic factor. Recently, this factor has been shown to exhibit neuroprotective effects on many CNS injuries. Spinal cord ischemic injury that frequently results in paraplegia is a major cause of morbidity after thoracic aorta operations. In the present study, we examined the neuroprotective role of G-CSF on spinal cord ischemia-induced neurological dysfunctions and changes in the mitogen-activated protein kinase (MAPK) and Akt signaling pathways in the spinal cord. Spinal cord ischemia was induced in male Wistar rats by occluding the descending aorta with a 2F Fogarty catheter for 12 min 30 s. Immediately after ischemia surgery, the rats were administered G-CSF (10 μg) or saline by intrathecal (i.t.) injection. The rats were divided into four groups: control, ischemia plus saline, ischemia plus G-CSF and G-CSF alone. The neurological dysfunctions were assessed by calculating the motor deficit index after ischemia surgery. The expressions of MAPK and Akt were studied using Western blotting and double immunohistochemistry. First, we observed that ischemia plus i.t. G-CSF can significantly reduce the motor function defects and downregulate phospho-p38 and phospho-c-Jun N-terminal kinase protein expressions—this can be compared with the ischemia plus saline group. In addition, G-CSF inhibited the ischemia-induced activation of p38 in the astrocytes. Furthermore, we concluded that i.t. G-CSF produced a significant increase in phospho-Akt and phospho-ERK in the motor neurons and exhibited beneficial effects on the spinal cord ischemia-induced neurological defects. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: G-CSF, MAPK, Akt, spinal cord ischemia, intrathecal.

Spinal cord ischemia is a distressing condition encountered in clinical practice. It commonly occurs after repair of thoracoabdominal aortic aneurysms or dissection. Paraplegia that occurs subsequently after aortic surgery can be a serious complication (Kouchoukos, 1991; Svensson et al., 1993). The preclinical stages of spinal cord ischemia remain unclear thus far and require further investigation. An effective method to prevent this condition has not yet been established by researchers. Thus, a preventive agent for spinal cord ischemia is immediately required.

Granulocyte colony-stimulating factor (G-CSF) is a 19.6-kDa glycoprotein and an important cytokine that is commonly used to treat neutropenia (Frampton et al., 1994). It is a granulocyte regulator with a major role in granulocyte production. Under normal body conditions, this cytokine is produced in different body locations such as the bone marrow stromal cells, endothelial cells, macrophages, and fibroblasts. G-CSF production in the body is induced by inflammatory stimuli. G-CSF acts through the granulocyte colony-stimulating factor receptor (G-CSFR), which is expressed on early myeloid progenitors, mature neutrophils, and monocytes-macrophages at the same time as on endothelial cells (Demetri and Griffin, 1991). Recently, G-CSFRs have also been observed on human T and B lymphocytes (Franzke et al., 2003; Morikawa et al., 2002). The lack of G-CSF or G-CSFR results in neutropenia, indicating the importance of G-CSF in steady-state granulopoiesis (Lieschke et al., 1994; Liu et al., 1996). In addition, G-CSF may exert effects on macrophages, resulting in an increase in the monocyte-macrophage numbers, enhancement of phagocytic function (Fattorossi et al., 2001; Bermudez et al., 1998), and regulation of inflammatory cytokine and chemokine production (Xu et al., 2000; Zavala et al., 2002). Boneberg and Hartung (2002) proposed that G-CSF is not only a neutrophil-directed cytokine but also an anti-inflammatory agent.

Recently, several reports have suggested that G-CSF has important non-hematopoietic functions in the CNS (Schneider et al., 2005b review). Further, several reports have indicated that the hematopoietic factor G-CSF can be used as a potential drug for neuronal injury, including
stroke and neurodegenerative disease (Schabitz et al., 2003; Shyu et al., 2004; Huang et al., 2007). Moreover, Schneider et al. (2005a) reported that G-CSF and G-CSFR are expressed in neurons and glial cells and are upregulated in response to neuronal injury (Kleinschnitz et al., 2004; Schneider et al., 2005a). In vivo and in vitro experimental reports have indicated that G-CSF treatment can produce neuroprotective effects via the anti-apoptotic pathway (Solaogu et al., 2006; Schneider et al., 2005a). The neuroprotective effects of G-CSF on spinal cord ischemia-induced motor function defects remain unclear. The most significant characteristic of intrathecal (i.t.) injections is that the required dose and side effects of the medication are reduced. In the present study, we examined the effect of i.t. G-CSF injections on spinal cord ischemia-induced neurological dysfunction.

It has been widely accepted that mitogen-activated protein kinase (MAPK) is an important intracellular signaling molecule that regulates external inflammation, transduction and injury to internal cellular responses (Chang and Kanin, 2001; Robinson and Cobb, 1997). Recent reports have further elucidated the role of MAPK activation in the inflammatory process following CNS injury (Harper and Wilkie, 2003; Zhu et al., 2002). In mammalian cells, three MAPK subfamilies have been defined: p42/44 extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (Herlaar and Brown, 1999). Several reports have indicated that activation of the ERK, JNK, or p38 pathways may be implicated in the mechanisms underlying CNS injury (Mori et al., 2002; Irving and Bamford, 2002; Shackelford and Yeh, 2006). Inhibition of MAPK (p38, JNK, or ERK) activity has therapeutic potential in neurological damage (Alessandrini et al., 1999; Horiuichi et al., 2003; Guan et al., 2006). The role of MAPKs in spinal cord ischemia is still unclear. Furthermore, Baumann et al. (2001) demonstrated the inhibition of MAPK activation during G-CSF stimulation. It was noted that G-CSF could attenuate the inflammatory response by reducing cytokine toxicity and neutrophil activation and infiltration (Boneberg and Hartung, 2002). Neuronal injury not only triggers an inflammatory response but also results in further neuronal damage and apoptosis. Further, Schneider et al. (2005a) observed that G-CSF counteracts programmed neuronal cell death by activation of the anti-apoptotic Akt transduction pathways. Beattie (2004) indicated that attenuation of the anti-inflammatory and anti-apoptotic responses to neuronal injury results in neuroprotection. The studies described above suggested that attenuation of the inflammatory and apoptotic responses to neuronal injury may therefore protect against neuronal injury. However, the cellular mechanisms of G-CSF on spinal cord injuries remain to be investigated. A suitable animal model has been developed for studying spinal cord ischemia-induced neurobehavioral deficits and for evaluating the neuroprotective mechanisms of i.t. G-CSF. The present results suggested that both spinal cord ischemia-induced neurological dysfunction and changes in the phosphorylation of the MAPK family (p38, JNK, and ERK) and in Akt protein expression were significantly attenuated by i.t. G-CSF injections.

**EXPERIMENTAL PROCEDURES**

**Implantation of i.t. catheters**

Male Wistar rats (400–450 g) were used in the experiments. As described in our previous study (Wu et al., 2007), i.t. catheters (PE5 tubes: 9 cm, 0.008-inch inner diameter, 0.014-inch outer diameter) were inserted via the atlanto-occipital membrane into the i.t. space at the level of the lumbar enlargement of the spinal cord and externalized and fixed to the cranial aspect of the head. The rats were then returned to their home cages for a 5-day recovery period. Each rat was housed individually on a 12-h light/dark daily cycle with food and water freely available. The rats that showed evidence of gross neurological injury or the presence of fresh blood in the cerebrospinal fluid (CSF) were excluded from the study. The use of animals conformed to the Guiding Principles in the Care and Use of Animals of the American Physiological Society and was approved by the National Sun Yat-sen University and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

**Induction of spinal cord ischemia in rats**

Spinal cord ischemia was induced in the male Wistar rats implanted with one i.t. catheter each. The rat spinal cord ischemia model used in this study was a modification of the model previously described by Taira and Marsala (1996) and Wu et al. (2007). In brief, the animals were anesthetized with 4% isoflurane in a plastic box in room air. After induction, 2.5% isoflurane in an air/O2 mixture was delivered to the rats through a mask. The tail artery was cannulated with a 22-gauge polytetrafluoroethylene catheter in order to monitor the distal arterial pressure and to administer heparin intra-arterially. The left carotid artery was cannulated with a 20-gauge polytetrafluoroethylene catheter for blood sample collection. The left femoral artery was exposed to induce spinal cord ischemia. Aortic occlusion was induced by inflation of a 2F Fogarty catheter inserted into the thoracic aorta for 12 min 30 s, and blood was collected from the cannulated left carotid artery and pumped into the peripheral stream during aortic occlusion. To induce spinal cord ischemia, an intraaortic Fogarty catheter was inflated with 0.06 ml of saline, and systemic hypotension (40 mm Hg) was induced by withdrawing arterial blood (11–13 ml). Immediately after the completion of the arterial cannulations, all the rats received 100 U of heparin (0.1 ml) through the thoracic aorta. After completion of all the surgical procedures, 0.4 ml of protamine sulfate (4 mg) was administered intraperitoneally. The animals were then returned to their cages for recovery of motor function and finally killed for spinal sample collection.

**I.t. G-CSF injection**

A 5-day recovery period was allowed after implantation of the i.t. catheter, and ischemic surgery was subsequently performed. Immediately after deflating the Fogarty catheter, G-CSF (10 µg/23 µl, Chugai Pharmaceutical Co., Kitaku, Tokyo, Japan) was injected via the i.t. catheter, followed by a flush with 3.5 µl of saline or normal saline (26.5 µl) alone. The rats were randomly divided into four groups: (1) control group, a balloon catheter was placed in the thoracic aorta without inflation and i.t. saline was injected; (2) ischemia alone group, a balloon catheter was placed in the thoracic aorta with inflation and i.t. saline was injected; (3) ischemia + G-CSF group, ischemia was induced and i.t. G-CSF was injected; (4) G-CSF alone group, i.t. G-CSF was injected without inducing spinal cord ischemia. The rats from each group were killed at 15, 30, 60, 180, and 360 min after ischemia (surgery) was induced, and the behavioral test was performed. The experimental protocol is summarized in Fig. 1.
portions of the spinal cords were homogenized in an ice-cold lysis cord homogenates were used at each time point. The dorsal ischemia was induced. From each group, four individual spinal and the ventral portions of the lumbar (L2–L5) spinal cords were removed. The method used for animal decapitation was in accord-
ance with the principles laid down by the Animal Care and Use Committee of our institute. For Western blot analysis, the rats from each group were killed at 15, 30, 60, 180, and 360 min after surgery. Ischemia was induced. From each group, four individual spinal cords were homogenized at each time point. The dorsal and ventral portions of the lumbar (L2–L5) spinal cords were removed. The method used for animal decapitation was in accordance with the principles laid down by the Animal Care and Use Committee of our institute. For Western blot analysis, the rats from each group were killed at 15, 30, 60, 180, and 360 min after ischemia was induced. From each group, four individual spinal cord homogenates were used at each time point. The dorsal portions of the spinal cords were homogenized in an ice-cold lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2% Triton X-100, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin) and then centrifuged at 68,000 r.p.m. (TXL-100, Bekman, Fullerton, CA, USA) for 30 min at 4 °C. The supernatant was decanted from the pellet and retained for Western blot analysis. The protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA) by the modified method of Lowry et al. (1951). Western blotting was performed as described in our previous study (Wen et al., 2005). In brief, an equal volume of sample buffer (2% SDS, 10% glycerol, 0.1% Bromophenol Blue, 2% 2-mercaptoethanol, and 50 mM Tris–HCl (pH 7.2)) was added to the sample, which was then loaded onto a 10% SDS–polyacrylamide gel and electrophoresed at 150 V for 60 min. For the analysis of the phosphorylated and non-phosphorylated forms of p38, JNK, ERK, and Akt proteins, 100 and 10 μg spinal homogenate samples, respectively, were prepared. After electrophoresis, the proteins were transferred to a polyvinylidene difluoro membrane (Immobilon-P, 0.45-μm pore size, Millipore, MA, USA) at 125 mA overnight at 4 °C in a transfer buffer (50 mM Tris–HCl, 380 mM glycine, 1% SDS, and 20% methanol). The membrane was then blocked for 50 min at room temperature with 5% non-fat dry milk and 0.1% Tween 20 in 20 mM Tris–HCl, 137 mM NaCl, pH 7.4 (TTBS), and incubated for 16 h at 4 °C with antibodies specific against the phosphorylated and non-phosphorylated forms of p38, JNK, ERK, and Akt. Phosphorylation of p38, JNK, ERK, and Akt was determined by Western blot analysis using anti-phospho-p38 (Thr180/Tyr182), JNK (Thr183/Tyr185), ERK (Thr202/Tyr204) and Akt (ser473) antibodies. All the antibodies (dilution 1:1000) were obtained from Cell Signaling Technology Inc. (MA, USA). The membrane was washed three times in TTBS for 10 min, blocked with 5% non-fat dry milk/TTBS, and then incubated for 1 h at room temperature with anti-rabbit antiserum horseradish peroxidase–conjugated secondary antibodies (dilution 1:2500). The blots were visualized in ECL solution (NEN, MA, USA) and finally exposed to X-omat LS (Kodak, Rochester, USA) and quantified by densitometry. The membranes were reprobed with monoclonal mouse anti-β-actin antibody (dilution 1:2500, Sigma, MO, USA) as the loading control.

**Preparation of spinal cord tissue homogenates and Western blotting**

The rats anesthetized with isoflurane were rapidly decapitated, and the ventral portions of the lumbar (L2–L5) spinal cords were removed. The method used for animal decapitation was in accordance with the principles laid down by the Animal Care and Use Committee of our institute. For Western blot analysis, the rats from each group were killed at 15, 30, 60, 180, and 360 min after ischemia was induced. From each group, four individual spinal cord homogenates were used at each time point. The dorsal portions of the spinal cords were homogenized in an ice-cold lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2% Triton X-100, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin) and then centrifuged at 68,000 r.p.m. (TXL-100, Bekman, Fullerton, CA, USA) for 30 min at 4 °C. The supernatant was decanted from the pellet and retained for Western blot analysis. The protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA) by the modified method of Lowry et al. (1951). Western blotting was performed as described in our previous study (Wen et al., 2005). In brief, an equal volume of sample buffer (2% SDS, 10% glycerol, 0.1% Bromophenol Blue, 2% 2-mercaptoethanol, and 50 mM Tris–HCl (pH 7.2)) was added to the sample, which was then loaded onto a 10% SDS–polyacrylamide gel and electrophoresed at 150 V for 60 min. For the analysis of the phosphorylated and non-phosphorylated forms of p38, JNK, ERK, and Akt proteins, 100 and 10 μg spinal homogenate samples, respectively, were prepared. After electrophoresis, the proteins were transferred to a polyvinylidene difluoro membrane (Immobilon-P, 0.45-μm pore size, Millipore, MA, USA) at 125 mA overnight at 4 °C in a transfer buffer (50 mM Tris–HCl, 380 mM glycine, 1% SDS, and 20% methanol). The membrane was then blocked for 50 min at room temperature with 5% non-fat dry milk and 0.1% Tween 20 in 20 mM Tris–HCl, 137 mM NaCl, pH 7.4 (TTBS), and incubated for 16 h at 4 °C with antibodies specific against the phosphorylated and non-phosphorylated forms of p38, JNK, ERK, and Akt. Phosphorylation of p38, JNK, ERK, and Akt was determined by Western blot analysis using anti-phospho-p38 (Thr180/Tyr182), JNK (Thr183/Tyr185), ERK (Thr202/Tyr204) and Akt (ser473) antibodies. All the antibodies (dilution 1:1000) were obtained from Cell Signaling Technology Inc. (MA, USA). The membrane was washed three times in TTBS for 10 min, blocked with 5% non-fat dry milk/TTBS, and then incubated for 1 h at room temperature with anti-rabbit antiserum horseradish peroxidase–conjugated secondary antibodies (dilution 1:2500). The blots were visualized in ECL solution (NEN, MA, USA) and finally exposed to X-omat LS (Kodak, Rochester, USA) and quantified by densitometry. The membranes were reprobed with monoclonal mouse anti-β-actin antibody (dilution 1:2500, Sigma, MO, USA) as the loading control.

**Double immunohistochemistry for glial fibrillary acidic protein (GFAP) plus p-P38, NeuN plus p-Akt, or NeuN plus p-ERK**

For immunohistochemistry, at 1 h after the ischemia surgery (or G-CSF injection), the rats were under deep anesthesia with isoflu- rane (5%). First, the rats were intracardially perfused with 500 ml of cold phosphate-buffered saline (PBS) containing 1% sodium nitrite and heparin (0.2 U/ml) and then with 4% paraformaldehyde in 500 ml of 0.1 M PBS (pH 7.4). The lumbar enlargements (L1–L2) were then harvested and post-fixed in the same fixative for 48 h and then transferred to a 30% sucrose solution overnight at 4 °C. Sections (4 μm) were prepared, air-dried on microscope slides for 1 h at room temperature, and preincubated (1 h) with 4% normal goat serum diluted in 0.01% Triton X-100, PBS. After washing three times in ice-cold PBS, the sections were incubated overnight at 4 °C with FITC-labeled mouse monoclonal anti-GFAP (an astrocyte cell marker) (Molecular Probes, OR, USA; green fluorescence) plus unlabeled rabbit polyclonal antibodies anti-phospho-p38 or Alexa Fluor 488 conjugated monoclonal anti-NeuN (a neuronal specific nuclear protein) (Chemicon, USA; green fluorescence) plus unlabeled rabbit polyclonal antibodies anti-p-Akt or anti-phospho-ERK in 0.01% Triton X-100, 2% normal goat serum in PBS. The sections were then reacted for 1 h at room temperature with rhodamine-labeled goat anti-rabbit antibody (red fluorescence) (Jackson ImmunoResearch Laboratories Inc., USA). The images were viewed using a Leica DM-1000 fluorescence microscope (Leica, Germany) at 400× magnification and captured using a SPOT CCD RT-slider integrating camera (Diagnostic Instruments Inc., USA). The laser wavelength was set at 488 nm for Alexa Fluor and FITC green fluorescence and 568 nm for rhodamine fluorescence. Samples from all the groups were numbered randomly, and these numbers were used to identify the samples through the evaluation to prevent bias. The double immunohistochemical observations were evaluated at 400× magnification by an investigator blind to the treatment groups by using three sections per rat.

**Statistical analysis**

All data are presented as the mean±S.E.M. The MDI were analyzed using one-way analysis of variance (ANOVA), followed Dunnnett’s post hoc test. In the immunoreactivity data, the intensity of each test band was expressed as the relative optical density (ROD) calculated from the average control optical density value.
for all the control X-ray films. In the p-p38 analysis, the density of the ischemic band (p-p38) at each time point was 100%. The density of the control p-JNK, p-ERK, and p-AKT band at each time point was 100%. We analyzed the protein expression of p-p38, p-JNK, p-ERK, and p-AKT of the control, ischemia, ischemia plus G-CSF, and G-CSF groups at each time point. Wherever applicable, data from testing was analyzed using one-way ANOVA followed by Dunnett’s test. A P value less than 0.05 was considered statistically significant.

RESULTS

The effect of i.t. G-CSF administration on spinal cord ischemia-induced neurological dysfunction

All rats from the control group and those treated with i.t. G-CSF exhibited a normal neurological outcome (MDI=0). Similar to that observed in our previous studies, all the ischemic group rats exhibited flaccid paraplegia after recovery from anesthesia and then developed spasms over the next 48 h. The MDI values in the ischemia+G-CSF group at 15, 30, 60, 180, and 360 min were 3.88±0.26, 3.44±0.29, 2.66±0.33, 1.77±0.44, and 1.50±0.55 respectively. The i.t. G-CSF treatment (10 μg, immediately after ischemia surgery) significantly inhibited spinal cord ischemia that induced neurological function deficits from 15 min to 360 min after the surgery (Fig. 2). Moreover, we also found that the MDI of the ischemia+G-CSF (n=6) and ischemia alone groups (n=5) at 48 h were 1.5±0.22 and 5.4±0.24, respectively.

The effect of i.t. G-CSF administration on spinal cord ischemia-induced changes in the expressions of p38, JNK, ERK, and Akt proteins

The expression levels of the non-phosphorylated (p38, JNK, ERK, and Akt) and phosphorylated (phospho-p38, phospho-JNK, phospho-ERK, and phospho-Akt) proteins were analyzed using Western blotting with the respective antibodies. As compared with the other groups (ischemia+G-CSF, G-CSF alone, and control groups), the expression of phospho-p38 proteins was significantly increased in the ischemia alone group from 15 to 180 min after spinal cord ischemia surgery. However, the upregulation of ischemia-induced phospho-p38 protein expression was completely inhibited by the i.t. G-CSF injection (Fig. 3A). No significant change in the total p38 (phosphorylated and non-phosphorylated forms) level was noted in any group (data not shown).

It was noted that the phospho-JNK expression level was approximately the same at 15 min in all the groups. In particular, the phospho-JNK expression level was significantly higher (approximately 200%) in the ischemia plus saline group at 30 min after surgery. Subsequently, it decreased and reached the 150% level from 60 to 360 min after surgery. In the ischemia+G-CSF group, i.t. G-CSF significantly inhibited spinal cord ischemia-induced phospho-JNK upregulation. From 180–360 min after spinal ischemia, G-CSF almost completely prevented the upregulation of p-JNK. The expression level of p-JNK was almost the same in the G-CSF alone and control groups (Fig. 3B). The total (phosphorylated and non-phosphorylated) JNK levels remained constant in all the treatment groups (data not shown).

Our results clearly indicate that the expression level of phospho-ERK protein was high at 15 min in the G-CSF alone group and was significantly high (approximately 200%) in the ischemia+GCSF group, and this level was decreased at 180 and 360 min. A transient upregulation of phospho-ERK was observed at 30 min in the ischemia alone group (Fig. 4A). No significant change in the total ERK (phosphorylated and non-phosphorylated) level was noted in any group (data not shown). A transient downregulation of anti-apoptotic p-Akt protein expression was observed at 15 min after ischemia-reperfusion; however, this was significantly prevented by i.t. G-CSF. Surprisingly, p-Akt protein expression was upregulated from 30 to 60 min after the ischemic insult. Moreover, the expression of p-Akt was significantly upregulated in the G-CSF alone and ischemia+G-CSF groups from 15 to 180 min after the G-CSF injection (and the ischemia surgery). G-CSF (i.t.) not only significantly upregulated p-Akt protein expression but also prolonged the spinal cord ischemia-induced p-Akt upregulation (Fig. 4B). No significant change in the total Akt level was noted in any group.

The effect of G-CSF on spinal cord ischemia-induced up-regulation of phospho-p38 in the astrocytes of the ventral lumbar spinal cord

As shown in Fig. 5, the cellular specificity of phospho-p38 regulation was confirmed. We used double-labeled immunofluorescent staining to detect the GFAP (astrocyte marker) (green, Fig. 5A, D, G and J) and phospho-P38 (red, Fig. 5B, E, H and K) in the ventral lumbar spinal cord. The merged images (yellow, Fig. 5C, F, I, and L) were used for detecting phospho-p38-positive astrocytes. In the control and G-CSF alone groups, the phospho-p38-positive astrocytes revealed extremely weak immunostaining. It is extremely clear that ischemia induced the upregulation of phospho-p38-immunoreactive at 1 h after ischemia surgery (Fig. 5E). Further, it is also clear that i.t. G-CSF inhibited the ischemia-induced up-
regulation of phospho-p38-immunoreactive (Fig. 5H). The merged images demonstrated pronounced colocalization of phospho-p38 and GFAP, indicating the upregulation of phospho-p38 expression in the astrocytes after spinal cord ischemia (Fig. 5F); however, this upregulation was prevented by i.t. G-CSF administration (Fig. 5L).

The effect of G-CSF on phospho-Akt and phospho-ERK expressions in the ventral spinal motor neurons

As shown in Fig. 6, it was confirmed that phospho-Akt was colocalized with motor neurons. In addition, we performed double immunofluorescence staining to detect the immu-
noreactivity of the neuron specific marker NeuN (green, Fig. 6A, D, G, and D) and phospho-Akt (red, Fig. 6B, E, H, and K) in the ventral part of the lumbar spinal cord at 1 h in the control, ischemia, ischemia \( \text{G-CSF} \), and G-CSF alone groups. The merged images (yellow, Fig. 6C, F, I, and L) were used for detecting phospho-Akt-positive neuronal cells. The upregulation of the p-Akt-immunoreactivity in the ischemia+G-CSF and G-CSF alone groups was compared with that in the control and ischemia alone groups (Fig. 6B, E, H, and K). Double immunofluorescence staining revealed that p-Akt was precisely colocalized with NeuN in the ventral spinal cord in the ischemia, ischemia+G-CSF, and G-CSF alone groups at 1 h after ischemia surgery (or G-CSF injection) (Fig. 6F, I, and L). Furthermore, in the ischemia alone group, only a few neuronal cells were p-Akt-positive (Fig. 6F). However, in the

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Fig. 4. Effect of G-CSF injection on (A) phosphorylated ERK (p-ERK) and (B) phosphorylated Akt (p-AKT) protein expression in ischemic rats. (A) A short-lived up-regulation of p-ERK expression was noted at 30 min in the ischemia alone group. Significantly upregulated phosphor-ERK in ischemia+G-CSF and G-CSF alone groups, from 15 to 60 min after G-CSF injection. (B) Our results clearly reveal that the i.t. G-CSF injection significantly upregulated p-Akt protein expression as well as inhibited the spinal cord ischemia-induced downregulation of p-Akt at 15th min. Ischemia-induced the upregulation of p-Akt expression from 30 to 60 min after surgery. The optical density of the protein bands was quantified using densitometry, and the relative optical density was calculated using the average value of samples from five rats and considering the density of the control band at each time point as 100%. (a) \( P<0.05 \) compared with the control group; (b) \( P<0.05 \) compared with the ischemia group.
G-CSF alone group, the large nuclei of the motor neurons in the ventral horn were immunostained with phospho-Akt (Fig. 6L). As shown in Fig. 7, double-immunofluorescence staining was used to confirm the neuronal phospho-ERK upregulation induced by G-CSF. NeuN-immunoreactive (green, Fig. 7A, D, G, and D) and phospho-ERK-immunoreactive cells (red, Fig. 7B, E, H, and K) were observed in the ventral part of the lumbar spinal cord at 1 h in the control, ischemia alone, ischemia+G-CSF, and G-CSF alone groups. The merged images (yellow, Fig. 7C, F, I, and L) indicated that phospho-ERK was colocalized with NeuN in the ventral spinal cord. In the ischemia alone group, clear upregulation of phospho-ERK was observed in the neuronal cells as compared with

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**Fig. 5.** Double-labeled immunofluorescent staining of phospho-p38 (red) and GFAP (green) in the ventral region of the lumbar spinal cords. The spinal cord sections (5 μm) at 1 h obtained from the control (A, B, C), ischemia (D, E, F), ischemia+G-CSF (G, H, I) and G-CSF alone (J, K, L) groups. These images represent multiple fields examined for each group from three independent immunofluorescence observations. The immunostaining images revealed cells labeled with GFAP (green) and phospho-p38 (red) in the spinal cord. The merged images of C, F, I, L (yellow) indicated the colocalization of p-p38 and GFAP-(astrocyte-specific marker) immunoreactive cells in the ventral spinal cord. In the control and G-CSF alone groups, the p-p38 immunoreactive cells revealed extremely weak staining in the ventral horn (B, K). p-p38 Activation was stronger in the ischemia alone group than in the ischemia+G-CSF, control, and G-CSF alone groups. In the merged images, double-fluorescence immunohistochemistry demonstrated the colocalization of the p-p38-immunoreactive and GFAP-immunoreactive cells in the ventral horn neuron in the ischemia alone group. Scale bars = 100 μm for all images. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
that in the control and ischemia alone groups. Fig. 7A, D, G, and J shows the large nuclei of NeuN-immunoreactive motor neurons.

**DISCUSSION**

In the present study, we demonstrated that an i.t. injection of the hematopoietic factor G-CSF significantly attenuated spinal cord ischemia-induced neurological defects. G-CSF significantly inhibited the spinal cord ischemia-induced upregulation of phospho-JNK and phospho-p38 MAPK. On the other hand, i.t. G-CSF prolonged and enhanced the spinal cord ischemia-induced activation of ERK and anti-apoptotic Akt. G-CSF (i.t.) alone significantly increased ERK and Akt activities, but did not change JNK and p38 activation. From double

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**Fig. 6.** Double-labeled immunofluorescent staining of p-Akt (red) and NeuN (green) in the ventral region of the lumbar spinal cord. The spinal cord sections (5 μm) were obtained at 1 h from the control (A, B, C), ischemia (D, E, F), ischemia+G-CSF (G, H, I), and G-CSF alone (J, K, L) groups. These images represent multiple fields examined for each group from three independent immunofluorescence observations. The immunostaining images revealed cells labeled with NeuN (green) and p-Akt (red) in the spinal cord. The merged images of C, F, I, and L (yellow) indicated the colocalization of p-Akt and NeuN-neuronal specific marker-immunoreactive cells in the ventral spinal cord. In the control group, the pAkt immunoreactive cells presented extremely weak staining in the ventral horn neurons. In the merged images, double fluorescence immunohistochemistry demonstrated the colocalization of p-Akt-immunoreactive and NeuN-immunoreactive cells in the ventral horn neuron in the ischemia, ischemia+G-CSF, and G-CSF alone groups. Moreover, neuronal p-Akt expression was weaker in the ischemia group than in the ischemia+G-CSF and G-CSF alone groups. Scale bars = 100 μm for all images. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
immunohistochemical observations, we observed that i.t. G-CSF upregulated phospho-Akt but inhibited spinal cord isch-emia-induced phospho-p38 expression in the motor neurons and astrocytes of the ventral region of the lumbar spinal cord. The investigation of the molecular mechanisms of neuropro-ective actions of i.t. G-CSF administration demonstrated that the attenuation of both the MAPK and Akt activities was related to the G-CSF-induced neuroprotective actions in spinal cord ischemia.

**The effect of G-CSF on anti-apoptotic Akt**

Since the original description of anti-apoptotic activities of Akt in neurons (Dudek et al., 1997), many reports have confirmed the important central regulatory role of this ki-
nase for neuronal survival (Brunet et al., 2001). From our study, it is extremely clear that i.t. G-CSF regulated the expression of the active form of Akt (phospho-Akt) protein in the spinal cord. Both ischemia-iG-CSF and G-CSF alone administration significantly upregulated phospho-Akt protein expression from 15 to 180 min. Moreover, i.t. G-CSF almost completely prevented the ischemia-induced downregulation of phospho-Akt at 15 min after the ischemic insult. From the immunohistochemical and Western blotting analysis, we proposed that i.t. G-CSF directly induced strong anti-apoptotic activity in the motor neuronal cells of the ventral region of the lumbar spinal cord.

It is extremely interesting to note the transient upregulation of phospho-Akt from 30 to 60 min after the ischemic insult. Similar to previous studies, Kitagawa et al. (1999) and Wagey et al. (1998) observed that the activated Akt protein levels were significantly increased in the nervous tissue in middle cerebral artery occlusion and amyotrophic lateral sclerosis. Moreover, Sakurai et al. (2003) observed the upregulation of phospho-Akt expression in the motor neurons of rabbit after transient spinal cord ischemia. We speculated that ischemia induced the transient upregulation of Akt activity as a compensatory mechanism of endogenous neuroprotection.

### The effect of G-CSF on ischemia-induced inflammatory p38 and JNK signaling

Several studies on the MAPK cascade and brain ischemia have been reported; however, the expression and distribution of phosphorylated p38, JNK, and ERK after an ischemic insult to the spinal cord have never been systematically reported (Irving and Bamford, 2002). Several studies have indicated that p38 MAPK activation plays a critical role in the regulation of inflammatory responses during the progression of neurodegenerative processes (Hertaar and Brown, 1999; Chaparro-Huerta et al., 2005; Dalrymple, 2002; Jeohn et al., 2002). The activation of p38 has been repeatedly associated with both acute CNS injury, such as stroke and trauma, and neurodegeneration (Xia et al., 1995; Kummer et al., 1997). The inhibition of p38 activity by a selective inhibitor could attenuate neuronal injury (Horiuchi et al., 2003; Legos et al., 2001). Similar to the findings of Wu et al. (2000) and Sugino et al. (2000), in the present study, Western blotting revealed that spinal p38 was rapidly phosphorylated from 15 to 180 min after an ischemic insult. Moreover, after the ischemic insult caused an increase in phospho-p38 protein expression, immunohistochemistry revealed that the increased phospho-p38 immunoreactivity was predominantly observed in GFAP-positive cells and that these cells were typically characterized as astrocytes. It has been known that astrocytes are the major cell type within the CNS (Korzus et al., 1997); they are capable of synthesizing various pro-inflammatory mediators and neurotrophic factors, and they exist between the nervous cells and vessels to transport selective substances (Kimelberg, 1995). In general, astrocytes become reactive and proliferative at the injury site following CNS injury caused by trauma, ischemia and chemical insults (Eng and Ghirmikar, 1994; Pekny and Pekna, 2004).

In the present study, we observed that the spinal cord ischemia-induced activation of p38 in astrocytes may play a role in neuroinflammation and neurodegeneration associated with cord ischemia injury. Increasing evidence suggested that activation of the JNK pathway occurs in response to similar stimuli that activate p38, which is associated with the mediation of neuronal death in central nervous ischemia-reperfusion injury (Irving and Bamford, 2002). Activated JNK in turn phosphorylates the nuclear and cytosol substrates, thereby leading to neuronal death, and the inhibition of JNK activity by a selective inhibitor could reduce apoptotic neuronal cell death (Borsello et al., 2003; Gao et al., 2005). Several lines of evidence revealed that neuronal injury is also associated with the upregulation of phospho-JNK protein expression (Hu et al., 2000; Sugino et al., 2000; Borsello et al., 2003). Moreover, after cerebral artery occlusion-induced mouse brain ischemia, increased phospho-JNK levels were detected that peaked at 30 min but remained significantly upregulated up to 6 h (Wu et al., 2000). Similar to previous reports, the present study reveals that spinal cord ischemia-induced activation of JNK in the spinal cord lasted at least up to 6 h. Similar to p38, the JNK signaling pathway has been demonstrated to regulate pro-inflammatory cytokine production (Waetzig et al., 2005). G-CSF can activated STAT3, and the subsequent inhibition of JNK activity results in the suppression of inflammation (Nishiki et al., 2004; Kim et al., 2006). The inactive form of JNK may demonstrate resistance to inflammatory neuronal death, suggesting that the prevention of JNK activation might also confer neuroprotection. In the present study, i.t. G-CSF inhibited the spinal cord ischemia-induced activation of p38 and JNK. We suggested that i.t. G-CSF produced neuroprotective effects by anti-inflammatory mechanisms via the inhibition of p38 and JNK activity.

### The effect of G-CSF on ERK

The present study revealed that ERK activity is only transiently increased at 30 min after the ischemic insult. Different to the effect of G-CSF on JNK and p38, i.t. G-CSF does not inhibit the ischemia-induced activation of ERK. Seger and Krebs (1995) indicated that ERKs are ubiquitously expressed and are activated primarily in response to growth factors or mitogens in dividing cells. The present results revealed that the G-CSF-induced increase in p-ERK was rapid but transient from 15 to 60 min after the i.t. G-CSF injection in the ischemia-iG-CSF and G-CSF alone groups. Schneider et al. (2005a) and Huang et al. (2007) observed that ERK was transiently activated by G-CSF in the cortical and dopaminergic neurons. The activation of ERK has been implicated to contribute to neuronal cell survival after a neurotoxic insult (Abe and Saito, 2000; Han and Holtzman, 2000). From immunohistochemical results, the upregulation of p-ERK expression in the motor neurons was observed in both the ischemia-iG-CSF and G-CSF alone groups. Further, we proposed that i.t. G-CSF-induced neuroprotection in spinal ischemia was related to ERK activation. In addition, our results revealed that ischemia-induced mild and transiently upregulated ERK activ-
ity at 30 min; this may be due to an endogenous compensatory mechanism in response to the ischemia insult.

The possible molecular mechanisms of G-CSF-induced neuroprotection

Inflammatory mechanisms are activated in response to CNS damage by the production of pro-inflammatory mediators by immune cells. This might be crucial for inducing neuronal death (Beattie, 2004; Amar and Levy, 1999). Many studies have shown that G-CSF activates the JAK-STAT signaling pathway, reduces the pro-inflammatory cytokine, TNF-α, interleukin (IL)-1β, IL-6, and IL-8 levels, and enhances the IL-1β receptor antagonist levels (Heard and Fink, 1999; Darnell, 1997; Boneberg and Hartung, 2002). Nishiki et al. (2004) demonstrated that G-CSF exerts the anti-inflammatory or immunomodulatory effect, thereby inhibiting the overproduction of pro-inflammatory cytokines from LPS-stimulated monocytes. Therefore, G-CSF might inhibit pro-inflammatory cytokine toxicity and neutrophil activation and infiltration. Our results indicated that G-CSF prevented the spinal cord ischemia-induced activation of p38 in the astrocytes. Furthermore, it induced the upregulation of Akt and ERK activities in the neuronal cells. Based on these results, we proposed that the potential neuroprotective effect of G-CSF in neuronal injury may be mediated by its anti-inflammatory and anti-apoptotic effects on the astrocytes and neuronal cells, respectively.

The effect of spinal anatomy on ischemia-induced pathological observations

In a clinical setup, the occurrence of spinal cord ischemia is very different from that of an accidental spinal cord injury. Spinal ischemia usually occurs after a thoracoabdominal aortic surgery and warrants the development of a prevention strategy because many thoracoabdominal aortic surgeries can be subjected to prior monitoring. Several studies have shown that the vascular anatomy of rats and humans is similar (Koyanagi et al., 1993; Scremin, 1995). However, in the present study, using the spinal cord ischemia animal model, we only observed the severity of hind limb paraparesis. Based on anatomical knowledge of the spinal cord of rats (Scremin, 1995), even if the aortic-occlusion-induced spinal cord ischemia is successful, the subclavian artery still has the ability to partly support the blood supply to the cervical and thoracic spinal cords. Furthermore, based on past studies and our present observations, the spinal cord ischemia animal model mainly affects the motor function of the lower half of the body (Taira and Marsala, 1996; Wu et al., 2007). On the other hand, the upper body appears to be unaffected by the disease.

CONCLUSION

In summary, a direct i.t. G-CSF injection significantly inhibited the spinal cord ischemia-induced neurological defects. To our knowledge, the present study is the first to demonstrate the effect of i.t. G-CSF on the regulation of the MAPK and Akt pathways in spinal cord ischemia. Both spinal cord ischemia-induced neurological defects and changes in the activity of the MAPK and Akt pathways were significantly and directly regulated by i.t. G-CSF administration. Further, we suggested that G-CSF has anti-inflammatory (p38 and JNK) and anti-apoptotic (Akt and ERK) actions causing substantial sparing of both neurons and glial cells and improves neurological function in spinal cord ischemic injury. These findings are noteworthy considering that G-CSF is one of the few growth factors that has been approved for clinical use, and the present results suggest that G-CSF might be beneficial for the treatment of spinal cord ischemic injury.

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