Granulocyte colony-stimulating factor (G-CSF) exerts neuroprotective effects via promoting angiogenesis after spinal cord injury in rats

Junko Kawabe[1], MD, PhD, Masao Koda[2], MD, PhD, Masayuki Hashimoto[1], MD, PhD, Takayuki Fujiyoshi[1], MD, PhD, Takeo Furuya[1], MD, PhD, Tomonori Endo[1], MD, PhD, Akihiko Okawa[1], MD, PhD, Masashi Yamazaki[1], MD, PhD,

[1]Department of Orthopaedic Surgery, Chiba University Graduate School of Medicine, 1-8-1 Inohana-Cho, Chuo-Ku, Chiba 260-8677, Japan
[2]Department of Orthopaedic Surgery, Chiba Aoba Municipal Hospital, 1273-2, Aoba-Cho, Chuo-Ku, Chiba 260-0852, Japan

Correspondence: Masao Koda, Department of Orthopaedic Surgery, Chiba Aoba Municipal Hospital, 1273-2, Aoba-Cho, Chuo-Ku, Chiba 260-0852, Japan
Tel: 81-43-227-1131, Fax: 81-43-227-1961
e-mail: masaomst@yahoo.co.jp

ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) has neuroprotective effects on the central nervous system. We previously demonstrated that G-CSF also exerts neuroprotective effects in experimental spinal cord injury (SCI) by enhancing migration of bone marrow-derived cells into the damaged spinal cord, increasing glial differentiation of bone marrow-derived cells, enhancing anti-apoptotic effects on both neurons and oligodendrocytes, and by reducing demyelination and expression of inflammatory cytokines. Because the degree of angiogenesis in the sub-acute phase after SCI correlates with regenerative responses, it is possible that G-CSF's neuroprotective effects after SCI are due to enhancement of angiogenesis. Our aim was to assess the effects of G-CSF on the vascular system after SCI. We utilized the contusive SCI rat model and randomly divided subjects between a G-CSF-treated group and a control group. Integrity of the blood spinal cord barrier was evaluated by measuring the degree of edema of the cord and the volume of extravasation. For histological evaluation, cryosections were immunostained with anti-von Willebrand factor and the number of vessels was counted to assess revascularization. Real time PCR was performed to assess expression of angiogenic cytokines, and recovery of motor function was assessed with function tests.

In the G-CSF treated rats, the total number of vessels was significantly larger and expression of angiogenic cytokines was significantly higher than those in the control group. The G-CSF-treated group showed significant recovery of hind limb function compared to that of the control group. These results suggest that G-CSF exerts neuroprotective effects via promotion of angiogenesis after SCI.

INTRODUCTION

There are two mechanisms of damage to the spinal cord after acute SCI: the initial mechanical injury followed by secondary injury. Various factors trigger the secondary damage that exacerbates neurologic deficit. Those factors include altered ion balance, lipid peroxidation, glutamate release, inflammatory processes, disturbance of pulsatile hydrodynamics and ischemia[33]. Acute SCI causes immediate mechanical damage to the microvasculature of the cord followed by a secondary injury to the vessels; this combination produces spinal cord ischemia which can be progressive[17, 29]. Thus, angiogenesis is critically important to reduce secondary damage to the spinal cord vasculature[4, 17].

Granulocyte colony-stimulating factor (G-CSF) is a 19.6 kDa glycoprotein that was identified initially as a serum activity that induced differentiation of a murine myelomonocytic leukemic cell line[31]. It is widely known as a growth factor for hematopoietic cells that promotes survival, proliferation and differentiation of cells of the neutrophil lineage[23]. It is used clinically in the treatment of neutropenia following anticancer therapy, and in the mobilization of peripheral blood-derived hematopoietic stem cells for transplantation[11].

Recently, the neuroprotective effects of G-CSF on the central nervous system (CNS) was reported[18, 24, 25, 34]. As we previously documented, G-CSF also exerts neuroprotective effects on experimental SCI (SCI). G-CSF promotes the migration of bone marrow-derived cells into the damaged spinal cord and glial differentiation of bone marrow-derived cells[13]. G-CSF has neuroprotective activity via anti-apoptotic effects on both neurons[20] and oligodendrocytes, and it attenuates inflammatory cytokine expression and demyelination.

In addition, G-CSF possesses angiogenic effects in various pathological models. Administration of G-CSF has been shown to accelerate angiogenesis in animal models of limb and myocardial ischemia[19]. As for the CNS, several studies have documented angiogenic effects of G-CSF in cerebral ischemia[16, 18, 21]. Lee *et al.* reported that the

vascular surface area, vascular branch points, vascular length, the number of BrdU$^+$ endothelial cells, and eNOS/angiopoietin-2 expression were all significantly increased in G-CSF-treated rats in the focal cerebral ischemia model[16]. Ohki et al. found that concomitant with an increase in circulating neutrophils, G-CSF increased plasma VEGF from neutrophils in vivo. Furthermore, blockade of the VEGF pathway abrogated G-CSF-induced angiogenesis, suggesting that G-CSF-induced angiogenesis is VEGF-dependent in the murine ischemic hindlimb model[21]. We speculated that G-CSF might possess angiogenic potential in SCI. The degree of angiogenesis during the sub-acute phase after SCI correlates with regenerative responses[17] and the newly formed vascular bridge might provide scaffolding to hasten axonal regeneration across the injury site[4]. Thus, angiogenesis might contribute to the regenerative response of neural tissue and enhance recovery of locomotor function after injury. Thus, we hypothesized that the neuroprotective effects of G-CSF after SCI were due to its enhancement of angiogenesis.

In this study, our aim was to assess the effects of G-CSF on both the vascular system and injury-induced edema, as well as maintenance of the integrity of the blood spinal cord barrier (BSB) and angiogenesis after SCI and its possible contribution to functional recovery.

MATERIALS AND METHODS

Experimental animals

All animals were treated and cared for in accordance with the Chiba University School of Medicine guidelines pertaining to the treatment of experimental animals. Sixty-three adult female Sprague-Dawley rats (10-12 weeks old; weight 200 – 240 g; Japan SLC, Inc. Hamamatsu, Japan) were used for the current experiments. Anesthesia was induced with inhalation of 5% halothane in 0.5 L/min oxygen and maintained with 1.2 - 1.4% halothane in 0.5 L/min oxygen. Laminectomy was carried out at the Th8/9 level and the dural tube was exposed without scratching the dura mater. Animals were positioned on the stereotaxic apparatus, vertebral bodies of Th7 and Th10 were grasped by two adjustable forceps to fix the spine. We induced moderate contusive SCI using the model IH-0400 impactor (Precision Systems and Instrumentation, Lexington, KY, 200Kdyne). Rats were left in the cages, warmed under a heat lamp, until they were awake. Upon awakening, rats were evaluated neurologically and were monitored for food and water uptake and urine output. Antibiotic was added prophylactically to drinking water. Rats were randomly divided into two groups, the G-CSF-treated group and the control group. We

administered recombinant human G-CSF (15 μg/kg/day; kindly provided by Kirin Brewery Co., Ltd, Pharmaceutical Division, Tokyo, Japan) to the treated group and the same volume of normal saline to the control group via tail vein one hour after injury, and successively for five days. The dose of G-CSF was determined by the basis of our preliminary experimental data. We performed preliminary experiments in other dose regimen (5, 15 and 50 μ g/kg/d for 5 days after injury) for contusive SCI. Those preliminary data suggested that 15 μ g/kg/d G-CSF exerts most strong effects. Thus we employed 15 μ g/kg/d dose regimen.

Integrity of Blood Spinal cord Barrier (BSB)

Integrity of the BSB after injury was evaluated by measuring the extent of edema of the cord after injury and volume of extravasation. In general, edema after SCI peaks at 3-5 days after injury. Thus we evaluated the G-CSF's effect on edema and BSB 3 days after SCI.

To assess the effect of G-CSF on edema following SCI, the water content of the spinal cord was measured using the wet-dry method (n=4 in each group). A ten mm segment of spinal cord including the lesion epicenter was removed three days after injury. We gently removed the hematoma or cerebrospinal fluid adherent to the spinal cord with tissue paper,

and weighed the fragment with a precision scale (accurate to 0.1mg) immediately to obtain the wet weight. Then, the samples were freeze-dried (FDU-810, Tokyo Rikakikai Co., LTD.) for 24 hours and weighed again, giving the dry weight. The water content of the spinal cord after injury was calculated by the following formula: percent spinal cord water content (%) = (wet weight − dry weight)/ wet weight $\times 100^{8,\ 32}$.

To evaluate the effects of G-CSF on BSB damage after SCI, we carried out quantitative detection of extravasated fluorescent dye (n=4 in each group)[26]. We used sodium fluorescein (Sigma, Taufkirchen, Germany) at a concentration of six mg/mL in PBS. Sodium fluorescein (MW 376.3) is not able to permeate normal BSB. Two-hundred μL of sodium fluorescein solution was injected via the tail vein three days after injury and allowed to circulate for 1.5 hr. Then, rats were perfused with saline to remove the sodium fluorescein from the vascular bed. Five mm segments of spinal cord including the lesion epicenter were removed and frozen quickly in liquid nitrogen. After homogenization in 100 μL of 0.5 M borate buffer, the sample was centrifuged at 3000 r/min for 15 min at 4°C. Ethanol (1.2 mL) was added to the supernatant to precipitate the protein. The solution was centrifuged at 13000 r/min for 20 min at 4 °C and then 100 μL of the supernatant was applied to the pellet and the

fluorescence was measured in a fluorescent plate reader at a wavelength of 485 nm.

Assessment of revascularization

For histological evaluation, animals were perfused transcardially with 4% paraformaldehyde in PBS under deep pentobarbital anesthesia one and two weeks after surgery. The spinal cord, including the lesion epicenter, was removed and fixed in 4% paraformaldehyde overnight. After dehydration in 20% sucrose in PBS for 48 hrs, the samples were embedded in O.C.T. compound (Tissue-Tek; Sakura Finetech. Tokyo, Japan) and frozen on ice. Axial sections (16 μm) were made on a cryostat at the lesion epicenter and two, four and six mm rostral and caudal from the epicenter and mounted on poly-L-lysine-coated glass slides (Matsunami, Tokyo, Japan) and dried for 48 hr in room air.

To evaluate angiogenesis after SCI, cryosections were immunostained with anti-von Willebrand Factor (VWF, 1:400, polyclonal rabbit anti-human, DakoCytomation, Denmark).as a marker for vascular endothelial cells (n=5 in each group). In the paper of Casella et al[4], angiogenesis was evaluated by basal lamina marker Laminin at various time points after SCI, and they described that the angiogenetic response after contusive SCI peaks 1week after the injury and diminishes thereafter. Kitamura et al[12] evaluated the

angiogenesis by immunohistochemistry for endothelial marker RECA-1 one week after contusive SCI. According to those previous papers, we employed one week after injury as the time point to evaluate the angiogenesis by immunohistochemistry for endothelial marker VWF. The sections were reacted with primary antibody overnight at 4 °C, and after three washes with PBS, reacted with Alexa 488-labeled anti-rabbit IgG antibody for 30 min at room temperature. The stained sections were then washed and covered with mounting medium. The positive signals were observed with a Zeiss LSM 510 confocal laser scanning microscope. The number of vessels (diameter > 20 μm) was counted in the lateral white matter (LWM), the ventral gray matter (VGM) and the cortico spinal tract (CST) to compare revascularization between two groups. All the quantitative histological evaluations were conducted by testers blinded to experimental groups.

Expression of angiogenic cytokines

Gene expression analysis of angiogenic cytokines was performed 12 hr after injury (n=4 in each group). In the preliminary experiments, there is no significant difference in expression of several angiogenic cytokines between the G-CSF and control rats at the other time points. Thus we chose the time point 12h after SCI for angiogenic cytokine expression

analysis. Animals were anesthetized deeply by intra-peritoneal injection of pentobarbital. Two segments of spinal cord were removed, including the lesion epicenter, and quickly frozen in liquid nitrogen, stored at −80 °C until use. Total RNA was extracted from the lesion segment of the spinal cord tissue using TRIzol reagent (Gibco Life Technologies, Rockville, MD) according to the manufacturer's protocol. Starting with 2.5 μg of total RNA, the samples underwent reverse transcription using the Superscript II RT preamplification System (Gibco LifeTechnologies) with an oligo (dT) primer. Real time PCR was used to assess expression of the following angiogenic cytokines: vascular endothelial growth factor (*VEGF*), angiopoietin-1 (*Ang1*), hepatocyte growth factor (*HGF*) and fibroblast growth factor 2 (*FGF2*). PCR was performed with 2.5 μL of cDNA in a 50 μL reaction mixture containing 25 μL of TaqMan Universal PCR Master Mix and PCR primers set (TaqMan Gene Expression Assays, Applied Biosystems, *VEGF*: Rn00582935, *Ang1*: Rn585552, *HGF*: Rn566673, *FGF2*:Rn00570809). The PCR analysis was performed using an ABI prism 7500 sequence detector (Applied Biosystems, Warrington, U.K.). All samples were run in duplicate, and the average values of the threshold cycle (Ct) were used for quantification. Ct values of the target genes were normalized to Ct values of endogenous

18S ribosomal RNA, and compared with a calibrator using the • • Ct method and converted to logarithmic values.

Assessment of locomotor function

We assessed the recovery of motor function weekly according to the 21-point Basso, Beattie, Bresnahan locomotor scale[2] from one to six weeks after injury in both groups (n = 11 in the G-CSF treated group, and n = 8 in the control group). In another subset of rats treated the same as above (n = 5 in the G-CSF treated group, and n = 5 in the control group), the inclined plane test was performed six and ten weeks after injury as previously described[22]. For the inclined plane test, the highest degree of inclination was defined as being that at which the animal could maintain its position for five sec on two separate trials. All behavioral evaluations were conducted by testers blinded to experimental groups.

Statistical analysis

The water content, extravasation study, the number of neovasculatures, expression of angiogenic cytokines and inclined plane test were subjected to the Student's t-test. Motor function scores were subjected to Repeated Measures ANOVA followed by post hoc test using Student's t-test. Data are presented as mean values±S.E. Values of p<0.05 were considered statistically significant.

RESULTS

G-CSF does not aggravate edema formation and permeability of vessels after SCI

The water contents of injured spinal cords were measured in order to estimate the effects of G-CSF on the extent of damage to the blood-spinal cord barrier (BSB), which leads to edema formation and results in aggravation of secondary injury. Water content in the G-CSF-treated rats was 74.5 ± 0.5 % and did not significantly differ from the control group 76.7 ± 6.1 % (p = 0.75) (Fig. 1A). We also injected sodium fluorescein solution via the tail vein and measured absorbance of extravasated fluorescein. In the G-CSF-treated rats, extravasation was 5.74 ± 1.73 ($\times$ 10^{-4} absorbance/mg) and that in control rats was 9.34 ± 2.55 ($\times$ 10^{-4} absorbance/mg). There was no significant difference between the two groups (p = 0.29) (Fig. 1B).

G-CSF enhances angiogenesis after SCI

Spinal cord vessels were immunostained for the endothelial marker von Willebrand Factor (vWF) one week after injury. We defined those vessels with diameters > 20 μm as meaningful neovasculature[4, 12] and counted the number of vessels in three different areas of the spinal cord (lateral white matter (LWM), ventral gray matter (VGM) and cortico spinal tract (CST)) in axial cryosections at two,

four and six mm caudal and rostral to the epicenter. In both groups, angiogenesis occurred more vigorously in VGM and CST than that in LWM (Fig. 2J). In the G-CSF-treated rats, the total number of vessels in the three parts was larger than that in the control group. In LWM, the number of vessels in the G-CSF group was significantly larger than that in the control group at four mm rostral and six mm caudal to the epicenter (p < 0.05) (Fig. 2. A, B, C) and in VGM there were significant differences between the number of vessels in the G-CSF group and that in the control group at four mm and six mm rostral and caudal to the epicenter (p < 0.01) (Fig 2. D, E, F). In CST, there was no significant difference between the groups in any of the sections (Fig. 2 I). However, the total number of each areas' vessels in the G-CSF group were larger than that in the control group (Fig. 2 J).

G-CSF boosts mRNA expression of angiogenic cytokines after SCI

Real time PCR was performed to detect expression of angiogenic cytokine genes, including vascular endothelial growth factor (*VEGF*), angiopoietin1 (*Ang1*), hepatocyte growth factor (*HGF*) and fibroblast growth factor2 (*FGF2*). The samples of cDNA were generated by reverse transcription from 10 mm segments from the injury site (12 hr after injury). Expression of cytokines' mRNAs was

compared to sham or control group and assessed by relative quantitative analysis. Expression of each angiogenic cytokines' mRNA increased in the G-CSF group. Especially, expression of *VEGF, HGF* and *FGF2* was significantly higher in the G-CSF group compared to the sham group (p < 0.01) (Fig. 3).

G-CSF promotes functional recovery after SCI

The recovery of motor function was assessed by using the Basso, Beattie, Bresnahan locomotor test[2] which ranges in values from zero to 21. All rats had a score of 21 prior to surgery, and the score dropped to 0 immediately after SCI. The G-CSF-treated group exhibited significantly higher locomotor scores four weeks after injury compared with the control group. The average score six weeks after injury was 12 ± 0.8 in the G-CSF group, which indicates frequent to consistent plantar weight support and occasional forelimb-hindlimb coordination, whereas the score was 8.6 ± 0.8 in the control group, which indicates plantar placement of the paw with weight support in stance only or occasional, frequent, or consistent weight supported dorsal stepping and no plantar stepping (p < 0.01) (Fig. 4 A). In addition, we assessed motor function of hindlimbs by using the inclined plane. The inclined plane test revealed that rats from the G-CSF group could stand on the plane at a

significantly steeper angle than the control group ten weeks after SCI. The average maximum angles ten weeks after injury were.50 ± 2.7 degrees in the G-CSF group and 31.7 ± 4.4 degrees in the control group (p < 0.01) (Fig4-B). There was no significant difference in results of inclined plane test between both groups six weeks after injury (not shown).

DISCUSSION

The data presented here show the beneficial effects of G-CSF on vascular systems after SCI. G-CSF significantly promoted angiogenesis in both the white and gray matter of the cord after injury. These results agree with other papers which reported the effects of G-CSF on the vascular system after cerebral ischemia[16, 18].

In our preliminary experiments, immunohistochemistry revealed that G-CSF receptors were rarely expressed by endothelial cells (not shown). Ohki *et al.* demonstrated that the angiogenesis-promoting effect of G-CSF is partly mediated by enhancement of mobilization of mature hematopoietic cells, specifically neutrophils and immature/mature endothelial cells and they showed that G-CSF stimulation significantly augments VEGF secretion from neutrophils *in vitro* and promoted angiogenesis in murine ischemic hindlimb models[21]. Thus, G-CSF might exert

its angiogenic effect indirectly via the up-regulation of angiogenic cytokines. To elucidate the effect of G-CSF on angiogenic cytokines, we carried out real time PCR to assess the expression of angiogenic cytokine genes, *VEGF, ANG1, HGF* and *FGF2*. Expression of *VEGF, HGF* and *FGF2* was significantly higher in the G-CSF-treated group than that in the sham-operated group.

VEGF is a potent stimulator of angiogenesis and affects blood vessel permeability[1]. It was reported that topical application of VEGF after transient cerebral artery occlusion significantly reduced ischemic brain damage, which was considered a protective effect involving angiogenesis of ischemic areas[10]. In addition, recent studies reported that VEGF might exert a direct neuroprotective effect in hypoxic ischemic injury. Ding Xin-min *et al.* documented that VEGF has direct neuroprotective effects on rat spinal cord neurons in hypoxia *in vitro*[7]. On the other hand, there are some reports which found negative effects of VEGF on the CNS. Benton reported that intraparenchymal application of VEGF might exacerbate SCI, likely through its effect on vessel permeability[3]. This discrepancy might be caused by general differences between the experimental models and / or unique microenvironments within the organs. Originally, VEGF was described as a vascular permeability factor[26]. Therefore, Benton's

report does not contradict our conclusion. In the present study, we measured water content of the spinal cord and the degree of extravasation. There were no significant differences between the two groups. In other words, G-CSF promoted angiogenesis after SCI without aggravation of neovasculature hyper-permeability. One can ask why vessel permeability and edema were not aggravated despite the increase of VEGF mRNA expression in the G-CSF-treated group? One possible explanation is the effects of other angiogenic cytokines.

As for HGF, recent studies have revealed that HGF administration enhances angiogenesis, improves microcirculation and inhibits the destruction of the blood-brain barrier (BBB)[5]. Kitamura *et al.* documented that administration of HGF using a replication-incompetent herpes simplex virus-1 (HSV-1) vector significantly promoted survival of neurons and oligodendrocytes, angiogenesis, axonal regrowth, and functional recovery after SCI[12]. FGF2, produced primarily by astrocytes, has been implicated in a multitude of physiological and pathological processes, including limb development, angiogenesis, wound healing, and tumor growth. In addition, FGF2 has also been found to decrease the permeability of the BBB *in vitro*[9]. Langford *et al.* documented that FGF2 is a prominent candidate to preserve BBB integrity[15]. Because

real time PCR showed that expression of *HGF* and *FGF2* after injury was significantly up-regulated in the G-CSF group, it is possible that VEGF-induced hyper-permeability of vessels might be negated by up-regulated *HGF* and *FGF2*.

Following trauma, angiogenesis is essential for the healing process and tissue regeneration outside the CNS. Angiogenic and anti-angiogenic therapies have each demonstrated functional efficacy in animal models after CNS insult, raising the question of whether angiogenesis is beneficial or detrimental to neurologic outcomes after SCI[30, 35]. Loy et al. reported that three to seven days after SCI, angiogenesis diminished cystic cavity formation and that fiber outgrowth was associated with new blood vessels by day 14[17]. Widenfalk et al. asserted the importance of angiogenesis for functional recovery and wound healing in SCI[33]. Angiogenesis might play an important role in reducing secondary damage. Furthermore, in future trials of combination therapy with cell transplantation, angiogenesis will be a critical factor for forming a scaffold within the spinal cord.

There are several possible limitations of the present study. The precise link between angiogenesis and functional recovery is still unclear. As described above, it is widely accepted that angiogenesis is necessary for wound healing process. How

angiogenesis itself promotes tissue restoration of damaged spinal cord remains to be clarified. How angiogenic cytokine up-regulation involves to spinal cord restoration and functional recovery is also unknown. There is a possibility that some kinds of angiogenic cytokine directly exert neurotrophic effects in addition to the promotion of angiogenesis. Further exploration is needed to answer those inquiries.

In conclusion, G-CSF promotes up-regulation of expression of several angiogenic cytokines and angiogenesis, resulting in promotion of hindlimb functional recovery.

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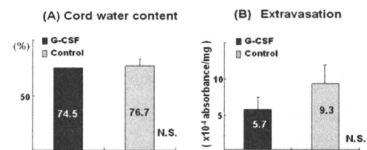
FIGURE LEGENDS

Figure 1. Assessment of blood spinal cord barrier (BSB) integrity (n=4 in each group). BSB integrity was evaluated by measuring cord water content (A) and detecting extravasation of sodium fluorescein (B). There was no significant difference in spinal cord water content or extravasation between the two groups.

G-CSF-treated group (D), whereas faint angiogenesis was seen in the control group (E). There was a significant difference between the two group at four and six mm rostral and caudal from the epicenter (F). In CST (G-H), there was no statistically significant difference between the G-CSF group (G), and the control group (H). The total number of vessels in the G-CSF group was larger than that in the control group (J). Bars = 100μm.

Figure 3. Expression of mRNAs for angiogenic cytokines assessed by real-time RT-PCR (n=4 in each group).

Expression of cytokine mRNAs was compared to sham group and assessed by quantitative analysis. Expression of each mRNA increased in the G-CSF group. Especially, *VEGF*, *HGF* and *FGF2* were expressed at significantly higher levels.
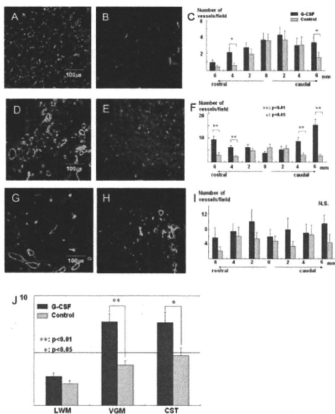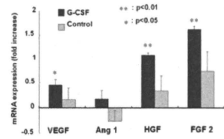
Figure 2. Angiogenesis in the spinal cord after SCI assessed by immunohistochemistry (n=5 in each group).

Vessels were immunostained with anti-vWF as an endothelial cell marker. Within the LWM (A-C), a relatively small number of vessels were observed in the G-CSF-treated group (A), and in the control group (B). The number of vessels larger than 20 μm showed significant differences at four mm rostral and six mm caudal from epicenter (C). In VGM (D-F), vigorous angiogenesis were observed in the
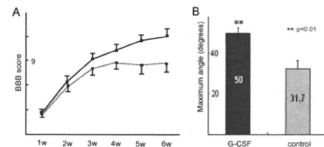

Figure 4. Hindlimb functional assessment with the Basso, Beattie, Bresnahan test.

The G-CSF-treated group (square) exhibited

significantly higher scores four weeks after injury compared with the control group (circle and dotted line)(n=8 in control group, n=11 in G-CSF group)(A). The mean value of the inclined plane test was significantly higher in the G-CSF group than in the control group (n=5 in each group)(50.0 to 31.7, B).

Transplantation of murine induced pluripotent stem (iPS) cell-derived astrocytes increases sensitivity to mechanical stimulus in a rat spinal cord injury model

Koichi Hayashi M.D., Ph.D.[1], Masayuki Hashimoto M.D., Ph.D.[1]*, Masao Koda M.D., Ph.D.[1], Atsuhiko Naito M.D., Ph.D.[2], Atsushi Murata M.D., Ph.D.[1], Akihiko Okawa M.D., Ph.D.[1], Kazuhisa Takahashi M.D., Ph.D.[1], Masashi Yamazaki M.D., Ph.D.[1]

[1]Department of Orthopaedic Surgery and [2]Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba260-8670, Japan

Address correspondence to: Masayuki Hashimoto, M.D., Ph.D.

Department of Orthopaedic Surgery, Chiba University Graduate School of Medicine

1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Tel: +81-43-226-2117, Fax: +81-43-226-2116, E-mail: futre@tg7.so-net.ne.jp

Abstract

Object: Clinical use of autologous induced pluripotent stem (iPS) cells could circumvent immune rejection and bioethical issues associated with embryonic stem cells. Spinal cord injury (SCI) is a devastating trauma with long lasting disability and current therapeutic approaches are not satisfactory. In the present study, we used the neural stem sphere (NSS) method to differentiate iPS cells into astrocytes which were evaluated after their transplantation into injured rat spinal cords.

Methods: iPS cell-derived astrocytes were differentiated using the NSS method and injected three and seven days after spinal cord contusion injury. Control rats were injected with DMEM in the same manner. Locomotor recovery was assessed for eight weeks and sensory and locomotion tests were evaluated at eight weeks. Then, immunohistological parameters were assessed.

Results: Transplant recipients lived for eight weeks without tumor formation. Transplanted cells stretched their processes along the longitudinal axis but they did not merge with the processes of host GFAP-positive astrocytes. Locomotion was assessed in three ways, but none of the tests detected statistically significant improvements compared to DMEM controls after eight weeks. Rather, iPS cell transplantation caused even greater sensitivity to mechanical stimulus compared to the DMEM control.

Conclusion: Astrocytes can be generated by serum treatment of neural stem sphere-generated cells

derived from iPS cells. However, transplantation of such cells is poorly suited for repairing SCI.

Keywords: iPS cells, astrocytes, sensitivity, neural stem sphere, neural stem cell, spinal cord injury

Abbreviations used in this paper: iPS, induced pluripotent stem; NSS, neural stem sphere; NSC, neural stem cells; SCI, spinal cord injury.

Introduction

Recently, induced pluripotent stem (iPS) cells were established by introducing three or four genes (such as *Oct3/4*, *SOX2*, *KLF4*, c-*MYC*) into mouse or human fibroblasts.[35, 36] iPS cells can be established from individual somatic cells, and they possess potent differentiative capacities. If autologous iPS cells could be established from patients, it would be possible to avoid bioethical concerns and immune rejection.

Nakayama and Inoue reported that astrocyte-derived factors modulate the differentiation of ES cells into neurons, a process they termed the "neural stem sphere" (NSS) method.[27,28,29] They also reported that ES-derived neural stem cells (NSC) could be differentiated almost exclusively into astrocytes by withdrawing fibroblast growth factor-2 (FGF-2) from the medium.[30] Recently, dopaminergic neurons derived from monkey ES cells (using the NSS method) were transplanted into a monkey model of Parkinson's disease and the technique promoted locomotion.[24] We hypothesized that the NSS method could also be applied to iPS cells to achieve targeted differentiation into neuronal cells.

Spinal cord injury (SCI) is a devastating type of trauma for patients due to the resulting long-lasting disability and the limited responsiveness to acute drug administration and rehabilitation. In assessing the pathology of SCI, it was noted that reactive astrocytes proliferate, form a glial scar and secrete inhibitory agents such as chondroitin sulfate proteoglycan (CSPG).[20] Because glial scar formation can become an obstacle to axonal regeneration,[6,7,10,20] astrocytes' actions have been regarded as harmful after SCI. More recently, there have been reports that astrocytes may be beneficial following SCI. For example, widespread infiltration of inflammatory cells results in severe motor deficits after SCI.[33] Reactive astrocytes can surround inflammatory tissue, and prevent inflammatory spread.[25] Knockdown studies of genetically modified GFAP-positive cells have reinforced the constructive role of astrocytes.[8] Ablation of reactive astrocytes has been found to reduce locomotor recovery[9] and exacerbate the inflammation and pathologies associated with autoimmune diseases of the CNS.[38] Disappearance of immature astrocytes in the region of spinal cord transection influences the regrowth of neurofilament-positive axons.[15] Transplantation of a purified population of a specific sub-type of astrocytes (derived from BMP-treated embryonic glial cell-restricted

precursors) promotes axon regeneration and functional recovery after acute transection injuries of the adult rat spinal cord.[4] Thus, transplantation of astrocytes following SCI could have beneficial effects on recovery.

In the present study, modified NSS methods were used to generate NSC from mouse iPS cells. Using that approach, we also differentiated neurons, astrocytes, and oligodendrocytes from iPS cell-derived NSCs. Conducting the transplant three days after spinal cord injury differed from previous studies. We chose acute transplantation in the present investigation in order to save damaged tissue at the lesion epicenter by replacing astrocytes soon after SCI. Although it has been well documented that the vast majority of neuronal death following SCI occurs in the first 24 hours, it is impossible to inject cells soon after SCI in the clinical setting. For that reason, we transplanted astrocytes derived from iPS cells three and seven days after SCI. We assessed locomotor recovery for eight weeks after SCI and assessed thermal and nociceptive thresholds eight weeks after SCI. This is the first report in which the NSS method has been used for iPS cell differentiation and transplantation of derived cells into injured spinal cords.

Methods

Cultivation of iPS cells and their differentiation into neural stem cells

Mouse iPS cells (iPS-MEF-Ng-20D-17; No. APS0001) were purchased from the Riken Cell Bank (Ibaraki,

Japan, http://www.brc.riken.jp/lab/cell/). These mouse iPS cells express GFP via the Nanog promoter.[35] We purchased feeder cells (SNL76/7) from DS Pharma Biomedical Co, Ltd (Osaka, Japan), and astrocyte-conditioned medium (ACM) from Sumitomo Bakelite Co, Ltd (Tokyo, Japan).

Figure 1 shows the schema for iPS colony generation and differentiation into neurons, oligodendrocytes, and astrocytes. To induce iPS colony formation, cells were seeded on a mitomycin C-treated feeder layer in ES medium (Dulbecco's modified Eagle's medium (DMEM), 15% knockout serum replacement, 1 mM non-essential amino acids, 0.1 mM • -mercaptoethanol, 1% penicillin-streptomycin) (all from GIBCO Invitrogen, Carlsbad, CA). Colonies of undifferentiated iPS cells, 300 - 500 μm in diameter, were picked up from the feeder layer using a Pipetman (Gilson, Inc. Middleton, WI) and transferred in ACM to non-treated bacteriological dishes containing an equal amount of DMEM/F2/N2 supplement (GIBCO), with 20 ng/mL recombinant FGF-2 (R&D Systems, Minneapolis, MN). The colonies were cultured for four days, giving rise to NSS, which were plated on Matrigel-coated dishes (BD Biosciences, Bedford, MA) and cultivated for seven days in NSC medium (Neurobasal medium supplemented with 2% B-27 (both from GIBCO), and 20 ng/mL FGF-2). At this stage, NSS gave rise to spherical clusters of NSCs in the outer layer which migrated from the surface of the NSS to the surrounding areas. The migrating NSCs were collected with a

Pipetman after NSS were detached using 0.25% trypsin treatment. The collected cells were expanded in NSC medium, and proliferating cells were frozen at –80 °C in DMEM, 10% fetal bovine serum (FBS: BIOWEST, Nuaillé, France) plus 10% DMSO. When required, the cryopreserved cells were thawed and expanded in NSC medium.

Differentiation of NSCs into neurons, astrocytes, and oligodendrocytes

To induce differentiation into neurons, NSCs were transferred to Matrigel-coated dishes and cultivated for seven days in ACM with 20 ng/mL FGF-2. This method was applicable to primary expanded or thawed NSCs. To induce differentiation into oligodendrocytes, we used a commercial oligodendrocyte differentiation kit (R&D systems, Minneapolis, MN) developed for ES cells. Briefly, to induce A2B5-positive cells from NSCs, expanded NSCs were plated on poly-L-ornithine/ fibronectin-coated plates for 12 days. The medium was changed every four days as follows: N2 plus/FGF medium for the first four days, followed by N2-plus/FGF/EGF medium for an additional four days, and N2 plus/FGF/EGF/PDGF-AA medium for the final four days. To direct differentiation into oligodendrocytes, the medium was changed to N2 plus/T3 medium, and the cells were cultivated for an additional seven days. To differentiate NSC into astrocytes, expanded NSCs (passage two to six) were transferred to Matrigel-coated dishes and cultivated for 14 days in DMEM with 10% FBS and 1%

penicillin-streptomycin without FGF-2.

Fluorescent immunocytochemistry

For *in vitro* experiments, cells cultured on chamber slides or NSS attached to chamber slides were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Immunocytochemistry was performed using standard protocols and antibodies as follows: rabbit polyclonal anti-nestin antibody (1:400; Chemicon, Temecula, CA, USA), mouse anti-•-tubulin III (Tuj-1, 1:800; Covance, Berkeley, CA), mouse anti-glial fibrillary acidic protein (GFAP, 1:400; Sigma-Aldrich), rabbit anti-s100 (1:400; Dako Cytomation Co. Ltd, Copenhagen, Denmark), mouse anti-A2B5 (1:100; R&D Systems, Minneapolis, MN), mouse anti-O4 (1:50; Chemicon), mouse anti-GalC (1:200; Chemicon), and rabbit anti-green fluorescent protein (GFP, 1:1600, Molecular Probes, Eugene, OR). Cell nuclei were stained with DAPI (1:1000, Molecular Probes). After reacting with primary antibodies, the sections were incubated with Alexa-Fluor 488-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes), Alexa-Fluor 488-conjugated anti-mouse IgM (Molecular Probes), and Alexa-Fluor 594-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes).

Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The samples consisted of undifferentiated iPS cells, NSCs (passage number three), and astrocytes derived from

NSCs (passage number five). Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) reagent and RNAeasy Mini Kit (Qiagen, Hilden, Germany). Using the Agilent 2100 Electrophoresis Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA), we ascertained that both 18 S and 28 S rRNAs were present without genomic contamination. Also, we characterized RNA by absorption spectroscopy, using the NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA) to confirm that the absorption value of A260/280 fell between 1.8 and 2.1. Total RNA (2 µg) was reverse transcribed into cDNA by Super Script III (Invitrogen). Samples were analyzed by ABI PRISM 7900HT Sequence Detection System and Taq-man Gene Expression Assay Products (Applied Biosystems Inc., Warrington, UK). Gene Symbols and Assays ID were as follows: Nanog (Mm02384862_g1), *Oct3/4* (Mm03053917_g1), Nestin (Mm00450205), *GFAP* (Mm01253033_m1). *GAPDH* (Mm99999915_g1) was used for the internal control gene. Standard curves and amplification plots of each gene were prepared using one of three RNAs (Nanog, *Oct3/4*: iPS cells; nestin: NSC; *GFAP*: astrocytes). We confirmed that all values fell within the range of the standard curve. To compensate for differences in RNA quantity, all data were normalized to *GAPDH* gene expression.

SCI and cell transplantation

Forty-five female Sprague-Dawley rats (eight weeks old; SLC Inc. Hamamatsu, Japan) were subjected to SCI. Anesthesia was induced by inhalation of 5% halothane in 0.5 L/min oxygen and maintained with 1.3% halothane in 0.5 L/min oxygen. A laminectomy was performed at the T9-T10 levels. The moderate contusion injury was introduced using Infinite Horizon impactor (2 mm diameter impactor head, 200 Kdyn; Precision Systems and Instrumentation, Lexington, NY). After injury, muscles and skin were sutured layer to layer and the rats placed in a warm cage overnight. All animals were given antibiotics (500 µL/ day; Bactramin, Chugai Pharmaceutical, Tokyo, Japan) by subcutaneous administration once a day for three days. Food and water were provided *ad libitum*. Manual bladder expression was performed twice a day until recovery of the bladder reflex.

iPS-derived astrocytes were detached from dishes using 0.25% trypsin, washed one time with DMEM, and resuspended in DMEM. In that time, we verified that iPS-derived astrocytes were not GFP-positive. Derived astrocytes were labeled for transplantation studies with a PKH26 Red Fluorescent Cell Linker Kit per the manufacturer's instruction (Sigma-Aldrich, St Louis, MO). In preliminary trials, we confirmed that PKH26-treated astrocytes could survive more than two months on PLL-coated dishes in DMEM with 10% FBS and 1% penicillin-streptomycin without FGF-2. Three or seven days after injury, the injured site was re-exposed and transplantation was performed. Astrocytes derived from iPS cells (100,000/ 5 µL; three day astrocyte group, n = 20,; seven day astrocyte group n = 9,) or DMEM (5 µL; three day DMEM group, n = 10;