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Potentiation of angiogenesis and regeneration by G-CSF after sciatic nerve crush injury

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ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) demonstrates neuroprotective effects through different mechanisms, including mobilization of bone marrow cells. However, the influence of G-CSF-mediated mobilization of bone marrow-derived cells on injured sciatic nerves remains to be elucidated. The administration of G-CSF promoted a short-term functional recovery 7 days after crush injury in sciatic nerves. A double-immunofluorescence study using green fluorescent protein-chimeric mice revealed that bone marrow-derived CD34+ cells were predominantly mobilized and migrated into injured nerves after G-CSF treatment. G-CSF-mediated beneficial effects against sciatic nerve injury were associated with increased CD34+ cell deposition, vascular endothelial growth factor (VEGF) expression, and vascularization/angiogenesis as well as decreased CD68+ cell accumulation. However, cell differentiation and VEGF expression were not demonstrated in deposited cells. The results suggest that the promotion of short-term functional recovery in sciatic nerve crush injury by G-CSF involves a paracrine modulatory effect and a bone marrow-derived CD34+ cell mobilizing effect.

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Introduction

The peripheral nerve system (PNS) possesses intrinsic potential for functional regeneration after injury. However, in spite of the endogenous regenerative capacity of the PNS, in many cases the regeneration is insufficient and recovery of function is incomplete. Recently, cell transplantation has become the focus of clinical research. Cell therapy has been shown to exert beneficial effects on peripheral nerve regeneration. Cell supplement, trophic factor secretion, extracellular matrix molecule synthesis and guidance, remyelination, microenvironment stabilization, and immune response modulation have recently been proposed as beneficial mechanisms after cell transplantation [1–7]. Among the applied cell types, bone marrow-derived cells, which contain hematopoietic, mesenchymal, and other types of cells, are candidates for cell therapy against peripheral nerve injury. Indeed, transplantation of whole bone marrow cells, bone marrow-derived hematopoietic stem cells, bone marrow-derived mesenchymal stem cells, or bone marrow-derived endothelial progenitor cells has been demonstrated to promote functional recovery in injured peripheral nerves [4–7]. These lines of evidence show the potential of bone marrow-derived cell transplantation to restore injured peripheral nerves and to promote functional recovery.

Granulocyte colony-stimulating factor (G-CSF) is widely known as a cytokine that induces survival, proliferation and differentiation of cells of hematopoietic lineage [8]. Furthermore, G-CSF can mobilize bone marrow cells into peripheral blood circulation, an action used clinically for patients with leukocytopenia and for donors of peripheral blood-derived hematopoietic stem cells for transplantation [9]. However, growing evidence has suggested that G-CSF also has important non-hematopoietic functions in other tissues including nerve tissues. Recently, a series of clinical and experimental studies have demonstrated the beneficial effects of G-CSF in several neurological diseases, including cerebral ischemia, spinal cord injury, Parkinson's disease, and peripheral nerve injury. It is proposed that G-CSF exerts beneficial effects through different mechanisms, including mobilization of bone marrow cells, anti-apoptosis, anti-inflammation, neuronal differentiation, and angiogenesis [2,3,10–15].

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Recently, we demonstrated that treatment with G-CSF promoted functional recovery in spinal cord injury and peripheral nerve injury, and concomitant treatment with G-CSF and stem cells augmented regeneration involving several beneficial actions including anti-apoptosis, anti-inflammation, and promotion of cell proliferation [2,3]. Bone marrow-derived cells mobilized by G-CSF may have the potential to migrate into and repair various injured tissues. The strategies of mobilized bone marrow-derived cells were applied and beneficial effects were demonstrated in ischemic myocardium, ischemic brain, and spinal cord injury [13,15,16]. However, the influence of G-CSF-mediated mobilization of bone marrow-derived cells on injured sciatic nerves remains to be elucidated. In green fluorescent protein (GFP) chimeric mice that underwent crush injury to the sciatic nerves, the results showed that G-CSF promoted the mobilization and migration of bone marrow cells into the injured nerves and the beneficial effects were associated with CD34+ cell deposition and increased vascularization/angiogenesis.

Material and methods

Bone marrow transplantation. Bone marrow cells were collected from 8- to 12-week-old male GFP transgenic mice (GFP-Tg) euthanized with pentobarbital. A total of 6×10^6 bone marrow cells derived from GFP-Tg mice were transplanted intravenously via the tail vein of lethally irradiated (800 cGy) male FVB/N mice [13]. In total, 24 animals received transplantation. Four animals were sacrificed 4 weeks after bone marrow transplantation and were used to evaluate the chimerism. The remaining 20 animals were subjected to further surgery.

Sciatic nerve crush injury and G-CSF treatment. Male FVB/N mice (25–30 g) were used in this study; permission was obtained from the Ethics Committee of Taichung Veterans General Hospital. Four weeks after transplantation, surgery and G-CSF treatment were performed. The mice were anesthetized with 2% isoflurane in induction followed by a maintenance dose (0.5–1%). The left sciatic nerve was exposed under a microscope using the gluteal muscle splitting method. A vessel clamp was applied 10 mm from the internal obturator canal for 20 min [3]. The animals were categorized into two groups: group I (crush, $n = 10$), the mice received one intra-peritoneal injection of normal saline per day for 5 consecutive days; group II (crush + G-CSF, $n = 10$), the mice were concomitantly injected with G-CSF (50 μg/kg) intra-peritoneally for 5 consecutive days. Normal mice which did not receive bone marrow transplantation were also subjected to the same surgery and G-CSF treatment ($n = 5$). All animals were sacrificed for examination 7 days after surgery.

Assessment of functional recovery. To evaluate sciatic nerve function, several measurements were taken from the red ink footprint [3]: (i) distance from the heel to the third toe, the print length; (ii) distance from the first to the fifth toe, the toe spread (TS); and (iii) distance from the second to the fourth toe, the intermediary toe spread (ITS). All three measurements were taken from the experimental (E) and normal (N) sides. The sciatic functional index (SFI) was calculated according to the equation: $SFI = -38.3(EPL - NPL/NPL) + 109.5(ETS - NTS/NTS) + 13.3(EIT - NIT/NIT) - 8.8$. The SFI oscillates around 0 for normal nerve function, whereas SFI around −100 represents total dysfunction.

Electrophysiological study. The sciatic nerves from individual groups were exposed. Electric stimulation was applied to the proximal side of the injured site; the conduction latency, and the compound muscle action potential (CMAP) were recorded with an active electrode needle 5 mm below the tibia tubercle and a reference needle 15 mm from the active electrode. The stimulation intensity and filtration ranges were 5 mA and 20–2000 Hz, respectively. The CMAP data and conduction latency were converted to ratios of the injured side divided by the normal side to adjust for the effect of anesthesia [3].

Immunohistochemistry. Serial 8 μm-thick sections of sciatic nerve were cut on a cryostat and mounted on superfrost/plus slides and were subjected to immunohistochemistry with antibodies against CD68, CD34, von-Willebrand factor (vWF), vascular endothelial growth factor (VEGF), and vimentin. The cell nuclei were stained with Dapi. The immunoreactive signals were observed by goat anti-mouse IgG (FITC) or anti-mouse IgG (Rhodamine). Among longitudinal consecutive resections, five consecutive resections contiguous to a maximum diameter were chosen to be measured. Of 100 squares with a surface area of 0.01 mm^2 each, 20 were randomly selected in an ocular grid and used to count the number of immunoreactive cells. The quantitative results are expressed as cell counts/0.05 mm^2. Immunohistochemical staining for isolectin B4 as an endothelial cell marker was visualized with fluorescence. For the determination of vWF, VEGF, and isolectin B4 reactivity, areas of activities (0.2 mm^2) from five consecutive resections appeared as density against the background and were measured by a computer image analysis system [3].

Statistical analysis. Data are expressed as means ± standard deviation. The statistical significance of differences between groups was determined by one way analysis of variance (ANOVA) followed by Dunnett's test. In SFI, the results were analyzed by repeated-measurement of ANOVA followed by the multiple comparison method of Bonferroni. A p value less than 0.05 was considered significance.

Results and discussion

Twenty-four mice tolerated the irradiation and bone marrow transplantation. There was no mortality associated with irradiation or transplantation. The FACS analysis showed that 87.1 ± 8.3% of the whole bone marrow cells were positive for GFP 4 weeks after bone marrow transplantation, indicating that GFP-Tg-derived bone marrow cells survived and reconstituted hematopoiesis in grafted mice. Further analysis revealed that CD34+ cells, an endothelial/hematopoietic progenitor-enriched cell population [1], constituted 9.1 ± 1.3% of GFP+ cells. After treatment with G-CSF for 5 days, white blood cell count was elevated significantly (8525 ± 1278 counts/mm^3) in the treated group when compared to the non-treated group (4750 ± 1500 counts/mm^3). These results suggest that G-CSF has a mobilization effect in bone marrow-transplanted mice.

To elicit the effect of G-CSF on peripheral nerve regeneration, irradiated/bone marrow-transplanted mice were subjected to crush injury in the sciatic nerve. Crush injury in the sciatic nerve caused deficits in neurobehavior and nerve electrophysiology. The escalation of the sciatic nerve functional index (SFI) in the G-CSF-treated group primarily implicated the improvement of peripheral nerve regeneration (Fig. 1A). Studies suggest that the amplitude of CMAP reflects the number of axons reinnervating the muscle and is related to the amount of acetylcholine release, and the nerve conduction latency is reciprocal to motor function improvement [17,18]. Electrophysiological recordings demonstrated an improvement in nerve conduction latency (Fig. 1B) and CMAP (Fig. 1C), indicating an increased recovery of nerve function after G-CSF treatment. The results suggest that G-CSF administration provokes the improvement of neurobehavior in animals with crush injury in sciatic nerves.

To elicit the potential beneficial mechanisms of G-CSF against crush injury in the sciatic nerve involved, the mobilization and deposition of bone marrow cells was analyzed (Fig. 2). No fluorescent signal was detected in sciatic nerves obtained from normal

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