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The combination of peripheral nerve grafts and acidic fibroblast growth factor enhances arginase I and polyamine spermine expression in transected rat spinal cords

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Abstract

Treatment with a combination of peripheral nerve grafts and acidic fibroblast growth factor improves hind limb locomotor function after spinal cord transection. This study examined the effect of treatment on expression of arginase I (Arg I) and polyamines. Arg I expression was low in the spinal cords of normal rats but increased following spinal injury. Only fully repaired spinal cords expressed higher Arg I levels 6–14 days following repair. In 10-day repaired spinal cords, high Arg I immunoreactivity was detected in motoneurons and alternatively activated macrophages in the graft area and graft–stump edges, and high levels of the polyamine spermine were expressed by macrophages within the intercostal nerve graft. Thus, in addition to enhancing the expression of Arg I and spermine in repaired spinal cords, our treatment may recruit activated macrophages and create a more favorable environment for axonal regrowth.

Keywords: Spinal cord; Arginase I; Acid fibroblast growth factor; Peripheral nerve graft; Alternatively activated macrophage; Polyamine spermine; Regeneration; Neuron; Rat

The failure of axons to regenerate following spinal cord injury is in part due to the intrinsic properties of the neuron [1], the absence of neurotrophic factors [2], and the presence of inhibitory factors in the spinal cord environment [3]. Unlike the central nervous system (CNS), the adult mammalian peripheral nervous system (PNS) readily regenerates after injury. One of the reasons is that macrophages and Schwann cells can remove myelin debris after injury. Injuries also trigger macrophages and Schwann cells to synthesize a cocktail of growth factors, cytokines, and growth-promoting surface molecules [4,5]. Therefore, reparative approaches using Schwann cell grafts [6,7], peripheral nerve grafts [8–11], or macrophages [12] implanted into the injured spinal cord have resulted in successful axonal regrowth or functional benefit. In addition,

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stem cell transplantation has shown good potential as a treatment for spinal cord injury [13].

Activation of intrinsic growth by neurotrophic factors will overcome inhibition due to CNS myelin. Stimulating the cAMP signaling pathway results in regeneration of injured axons and modulation of myelin-associated inhibition [14,15]. Myelin-associated inhibition of neurite growth was overcome by increasing the level of arginase I (Arg I), which occurred in response to increase in cAMP [16]. Indeed, the steps from elevation of cAMP through upregulation of Arg I transcription require activation of the transcription factor cAMP response element-binding protein (CREB) [17]. Arg I hydrolyzes arginine to urea and ornithine. Ornithine decarboxylase (ODC) and other enzymes then convert ornithine into the polyamines putrescine, spermidine, and spermine [18]. Treatment with polyamines promotes axonal regeneration in the peripheral nerves [19]. Furthermore, the addition of putrescine alone to cultured neurons has the same effect on myelin-associated inhibition as does overexpression of Arg I [16].

Arg I is upregulated in macrophages and serves as a marker of macrophages activated by interleukin (IL)-4 or IL-13 [20]. These activated macrophages (also known as alternatively activated macrophages) differ from classical immune-activated macrophages. The induction of Arg I prevents macrophages from producing nitric oxide (NO), and thus these macrophages are relatively inept killers of intracellular pathogens [21]. Alternatively activated macrophages may be involved in tissue remodeling since they also produce several extracellular matrix proteins [22].

Our repair strategy (a combination of peripheral nerve grafts with acidic fibroblast growth factor [aFGF] treatment) improves hind limb locomotor function of spinal cord-transected rats [8–11]. The full repair procedure improves hind limb locomotor function of spinal cord-transected rats, but partial procedures without aFGF or white matter-to-white matter bridging plus aFGF are without effect [8]. These findings indicated that some molecular mechanisms triggered by the full repair procedure in the early stage of spinal cord injury are important to functional recovery. In this report, these mechanisms were examined by assessing Arg I and spermine expression in spinal cord injured animals after different treatments.

Materials and methods

Experimental animals and surgical procedures. Adult 225–250 g female Sprague–Dawley rats were used in these studies. All procedures involving animals were approved by the Animals Committee of Taipei Veterans General Hospital. In the transected group (Group T), the spinal cords of rats were completely transected at T8 and 5 mm of spinal cord tissue was removed. In the repair group (Group R), an *in vivo* repair strategy developed by Cheng [8] was used. Peripheral intercostal nerve segments combined with aFGF in a fibrin glue carrier were implanted to bridge the 5 mm gap in the severed spinal cords. The transected rats that received only peripheral intercostal nerve grafts in fibrin glue carrier were designated "Group I." Those which received only aFGF in fibrin glue carrier were designated "Group F." Post-operative care, monitoring, and behavioral evaluation have been described previously [8,23]. At 2, 6, 10, and 14 days following injury, the rats received an overdose of sodium pentobarbital. The procedure for collecting spinal cord tissues from each group of experimental animals is illustrated in Fig. 1A. Protein samples designated "p" were prepared from a 1-cm long spinal cord segment rostral to the injury site from each rat and protein samples designated "d" were prepared from a 1-cm long spinal cord segment caudal to each injury site with a scar (Groups T and F) or nerve graft (Groups R and I).

Western blot analysis. Spinal cord segments were homogenized in 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 5% glycerol, and protease inhibitors (Roche Applied Science, Mannheim, Germany). Homogenates were then centrifuged at 12,000*g* for 20 min at 4 °C, and the supernatants (protein extracts) were removed and collected. The Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Hercules, CA, USA) was used to measure protein concentration. Equal amounts of proteins were loaded and separated on 10% SDS–PAGE gels. Electrophoresis was performed according to standard procedures. After electrophoresis, gels were transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA) and incubated overnight at 4 °C with antibodies against Arg I (1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or β-actin (1:2000, Abcam, Cambridge, MA, USA). Blots were incubated with a donkey anti-goat IgG HRP (horseradish peroxidase)-conjugated secondary antibody (1:5000, Santa Cruz) or a goat anti-mouse IgG HRP-conjugated secondary antibody (1:5000, Santa Cruz) for 1 h, and HRP detection was performed using SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Statistical analysis. Western blot results were obtained from three independent experimental animals in each group. Average of densitometric values of different bands of Arg I was evaluated separately after normalization to β-actin, and the results are presented as means and standard deviation (SD). Statistical comparisons were performed by one-way ANOVA, followed by Holm–Sidak method. The differences were considered significant when $P < 0.05$.

Immunohistochemistry. At the specified post-injury times, the rats received an overdose of the anesthetic sodium pentobarbital and were perfused intravascularly with 0.9% saline and 4% paraformaldehyde in PBS. The spinal cords were removed and post-fixed overnight in the same fixative at 4 °C. On the following day, the spinal cords were dehydrated in serially increasing concentrations of ethanol, transferred to xylene, and finally embedded in paraffin. The spinal cords were sagittally sectioned (5 μm thick) and placed on slides. The slides were dried and immersed three times in xylene, 5 min each, then rehydrated in serially diluted ethanol and finally in PBS. The slides were subjected to antigen retrieval by microwave heating for 5 min in 10 mM sodium citrate (pH 6.0) and stained using specific antibodies. The primary antibodies used were as follows: goat anti-Arg I (1:1000, Santa Cruz); rabbit anti-ionized calcium-binding adapter molecule-1 (IBA1, 1:500, Wako Chemicals, Osaka, Japan), which stains resting as well as activated and phagocytic microglia/macrophages [24]; mouse anti-ED1 (1:1000, Serotec, Oxford, UK), which labels activated and phagocytic microglia/macrophages but not resting and non-phagocytic microglial cells [25]; rabbit anti-spermine (1:250, Chemicon, Temecula, CA, USA); rabbit anti-cow S100 antibody (1:500, DAKOCytomation, Glostrup, Denmark), which can label Schwann cells; and rabbit anti-human fibronectin antibody (1:500, Sigma–Aldrich Corp., Saint Louis, MO, USA); mouse anti-ODC (1:500, Sigma–Aldrich Corp.). The bound antibodies were visualized using the avidin–biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories Inc., Burlingame, CA, USA) and with appropriate chromagens. The secondary antibodies used for fluorescence microscopy were FITC-conjugated anti-rabbit (1:200, Jackson Laboratories, Bar Harbor, ME, USA), and Cy3-conjugated anti-goat immunoglobulin (1:200, Jackson). Staining specificity of Arg I was confirmed by adding negative control peptide for Arg I (Santa Cruz).

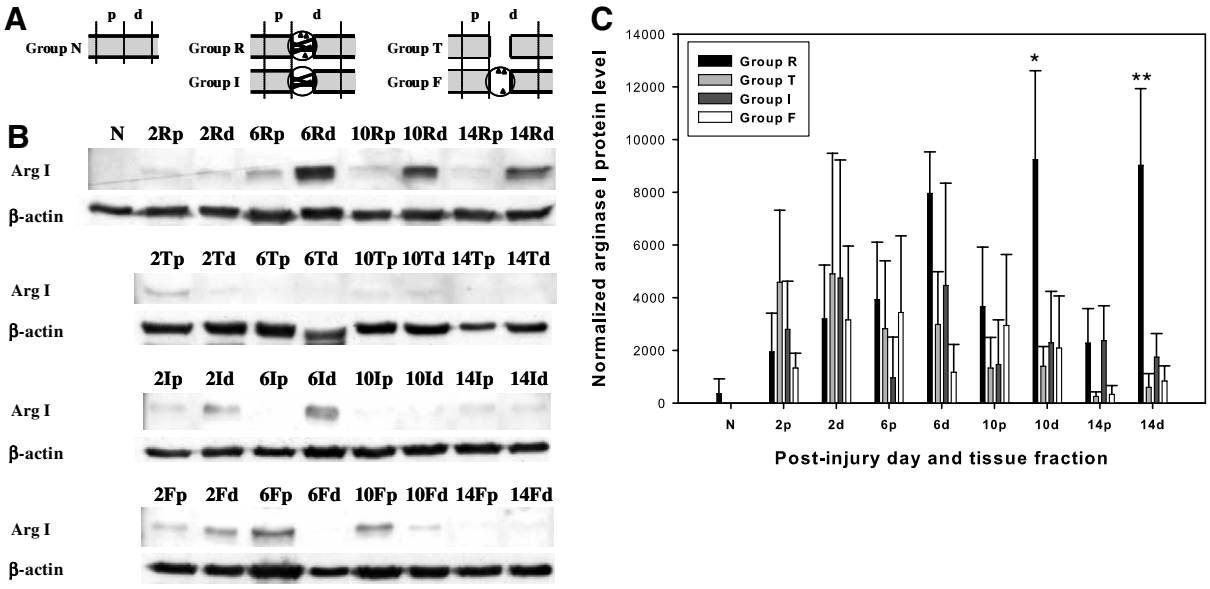

Fig. 1. Arginase I was upregulated in graft area and wound edges of repaired spinal cords. (A) Spinal cord segments of normal (N), repaired (R), transected (T), intercostal nerve grafted (I), and aFGF-treated (F) rats were collected and prepared as described in Materials and methods. Gray segments: spinal cords; lines in the gap between spinal cord stumps: grafted nerves; circle: fibrin glue mixture; triangle: aFGF. (B) Western blot analysis of arginase I (Arg I) and β-actin protein level. (C) Normalized Arg I protein level. All data were expressed as means ± SD ($n = 3$ in each group) $^{*}P = 0.008$, $^{**}P < 0.001$ compared with other groups in the same post-injury day and tissue fraction.

Results

Induction of Arg I requires the combination of aFGF and peripheral nerve graft

Western blot analysis showed that, Arg I was expressed in low amounts in normal spinal cords and upregulated in injured cords (Fig. 1). The peak activation time point in the Groups I and F spinal cords was less than 6 days post-injury. This upregulation in T, I, and F spinal cords did not continue beyond 10 days after injury. Only the fully repaired spinal cord continued to express Arg I more than 10 days following injury. Arg I protein levels in 10- and 14-day graft and distal stump of repaired spinal cords were significantly higher than samples of other groups (Fig. 1C).

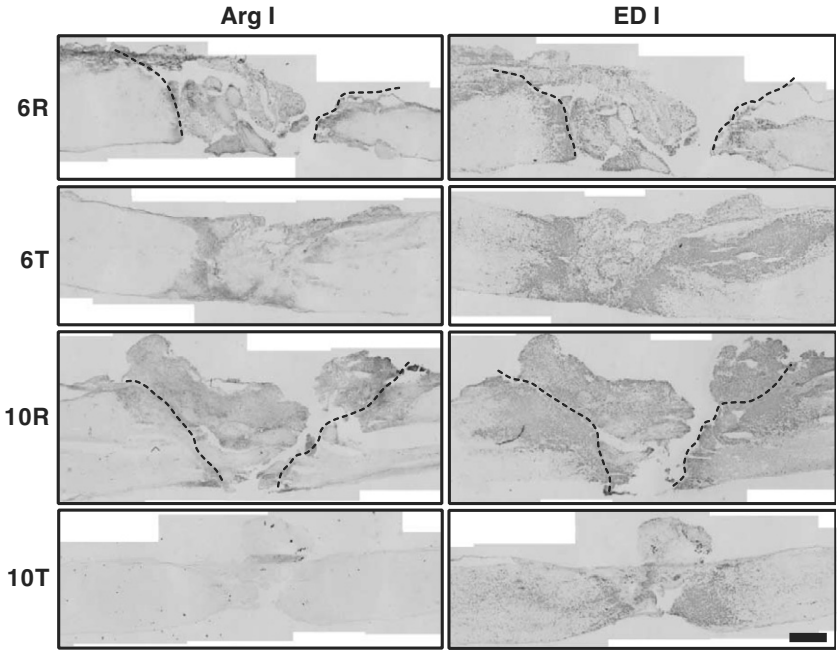

Fig. 2. Arg I was localized within the graft area and stumps of 6- and 10-day repaired spinal cords. Sagittal sections of 6- and 10-day repaired (6R and 10R) and transected (6T and 10T) spinal cords are positioned so that the left side of the tissue is rostral to the lesion. Sections were stained for Arg I and ED1 (macrophages). Dotted line: graft–stump boundary. Scale bar, 1 mm.

Neurons and alternatively activated macrophages express Arg I

Six and ten days after repair, large numbers of Arg I-positive cells were seen around the wound edges and along the bridge in the graft area (Fig. 2). This accumulation of strong Arg I signal within the graft area is responsible for the higher amount of Arg I in graft-containing fraction "d" than in fraction "p" by Western blot analysis in repaired spinal cords (Fig. 1). ED1-positive activated microglia and macrophages were prominent around the lesion sites and graft area, which was similar to the distribution of Arg I expression. Although many macrophages were detected in the injury site of 6-day transected spinal cords, the Arg I signal was relatively low. In 10-day transected spinal cords, the number of macrophages further decreased and the Arg I signal was undetectable (Fig. 2).

Motoneurons about 5 mm rostral to the grafts were Arg I-positive (Fig. 3A), whereas IBA1-positive microglia around the motoneurons were Arg I-negative (Fig. 3B and C). Many Arg I-positive alternatively activated macrophages with abundant translucent cytoplasm and phagocytic appearance were found in the graft area (Fig. 3D–F). A few fibronectin-positive cells were stained for Arg I in graft area (Fig. 3G–I). However, other cells including Schwann cells were not stained for Arg I (Fig. 3J–L).

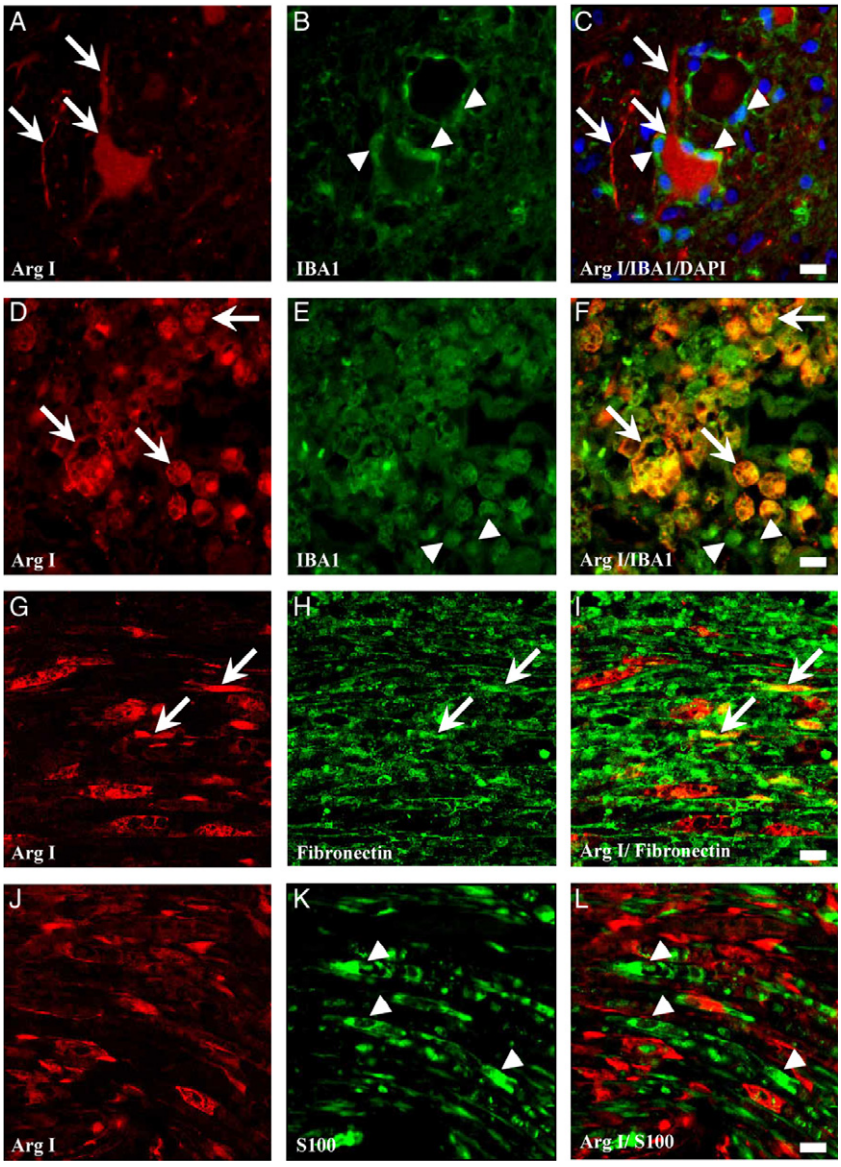

Fig. 3. Arg I was colocalized with motor neurons and macrophages in 10-day repaired spinal cords. (A–C) Colocalization studies of 10-day repaired spinal cords showed that Arg I is colocalized with motor neurons, axons, and other neurites (arrows) in the proximal stump. Microglia around the motor neuron are not stained for Arg I (arrowheads). (D–F) Arg I is highly colocalized with IBA1-positive phagocytic macrophages in graft area (arrows) but not with some smaller macrophages (arrowheads). (G–I) Only a few fibronectin-positive cells are stained for Arg I in graft area (arrows). (J–L) S100 is a cellular marker for Schwann cells. Schwann cells in the nerve graft are not stained for Arg I (arrowheads). Scale bar, 10 μm.

Alternatively activated macrophages express polyamine spermine

Next we examined the levels of the polyamine spermine in spinal cords by immunostaining. High levels of spermine were found in the graft area and grafted nerves in the 10-day repaired spinal cord but not in samples from 10-day transected spinal cords (Fig. 4A and B). It seemed that macrophages within the grafted nerves express higher levels of spermine. These spermine-positive macrophages in the grafted intercostal nerves were both colocalized with Arg I (Fig. 4C–E) and ODC (Fig. 4F–H).

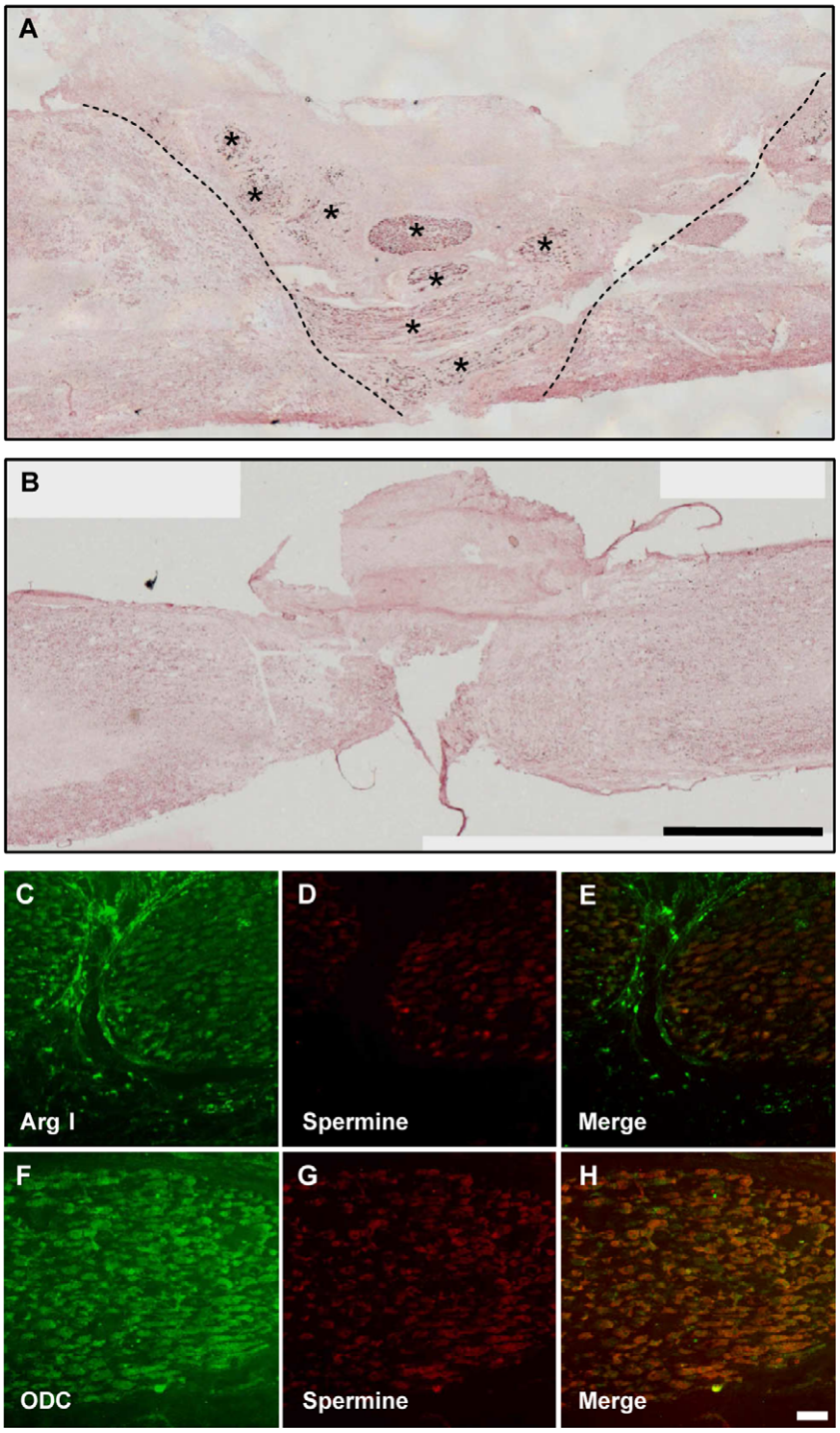

Fig. 4. High levels of spermine were expressed by macrophages in the intercostal nerve graft. Sagittal sections of 10-day repaired spinal cords were stained for spermine. High immunoactivity of spermine was detected in the grafted area and cells within the intercostal nerves had intense spermine signals (asterisks in A). By contrast, low immunoreactivity of spermine was detected in 10-day transected spinal cords (B). Immunoreactivity of spermine was colocalized with Arg I (C–E) and ODC (F–H). Dotted line: graft–stump boundary. Scale bar: 1 mm for A and B; 10 μm for C–H.

Discussion

The expression of Arg I is induced in sympathetic neurons 2 days after axotomy and remains high for 5 days [26]. In addition, Arg I expression in cultured cerebellar neurons treated with either brain-derived neurotrophic factor (BDNF) or dibutyryl (db) cAMP was upregulated for at least 21 h [16]. In our studies, Arg I-positive motoneurons and Arg I-positive axons were found in 10-day repair spinal cords. Thus our repair strategy could prolong the expression of Arg I in spinal cord neurons for at least 10 days. Upregulation of Arg I in neurons inhibited neuronal apoptosis [27] and could overcome myelin inhibition of neurite growth [16]. This is one possible reason for better functional recovery following our repair strategy.

The amount of ED1-positive activated macrophages in 10-day repaired spinal cord is higher than in 10-day transected spinal cord. Nerve segments may enhance phagocytic activity of macrophages in the graft area [28]. It has been suggested that the different immune responses triggered in the PNS and CNS after injury might account for the different regenerative ability. One strategy to encourage regeneration is to introduce macrophages into the injury site [12]. Incubated autologous macrophage cell therapy was claimed to be better than natural recovery in patients with acute spinal cord injury in phase I clinical trials [29]. After lens injury, macrophages activated by zymosan enhanced retinal ganglion cells' axonal regeneration and oncomodulin was a potent macrophage-derived growth factor for retinal ganglion cells [30]. By contrast, depletion of macrophages improved hind limb recovery [31]. Activation of CNS macrophages achieved by microinjections of zymosan in spinal cords resulted in axonal injury [32]. Overproduction of nitric oxide (NO) was found in zymosan-treated rats [33], but upregulation of Arg I limited the production of NO [34]. We suggest that the number of recruited macrophages as well as the type of macrophage activation is important for functional outcome in our repaired rats. Alternatively activated macrophages may contribute to the repair processes in our repair strategy.

IL-4 and IL-13 induce alternative activation of macrophages [35,36]. The regulation of IL-4 and IL-13 and their main sources in the body is not well defined. Schwann cells, which are abundant in the grafted nerves in our repaired spinal cords, appeared to be one source of IL-4 [37]. Alternatively activated macrophages upregulate the expression of Arg I and may lead to polyamines and proline biosynthesis, promoting cell growth, collagen formation, and tissue repair [38]. Adding polyamines can promote axon extension in cultured neurons [16] and in peripheral nerves *in vivo* [19], but their effects on spinal cord injuries in animals still need to be tested. We found that high levels of spermine were detected in macrophages within the intercostal nerve graft of repaired rats. Therefore, polyamines released by these macrophages might promote axonal regrowth in repaired spinal cords. aFGF induces ODC, the initial enzyme in polyamine biosynthesis, in PC12 cells [39]. We found that macrophages in the grafted nerves expressed Arg I and ODC. This regulation may give rise to the higher expression of spermine in macrophages within the grafted nerves.

In summary, the combination of peripheral nerve grafts and aFGF not only enhanced the expression of Arg I in motoneurons and axons, but also increased recruitment to graft area of alternatively activated macrophages, which express high levels of spermine. These cellular and molecular responses might promote axonal regeneration and be valuable accelerants to nerve regeneration after injury.

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