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# Combined treatment using peripheral nerve graft and FGF-1: Changes to the glial environment and differential macrophage reaction in a complete transected spinal cord

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**Abstract**

We used a complete spinal cord transection model in which the T8 spinal segment was removed to study the effect of combined treatment of peripheral nerve graft and application of FGF-1 on the glial environment. The combined treatment resulted in reduced astrocytic glial scarring, reactive macrophage gliosis, and inhibitory proteoglycan in the back-degenerated white matter tract. While the macrophage activities in the back-degenerative tract were down-regulated, those in the grafted peripheral nerves and in the distal Wallerian degenerative tracts were not. We concluded that the combined treatment changed the glial environment in the back-degenerative tract, and differentially regulated the macrophage activities in the system, in favor of CNS regeneration.


*Keywords:* FGF-1; Back-degeneration; Astrocytic scarring; Reactive gliosis; Macrophage; Ketatan sulphate proteoglycan (KSPG)

Axons in transected spinal cords would regenerate when neuronal survival and neurite outgrowth are promoted by a neurotrophic factor [18], or when inhibitory surface molecules are removed [25]. Astrocytes and macrophages are the major cellular constituents of glial scarring and a source for inhibitory proteoglycan in the injury site. After insult to the axons, astrocytes response by hypertrophy and process extension, and form a tightly compacted limiting glial margin, the astrogliotic scar [17]. Macrophages migrate to the trauma site, secrete inhibitory proteoglycans including the keratin sulphate proteoglycan (KSPG), which are inhibitory to the growth of axons [11]. Interaction of macrophages and astrocytes at the trauma site significantly influence the outcome of axon degeneration and cavity formation [7]. Increased axon penetration was associated with down-regulated astrocytic reaction due to deletion of the GFAP gene or reduced astrocyte numbers [24]. Keratanase treatment results in the misrouting of mossy fibers during regeneration, demonstrating its role in CNS regeneration [3]. Depletion of activated macrophage results in partial CNS functional recovery [22].

Peripheral nerves are known good substrate for bridging CNS trauma [1], and Schwann cell transplantation in CNS trauma sites improved regeneration [8,10,20]. In our previous test of growth factors added to grafted peripheral nerve bridge to further promote neurite outgrowth, empirically, we found that FGF-1-graft combination resulted in functional recovery [4]. The transected gap was bridged with intercostal nerves, encapsulated with FGF-1 added fibrin glue, followed by securing the site

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with wire [4]. The Basso, Beattie and Bresnahan (BBB) score was significantly improved; detection of somatosensory (SSEP) and motor evoked potentials (MEPS) showed both sensory and motor information across the damaged site [15]; and regeneration of axons throughout the graft and into the distal stump were demonstrated by retrograde labeling in the cortex and brain stem nuclei and anterograde labeling in the distal stump [4,16]. With the same operation procedure in which rats were randomly divided into treatment groups including the transection control and the laminectomy control, only the FGF-1-graft combination improved hind-limb locomotor function, and the graft alone or FGF-1 alone did not [15]. Therefore, the functional recovery in the combined treatment group is a result of a synergistic effect rather than a mere summation of effects of the individual components. A repair operation of this kind demonstrates the possibility of CNS regeneration and is unique in its strategy. However, the molecular mechanism as to how this operation worked remains to be elucidated. We seek to examine the difference between the transected rats and the rats receiving the seemingly paradoxical combined treatment, in order to look for the target molecules or cells that have been altered.

'Back-degeneration' or 'axon die-back' is secondary degeneration of axons after axotomy that progresses from a small area of direct trauma to a greatly enlarged injury site surrounded by glial scar tissue [7], and contributes to the tissue loss in the white matter. Back-degeneration could be caused sufficiently by injection of activated macrophages to the white matter tract [7]. The cellular phenotypes in this area dictate the first environment that the regenerating axons encounter. In particular, reactive gliosis and inhibitory extracellular matrix at the lesion site are associated with failure of axon regrowth [5]. In this study we used the caudal dorsal column as our model for observation of 'back-degeneration'. The dorsal column is structurally distinct, and unlike a lot of white matter tracts it contains mostly ascending axons. This gives the advantage of observing the caudal back-degenerative stump and the rostral Wallerian degenerative stump separately.

Inflammatory reaction in PNS is known to be a pre-requisite for regeneration, while inflammation in the CNS axons results in post-injury secondary pathology. In addition to providing a less inhibitory environment due to the nature of its myelin contents, macrophages and Schwann cells in PNS grafts synthesize an arsenal of factors to promote regeneration [6,21]. Recently, regeneration of CNS has been reported with treatments altering the macrophage activities. These include injection of autologous macrophage [26], depletion of hematogenous macrophage [22], and systemic administration of minocycline [13]. However, some reports claim the opposite effect or a minimal effect for manipulating the macrophage population [31]. Although still controversial, consensus is forming that different activation states of macrophage exist, and some are beneficial for CNS regeneration and some are detrimental. In our combined repair strategy, grafted intercostals nerves located adjacent to the CNS stump, and nervous tissue of both PNS and CNS origin, were under the influence of invaded macrophages and exogenous FGF-1. The macrophage activities in these two parts were compared.

Adult 200–250 g female Sprague-Dawley rats were used in this study. All procedures involving animals were approved by the Animals Committee of Taipei Veterans General Hospital. Operation procedures have been detailed elsewhere [4]. Briefly, 5 mm of 8th thoracic spinal segment (T8) was removed from the transected rats (the T group). For the combined treatment, intercostal nerve segments combined with FGF-1 in a fibrin glue carrier were implanted to bridge the 5 mm gap in the severed spinal cord (the R group). Another group of rats were transplanted with intercostal nerve segments in fibrin glue with no addition of FGF-1 (the I group). Post-operative care and monitoring have been described [4]. A 2 cm segment, including the middle scar or graft area, was collected at 14 or 28 days after operation, and processed for cryo-section and immunohistochemistry. For statistical analysis at least 4 rats were used for T and R groups for the time point of interest. Comparisons were performed by oneway ANOVA.

Animals were perfused and the spinal cords rapidly dissected. Tissue block was cryo-protected and transversely or longitudinally cryo-sectioned at 20 μm thickness. Transverse sections were systematically labeled and the distance from the trauma center was calculated accordingly. The trauma center was decided by the midpoint of two graft-stump or scar-stump junctions. Primary antibodies (Abs) used were: mouse monoclonal 5D4 anti-keratan sulphate proteoglycan (KSPG) (1:100; Seikagaku); mouse monoclonal anti-ED-1 (1:100; Serotec), for activated and phagocytic microglia/macrophage; rabbit polyclonal anti-glial fibrillary acidic protein (GFAP), astrocyte marker (1:1000; Sigma); and rabbit polyclonal anti-collagen IV (1:100; Progen), rabbit polyclonal anti-fibronectin (1:500, Dako), and mouse monoclonal anti-beta III tubulin (1:500, Covence). The bound antibodies were visualized using the avidin–biotin–peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories) and with appropriate chromagens. The secondary antibodies used for fluorescence microscopy were Alexa 488-conjugated anti-rabbit (1:200; Molecular Probes) and Cy3-conjugated anti-mouse immunoglobulin (1:200; Jackson). Primary antibody omission controls were performed for all immunostaining as a control for nonspecific binding. Fluorescent photography was performed with a Zeiss Axioscope microscope with appropriate filter sets. Sections used in the same experiment were photographed under the same setting for quantification.

Digital images were captured from the caudal stumps from the longitudinal sections that were 20 μM to the midline. A rectangle of 600 by 600 pixels was placed on the area of dorsal column so the left border was 2 mm from the trauma center, and the top border was the dorsal border of the longitudinal section. All pixels within the rectangles were analyzed with ImageJ software (public domain Java image processing program, http://rsb.info.nih.gov/ij/) on a 0 (black) to 255 (white) scale. The average value of the background was 149. The number of pixels that had a value of more than 149 were calculated and used to estimate the immunoreactivity within the rectangle.

ED1 immunostaining and macrophage morphology were jointly considered. Cells were manually scored on an arbitrary grade of 1–7, as follows (see also online Supplementary data).

Cells with long branches and low ED1 immunoreactivity were grade 1. Cells that had shortened branches and a higher ED1 immunoreactivity were grade 2. Cells with shorted branches and bigger but long cytoplasmic ED1+ area were grade 3. Cells with rounded ED1+ cytoplasm but short branches were grade 4. Cells with strong staining in rounded cytoplasm and almost no branches were grade 5. Cells of typical round morphology of brain macrophage were grade 6. Cells of typical aggregated phagocytic morphology were grade 7. A series of grades was plotted against the distance of sections from the transection center.

14 days post-operation (p.o.), in the T group ED-1 positive cells aggregated at the back-degenerated caudal dorsal column (Fig. 1A, panel T). Elevated GFAP immunoreactivity was located around the area that was invaded by ED-1 positive cells. Extracellular matrix protein such as KSPG and collagen IV were deposited in and around this cavity area (Fig. 1A, panel T). The immunoreactivities for KSPG, GFAP, ED-1, and collagen IV in I group were similar to the T group (Fig. 1A, panel I). In the R group, tissue in the dorsal column had much less immunoreactivities for KSPG, GFAP and collagen IV. The end fragment of the dorsal column had much fewer ED-1 positive cells (Fig. 1A, panel R). When analyzed statistically, GFAP and KSPG immunoreactivities in the end fragments of the caudal dorsal column in the R group were significantly down-regulated, compared to the T group (Fig. 1B). The repair operation also changed the cell morphology. Immunoreactivity for KSPG was present in the T and I groups, 5 mm from the trauma center in the back-degenerative dorsal column. The GFAP positive, supposedly astrocytes, extended processes and showed interdigitation around individual cavities, and form a tightly compacted limiting glial margin (Fig. 2A, T and I panel). Some multiple-nuclear ED-1 positive cells were present in the same area (Fig. 2A, T and I panels). In the R group, KSPG immunoreactivity was down-regulated compared to the T and I groups. GFAP-positive astrocytes formed a loose net of processes and the number of ED-1 positive cells were very few at 5 mm from trauma center (Fig. 2A, R panel). In the R group only, type III beta tubulin positive axons were seen in the grafted nerves on a bed of fibronectin positive matrix, demonstrating the growth of axons at this time point through the graft (Fig. 2B).

Four weeks post-operation the grafted nerves were full of ED-1 positive cells, while scars generated by the removal of T8 spinal segments were devoid of them (Fig. 3A, c). As the ascending dorsal column was used as our model, the back-degenerative tract was in the caudal stump. Macrophage activities in the back-degenerative tract were regulated the opposite way to that in the grafted nerve, i.e., down in the R group, and up in the T group (Fig. 3A, 3B, bd). Within an average distance of 3–7 mm from the trauma center in the back-degenerative tract (Fig. 3B, bd), the grades of activities of macrophages averaged about 2 in R group, which presented shortened branches and a higher ED1 immunoreactivity than non-activated microglia. In the T group the grades of activities of macrophages averaged about 6–7, which presented round morphology of brain macrophage, to aggregated phagocytic morphology (Fig. 3B, bd). The differential regulation of macrophage activities was not observed in the distal Wallerian degenerative (rostral) stump (Fig. 3A, 3B, w). In the Wallerian degenerative tract (Fig. 3B, w) macrophages were both of grade 7, which presented aggregated phagocytic morphology, for both treatments.

FGF-1 was reported to have an effect in neuroprotection and in promoting neurite outgrowth [27]; transplantation of Schwann cells or PNS graft into a CNS trauma site improves regeneration [8]. Previous studies using the same operation as Cheng et al. [4] suggested that synergistic action rather than a summation of the individual effects caused the functional recovery [15]. The mechanism of our combined treatment remains largely unknown. One of the components, FGF-1, has never been regarded as a ‘classic’ neurotrophic factor. FGF-1 was shown to regulate astrocyte antioxidation [29]. To date, the neurite promoting effects of FGF-1 have been demonstrated in the traumatized spinal cord, in the organo slice, and in an explant culture, where a large number of glia were present [14,19,27], but not in neuron cultures where astrocytes were rare [30]. Therefore, it is possible that the neurite promoting effect of FGF-1 applied to a trauma site arises from a secondary effect on accompanying non-neuronal cells, or from the environment that axons would regenerate *in vivo*, in addition to a direct survival and neurite promoting effect. Indeed, with our combined treatment, the astrocytes had lower GFAP immunoreactivity and were less interdigitated, and accompanying macrophage activities and KSPG deposition were down-regulated. We also demonstrated at the same time many regenerating neurites had extended into the graft.

Our data qualitatively demonstrated that, while transplantation of peripheral nerves did not inhibit the glial reaction in the back-degenerative tract, addition of FGF-1 to the graft–fibrin system resulted in down-regulation of the glial reaction. In several studies using Schwann cells, with or without a neurotrophic factor for spinal repair, increased astrocytic scarring and inhibitory proteoglycan were reported [8–10]. Even with a promising therapy such as elevated cAMP level plus Schwann cell injection in which the rats gained functional recovery, when axons were capable of venturing into the graft, the levels of immunoreactivity for GFAP or proteoglycan were not decreased in the graft–CNS interface [20]. Our results differ from others using Schwann cells in that our post-traumatic astrocyte reactions were down-regulated, and levels of inhibitory proteoglycan lowered, when FGF-1 was applied, in addition to PNS graft. For penetrating the glial scar, approaches such as increasing the intrinsic ability of axons to regenerate [20], or removing the inhibitory molecules with enzyme treatment [2], were successful. While other Schwann cell therapy carried out the task by simultaneous elevation of cAMP, and thus promoted axon outgrowth ability, our treatment was effective partly because it altered the glial environment. This is the first to report a combined treatment using a growth factor that results in the reduction of inhibitory proteoglycan.

Our results demonstrated that, with the repair operation, the macrophage activities were inhibited in the CNS tract in the back-degenerative area, while in the same rat the macrophage activities in the PNS graft, and in the distal Wallerian degenerative stump of the dorsal column, were active. This demonstrated

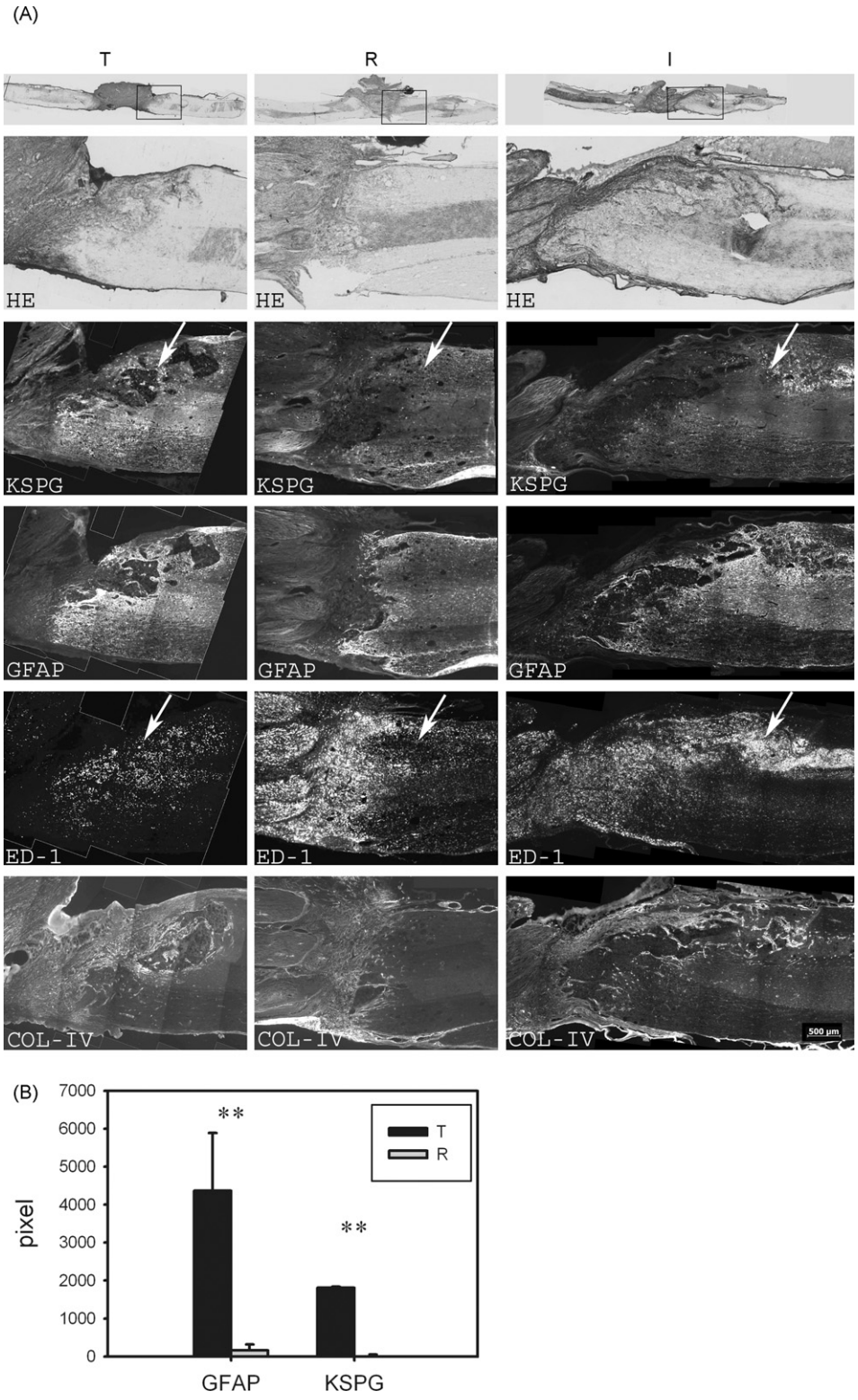


Fig. 1. (A) Immunoreactivities of KPSG, GFAP, ED-1 and collagen IV at 14 days p.o. The square in lower magnification photography for hematoxylin–eosin (HE) staining indicates the area that the rest of the photos depict. T panel: transected; R panel: FGF-1+graft; I panel: graft only. Representative longitudinal sections 20 uM lateral to the midline were double-stained with KSPG/GFAP, and their two adjacent sections were stained with ED-1/collagen IV, and HE, respectively. Arrows point to the back-degenerated or preserved dorsal column. (B) KSPG deposition and GFAP immunoreactivity were down-regulated in the R group. The area from the end fragment of the caudal dorsal column in the longitudinal section was analyzed. $N = 4$; $^{**}P < 0.001$.

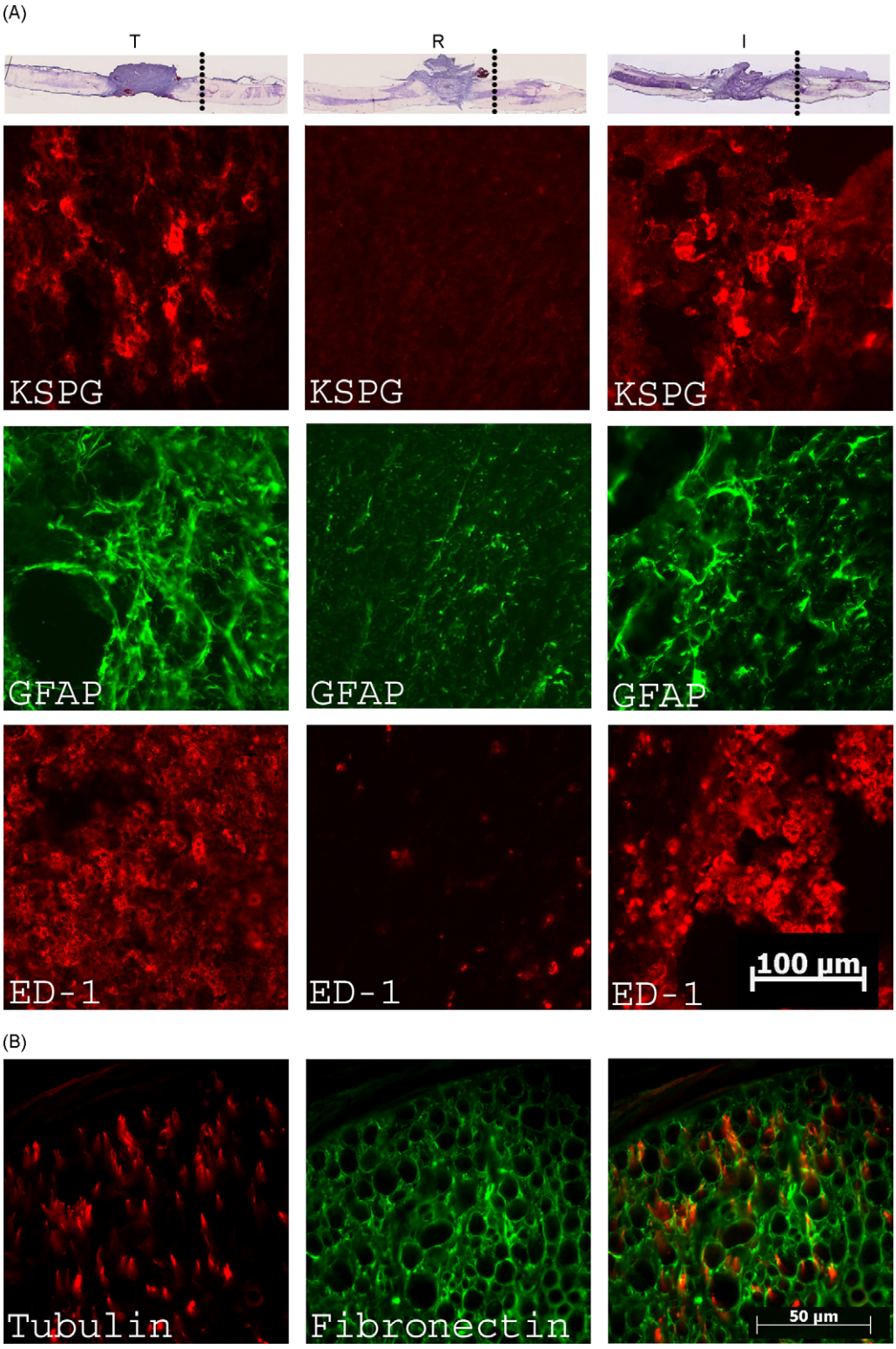


Fig. 2. (A) Morphology of GFAP+ astrocytes, presence of ED-1+ macrophage, and KSPG immunoreactivity at 14 days p.o. in the dorsal column 5 mm from the trauma center, as indicated by dotted line in HE stained longitudinal sections. Sections were double-stained with KSPG and GFAP, and photographed separately, and their respective adjacent sections stained with ED-1. (B) Cross-section of transplanted nerves in R group showed deposition of fibronectin and penetrating neurites stained with type III beta tubulin.

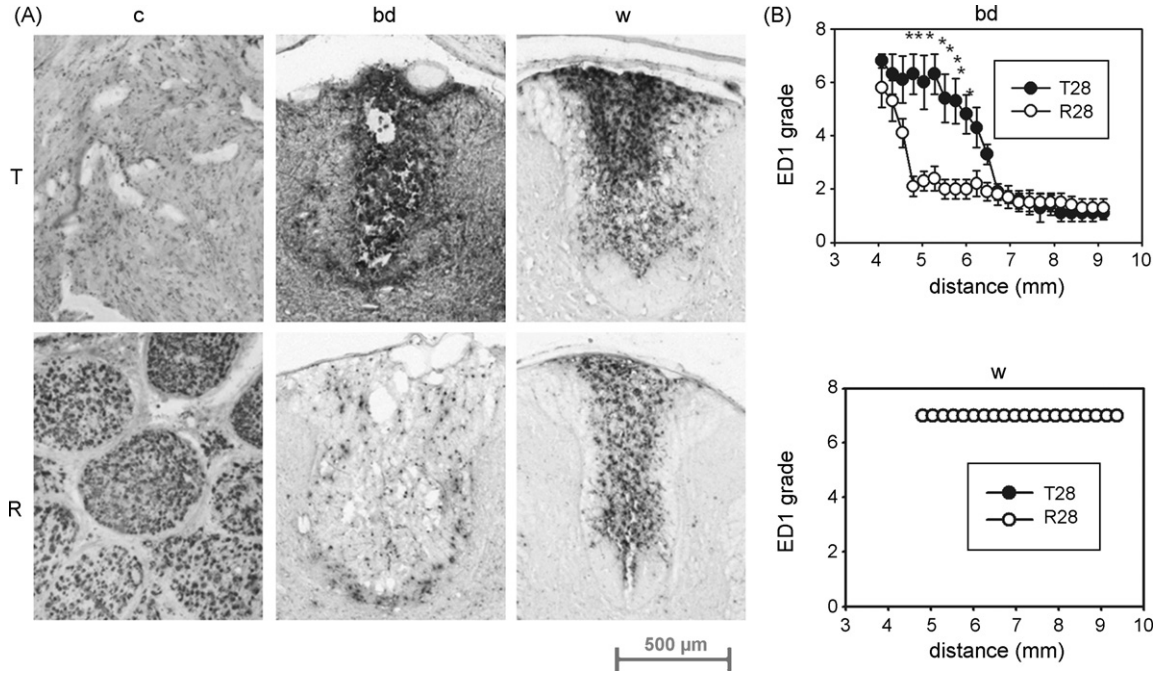


Fig. 3. (A) ED-1 positive macrophages distribution in the system. The left panel (c) showed the distribution of ED-1+ cells in the scar center (for T group) and in the nerve graft (for R group). The middle panel (bd) showed distribution of ED-1 cells in the back-degenerated dorsal column. The right panel (w) showed ED-1+ cells in the rostral Wallerian degenerative tract. Scale bar = 500 uM. (B) The macrophage activities were analysed by an arbitrary scale of 1–7 and plotted against the distance from the trauma center. $^{*}P < 0.01$.

a local, not a systematic regulation of the macrophage activities. As the grafted intercostal nerves were distal to the cut, they were expected to undergo Wallerian degeneration. Wallerian degeneration in PNS results in clearance of myelin by Schwann cells and macrophage phatocytosis, and cytokine secretion by these cells attracts axon regrowth. The denervated Schwann cells secrete an arsenal of growth factors [28], and in our sealant glue were further supplemented with FGF-1. Macrophage activities in the grafted nerves could be regulated by addition of FGF-1 and secrete beneficial factors. Indeed, we have recently found large amounts of macrophage in the graft nerves with our combined treatment were arginase positive and secreted polyamine spermidine [12], which could promote neurite outgrowth. Therefore, the PNS Wallerian degenerative activities could contribute to the regulation of the nearby back-degenerative tract. Furthermore, contrary to previous knowledge, the CNS distal stump could degenerate by macrophage phagocytosis and inflammation [23]. In our repaired model the macrophages in the Wallerian degenerative distal stump were active, and therefore could aid in the removal of myelin and help regeneration.

The above stated differential regulation between graft and host, and between proximal and distal stumps, is unique among other treatments when immune reaction is manipulated, as others were systemic, at least when macrophages were considered [13,22,26]. For example, methylprednisolone, a standard care for acute SCI, caused non-selective, long-lasting reduction for the macrophages, and resulted in poor functional outcome [31]. Therefore, manipulation of macrophage for CNS regeneration is not just a matter of a reduction of macrophage numbers, but specific activities of macrophage in respect to its location and function could yield better results.

We conclude that various glial phenotypes in the back-degenerated tract were altered favorably for CNS regeneration by our combined treatment. Local inhibition of back-degeneration related macrophage activities, and retention of beneficial macrophage activities in other parts could also favor CNS regeneration.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2007.11.067.

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