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ORIGINAL ARTICLE

Vaccination with dendritic cells pulsed with homogenate protein of spinal cord promotes functional recovery from spinal cord injury in mice

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Study design: Randomized, double-blinded animal experiment for neural functional recovery from spinal cord injury (SCI) through vaccination with immature dendritic cells (DCs) pulsed with homogenate protein of spinal cord (hpDCs) in mice.
Objective: To study the effect of hpDCs in the recovery from SCI in mice.
Method: Immature DCs pulsed with homogenate protein of spinal cord, myelin basic protein (MBP) or phosphate-buffer solution (PBS) were injected into spinal cord-injured mice locally or peritoneally. The functional recovery of spinal cord (open-field locomotor rating scale of Basso, Beattie and Bresnahan, BBB score) was measured weekly. The areas of injured region and cyst as well as the thickness of the glial scar were measured and the expressions of glial fibrillary acidic protein, neurofilament and nestin were detected to confirm the BBB scores.
Results: Eighty-four days after injection, the BBB score of the hpDCs group (peritoneally injected mice) reached 18.2 ± 1.1, significantly higher than that the scores of the mbpDCs and control groups (16.3 ± 2.1 and 10.0 ± 2.0, respectively). The areas of injured region and cyst as well as the thickness of the glial scar of the hpDCs group were less than that of the control group. Meanwhile, the expression of nestin lasts up to 56 days after injection in the hpDCs group, while it disappeared in the mbpDCs and PBS groups.
Conclusion: Implanting DCs pulsed with homogenate protein of spinal cord, but not mbpDCs or PBS alone, locally or peritoneally, have a significant effect on functional recovery and neural preservation from SCI.
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Keywords: spinal cord injury; dendritic cells; homogenate protein of spinal cord

Introduction

The devastating outcomes of spinal cord injury (SCI) result from direct as well as secondary damage to neurons and fibers in the injured site. The secondary damage is caused mainly by the activity of injury-evoked destructive self-compounds, such as physiological substances with an excess of toxin over their normal levels or degradation products of self-compounds.[1] Thus, neuroprotection of the spared neurons and regeneration of the damaged fibers may improve the functional recovery from SCI.[2,3]

Studies have indicated that a properly controlled T cells-mediated, antigen-specific immunity in SCI has a pivotal function in regrowth of the injured spinal cord and its protection from secondary degeneration. Passive or active immunization with T cells specific to central nervous system-associated myelin antigens reduces secondary degeneration in rat and mouse models of optic nerve crush or spinal cord contusion.[4,5] Moreover, local implantation of macrophages activated by an autologous sciatic nerve improves the functional recovery in a completely transected spinal cord or an optic nerve because of regenerative growth.[6]

Dendritic cells (DCs) are immune cells whose principal function is antigen presentation.[7] Their capacity of immune

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regulation is determined by the state of maturation as well as the number and context in which they are activated. The decisive signal is the expression of CD86 (B7-2) and MHC class II (MHC-II) molecules concurrently with the release of proinflammatory cytokines, especially interleukin-12, interleukin-6 and tumor necrosis factor-α from the DCs.

A study indicates that vaccination with DCs pulsed with myelin basic protein (MBP) or peptides derived from MBP can improve functional recovery from SCI in rats, although the mechanism is unclear.[8] Homogenate protein of spinal cord, which is easy to prepare, contains all kinds of autoimmune antigens theoretically, if it is true. The purpose of this study is to find out whether immature DCs pulsed with homogenate protein of spinal cord (hpDCs) could improve functional recovery from SCI in mice and to compare their effect with immature DCs pulsed with MBP (mbpDCs).

Materials and methods

Animals

Adult BALB/c mice (4–6 weeks old, 18–23 g) were supplied by the Animal Breeding Center of Third Military Medical University (Chongqing, PR China). They were housed in a light- and temperature-controlled room and matched for age. All animals were handled according to guidelines of the National Institutes of Health and Third Military Medical University for the management of laboratory animals. We certified that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

Spinal cord injury

According to Fehlings' method,[9] all the mice were anesthetized by intraperitoneal injection of 10% chloral hydrate ($5\,ml\,kg^{-1}$), and the spinal cord was exposed by laminectomy at the level of T10. The spinal cord at the T10 level was clamped vertically to the longitudinal axis of the spinal cord by a microsurgery artery clamp (pressure is 2 g), which resulted in severe injury.

Antigens

A homogenate of the spinal cord was harvested from BALB/c mice (aged 4–6 weeks, weighing 18–23 g). The brief process is described as follows: BALB/c mice were killed by overdose anesthesia and T7-10 were sectioned. The spinal cord segments were ground and filtered (200 μm), then ground by an ultrasonic homogenizer, and finally centrifuged at 15 000 r.p.m. for 20 min. The supernatant was collected and the concentration of protein was measured. The concentration of total protein used here was $1\,\mu g\,\mu l^{-1}$. MBP was bought from Sigma Company (St Louis, MO, USA).

Preparation of mouse DCs

Dendritic cells were obtained from bone marrow by a method described earlier[10] with some modifications. The brief process is described as follows: femurs and tibias were removed from the dead mature male BALB/c mice (4–6 weeks old), with the muscles and connective tissues stripped, placed in 70% ethanol for 5 min for disinfection, and then washed with phosphate-buffer solution (PBS). Both ends of the bones were cut with scissors and the marrow was flushed out with calcium-free and magnesium-free PBS using a syringe with a 23-gauge needle. Cell aggregates were broken down by vigorous pipetting. Red blood cells were lysed with ACK buffer (containing (in $mmol\,l^{-1}$): 150 NH_4Cl, 10 $KHCO_3$, 0.1 Na_2EDTA, pH 7.2–7.4). Bone marrow cells were counted and placed in $2-5\times10^6$ cells ml^{-1} in a 250 ml flask (total 5 ml). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with $100\,\mu g\,ml^{-1}$ penicillin and streptomycin, $2\,mmol\,l^{-1}$ L-glutamine, $50\,\mu mol\,l^{-1}$ β-mercaptoethanol, $1\,mmol\,l^{-1}$ pyruvate, 1:100 nonessential amino acids, and 10% heat-inactivated and filtered fetal calf serum (referred to hereafter as DC medium). The cytokine's recombinant murine granulocyte macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA) and recombinant murine interleukin 4 (PeproTech), both at $20\,ng\,ml^{-1}$, were added on day 0. On days 2 and 4, the culture medium was replaced by DC medium supplemented with cytokines, and the floating cells were discarded. On day 7, the cells were collected with a cell scraper (Sarstedt, Newton, NC, USA) for adherent cells and centrifuged, and the pellet was resuspended in fresh DC medium (without adding cytokines; 2×10^6 cells ml^{-1}) containing homogenate protein or MBP ($80\,\mu g\,ml^{-1}$). The cells were pulsed with antigen (incubated for 2 h), washed with fresh DC medium, and kept on ice until injected. Just before injection, the cells were centrifuged and resuspended in PBS (5×10^5 cells in 5 μl of PBS for local injection; 5×10^5 cells in 0.3 ml of PBS for intraperitoneal injection).

Experimental protocol for DC administration

The first evaluation of an open-field locomotor rating scale of Basso, Beattie and Bresnahan (BBB score) was carried out when the clamped mice resuscitated completely from anesthesia (about 4 h after surgery), and the inclusion criterion was BBB score <2. Three groups were established as the hpDCs group, mbpDCs group and PBS group, and each group was divided into two subgroups according to the injection site (lesion site or peritoneum). Cells (hpDCs or mbpDCs) were adjusted to the appropriate number and volume just before injection. For local injection, the wound was reopened and the mouse was fixed on a stereotaxic apparatus (Reward, China). Cells were injected into the center of lesion and the depth of injection was 0.5 mm using a Hamilton syringe. The wound was sutured when the injection was accomplished. For peritoneal injection, cells were injected into the peritoneum at the site of the right mid-abdomen. All injections were undertaken 24 h after SCI, and the concentrations of cells were recorded. The control mice were injected locally or peritoneally as described above with the same volume of PBS as the treated mice.

Assessment of functional recovery from spinal cord contusion

Functional recovery was determined by BBB score on a scale of 0 (complete paralysis) to 21 (normal mobility). Blind

scoring ensured that the observers were not aware of the treatment received by each mouse. The locomotor activities of the trunk, tail and hindlimbs were evaluated weekly in an open field for 4 min. Before each evaluation, the mouse was examined carefully for perineal infection, wounds in the hindlimbs, and tail and foot autophagia.

Histology
On days 28, 56 and 84 after injection, the mice were anesthetized with an overdose of chloral hydrate and perfused intracardially with 100 ml of cold 0.1 mol l^{-1}. PBS, pH 7.4, at 4 °C and then with 200 ml of 4% paraformaldehyde (prepared in 0.1 mol l^{-1} PBS, pH 7.4, containing 5% glucose). A 15-mm block of the spinal cord, with the injury site in the middle, was excised, postfixed overnight in 10% phosphate-buffered formaldehyde, dehydrated overnight in ethanol, and embedded in a paraffin block. Serial longitudinal sections (5 μm) were made. Each block was stained with hematoxylin and eosin. From each spinal cord, approximately 50 sections were inspected.

Immunohistochemistry
Serial longitudinal sections (5 μm) were analyzed by immunohistochemical staining for glial fibrillary acidic protein (GFAP, 1:200) to delineate the site of injury, for neurofilament (NF-200, 200 kDa, 1:250) to detect NF, and for nestin (1:250. All antibodies were bought from Boster, Wuhan, PR China) to detect neural stem cells and progenitor cells. The antigens were repaired according to the instructions of the manufacturer of the antibody (NF using microwave and nestin using trypase). Slides were overlaid with serum (4–10%) for 60 min before incubating with primary antibody. After incubating overnight at 4 °C, the sections were rinsed three times with buffer before overnight incubation (4 °C) with biotinylated secondary antibody (1:400). The slides were rinsed three times, and endogenous peroxidase activity was quenched by applying 6% $MeOH/H_2O_2$ for 15 min. Bound antibody was visualized by applying Elite ABC (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature, followed by diaminobenzidine (DAB) or DAB-ammonium nickelous sulfate staining (NF, Vector Laboratories). Then, the sections were dehydrated, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Shanghai, PR China). From each spinal cord ($n = 4$ for each group), 50 sections were inspected, of which the 5th, 25th and 45th sections (representing the bilateral and midsagittal areas of interest) were selected for further quantitative analysis. Cyst sizes were determined by semiautomated image analysis. Borders of the spinal cord sections were defined manually, and the numbers of blank pixels (that is, with no tissue inside), each 1.8×1.8 μm, were determined automatically (Image-Pro Plus program, Media Cybernetics, Silver Spring, MD, USA), yielding the sizes of the cysts.

Retrograde labeling of axons
At 84 days after injection, three mice from each group were reanesthetized, and spinal canal of T12 was opened as mentioned above. Horseradish peroxidase (Xitang biocompany, Shanghai, China) was applied below the site of contusion at T12.[11] The number of stained axons in the proximal spinal cord was taken to represent the number of intact axons ascending from the distal end and traversing the area of contusion.[12] After 5 days, the mice were again deeply anesthetized, and their spinal cords proximal to the lesion sites were excised and made into paraffin sections. All sections (5 μm) taken from the spinal cord were analyzed by DAB-niekel sulfate amine staining. The total number of labeled axons was counted in each section.

Antegrade labeling of axons
The mice were also subjected to antegrade labeling of axons with biotinylated dextran amine with the method mentioned above, but the injection site was at T7 and the distal spinal cord was processed.

Statistical analysis
Behavioral and morphological data were analyzed by two-tailed Student's *t*-test. Because the open-field motor scores were measured at different times after injury, they were also analyzed by two-factor repeated measures of analysis of variance.

Results

HpDCs improving functional recovery from SCI
Typical paraplegia was observed in all animals after contusion. However, the mice injected with hpDCs and mbpDCs recovered from SCI more rapidly and significantly than the mice injected with PBS. As soon as 7 days after injection ($P < 0.05$, Table 1), the differences increased with the passage of time. Twenty eight days after injection, the score of the hpDCs group reached and persisted at the highest plateau, with the score of mbpDCs group at a relatively lower plateau,

Table 1 BBB scores of control, mbpDCs and hpDCs groups

	Control		*mbpDCs*		*hpDCs*	
	Locally	*Peritoneally*	*Locally*	*Peritoneally*	*Locally*	*Peritoneally*
28 dpi	7.8 ± 1.2	7.5 ± 1.7	14.8 ± 2.0[a]	14.0 ± 2.2[a]	16.2 ± 2.0[b]	16.0 ± 2.2[b]
56d pi	10.0 ± 1.2	9.0 ± 1.4	16.3 ± 1.8[a]	16.4 ± 2.3[a]	18.2 ± 1.8[b]	18.1 ± 1.3[b]
84 dpi	10.0 ± 1.2	10.0 ± 2.0	16.5 ± 1.8[a]	16.3 ± 2.1[a]	18.5 ± 1.8[b]	18.2 ± 1.1[b]

Abbreviation: dpi, days post injection.
[a]Compared with control group, $P < 0.05$.
[b]Compared with control group, $P < 0.01$, compared with mbpDCs groups, $P < 0.05$.

whereas the score of the PBS group reached and persisted at the lowest plateau 35 days after injection (Figure 1). Eighty-four days after injection, the scores of the PBS, mbpDCs and hpDCs groups (subgroups of local injection) were 10.0 ± 2.0, 16.3 ± 2.1 and 18.2 ± 1.1, respectively. No significant difference was observed between the locally or peritoneally injected groups at the same time points.

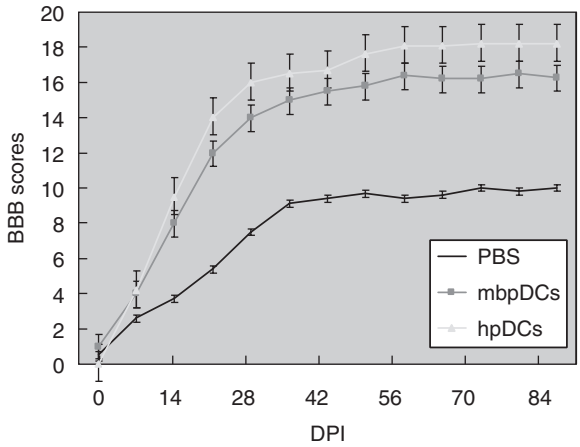

Figure 1 Spontaneous recovery from spinal cord contusion of peritoneal injection subgroups at T10. At 7 days after injection, the scores of the mbpDCs and hpDCs groups were significantly higher than that of the control group ($P<0.05$). At 28 days after injection, the mbpDCs and hpDCs groups reached higher plateaus (14.8 ± 2.0, 16.2 ± 2.0) more rapidly and significantly than did the phosphate-buffered saline (PBS) group, which reached a lower plateau 35 days after injection (10.0 ± 1.2, $P<0.01$). The difference between the mbpDCs and hpDCs groups was observed at 28 days after injection ($P<0.05$).

Histological analysis

The spinal cords were removed with the lesion sites in the middle 28, 56, 84 days after injection. All the cords were sectioned longitudinally and stained with hematoxylin and eosin, and observed with light microscopy. The characteristics of the PBS group are described as follows: 28 days after injection, the lesion site was full of debris of tissues, foam cells and the normal longitudinal structure of conduction tracts lost, whereas there was no distinct boundary between the lesion and normal sites (Figure 2a). Fifty-six days after injection, cysts in the lesion site formed distinct boundary, and the glial bundles extended into the cysts (Figure 2b). Eighty-four days after injection, the boundary of the cysts formed completely and the glial scar became more compact, whereas there were no tissues in the cysts (Figure 2c). There was no difference in the pathological change between the mbpDCs and hpDCs groups, but differences were found in the areas of lesion sites and cysts, as well as in the thickness of the glial scar (Table 2).

Immunohistochemistry

Nestin. The expression of nestin is transiently positive 4 weeks after SCI. In this study, however, immunohistochem-

Table 2 Measurements of lesion site, cyst and thickness of glial scar of control, mbpDCs and hpDCs groups

	Lesion site (mm)	*Cyst (mm)*	*Thickness of glial scar (μm)*
Control group	2.3 ± 0.3	1.7 ± 0.2	12.5 ± 2.3
mbpDCs group	1.9 ± 0.3[a]	1.6 ± 0.3[a]	11.7 ± 2.0
hpDCs group	1.9 ± 0.2[a]	1.5 ± 0.2[b]	10.7 ± 2.0[a]

[a]Compared to control group, $P<0.05$.
[b]Compared to control group, $P<0.01$.

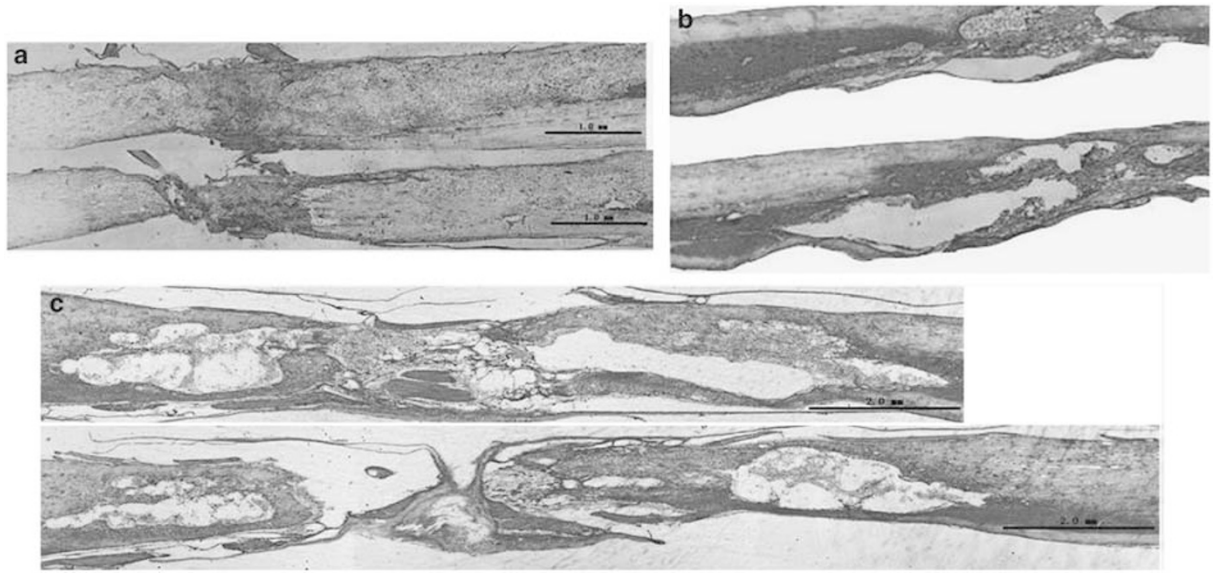

Figure 2 The lesion sites of control group. Representative micrographs from the phosphate-buffered saline (PBS) group are shown. Exemplify formation of cysts in the neural tissues of a PBS-treated mice. At 28 days after injection, cysts formed with unclear edge (**a**). The edges became more definite with the passage of time. At 56 days after injection, the edge formed definitely. (**a**: 28 days after injection; **b**: 56 days after injection; **c**: 84 days after injection. hematoxylin and eosin, ×10).

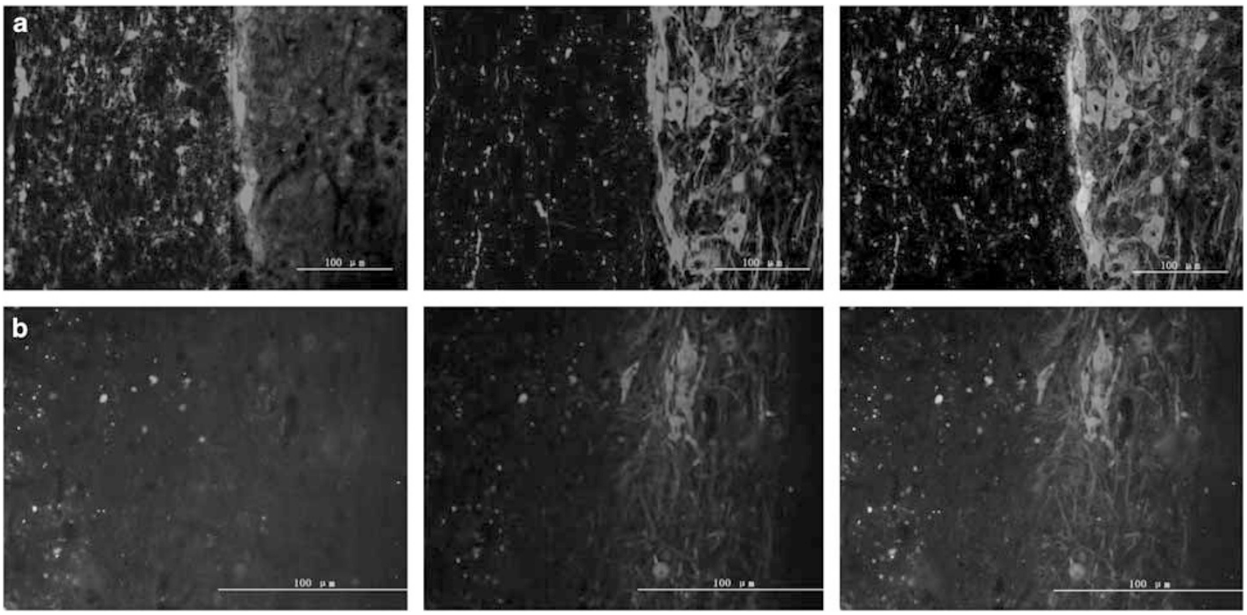

Figure 3 Nestin and neurofilament (NF)-stained spinal cord 56 days after injection. The expression of nestin of the hpDCs group (**a**) was stronger than that of the mbpDCs group (**b**), suggesting that the hpDCs group was more potential in neural regeneration. (Nestin and NF fluorescent stain, red-NF, green-nestin, ×100.)

istic fluorescence of nestin and NF showed that there were nestin-positive cells in the hpDCs group until 56 days after injection (Figure 3a), whereas the PBS or mbpDCs groups were nestin negative since 28 days after injection (Figure 3b). This fact showed that the expression of nestin was prolonged in the hpDCs group.

Neurofilament. On 28 and 56 days after injection, the axons around the lesion sites were destroyed and debris were phagocytosed, whereas cysts formed and no axon was spared in the lesion sites center. The axons stopped at the wall of the glial scar and never reached into the cyst in the PBS group (Figure 4a). No difference was observed between the mbpDCs and hpDCs groups (Figure 4b).

Glial fibrillary acidic protein. On 56 days after injection in the PBS group, cysts formed with a definite edge, and no $GFAP^{+}$ cells were stained in the cyst. Proliferative, hypertrophic and compact astrocytes were found near the wall of the cyst. The expression of GFAP decreased with the distance to the cyst, and the astrocytes became normal. The expression of GFAP in the hpDCs group was less and slower than that in the DCs group. The distribution of astrocytes was looser than in the DCs group (Figure 5).

Retrograde and antegrade labeling of axons. Retrograde labeling with horseradish peroxidase and antegrade labeling with biotinylated dextran amine of the axons showed that axons stopped at the lesion site and that no difference was observed among the three groups (Figure 6).

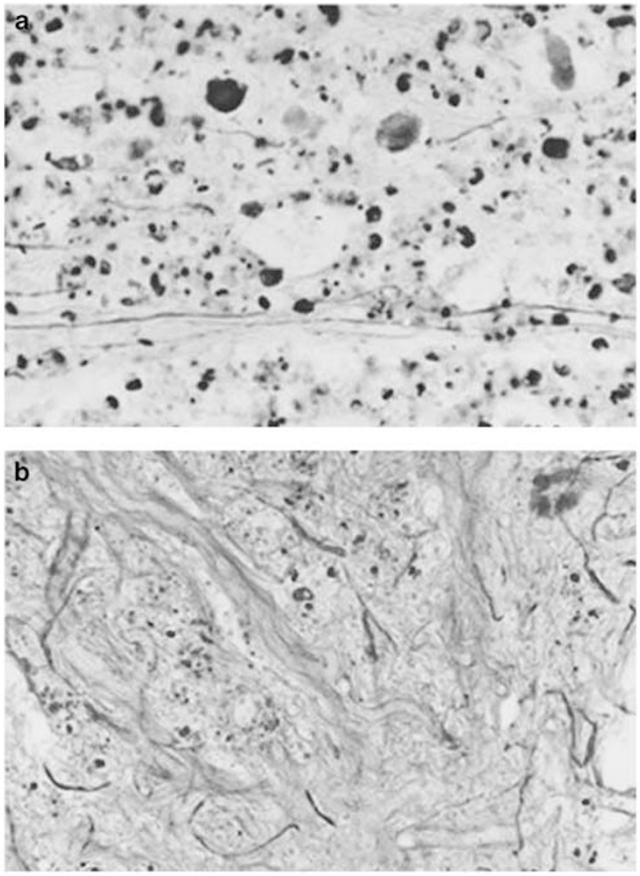

Figure 4 At 56 days after injection, expression of neurofilament (NF). NF-positive debris were stained in the lesion site, and no difference was observed between the phosphate-buffered saline (PBS) and hpDCs groups (**a**: PBS group; **b**: hpDCs group. diaminobenzidine ammonium nickelous sulfate stain, ×200).

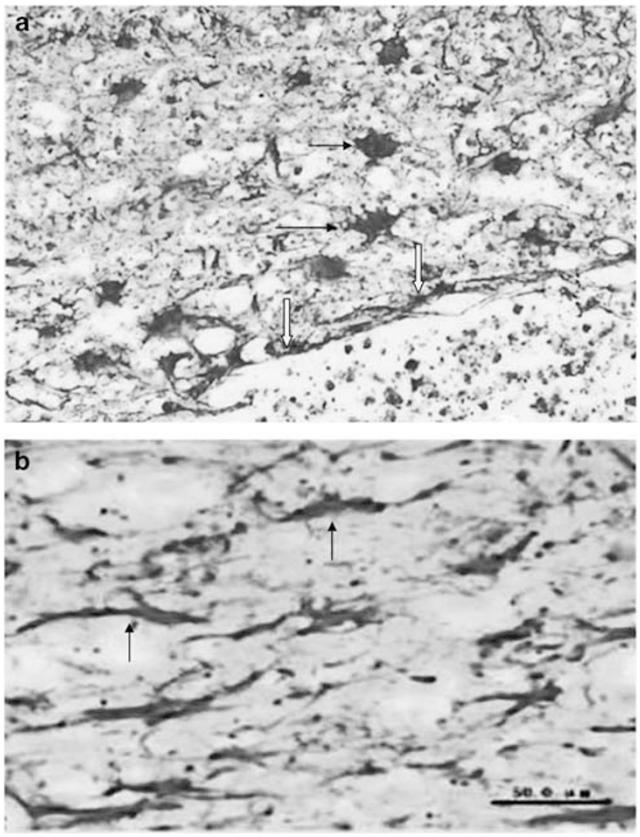

Figure 5 Glial fibrillary acidic protein (GFAP) immunohistochemical stain showed different proliferation and hypertropia between the mbpDCs and hpDCs groups. At 56 days after injection, the mbpDCs group (**a**) GFAP$^+$ cells were more proliferative and hypertrophic than those of the hpDCs group (**b**). (→, GFAP$^+$ cells. ⇨, wall of cyst. Diaminobenzidine ammonium nickelous sulfate stain, ×200.)

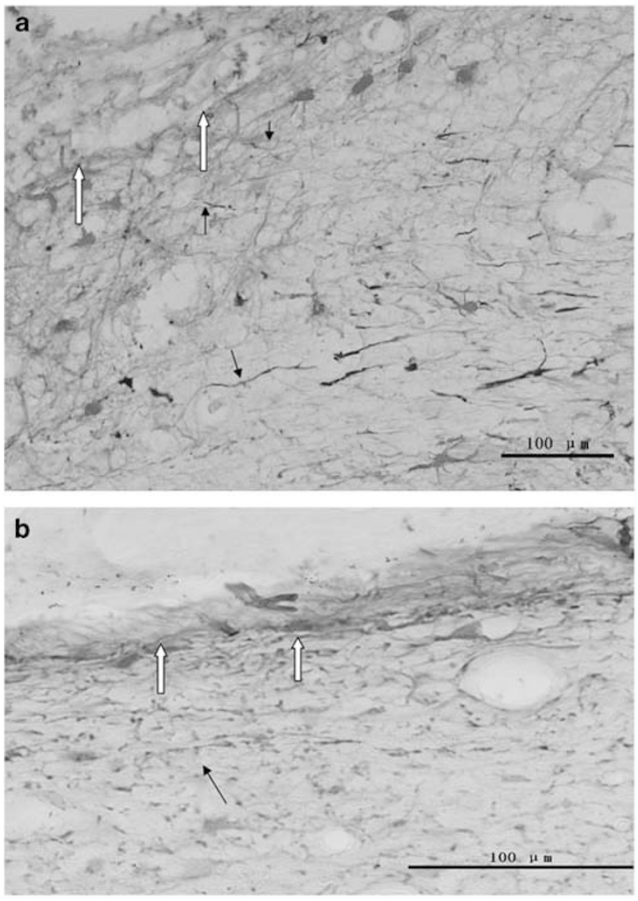

Figure 6 Biotinylated dextran amine (BDA) and horseradish peroxidase (HRP) labeling of axon. At 56 days after injection, antegrade labeling with BDA (**a**) and retrograde labeling with HRP (**b**) of axons in the hpDCs group. Axons (→) stopped at the wall of cyst (⇨) and never reached into the cyst. (Ammonium nickelous sulfate stain, glial fibrillary acidic protein (GFAP) stained by streptavidin–biotin complex , ×200.)

Discussion

The results of this study show a significant improvement in locomotor function after contusive SCI in mice treated by local or peritoneal injection of bone marrow-derived DCs pulsed *in vitro* with homogenate protein. The beneficial effect of the treatment was also evident morphologically, with better preservation of neural tissues on histology and a decrease in the sizes of cysts in the spinal cords of treated mice examined by immunohistochemistry.

The endogenous autoimmune response can be boosted by innate and adaptive immune manipulations,[13] whereas DCs were found to populate specific areas of the central nervous system, including the meninges and choroid plexus.[14] DCs are potent to initiate specific immune responses. Yoles *et al*.[15] have found that stimulation of an adaptive immune response against central nervous system self-antigens after an injury is a normal part of the body's own healing mechanism and a central feature of the proposed concept of 'protective autoimmunity.'[16,17]

We show here that function recovery from SCI is improved by implanting hpDCs locally or peritoneally. The locomotor activity of the mice used in the experiment was assessed before treatment, and only mice with a BBB score lower than 2 were used. This homogeneous group of mice was randomly divided into three groups, which were treated with the hpDCs, mbpDCs or PBS, respectively. This approach minimizes the variations in insulting severity among contused mice within the same group and between the treated and control groups. Interestingly, the outcome in the treated mice was different, whereas the mice treated with hpDCs showed the best recovery, whereas only a slight improvement was observed in the control group.

Treatment with mbpDCs or hpDCs has different effects on the injured spinal cord. DCs pulsed with homogenate protein of the spinal cord were more beneficial than mbpDCs, suggesting that hpDCs cause more potent protective immunity reactions. We presume that there is more than one kind of autoantigen *in vivo*, which is beneficial to neural recovery from SCI, rather than a single antigen, for example, MBP alone. We are designing new experiments to seek out the mechanisms.

Our results showed that vaccination with hpDCs decreased the areas of lesion sites and cysts, as well as reduced the

density of the glial scar. Glial cells have important functions in the functional recovery from SCI and their number and disposition are both important. The fact that vaccination with hpDCs decreases the density of the glial scar to a relatively low level may reduce the barrier of the glial scar to regrowth of the axons and decrease the inhibitory factors produced by the glial cells, although we do not find the axons growing into glial scars. It is evident that hpDCs improve the functional recovery of neural tissues not only immunologically but also morphologically.[18,19] The local mechanisms of protection acting on the neural tissues are not understood fully, but increasing data support a key function for resident microglia and infiltrating macrophages in this process. Rapalino *et al*.[20] thought that the local implantation of activated macrophages into completely transected spinal cords leads to regeneration of severed axons with partial functional recovery of otherwise completely paralyzed rats. The injected activated macrophages are reminiscent of antigen-presenting cells. The findings in this study suggest that the effects of hpDCs on the injured spinal cord are mediated through systemic immune mechanisms, because there were no significant differences among the effects of DCs administered locally or peritoneally to the spinal cord.

Conclusion

The results of this study clearly show a beneficial effect of hpDCs on functional and morphological recovery from SCI in BALB/c mice, and both local and peritoneal injections have the same effect. The results show that hpDCs improve functional recovery and decrease the areas of cysts as well as the density of glial scar. It is of more importance that hpDCs prolong the duration of expression of nestin. HpDCs are more potent than mbpDCs in functional recovery from SCI.

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