Histological repair of damaged spinal cord tissue from chronic contusion injury of rat: A LM observation

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Summary. The spinal cord has an intrinsic, limited ability of spontaneous repair; the endogenous repair of damaged tissue starts a few days after spinal cord injury (SCI). To date, however, detailed observation in histology at the injury site has not been well documented. In the present study we analyzed the histological structure of the repaired tissue from injury site of rats 6 or 14 weeks after contusion injury (NYU impactor device, 25 mm height setting) on T10, and rats 8 weeks after transplantation of lamina propria (LP) or acellular lamina propria. We found that the initial repaired tissue can be histologically divided into three different zones, i.e., fibrotic, cellular and axonal. The fibrotic zone consists of invading connective tissue, while the cellular zone is composed of invading, densely compacted Schwann cells. Schwann cells migrate from dorsal roots laterally toward and merge underneath the fibrotic zone, forming the U-shape shell of the cellular zone. The major component of the axonal zone is regenerating axons. Schwann cells myelinate regenerating axons in all three zones. In rats with combination treatments including scar ablation and LP transplantation, both cellular and axonal zones significantly expand in size, resulting in the disappearance of the lesion cavity and the integration of repaired tissue with spared tissue. Olfactory ensheathing cells from transplanted LP may promote the expansion of the cellular and axonal zones through stimulating host Schwann cells, indirectly contributing to tissue repair and axonal regeneration. The ependyma-derived cells may be directly involved in tissue repair, but not contribute to the formation of myelin sheaths.

Key words: Spinal cord injury, Tissue repair, Transplantation, Olfactory ensheathing cell, Ependyma-derived cells, Rat

Introduction

Our observation and others have demonstrated that the spinal cord has endogenous ability of repair following injury (Schwab and Bartholdi, 1996; Beattie et al., 1997), although this ability is limited and the procedure is slow. Endogenous repair of damaged tissue takes place a few days after spinal cord injury (SCI). Following spinal cord contusion injury (such as 25 mm height setting) in rats, the whole gray matter and dorsal white matter were destroyed at the injury epicenter. Two weeks later the lesion cavity appears and the glial scar forms (Liu et al., 1997), which lines the spared tissue surrounding the cavity. At the same time a newly formed, endogenous repaired tissue, which is previously called connective tissue scar, fibrotic scar, or collagenic scar tissue, can be observed at the dorsal part of the lesion cavity (Zhang et al., 2005).

This initial, small repaired tissue is located at the dorsal part of the spinal cord, neighbors laterally on the spared tissue and faces ventrally the lesion cavity, which is filled with fluid and macrophages. It is loosely connected to the ventral spared tissue with trabeculae, which contain fibroblasts, blood vessels and even nerve fibers (Beattie et al., 1997; Zhang et al., 2005). The connective tissue-like component of the endogenous repaired tissue starts to form one week following spinal cord injury and it is associated with the invasion of fibroblasts from pia mater due to the destruction of glial limitans. The endogenous repaired tissue is structurally different from either normal spinal cord tissue or regular connective tissue scar in the skin wound, since in addition to blood vessels, fibroblasts, collagen fibers and

small cysts, it most importantly contains a number of Schwann cells which invade from the dorsal roots, and regenerating axons (Blight and Young, 1989; Bunge et al., 1994; Bruce et al., 2000; Hermanns et al., 2001; Zhang et al., 2005). To our knowledge, detailed observation in histology at the injury site has not been well documented yet in chronic injury model. Because our research has been focused on the chronic model and we found 6 weeks after contusion injury, both morphological and functional situations are relatively stable, we select this time point for the intervention treatments. In this report we will concentrate on the histological analysis of this endogenous repaired tissue and the possible relationship with histological recovery of damaged cord tissue.

Over the past decade, transplantation of olfactory ensheathing cells (OEC) has emerged as a very promising experimental therapy to promote repair of spinal cord injury, because OECs can secrete extracellular molecules and neurotrophic factors to promote axonal regeneration (Santos-Benito and Ramon-Cueto, 2003). OECs also can induce the migration of host Schwann cells into the damaged area (Ramer et al., 2004; Boyd et al., 2005; Belegu et al., 2007; Cao et al., 2007). More importantly, lamina propria (LP) of the olfactory mucosa is one of the best sources of OECs. It is easily accessible via a simple biopsy through the external nares, and also can prevent the problems of rejection or immunosuppression. All these advantages support a potential therapeutic role for LP transplantation that may be applicable to human spinal cord injury (Lu and Ashwell, 2002; Richter et al., 2005). In our previous study we have found that combination treatments including scar ablation and transplantation of LP into the lesion cavity resulted in substantial repair of damaged cord tissue and axonal regeneration in a chronic contusion injury model (Zhang, 2008). Another purpose of this study is to further investigate the role of the repaired tissue in structural recovery of damaged cord tissue promoted by intervention treatments.

Materials and methods

Animal and spinal cord injury

Adult, female Long-Evans rats (weigh 200-220 g) were employed for this study. All rats underwent contusion injury (25 mm height setting) to spinal cord thoracic (T) level T10 produced by using the NYU (New York University) impactor device (Constantini and Young, 1994) under deep anesthesia with sodium pentobarbital (50 mg/kg, ip). To relieve pain analgesia buprenorphine was administered (0.02 mg/kg, sc, b.i.d.) for 2 days after injury. Antibiotic cephazolin was used (20 mg/kg, im, b.i.d.) for 7 days to prevent bladder and wound infection. In addition, 0.9% saline solution was administered for 7 days to prevent dehydration (10 ml, sc, daily). Urinary bladders were manually emptied

twice a day until adequate spontaneous voiding returned. Two or six weeks after contusion injury or 8 weeks after LP or ALP (acellular lamina propria) transplantation rats were transcardially perfused under deep anesthesia (pentobarbital 90 mg/kg, i.p.) with saline followed by phosphate-buffered 4% paraformaldehyde. Each group contains at least 6 animals. All surgical procedures, including interventions and pre- and post-surgery care, were approved by the Institutional Animal Use and Care Committee of Colorado and are consistent with the *Guide for the Care and Use of Laboratory Animals* (1996).

Preparation of LP and ALP for transplantation

Tissue for transplantation was collected from young female Long-Evans rats. Freshly prepared LP of the olfactory mucosa was separated from the epithelium using Dispase II (Roche, Indianapolis, IN) digestion (45 min, 37°C) (Feron et al., 1999). The tissue was rinsed, cut into 0.5x0.5 mm pieces and kept on ice in L-15 (Gibco, Grand Island, NY). Preparation of ALP (acellular lamina propria) is the same as that of LP, but the final step is to freeze the LP at -20°C over night in order to kill all the living cells within the LP.

Removal of glial scar

Before transplantation the glial scar was removed photochemically from the injury site of rats six weeks after contusion as described before (Zhang et al., 2007). Briefly, under deep anesthesia with sodium pentobarbital, the wound was reopened and the injury site of the spinal cord was re-exposed. One (1.0) µl of 2% rose Bengal solution in saline was slowly injected into the lesion cavity through a glass pipette connected to a Digital Microdispenser (The Drummond Scientific Co., Broomall, PA, USA), which was fixed in a stereotaxi. The rose Bengal within the cavity was allowed to diffuse for 5 minutes after injection; the injury site was illuminated for 5 min with the full spectrum light of a halogen bulb (150 W, 7 cm distance). To prevent unnecessary damage to the cord tissue by the heating source from the halogen light, the exposed part of spinal cord was bathed with room temperature saline solution with fresh change each 30-40 seconds during the illuminating time.

Transplantation of LP or ALP

Immediately after photochemical scar ablation, the dura was incised longitudinally and a 1.0 mm long opening of the lesion cavity on the median line of spinal cord at the injury epicenter was made with a sharp surgical blade. Two pieces of 0.5 x 0.5 mm LP or ALP were carefully delivered into the cavity through the opening. After that the dura was closed by suturing with a 10-0 Novafil nylon suture (Ethicon, Somerville, NJ, USA) to reduce the invasion of connective tissue scar.

Then the muscles and skin were closed with proper sutures, respectively.

Histology and immunohistochemistry

Immediately after perfusion, spinal cords were removed and sufficiently fixed with 4% para-formaldehyde fixative. The cord blocks were dehydrated with gradient alcohol and cleared with xyline and embedded in paraffin wax. The 10 μm thick paraffin sections were used for routine staining (hematoxylin and eosin), MTT (Masson trichrome) staining or immnuno-staining with several primary antibodies.

For immunostaining, deparaffinized and rehydrated sections were boiled in the Antigen Retrieval Citra solution (BioGenex, San Ramon, CA) for 15 min and were incubated with 3% H_2O_2 in 50 mM Tris-buffered saline containing 0.1% Triton X-100 (TTBS) pH 7.5, for 30 min at room temperature (RT) to quench the endogenous peroxidase. To block non-specific antigens the normal goat serum (2% in TTBS) was added (60 min at RT) to the tissue. After being incubated with primary antibodies in TTBS with 1% normal goat serum in a humidified chamber overnight at RT, the sections were rinsed and incubated in 1:200 biotinylated goat anti-mouse IgG (Jackson) for 60 min and then in 1:500 streptavidin peroxidase (Jackson) for 60 min in the same buffer. To visualize the immunoreactive product the sections were incubated in 3,3'-diaminobenzidine solution (DAB, 0.5 mg in 1 ml TTBS) with 0.01% H_2O_2 for 1-3 min. After counter-staining with hematoxylin the sections were rinsed, dehydrated, cleared, and coverslipped. Negative controls were treated similarly, except the lack of the primary antibodies. The primary antibodies used for the present study are monoclonal antibody against 49 kD glial fibrillary acidic protein (GFAP, 1:2000; Boehringer, Germany) of astrocytes, and monoclonal antibody P0 (1:1000; courtesy of Dr. Archelos, Austria) against the peripheral myelin protein. In addition, to determine the origination of Schwann cells within the damaged cord tissue, and the relationship of Schwann cells with axons (either regenerating and spared) and spared tissue, antibodies p75[NTR] against Schwann cells, GFAP against astrocytes, and SMI-31 against neurofilaments were simultaneously used for observation under the fluorescent microscope.

Results

Structure of the initial repaired tissue

The initial, endogenous repaired tissue is small in size and located at the dorsal part of damaged cord segment at the early stage after contusion injury. According to various cell elements, this initial repaired tissue can be histologically divided into three irregular zones: fibrotic zone, cellular zone, and axonal zone.

Fibrotic zone

Residing under the dorsal pia matter, the fibrotic zone consists mainly of invading fibroblasts, collagen fibers, and newly formed blood vessels; it also contains some invading Schwann cells, regenerating axons (myelinated or ensheathed by Schwann cells), macrophages and sometimes bubble-like structures, which may be related to the degenerating axons. Regenerating axons, myelinated or unmyelinated, are usually assembled into small bundles, surrounded by a thin layer of fibroblasts. This style looks similar to what is seen in the peripheral nerves. The whole fibrotic zone appears as a kind of loose connective tissue, distinguishable from the close cellular zone (Figs. 1, 2, 3, 6, 7).

Cellular zone

The cellular zone is composed of densely compacted, reactive young cells, and forms a clear U-shaped shell surrounding the fibrotic zone together with the pia matter at the dorsal border of the damaged cord. These young cells appear as immature Schwann cells and were often observed connecting to the cells from the dorsal roots, indicating that these young cells may migrate from the dorsal roots. The cellular zone also contains blood vessels and some myelinated axons detected by P0 antibody, but less than that in axonal zone in count. At the earlier stage of tissue repair the cellular zone is thin and small, but it can become thick and expand in size with time or following intervention treatments (Figs. 1, 2, 3, 6, 7).

Axonal zone

Usually the axonal zone neighbors on the cellular zone ventrally and faces the lesion cavity. It has the smallest size in untreated spinal cord when compared with the other two zones. It has a characteristic feature that it consists mainly of myelinated axons positively stained by P0 antibody. The axons may form small bundles usually without fibroblasts surrounding them. The bundles can be arranged loosely or densely, depending on the amount of axons. At the later stage of the spinal cord injury, axons in this zone may increase in number. However, the increasing is limited and slow if the injured spinal cord receives no treatment (Figs. 1, 2, 3, 6, 7).

Under the fluorescent microscope, Schwann cells labeled with antibodies p75[NTR] were found distributed in the dorsal and ventral roots, endogenous repaired tissue including fibrotic zone, cellular zone and axonal zone, and spared tissue, indicating that these Schwann cells migrate from the spinal roots. In the cellular zone, Schwann cells appear denser than the other zones; in the axonal zone Schwann cells are present together with numerous regenerating axons detected with antibody

SMI-31 (Fig. 3), consistent with images observed in P0-immunostaining samples (Fig. 7).

Blood vessels at the injury site

Blood vessels are also distributed in all three zones, and some of the blood vessels including capillaries are located close to the regenerating axons. However, these blood vessels lack an integrated blood-brain barrier (BBB), demonstrated by the GFAP immunostaining showing that these blood vessels are not surrounded by the perivascular feet of astrocytes as seen in the uninjured spinal cord tissue or in the spared cord tissue of injury site (Fig. 4).

Central canal and ependyma-derived cells

At the rostral and caudal ends of injury site, the central canal was often found intact morphologically; it was surrounded by numerous reactive, young cells and was rich in blood vessels. These ependyma-derived cells usually form cellular clusters or rosettes with accessory
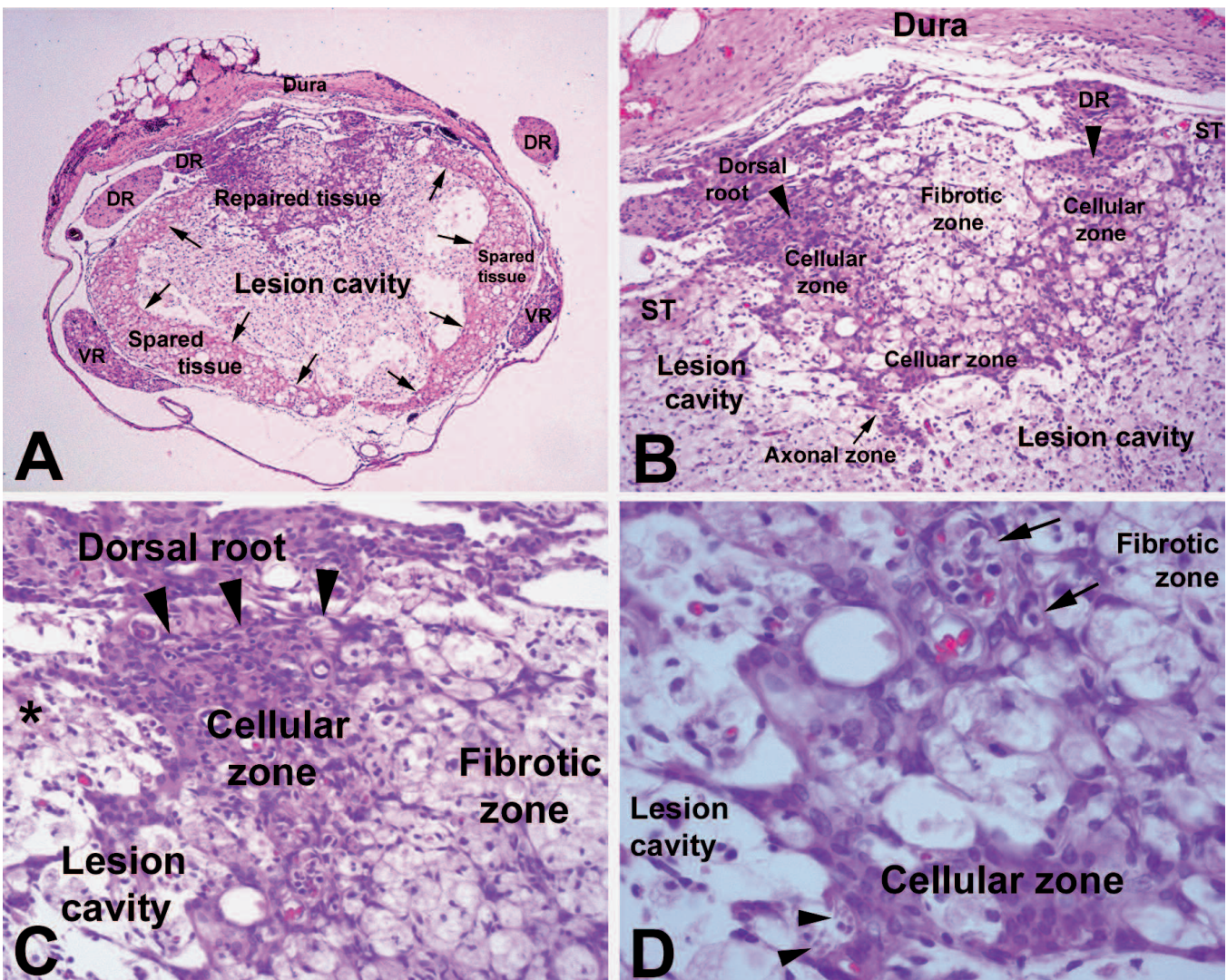
Fig. 1. Initial repaired tissue in cross section from injury epicenter (6 weeks after 25 mm contusion; H.E. staining). **A.** The repaired tissue located at the dorsal part of damaged cord, connects bilaterally to the spared tissue, and neighbors the lesion cavity, which is surrounded by the glial scar (arrows) and spared tissue. **B.** Under higher magnification, the repaired tissue can be divided into three different zones: fibrotic, cellular and axonal. The fibrotic zone is surrounded by a U-shape cellular zone. The axonal zone does not appear clearly due to its small amount of axons at this moment. **C.** The cellular zone, connecting to both spared tissue (*) and fibrotic zone, consists mainly of Schwann cells migrating from dorsal root (arrowheads). **D.** Regenerating axons (myelinated fibers) with H.E. staining are difficult to identify at the axonal zone (arrowheads), cellular zone and fribrotic zones (arrows). DR: dorsal root; VR: ventral root. Magnifications: A, x 4; B, x 10; C, x 20; D, x 40
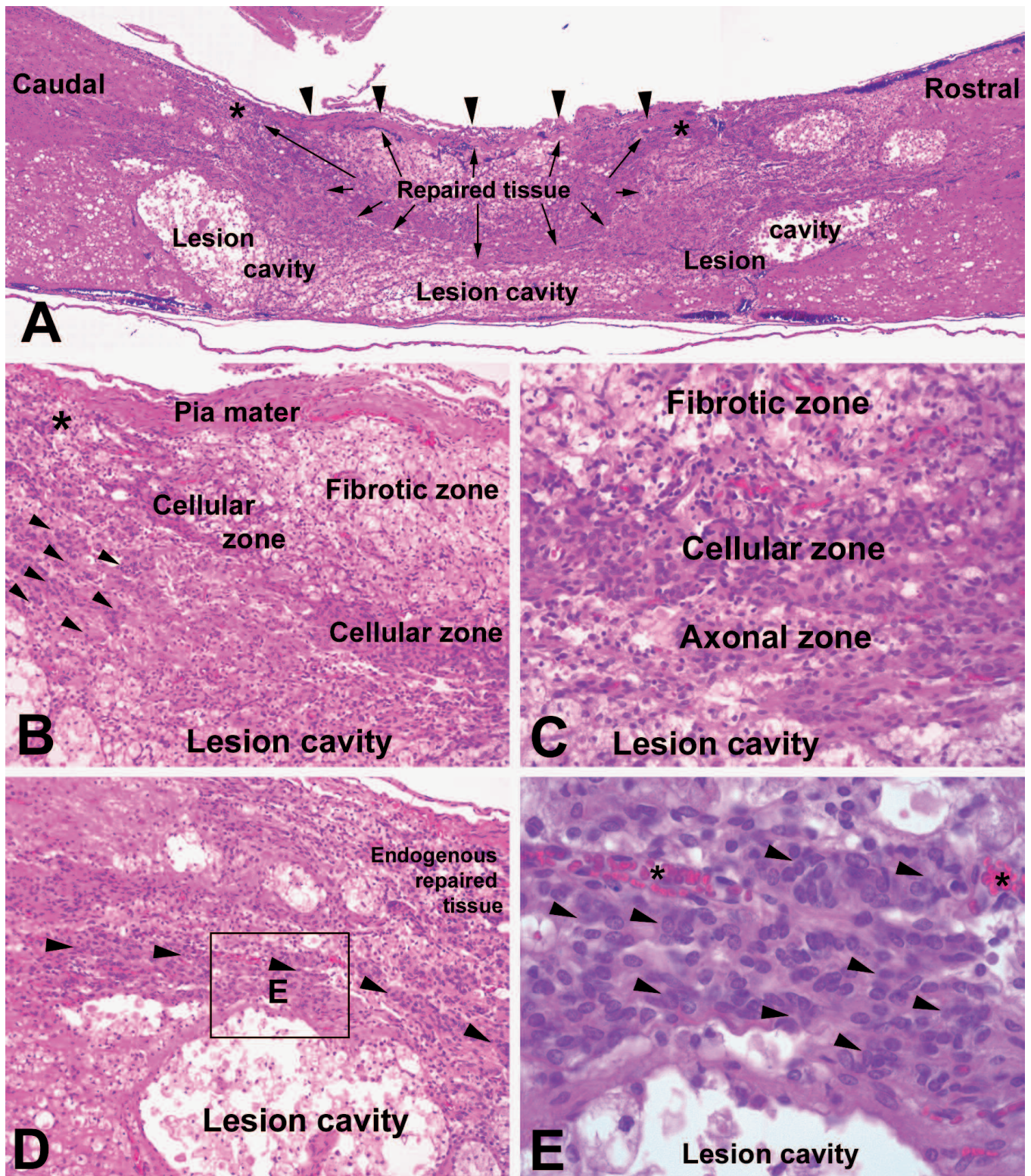
Fig. 2. Initial repaired tissue in sagittal section from injury epicenter (6 weeks after 25 mm contusion; H.E. staining). **A.** The endogenous repaired tissue, outlined by the arrows, resides under the thicker pia mater (arrowheads) and neighbors the lesion cavity. Two asterisks (*) mark the ends of the repaired tissue at the rostral and caudal sides. **B.** Caudal end (*) of the repaired tissue shows the relationship of its three zones. In addition, many cellular cords (arrowheads) can also be seen within the lesion cavity. **C.** At the middle part of the repaired tissue, like at the cross section, 3 zones can be clearly identified. **D.** Arrowheads indicate that cellular cords of ependyma-derived cells may move toward the epicenter from the caudal end of the cord. **E.** Higher magnification of boxed area in D shows that cellular cords are composed of young, lower-differentiated cells, which form cord-like or rosette structures (arrowheads). * in E: blood capillaries. Magnifications: A, x 4; B, D, x 10; C, x 20; E, x 40

lumina. Among these cells few of P0-positive myelin sheaths were observed (Fig. 5). Interestingly, in sagittal section, the ependyma-derived cells were found filling up the remote lesion cavity or forming cord-like structures to move toward the lesion epicenter (Fig. 2). In addition, some ependymal cells are shown to contain immunoreactive product of GFAP, illustrating that they may be a type of young astrocytes or have potential to differentiate into mature astrocytes (Fig. 5).

Tissue repair after LP or ALP transplantation

Eight weeks after scar ablation and LP transplantation into the lesion cavity, the repaired tissue significantly developed and expanded to fill up the lesion cavity and integrated with the spared tissue, resulting in the disappearance of the lesion cavity and histological repair of damaged spinal cord tissue at the injury epicenter. On the contrary, the morphology of the endogenous repaired tissue as well as the lesion cavity at the injury epicenter did not show significant change in control rats without scar ablation and LP transplantation (Fig. 6).

In detail, usually the fibrotic zone decreases in size or contain more regenerating axons with PNS style, demonstrated by P0-positive myelin sheaths. However,
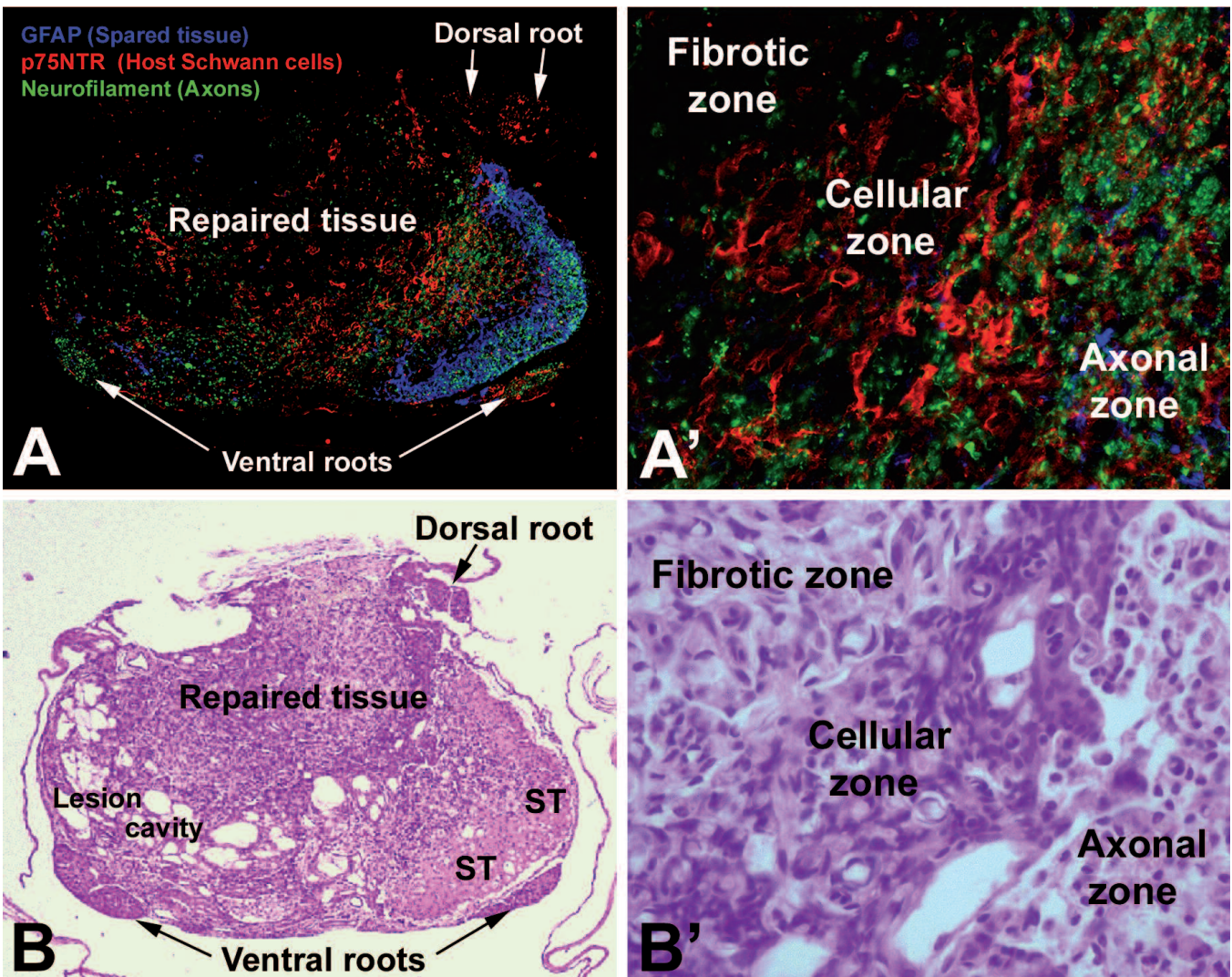
Fig. 3. Fluorescent images of Schwann cells and axons. Neighboring cross paraffin sections of injury epicenter from a control rat without LP transplantation were immunostained with antibodies against p75^{NTR} (Schwann cells), neurofilament and GFAP **(A)** and routinely stained with hematoxylin and eosin **(B)** as comparison. **A' and B'** are the local magnifications of **A and B**, respectively. Schwann cells derived from the dorsal root are distributed in three zones of the repaired tissue (with higher density in the cellular zone) and spared tissue. Axons are also present in three zones of the repaired tissue and spared tissue, but the axonal zone (regenerating axons) and spared tissue (spared or regenerating axons) have a higher density of axons. ST: spared tissue. Magnifications: A, B, x 4; A', B', x 40

both cellular zone and axonal zone can significantly expand. As seen before intervention treatments, the cellular zone is mainly composed of densely compacted, root-derived Schwann cells with some regenerating axons (Figs. 6, 7). The axonal zone consists of huge amount of regenerating axons with non-PNS feature. Cells from both cellular and axonal zone were found to integrate with spared tissue (Fig. 6).

In rats with contusion injury only or with scar ablation plus LP transplantation, P0-positive myelin sheaths can be found far behind the lesion epicenter (more than 10 mm) both rostrally and caudally. These P0-positive myelin sheaths usually are distributed along with the small cyst, and most of them are located in the dorsal funiculus (Fig. 7).

Eight weeks after transplantation, ALP at the implantation site is found still separated and surrounded by the repaired tissue. The ALP itself morphologically appears as a dense connective tissue with intercellular matrix, scattered fibroblasts, some macrophages and few blood vessels. No Schwann cells, OECs, myelin sheaths and axons can be found within the ALP. In addition, the lesion cavity did not decrease in size, and a developed cellular zone and axonal zone can not be observed at the
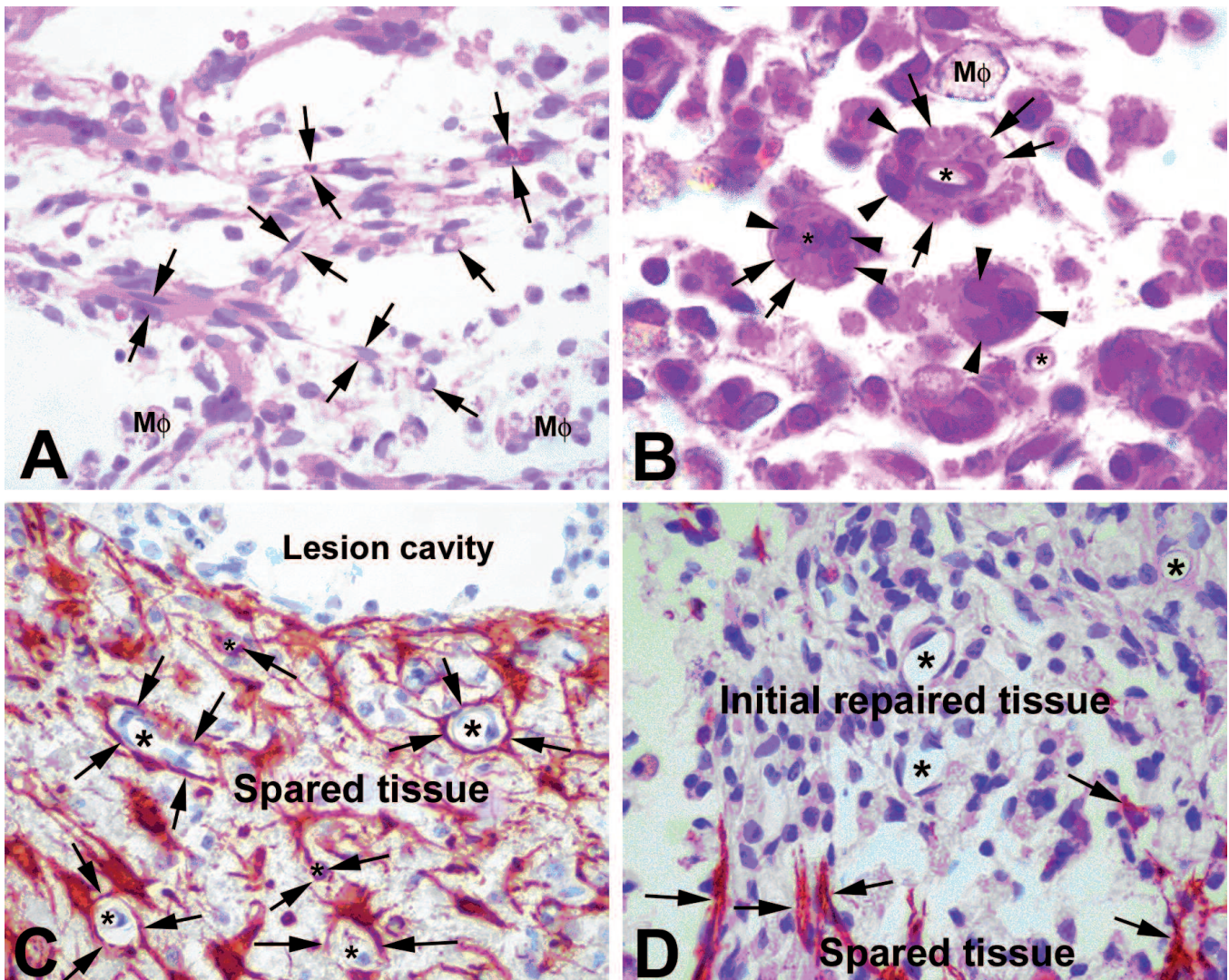
Fig. 4. Blood vessels at the injury site. **A and B.** H.E. staining. **C and D.** GFAP immunostaining. After spinal cord injury (2 weeks) the initial repaired tissue contains newly formed network of blood capillaries (arrows in **A**). The blood capillaries (*) at the injury site are surrounded by regenerating axons (arrows in **B**), which are myelinated or ensheathed by Schwann cells (arrowheads). Even after injury the blood vessels (* in **C**) in the spared tissue are still surrounded by a layer of astrocytic feet (arrows), keeping the BBB (blood-brain barrier) structure intact. Blood vessels (* in **D**) in the repaired tissue, however, do not have perivascular feet of astrocytes (arrows) surrounding them, indicative of lack of intact BBB structure. Mφ: Macrophage. Magnifications: A, C, D, x 40; B, x 100

injury site (Fig. 8), suggesting that active tissue repair did not take place at the injury site after ALP transplantation.

Discussion

In the present study we report, for the first time, the initial, endogenous repaired tissue can be histologically divided into three zones, i.e., fibrotic, cellular and axonal at the injury site in chronic contusion model of rat. The development and expansion of both cellular zone and axonal zone directly contribute to the histological repair of damaged spinal cord tissue at the injury site. This point of view is derived not only from the present study but also from our long term observation of the injury site, and may be helpful to understand mechanisms of tissue repair which is related to the therapeutic strategy to cure spinal cord injury.

After contusion injury (25 mm height setting), the dorsal white matter and whole gray matter were destroyed at the injury epicenter. In response to the trauma injury, fibroblasts at the dorsal pia mater were
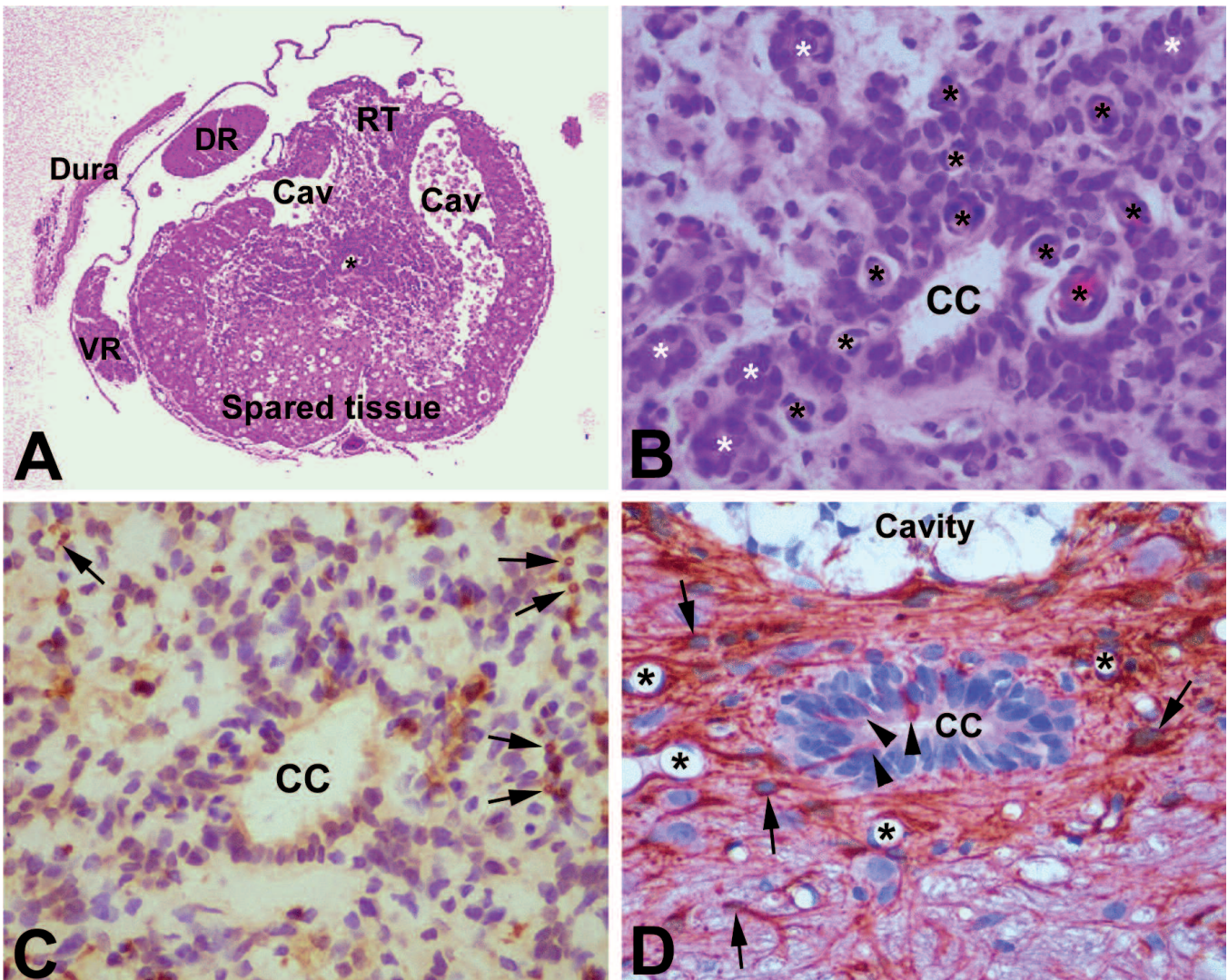

Fig. 5. Central canal and ependyma-derived cells (6 weeks after 25 mm contusion injury): H.E. staining (**A**, **B**), P0 immunostaining (**C**) and GFAP immunostaining (**D**). **A.** Injured spinal cord at low magnification shows the central canal (*) and its surrounding tissue rostral to the injury site. Spared tissue, lesion cavity (Cav), and repaired tissue (RT) can be seen. **B.** Higher magnification of a local area in A shows the central canal (CC), surrounding ependyma-derived cells and blood vessels (black asterisks). White asterisks indicate the rosette-like structures formed by ependyma-derived cells. **C.** Around the central canal (CC), a few P0-positive myelin sheaths (arrows) can be recognized. **D.** Among ependymal cells lining the central canal (CC), a few cells are GFAP positive (arrowheads). Surrounding astrocytes are pointed by arrows and blood vessels are labeled with asterisks (*). DR: dorsal root; VR: ventral root. Magnifications: A, x 4; C, x 40; B, D, x 60
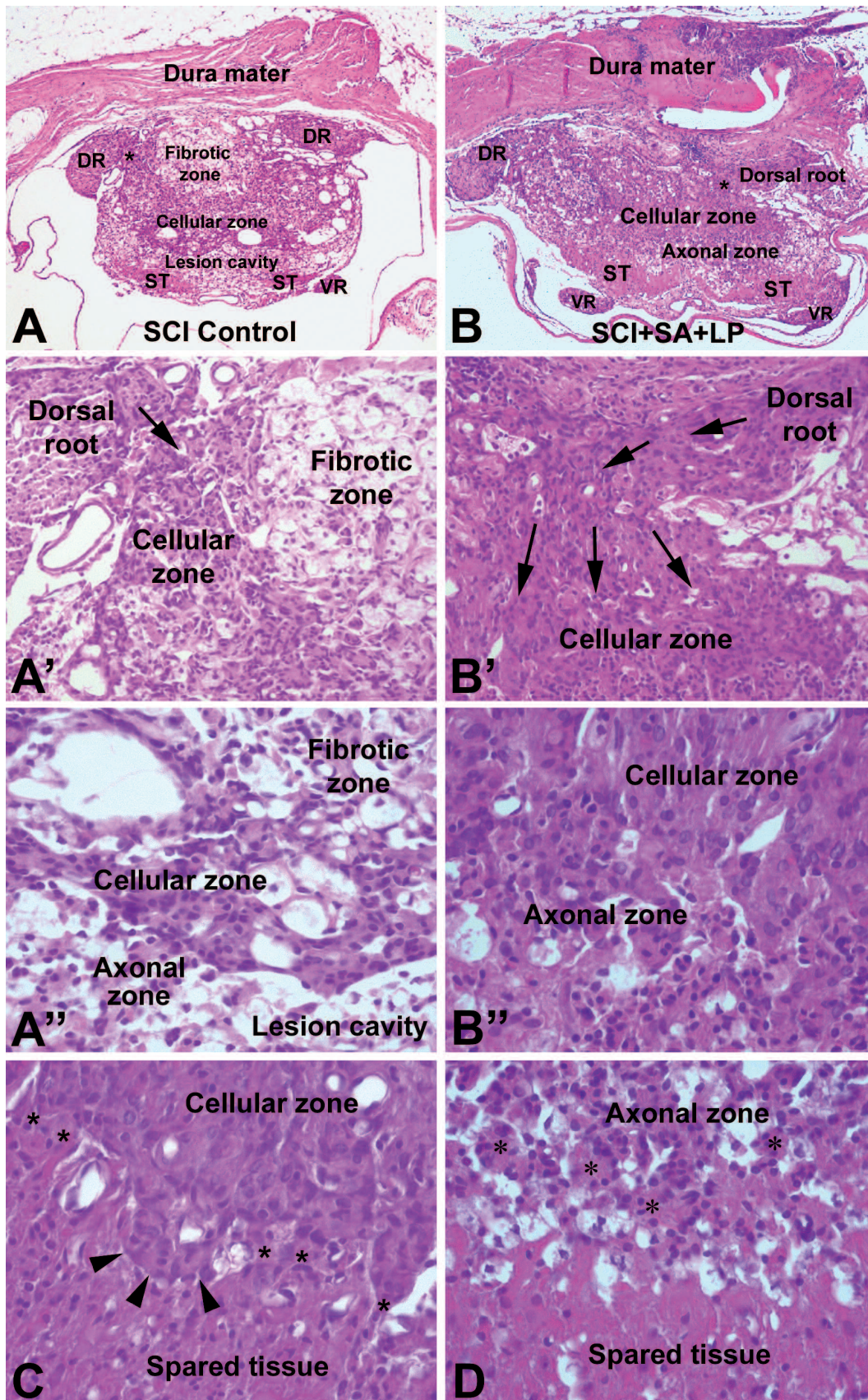
Fig. 6. Comparison of tissue repair (H.E. staining). In SCI control rats, the endogenous repaired tissue appears quiet, the fibrotic zone looks similar to a loose connective tissue, and the lesion cavity is still present 14 weeks after injury **(A, A', A'')**. Schwann cells in cellular zone near the dorsal root (arrow in **A'**) do not show to be densely compacted, indicating that migration of Schwann cells from the dorsal root to the injury site may have been reduced or stopped (A'). On the contrary, with scar ablation and LP transplantation, the repaired tissue characterized by obvious development of cellular zone and axonal zone occupies the damaged area, resulting in the disappearance of lesion cavity 8 weeks after treatments **(B)**. At the same time, Schwann cells keep actively migrating from the dorsal root into the cellular zone **(B')**; arrows in **B'** point out the possible direction of Schwann cell migration. In addition, the cellular zone consist of densely compacted young Schwann cells; the axonal zone contains numerous mature Schwann cells with axons **(B'')**. Asterisks in **A and B** indicate the border between dorsal root and cord tissue (cellular zone). **C** shows the integration of cells from the cellular zone and spared tissue (asterisks); a newly formed junction of these two tissue types is indicated by arrowheads. Integration of repaired tissue and spared tissue can happen between cells from axonal zone (asterisks in **D**) and spared tissue. ST: spared tissue; DR: dorsal root; VR: ventral root. SCI: spinal cord injury; SA: scar ablation; LP: transplantation of lamina propria. Magnifications: A, B, x 4; A', B', x 20; A'', B'', C, D, x 40
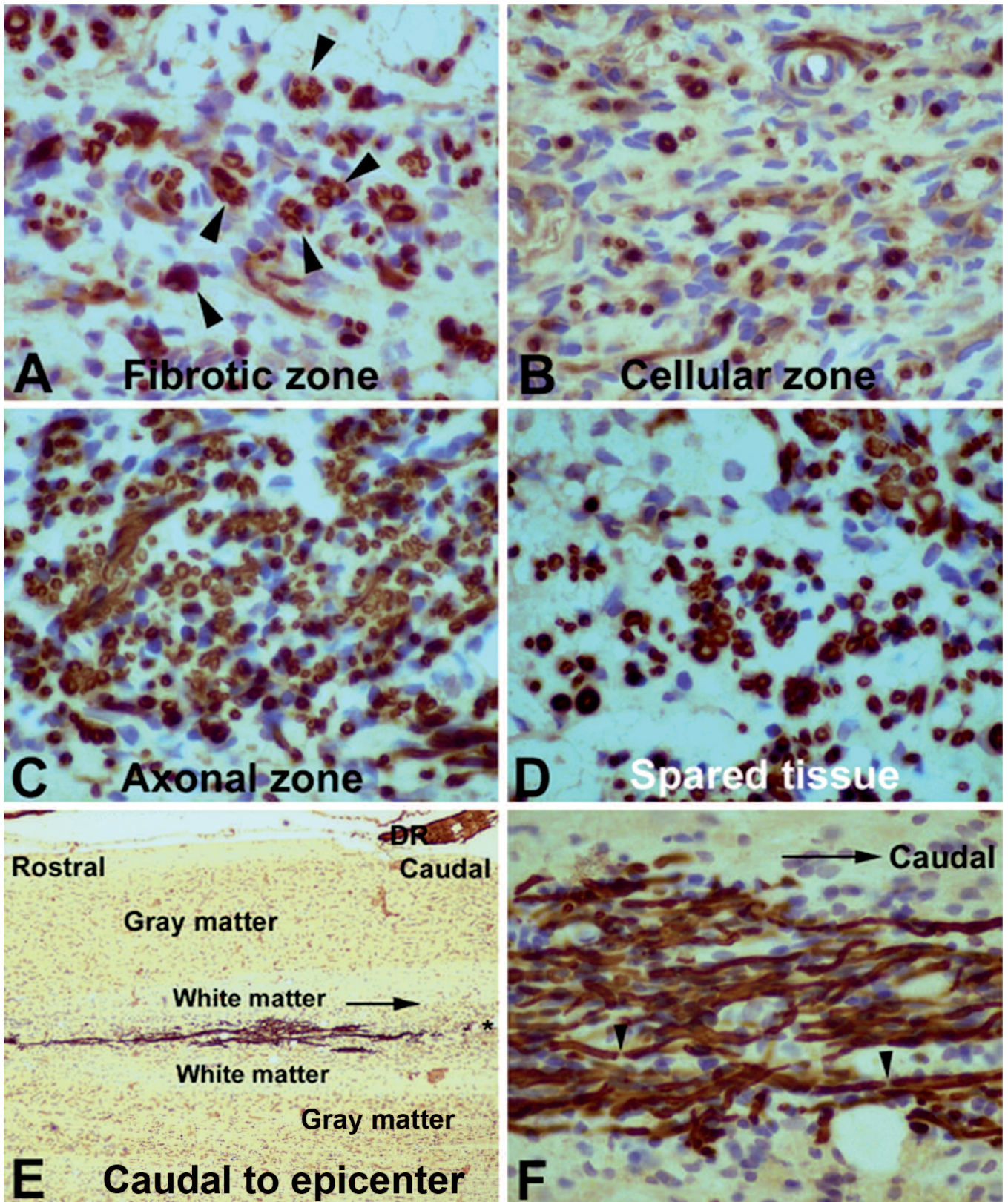
Fig. 7. P0-positive myelin sheaths in different areas (P0 immunostaining; **A-D**, cross section; **E, F**: horizontal section). In the fibrotic zone, P0-positive myelin sheaths (myelinated fibers, dark brown in color) form small fascicles encircled by a layer of fibroblasts (arrowheads in **A**), showing the PNS style. P0-positive myelin sheaths in the cellular zone do not form fascicles but scatter among densely compacted Schwann cells **(B)**. In the axonal zone, numerous P0-positive myelin sheaths are aggregated and are dense in appearance **(C)**. In the spared tissue, the P0-positive myelin sheaths (regenerating or demyelinated axons) are distributed unevenly **(D)**. E shows P0-positive myelin sheaths found 15 mm caudal to the injury site. They are distributed along with a small longitudinal cyst (*) in the dorsal funiculus. The arrows indicate the direction of Schwann cell movement. F is the local magnification of E, clearly showing the P0-positive myelin sheaths. The node of Ranvier is pointed by arrowheads. Nuclei of both Schwann cells and other cells of spinal cord were stained in purple with hematoxylin. DR: dorsal root. Magnifications: A-D, F, x 40; E, x 4

activated and invaded the injury site to fill the lesion cavity, thus constructing a special connective tissue, fibrotic zone of the initial, endogenous repaired tissue. At the same time numerous young Schwann cells also invaded the injury epicenter from dorsal roots bilaterally (Blakemore, 1975, 2005; Zhang et al., 2005, 2007). These invading Schwann cells migrate forward and converge underneath the fibrotic zone, forming a U-shape cell shell to surround the fibrotic zone together with the dorsal pia mater.

As well known, fibroblasts are a cell type with most active property in the body. Any slight trauma or inflammation can induce active proliferation and migration of fibroblasts into the injury site (Ross, 1968; Shekhter and Berchenko, 1978). Following spinal cord injury the invasion of fibroblasts into the damaged area may play a role in wound healing temporarily; however fibroblasts are not members of nervous system family. Schwann cells are members of nervous system family, although they belong to PNS. One of the effects of invading Schwann cells may be related to the prevention of invading fibroblasts from unlimited spreading within
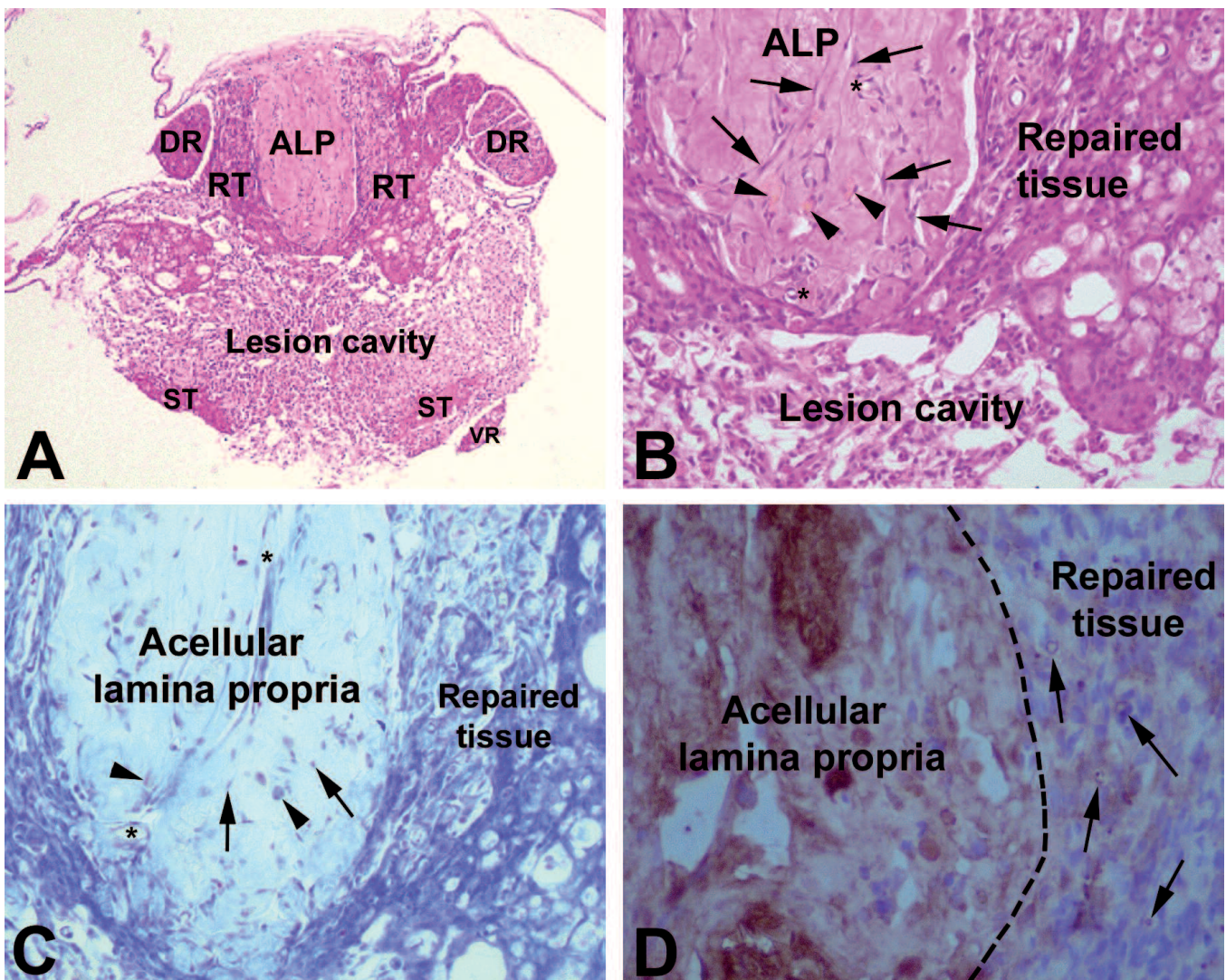
Fig. 8. Transplanted ALP at injury site. **A and B.** H.E. staining. **C.** MTT (Masson trichrome) staining. **D.** P0 immunostaining. Eight weeks after transplantation, the endogenous repaired tissue did not show marked development and expansion, and the lesion cavity is still present at the injury site **(A)** when compared with that from treatment rats shown in Fig. 6. The transplanted ALP is seen separated and surrounded by the repaired tissue (RT in **A**), and few fibroblasts (arrows), macrophages (arrowheads) and blood vessels (*) scattered among the intercellular matrix without abundant collagen fibers **(B, C)**. In addition, no P0-positive myelin sheath can be detected in ALP while some of them were found in the repaired tissue (arrows in **D**). Note that the border between ALP and repaired tissue is marked by a broken line **(D)**. RT: repaired tissue; ST: spared tissue; DR: dorsal root; VR ventral root. Magnifications: A, x 4; B, C, x 20; D, x 40

the spinal cord. This is probably the reason why the invading Schwann cells form a U-shape structure seen in the cross section as well as in the longitudinal section (more widely open U-shape). We have never found an extensive invasion of fibroblasts in our injury model. This may be the result from limitation by the invading Schwann cell belt. But the mechanisms remain to be known.

About two weeks after contusion, injured axons started to spontaneously grow into the initial repaired tissue (Schwab and Bartholdi, 1996; Beattie et al., 1997). At this moment Schwann cells migrated from the cellular zone into the fibrotic zone to myelinate the regenerating axons there. These myelinated fibers form small bundles, which are surrounded by a layer of fibroblasts, leading to the appearance with a characteristic feature of PNS. Schwann cells also myelinate regenerating axons growing into the cellular zone. More importantly Schwann cells migrate out of the cellular zone to myelinate the regenerating axons underneath the cellular zone. These myelinated fibers outside the cellular zone form a different area rich in regenerating axons, the axonal zone. But these regenerating axons bundles do not have PNS characteristic features.

Prominent tissue repair at the lesion epicenter was found in rats receiving scar ablation and LP transplantation. The tissue repair was characterized by extensive development and expansion from the initial repaired tissue; the expanded repaired tissue occupied the most space of lesion cavity, resulting in the significant reduction or disappearance of the lesion cavity and integration of repaired tissue with spared tissue. Therefore no more typical glial scar can be recognized at this area, suggesting the tissue repair is substantial and complete. During the tissue repairing, either cellular zone or axonal zone can extensively expand solely. The integration of repaired tissue with spared tissue might occur either between cells of cellular zone and spared tissue, or between cells of axonal zone and spared tissue. All these facts further verify that our combination treatments can significantly promote the histological repair of damaged cord tissue at the lesion epicenter. However, blood capillaries at the injury site lack intact BBB structure, the potential meaning remains unknown.

Recent studies have revealed that Schwann cells but not OECs myelinate the regenerating axons (OECs may ensheath axons, which become unmyelinated fibers), and OECs stimulate the host Schwann cells to migrate to the injury site and myelinate the demyelinated or regenerating axons, promoting the axonal regeneration and repair of damaged cord tissue (Takami et al., 2002; Ramer et al., 2004; Boyd et al., 2005; Andrews and Stelzner, 2007; Belegu et al., 2007; Buss et al., 2007; Cao et al., 2007). Evidence from the present study exhibited that transplanted LP did not always directly contact the spared tissue. In many cases, the transplanted LP was surrounded by fibrotic and/or cellular zone, but

the other part of enhanced cellular zone or axonal zone made a perfect integration with spared tissue, resulting in complete tissue repair. These findings support the ideas mentioned above that transplanted OECs may not be necessarily directly involved in the tissue repair, but stimulate through neurotrophins the host Schwann cells to make the cellular zone spreading and stimulate these young cells to become mature cells and myelinate the regenerating axons. In addition, the results that ALP transplantation did not cause any prominent modification of initial repaired tissue, may negatively confirm the indirect role of OECs in tissue repair. P0-positive myelin sheaths were found at remote ends in rats with and without LP transplantation, revealing that Schwann cells can migrate for a long distance to myelinate regenerating or demyelinated axons behind the injury site (Blight and Young, 1989).

It has been reported that ependymal cell population largely contains adult spinal cord stem cells. After spinal cord injury they may proliferate and differentiate into astrocytes, oligodendrocytes or other type of cells, involved in the tissue repair (Dervan and Roberts, 2003; Attar et al., 2005; Kulbatski et al., 2007; Meletis et al., 2008; Parr et al., 2008). The astrocytes differentiated from the ependymal cells may contribute to the formation of glial scar, but do not express inhibitory CSPG (Meletis et al., 2008). In this study we found some ependymal cells are GFAP-positive, suggesting that they may contribute to the formation of the glial scar. More importantly, we found numerous ependyma-derived cells forming rosette or cord-like structures to fill up the remote lesion cavity or move toward the lesion epicenter. This is evidence that ependyma-derived cells are directly involved in the tissue repair, but they may not contribute to the formation of P0-positive myelin sheaths. Unlike in some lower species, such as *eel Anguilla*, a new central canal can form after spinal cord transection (Dervan and Roberts, 2003), we have never found a reconstructed central canal at the repaired tissue in our rat model. In response to the spinal cord injury, ependymal cells undergo proliferation, migration and differentiation (Ke et al., 2006); this may be associated with certain factors or signals produced by injury. Should we find a similar way to enforce these *in vivo* activities of ependymal-derived cells, it would be very helpful to speed the tissue repair and axonal regeneration.

In summary, the initial repaired tissue at the injury site can be histologically divided into three zones, i.e. fibrotic, cellular and axonal zones. The fibrotic zone is formed by invading connective tissue from the pia mater. The cellular zone consists of densely compacted young Schwann cells migrating from the dorsal roots. Schwann cells form a U-shape cell shell surrounding the fibrotic zone and prevent it from unlimited spreading. In addition to myelinating regenerating axons within the cellular zone, Schwann cells also move out to myelinate the regenerating axons in the fibrotic zone and axonal zone, as well as myelinate the regenerating or

demyelinated axons in the spared tissue. Extensive development and expansion of cellular zone or axonal zone directly result in histological repair of damaged cord tissue at the injury site. Transplanted OECs may stimulate the expansion of cellular and axonal zones through the proliferation of host Schwann cells, indirectly contributing to the tissue repair and axonal regeneration. The ependyma-derived cells are directly involved in tissue repair, but may not contribute to the formation of P0-positive myelin sheaths.

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