Endogenous radial glial cells support regenerating axons after spinal cord transection

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During the development of central nervous system, radial glial cells support target-specific neuronal migration. We recently reported that after implantation of chitosan channels with complete spinal cord transection, the tissue bridging the spinal cord stumps contained axons and radial glial cells. The purpose of this study was to clarify the role of the radial glial cells in the tissue bridges. Chitosan channels were implanted in rats with thoracic spinal cord transection. After 14 weeks, all animals had tissue bridges in the channels that contained many radial glial cells in longitudinal arrangement, some of which were in contact with axons in the bridges. We suggest that radial glial cells can guide regenerating axons across the bridge in the channel after spinal cord transection.

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Introduction

During the development of central nervous system (CNS), neuroepithelial-derived radial glial cells develop two unique characteristics: as a scaffold to support target-directed neuronal migration [1–3], and as neural precursor cells [4–6]. Radial glial cells are a feature of the developing CNS, and only a few persist in the adult [7]. They are capable of differentiating into astrocytes, oligodendrocytes, neurons, and macrophages in the appropriate niche within the adult mammalian CNS [5,8]. A recent study showed that after spinal cord injury in the adult rat, radial glial cells were derived from subpial astrocytes, and then extended into the damaged white matter, likely participating in restoration of neural tissue and regeneration [9]. However, the specific function of radial glial cells in adults is still unknown. In contrast, in birds and amphibians, radial glial cells are always present, contributing to neurogenesis, providing neurotrophic support such as insulin-like growth factor-1, and playing a role in regeneration [7,10,11].

To repair the experimental spinal cord injury, our group has applied synthetic guidance channels to promote axonal regeneration across the lesion site [12–15]. Many biomaterials have been used to create synthetic guidance channels. We have focused on channels composed of chitosan, a biodegradable polysaccharide [16], because of its excellent biocompatibility [13,15,17]. Recently, we reported that implantation of extramedullary chitosan channels seeded with neural stem/progenitor cells after complete spinal cord transection created a large tissue bridge containing many transplanted cells and host axons. Most of the transplanted cells in the bridge differentiated into mature phenotypes such as oligodendrocytes or astrocytes [13,15]. We found that implantation of chitosan channels without the precursor cells after spinal cord transection also created a tissue bridge composed mainly of collagen and containing host axons [13]. Interestingly, we found that there were many radial glial cells in the tissue bridge after implantation of the channels seeded with precursor cells, and most of these radial glial cells did not arise from the transplanted cells [13]. Thus, we speculate that endogenous radial glial cells migrated into the bridges from the host spinal cord stumps.

The purpose of this study was to clarify the role of radial glial cells in the tissue bridge created by implantation of an extramedullary chitosan channel after complete spinal cord transection. This analysis of the role of the radial glial cells in the tissue bridges was carried out after implantation of chitosan channels without transplanted cells and was compared with rats with spinal cord transection but without channels.

Materials and methods

Production of channels

The chitosan channels were produced as reported earlier [13,16]. The channels were 10 mm in length, approximately 4.1 mm in outer diameter with wall thickness of 0.2 mm. They were sterilized with γ radiation and then coated with 5 μg/ml of laminin solution (Invitrogen, Burlington, Ontario, Canada).

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Animals

Twenty-five adult female Sprague–Dawley rats (Charles River, St Constant, Quebec, Canada) weighing 200–320 g were used in this study. The animal protocols were approved by the Animal Care Committee of the Research Institute of the University Health Network in accordance with policies established by the Canadian Council on Animal Care. The rats with spinal cord transection (described below) were divided into two groups: the channel group received implantation of a laminin-coated channel followed by spinal fixation as described below ($N = 12$), and the control group had spinal cord transection followed by spinal fusion but without implantation of a channel ($N = 11$). All animals were maintained for 14 weeks after spinal cord transection. Two uninjured animals were transcardially perfused with 4% paraformaldehyde to obtain sections of the uninjured spinal cord.

Spinal cord transection and channel implantation

Channels were implanted after spinal cord transection at the T8 level and spinal fixation was performed as shown Fig. 1a and described earlier [12,13]. Briefly, a laminectomy was performed at the levels of T7, 8, and 9, and the duramater was longitudinally incised in the midline. The spinal cord at T8 and the adjacent nerve roots were completely divided, and then each stump was inserted into one end of the channel. After insertion, the distance between the stumps was approximately 3 mm. The control group had spinal cord transection only (Fig. 1b). A spinal fusion was then performed in all animals from T6-T10 with surgical wire, and the operative field was covered by a synthetic expanded polytetrafluoroethylene membrane (Preclude Goretex Dura Substitute, Gore, Gift from Gore and Associates, Inc., Flagstaff, Arizona, USA) to prevent extradural scarring and invasion of fibrous tissue.

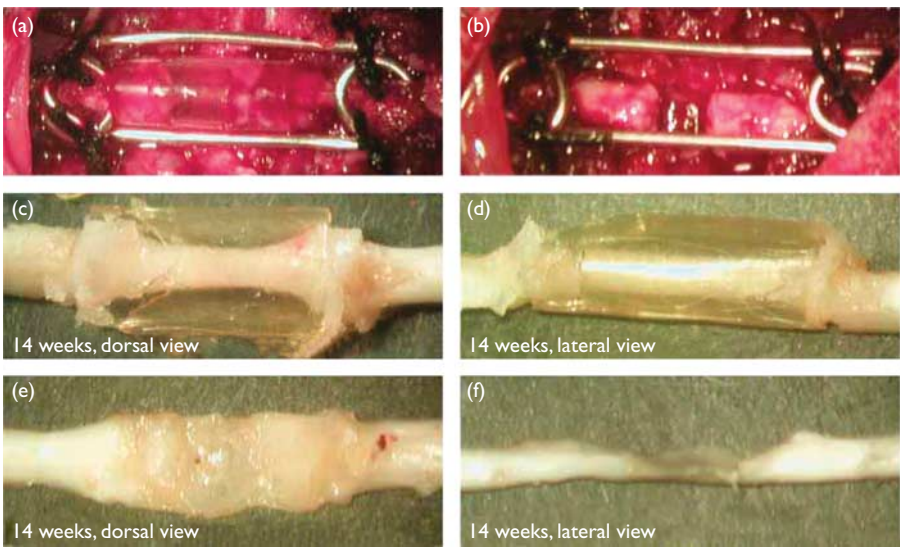
Tissue preparation

Fourteen weeks after channel implantation, the rats were transcardially perfused with 4% paraformaldehyde. A 2 cm length of spinal cord encompassing the implanted channel or transection site was carefully removed. Four animals in each group were randomly selected for immunohistochemistry of frozen sections. The sections were cryoprotected with 30% sucrose in 0.1 M phosphate buffer at 4°C and then frozen and embedded in frozen section medium compound (Stephens Scientific, Riverdale, New Jersey, USA). Parasagittal sections with a thickness of 20 µm were cut on a cryostat and mounted on cold (−20°C) slides. The mid-thoracic spinal cord was removed from the two uninjured spinal cords, and parasagittal sections were made from one and transverse sections were made from the other in the same way as described above.

Immunohistochemistry

The following antibodies were used: mouse anti-3CB2 monoclonal immunoglobulin M (IgM) antibody (3CB2; Hybridoma Bank, University of Iowa, Iowa, USA) to

Fig. 1



Surgical procedure for extramedullary chitosan channel implantation after spinal cord transection (a) or control with spinal cord transection alone (b). The channel is 10 mm in length and the distance between the stumps is approximately 3 mm. At 14 weeks, gross appearance of the implanted channel (c and d) or control group after spinal cord transection alone (e and f), from the dorsal and lateral aspects. In each panel, rostral is on the left. (a) Dorsal view of the transected spinal cord stumps placed within the transparent chitosan channel and the spinal fusion with wire. (b) Dorsal view of the transected spinal cord stumps. The wire for spinal fusion can be observed. (c and d) There is a tissue bridge inside the channels in the channel group. (e and f) In the spinal cord transection alone control, there is pale connective tissue between the stumps.

visualize radial glial cells [18]; mouse anti-RC1 monoclonal IgM antibody (RC1; Hybridoma Bank) to visualize radial glial cells [18]; mouse antirat nestin monoclonal antibody (nestin; BD Bioscience Pharmingen, Mississauga, Ontario, Canada) to visualize progenitor cells; mouse antineurofilament 200 monoclonal antibody (NF200; Sigma, St. Louis, Mississippi, USA) to visualize neurons and axons; and polyclonal rabbit antiglial fibrillary acidic protein antibody (GFAP; 1 : 200 dilution, DakoCytomation, Mississauga, Ontario, Canada) to visualize reactive astrocytes. In double labeling immunohistochemistry of nestin/3CB2 and nestin/RC1, the slides were rehydrated and blocked in 2% normal goat serum (NGS) with 0.3% triton X at room temperature (RT), and then incubated with nestin (1 : 100) overnight at 4°C. The sections were then washed with 0.1 M phosphate buffer saline and incubated with goat antimouse Alexa 488 (1 : 500, Vector Laboratories, Burlington, Ontario, Canada) for 1 h at RT. Later, the sections were washed and blocked in 5% NGS with 0.3% triton X at RT, and then incubated with either 3CB2 (1 : 50) or RC1 (1 : 20) overnight at 4°C, followed by washing and incubation with goat antimouse IgM Alexa 568 (1 : 500, Vector Laboratories) for 1 h at RT. For double labeling of NF200/3CB2 and GFAP/3CB2, the slides were rehydrated, except for the NF200 slides, which were treated with cold 100% methanol for 20 min on ice and washed three times with phosphate buffer saline. The sections were then blocked in 10% heat-inactived goat serum with 0.3% triton X at RT and incubated overnight at 4°C with NF200 (1 : 500) and GFAP (1 : 200). The next day the sections were washed; NF200 sections were incubated with goat antimouse Alexa 488 (1 : 500, Vector Laboratories) and GFAP sections with goat antirabbit 488 (1 : 500, Vector Laboratories) for 1 h at RT. After all sections were washed and blocked in 5% NGS with 0.3 triton X, they were incubated with 3CB2 (1 : 50) or RC1 (1 : 20) overnight at 4°C, followed by washing and incubation of goat antimouse IgM Alexa 568 (1 : 500) for 1 h at RT. The appropriate single positive and negative controls were run simultaneously with the double immunohistochemistry slides. The sections were coverslipped with the nuclear dye 4′,6-diamidino-2-phenylindole mounting media (Vector Laboratories) to counterstain the nuclei.

All sections were examined and photographed using a Zeiss-LSM 510 (Zeiss, Oberkochen, Germany) confocal microscope. Z-stack images were taken based on 20–40 optical sections, at 0.5 μm height per section. The entire thickness of the section was then visualized using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The color tone was modestly adjusted using Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, California, USA). Three-dimensional (3D) imaging was constructed using Imaris software (Bitplane, Exton, Pennsylvania, USA).

Results

Tissue bridges

At 14 weeks after channel implantation, all animals in the channel implantation group had tissue bridges within the implanted channels (Fig. 1c and d). In contrast, in the control group without channels, the connective tissue bridge was thin, pale, and disorganized (Fig. 1e and f). These findings were similar to those reported earlier [13].

Radial glial cells were immunolabeled in the uninjured spinal cords

In the uninjured spinal cord, radial glial cells were detected by labeling with 3CB2 or RC1 mainly in the white matter near the pial surface of the spinal cord, and most were colocalized with nestin-positive progenitor cells (Fig. 2a and b). Some were colocalized with GFAP-positive astrocytes in the white matter as reported earlier [9,19] (Fig. 2c). Morphologically, individual radial glial cells in the uninjured spinal cord had several short ramified processes (Fig. 2b and c).

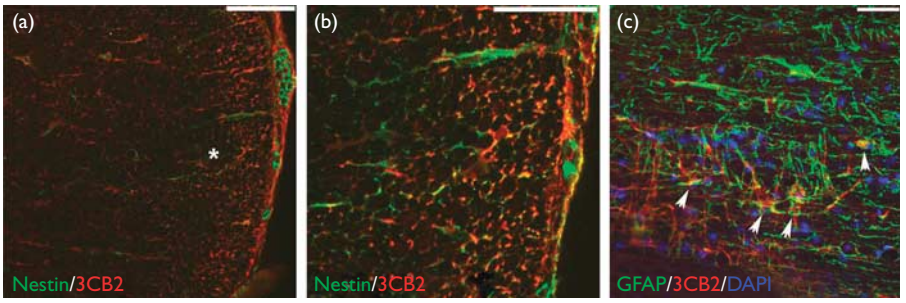
The tissue bridge in the channel group contained radial glial cells and progenitor cells

At 14 weeks after channel implantation, there were some nestin-positive progenitor cells and many 3CB2-positive or RC1-positive radial glial cells not only in the white matter but also in the gray matter within 3 mm rostral or caudal to the ends of the rostral or caudal stumps, respectively. There were more radial glial cells in the channel group than in the uninjured spinal cords. In the bridges between the stumps, there were a large number of 3CB2-positive or RC1-positive radial glial cells arranged longitudinally, and less than 10% were colocalized with nestin (Fig. 3a and b). There were also some NF200-positive axons arranged longitudinally in the bridge (Fig. 3c). Double immunochemistry showed that 3CB2-positive radial glial cells were longitudinally arranged adjacent to NF200-positive axons (Fig. 3c and d), and 3D images showed that these cells were in contact with axons (Fig. 3e). There were a few GFAP-positive astrocytic components in the tissue bridges, but not colocalized with radial glial cells (Fig. 3f). Morphologically, individual radial glial cells in the tissue bridges in the channel group had thick and long processes that extended rostrally and caudally (Fig. 3a–f). In contrast, the morphology of individual radial glial cells in the stumps was similar to that in the uninjured spinal cords (data not shown).

Radial glial cells and progenitor cells in the stumps in the control group

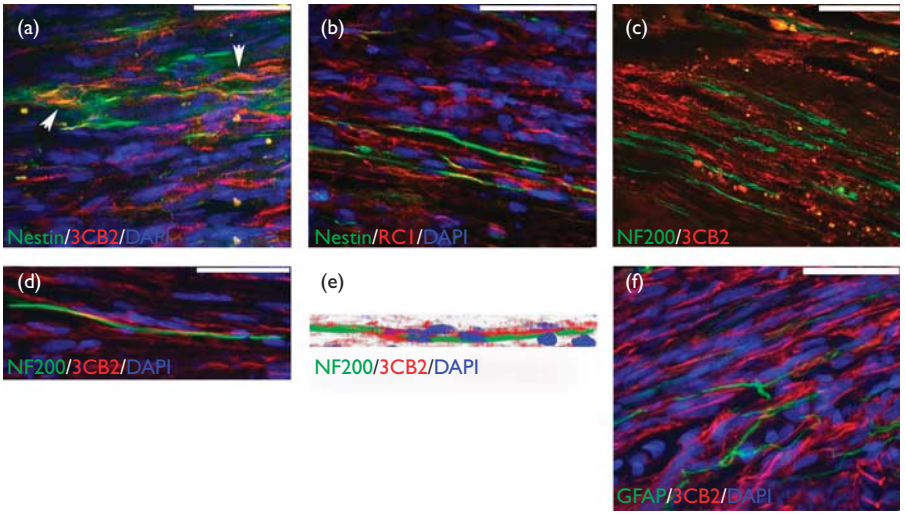
In the control group without channels 14 weeks after spinal cord transection, there was a narrow connective tissue bridge between the stumps of the spinal cord (Fig. 1e and f). There were many nestin-positive precursor cells and 3CB2-positive or RC1-positive radial glial cells in the stumps within approximately 2 mm of the

Fig. 2



Confocal photomicrographs of 20-μm transverse (a and b) and parasagittal frozen sections (c) in the uninjured spinal cords. Confocal laser scanning Z-stack images taken with a confocal microscope were composed of 20–40 confocal laser scanning micrographs per section. In panel (c) rostral is on the left. Nestin (a and b) and glial fibrillary acidic protein (GFAP) (c) signals are in green; 3CB2 (a–c) is in red; and 4′,6-diamidino-2-phenylindole (DAPI) signal is in blue (c). Colocalized signals with green and red or blue are in yellow or azure, respectively (a–c). (a) There are some nestin-positive or 3CB2-positive cells in the white matter near the pial surfaces in the transverse sections. (b) The dotted area with an asterisk in (a) is shown at a higher power in (b). 3CB2-positive radial glial cells generally overlap with nestin-positive cells. (c) Some radial glial cells overlap with GFAP-positive astrocytes in the white matter (arrows shown in c). Scale bars in a = 100 μm and b, c = 50 μm. (a) Under low magnification and (b and c) under high magnification.

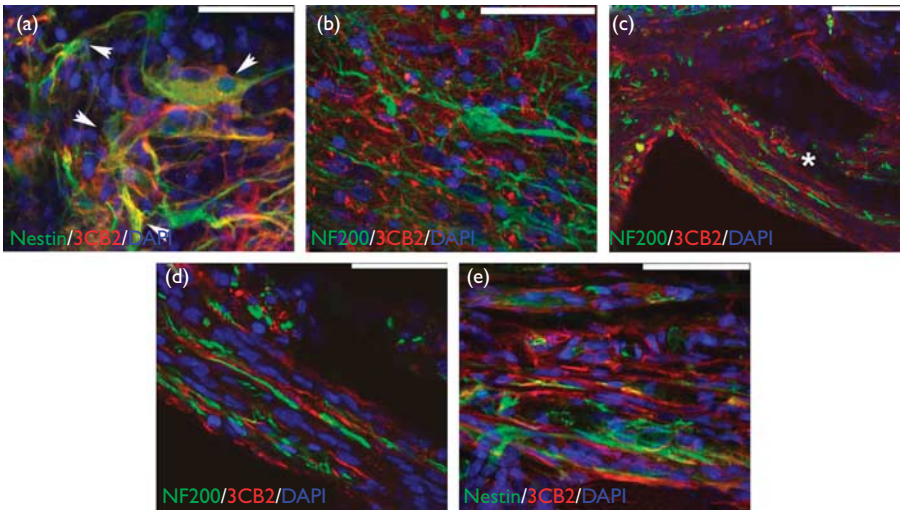
Fig. 3



Confocal photomicrographs of 20-μm parasagittal frozen sections (a–f) from the tissue bridge created between rostral and caudal stumps in the channel group. All are Z-stack images. In each panel, rostral is on the left. (e) is a merged 3D Z-stack image of the confocal laser scanning micrographs at 0.5 μm in height per parasagittal section of the tissue bridge shown in (d) using Imaris software. Nestin (a and b), NF200 (c–e) and GFAP (f) signals are in green; 3CB2 (a, and c–f) and RC1 (b) are in red; and 4′,6-diamidino-2-phenylindole (DAPI) signal is in blue (a, b, and d–f). Colocalized signals with green and red or blue are in yellow or azure, respectively (a–f). (a) In rats with channels, some 3CB2-positive radial glial cells overlap with nestin-positive cells in the tissue bridge (arrows shown in a). (b) RC1-positive radial glial cells are linearly related to nestin-positive cells in the tissue bridge, but do not overlap. (c and d) 3CB2-positive radial glial cells are linearly aligned to NF200-positive axons [(c) under low magnification and (d) under high magnification], but do not overlap. (e) Three-dimensional image shows that 3CB2-positive radial glial cells are in contact with NF200-positive axons. (f) 3CB2-positive radial glial cells do not overlap with GFAP-positive astrocytes in the tissue bridge. Scale bars in a, b, d, and f = 50 μm and c = 100 μm.

rostral and caudal ends of the stumps, similar to the stumps in the channel implantation group. In addition, the morphology of individual radial glial cells in the stumps was identical to that in the stumps in the channel group. Most of the radial glial cells in the stumps were colocalized with nestin-positive progenitor cells (Fig. 4a). There were some NF200-positive neurons and axons in the stumps, but not colocalized with the radial glial cells (Fig. 4b). In the stumps, there were a large number of GFAP-positive astrocytes, and some 3CB2-positive radial glial cells were colocalized with GFAP-positive astrocytes (data not shown). In the connective tissue bridges, there were some 3CB2-positive or RC1-positive radial glial cells and a small number of NF200-positive axons (Fig. 4c).

Fig. 4



Confocal photomicrographs of 20-μm parasagittal sections from the stumps (a and b) and connective tissue created between rostral and caudal stumps (c–e) in the control group. In each panel, rostral is on the left. Nestin (a and e), NF200 (b–d) signals are in green; 3CB2 (a–e) is in red; and 4′,6-diamidino-2-phenylindole (DAPI) signal is in blue (a–e). Colocalized signals with green and red or blue are in yellow or azure, respectively (a–e). (a) 3CB2-positive radial glial cells generally overlap with nestin-positive cells in the stump (arrows shown in a). (b) 3CB2-positive radial glial cells do not overlap with NF200-positive neurons and axons in the stump. The dotted area with an asterisk in (c) is shown at a higher power in (d). Several NF200-positive axons are detected in the connective tissue, and some 3CB2-positive radial glial cells are linearly aligned to NF200-positive axons. (e) 3CB2-positive radial glial cells are linearly related to nestin-positive cells in the connective tissue, but most of the radial glial cells do not overlap with nestin-positive cells. Scale bars in a, b, d, and e = 50 μm and c = 100 μm.

Some radial glial cells were longitudinally arranged adjacent to NF200-positive axons, and this phenomenon was identical to that in the tissue bridges in the channel group (Fig. 4d). Similarly, most of the radial glial cells were negative for nestin in the connective tissue bridges (Fig. 4e).

Discussion

In this study, there were many radial glial cells in the tissue bridges created by implantation of a synthetic guidance channel after spinal cord transection in adult rats. 3CB2 and RC1 are immunochemical markers for radial glial cells and possibly neural progenitor cells [9,18,20]. We confirmed that 3CB2-positive or RC1-positive radial glial cells were neural progenitor cells because they colocalized with antinestin antibody in the uninjured spinal cords, similar to the findings of Shibuya *et al.* [9]. In our earlier study, we found that the tissue bridges in rats with implanted channels contained several hundred myelinated axons [13]. In this study, radial glial cells migrated into the tissue bridges in the channels and guided regenerating axons that traversed the bridges. Interestingly, we found that radial glial cells could also guide axons in the connective tissue bridges in the control group. These features are similar to the role played by these cells during development when they act as scaffolds to support migration of neurons and their processes in the developing brain [2,3] and spinal cord [21,22]. As most of the radial glial cells guiding the axons in the channel and control groups were not nestin-positive, there is a possibility that after spinal cord transection, radial glial cells with long processes may be recruited and developed from nestin-positive immature endogenous radial glial cells in the white matter of the spinal cord, although the exact cell of origin is unknown.

In 2005, Grumet's group transplanted radial glial cells cultured from the embryonic rat brain into the injured adult rat spinal cord after mild cord contusion. They found slight functional recovery in the early period after transplantation and showed that transplanted radial glial cells migrated rostrocaudally from the injection sites and surrounded the cystic cavities created by the contusion injury [2]. Neurofilament-positive axons near the injury sites were longitudinally aligned with the radial glial cells, which is similar to our findings. In this study, endogenous radial glial cells also supported regenerating axons in the tissue bridge, and thus our findings would support the transplantation of radial glial cells as a strategy for repair of experimental spinal cord injury.

Conclusion

Radial glial cells have the ability to migrate from the spinal cord stumps into the tissue bridge that develops between the stumps of the completely transected spinal cord to support axons that regenerate and grow into the bridge. There were more radial glial cells in the tissue bridges that formed within the implanted chitosan channel than in the connective tissue bridges that formed in the absence of channels. Radial glial cells act as cellular scaffolds for axonal guidance.

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