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Lecture

Patterns of outgrowth of regenerating axons through spinal cord lesion

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Abstract

We found that bone marrow stromal cells (BMSCs) do not survive for long enough to serve as a scaffold for regenerating axons after transplantation in the injured spinal cord of rats. However, axonal regeneration was facilitated, possibly by trophic factors secreted from transplanted BMSCs. Regenerating axons were not associated with astrocytes, but surrounded by Schwann cells (SCs), and embedded in collagen fibril matrices just as the axons of peripheral nerves.

Experiments involving the transplantation of SCs themselves indicated that, besides exogenous SCs, intrinsic SCs infiltrated the lesion and formed myelin sheaths on regenerating axons in the same manner as described with BMSC transplantation.

The transplantation of olfactory ensheathing cells (OECs) showed that OECs themselves enclosed regenerating axons in the same manner as SCs. No study has been carried out to address whether such Schwann-like cells were derived from transplanted OECs or intrinsic SCs. However, the possibility cannot be excluded that intrinsic SCs contributed to surround regenerating axons.

Neural stem cells (NSCs) derived from iPS cells survived long-term, emanating numerous axons that extended over a long distance through the host spinal cord tissue. However, no myelination occurred on regenerating axons, and no behavioral improvement was observed. It would be difficult to manipulate iPS-derived NSCs to appropriately integrate them into the host spinal cord tissue. In this respect, iPS cells have crucial problems concerning whether they can be integrated appropriately into the host tissue.

Muse cells (multilineage-differentiating stress-enduring cells) were separated as SSEA3-positive cells from BMSCs. Transplanted Muse cells survived long-term, but they were not as effective as non-Muse cells or BMSCs for the treatment of infarcted brains, suggesting that trophic factors from non-Muse cells and BMSCs are involved in those effects.

These findings indicate that intrinsic SCs and trophic factors released from transplants may play important roles in nerve regeneration of the spinal cord. Differing from the generally believed pattern of regeneration, glial cells are not necessarily needed as the scaffolds for growing axons in the spinal cord.

Key words : spinal cord injury, axonal regeneration, bone marrow stromal cell, Schwann cell, olfactory ensheathing cell, iPS cell, Muse cell

Introduction

It was previously believed that there was no nerve regeneration in the injured spinal cord. No distinct nerve regeneration was reported in studies involving experimental spinal cord injuries before the early 1980's. However, sciatic nerve transplantation into the spinal cord or medulla oblongata demonstrated that CNS neurons within the spinal cord or medulla oblongata extended regenerating axons through the transplanted peripheral nerve segment (David and Aguayo, 1981). This experiment clearly showed that CNS neurons have an ability to produce regenerating axons when an appropriate environment is provided.

Since then, the transplantation of various kinds of cell and tissue has been studied in an attempt to provide a suitable environment for CNS neurons to produce regenerating axons in the spinal cord lesion.

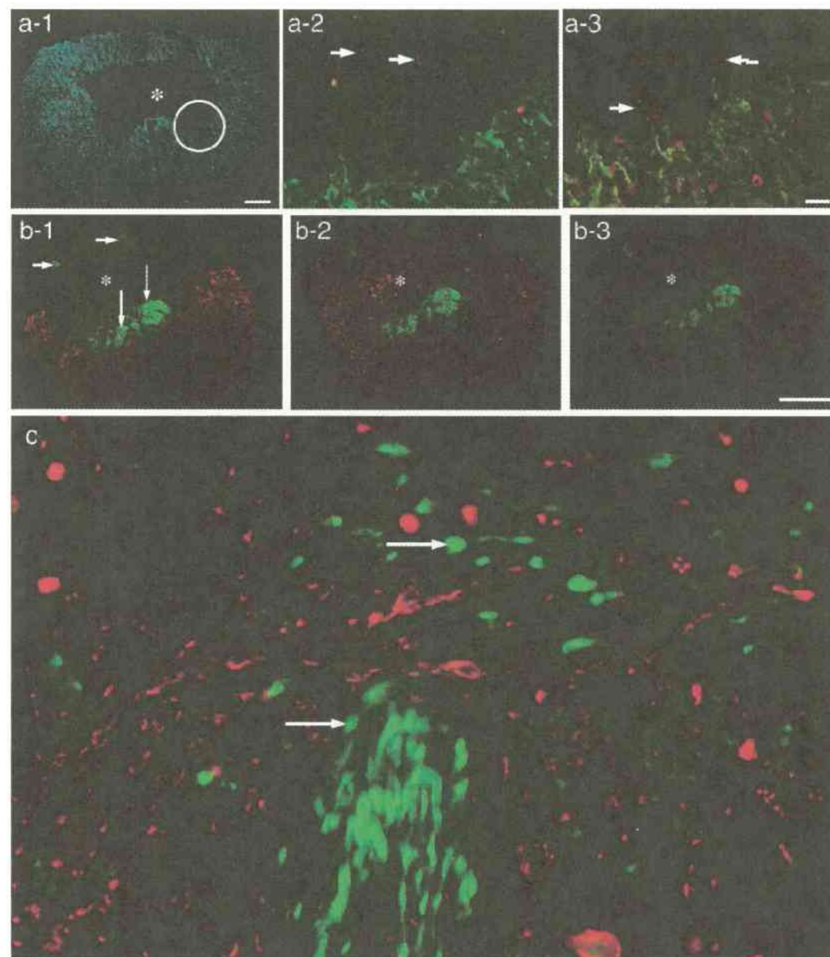
The premise of cell transplantation studies is that transplanted cells should be integrated into the host spinal cord tissue to survive long-term, serving as a scaffold for the extension of regenerating axons through the lesion. This premise is based on the hypothesis that transplanted cells or tissues can replace the injured host tissues. However, this is not necessarily the case. In our studies, transplanted cells disappeared within 2–3 weeks after transplantation. Nevertheless, the locomotor behaviors and tissue repair, including cavity formation and axonal regeneration, were improved: cavity formation was repressed and axonal regeneration was promoted. These findings suggested that, although they did not survive long enough to replace the injured host tissue, transplanted cells accelerated tissue recovery, probably due to the secretion of certain trophic factors effective for tissue repair, including axonal regeneration, in the spinal cord.

Transplantation of bone marrow stromal cells

We have been studying transplantation of bone marrow stromal cells (BMSCs) in rat spinal cord injuries. Rat bone marrow cells from femurs and tibial bones were cultured, and cells that proliferated on the base of the dish were harvested for transplantation. BMSCs were transplanted by directly injecting them into the injured spinal cord, or by infusing into the CSF via the fourth ventricle. In either case, the locomotor behaviors as evaluated by the BBB score were improved, and the spinal cord lesion was well repaired, resulting in reduced cavity formation. A notable finding was that extensive axonal regeneration occurred in the spinal cord lesion: regenerating axons extended through the space devoid of astrocytes in the spinal cord lesion. The astrocyte-devoid area contained collagen fibril matrices in which regenerating axons extended vigorously. It was suggested that astrocytes could not invade collagen fibril-filled areas. In our studies, transplanted cells disappeared from the spinal cord regardless of the method of cell transplantation; however, extensive nerve regeneration occurred through the spinal cord lesion, and behavioral functions as estimated by the BBB score were improved (Ohta et al., 2004; Ide et al., 2010; Nakano et al., 2013). Another notable finding was that regenerating axons extended through the spinal cord lesion, in which no invasion or proliferation of astrocytes was found (Fig. 1). There was no finding indicating that regenerating axons were blocked at the border of the lesion (Fig. 2). Regenerating axons were surrounded by SCs and embedded in collagen fibril matrices (Fig. 3). This finding indicated that regenerating axons were peripheral nerves.

Regenerating axons extended without being blocked at the caudal or rostral border of the lesion. Although no data were available concerning synaptic connections of regenerating axons with host neurons, the prominent locomotor recovery suggested that there may be effective neural connections between regenerating axons and host neurons. It had been believed that regenerating axons extended through the repaired CNS tissues consisting of astrocytes and oligodendrocytes, and that regenerating axons may extend caudally or rostrally through the repaired astrocytic tissues. However, such patterns of nerve regeneration were not found to be common in spinal cord injuries. As described above, regenerating axons extended through the collagen fibril matrices as peripheral nerves. Basically the same findings of axonal regeneration were seen in the spinal cord lesion of control rats, although the axonal population was much smaller than that of the cell-transplanted spinal cord.

We suggest that this may be a basic and intrinsic pattern of axonal regeneration in the spinal cord lesion. Our standpoint is that it would be optimal for the treatment of spinal cord injury if cell transplantation could elicit and enhance the "intrinsic properties" of nerve regeneration of the spinal cord. In this respect, the generally believed



a-1. GFAP-immunohistochemistry of the section from the epicenter of the lesion before transplantation. Sections were stained with FITC-labeled anti-mouse IgG antibody for GFAP. There is a cavity (asterisk) in the center of the spinal cord, and astrocytes (green) surround the cavity. Astrocyte scar is not distinct on the border of the cavity. Scale: 200 μm

a-2. Double-staining for GFAP (green) and neurofilaments (red) of the section adjacent to a-1. The area corresponding to the circle in a-1 was enlarged. Axons were stained by Cy-3-labeled anti-rabbit IgG antibody. Some axons (arrows) are associated with oligodendrocytes as shown in a-3.

a-3. Double-staining for GFAP (green) and GST-π (red) of the section adjacent to a-1. The area corresponding to the circle in a-1 was enlarged. Oligodendrocytes were stained by Cy-3-labeled anti-rabbit IgG antibody. Short cell strands of oligodendrocytes (arrows) extend into the cavity, without accompanying astrocyte processes. No distinct astrocyte scar was formed. Scale: 20 μm (a-2 and 3)

b-1. BMSC transplantation. GFP-transgenic BMSCs (green) are located as cell assemblies (long arrows) in the center of the spinal cord. Astrocytes immunostained by Cy-3-labeled antibody (red) are seen in the ventral part of the spinal cord, while the dorsal side (asterisk) of the BMSC assemblies is devoid of GFAP immunoreactivity. Small BMSC clusters (short arrows) are also seen at the periphery of the spinal cord.

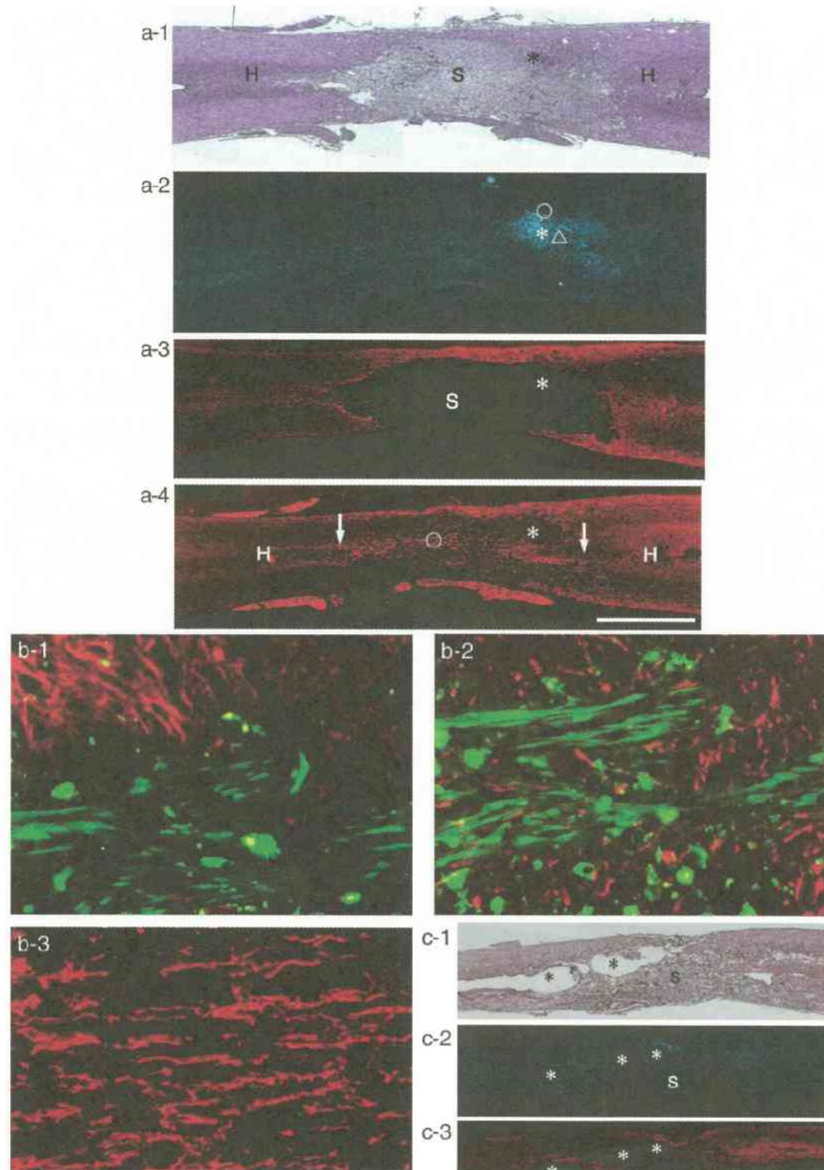
b-2. Immunohistochemistry for neurofilaments of the section adjacent to b-1. Numerous axons stained by Cy-3-labeled antibody (red) are seen in the astrocyte-devoid area (asterisk) of the spinal cord.

b-3. Immunohistochemistry for GST-π of the section adjacent to b-2. No distinct staining for oligodendrocytes is found in the astrocyte-devoid area (asterisk) of the spinal cord. Scale: 500 μm (b-1, 2 and 3)

c. Higher magnification of the astrocyte-devoid area of the section obtained from the same series of sections (b-1, 2, and 3), but at the different level from them. Immunohistochemistry for neurofilaments. Abundant axons (red) are found around engrafted BMSCs (green, arrows) in the astrocyte-devoid area of the spinal cord. Scale: 50 μm (From Ide et al., 2010)

Fig. 1 Spinal cord before cell transplantation (2 weeks after crush injury) (a-1, 2, and 3), and 1 week after BMSC transplantation (b-1, 2, 3, and c).

premise that transplanted cells should survive to serve as a scaffold for regenerating axons, or as a source of neuronal cells to replace the injured host tissues, might not necessarily be applicable to spinal cord regeneration studies.



a-1. HE staining. The lesion is filled with tissues (S) apparently different from spinal cord parenchyma (H). No distinct cavity is formed. An asterisk indicates the site of engrafted BMSCs shown in a-2.

a-2. The section adjacent to a-1. Engrafted BMSCs (green) are located as cell assemblies (asterisk) at the border of the lesion shown in a-1.

a-3. GFAP immunohistochemistry (red) in the section adjacent to a-2. A large astrocyte-devoid area (S) extends rostro-caudally. Engrafted BMSCs (site indicated by asterisk) are located at the border of astrocyte-devoid area.

a-4. Immunohistochemistry for neurofilaments in the section adjacent to a-3. It is remarkable that numerous axons (red) extend in bundles along the whole length of the astrocyte-devoid area shown in a-3. These axons appear in continuity with host nerve networks in the transitional zones on the rostral as well as caudal side (arrows). Asterisk indicates the site of engrafted BMSCs shown in a-2. H: spinal cord parenchyma. Scale: 2 mm (a-1, 2, 3 and 4)

b-1. Magnification of the part marked with a circle in a-2, showing the relationship between engrafted BMSCs (green) and astrocytes (red, GFAP immunohistochemistry). Most BMSCs extend longitudinally, and are only partly in association with astrocytes.

b-2. Magnification of the part marked with a triangle in a-2, showing the relationship between engrafted BMSCs (green) and axons (red, neurofilament immunohistochemistry). Regenerating axons are in intimate association with engrafted BMSCs.

b-3. Magnification of regenerating axons marked with a circle in a-4. Axons extend longitudinally through the astrocyte-devoid area. Scale: 50 μm (b-1, 2, and 3)

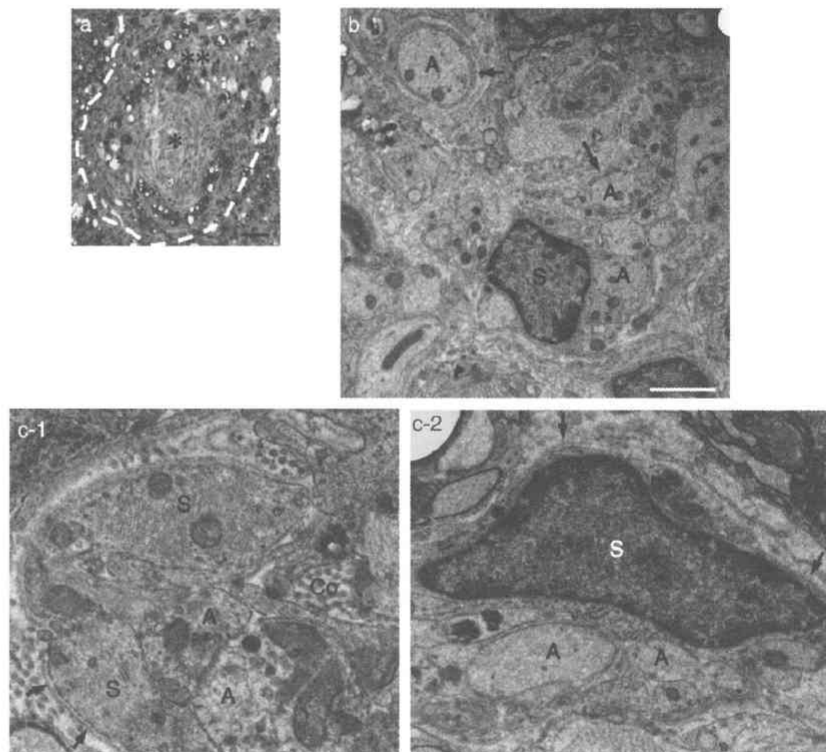
c-1. Control (vehicle injection). Horizontal section. HE staining. There are cavities (asterisks) and a tissue area (S) different from the host spinal cord tissue in the lesion.

c-2. The section adjacent to c-1. GFAP staining. Astrocytes are found around cavities (asterisks).

c-3. The section adjacent to c-2. Neurofilament staining. Some axons (red) remain around cavities (asterisks); however, few axons are found in the lesion (S). Scale: 2 mm (c-1, 2 and 3)

(From Ide et al., 2010)

Fig. 2 One week after transplantation. Horizontal sections. Left to right: rostro-caudal direction. These micrographs show that regenerating axons extend longitudinally through the astrocyte-devoid area.



a. Epon section. A cell assembly (asterisk) presumed as engrafted BMSCs is located in the white matter of the spinal cord. The border of host white matter containing degenerated myelin sheaths is marked by a dotted line. The dorsal part (double asterisks) of the cell assembly is lacking in the host white matter, corresponding to the astrocyte-devoid area seen in Fig. 1b. Scale: 50 μm

b. Electron micrograph taken from the area dorsal to the cell assembly in "a". There are numerous unmyelinated axons (some are labeled "A") covered by cell processes (arrows) or a cell body (S), suggesting that these axon-associated cells might be Schwann cells. These unmyelinated axons correspond to the abundant axons demonstrated by immunohistochemistry in Fig. 1b-2 and c, and Fig. 2a-4 and b-2 and 3. Scale: 2 μm

c-1. Magnification of an unmyelinated axon bundle. Two axons (A) are surrounded by cell processes (S) with basal laminae (arrows) on their surface. This feature indicates growing axons surrounded by immature Schwann cell processes. Collagen fibrils (Co) are seen around the nerve bundle. Scale: 500 nm

c-2. This micrograph shows that an immature Schwann cell (S) covered by basal lamina (arrows) surrounds unmyelinated axons (A). Scale: 1 μm
(From Ide et al., 2010)

Fig. 3 Conventional electron microscopy. One week after transplantation.

Transplantation of Schwann cells and olfactory ensheathing cells

On the other hand, the transplantation of SCs and olfactory ensheathing cells (OECs) has been extensively studied for the treatment of spinal cord injuries.

SCs are associated with axons of peripheral nerves, and form a myelin sheath on thick axons of more than 1 μm in diameter. Transplantation of peripheral nerve segments provides an appropriate environment for axonal regeneration in the CNS, as demonstrated by David and Aguayo (1981). This indicates that SCs serve as an effective conduit for regenerating axons in the CNS. Since this study, the transplantation of cultured SCs has been extensively studied for the treatment of spinal cord injury. These studies are based on the hypothesis that transplanted SCs survive long-term in the spinal cord lesion, serving as a scaffold for growing axons. However, the report by Hill et al. (2006) showed that transplanted SCs disappeared from the spinal cord shortly after transplantation, followed by the infiltration of intrinsic (endogenous) SCs ($P75^+$ cells) into the lesion. This study showed that the transplantation of exogenous SCs induces the proliferation of intrinsic SCs, which may play an important role in spinal cord repair. The appearance of intrinsic SCs after cell transplantation is the same as that with BMSC transplantation. A certain fraction of transplanted exogenous SCs survived by immunosuppression with cyclosporine.

OECs surround axons of the olfactory nerve, extending from the olfactory mucosa to olfactory bulb. They are unique in that they have dual properties characteristic of astrocytes and SCs at the same time. They are considered, owing to these characteristics, to be able to contribute to axonal regeneration in the spinal cord lesion: as SCs, they serve as a conduit for regenerating axons, and as astrocytes they assist regenerating axons to enter the host spinal cord tissue. In his transplantation study, Raisman (2007) reported that regenerating nerves entered the OEC graft, and traversed the lesion to re-enter the host spinal cord. Axons were ensheathed by Schwann-like cells and enclosed in a perineurial-like sheath of fibroblasts in the OEC graft. These axons became myelinated by Schwann-like cells during the course through the OEC graft, and by oligodendrocytes after re-entering the host spinal cord. This description suggested that the engrafted OECs served as a scaffold for the growth of regenerating axons, and that engrafted cells might differentiate into Schwann-like cells and/or perineurial cells.

OECs are closely associated with astrocytes, suppressing astrocytic glial formation at the lesion. This effect opens a door for regenerating axons to enter the host spinal cord tissue beyond the lesion borders. Since OECs were not labeled before transplantation, their fates could not be precisely determined in the spinal cord lesion.

On the other hand, Torres-Wspin and colleagues (Torres-Wspin et al., 2014) reported that OECs survived until 7 days after transplantation, but disappeared in the following 2 weeks. Even though they did not survive for a long time, tissue protection occurred in the lesion. These findings indicate that OECs are similar to BMSCs in their fates after transplantation. As far as OECs obtained from rats are concerned, the lifespan may be as short as that of BMSCs. Based on this hypothesis, it is probable that OECs did not serve as a scaffold for regenerating axons. Schwann-like cells and perineurial-like cells observed in the OEC grafts may not be derived from engrafted OECs, but, instead, derived from the host tissue as suggested in BMSC transplantation.

Transplantation of iPS-derived neural stem cells

Since the introduction of iPS cells, it has been anticipated that the transplantation of iPS-derived NSCs might be efficient for spinal cord injury. It has been suggested that engrafted NSCs differentiate into neural cells including, neurons, astrocytes, and oligodendrocytes to replace the injured tissues, and that neurons derived from iPS cells regenerate axons to re-establish neural connections in the host spinal cord tissue. However, there was previously no report concerning the effects of iPS-derived cell transplantation on the spinal cord injury.

The recent study by Lu and colleagues (Lu et al., 2015) reported findings on the transplantation of iPS-derived NSCs into the spinal cord injury. Human iPS cells were obtained from dermal fibroblasts of a healthy 86-year-old male and driven toward a NSC lineage. NSCs were labeled with GFP using a lenti-viral vector. GFP-expressing NSCs were embedded in fibril matrices containing a growth factor cocktail to promote graft survival and retention in the lesion site. Cells were transplanted into a C5 spinal cord hemisection lesion site 2 weeks after the original spinal cord injury. Rats underwent weekly functional assessments and were sacrificed 3 months after transplantation. The results were as follows: iPS-derived NSCs survived and differentiated into neurons and glia, and extended tens of thousands of axons from the lesion site over virtually the entire length of the rat CNS. These iPS-derived axons extended through the space between myelinated axons of white matter of the injured spinal cord, frequently penetrating gray matter and forming synapses with host neurons. No myelination occurred on such axons. There was no improvement of behavioral functions.

This study suggests that iPS-derived NSCs have many serious problems with clinical applications, as follows:

1. It is difficult to control the production of regenerating axons from the engrafted NSCs. The number of axons appeared to be several times larger than that in the normal white matter. It is desirable for regenerating axons with no synapses to be eliminated (spliced) in due course.
2. Although some synaptic connections appeared to be formed between regenerating axons and host neurons, such neural connections showed no influence on locomotor functions.
3. Regenerating axons should be myelinated for normal functioning.
4. The most disappointing finding was that there was no behavioral recovery 3 months after transplantation in rats.

Transplantation of Muse cells

Muse cells were identified and separated from mesenchymal cells (Kuroda et al., 2010). At present, Muse cells are isolated from BMSCs as stage-specific embryonic antigen 3 (SSEA-3)-positive cells. So far, no study has been reported concerning the effect of Muse cells on spinal cord injury. The recent study by Yamauchi and colleagues (Yamauchi et al., 2015) deals with the effects of Muse cells on the functional recovery of rats with ischemic stroke. Muse and non-Muse cells were separated from human BMSCs, and both were labeled with GFP using a lenti-viral vector. On the other hand, immunodeficient mice were subjected to permanent middle cerebral artery occlusion to induce an ischemic lesion in the brain. GFP-expressing Muse and non-Muse cells were transplanted into the ipsi-lateral striatum of mice with infarction 7 days later. Muse cell-transplanted mice exhibited no functional recovery for up to 4 weeks, but a slight improvement at 5 weeks after transplantation. Non-Muse cell-transplanted mice displayed more effective recoveries than mice transplanted with Muse cells. In parallel, they performed the transplantation of BMSCs in the same manner. BMSC-transplanted mice showed the most favorable recovery of behavioral functions. Muse cells survived in the brain tissue longer-term compared with non-Muse cells or BMSCs. This report explains the results as follows: non-Muse cells and BMSCs did not replace the injured tissue, but had influences by secreting trophic effects. On the other hand, Muse cells were integrated into the peri-infarct cortex, and spontaneously differentiated into neuronal cells to replace lost neurons. No data were available concerning axonal production from integrated Muse cells in this report. If Muse cell-derived neurons produce axons, contributing to neural connections in the brain, it is expected that locomotor behaviors will be improved in due course.

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