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Action of Schwann Cells Implanted in Cerebral Hemorrhage Lesion[1]

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Objective To investigate whether there is neogenesis of myelin sheath and neuron after transplantation of Schwann cells into cerebral hemorrhage lesion. **Methods** Schwann cells were expanded, labeled with BrdU *in vitro* and transplanted into rat cerebral hemorrhage with blood extracted from femoral artery and then injected into the basal nuclei. Double immunohistochemistry staining and electron microscopy were used to detect the expression of BrdU/MBP and BrdU/GAP-43 and remyelination. **Results** BrdU/MBP double positive cells could be seen at 1 week up to 16 weeks after transplantation of Schwann cells. Thin remyelination was observed under electron microscope. GAP-43 positive cells appeared after 12 weeks and were found more in Hippocamp. **Conclusions** Grafted Schwann cells participate in remyelination and promoter nerve restore in rat cerebral hemorrhage.

Key words: Schwann cell; MBP; GAP-43

INTRODUCTION

It is well known that Schwann cells are able to promote axonal renewal and remyelination in peripheral nervous system since they can secret a variety of neurotrophic factors, extracellular matrix and cell adhensive factors. It have attracted more and more attention in repairing and regenerating the central nervous system[1]. For many years, we have been studying on the impact of transplanted Schwann cells in injured center nerve system. We also have investigated the generation of rat brain stem reticular structure pro-injured by electric needle stimulus[2]. The present study transplanted Schwann cells, which were derived from sciatic nerves of newborn rats, into the rat cerebral hemorrhage induced by injecting autologous blood extracted from femoral artery to explore remyelination and neural restore.

MATERIALS AND METHODS

Culture of Newborn Rat Schwann Cells in vitro[3]

Adult and 1 to 2-day-old newborn Wistar rats were obtained from the Experimental Animal Institute of Chinese Academy of Medical Sciences. Ten newborn rats were used to prepare Schwann cells. The bilateral sciatic nerves obtained under aseptic conditions were placed into L-15 medium. After epineurium and connective tissue were removed under microscope, the nerves were cut into 2 mm^3 segments and digested with 0.25% trypsin and 0.125% collagenase for 15 min at 37℃, then centrifuged at 1000 rpm/min for 6 min. The precipitate was re-suspended with DMEM supplemented with 10% fetal bovine serum, 2 μmol/L forskolin (Sigma, USA) and 20 mg/L bovine pituitary extract (Sigma), at 37℃ in 5% CO_2. The medium was changed twice a week. When cells were in good growth state, 5'-bromodexyuridine (BrdU, Sigma, USA) at a final concentration of 10 μmol/L was added into the medium and kept for 24 h.

Rat Cerebral Hemorrhage Model

The adult Wistar rat was anesthetized with pentobarbital (400 mg/kg, IP), its head was fixed in a stereotactic frame, skull was drilled 3 mm from left frontal fontanelle, its femoral artery was exposed, 100 μL blood was drawn with hypodermic needle containing pre-treated heparin. Its head was fixed in a

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stereotactic frame again. Hypodermic needle was fixed in a microinjector, introduced into the brain basal nuclei according to rat stereotactic coordinates 6 mm below the surface of skull, 40 μL blood was for 20 min and kept for 10 min.

Transplantation of Schwann Cells and Preparation of Tissue Sections

Animals were randomly divided into an experiment group and a control group. In the experiment group, 10 μL (1×10^6 cells) Schwann cells labeled with BrdU was injected into the same hole 3 days after cerebral hemorrhage, 5 mm, 6 mm, and 7 mm below the surface of skull. In the control group, 10 μL PBS buffer was injected into the same hole, no Schwann cells were transplanted. For the two groups, 3 days and 1, 2, 4, 8, 12, 16 weeks after transplantation of Schwann cells, the rats were anesthetized by injecting 10% choloral hydrate into abdominal cavity, and 250 mL physiological saline was infused into the heart via cannula, followed by 4% paraformaldehyde-phoric acid (0.1 mol/L). The rat brain was removed 1 h later and fixed in the above-mentioned solution at least for 6 h. The brain was transferred to 20% sucrose solution for dissolving fixation fluid, and stored at -70℃. The frozen brain was dissected using a cryoultramicrotome (Thermo, UK) into 12 μm thick sections. The sections were smeared and dried with cold wind, and stored at -70℃.

BrdU/MBP and BrdU/GAP-43 Immunohistochemistry Double Staining

BrdU labeling, MBP and GAP-43 expressions were measured by immunohistochemistry double staining. The sections were dried at room temperature for 2 h and placed into sodium citrate buffer (0.9 mL of 0.1 mol/L citric acid, 4.1 mL of 0.1 mol/L sodium eitrate and 45 mL of H_2O) and heated at 92℃-98℃ for 10 min. After washed with PBS buffer, the sections were treated with normal goat serum for 20 min to block nonspecific reactions. The primary antibody (1:100 mouse anti-BrdU monoclonal antibody, Sigma, USA) was added to the sections and kept overnight at 4℃. Then the sections were rinsed with PBS buffer and exposed to secondary antibody (1:200 goat anti-mouse IgG-biotin, Vector, USA) at 37℃ for 40 min. The sections were kept in alkaline phosphatase–labeled streptavidin at 30℃ for 4 h. Color reaction was carried out with NBT. The sections were rinsed thrice with PBS buffer, exposed to antibodies of MBP (1:300, Chemicon, USA) and GAP-43 (1:100, Oncogene, USA) overnight at 4℃. The sections were rinsed thrice, kept in HRP-labeled streptavidin at 37℃ for 4 h. DAB was used for color development.

Electron Microscopy

Some brain samples were placed in 3.6% glutaraldehyde for 3 hours. The specimens were then stored in phosphated-buffered saline at 4℃. After the specimens were fixed in osmium tetroxide and embedded in Epon, they were cut transversely into semithin (1 μm) and ultrathin sections with an ultramicrotome. Then semithin sections were stained with thionin, and the ultrathin sections were stained with uranyl acetate and lead citrate and analyzed by electron microscopy.

RESULTS

In vitro Newborn Rat Schwann Cells

Two types of cells, namely Schwann cells and fibroblast cells, were visualized under a phase contrast microscope. The Schwann cells were shuttle-shaped with oval nuclei and two thread-like processes at body poles. The fibroblast cells were flat-shaped with round nuclei and multi-processes. More than 95% cells were Schwann cells (Fig. 1).

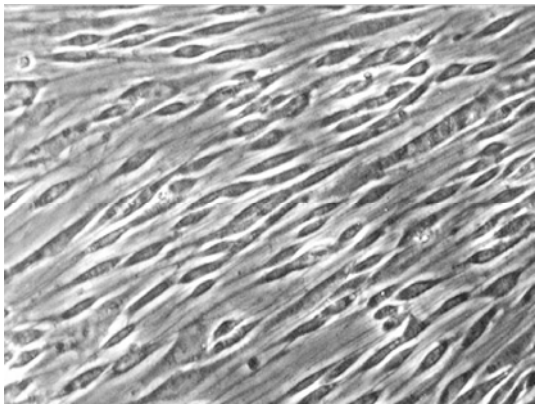

FIG. 1. Schwann cells are shuttle-shaped with oval nuclei and two thread-like processes at two cell body poles, while fibroblast cells are flat-shaped with round nuclei and multi-processes. More than 95% cells are Schwann cells under phase contrast microscope (240×).

Regenerative Myelin

BrdU/MBP double-immunoactivity was present around cerebral hemorrhage. Some indigo nuclei were flat or lunate, the appearance was irregular (Fig. 2). These cells were seen 1 week after transplantation until 16 weeks. Electron microscopy of the ultrathin sections confirmed the presence of newly regenerated myelin (Fig. 3).

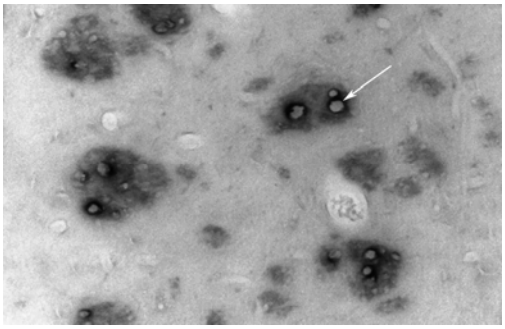

FIG. 2. BrdU/MBP double-immunohistochemical staining. BrdU and MBP expression 1 week after implantation of Schwann cells into rat cerebral hemorrhage. Some indigo nuclei are flat or lunate (arrow).

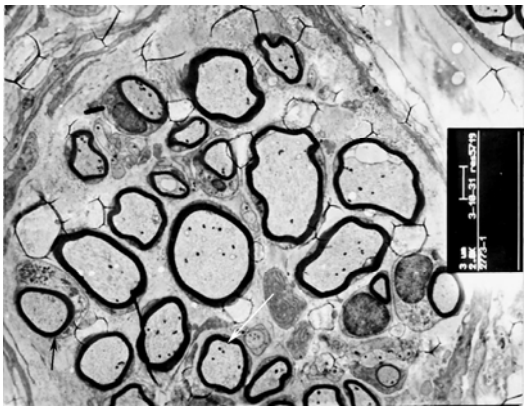

FIG. 3. Newly regenerated myelin (arrow) on electron micrograph.

Regeneration of Injured Neurons

BrdU/GAP-43 double-immunohistochemical staining showed that BrdU positve cells were round and indigo positive 1 week after transplantation of Schwann cells. GAP-43 positive cells appeared after 12 weeks, brown granules were scatted or coagulated under membrance in some neurons, which were markedly seen in Hippocampus (Fig. 4).

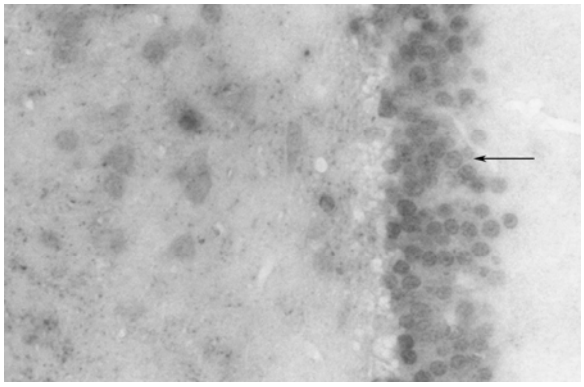

FIG. 4. GAP-43 protein expression 12 weeks after transplantation of Schwann cells to rat cerebral hemorrhage. Scatted or coagulated brown granules under cell membrance in some neurons, particularly in Hippocampus neurons (arrow) (400×).

DISCUSSION

The present data have proven that transplantation of Schwann cells can promote the repair of injured center nerve system. In the present study, we choosed the cerebral hemorrhage animal model. There are several kinds of intercerebral hemorrhage model established by injecting drugs to damage vessels[4-6] or by injecting autologous blood extracted from heart[7] *etc*. Collagenase Ⅳ injected to the special area of brain can damage blood vessels and cause intercerebral hemorrhage. Though its performance is easy, the volume and position of hematoma have a large difference. Autologous blood injection is time-consuming and causes easily rat death. Therefore, we selected the rat cerebral hemorrhage model compared with others, it is a better animal model.

Schwann cells are glial cells in peripheral nervous system. If Schwann cells are transplanted into the central nervous system, their survival is important. In a previous study, we injected Schwann cells into brain with electrical injury and found that Schwann cells labeled with BrdU survived, actively proliferated and distinctly migrated toward the cortex at 8 months after transplantation. The present study found that the number of Schwann cells with BrdU positive was not reduced around lesion, and was small in cortex, demonstrating Schwann cells could survive and migrate. We also found newly generated myelin in the present study, suggesting that some remyelination might occur in double positive BrdU/MBP, due to implanted Schwann cells.

GAP-43 largely synthesized in nerve tissue during the development and regeneration of neurons, is the molecular substance of nerve reconstruction and regeneration and takes part in axonal growth and synapse formation[8-10]. Chen *et al.*[11] found that GAP-43 expression reached its peak at week 2 after spinal cord lesion and then dropped gradually. Its expression in the 12th week was not significantly different from that in the 1st week. Hsu *et al.*[12] investigated the early events associated with axonal regrowth after spinal cord hemisection and implantation of a Schwann cell-seeded minichannel in adult rats and found that GAP-43 expression in injured axons remained high throughout the first 2 weeks after implantation. Wan *et al.*[13] showed that GAP-43 expression reached its peak at week 4 after transplantation of Schwann cells into injured brain with electric needle, and disappeared at week 12 thereafter. In the present study, GAP-43 expression reached its peak at week 12 after transplantation of Schwann cells, and start to decrease at week 16, implying that implanted Schwann cells could

promote regeneration of neurons and extension of injured axons. We explained that the different GAP-43 expression in the two studies depended on animal models. In electric injury animal model, nerve tissue around the lesion get necrosis directly by electric wave, which may induce rapid Schwann cell response and turn on repair system. But in our present animal model, injured nerve tissue around lesion was due to ishemic and hematoma and the process of pathological response needed a long time, GAP-43 expression appeared at the 12th week. How GAP-43 promotes regeneration and reconstruction is not yet clear. Recent studies suggest that GAP-43 is a substrate of protein kinase C (PKC), which is able to mediate reconstruction of axonal cytoskeleton and promote the budding of injured axons.

The results of Schwann cells transplanted in the central nervous system substantially vary with different experimental methods used. Xu *et al.*[14-15] tested the ability of Schwann cells to enhance axonal regeneration in adult rat spinal cord by grafting Schwann cells into transected spinal cords and found that both myelinated and unmyelinated nerve fibers vigorously thread into the channel containing Schwann cells, and the axonal regeneration was also closely related with the survival of implanted Schwann cells. As to the outcome of imtracerebral tranplantation of Schwann cells, no consensus has reached so far. Stichel *et al.* [16] injected Schwann cell suspension into the acutely transected postcommissural fornix in adult rats and found that Schwann cells were rapidly distributed in extensive cortical areas and could survive for 8 months with poor proliferation. Induced by Schwann cells, the dissected nerve fibers cross the lesion site and reach their target tissues via their original routes. Regenerated nerve fibers could be remyelinated by oligodendrocytes. Stichel *et al.*[17] injected Schwann cell suspension into subacutely and chronically injured axons of transected postcommissural fornix in adult rats and found that Schwann cells could elicit a growth response and attract axons in a subacute and chronic traumatic lesion, but failed to stimulate re-growth of the post-commissural fornix projection at any nonacute postlesion stage. All these findings indicate the use of Schwann cells as stimulators of axon regrowth seems to depend on the neuronal cell types and the appropriate postinjury time points. However, Yasushi *et al.*[18] have achieved different results. Hill *et al*.[19] reported that after Schwann cell-labeled human placental alkaline phosphatase (PLAP) was transplanted to spinal cord contusion lesion, few PLAP-Schwann cells could survive for 2 weeks after acute transplantation while delaying transplantion 7 days after injury improves survival. Wiby *et al.*[20] co-cultured Schwann cells with astrocytes *in vitro* and found that these cells formed separate territories with sharp boundaries between them. Schwann cells migrate poorly when they are placed on astrocyte monolayers, implying that N-cadherin-mediated adhesive astrocytes inhibit the widespread migration of Schwann cells in the central nervous system.

The mechanism underlying central nervous system injury and its recovery remains poorly understood. Numerous questions concerning cellular and molecular changes of pathology and physiology need to be addressed[21]. Implantation of Schwann cells could promote the regeneration of injured central nervous system, hoping that it might become a new therapeutic way for central nervous system injury.

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