Differentiation of Rat Neural Stem Cells and Its Relationship With Environment[1]

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Objective To explore the differentiation fates of rat neural stem cells (NSCs) in different environmental conditions. **Methods** NSCs derived from 16-day-old rat embryo were proliferated *in vitro* and implanted into the brain of rats with intra-cerebral hemorrhage. At the same time some NSCs were co-cultured *in vitro* with Schwann cells derived from newborn rats. MAP-2, GFAP and GalC (which are the specific markers of neural cells, astrocytes and oligodendrocytes respectively), BrdU and β-tubulin were detected by immunohistochemical and immunofluorescent methods. **Results** BrdU positive cells that were implanted into the brain distributed around the hemorrhagic area. The majority of them were GFAP positive astrocytes while a few of them were β-tubulin positive neural cells or GalC positive oligodendrocytes. After being co-cultured with Schwann cells *in vitro*, NSCs are predominately shown β-tubulin and MAP-2 positive, and only a minority of them were GFAP or GalC positive. **Conclusions** The hemorrhagic environment *in vivo* induces NSCs to differentiate mainly into astrocytes while co-culture with Schwann cells *in vitro* induce the majority of NSCs to differentiate into neural cells.

Key words: Stem cells; Differentiation; Stroke; Immunohistochemistry; Schwann cells; Co-culture

INTRODUCTION

Neural stem cells (NSCs) are known to be able to differentiate into neural cells and glial cells[1,2]. Their fates, however, vary under different conditions. When brain tissue is injured due to trauma, stroke, *etc*., the "dormant" neural stem cells proliferate and finally differentiate into terminal cells—neural cells and glial cells[3]. Being stimulated by different neurotrophic factors, the proliferating rate, differentiating direction and extent of NSCs cultured *in vitro* are quite different. For instance, the stimuli of epithelium growth factor (EGF) are needed for NSCs to become the progenitors of neurons, while basic fibroblast growth factor (bFGF) is required for these progenitors to become precursors of neurons. To further differentiate, much more other factors are needed[4]. The exact mechanism of direct differentiation of NSCs as well as the factors and environmental conditions affecting their differentiation fates are not yet well known. To apply NSCs implantation clinically, it is very important to explore these unknown fields. In this study, rat NSCs were implanted into

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striatum after cerebral hemorrhage or co-cultured with Schwann cells (SCs) *in vitro*. Their different results were compared.

MATERIALS AND METHODS

Materials

Sprague-Dawley (SD) rats were purchased from the Animal House, Chinese Institute of Medical Sciences. L-15 medium, Dulbeco's modified Eagle's medium (DMEM)/F12 and B27 supplement were obtained from GIBCO/BRL; Recombined human bFGF and recombined human EGF were obtained from Promega; Bisbenzimide Hoechst 33342 was bought from SIGMA. Monoclonal anti-β-tubulin (1:500, ZYMED), polyclonal anti-glial fibrillary acidic protein (GFAP, 1:100, CHEMICON), polyclonal anti-galactocerebroside (GalC, 1:50, CHEMICON), monoclonal anti-bromodeoxyuridine (BrdU, 1:100) and monoclonal anti-microtubule associated protein (MAP-2, 1:800) were used as indicated. Microscope used was Nikon Eclipse E600.

Methods

Culture of rat embryonic NSCs The cortex of 16-day-old SD rat embryo was dissected and mechanically dissociated into single cells. Cells were seeded in DMEM/F12 supplemented with 20 ng/mL EGF, 12.5 ng/mL bFGF and 1×B27. Cultures were incubated at 37℃ in 5% CO_2 and were serially passed every 4-7 days followed by re-seeding in fresh medium.

Intracerebral hemorrhage The method was reported previously[5]. Briefly, a rat was anesthetized with pentobarbital (50 mg/kg IP) and fixed in a stereotactic frame. A 30-gauge needle with 1 mL syringe pretreated with heparin was inserted into the cardiac ventricle to draw out 0.1 mL blood, and then into the right caudate nucleus (3 mm lateral to midline, 0.5 mm anterior to the coronal suture, 6 mm below the surface of the skull). Seventy-five μL blood was injected into the caudate nucleus over 7 min. Ten minutes later the needle was drawn out very slowly, bone hole was sealed with bone wax and the scalp incision was sutured.

Culture of neonatal SCs Wan's method was used[6]. Sciatic nerves of 1 to 3 days old SD rat pups were dissociated bilaterally and placed into L-15 medium. The epineurium and connective tissue were removed under microscope, the sciatic nerves were cut into 2 mm^3 fragments and digested with 0.25% trypsin and 0.125% collagenase for 15 min at 37℃, and then were centrifuged at 1 000 rpm/min for 5 min. The supernatant was discarded, cells were resuspended in DMEM containing 10% fetal bovine serum, 2 mol/L forskolin and 20 μg/mL bovine pituitary extract. Finally, cells were placed in dishes pre-coated with poly-lysine and incubated at 37℃ in 5% CO_2 for 24 h. The medium was replaced by serum-free DMEM/F12 and the cells were incubated continuously.

NSCs transplantation Three days after cerebral hemorrhage, BrdU was added to the culture at a final concentration of 50 μmol/L. Twenty-four hours later NSCs were rinsed with PBS and mechanically dissociated into single cell suspension. The cell concentration was adjusted to 2×10^7 cells/100 μL. Rat was anesthetized and fixed again. The needle was inserted into the old bone hole or the contralateral stereotactic coordinates to the midline. After the needle was inserted below the surface of skull for 5 mm, 6 mm and 7 mm, 5 μL cell suspension was injected. Ten minutes later the needle was drawn out very slowly. In the control group, NSCs were injected into right caudate nucleus of 8 normal rats.

Co-culture of NSCs and SCs NSCs were rinsed with Hank's and some cells were

seeded into dishes in which Schwann cells were growing well. The medium was half changed every 2 d. The rest of NSCs were cultured in DMEM/F12 without neurotrophic factors as the control group.

Immunofluorescent and immunohistochemical staining Cells were rinsed with PBS, fixed with 4% paraformaldehyde for 30 min and non-specific reaction was blocked. After incubated with primary antibody at 4℃ overnight, cells were incubated with FITC or TRITC conjugated secondary antibody. As to immunohistochemical staining, 6 days after intracerebral hemorrhage, rat was reanesthetized and perfused through the heart with 250 mL 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline. The brain was fixed in the same fixative overnight and cut coronally through the needle entry site 2 mm anterior and posterior to that plane. Brain slices were dehydrated, embedded in paraffin and 5 μm sections were cut. After being incubated with primary antibody at 4℃ overnight, brain slides were reacted with secondary and tertiary (if necessary) antibodies at room temperature for 15 min, and then stained with DAB.

RESULTS

Migration and Differentiation of Implanted NSCs

Six weeks after transplantation, numerous BrdU positive NSCs that were implanted into caudate nucleus contralateral to the hematoma migrated toward the lesion (Fig. 1a). The majority of these cells were GFAP positive astrocytes and no β-tubulin positive neurons were seen (Fig. 1b). Cells implanted ipsilateral to the hematoma distributed around the lesion. The majority of cells were astrocytes and a few of them were neurons or GalC positive oligodendrocytes. In the control group, BrdU positive cells mainly migrated into the subventricular zone and remained in an undifferentiated state (nestin staining positive).

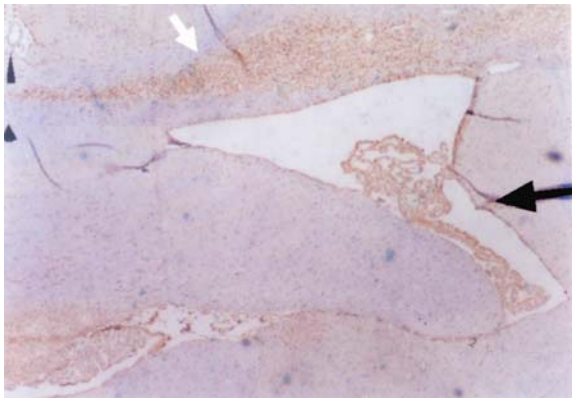


FIG. 1a. Six weeks after transplantation, numerous BrdU positive NSCs that were implanted into caudate nucleus contralateral to the hematoma migrated toward the lesion and formed a triangle-like troop with its tip directed to the lesion side (×40) (white arrow: BrdU positive NSCs, black arrow: lateral ventricle ipsilateral to the transplantation, white arrowhead: midline of the brain, black arrowhead: callus).

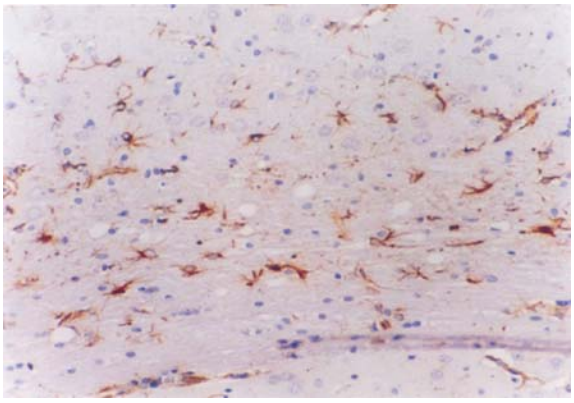


FIG. 1b. The majority of these cells were GFAP positive astrocytes (×80).

Differentiation of Co-cultured NSCs

Phenotype visualized by phase contrast microscope Several hours after being co-cultured with SCs, neural spheres stopped floating in the media and were attached to the bottom. Two days later they obviously differentiated. NSCs migrated out from the spheres and stuck out numerous thin and long processes radially (Fig. 2). Their processes prolonged continuously. In the control group, though the neural spheres were also attached to the bottom, their growing state deteriorated daily: cells looked atrophic and were attached to the bottom loosely, and no obvious cell proliferation or differentiation was seen. Most cells died within 7 d.

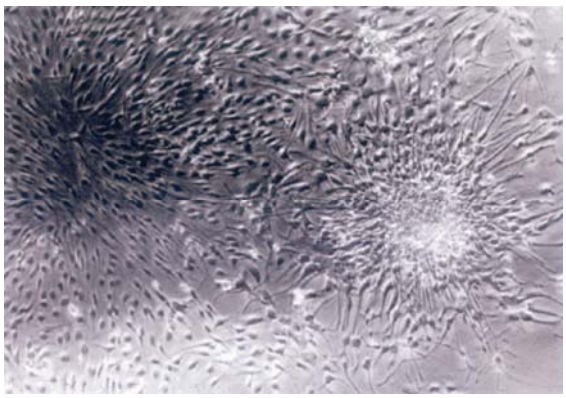


FIG. 2. After being co-cultured with SCs, NSCs migrated out from the spheres and stuck out numerous thin and long processes radially (×80).

Immunofluorescence Seven days after being co-cultured with SCs, majority of the differentiated NSCs were stained positive by MAP-2 (Fig. 3), indicating that they predominately differentiated into neural cells. NSCs were still stained positive by nestin, though they had initially differentiated. Nestin staining showed that majority of the differentiated NSCs presented typical shape of neurons: irregular cell body with several thin and short dendrites as well as one thick and long axon (Fig. 4a), while a few of them looked like astrocytes or oligodendrocytes (Fig. 4b). It was confirmed by GalC and GFAP staining (Fig. 5).

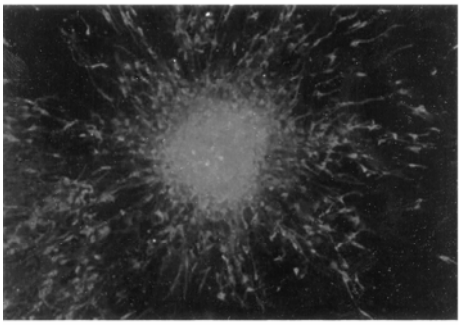
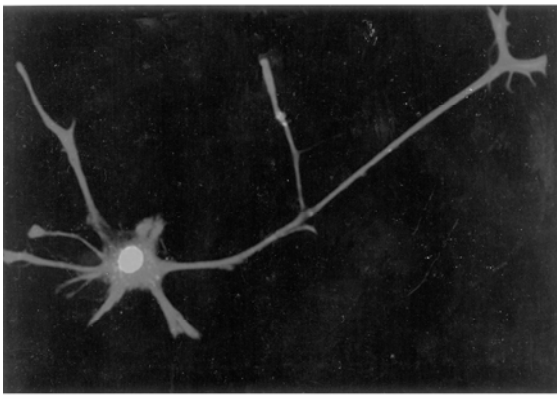


FIG. 3. Majority of the co-cultured NSCs were MAP-2 staining positive, indicating that they mainly differentiated into neural cells (×40).



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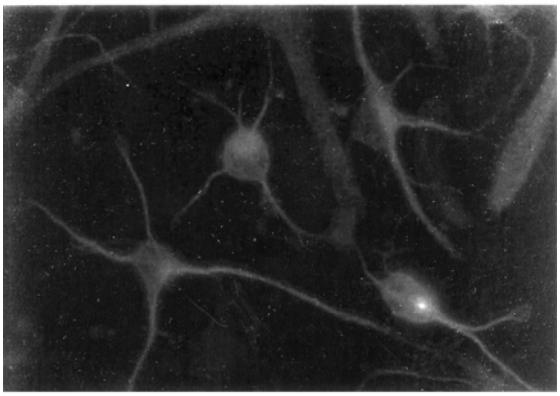


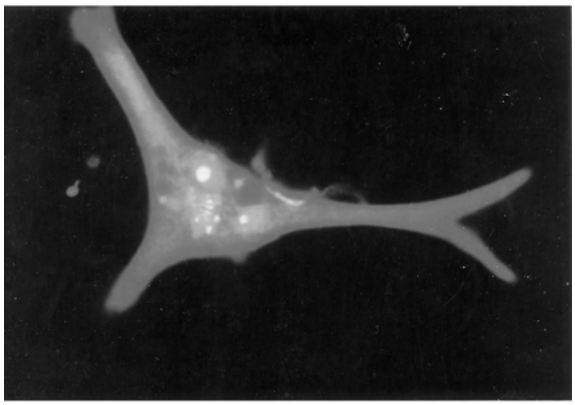
4b

FIG. 4. Nestin staining of differentiated NSCs. Majority of them showed typical shape of neurons: irregular cell body with several thin and long processes (4a, ×160). A few of them looked like oligodendrocytes (4b, ×160).

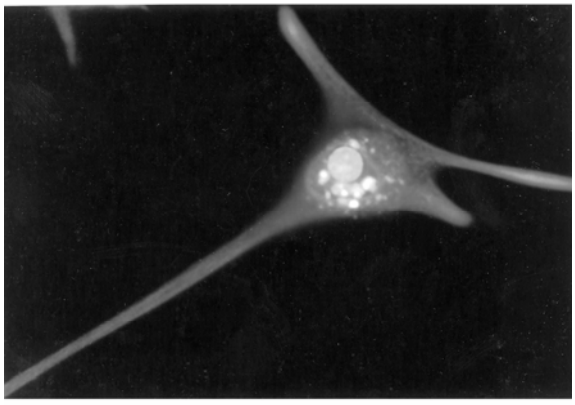
DISCUSSION

The differentiating mechanism of NSCs is very complex. Their final differentiation is

affected by various factors and environmental conditions. Briefly, it depends on the culture media component *in vitro* and the interaction of cells *in vivo*[7-10]. Many factors play important roles in NSCs differentiation. We previously reported that serum induced the NSCs to differentiate into neurons, astrocytes and oligodendrocytes[5]. Lillien *et al.* demonstrated that retina precursors of embryonic rat were induced by EGF to differentiate into neurons and glial cells[11]. It was interesting that Qian found that different concentrations of FGF2 had different effects on the differentiating directions of NSCs[12]. Being incubated in medium containing 1-10 ng/mL FGF2, the neural precursors of cortex differentiated into neurons and oligodendrocytes. If some other factors were added, they differentiated toward astrocytes instead[7]. Till now, people usually study the effect of a single factor at one time. While being a complex organic body, a man's biological regulation is affected by numerous factors both inside and outside of his body. We found that changed environmental conditions determined different fates of NSCs. Being a kind of glial cells in peripheral nervous system (PNS), SCs were very important for PNS repairing, mainly depending on the microenvironment constructed by various neurotrophic factors secreted by SCs. This microenvironment was beneficial to neural regeneration[8]. We co-cultured NSCs and SCs *in vitro* to mimic the PNS microenvironment *in vivo*. The results showed that majority of the co-cultured NSCs differentiated into neural cells. Most of them grew out secondary and tertiary branches.



5a



5b

FIG. 5. Immunofluorescence confirmed that a few of NSCs differentiated into GFAP staining positive astrocytes (5a, $\times 280$) and GalC staining positive oligodendrocytes (5b, $\times 160$).

These findings indicate that the microenvironment constructed by SCs is favorable for NSCs to maturate in phenotype. It is not clear which one (ones) of more than 20 kinds of proteins produced by SCs exerts the dominant effect in this process. From the relative reports, we deduce that it may be the result of cooperation between many factors. The effect of extracellular matrix and cell adhesive factor should not be ignored due to the fact that when only the supernatant of SCs to culture NSCs were used, cells grew not very well even though NSCs differentiated.

To explore the differentiation of NSCs in damaged brain, we transplanted NSCs into brain tissue after cerebral hemorrhage. Under such an environmental condition, NSCs mainly became astrocytes, quite different from the result when they were co-cultured with SCs *in vitro*. This may be related with various factors released generally and regionally after lesion *in vivo*. The exact mechanism needs to be further studied.

In summary, the mechanism of direct differentiation of NSCs is very complex, and is also very important for future NSCs transplantation therapy of various neurological disorders.

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