Differentiation of Neuroepithelial Progenitor Cells Implanted into Newborn Rat Brain Striatum

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It has been demonstrated that multipotent neuronal progenitor cells can be isolated from the developing or adult CNS and proliferated in vitro in response to epidermal growth factor. The present study was undertaken to investigate the differentiation of neuronal progenitor cells after transplantation into the neonatal rat forebrain striatum. Primary cultured progenitor cells were labeled with 3,3’-dioctadecyl-
cloxacarbonyl- amine perchlorate (DiO). DiO labeled progenitor cells were implanted into neonatal rat striatum. Implanted DiO labeled progenitor cells were differentiated into astrocytes and GABAergic neurons. These results suggest that implanted progenitor cells can be differentiated into neurons in host forebrain striatum. In addition, our data show that DiO labeling is a useful technique for tracing implanted progenitor cells.

Key Words: Cell differentiation, Stem cells, Neurons, Astrocytes, Transplantation

INTRODUCTION

Transplantation of fetal central nervous system (CNS) tissue has been evaluated as a potential therapeutic strategy for various CNS insults, including Parkinson’s disease (Nikkhan et al, 1994), spinal cord injury (Anderson et al, 1995), cerebral ischemia (Hodges et al, 1996), and traumatic brain injury (Sizelion et al, 1996). It has been reported that fetal rat cortical tissue survived and grew when transplanted into injured, adult rat cortex following lateral fluid percussion (LFP) brain injury (Soares et al, 1999). In addition, fetal rat cortical grafts transplanted into the cortex at 24 h after LFP brain injury, alone or in combination with nerve growth factor infusion, were found to reduce both cognitive and motor function deficits (Simson et al, 1996).

While dramatic results have been demonstrated using fetal tissue transplantation in rodent models of CNS injury, serious ethical, moral and technical problems remain using fetal tissue as a therapeutic strat-

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Although tumors have not been reported in any animals grafted with these cells, transforming cells with oncogenes may raise serious safety issues if these cells are to be used for clinical transplantation.

There are other types of cells which can be expanded in culture without the use of oncogenic transformation. It has been demonstrated that multipotent precursors can be isolated from the developing or adult CNS and proliferated in vitro in response to certain mitogens including epidermal growth factor (EGF) (Reynold & Weiss, 1992; Park et al., 1998), and basic fibroblast growth factor (bFGF) (Joh et al., 1996). Although the exact characteristics of these precursors are still to be established, previous studies have shown that EGF-responsive cells from embryonic mouse striatum can be passaged over 30 times with a logarithmic increase in the total number of viable cells while still retaining the capacity to differentiate into various types of neurons and glia (Reynold & Weiss, 1993). The present study was undertaken to characterize the neuronal cells differentiated from multipotent progenitor cells in vitro, and to investigate the differentiation of progenitor cells after transplantation into the neonatal rat striatum.

METHODS

Primary progenitor cell cultures

Primary progenitor cell cultures were prepared from fetal rat forebrain (Sprague-Dawley rats, gestation days 17–19) by a method similar to glial cell cultures (McCarthy & de Villis, 1980; Reynolds & Weiss, 1992). Briefly, the forebrains were isolated under a dissecting microscope and the meninges were carefully removed. The tissues were triturated using 10 ml pipettes, and were incubated in Ca⁺⁺ and Mg⁺⁺-free Hanks’ balanced salt solution (HBSS, Gibco, Grand Island, NY, USA) containing 0.13% trypsin (Sigma, St. Louis, MO, USA) for 15 min in a magnetically-stirred flask at 37°C. The dissociated cells were filtered through Nitex (Tetko; Elmsford, NY, USA) #210 followed by #130. The cell filtrates were centrifuged at 100 × g for 5 min. The resulting pellets were resuspended in fresh HBSS and refiltered through Nitex #130 and repelleted. The pellets were resuspended again in serum-free medium (SFM) and filtered through Nitex #40. Cells were counted in a hemocytometer and were then plated at a density of 2.5 × 10⁵/ml on Linbro 6-well culture plates (ICN-Flow Labs; Horsham, PA, USA). Primary cultures were maintained in SFM at 37°C in a humidified atmosphere of 95% air/5% CO₂ for 4–6 days until floating cell clusters were formed.

SFM was composed of Dulbecco’s modified Eagle medium (DMEM, Gibco) containing 50 nM hydrocortisone (Sigma), 100 nM putrescine (Sigma), 30 nM selenium (Sigma), 500 ng/ml prostaglandin F₂α (Sigma), 20 μg/ml transferrin (Sigma), 20 μg/ml insulin (Sigma), 20 ng/ml EGF (Sigma), 50 unit/ml penicillin/streptomycin, and 5 mM HEPES (Sigma).

Characterization of progenitor cells and neuronal cells derived from progenitor cells

To characterize progenitor cells, floating cell clusters were collected and replated (secondary culture) on poly-L-lysine (PLL, Mw: 70,000–150,000, Sigma) coated chambered slides (Nunc, Naperville, IL, USA), and allowed to attach for 1 hr; immunocytochemistry was then performed using anti-vimentin antibodies (Boehringer Mannheim, Indianapolis, IN, USA). To confirm progenitor cells that could give rise to neuronal cells, astrocytes, and oligodendrocytes, immunocytochemistry for neuron-specific enolase (NSE), glial fibrillar acidic protein (GFAP), and galactocerebroside (GC) was performed after two weeks in secondary culture. Culture medium was exchanged every 4–5 days with fresh SFM.

To characterize the neuronal cells derived from progenitor cells, immunocytochemistry for tyrosine hydroxylase (TH), γ-aminobutyric acid (GABA), substance P, and serotonin were performed after 2 weeks in secondary culture.

Labeling and transplantation of progenitor cells

Cultured progenitor cells were harvested in 10 mL cornical tube and labeled with DiO (3,3′-diiodoacetyl-cyclohexylamide perchlorate, Molecular Probes Inc., Eugene, Ore, USA) prior to transplantation. This dye is brightly fluorescent, non-cytotoxic, and label the plasma membrane with high specificity (Snider et al., 1992). After introduction, they diffuse laterally within the plasma membrane, resulting in uniformly staining entire cell. For DiO labelling, harvested cells were incubated for 5 min with fresh SFM containing 5 μg/
mL DiO. DiO-labeled cells were centrifuged at 100 × g for 5 min. The resulting cell pellets were resuspended in SFM and repelleted. This protocol resulted in a labelling efficiency close to 100%. The pellets were resuspended again in 100 μl SFM and used for transplantation. The final cell density of the suspensions was more than 5 × 10⁶ cells/mL. The 3 μl of the cell suspension was injected with a 10 μl Hamilton syringe into the anesthetized postnatal 1–2 day old rat striatum using stereotaxic frame at the following coordinate: 0.5 mm anterior from bregma, 2.5 mm right lateral from midline, and 4 mm deep from dura. Cell suspension injection rate was 1 μl/min and needle was allowed to remain in place for 5 min after injection to prevent backflow.

Tissue processing

One week after transplantation, animals were terminally anesthetized (pentobarbital: 100 mg/kg), and perfused intracardially with 20 mL PBS, followed by 40 mL of 4% paraformaldehyde. The brains were removed and postfixed for at least 48 hr in the 4% paraformaldehyde solution. Following postfixation, the brains were sectioned on a vibratome at 40 μm thickness and sections were mounted on gelatin-coated slides, and allowed to air dry. Sections were divided into 8–10 series and were permeabilized in saponin solution (0.03% saponin in PBS). Separated series were processed for indirect immunohistochemistry. Markers used for immunocytochemistry were antibodies directed against NSE, GFAP, GC, tyrosine hydroxylase, GABA, substance P, and serotonin.

Immunocytochemistry

We used indirect immunocytochemistry. Briefly, cultured cells on chambered slides or tissue sections were fixed in 4% paraformaldehyde (Sigma) in 20 mM phosphate buffered saline (pH 7.4, PBS) for 20 min at 4°C and permeabilized in 0.1% triton-x100 (Sigma) for 10 min at 4°C. Non-specific binding sites were blocked with blocking solution consisting of 10% fetal bovine serum (Gibco) in 20 mM PBS (30 min, 37°C).

Primary antibodies were diluted with blocking solution to the working concentrations and incubated for 1 hr at room temperature: rabbit anti NSE (Sigma), anti GFAP (Sigma), and anti GC (Sigma); 1 : 100, mouse anti serotonin (Boehringer Manheim); 1 : 10, mouse anti TH, anti GABA, anti substance P; 1 : 500, mouse anti neurofilament (NF) (Boehringer Mannheim); 1 : 1,000. Then the slides were washed with 20 mM PBS.

For mouse monoclonal antibodies, fluorescein isothiocyanate (FITC) - conjugated goat anti mouse IgG secondary antibody (Boehringer Mannheim, diluted 1 : 500 in blocking solution) was incubated on slides for 30 min at room temperature; for rabbit polyclonal antisera, goat anti-rabbit IgG conjugated to rhodamine (Rhod) was used for secondary antibody (diluted 1 : 200). After washing with 20 mM PBS, coverslips were mounted on slides with 50% glycerol in PBS with 0.05% propyl gallate (Sigma). All slides were examined with a fluorescent microscope equipped for epifluorescence (Olympus, Tokyo, Japan).

For double immunocytochemistry, mouse monoclonal antibodies and rabbit polyclonal antisera were combined. For the staining of oligodendrocytes membrane surface antigens (GC), the permeabilization step was omitted.

RESULTS

Characteristics of primary cultured progenitor cells

After 1–2 days in primary culture, most of the plated cells had died (Fig. 1A). However, some round phase contrast-bright cells began to proliferate and to form small clusters. Between 4–6 days in culture, these proliferating clusters enlarged and floated freely in suspension (Fig. 1B).

To characterize the EGF-responsive primary cultured cells, floating cell clusters were replated on PLL coated chambered slides and allowed to attach for 1 hr. After appropriate fixation, immunocytochemistry was performed using several different antibodies. Virtually all cells expressed nestin. However, at this time, almost no cells showed immunoreactivity for NSE, GFAP, or GC antibodies (data not shown). Furthermore, these clusters were negative for certain cell-specific markers such as fibronectin (fibroblast) or OX-42 (microglia/macrophage).

Replated clusters attached immediately to the PLL substrate. In the presence of EGF, clusters continued to proliferate, and cells migrated from clusters. To determine the potentiality of EGF-responsive progenitor cells, secondary cultures were maintained for up to 2 weeks and processed for immunocytochemistry.
Fig. 1. Light microscopic appearance of neuronal stem cells from newborn rat forebrains. (A) Single neuronal stem cell (arrowhead) after 2 days in primary culture (×200). (B) A cluster of cells (arrowhead) after 7 days of primary culture (×200).

Fig. 2. Immunocytochemical characterization of neuronal stem cells. (A) After 2 weeks of secondary culture, cells were processed for indirect immunocytochemistry using anti GFAP and anti GC antibodies and for double immunocytochemistry using anti NSE and anti NF antibodies. (A) GFAP-positive cells with flat, polygonal shape (arrowhead, ×400). (B) GC-positive cells (arrowhead, ×400). (C) NSE-positive cells (×400). (D) NF-positive cells (×400). Each arrowhead in C and D indicates identical cells.
Within 3~5 days, some cells expressed GFAP. After 2 weeks in culture, many cells expressed GFAP and these cells had a flat, polygonal morphology (Fig. 2A). Some cells were immunoreactive either GC (Fig. 2B) or NSE (Fig. 2C) after 2 weeks in culture. GC positive cells had a typical oligodendrocytic morphology. NSE positive cells were found at the center of clusters and had a round soma with fine, long processes. All NSE positive cells were positive for NF in double immunocytochemistry (Fig. 2D).

Characterization of neuronal cells derived from progenitor cells

To determine what kind of neurotransmitters neuronal cells had, double immunocytochemistry for NSE and TH or serotonin, GABA and substance P were performed. Some NF positive cells expressed either GABA or substance P (Fig. 3), but serotonin, glutamate, or TH positive cells were not observed.

DiO labeled progenitor cells show same migration pattern as unlabeled cells (Fig. 4). We confirmed DiO labeled progenitor cells were differentiated into astro-
cytes, oligodendrocyte, and neurons after 2 weeks in culture.

**Immunocytochemical identification of transplanted cells**

Rats implanted with DiO-labeled progenitor cells were sacrificed 7 days after implantation and assessed for implanted cell survival, migration and differentiation. Quantitative estimation of survived DiO-labeled cells revealed only a small proportion of total implanted DiO-labeled cells (<0.5%).

DiO-labeled cells were distributed up to 2 mm from the site of implantation, although most cells remained near the injection track. Implanted cells were not detected outside the striatum in any of the recipient rats.

Astrocytes were differentiated from implanted progenitor cells (Fig. 5). Some of DiO-labeled cells expressed GFAP densely and these cells had polygonal shape morphology. The surrounding host brain also expressed GFAP-positive cells with lower density, in addition, the density diminished with distance from the graft.

Neuronal cells differentiated from implanted progenitor cells could be observed, although the number of these cells was small. These cells had GABA as a neurotransmitter (Fig. 6). Individual GABA-positive cells had mono or bipolar shape and displayed large round nuclei consistent with a neuronal phenotype.
Fig. 6. Expression of GABA positive cells in the striatum of a mouse 7 days after DiO labeled neuronal stem cells implantation. (A) DiO labeled cells at implantation site. (B) GABA positive cells at implantation site. Each arrow and arrowhead in A and B indicate identical cells. (C) DiO labeled cells migrate from implantation site. (D) GABA positive cells migrate from implantation site. Each arrow and arrowhead in A and B indicate identical cells.

DISCUSSION

Most of the neuronal and glial cell types found in the mature CNS originate from precursor cells in the ventricular zone of the fetal brain (Davis & Temple, 1994). Recently, several studies have examined cell lineage in the vertebrate CNS to determine whether neuronal and glial cell types originated from a common precursor or from distinct progenitors. In the cortex, retina, and optic tectum, a single precursor cell can give rise to both neurons and glia as well as different types of neurons, even at the late stages of neurogenesis (Cameron & Rakic, 1991). These results imply that multipotential precursor cells persisted throughout the CNS developmental stages.

The results presented here demonstrated that a single progenitor cell from a fetal rat forebrain proliferated in the presence of EGF (Fig. 1), and subsequently differentiated into neurons, astrocytes, and oligodendrocytes (Fig. 2). In our culture system, neuronal cells derived from progenitor cells contain either GABA or substance P as a neurotransmitter (Fig. 3): these are two of the major neurotransmitters of the adult striatum in vivo (Jacobson, 1991). In contrast, these cultures did not contain cells that were immunoreactive for glutamate, serotonin, or TH. The reason for the restricted expression of phenotype is unknown, but it is possible (i) that EGF-responsive progenitor cells are limited to produce only cells containing GABA and substance P or (ii) that other phenotypes may appear at different times or under different culture conditions (Acheson et al, 1986; Frade et al, 1996).

It is generally acknowledged that extrinsic factors such as cytokines, cell to cell contact and extracellular matrix are important in generating the cellular di-
versity observed in the CNS (Cameron & Rakic, 1991). Johe et al (1996) reported that platelet-derived growth factor supports neuronal differentiation and that ciliary neurotrophic factor acts on stem cells to generate astrocytes and oligodendrocytes. The survival and development of neurons are influenced not only by soluble molecules such as cytokines, but also by neurotrophic factor or cell adhesion molecules (Barres et al, 1994).

The recent interest in transplantation of neuronal cells raises the question of a reliable method for tracing the grafted cells, and distinguishing grafted cells from host cells. One common method for labeling of neuronal stem cells prior to transplantation is the use of bromodeoxyuridine or reporter gene such as lac Z. But cytotoxicity of bromodeoxyuridine (Sheftly et al, 1994) and downregulation of lac Z gene after transplantation have been reported (Onifer et al, 1993b).

DiO is a fluorescent dye that has been used as a tracer for the mapping of axonal projections in the PNS and CNS (Snider et al, 1992). The presence of DiO in the cell membrane does not affect cell viability and cell surface properties including cell-cell recognition and cell migration (Serbenzia et al, 1990). Motoneurons labeled with DiO have been reported to remain viable for up to 4 weeks in culture (Kuffler, 1990) and up to 1 year in vivo (Pomeranz et al, 1991). DiO has rhodamine wavelengths and does not cross over the fluorescein wavelength. Moreover DiO labeled tissue is visible in vibratome and frozen sections. Based on these characteristics, along with the fact that the dye is well retained and loaded uniformly in the membrane, we chose DiO for labelling of transplanted progenitor cells.

We demonstrated that cultured progenitor cells can be robustly labeled with DiO prior to transplantation (Fig. 4) and DiO labeled cells are identifiable within the host brain (Fig. 5 and Fig. 6). However we could trace the DiO labeled cells only for up to 7 days. This was probably due to the fact that after transplantation, a certain percentage of the cells died and released DiO into extracellular space. Therefore, if the survival rate of grafted progenitor cells can be increased, DiO labeling method can be used as a more useful technique for tracing implanted progenitor cells.

The present results show that, although only few cells were found, cultured progenitor cells implanted into the striatum became differentiated into astrocytes and neurons (Fig. 5 and Fig. 6). Some of implanted cells were migrated from injection site, but all surviving cells were found within the host striatal parenchyma. The neuronal cells differentiated from transplanted progenitor cells had GABA as a neurotransmitter (Fig. 6). We can not explain why only astrocytes and GABAergic neurons are differentiated from transplanted progenitor cells in neonatal host brain. However, it is possible that regionally specific environmental cues influenced the developmental fate of the transplanted progenitor cells. Difiglia et al (1988) & Liu et al (1993) reported that striatal neurons transplanted into striatum or cortex, and these cells adopted morphologies and characteristics of striatal cells or cortical cells, respectively.

In our experiments, survival rate of implanted progenitor cells were low (<0.5%). It is likely that serum factors, at the site of injection, possibly reduced the transplanted progenitor cells survival. It has been reported that serum factors have cytotoxicity on neuronal cells differentiated from progenitor cells in vitro. If as little as 1% serum is added to the medium, the number of neuronal cells differentiated from progenitor cells decreases dramatically and almost no neurons are present by 7 days in culture (Svendsen et al, 1996; Park et al, 1998). It will be necessary to study further about increasing the survival rate of transplanted progenitor cells and which factors affect the differentiation of transplanted progenitor cells.

In conclusion, our findings indicate that implanted progenitor cells can be differentiated into astrocyte and GABAergic neurons in host forebrain striatum. In addition, our data show that DiO labeling is a useful technique for tracing implanted progenitor cells.

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