ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol Exp 2007, 67: 367–377

A simplified method for generating oligodendrocyte progenitor cells from neural precursor cells isolated from the E16 rat spinal cord

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Abstract. Conditioned medium obtained from B104 neuroblastoma cells (B104CM) has been used widely for inducing oligodendrocyte progenitor cells (OPCs) from neural precursor cells (NPCs). Our previous studies have demonstrated that E16 rat spinal cord-derived NPCs could be induced to differentiate into OPCs using a combination of B104CM and basic fibroblast growth factor (bFGF). Here we report the development of a more efficient and reliable approach to generate large quantities of highly purified OPCs from spinal cord-derived NPCs using a combination of platelet derived growth factor (PDGF) and bFGF. We demonstrated that, after the two factors application, over 90% cells displayed typical bipolar or tripolar morphology and expressed markers for OPCs including A2B5 (90.36 ± 4.59%), NG2 (93.63 ± 3.37%) and platelet derived growth factor alpha receptor (PDGFR; 90.35 ± 1.95%). Our results indicated that the PDGF/bFGF combination is more efficient in generating OPCs than the B104CM/bFGF. And it is a more potent combination of factors in promoting proliferation of OPCs.

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Key words: cell culture, differentiation, neural precursor cells, oligodendrocyte progenitor cell, PDGF, bFGF, B104CM

INTRODUCTION

Oligodendrocyte progenitor cells (OPCs) play an important role not only as progenitor cells that give rise to myelinating cells in the central nervous system (CNS), but also as an active participant in the neural network (Lin and Bergles 2004). Recent studies have demonstrated that OPC transplantation could be a potential therapy for CNS injuries and demyelinating diseases (Cao et al. 2005, Faulkner and Keirstead 2005, Nistor et al. 2005, Tontsch et al. 1994). For both experimental and therapeutic purposes, it is essential to obtain large quantities of highly purified OPC populations. To date, a number of methods have been reported to isolate OPCs from the neonatal rodent CNS such as shaking (McCarthy and de Vellis 1980) and immunopanning (Raff et al. 1983). These protocols, however, are restricted in practice due to their complicated procedures, lower cell yields and high costs. Recently, embryonic neural precursor cells (NPCs) have been used as a potential source for generation of OPCs for myelin repair (Bogler et al. 1990, Broughton et al. 2007, Brustle et al. 1999, Espinosa-Jeffrey et al. 2002, Lin and Bergles 2004, Smith and Blakemore 2000, Zhang et al. 1998, 1999). These studies suggest that establishing a self-renewing OPC population from NPCs may be a better approach to obtain OPCs because NPCs can be expanded repetitively *in vitro* while retaining their ability to differentiate into both neurons and glial cells (Reynolds and Weiss 1996, Weiss et al. 1996b)

The above-mentioned strategies to induce differentiation of NPCs into OPCs *in vitro* usually employ the supplementation of medium with a variety of growth factors and/or conditioned medium derived from the B104 cell line (B104CM) or glial cells, commonly in combination with serum. We previously reported that E16 rat spinal cord-derived NPCs could be induced to differentiate into OPCs using a combination of B104CM and basic fibroblast growth factor (bFGF) (Fu et al. 2005b, Hu et al. 2004) As the components of B104CM are complicated and its biological activity is critically dependent on the status of the cell line, it would be hard to control the production of OPCs from one passel to another, and is not appropriate to use these cells obtained by such undefined neuroblastoma-produced compounds for future clinical applications. Thus, there is an imperative need to improve methods for generating OPCs from NPCs.

Previous studies have demonstrated that purified OPCs from postnatal rat optic nerve, cultured in serum-free medium containing platelet-derived growth factor (PDGF) and bFGF, could undergo continuous self-renewal in the absence of differentiation (Noble et al. 1990, Tang et al. 2000). Furthermore, Kang et al recently reported that neurospheres differentiated from rat adipose tissue-derived stromal cells, treated with the combination of PDGF, bFGF and Shh, could be induced into OPCs (Kang et al. 2006).

In this study, we sought to determine whether PDGF, a major component of B104CM, could be used as a substitute for B104CM and whether large quantities of OPCs could be generated from spinal cord-derived NPCs using a combination of two growth factors, i.e., PDGF and bFGF.

METHODS

Animals and reagents

All E16 rats were obtained from female pregnant Wistar rats bred in the Animal Care Facility at Shanghai Jiaotong University School of Medicine. All animal procedures used in this study were approved by Shanghai Jiaotong University School of Medicine Animal Care and Use Committee.

All cell culture medium and supplement reagents were purchased from Gibco-BRL (Life Technologies, Gaithersburg, MD, USA) and Sigma (St. Louis, MO, USA) unless otherwise stated in the text.

The medium used for NPC culture (NPC-medium) was composed of DMEM/F12 (1:1), 1% N2, 1% B27, 3 μg/ml Heparin, 2 mM Glutamine, (referred as basal-NPC-medium) and freshly supplemented with 20 ng/ml bFGF and 20 ng/ml epidermal growth factor (EGF). For OPC culture, the medium (OPC-medium) consisted of either (1) basal-OPC-medium (basal-NPC-medium containing 0.1% bovine serum albumin (BSA) and 10 ng/ml Biotin) supplemented with 10 ng/ml PDGF-AA (Chemicon Inc., Temecula, CA, USA) and 10 ng/ml bFGF (referred as PDGF/bFGF), or (2) basal-OPC-medium supplemented with B104CM (30%) collected from B104 neuroblastoma cells and 10 ng/ml bFGF (referred as B104CM/bFGF). B104 cells were a generous gift from Dr. Ian Duncan (University of Wisconsin) and B104CM was prepared according to Louis's method (Louis et al. 1992).

Spinal cord-derived NPCs culture

The NPCs in suspension culture (neurospheres) were prepared from Wistar E16 rat spinal cords according to the method of Reynolds and Weiss (Reynolds and Weiss 1996, Weiss et al. 1996a) with appreciable modifications (Fu et al. 2003, 2005a). In brief, following mechanical dissociation of primary tissue to a single cell suspension, the cells were seeded into a T25 Corning tissue culture flask (Corning Inc., Corning, NY, USA) containing NPC-medium at a density of 1×10^5 cells/ml and maintained at 37°C in an incubator with 5% CO_2. Within 5–7 days, neurospheres were formed and passaged by mechanical triturating. To examine differentiation of spinal cord-derived NPCs *in vitro*, cells were seeded onto glass coverslip (12 mm in diameter, Fisher, Pittsburgh, PA, USA) coated with 200 μg/ml poly-L-lysine (PLL) on 35-mm dish (Falcon, Becton Dickinson, NJ, USA) at a density of 5×10^4 cells/coverslip and cultured in basal-NPC-medium containing 1% fetal bovine serum (FBS). The cultures were allowed to differentiate for 7 days before being fixed for immunostaining as described below.

Generation of OPCs from neurospheres

Freshly dissociated spinal cord-derived NPCs were seeded at 1×10^5/ml in basal-NPC-medium supplemented with reduced does (10 ng/ml) of bFGF/EGF to keep the neurospheres small. The cultures were fed every 2 days by removing one third of volume of the NPC-medium and adding back the same volume of fresh OPC-medium supplemented with PDGF/bFGF or B104CM/bFGF. With time, the majority of cells in the neurospheres migrated out and attached to the bottom of the flask, and the cells with bipolar or tri-polar process showed morphological characteristics of OPCs. Simultaneously, some necrotic spheres generated and floated within the medium. To enhance the purity and vitality of the attached OPCs-like cells, the medium was all replaced with fresh OPC-medium (with PDGF/bFGF or B104CM/bFGF) at this time and the cultures were allowed to proliferate until visible spheres (referred as oligospheres) of 50–200 cells/sphere had formed. Generated oligospheres were then dissociated and passaged.

Passage of OPCs using Accutase

For passaging OPCs, the supernatant fluid was gently removed and cell cultures were incubated with 1.5–2.0 ml Accutase solution (Innovative Cell Technologies, San Diego, CA, USA) for 8 min at 37°C. When OPCs/oligospheres started to detach from the bottom of the flask, 3 ml HBSS was added to each flask and cells detached entirely by gently shaking. The cell suspension was harvested, gently triturated using a fire-polished Pasteur pipette to dissociate spheres and then centrifuged at 800–1 000 rpm for 6 min at room temperature (RT). Dissociated cells were resuspended in 200 μl basal-OPC-medium and gently triturated approximately 10–20 times using a pipette. Finally, cells were reseeded into a T25 flask containing fresh OPC-medium at the density of $2\times10^4/cm^2$. On the 7th day after plating, new oligospheres were formed and dissociated by Accutase again for further passage.

Differentiation of OPCs

To induce OPCs differentiation, cells were seeded onto 200 μg/ml PLL-coated coverslips at a density of 3×10^4 cells/coverslip. For oligodendrocyte differentiation, PDGF/bFGF was withdrawn from the OPC-medium and 1% FBS, 30 μM thyroid hormone [triiodothyronine (T3)] were added. The OPCs were allowed to differentiate for 3, 4, 7, and 10 days, respectively. Antibodies against O4, O1, receptor interaction protein (Rip) and myelin basic protein (MBP) were used to identify oligodendrocytes at different developmental stages. For type 2-Astrocytes differentiation, OPCs were cultured in basal-OPC-medium supplemented with 10% FBS for 3 days. Antibodies against A2B5 and glial fibrillary acidic protein (GFAP) were used to identify type 2-Astrocytes.

[^{3}H]thymidine incorporation assay

The rate of OPC proliferation was measured by [^{3}H]thymidine incorporation assay. Dissociated oligosphere cells (at passage 3) were seeded on 96-well plate at a density of 2×10^4/well in basal-OPC-medium containing PDGF/bFGF, B104CM/bFGF or no factor (control) for 48 h, and 1 μCi/well [^{3}H]thymidine (Shanghai Institute of Atomic Nucleus, Shanghai, PR

China) was added to the medium for 18 h. After incubation, the cultures were harvested with automated multi-well harvester. [^{3}H]thymidine incorporation was determined by liquid scintillation counting. Scintillation counts were expressed as counts per minute (CPM).

Immunocytochemical detection

Before being inmmunostained, cell cultures were fixed with 4% Paraformaldehyde (PFA) in PBS (0.01M, pH7.4) for 10 min at room temperature (RT) and then blocked in 10% normal goat serum (NGS) in PBS (for cell surface markers) or 0.3% Triton X-100-containing 10% NGS in PBS (for intracellular antigens) or 10% normal donkey serum (NDS) in PBS (for O1, O4 and A2B5 staining), respectively.

To identify spinal cord-derived NPCs, Free-floating spheres were fixed with 4% PFA/PBS overnight, washed in PBS, and then cryoprotected in 30% sucrose/PBS. Spheres were embedded in OCT (Sakura Fine Tec Inc., Torrance, CA, USA) and sectioned with a cryostat. The spheres sections or 4% PFA-fixed cell cultures were blocked in above appropriate solutions for 60 min at RT and then incubated with the following first antibodies overnight at 4°C. Mouse anti-nestin antibody (1:100; Pharmingen, San Diego, CA, USA) was used as a marker to identifying undifferentiated NPCs, anti-βIII tubulin antibody (IgG, 1:800; Sigma) or anti-MAP-2 (IgG1, 1:500; Sigma) for neurons, anti-Rip antibody (IgG, 1:25; a kind gift from Dr. R. Whittemore, University of Louisville) for oligodendrocytes and anti-GFAP antibody (IgG, 1:200; Sigma) for astrocytes.

To identify OPCs and OPC-derived cell types, the following primary antibodies were used: mouse anti-rat A2B5, O4, O1 (IgM, 1:100; all from R&D, Minneapolis, MN, USA), Rip, myelin basic protein (MBP, IgG1, 1:40; Oncogene, Boston, MA, USA), GFAP and rabbit anti-rat NG2 antibody (1:200; Chemicon, Temecula, CA, USA) and rabbit anti-rat platelet derived growth factor alpha receptor (PDGFR) antibody (1:100; Neomarkers, Fremont, CA, USA).

After rinsing in PBS, slides or coverslips were incubated for 60 min at 37°C with the appropriate secondary antibody at the following dilution: the FITC-conjugated goat anti-mouse IgM (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or IgG (1:120, Sigma); the Rhodamine-conjugated goat anti-mouse IgM (1:200, Santa Cruz Biotechnology Inc.) or IgG (1:50, Cappel, Costa Mesa, CA, USA); the FITC-conjugated goat anti-rabbit IgG (1:80, Sigma). The slides or coverslips were rinsed with PBS and mounted with Gel/Mount aqueous mounting media (Biomeda Corp., Foster City, CA, USA) containing Hoechst 33342 (1 μg/ml, Sigma), a fluorescent nuclear dye.

Fluorescent images were acquired using an Olympus BX60 microscope equipped with a digital camera and SPOT 4.0.1(G) software. For cell counts, at least five randomly selected fields with more than 500 cells were counted. In all experiments, primary antibody omission controls were used to confirm the specificity of the immunofluorescence labeling.

Statistical analysis

Two-tailed *t*-test was used to determine statistical significant. Significance was calculated with *P* value of <0.05 and highly significance was determined by $P<0.01$.

RESULTS

Properties of bFGF/EGF-expended eurospheres

Neurospheres are free-floating multipotent cell clusters that express the stem cell marker nestin during proliferation and can be induced to differentiate into neuronal and glial phenotypes (Reynolds and Weiss 1996, Weiss et al. 1996a). Our results showed that spinal cord-derived NPCs formed free-floating clusters of cells when grew in bFGF/EGF-containing NPC-medium for 5–7 days (Fig. 1A). These neurosphere cells were immunopositive for nestin, an intermediate filament protein mainly expressed by stem or precursor cells (Fig. 1B) and could be passaged multiple times and cyro-preserved while being maintained in a proliferating state in culture with bFGF and EGF (Fu et al. 2003, 2005a). When the spheres were triturated into single cells and plated onto PLL-coated coverslips in basal-NPC-medium containing 1% FBS, they differentiated into astrocytes, neurons and oligodendrocytes as demonstrated by immunocytochemical detection of the specific cellular markers GFAP, MAP-2 and Rip, respectively (Fig. 1C–E).

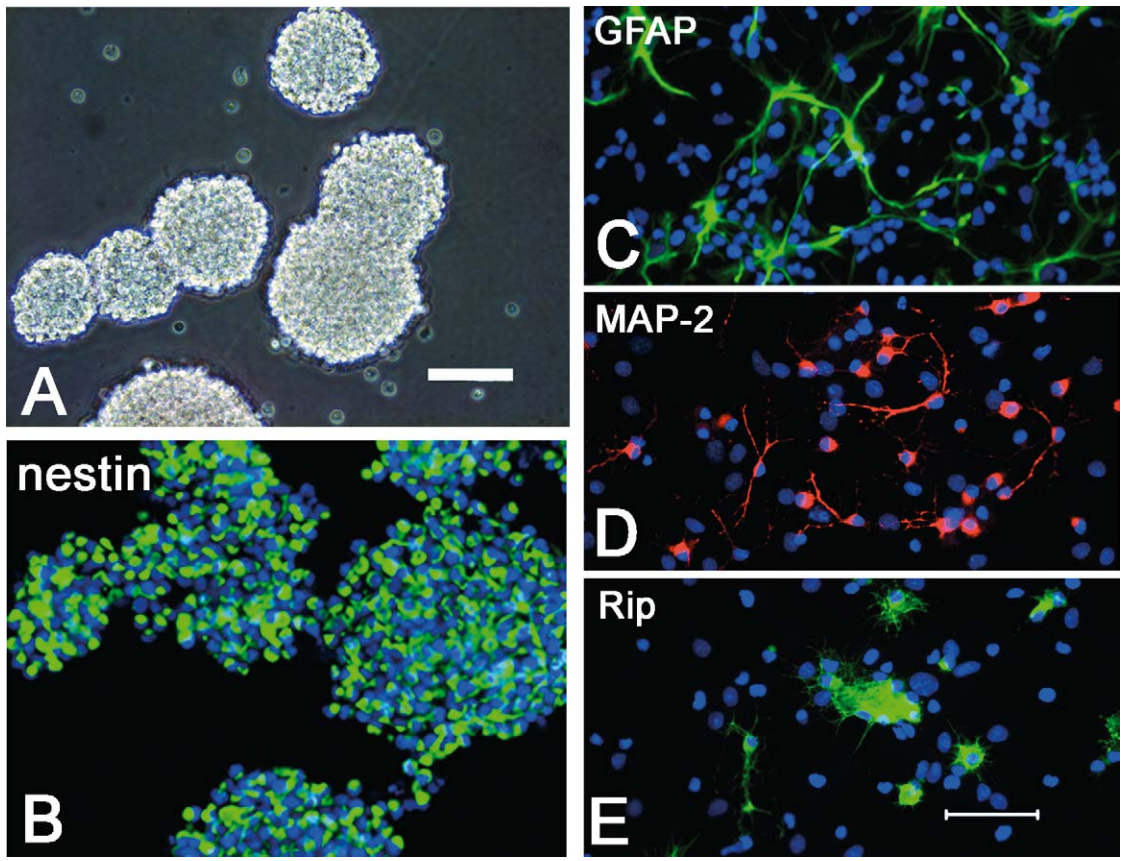

Fig. 1. Morphological and immunocytochemical characteristics of spinal cord-derived NPCs *in vitro*. (A) Phase contrast photomicrograph of spinal cord-derived neurospheres at Passage 2 grown in NPC-medium for 4 days. (B) Cells in the neurospheres were immunopositive for nestin (green). (C–E) When cultured in NPC-medium containing 1% FBS in the absence of bFGF/EGF for 7 days, NPCs differentiated into astrocytes (GFAP+, C), neurons (MAP-2+, D), and oligodendrocytes (Rip+, E). All cell nuclei were labeled with Hoechst 33342 (blue). Scale bars are 200 μm (A) and 50 μm (B–E).

PDGF/bFGF induced generation of OPCs from neurospheres

We previously reported that E16 rat spinal cord-derived NPCs could be induced to differentiate into OPCs in the presence of B104CM/bFGF. To determine whether PDGF, a major component of B104CM, can be used as a substitute for B104CM in generation of OPCs from neurospheres, freshly dissociated neurosphere cells were seeded in basal-NPC-medium supplemented with bFGF/EGF (each at 10 ng/ml) for 2 days. Within this period small neurosheres had been developed, which were loosely attached to the surface of the flask. On Day 3, the cultures were fed with PDGF/bFGF (each at 10 ng/ml)-supplemented OPC-medium by replacing one third of the NPC-medium every 2 days. With time in this transition medium, many single cells detached from neurospheres and migrated onto the substrate. These cells displayed bipolar or tripolar morphology, as seen in Fig. 2A. After active cell migration, the remaining spheres gradually contained mainly dead cells and cell fragments, lost their 3-dementional morphology, and then lifted from the bottom of the flasks. At this time (7-day postplating), these degenerating spheres were removed from the culture so that only healthy cells that attached to the bottom of flask were maintained (Fig. 2B). The adhering cells were allowed to grow in fresh OPC-medium with PDGF/bFGF for another 7 days until oligospheres had formed (Fig. 2C). To compare the effect of PDGF/bFGF with that of B104CM/bFGF, parallel cell cultures were fed with B104CM/bFGF-supplemented OPC-medium as described above at the same time. The generating oligospheres were then dissociated into single cells using Accutase and the number of cells in different growth factors-containing medium was quantified. As

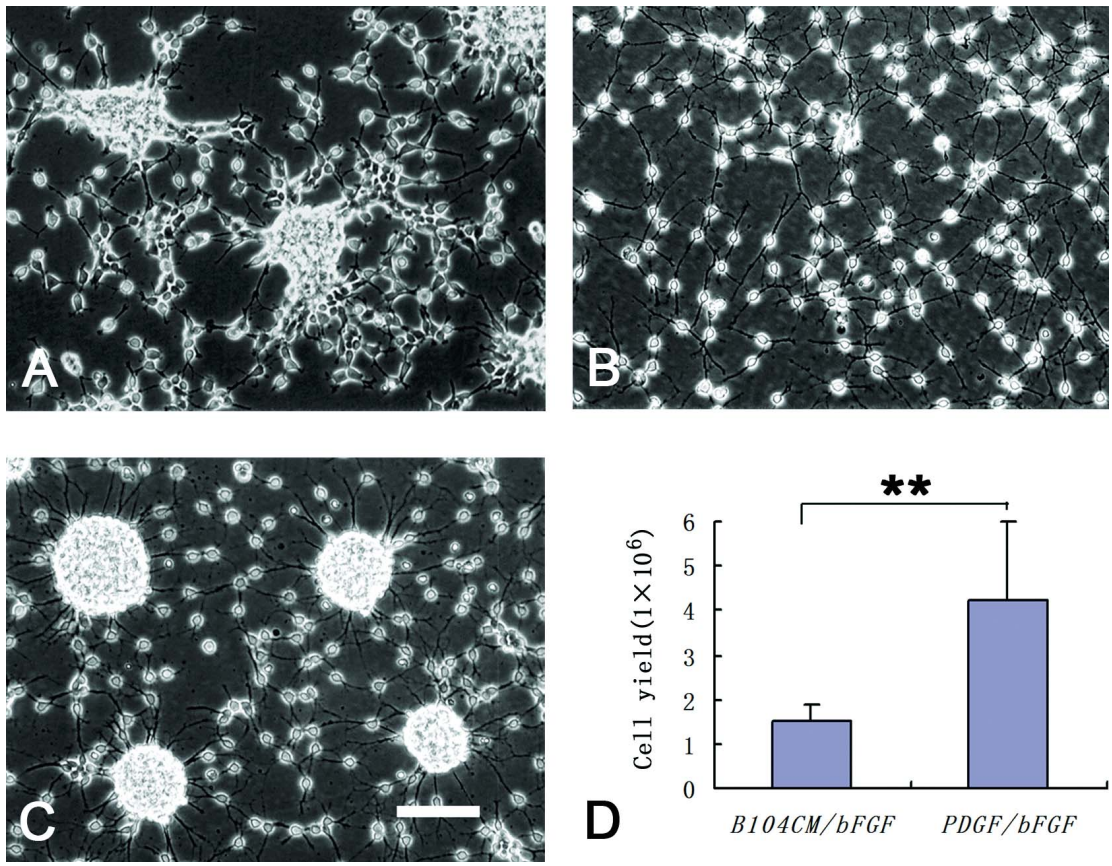

Fig. 2. Generation of OPCs from neurospheres. (A) After culture medium was changed from NPC-medium to PDGF/bFGF-containing OPC-medium for 4 days, many single cells detached from neurospheres and migrated out onto the substrate of flask. (B) Adhering cells with bipolar or tri-polar processes remained in the bottom of flask after the removal of degenerated spheres. (C) After 2 weeks in the presence of PDGF/bFGF, new oligospheres formed along with bipolar or tri-polar OPCs attaching to the bottom of flask. (D) Comparison of cell yields of OPCs from neurospheres between treatments with B104CM/bFGF and PDGF/bFGF (**P<0.01).

presented in Figure 2D, the number of oligosphere cells in the presence of PDGF/bFGF (4.21×10^6) was 2.77-fold greater than that in the presence of B104CM/bFGF (1.52×10^6), even though the neurosphere cells were originally seeded at the same density. These results indicate that both PDGF/bFGF and B104CM/bFGF could induce spinal cord-derived NPCs to differentiate into OPCs *in vitro*. However, the PDGF/bFGF combination is more efficient in generating OPCs from NPCs than the B104CM/bFGF combination (4.21×10^6 *versus* 1.52×10^6, P<0.01).

Morphological and immunocytochemical characteristics of PDGF/bFGF-induced OPCs

OPCs generated in this culture system were identified by morphological and immunocytochemical analyses using the following various antibodies: NG2, A2B5, PDGFR, nestin, O4, GFAP and βIII-tubulin. As is shown in Fig. 3 (A–F), the PDGF/bFGF-induced OPCs displayed typical bipolar or tripolar morphology and expressed the markers for OPCs including A2B5 (94.36 ± 4.59%), NG2 (93.63 ± 3.37%) and PDGFR (90.35 ± 1.95%). PDGFR-positive cells were also recognized by nestin antibody, but not neuronal marker βIII-tubulin. A smaller percentage (17.86 ± 1.89%) of O4 expressing cells was also detected. In addition, a small percentage of cells expressed GFAP (5.81 ± 3.4%) and was double-stained with PDGFR (data not shown). These data suggest that the current method with the combination of PDGF and bFGF is sufficient to generate highly purified OPCs from the E16 rat spinal cord-derived NPCs (Fig. 3E).

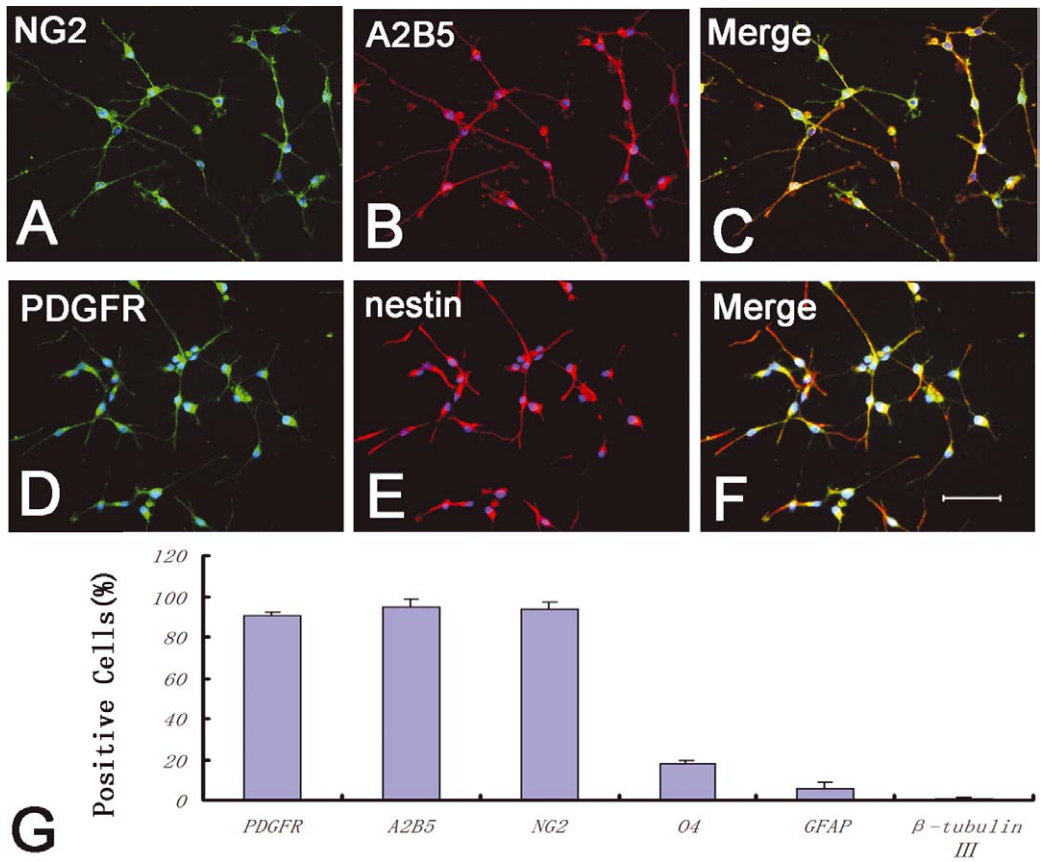

Fig. 3. Immunocytochemical characteristics of OPCs. Dissociated OPCs were seeded onto coated coverslips at a density of 3×10^4 cells/coverslip in PDGF/bFGF-containing OPC-medium for 48 h. (A–C) Double immunostaining for NG2 and A2B5 showed that OPCs were NG2+ (green, A) and A2B5+ (red, B). (D–F) Double immunostaining showed that PDGFR-positive cells (green, D) were also recognized by the nestin antibody (red, E). (C) and (F) show merge pictures. All cell nuclei were labeled with Hoechst 33342 (blue). Scale bar is 50 μm (A–F). (G) Quantitative data show percentages of OPCs expressing each antigen. Error bars indicated SD of three independent experiments.

Enhance proliferation of OPCs by PDGF/bFGF

To examine the proliferation rate of OPCs, dissociated oligosphere cells (5×10^5) were seeded into a T25 flask in 5 ml fresh PDGF/bFGF-containing OPC-medium for multiple passages. Every 7 days, newly formed oligospheres were dissociated and passaged by using Accutase and reseeded at the same density. The mean proliferation rate of OPCs in two different factor combinations, i.e. PDGF/bFGF and B104CM/bFGF, is shown in Fig. 4A. In the presence of PDGF/bFGF, the proliferation rate of OPCs yielded a 435.47-fold (from P0 5×10^5 to P5 218×10^6) within 35 days, compared to a 99.03-fold expansion (from P0 5×10^5 to P5 49.52×10^6) when B104CM/bFGF was applied within the same time period. And the statistically significant differences between the two combinations were found at passages 2, 3, 4 ($P<0.01$) and passages 5 ($P<0.005$). PDGF/bFGF also significantly increased thymidine incorporation of OPCs by 1.92-fold compared to B104CM/bFGF (77 103.5 ± 11 991.70 CPM *versus* 40 105.7 ± 9 146.76 CPM, $P<0.01$), and 14.70-fold compared to the control (77 103.5 ± 11 991.70 CPM *versus* 5 243.33 ± 4 206.55 CPM, $P<0.005$) (Fig. 4B). These results indicate that PDGF/bFGF is a more potent combination of factors than the B104CM/FGF in promoting proliferation of OPCs.

Differentiation potential of PDGF/bFGF-induced OPCs

To determine the differentiation capacity of PDGF/bFGF-induced OPCs *in vitro*, OPCs were plated onto PLL-coated coverslips and parallel cultures

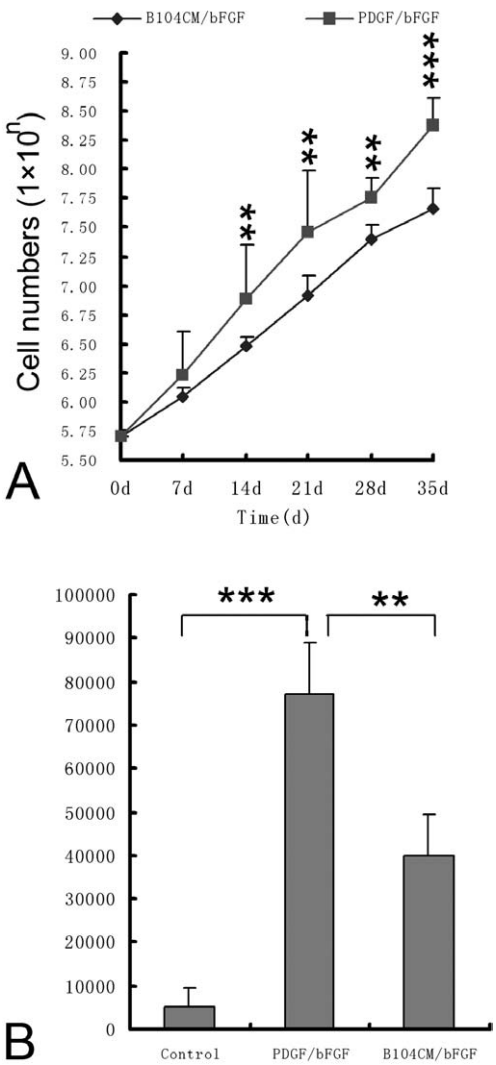

Fig. 4. Comparison of OPC proliferation rates in different growth factor combinations. (A) Comparison of estimated total numbers (1×10^n) of OPCs grown in medium containing either B104CM/bFGF or PDGF/bFGF for 35 days. For every 7 days, oligospheres were dissociated and reseeded with a density of 1×10^5 cells/ml in 5 ml medium (***P*<0.01, ****P*<0.005). (B) [^{3}H]thymidine incorporation assay showed a significant increase in [^{3}H]thymidine incorporation in the PDGF/bFGF-induced OPC group compared to the B104CM/bFGF or no growth factor groups. Values represent the average CPM for each culture condition. Error bars indicated SD of three independent experiments. (vs. B104CM/bFGF or no growth factor group, ***P*<0.01, ****P*<0.005).

were maintained in 1% FBS/T3 or 10% FBS-containing basal-OPC-medium to induce their differentiation either into oligodendrocytes or type-2 astrocytes, respectively. In the presence of 1% FBS/T3, OPCs readily differentiated into O4, O1, Rip and MBP-immunoreactive cells (Fig. 5A–D). All cells displayed typical highly branched morphology of oligodendrocytes. Quantitative data showed that the percentage of O4, O1, Rip and MBP-positive cells changed along with the *in vitro* differentiation of oligodendrocytes (Fig. 5E). Generally speaking, the percent labeling of immature oligodendrocyte markers such as O4 decreased with time whereas the percent labeling of more mature oligodendrocyte markers such as MBP increased correspondingly. In the presence of 10% FBS, over 95% of the cells were flattened with multiple processes and positive for both A2B5 and GFAP (Fig. 5F–H). These cells were morphologically and immunocytochemically type-2 astrocytes. No neurons were observed under these differentiating conditions. Thus, OPCs induced by PDGF/bFGF were bipotential and could differentiate into both oligodendrocytes and type-2 astrocytes. This pattern of differentiation was similar to that seen in O-2A cells or the CG-4 cell line (Avellana-Adalid et al. 1996, Hunter and Bottenstein 1991, Liu et al. 2000, Raff et al. 1983).

DISCUSSION

We previously demonstrated that the E16 rat spinal cord-derived NPCs could be induced to differentiate into OPCs using a combination of B104CM and bFGF (Fu et al. 2005b, Hu et al. 2004). These OPCs appeared to be identical to O-2A cells and the CG-4 cell line in terms of morphological characteristics, antigenic profiling and differentiation potentials as reported previously (Avellana-Adalid et al. 1996, Hunter and Bottenstein 1991, Raff et al. 1983). However, the use of B104CM may not be appropriate for clinical application since many of the B104CM components are unknown. In addition, the biological activities of B104CM may vary between different preparations since the number and status of B104 cells are hard to control for each preparation. In the present study, we examined the possibility that the effect of B104CM can be substituted by PDGF, a factor that plays a central role in OPC differentiation (Asakura et al. 1997, Baron et al. 2005). The main advantage of using the current method is that the exact composition and concentration of each growth factor required for OPC induction are known and can be precisely administered.

One advantage of using the PDGF/bFGF combination is that it generates significantly high numbers of OPCs than that of B104CM/bFGF, when the neurosphere cells were originally seeded at the same densi-

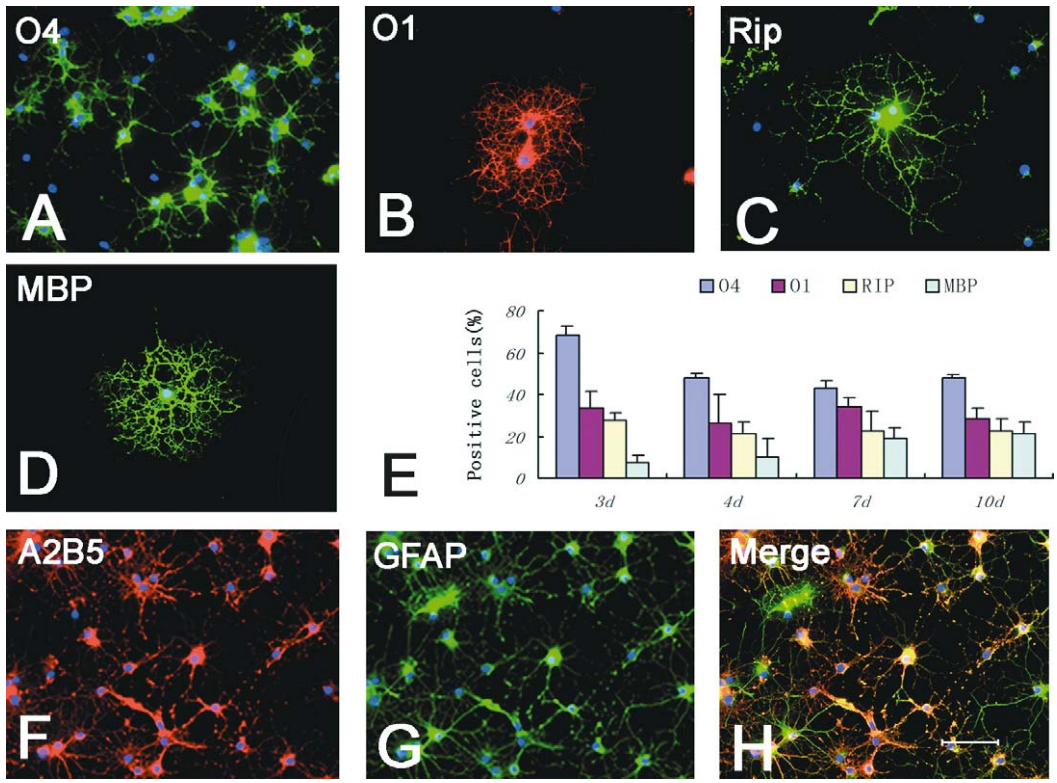

Fig. 5. Immunocytochemical identification of OPCs differentiation potential. (A–D) In the presence of 1% FBS/T3, OPCs readily differentiated into oligodendrocytes and expressed O4 (A), O1 (B), Rip (C), and MBP (D). (E) Quantitative data show the percent marker expression *versus* days *in vitro* of OPCs differentiation. (F–H) In the presence of 10% FBS, over 95% of cells were flattened with star-shaped morphology and were positive for A2B5 (F) and GFAP (G), characteristics of type 2-astrocytes. All cell nuclei were labeled with Hoechst 33342 (blue). Scale bar is 50 μm (A–D, F–H).

ty (Fig. 2D). And the proliferating rate of OPCs was much higher after PDGF/bFGF induction than the B104CM/bFGF induction (Fig. 4A). Since there was a significant increase in [^{3}H]thymidine incorporation in OPCs after PDGF/bFGF administration (Fig. 4B), we attribute the higher yield of OPCs in the presence of PDGF/bFGF to enhanced proliferation of OPCs rather than enhanced survival of these cells.

To ensure the success of OPC induction from neurospheres, several methodological improvements were made in the present study. First, we cultured NPCs in reduced does of bFGF/EGF (10 ng/ml) for 48 h before induction to keep the neurospheres small. If neurospheres were too large, the PDGF/bFGF in medium would not be accessible to cells in the center of neurospheres. This would result in massive cell death and therefore affect the efficiency of OPC induction. Thus, to select an appropriate neurosphere size for generating OPCs is critical. Second, it is important to avoid subtle changes of the culture condition. In this study, the transition of the culture medium from the NPC-medium to OPC-medium was carried out gradually. This is because both NPCs and OPCs are sensitive to culture condition. When neurospheres cultured in NPC-medium were switched abruptly to an OPC-medium, massive cell death could be induced. We thus replaced only one third of the bFGF/EGF-containing NPC-medium with the same volume of fresh OPC-medium containing PDGF/bFGF every two days. Until the majority of cells in the neurospheres had migrated out and bestrewed the bottom of the flask, all of the culture medium was removed and 5 ml fresh OPC-medium was added. With replacement of the medium, the debris, dead cells and degenerating neurospheres were removed, leaving only healthy cells in the environment. This procedure is important for improving the purity and vitality of OPCs. Third, to prevent OPC processes from damage during passaging, we used the enzyme Accutase instead of classical trypsin and mechanical trituration to dissociate oligospheres. We prefer not to use trypsin to dissociate OPCs because it is difficult to control the concentration and timing of trypsin dissociation. At low concentration,

trypsin may be insufficient to detach adherent cells whereas at high concentration, the enzyme may damage receptors and adhesion molecules and therefore affect cellular responses to corresponding nutrition factors and ultimately oligosphere formation. Accutase is a proprietary formulation containing proteolytic and collagenelytic activities which was developed for rapid dissociation of cells with high viability such as those used for FACS analysis (Wachs et al. 2003). We found that Accutase not only efficiently lifted the adherent cells from the bottom of the flask but also dissociated cells from oligospheres. As a result, the vitality and yield of OPCs were enhanced greatly after Accutase dissociation. We also found that mechanical dissociation with repetitive pipetting should be reduced. This is because OPCs possess many symmelrical bipolar or tri-polar processes. When OPCs proliferate and form oligospheres, their processes may twist with each other and repetitive pipetting may result in the damage of their processes and, therefore, cell death.

Finally, we found that the source of NPCs was a critical factor that affects OPC induction. Our previous study reported that NPCs isolated from E16 rat brain and spinal cord represented two distinct populations of precursor cells that exhibited different morphology, growth factor-responsiveness, and differentiation potentials (Fu et al. 2005a). After 10 days *in vitro*, more oligodendrocytes were generated from spinal cord-derived NPCs than from brain-derived ones (data not shown). It is possible that spinal cord-derived neurospheres contain more glia-restricted progenitors which can be induced more easily to differentiate into OPCs. Indeed, we found that, in the same culture condition, the number of oligosphere cells generated from spinal cord-derived neurospheres was greater than that from brain-derived neurospheres (data not shown). Also, the time required for the induction of OPCs from spinal cord-derived neurospheres was also shorter than that from brain-derived ones (data not shown).

CONCLUSION

In conclusion, the present study reported an efficient and reliable method to generate large quantities of highly purified OPCs from NPCs isolated from the E16 rat spinal cord using a combination of PDGF and bFGF. These OPCs can be passaged for many generations and maintain their morphological and immunocytochemical properties. OPCs obtained through this method would be useful in studies of oligodendrocyte cell biology as well as cellular transplantation for treating many CNS diseases.

ACKNOWLEDGMENTS

The authors are grateful for the support from the 973 Project (2003CB515302), Shanghai Science and Technology Developing Foundation (00JC14021), and Shanghai Educational Committee Technology Foundation (99ZD08). We are also grateful for the Daniel Heumann Fund for Spinal Cord Research, the Kentucky Spinal Cord and Head Injury Research Trust (nr 4-16) and the James R. Petersdorf Endowment Funds (the Norton Healthcare, Kentucky Spinal Cord and Head Injury Research Trust Board, Bucks for Brains of State of Kentucky, and University of Louisville). We thank Animal Care Center at Shanghai Jiaotong University School of Medicine for providing the pregnant rats.

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Received 15 May 2007, accepted19 July 2007