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Research report

Isolation and culture of human oligodendrocyte precursor cells from neurospheres

Yabin Lu[a,b,1], Yinxiang Yang[a,1], Zhaoyan Wang[a], Caiying Wang[a], Qingan Du[a], Qian Wang[a], Zuo Luan[a,*]

[a] Department of Pediatrics, Navy General Hospital, No. 6, Fucheng Road, Beijing 100048, China
[b] Department of Biochemistry and Molecular Biology, Capital Medical University, Beijing 100069, China

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ABSTRACT

Culture of human oligodendrocyte precursor cells (OPCs) can help understand the regulatory mechanism of differentiation and myelination of oligodendrocytes. However, existing culture methods have limitations, particularly the lack of a source of human donor tissue and high cost. We sorted cells with the $A_2B_5^+$PSA-NCAM$^-$ phenotype from neurospheres instead of human donor tissues through immunomagnetic sorting and subsequently cultured the isolated cells in OPC medium. Of all the isolated cells, 15.69% were of the $A_2B_5^+$PSA-NCAM$^-$ phenotype. More than 90% of the isolated OPCs expressed the OPC-specific markers O4, PDGFαR, and Sox10, and less than 5% of cells expressed GFAP and Tuj-1. After induction, the isolated cells had the capacity to differentiate into oligodendrocytes. Furthermore, the OPCs could be stably passaged *in vitro* for at least four generations and all the cells had high expression levels of O4 and Sox10 and very low expression levels of GFAP and Tuj-1; moreover, the cells had the capacity to differentiate into oligodendrocytes. After four passages, OPCs can proliferate at least 14 times above. In addition, in the presence of B27, only one cytokine, namely, bFGF, was sufficient to maintain proliferation, and this greatly reduced the experimental cost. Cells of the $A_2B_5^+$PSA-NCAM$^-$ phenotype have already been identified as OPCs. We developed and characterized a reproducible, simple, and economical method for the isolation and culture of human OPCs. This method will contribute to studying the function of OPCs in development, disease, and treatment.

1. Introduction

Oligodendrocytes (OLs) are cells derived from oligodendrocyte precursor cells (OPCs) and occur in the central nervous system (CNS). They play a crucial role in myelin sheath formation and facilitation of rapid conduction of neuronal action potentials (Miller, 1996). OPCs differentiate through a series of developmental stages, such as pro-OLs, immature OLs, and mature OLs (Stangel and Hartung, 2002). If OPCs are injured during development or remyelination, myelination of brain white matter is delayed or damaged, which can lead to nerve function deficit (Lin and Bergles, 2004; Nishiyama, 1998). OPC differentiation has been found to have important significance in the promotion of myelin repair (Artemiadis and Anagnostouli, 2010; Wu and Ren, 2009). Therefore, understanding the mechanism by which OPC differentiation is regulated would provide insights into the process of myelin-associated diseases. It is important to establish a method for the in vitro isolation, amplification, and differentiation of OPCs.

The specific immunophenotype of OPCs is distinct from those of the human fetal CNS tissue and adult human white matter. Several methods have been described for the isolation of human OPCs from the CNS, such as fluorescence-activated cell sorting (FACS) by exploiting cell surface-specific antigens, immunomagnetic sorting, or a combination of the two (Dincman et al., 2012; Windrem et al., 2004). Because the source of human donor tissue is very limited, it is difficult to obtain a large number of cells for research. Another method for obtaining human OPCs is by neural stem cell- or embryonic stem cell-induced directional differentiation. This method often needs complicated procedures of induction. Furthermore, obtaining OPCs of high purity via this method takes at least 21 days (Monaco et al., 2012); therefore, the experimental cost increases, limiting the potential productivity of OPCs. Therefore, a simple and economical method for the isolation and culture of human OPCs is needed to overcome these limitations.

* Corresponding author. Fax: +86 10 66958303.
E-mail address: luanzuo@aliyun.com (Z. Luan).
[1] These authors contributed equally to this work.

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In this study, we described a simple and reproducible method for the isolation and culture of human OPCs. OPCs were isolated from neurospheres derived from human fetal brains by immunomagnetic sorting. Neurospheres were selected as the source of OPCs because they can be stably passaged *in vitro*; thus, they can serve as a stable source of sorting cells. First, we used microbeads to deplete polysialylated neural cell adhesion molecule (PSA-NCAM)$^+$ neurons. Then, $A_2B_5^+$ cells were isolated from the larger PSA-NCAM$^-$ cell population via magnetic sorting. Previous studies have reported subpopulations of $A_2B_5^+$PSA-NCAM$^-$ marker cells in brain tissues, and this cell subpopulation has been identified to be OPCs, which exhibit extensive myelin production in shiverer mice (Strathmann et al., 2007; Windrem et al., 2004). After sorting from neurospheres, the cells showed stably high expression levels of O4, NG2, and Sox10 and very low expression levels of GFAP and Tuj-1. After induction, the cells differentiated into oligodendrocytes, indicating the oligodendrocytic bias of the sorted cells. Furthermore, these cells can be stably cultured and passaged at least four times and proliferate at least 14 times *in vitro*. Therefore, we can use this reproducible and simple method to stably isolate and culture human OPCs in vitro.

2. Materials and methods

2.1. Reagents and supplies

All reagents and supplies were obtained commercially. Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, C11330500BT), Neurobasal-A medium (Gibco, 10888-022), N2 supplement (Gibco, 17502-048), B27 supplement (Gibco, 17504-044), recombinant human FGF-basic (PeproTech, AF-100-18B), recombinant human EGF (PeproTech, AF-100-15), heparin (Sigma, H3149), penicillin/streptomycin (Invitrogen, 15140), 0.25% trypsin (Gibco, 15050-065), trypsin inhibitor (Sigma, T6522), L-glutamine (Gibco, 35050-061), ethylenediaminetetraacetic acid (EDTA) disodium salt dehydrate (Amresco, 6381-92-6), Poly-D-lysine hydrobromide (Sigma, p6407), laminin (Invitrogen, 23017-015), OPC differentiation medium (OPCDM; ScienCell, 1631), cell strainer (BD Falcon, 352340), MS Column (Miltenyi Biotec, 130-091-506), fetal bovine serum (FBS; Gibco, 10099-141), anti-PSA-NCAM microbeads (Miltenyi Biotec, 130-092-966), anti-PSA-NCAM antibodies (Miltenyi Biotec, 130-093-273), anti-A2B5 microbeads (Miltenyi Biotec, 130-093-388), Anti-A2B5 antibodies (Miltenyi Biotec, 130-093-581), mouse anti-O4 antibodies (1:300 dilution; R&D, MAB1326), mouse anti-Sox10 (R&D, MAB2864, 1:50), rabbit anti-GFAP (Invitrogen, 18-0063, 1:80), mouse anti-Tuj-1 (1:500 dilution; Abcam, ab7751), rabbit anti-Galc (Millipore, AB142, 1:50), rabbit anti-PDGFαR antibodies (1:400 dilution; Cell Signaling, 5241), Alexa Fluor 488-AffiniPure Goat Anti-Mouse IgG + IgM (H + L) (1:800 dilution; Jackson Immunoresearch, 111-545-042), and Alexa Fluor 594-AffiniPure goat anti-rabbit IgG (H + L, 1:800 dilution; Jackson Immunoresearch, 111-585-144).

2.2. Neurosphere culture

Neurospheres were cultured and passaged using the methods described previously (Tarasenko et al., 2004). Human fetal CNS tissues obtained from 10- to 13-week-old embryos were provided by Navy General Hospital, Beijing, China. All women had requested to terminate their pregnancy. After being fully informed according to the guidelines approved by the Ethics Committee of the hospital, they consented to donate the aborted fetuses. Surface regions of the cortex were randomly chosen. Cortex tissues were dissected and mechanically dissociated by repetitive blowing into a single-cell suspension. Primary cells were cultured at a density of 1×10^6 cells/mL in primary culture medium containing DMEM/F12, 1% L-glutamine, 1% N2 supplement, 2% B27 supplement, 20 ng/mL bFGF, 20 ng/mL EGF, 5 μg/mL heparin, and 1% penicillin/streptomycin. Every three to four days, two-thirds of the medium was removed and replaced with fresh primary culture medium. The expanded cells formed colonies and were cultured as free-floating "neurospheres" after 5–7 days of culture. As not all the cells in the tissues had survived, the cells that remained alive after dissection and mechanical dissociation could form neurospheres. The neurospheres were passaged every 10–12 days using 0.025% trypsin. The reaction was stopped by using 1.2 mg/mL of trypsin inhibitor. Neurospheres were repeatedly blown in order to obtain single-cell suspensions. The resultant cells were seeded into a flask, and the medium from passage 1 was used but without B27 supplement.

2.3. Magnetic cell sorting (MACS) for OPC

Cells were sorted according to the instructions provided by microbeads. Neurospheres were digested into single cells using 0.025% trypsin solution. Cells were passed through a 40 μm nylon mesh to remove cell clumps, which may clog the column. The cell number was then determined, and the cell suspension was centrifuged at $300 \times g$ for 10 min. The supernatant was completely aspirated, and 10^7 cells were resuspended in 60 μL of MACS buffer, which consisted of phosphate-buffered saline (PBS; pH 7.2), 0.5% FBS, and 2 mM EDTA. The suspension was mixed well and incubated for 10 min in the refrigerator (2–8 °C). Then, 20 μL of anti-PSA-NCAM microbeads were added to the cell suspension containing 10^7 cells, mixed well, and incubated for 15 min in the refrigerator. The cells were washed by adding 1–2 mL of MACS buffer and centrifuged at $300 \times g$ for 10 min. During centrifugation, the MS column was placed in the magnetic field of a suitable MACS Separator and equilibrated with 0.5 mL of MACS buffer. The supernatant was completely aspirated and the cells (up to 10^8 cells) were resuspended in 500 μL of MACS buffer. The cell suspension was applied on to the column. Unlabeled cells that passed through the column were collected, and the column was washed with the appropriate amount of buffer. The total effluent, *i.e.*, the unlabeled cell fraction, was then collected from the column. Washing was carried out three times by adding 500 μL of MACS buffer. The cell suspension was centrifuged at $300 \times g$ for 10 min. The supernatant was completely aspirated, and the cell pellet (10^7 cells) was resuspended in 60 μL of MACS buffer. Then, 20 μL of anti-A2B5 microbeads were added for 10^7 cells and the mixture was incubated for 15 min in the refrigerator. This mixture was passed through the MS column and washed thrice according to the same procedure. The column was then removed from the separator, placed on a suitable collection tube, and 1 mL of buffer was pipetted on to the column. The magnetically labeled cells were immediately flushed by firmly pushing the plunger into the column. The cell suspension was then centrifuged at $300 \times g$ for 10 min to obtain the A2B5$^+$PSA-NCAM$^-$ cell population.

2.4. Culture, passage, and differentiation of OPCs

OPCs were plated into a flask at a density of 4×10^4 cells/cm^2 and cultured in OPC medium for propagation. OPC medium was prepared by adding 2% B27 supplement (2%), 20 ng/mL bFGF, 1% penicillin/streptomycin, 5 μg/mL heparin, and 2 mM L-glutamine to Neurobasal-A medium. Every three days, two-thirds of the medium was removed and replaced with fresh OPC medium. After 7–10 days of proliferation, the OPCs reached 80–90% confluence. They were gently blown with a pipette and plated into a flask.

For differentiation, the OPCs were plated on to PDL/laminin-coated well plates at a density of 4×10^4 cells/cm^2 and cultured