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METHODS AND REAGENTS

Isolation of human adult olfactory sphere cells as a cell source of neural progenitors☆

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Abstract
Olfactory stem cells are generated from olfactory mucosa. Various culture conditions generate olfactory stem cells that differ according to species and developmental stage and have different progenitor or stem cell characteristics. Olfactory spheres (OSs) are clusters of progenitor or stem cells generated from olfactory mucosa in suspension culture. In this study, adult human OSs were generated and their characteristics analyzed. Human OSs were adequately produced from olfactory mucosa with area over 40 mm^2. Immunocytochemistry (ICC) and fluorescence-activated cell sorting showed that human OSs were AN2 and A2B5-positive. Immunofluorescence analysis of cell type-specific ICC indicated that the number of Tuj1-positive OS cells was significantly elevated. Tuj1-positive cells displayed typical neuronal soma and dendritic morphology. Human OS cells were also immunopositive for MAP2. By contrast, few RIP-, O4-, and GFAP-positive cells were present. These RIP, O4, and GFAP-positive cells did not resemble bona fide oligodendrocytes and astrocytes morphologically. In culture to induce differentiation of oligodendrocytes, human OS cells also expressed neuronal markers, but neither oligodendrocyte or astrocyte markers. These findings suggest that human OS cells autonomously differentiate into neurons in our culture condition and have potential to be used as a cell source of neural progenitors for their own regenerative grafts, avoiding the need for immunosuppression and ethical controversies.

Abbreviations: A2B5, A2B5 antigen; AN2, AN2 proteoglycan; CNS, central nervous system; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's modification of Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; ICC, immunocytochemistry; MAP2, microtubule associated protein 2; NF, neurofilament; Olig2, oligodendrocyte transcription factor 2; OPC, oligodendrocyte progenitor cell; OSCs, olfactory sphere cells; PFA, paraformaldehyde; TH, tyrosine hydroxylase; Tuj1, neuron specific class III beta-tubulin; RIP, receptor interacting protein.

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Introduction

The olfactory neurons renew itself throughout life and have potential application in central nervous system (CNS) repair (Barnett and Riddell, 2004; Li et al., 1997; Roisen et al., 2001). Olfactory mucosa is easily accessible, and can be obtained by a simple biopsy performed through the external nares (Feron et al., 1998). Olfactory stem cells are generated from olfactory mucosa (Murdoch and Roskams, 2008; Murrell et al., 2005; Tome et al., 2009). Various culture conditions generate olfactory stem cells that differ according to species and developmental stage and have different progenitor or stem cell characteristics (Lindsay et al., 2013; Murdoch and Roskams, 2008; Murrell et al., 2005; Tome et al., 2009; Zhang et al., 2004). Murrell et al. reported that cells from human olfactory mucosa generate neurospheres that are multipotent *in vitro* and human olfactory neurospheres gave rise to neurons and glia and self replicating (Murrell et al., 2005, 2008).

Olfactory spheres (OSs) are clusters of progenitors or stem cells generated from olfactory mucosa in suspension culture. We previously reported that adult rat olfactory sphere cells (OSCs) were generated, expressed oligodendrocyte progenitor cell (OPC) markers, and differentiated into oligodendrocytes *in vivo* and *in vitro* (Ohnishi et al., 2013). Under standard culture conditions, adult rat OSCs rarely differentiate into neurons. In the present study, human adult OSCs were generated and their characteristics investigated. Human adult OSCs also expressed OPC markers, but they were directed toward neuronal differentiation in our culture condition. These findings suggest that human OSCs have potential to be used as a cell source of neural progenitors.

Materials and methods

Experimental design

This study was approved by the Ethics Committee of the Osaka University Medical School in Osaka, Japan. All procedures were performed after obtaining written, informed consent, which included permission to culture and analyze a biopsy from the tissue to be grafted. Human olfactory mucosa was obtained from surplus mucosa, which was not used for transplantation surgery into spinal cord lesions (Iwatsuki et al., 2013; Lima et al., 2010, Lima et al., 2006).

Collection of olfactory mucosa in humans

The otolaryngologists harvested the olfactory mucosa. A transnasal endoscopic approach and instrumentation were used. After cleaning the nasal and olfactory space, vasoconstrictors were injected into the mucosa. A submucoperiosteal tunnel was created in the medial nasal septal side of the olfactory groove and superior concha. Reabsorbable packing was placed in the olfactory groove to avoid postoperative nasal bleeding.

Olfactory sphere culture

The procedure of generating human OSs essentially followed that reported by Ohnishi et al. (Ohnishi et al., 2013). In brief, the olfactory mucosa was carefully dissociated mechanically and then treated for 60 min at 37°C with an enzyme solution comprised of collagenase (Wako, Osaka, Japan), dispase (Sanko Junyaku, Tokyo, Japan), DNase I (Sigma-Aldrich, St Louis, MO), and hyaluronidase (Sigma-Aldrich) in DMEM/F12 medium (DF; Invitrogen Corporation, Carlsbad, CA). Culture dishes were coated with poly(2-hydroxyethyl methacrylate) (Sigma-Aldrich, P3932) to prevent attachment of cells to the bottom of the dish. Dissociated cells were plated onto poly(2-hydroxyethyl methacrylate)-coated dishes in DF medium containing 10% FBS (Gibco), B27 supplement (Invitrogen), 20 ng/mL basic fibroblast growth factor (Sigma-Aldrich), 20 ng/mL epidermal growth factor (Sigma-Aldrich), and antibiotic-antimycotic solution (Invitrogen, 15240) at approximately 5×10^6 cells per 10-cm dish. Cultures were incubated at 37°C in a 5% CO_2 atmosphere, and the medium was changed every 3 to 4 days. Cell clusters were classified as OSs if they were spherical in shape and had a diameter of at least 50 μm (Fig. 1A).

Differentiation culture

After 10 to 14 days in culture, OSs were treated with trypsin-EDTA (Invitrogen), plated onto poly-L-lysine/laminin-coated 4-well chamber slides (Becton Dickinson, Franklin Lakes, NJ) at approximately 1×10^3 cells per well, and cultured for 14 to 21 days in DF medium supplemented with 10% FBS (Gibco), N2 (Invitrogen), B27, and antibiotic-antimycotic solution. To induce differentiation into oligodendrocytes, OSCs were cultured in Sato differentiation medium (DMEM supplemented with 2 mM glutaMAX-I, 1 mM sodium pyruvate, 30 nM 3,5,3′-triiodothyronine, 30 nM thyroxine, 1% N2 supplement, 50 U/mL penicillin and 50 μg/mL streptomycin) for 14 to 21 days.

Immunocytochemistry

OSCs were fixed with 4% paraformaldehyde (PFA) and incubated for 1 h at room temperature in blocking solution, incubated overnight at 4°C with the primary antibody, washed, and incubated overnight at 4°C in the appropriate species-directed secondary antibody. The primary antibodies were as follows: anti-AN2 biotin conjugated (1:11; Miltenyi Biotec Inc., Auburn, CA), anti-A2B5 biotin conjugated (1:11; Miltenyi Biotec Inc.), anti-β3-tubulin (Tuj1, 1:200 mouse monoclonal antibody; Abcam, Cambridge, UK or 1:200 rabbit polyclonal antibody; Abcam), anti-glutamic acid decarboxylase 67 (GAD67, 1:500 mouse monoclonal antibody; Millipore), anti-glial fibrillary acidic protein (GFAP, 1:300 mouse monoclonal antibody; Cell Signaling Technology), anti-microtubule-associated protein 2 (MAP2, 1:200 rabbit polyclonal antibody, Abcam), anti-neurofilament-L (NF, 1:100 rabbit monoclonal antibody; Cell Signaling Technology), anti-receptor-interacting protein (RIP, 1:100 rabbit monoclonal antibody; Cell Signaling Technology), anti-oligodendrocyte transcription factor 2 (Olig2, 1:300 sheep polyclonal antibody; Abcam), and anti-O4 (1:200 mouse monoclonal; Neuromics, Edina, MN), anti-tyrosine hydroxylase (TH, 1:100 rabbit polyclonal antibody; Abcam).

The secondary antibodies used were anti-biotin FITC conjugated (1:100; Miltenyi Biotec Inc.), DyLight 488-conjugated goat