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Culturing of Specialized Glial Cells (Olfactory Ensheathing Cells) of Human Olfactory Epithelium

E. A. Savchenko*, N. A. Andreeva, T. B. Dmitrieva*, I. V. Viktorov*,**, and V. P. Chekhonin***

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A monolayer of dissociated glial cells of human olfactory epithelium was cultured in Petri dishes and 12-well plates using a polylysine-laminin substrate. Primary cultures were subcultured after 10-15 days. The cell cultures were analyzed by phase contrast microscopy at all stages of culturing. A cytological study involved histological methods (trypan blue staining) and immunocytochemical visualization of GFAP, nestin, and low-affinity nerve growth factor receptors. At the final stage of culturing (5 passages) the monolayer cultures included 2 types of cells: GFAP- and p75-positive glial cells and nestin-positive fibroblasts.

Key Words: *olfactory epithelium; glial cells; gliofibrillar acid antigen; low-affinity receptors for nerve growth factor; nestin*

Previous studies showed that the olfactory epithelium and glomerular layer in mammalian olfactory bulbs include glial cells. These cells stimulate axonal regeneration of neurons in the central and peripheral nervous system [12,13]. In English literature these cells are designated as olfactory ensheathing cells (OECs, accessory or parietal cells of the olfactory epithelium). There is no analogue to this term in Russian literature.

Experiments with transplantation of OECs during rhizotomy and spinal cord injury showed that precultured OECs retain the ability to stimulate regeneration and growth of neuronal axons in the central and peripheral nervous system [1,2,4-6,10-15].

To understand functional activity of OECs as stimulators of axonal regeneration, it is necessary to study structural and functional organization of the olfactory epithelium. Axons of receptor neurons form the olfactory nerve and terminate in synapses on mitral cell dendrites of the olfactory bulb, glial cells, sustenocytes and basal stem cells. Fibroblasts are also present in periaxonal connective tissue.

The lifetime of receptor neurons in the olfactory epithelium is 4-6 weeks. Then these cells undergo degeneration and are replaced by new receptor neurons developing from basal stem cells. The death and renewal of olfactory neurons proceed gradually. Rapid growth of axons in newly formed olfactory neurons is provided by OECs that produce a variety of neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell-derived neurotrophic factor (GDNF), extracellular matrix protein laminin, nerve cells adhesion molecules (N-CAM and PSA-NCAM), and other factors [7,9,10].

Apart from olfactory epithelium, the glomerular layer of human olfactory bulbs can serve as a source of OECs [9]. However, this method of OEC isolation requires craniotomy and cannot be used in clinical transplantation. OECs isolated from the olfactory epithelium of the recipient are a universal material for autotransplantation after damage to axons in spinal cord neurons.

We developed a method of long-term culturing of OECs from human and rat olfactory epithelium for transplantation after spinal cord injury.

*Department of Biological Psychiatry, V. P. Serbskii State Research Center of Social and Forensic Medicine; **Laboratory of Experimental Neurocytology, Institute of Brain, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** chekhonin@ telemednet.ru. V. P. Chekhonin

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MATERIALS AND METHODS

Olfactory epithelial tissue was obtained from patients with spinal cord injury at the Neirovita clinics. The procedure was approved by the Ethics Committee (Russian State Medical University, Russian Ministry of Health).

Fragments of the olfactory epithelium (10×5 mm) were dissected from the upper region of the superior nasal field under local anesthesia with 2% lidocaine. The tissue was maintained in Ca^{2+},Mg^{2+}-free Hanks solution (HBSS) supplemented with antibiotics and antimycotics (1:100, Gibco). Tissue samples of the olfactory epithelium were delivered to the laboratory within 2 h after isolation, repeatedly washed with the same solution, and devascularized. The tissue was minced, incubated in 0.1% solution of collagenolytic complex (CPK, IMTEK, Research-and-Production Center of Cardiology, Russian Ministry of Health) at 36°C for 40, washed with HBSS, and dissociated by repeated pipetting in a nutrient medium. The cell suspension was centrifuged at 1000 rpm for 3 min. The pellet was resuspended in 1 ml nutrient medium. The medium contained minimum Eagle medium with D-valine (MEM D-valine, PromoCell GmbH), 10% fetal bovine serum (FBS, Gibco), 2 mM glutamine (Gibco), 0.8% glucose, mixture of insulin, transferrin, and sodium selenite (1:100, Gibco), 10 mM HEPES, and growth factor (2 ng/ml human neuregulin-1 β-1/heregulin 1-β-1 EGF domain, R&D Systems).

The number and viability of dissociated cells were estimated in a Goryaev chamber. The cell suspension (50 μl) was assayed after staining with 0.1% trypan blue. This suspension (100,000 cells/ml) was cultured

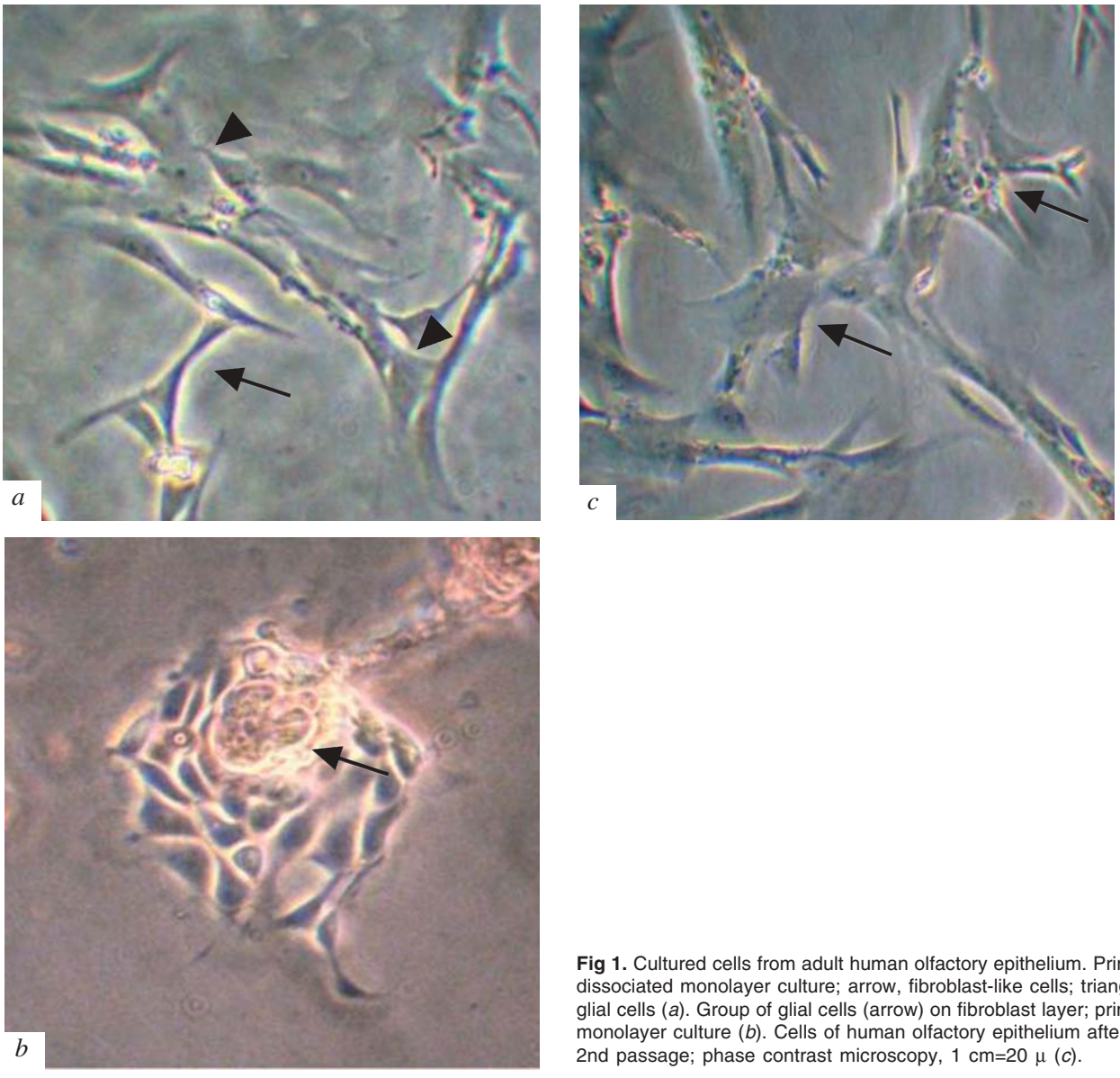

Fig 1. Cultured cells from adult human olfactory epithelium. Primary dissociated monolayer culture; arrow, fibroblast-like cells; triangles, glial cells (*a*). Group of glial cells (arrow) on fibroblast layer; primary monolayer culture (*b*). Cells of human olfactory epithelium after the 2nd passage; phase contrast microscopy, 1 cm=20 μ (*c*).

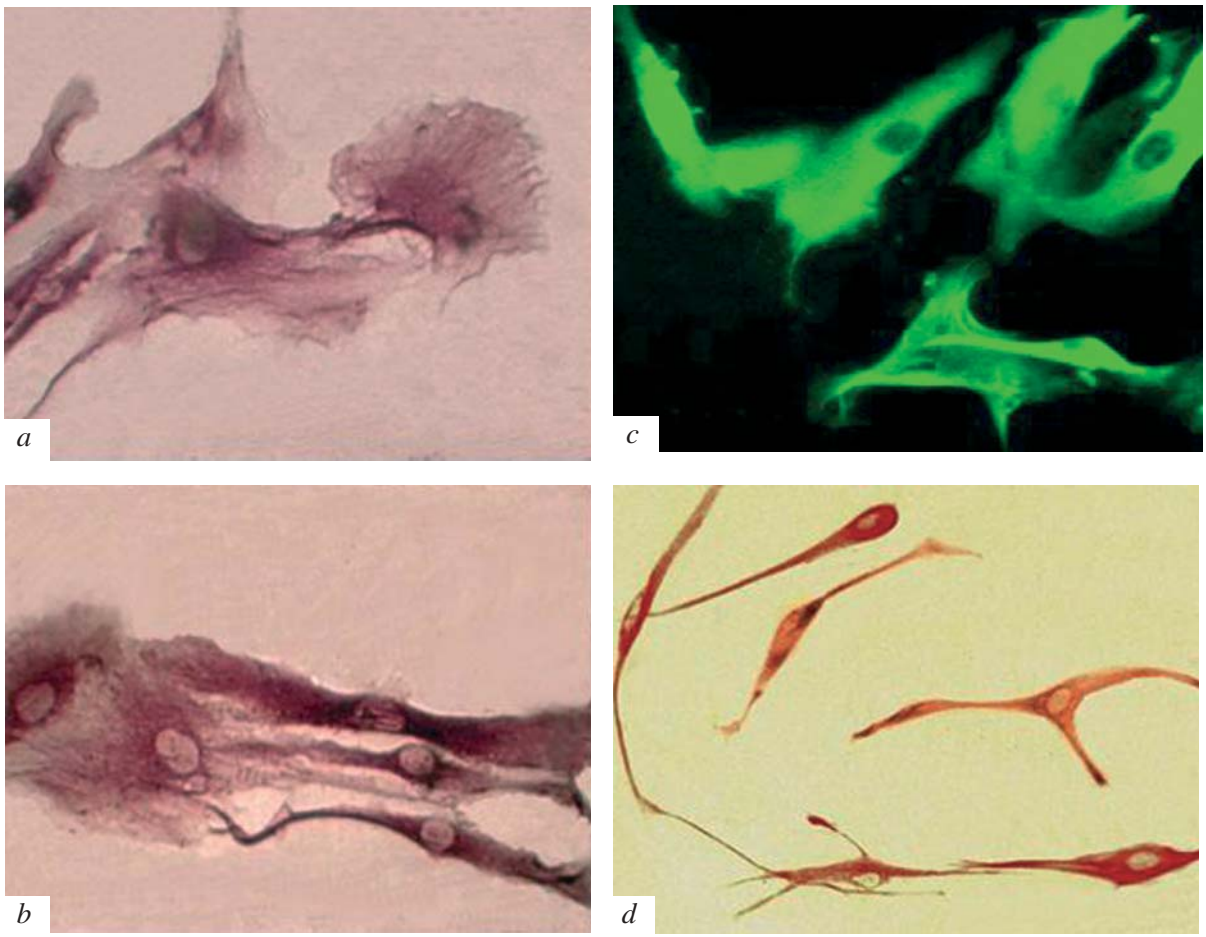

Fig. 2. Immunocytochemical study of glial cells and fibroblasts from adult human olfactory epithelium. Positive staining of glial cells with antibodies to gliofibrillar acid protein (1 cm=15 μ, *a*, *b*); positive staining of OECs with fluorescein-conjugated antibodies to low-affinity receptors for NGF (p75, 1 cm=15 μ, *c*); nestin-positive fibroblast-like cells (1 cm=30 μ, *d*).

in 12-well plates with a polylysine-laminin substrate [13] at 5% CO_2 and 36.5°C for 10-15 days. The nutrient medium was replaced at 3-4-day intervals. During the next passages, cultures were washed with HBSS and treated with CPK for 3-4 min. The effect of enzymes was blocked with minimal DMEM (PromoCell GmbH) containing 3% FBS. After the 4th passage, the cells were washed out from the substrate. The cell suspension was centrifuged at 1000 rpm for 3 min. The pellet was washed with DMEME and resuspended in the medium. The cells were cultured in 25 cm^2-flasks (10,000-12,000 cells/cm^2) or 12-well plates.

During each passage, an aliquot of the suspension was cultured on cover slips (22×22 mm) in Petri dishes for 1 week for cytochemical analysis. After the 4th passage, the cells were routinely frozen and stored in liquid nitrogen; no less than 90% cells in control samples retained viability after defrosting.

Immunocytochemical study. For identification, OECs were fixed with 4% paraformaldehyde in phosphate buffer (0.05 M, pH 7.4) at 4°C for 30 min. For immunocytochemical visualization, the cultures were washed 3 times with phosphate buffer and incubated with primary mouse antibodies to one of the antigens at 4°C for 24 h. We used antibodies to gliofibrillar acid protein (GFAP, 1:100; monoclonal antibodies synthesized at the Laboratory of Immunochemistry, V. P. Serbskii State Research Center of Social and Forensic Medicine), nestin (10 μg/ml, Chemicon Int., CA), and low-affinity receptors for nerve growth factor (p75, 10 μg/ml, Chemicon Int., CA). The preparations were washed with phosphate buffer and consecutively treated with biotinylated secondary horse anti-mouse IgG antibodies (Vector Lab., dilution 1:100; room temperature, 2 h) and components of the avidin-biotin complex (Vector Stain Lab.; room temperature, 1 h) according to manufacturer's recommendations. Peroxidase activity was developed using 0.025% diaminobenzidine (5 mg 3,3- diaminobenzidine per 4 ml HCl, 20 ml phosphate buffer, and 60 μl 3% H_2O_2).

Fluorescein isothiocyanate- or rhodamine-conjugated donkey anti-mouse IgG antibodies (dilution 1:200, Sigma) were used for immunofluorescence stu-

dy. The preparations were examined and photographed under a Leica DLMB fluorescent microscope.

RESULTS

In the primary culture the majority of dissociated cells of the olfactory epithelium adhered to the substrate over the 1st day of culturing. Flattened processes were revealed on day 3-4 of culturing. Some cells remained nonadherent and formed cytospheres. They adhered to the substrate on day 3-4 of culturing. Similar cytospheres appeared on the layer of flattened fibroblasts and looked like fungiform outgrowths (Fig. 1, *b*); the size of these cytospheres increased due to cell proliferation.

During the next passages, these cells were removed from the substrate with proteolytic enzymes, dissociated, and cultured in 12-well plates or 25-cm^2 flasks. Under these conditions dissociated cells formed a monolayer.

During culturing the total number of cells increased by 2-2.5 times and the composition of cell cultures varied. Primary cultures consisted of fibroblast-like cells (Fig. 1, *a*) and included zones of the monolayer with fungiform outgrowths (Fig. 1, *b*), while after the first and subsequent passages the population of polygonal cells with multiple processes reacting with anti-GFAP antibodies increases (Fig. 1, *c*, Fig. 2, *a*, *b*). GFAP-positive cells were also visualized with anti-p75 antigens (Fig. 2, *d*). A small number of nestin-positive fibroblasts (not more than 10%) was also seen (Fig. 2, *c*).

These data on cellular composition of monolayer cultures of human olfactory epithelium are consistent with the results of the experiments with cultured cells of rat olfactory epithelium [3]. These cells were used for transplantation during experimental trauma of the spinal cord.

The proposed method for culturing of human olfactory epithelium allows obtaining a sufficient amount of cells for clinical autotransplantation in humans with spinal cord injury.

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