Multipotent Stem and Progenitor Cells of the Olfactory Epithelium

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In recent decades, a wide spectrum of fetal and embryonic stem and progenitor cells were used for cell therapy of diseases of the central nervous system, but the olfactory glial ensheathing cells exhibited certain advantages due to their biological properties and capacity to stimulate regeneratory processes in spinal injury. The therapeutic effect of a heterogeneous complex of olfactory epithelial cells is more pronounced; apart from glial ensheathing cells, this complex includes fibroblasts, Schwann cells, stem and progenitor cells of this structure. The use of minimally invasive methods for isolation of human olfactory epithelial tissue is important for clinical practice, because they provide cells for autologous transplantation and rule out graft rejection immune reaction and the risk of transmission viral infection and transfer of genetic defects, which can be associated with allotransplantation.

Key Words: *olfactory epithelium; progenitor cells; stem cells; multipotent cells*

Stem cells are undifferentiated multipotent cells with high proliferative activity and unlimited capacity to autoreproduction in symmetrical mitosis. In asymmetrical mitosis stem cells generate daughter precursor cells (progenitor cells) giving rise to definitive somatic cells. Adult mammalian brain (including human brain) contains neural stem cells in the subependymal zone of the lateral ventricles and in the subgranular zone of the hippocampal fascia dentata [17,32,33,38-40,63,67,95,102,120]. Due to the capacity to differentiate into neurons and glia, transplantation of neural stem and progenitor cells opens new vistas for the treatment of many diseases and injuries of human central nervous system [1,2,4,6,24,26,31,82,84,98,103,105,107,109,117, 121]. However, the use of human fetal stem cells for this purpose is associated with many ethical and religious problems [49,85], while transplantation of neural stem cells located in structures of mature brain is in fact impossible, because these cells can be obtained only by invasive neurosurgical methods. Until now the use of the olfactory epithelial (OE) glial and stem cells seems to be the only possible approach to the solution of this problem.

Transplantation of adult animal and human OE cells, mainly the olfactory ensheathing cells (OEC), is used for experimental and clinical correction of spinal injuries [12,16,56-58,69-72,75-78,81,99, 108,115]. However, recent studies indicate that transplantation of "pure" OEC population is insufficient for attaining the maximum positive effect [43,50,97,122]. More complete recovery of the structure, sensory and motor functions of injured spinal cord can be attained using a complex of cells, including, in apart from OEC, fibroblasts, astrocytes, Schwann cells, and probably OE multipotent stem and progenitor cells [11,12]. This assumption is based also on the results of transplantation of OE tissue fragments in spinal injury [73] or transplantation of complete suspension of dis-

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sociated cells of this structure, containing all above-listed cells [51,70,75,76].

Hence, the problem of efficiency and possibility of using various OE cells and tissues for transplantation therapy in cerebral and spinal injuries, no doubt, requires further experimental studies.

The OE occupies limited areas of the nasal mucosa and is located in the upper compartments of the superior nasal passage and nasal septum. The OE includes olfactory receptor neurons (ORN), supporting and stem cells forming three OE compartments: surface, including the supporting cells and ORN apical dendrites; median, containing mature and immature (transitional) ORN forms; and basal compartment with layers of globose and flat horizontal stem cells [21]. OE includes also the Bowman gland ducts opening into the nasal cavity. Lamina propria (connective tissue lining the OE) contains ORN axons and ensheathing glial cells forming their membranes, olfactory nerve fibroblasts, Bowman glands, Schwann cells of the sympathetic and trigeminal nerve fiber membranes, and blood vessels [10,16].

The OE, similar in some respects to the fetal neuroepithelium [21], is the only structure in the nervous system, where death and regeneration of nerve cells (ORN) are permanent processes. ORN lifespan is 4-6 weeks, after which they die via apoptosis and are replaced by new cells of the same type originating from basal stem cells [46-48]. OE is a unique object for the study of neural stem cells; *in vivo* and *in vitro* studies of these cells appreciably extended our notions on the role of molecular biological and molecular genetic factors in ORN development and regeneration [14,19,21,23, 25,60,106].

Stem cells in developing and mature OE are in a state of constant mitotic division. Daughter cells forming as a result of asymmetrical division (progenitor cells) pass through a succession of several stages during migration and differentiation into mature ORN. Due to this OE contains cytokeratin-positive horizontal and cytokeratin-negative globose stem cells, primary progenitor cells expressing proneuronal Mash 1 (mammalian achaete scute homolog 1) gene, secondary progenitor cells (immediate neuronal precursors — INP) expressing Neurogenin (Ngn1) proneuronal gene. Daughter cells forming as a result of INP progenitor division are differentiated into ORN [14,19,21]. *In vivo* experiments showed that globose stem cells serve as the immediate precursors of ORN [79]; these cells also form OE supporting cells [14]. The role of horizontal stem cells in OE cytogenesis remained unclear for a long time. Cultures of these cells were obtained and it was shown that horizontal stem cells formed a heterogeneous population including two subpopulations: globose stem cell precursors and glial OEC precursors [23].

Stem cell division and successive stages of their transformation into mature ORN are regulated by signal molecules and transcription factors expressed in fetal development of OE as a result of natural death of ORN or their induced destruction. This was experimentally confirmed by studies of OE in transgenic mice. ORN completely degenerate by the apoptosis mechanisms in Mash $1^{-/-}$ transgenic mouse embryos, which is paralleled by activation of proliferation of the retained OE stem cells [21].

Activation of OE stem cell proliferation and subsequent regeneration of this structure are observed after crossing of the olfactory nerve or removal of olfactory bulbs. After unilateral excision of the olfactory bulb mouse ORN die by the apoptosis mechanisms within 2-3 days because of retrograde degeneration, the thickness of OE decreasing and minimized on day 5 [29]. Stem cell proliferation is paralleled by ORN neoformation and OE regeneration. Globose and horizontal stem cells generate about 8-10 million nerve cells over 2-3 weeks after bulbectomy and restoration of OE structure, after which the rate of stem cell proliferation decreases to the initial level [18,22,28,55,111,118]. It was hypothesized that intact ORN suppress proliferation of OE basal stem cells, while in apoptosis ORN express factors initiating proliferation of these cells [21]. Among these factors are proapoptotic signals [29], leukemia inhibitory factor (LIF) [13], bone morphogenetic proteins (BMP) [14,118], (transforming growth factor-α [34], neuron growth factors FGF-2, TGF-β_2, and PDGF (fibroblast growth factor-2, transforming growth factor β_2, and platelet growth factor) [30,89].

Similar destruction and regeneration processes in OE were described after toxic exposure to zinc sulfate [119] and methylbromate vapor. Toxic effect of methylbromate leads to complete death of ORN, supporting cells, and partial injury of Bowman gland and their duct cells, globose and horizontal basal cells, which are retained only at the interface with the lamina propria [59,61,112,114]. OE regenerate due to retained progenitor cells generating ORN, horizontal and globose basal cells, and multipotent cells of glandular ducts. These latter, in turn, form Bowman glands and supporting cells. The results of clonal analysis of OE cells regenerating after toxic treatment with methylbromate showed the presence of three cell clone categories in OE, which contain the precursors of basal globose cells, Bowman gland duct cells, and supporting cells of OE, respectively [60].

Methods for isolation and culturing of experimental animal and human OE cells were developed. Monolayer dissociated cultures were obtained, containing stem and progenitor cells of mice [21, 23,25], rats [60,41], chicken embryos [27], and humans [80,88,106,127-130]. Common methods for enzymatic dissociation and culturing of the cerebral neural stem cells were used for culturing OE stem and progenitor cells. Serum-containing and serum-free media for culturing of these cells are based on DMEM/F12 and can include various combinations of growth factors: epidermal growth factor, fibroblast growth factor-2, NT3, vitamins, hormones, and antioxidants (components of commercial N2 and B27 supplements). Primary dissociated OE cultures are heterogeneous and contain various types of cells of this structure. In order to isolate "pure" populations of OE stem and progenitor cells, methods of immunoadsorption with antibodies to antigens specific for these cells are used (Trk-pan, NCAM-1$^+$ and ICAM-1/$\beta_1$integrin$^+$, GBC-2) [23,25, 27,93].

The dynamics of the development of animal and human dissociated OE cell culture is similar. The fundamental data obtained in animal studies formed the basis for modern concepts on the molecular biological mechanisms of development and differentiation of OE stem cells. However, the great basic and applied significance of the data obtained in isolation and long culturing of human OE multipotent stem and hemopoietic cells, which can be used as autologous neural stem cells in clinical neurotransplantology, is obvious.

Human dissociated OE cells collected during biopsy or post mortem form a confluent monolayer over week 1 of culturing; this monolayer consists of flat, spindle, stellate, and globose cells. This heterogeneous cell population includes ORN, glial and supporting cells, fibroblasts, and endotheliocytes, among which the stem and progenitor cells constitute just a negligible portion. For this reason these cells are preserved in just 5-10% OE cultures [106]. Similar data were obtained in culturing of OE fragments from 14-day-old mice in a medium with FGF-2. The formation of ORN in these cultures continued for 4 days in only 5-8% explants, this indicating the presence of a negligible population of proliferating stem cells (about 1 stem cell per 2500 cells of other types) [21].

Actively proliferating human OE cells obtained by biopsy form small groups of about 50 cells in the monolayer [88]. As the number of these cells increases, mushroom-like growth appear, after which spheroid aggregations (neurospheres) are detached from the monolayer. One neurosphere consisting of about 1000 cells forms per 1-4 mm^2 culture flask surface and one culture can contain 500-2000 neurospheres after 7-10 days of culturing. The number of cells in floating neurospheres can reach 40,000 during subsequent culturing. In some dissociated OE cell cultures the formation of neurospheres continues during several months [88].

The cells obtained by dissociation of primary neurospheres and reinoculated into new culture flasks form a monolayer in which secondary neurospheres form. Third- and next-generation neurospheres can be thus obtained. Immunocytochemical studies showed that cells of primary neurospheres express predominantly nestin (neurofilament protein) characteristic of cerebral neural stem cells, some neurospheres contain also cells expressing acid glial fibrillary acidic protein (GFAP) and β-tubulin III. Differentiating cells in dissociated cultures of primary neurospheres are also heterogeneous and express β-tubulin III, GFAP, and O4GalC (oligodendrocyte marker), this indicating the multipotency of these cells, most expressed after their transplantation. Modification of culturing conditions, such as the use of serum-supplemented or serum-free DMEM containing insulin, transferrin, and selenium, addition of nerve growth factor, ciliary neurotrophic factor, or retinoic acid, did not change the composition of cultured cells expressing the antigens, but modified their percent ratio.

The dynamics of cell development and formation of neurospheres in a monolayer of human OE cells obtained at autopsy 6-18 h post mortem was described [106]. Ultrastructural studies of OE fragments directly after isolation showed good preservation of cells in this structure, including the terminals of ORN apical dendrites with kinocilia. Primary monolayer of dissociated OE cells formed during week 1 of culturing and contained a heterogeneous cell population, including the neural and glial cells, which died by apoptosis mechanisms during week 3 of culturing. As a result, the culture retained mitotically active cells, and their number doubled every 2-3 days. These cells denoted as NSFC (neurosphere forming cells) [106] were characterized by low adhesive activity and were easily separated from the substrate. During the subsequent 2 weeks neurospheres consisting of about 20-80 cells formed in cultures; they were similar to stem cell neurospheres isolated from other brain structures. Total duration of culturing was more than 8 months, during which 70 passages were made. Immunocytochemical studies showed that the majority of neurospheres contained neural stem and progenitor cells expressing β-tubulin III, nestin, MAP2ab, NCAM, peripherin (protein of cellular

intermediate filaments neural crest derivatives, and tyrosine kinase receptor markers for neurotrophins (Trk A, B), though some neurospheres also contained glial, GFAP- and A2b5-positive cells [106, 127,129, 130]. Clonal analysis of NSFC in monolayer cultures [94] showed that proliferating nestin-positive cells formed primary neurospheres. Dissociation of these neurospheres led to the formation of secondary, tertiary, *etc.* neurospheres in all subsequent subcultures (70 NSFC cultures were obtained in the study [94]). Similarly to cerebral stem cells, solitary isolated NSFC can form a cell clone producing a new neurosphere during a week. This NSFC capacity to unlimited self-reproduction was explained after studies of telomerase activity and apoptosis in these cells [80]. It was found that progenitor NFSC, whose mitotic cycle took 18-20 h in each of 200 passages, retained initial telomerase activity. Evaluation of activities of nine different caspases (cysteine proteases) revealed no increase in activities of these enzymes in all studied NFSC clones, which explains low level of apoptotic death of these cells.

Neuronal differentiation of stem cells was attained in adult human OE dissociated cultures [3]. In addition to flat polygonal cells positively reacting with antibodies to GFAP, nestin, and low-affine receptors for nerve growth factor (p75, LNGF), due to which they were identified as the olfactory nerve glial ensheathing cells and fibroblasts [5,72, 100], these cultures contained colonies of oval cells, forming neurospheres. Repeated reinoculations in serum medium containing D-valin led to the formation of multipolar astrocytes with long branched axons in these cultures; these cells were stained with antibodies to specific neuronal enolase.

Cultures of isolated globose [25] and horizontal [23] basal stem cell of OE were obtained by immunoadhesion using antigens specific for these cells.

Cells expressing green fluorescent protein (GFP) were isolated from OE of transgenic mice [25] and treated with first GBC-2 antibodies specific for OE globose basal stem cells [42] and with second antibodies conjugated with fluorescein or R-phycoerythrin. Globose stem cells were isolated on a flow fluorescent sorter. The resultant cells were transplanted into the nasal cavity of C57Bl/6 mice, in which OE was pre-destroyed by methylbromate vapor. Transverse sections of OE were examined in a fluorescent microscope 2-4 months after transplantation. The results indicated that GFP-positive globose stem cells incorporated in recipient mouse OE and formed isolated colonies. The cells formed as a result of proliferation and differentiation of transplanted globose stem cells were identified and located by cytological and immunocytochemical methods using specific immune markers: antineurotubulin (Tuj-1), mature ORN labeling antiolfactory protein [87], SUS-4 monoclonal antibodies specific for supporting cells and Bowman glandular duct cells. Transplanted globose cells exhibited properties of multipotent stem cells and generated secondary progenitor globose cells, ORN, supporting cells, Bowman gland cells, and even ciliary cells of the respiratory epithelium. The regenerating axons of new ORN incorporated in the olfactory nerve and grew into the olfactory bulbs. Hence, transplanted globose stem cells promoted the structural and functional recovery of OE destroyed by methylbromate.

Another type of OE multipotent stem cells, basal horizontal cells, were studied in detail. According to immunocytochemical findings, these cells express some antigens, the most specific of which are keratins 5 and 14 homologues, not characteristic of other OE cells [54]. In subsequent studies solitary basal horizontal cells were isolated by immune adhesion on magnetic microcarriers and by fluorescent sorting using other antibodies specific for them (NCAM-1 and ICAM-1/$\beta_1$integrin) [23]. It was shown *in vitro* that horizontal progenitor cells with a high proliferative potential, cultured in media with growth factors (NGF, TGF-α, EGF), generated secondary horizontal NCAM-1$^+$ progenitor, globose stem and glial cells. The *in vitro* proliferation and differentiation of horizontal stem cells is determined by a complex of microenvironment factors (extracellular matrix): cell adhesion molecules (NCAM-1), integrins (β_1, β_4, α_1, α_3, α_6), and components of the lamina propria (collagen, laminin, and fibronectin).

Multipotency of OE stem and progenitor cells, described for their culturing [14,20,21,23] and regeneration [25,112], was also detected in a series of original *in vitro* and *in vivo* experiments [88].

In these experiments human OE neurospheres were cultured simultaneously with somatic cells in isolated compartments of culture dishes divided by a semipermeable membrane (Transwell culture dishes, Costar). Sections of rat liver, heart, and skeletal muscles were placed into the upper compartment and human OE neurospheres into the lower one. The cultures were fixed after 4 days and stained with antibodies specific for inductor tissue cells. Due to humoral factors released by inductor tissues, the neurosphere target stem cells acquired the capacity to express proteins characteristic of inductor cells: hepatocytes (ferritin, albumin), cardiomyocytes (sarcomer α-actin, cardiotroponin), and striated muscles (myosin, tropomyosin).

It was also shown that intravenous injection of rat OE cell suspension led to the formation of multiple leukocyte types in the spleens of irradiated recipient rats; these leukocytes were identified by immunochemical markers of these cells (RT7.2, CD34, CD11b, CD3, CD45RA). In addition, polymerase chain reaction (PCR) detected the determinant gene of the donor male (SRY) in the recipient female lymphocytes.

In another series of experiments, stem cells were transplanted into chicken embryos after 20-24-h incubation [88]. Suspension of human dissociated OE neurosphere cells (1000-2000 cell/μl, 2 μl) labeled with Dil (dioctadecyl-tetramethylindocarbocyanine perchlorate; nuclear marker) and CMFDA (cytoplasmatic marker) or of Lac Z transgenic mouse OE cells, containing β-galactosidase, was injected into the primary stripe area.

Three days after transplantation of CMFDA-labeled human OE stem cells the chicken embryos were fixed, labeled with propidium iodide, and dissociated cells were analyzed by flow cytophotometry. Of the total number of embryonic cells (45 million), 446 000 cells (0.97%) were the transplanted human OE cells, this indicating integration of these cells with chicken embryo tissues. Subsequent DNA analysis showed that transplanted human OE cells formed numerous populations in chicken embryonic tissues.

Immunocytochemical studies with antibodies to β-galactosidase, carried out 4 days after transplantation of Lac Z transgenic mouse OE cells in chicken embryos, showed X-gal in the allantois, amnion, notochord, somites, Rathke's pouch, mesenchyma of the head, limb and tail buds, trunk muscles, mesonephros, liver, intestine, heart, aorta, pharyngeal arch, acoustic and visual bubbles, spinal tube and brain structures, and spinal ganglia. Donor cells acquired the structure characteristic of the adjacent tissue in all the studied recipient tissues and expressed the phenotypical markers intrinsic of recipient tissues. Hence, the results indicate that after xenotransplantation the OE multipotent stem and progenitor cells differentiate into the cells, descending from all three embryonic leaflets (endoderm, mesoderm, and ectoderm) under the effect of embryonic inductors. The significance of these results was confirmed by appropriate controls. The probability of fusion [8,123] of transplanted OE stem and progenitor cells with recipient lymphocytes and somatic cells was ruled out.

The possibility of directed differentiation of cultured OE stem cells under the effect of transcription factors, regulating the formation of motoneurons during the early period of chicken and mouse embryo development (Olig2, Ngn2, HB9), was shown [127]. Human OE progenitor cells acquired the phenotypical signs of motoneurons and expressed Isl1/2 marker specific of them in medium containing, in addition to these transcription factors, retinoic acid, forscolin, and Sonic Hedgehog morphogenetic protein. During coculturing with chicken embryo myocytes, axons of new motoneurons formed neuromuscular synapses, the presence of which was confirmed by immunocytochemical reactions to acetylcholintransferase and synapsin 1.

Numerous research reports and published reviews during the latest decade indicate that transplantation of multipotent stem and progenitor cells isolated from human and animal fetal and adult brain is intensely used in experiments and in some cases clinically for cell therapy of brain diseases and spinal injuries [1,2,6,7,26,33,65,82-84,91,92, 107,109,110]. It was shown on experimental models of intracerebral allo- and xenotransplantation that transplanted cells integrated in the recipient tissues, migrated directly to the focus of brain injury, and differentiated into nerve and glial cells. Transplanted stem and progenitor cells produce a complex of neurogrowth factors and cell adhesion molecules, promoting the regeneration of damaged neurons and growth of their axons, which leads to partial restoration of impaired cerebral and spinal functions. However, the therapeutic potential of OE stem and progenitor cells attracted attention of scientists just in recent years [37,81,106]. The presence of a population of constantly proliferating neural stem and progenitor cells in the olfactory epithelium, the possibility of their extracranial isolation, long-term culturing, cloning, and cryopreservation indicate that these cells can be used as autologous material for transplantation in traumatic injuries and degenerative diseases of the central nervous system.

Removal of some human OE cells does not lead to lasting disorders of olfaction even after repeated biopsy [68]. Fragments of human OE 1-2 mm^2 in size were collected from patients aged 20-78 years during turbinectomy, plastic repair of the nasal septum [88], and sinus puncture [125,128]. The maximum intraoperative removal of OE in the patients was 3×5 or 2×10 mm^2 [35]. The location and methods for endoscopic isolation of human OE are amply described [15,37,125]. In addition to intraoperative biopsy, human OE cells can be isolated and cultured post mortem, due to which cloned cultures of OE cells for allotransplantation can be prepared [106,127-130].

Human OE progenitor cells were transplanted to adult rats after unilateral crossing of the spinal

cord at the level of cervical segments [126]. The transplanted cells were labeled with GFP and suspended in matrix (Matrigel®). The group of control animals included rats with similar spinal injury without subsequent transplantation of OE cells and rats injected with just cell-free matrix to the site of injury. Immunocytochemical studies carried out 2 weeks after transplantation showed GFP-positive cells at the site of spinal injury, their number decreasing by week 12. The use of BDA (biotinylated dextranamine) anterograde axonal tracer visualized the growth of regenerating rubrospinal axons along segments 5-6 below the site of spinal injury and cell transplant location. The appurtenance of these fibers to the rubrospinal tract was confirmed by a retrograde tracer (fluorogold), which showed a greater number of red nucleus neurons in comparison with the control. The rats transplantated OE progenitor cells showed better performance in the behavioral motor tests in comparison with the controls. No signs of regeneration of the damaged spinal cord and recovery of motor functions were detected after transplantation of the cell-free matrix. Presumably, the positive effects of OE progenitor cell transplantation were due to the release of brain neurotrophic factor (BDNF) by these cells, because in many experiments injection of this factor or transplantation of BDNF-expressing OE fibroblasts and glial cells prevented retrograde degeneration of the red nucleus cells in rats with spinal injury [74,90].

Some data indicate the possibility of using OE cells, including stem cells, for the diagnosis and studies of the pathogenesis of mental and degenerative diseases of human central nervous system [9,66,78,96]. Clinical studies showed that decrease of olfaction is observed at the initial stages of Alzheimer's disease and can precede mnestic disorders characteristic of this disease [45,116]. These symptoms are associated with impairment of the olfactory bulb, preperiform cortex, and other cerebral structures linked with the olfactory functions [62, 104,123]. Similar olfaction disorders were detected at the initial stages of Parkinson's disease, this suggesting their use for the early diagnosis of this disease [64], associated with a decrease in the content of neural stem cells in OE [53].

Schizophrenia and some other mental disorders are also associated with olfactory disorders, manifesting by decreased capacity to differentiate the smells, loss of olfactory memory, and organic changes in the olfactory neurons [13,44,52,86]. Comparative study of OE cultures derived from patients with schizophrenia and patients without this disease showed that OE from schizophrenics was characterized by a lesser capacity to adhere to the substrate (fibronectin), contained a greater number of dividing cells and cells dying by the apoptosis mechanisms. Dopamine treatment reduced the number of mitotic cells in both groups of cultures, reducing apoptotic cell death in OE cultures from schizophrenics and increasing this parameter in control cultures [36,96]. The authors suppose that these characteristics can be used for early and differential diagnosis of schizophrenia.

Hence, *in vivo* and *in vitro* studies of OE open new vistas for the analysis of cytological, biomolecular, and molecular genetic regularities of stem cell development and differentiation. In addition, OE can serve as a unique source of cells (including the neural stem and progenitor cells) requiring no invasive neurosurgical intervention for allo- and autotransplantation in diseases and injuries of the central and peripheral nervous system.

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