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Development of Strategies for Appropriate Laboratory Incubation of Adult Stem/Progenitor Cells and Explants from Different Tissues

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ABSTRACT

The main goal of the current study was directed to development of strategies for application of different types of adult stem/progenitor cells and explants from several different tissues, for differentiation of appropriate mature lineages, by appropriate laboratory incubation, varying according to the respective initial tool. Different laboratory techniques for *in vitro*-incubation of stem/progenitor cells for therapeutic needs in deficiency of limbal ocular cornea stem cells were developed and tested. For this goal, stem/progenitor cells and tissue explants from limbus of human ocular cornea, but also from several other sources of the adult organism (bone marrow stroma, adipose tissue, cartilage, synovial and oral mucosa), were cultivated in respective appropriate laboratory conditions. In agreement with the literature data, the used epithelial stem/progenitor cells and tissue explants from oral mucosa were proved as the most nearto the epithelial stem/progenitor cells and explants from limbus of ocular cornea, mainly on the expression of specific molecular markers, which allows application of similar methods for *in vitro*-cultivation. For study of the underlying events, which determine the differentiation of cell progenitors toone or another direction in respective incubation conditions, primary investigations on the cytoskeleton components, but also on different intra- and extra-cellular inter-molecular interactions were also performed.

INTRODUCTION

The abilities of stem cells to differentiate into different cell lineages, suggest their usability as appropriate cellular vectors for treating of different injuries, including different types of malignancies, and characterize them as strong candidates for delivering of genes and restoring of different tissues and organs structure and function, by processes of *trans*-differentiation and/or dedifferentiation according both the respective tool and incubation conditions [Lisingnoli *et al*., 2001; Molofsky *et al*., 2004; Pitaru *et al*., 1993; Torregianni *et al*., 2012]. In this way, undifferentiated stem/progenitor cells could circulate in the body and contribute to repair if needed. On the other hand, by taking in consideration their high replication rate and self-renewal capacity, these cells have been proposed as strong candidates for different therapeutic procedures of various tissues and organs, by appropriate internal (genetic), epigenetic and external manipulations.

According many literature data different cytoskeleton components, but also various and intra- and extra-cellular inter-molecular interactions with the participation of these bio-molecules, have been indicated to underline the control of cell growth, proliferation and differentiation, by cascade regulatory pathways [Aamodt and Culotti, 1986; Buchwitz *et al.*, 1999; Jaumot *et al.*, 1994; Nguyen-Ngoc *et al.*, 2007; Oakata *et al.*, 1995; Savage and Chalfie, 1991].

In this connection, the main goal of the current study was directed to the possibility for application of adult stem/progenitor cells from different tissues about appropriate transplantation and therapeutic aims by appropriate laboratory incubation techniques.

MATERIALS AND METHODS

Laboratory *in vitro*-incubation of stem/progenitor cells and tissue explants from human ocular limbus

Isolated tissue explants from human ocular cornea limbus and oral mucosa were incubated indifferent combinations of the growth Modified Minimal Essential Medium (DMEM) and Ham's (Sigma-Aldrich). Those media mixtures were supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic mixture (100 UI/ml Penicillin, 0.25 mg/ml Streptomycin and 0.25 mg/ml Amphotericin-B). Subsequently, L-Glutamine, 10ng/ml Epidermal Growth Factor (EGF -

Sigma-Aldrich), 5 µg/ml Insulin, 0.4 µg/ml Hydrocortisone,24 µg/ml Adenine, as well as 2% ml/ml conditioned cultural fluid of previously cultivated in it 3T3 cells(fibroblasts from embryos of Balb/c experimental mice); on monolayer of 3T3 fibroblasts in their role of feeder cells, with previously stopped further proliferation by Mitomycin-C, as well as on vitelline membrane, previously treated by Glutamine-Glutaraldehyde solution [Bratanov *et al.*, 2011; Valkova *et al.*, 2013], respectively. Cells from limbus ocular cornea explants were derived by their treatment with Dispase and Trypsin/EDTA (0.05% Trypsin and 0.02%EDTA), after which they were similarly incubated and proceeded.

Laboratory *in vitro*-incubation of stem/progenitor mesenchymal stromal cells (MSCs) from human bonemarrow material

After isolation of normal mesenchymal stem cells (MSCs) from human bone marrow stroma, their phenotypic characteristics were proved by flow-cytometry assay: negative on markersCD31, CD34 and CD45, but positive on markers CD90, CD105, and CD106. Subsequently, the so derived MSCs were seeded in initial density 3 x 10^4 cells/ml, in basic α-MEM (Minimum Essential Medium, alpha modification), supplemented with 15% FBS,50µg/ml freshly prepared Ascorbic acid (Vitamin C)and 100µg/ml Penicillin.

Laboratory *in vitro*-incubation of mesenchymal stem/progenitor cells (MSCs) from human adipose tissue

Isolated fat tissue was digested by treatment with the enzyme Collagenase (Sigma), in α-MEM (Minimum Essential Medium, alpha modification) without serum, at 37^0C for 45 minutes. After centrifugation at 600 g for 10 minutes, the cell pellet was resuspended, the so derived stem/progenitor cells were seeded at a density 2 x 10^3 - 4 x 10^3cells/ml in basic α-MEM (Sigma), supplemented with FBS (Sigma).

Laboratory *in vitro*-incubation of mesenchymal stem/progenitor cells (MSCs) from human cartilage tissue

Isolated cartilage was digested by treatment with 0.5% Pronase (Sigma) at 37^0C for 1 hour, supplemented by treatment with 0.2% Collagenase (Sigma) for 45 minutes, in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) without serum. After centrifugation, the pellets

were resuspended and the cells were seeded at a density 25 x 10^3cells/ml in DMEM, supplemented with 10% FBS (Sigma) and antibiotics mixture (100 UI/ml Penicillin and100 mg/ml Streptomycin).

Laboratory *in vitro*-incubation of tissue explants from human *synovial membrane*

Isolated synovial pieces were seeded and incubated in DMEM, supplemented with 10% FBS (Sigma) and antibiotic mixture (100 UI/ml Penicillin and100 mg/ml Streptomycin).

Laboratory *in vitro*-incubation of stem/progenitor cells and tissue explants from human oral mucosa

Tissue explants from human oral mucosa were incubated in different combinations of the growth media DMEM and F12 (Sigma-Aldrich). The media mixtures were then supplemented with 10% Fetal Bovine Serum(FBS) and antibiotic mixture (100 UI/ml Penicillin,0.25 mg/ml Streptomycin and 0.25 mg/ml Amphotericin-B), and L-Glutamine, 10 ng/ml Epidermal Growth Factor (EGF - Sigma-Aldrich), 5µg/ml Insulin, 0.4 µg/ml Hydrocortisone, 24 µg/ml Adenine, as well as 2% ml/ml conditioned culturalfluid of previously cultivated in it 3T3 feeder cells(fibroblasts from embryos of Balb/c experimental mice), were added. Separated cells from oral mucosa explants were derived by their treatment with Dispase and Trypsin/EDTA (0.05% Trypsin and 0.02%EDTA), after which they were similarly cultivated and proceeded.

Lightmicroscopy observations

All cultures were incubated at 37^0C, in 5% CO_2-incubator with 95% air humidity, and were observed as native preparations by inverted light microscope (Leica), supplied with megapixel CCD-camera.

Determination of cytoskeleton components and interactions with their participation, underlining the cell fate directions

Protein material from human synovial fluid (SF), and from nuclear extract (NE) of human malignant cervical carcinoma cells HeLa, respectively, were isolated and tested. SF is known as a feeder source for different types of adult normal stem/progenitor cells (from *synovia*, cartilage, bone, muscle and *Hoffa*sub-*Patella* adipose tissues, as well as separated immature bonemarrow

and blood cells), in the respective anatomic area, but this liquid is also supplemented of different cellular metabolites. On the other hand, NE is known as rich of proteins, able to connect directly with nucleic acids and, hence, to influence directly the expression on any genes, but suppress others at the same time. The current investigation is directed to find the common proteins in both protein mixtures, which is necessary for characterization of molecules, which supports growth and proliferation capacity of cells by appropriate cascade pathways, but also underline in the control and regulation of the same processes by other cascade mechanisms. So, the isolated proteins from both mixtures are subjected on separation by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), subsequent Coomassie-Blue staining of the gel, for identification of protein bands according molecular weight, washing, chemical treatment of each band for digestion of each protein molecule, and subsequent label-free tandem mass spectrometry, combined with liquid chromatography (LC-MS/MS) assay.

RESULTS

Corneal epithelial cells and tissue explants were *invitro*-incubated on feeder cell monolayer of 3T3mouse embryonic fibroblasts; in growth medium with cultural fluid of the same feeder cells, as well as on chemically-modified vitelline membrane, respectively
(Figure1). In all cases, cells with different morphology and in different phases of differentiation were established: early epithelial cell progenitors with round shape, oval nucleus, and poor cytoplasmic contention, as well as polygonal-shaped mature epithelial cells.

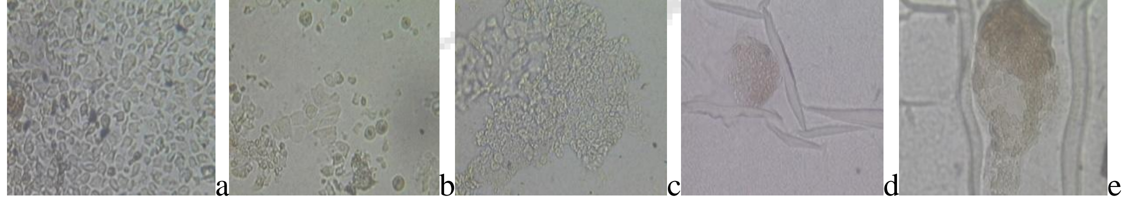

Figure 1. Adult stem/progenitor cells from limbus of human ocular cornea, as well as tissue explants from the same biological material, incubated in different laboratory conditions: a) epithelial stem/progenitor cells from limbus of human ocular cornea, incubated on a confluent monolayer of 3T3 mouse embryonic fibroblasts in the role of feeder cells, after previous treatment with Mitomycin-C; b) epithelial stem/progenitor cells from limbus of human ocular cornea, cultivated in the absence of 3T3 mouse embryonic fibroblasts monolayer, but in the

presence of cultural fluid, supplemented by metabolites by previous incubation of these feeder cells in it; c) non-trypsinized tissue explant from limbus of human ocular cornea, cultivated in the absence of 3T3 mouse embryonic fibroblasts monolayer, but in the presence of cultural fluid, supplemented by metabolites by previous incubation of these feeder cells in it; d)non-trypsinized tissue explant from limbus of human ocular cornea, cultivated on chemically-modified hen's egg vitelline membrane; e) cells from non-trypsinized tissue explant of human ocular cornea limbus, incubated on chemically-modified hen's egg vitelline membrane (Native light-microscopy preparations, magnification: X100).

These techniques were usable in analogical laboratory incubation of other types of adult stem/progenitor cells and explants, isolated from several other tissues of adult organism (Figure 2). Maximal analogy to the methods for laboratory cultivation of stem/progenitor cells and tissue explants from limbus of ocular cornea were established with the same technologies, applied to stem/progenitor cells and explants from oral mucosa (Figure 3).

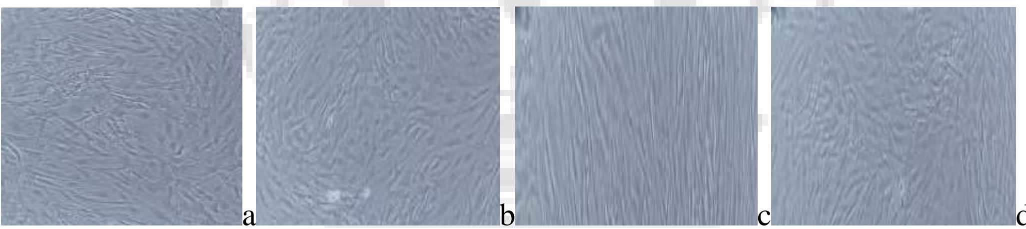

Figure 2. Adult stem/progenitor cells (MSCs) and tissue explants, isolated from different initial sources and incubated in appropriate laboratory conditions: a) MSCs from human bonemarrow stroma; b) MSCs from human adipose tissue; c) MSCs from human cartilage; d) non-treated by proteolytic enzymes tissue explant from human *synovia* (Native light-microscopy preparations, magnification: X100).

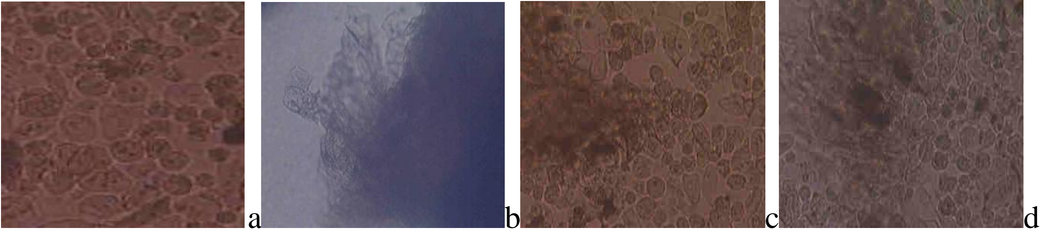

Figure 3. Adult stem/progenitor cells from human oral mucosa and cell proliferate from tissue explants of the same biological material: a) separated cells, derived tissue explant from the same source, after previous treatment by the proteolytic enzymes Trypsin and Dispase; b) 24-hours culture of non-trypsinized explant – separation of subpopulations could be seen; c) 48-hours culture of non-trypsinized explant – as a proof for active cell proliferation, but also about their stem cell phenotype, could be accepted the increased amounts of cells around the explants; d) 72-hours culture of non-trypsinized tissue explant – confluent cell monolayer with formation of cell proliferates around the explants could be observed (Native light-microscopy preparations, magnification: X100).

Proteins p63 and Vimentin as markers of adult stem cell phenotype were proved in other bio-materials, known as containing different types of adult stem/progenitor cells (from synovia, cartilage, bone, muscle, adipose tissues, bonemarrow, blood, etc.), as is the synovial fluid (SF) from human knee (Figure 4).

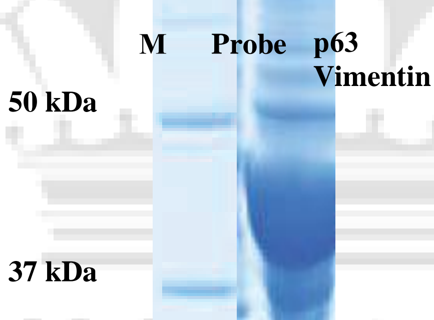

Figure 4. Establishment of proteins p63 and Vimentin as markers of adult stem cell phenotype, in synovial fluid (SF) from human knee joint, known as a bio-material, supplemented different proteins and metabolites from the living action of different types adult stem/progenitor cells; M – marker (Coomassie-blue staining).

Common protein molecules between this biological fluid, on the one hand, as well as from nuclear extract (NE) of human malignant cervical carcinoma cells HeLa, on the other, were searched and proposed (Figure 5).

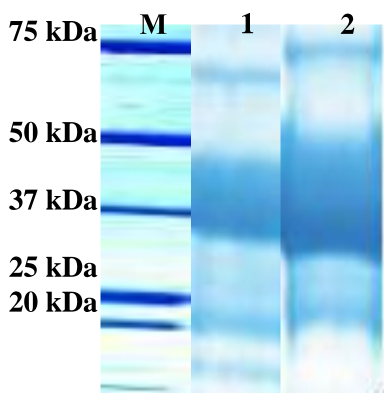

Figure 5. Protein molecules in lysates from different biological materials with human origin, containing adult stem/progenitor cells, as well as metabolites of their living activity:M – marker(line 1); line 2 – from synovial fluid (SF) of human knee joint; line 3– from nuclear extract (NE) of human malignant cervical carcinoma HeLa cells. Proteins, distributed on line 2 and line 3, are characterized as common between the SF, which is known as a biological material, containing different types of stem/progenitor cells, and from NE from actively proliferating malignant cells, respectively, suggesting the support of active cell proliferation, on one hand, but also its control, on the other (Coomassie-blue staining).

SF is known as a feeder source for different types of adult normal stem/progenitor cells (muscle, bone, cartilage, adipose tissue, bone marrow material, blood, etc.), in the respective anatomic area, but this liquid is also supplemented with different cellular metabolites. On the other hand, NE from HeLa malignant cervical carcinoma cells is known as rich in proteins, able to connect directly with nucleic acids and, hence, to influence directly the expression on any genes, but suppress others at the same time. Thus, the proteins, common between both biological probes tested, could be suggested to support active cell proliferation, on one hand, as well as to control it, on the other.

DISCUSSION

The data obtained are in support of the messages about expression of some markers from oral mucosa epithelial stem/progenitor cells, which have also been proved in epithelial cell progenitors from limbus of ocular cornea [Grueterich *et al*., 2002; Pellegrini *et al*., 1999].In addition, these results obtained confirmed the literature data about them, importance of the

feeder cells and biomembranes, respectively, as appropriate bio-substrates for incubation and proliferation of stem/progenitor cells and tissue substrates in laboratory conditions [Anderson *et al*.,2001; Grueterich *et al*., 2002; Grueterich *et al*., 2003; Shortt *et al*., 2007]. These results suggested possibilities for application of other types of adult stem/progenitor cells from different tissues of the adult organism for derivation of different mature cells by respective appropriate laboratory incubation, which is also in agreement with the respective literature findings [Huang *et al.*, 2015; Lisingnoli *etal*., 2001; Torregianni *et al*., 2012]. Also, the data from the current study supported the literature references about the maximal similarity of epithelial stem/progenitor cells and tissue explants from oral mucosa to these from ocular cornea limbus, in particular on the expression of specific molecular markers, which allows application of analogical laboratory methods for *in vitro*-incubation [Jaumot *et al.*, 1994].

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