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Research Article

Directions of *in vitro*-differentiation of adult stem/progenitor cells, depending of the respective sources and incubation conditions

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Abstract

The main goal of the presented study was directed to derivation of one or another type of normal mature cells from adult stem/progenitor cells, by incubation in different laboratory conditions, as well as to reveal the molecular mechanisms, underlining these maturation variations. In *in vitro*-cultivation of corneal epithelial cells and tissue explants on feeder cell monolayer of 3T3 mouse embryonic fibroblasts, 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. These features were observed in incubation in the presence only of cultural fluid from embryonic 3T3 cells, but also on confluent mono-layers of 3T3 fibroblasts, with formation of colonies of stem/progenitor cells, as well as on vitelline membrane, previously treated with Gelatine-GlutaraldehydeBecause epithelial stem/progenitor cells from human oral mucosa were proved to express limbal epithelial stem cell markers, they were *in vitro*-cultivated in similar laboratory conditions as limbal epithelial stem/progenitor cells. These results were in agreement with respective literature data, and could be explained with eventual existence of separated stem/progenitor cell sub-populations, which are able to differentiate in respective directions in appropriate cultivation conditions. For study of the underlying events, which determine the differentiation of cell progenitors to one or another direction in respective incubation conditions, investigations on the direct and/or indirect intra-molecular interactions, should be performed.

Keywords: Adult Stem/Progenitor Cells, Incubation Conditions, Cell Differentiation.

Introduction

The impaired function of stem/progenitor cells in different self-renewing tissues has been found to limit the longevity of experimental animals and humans (Coulombel, 2005; Molofsky *et al*., 2004; Vogelstein and Kinzler, 2004). Their possibilities to differentiate in various directions by processes of *trans*-differentiation and/or dedifferentiation according both the respective tool and incubation conditions, determine them as strong candidates for regeneration of different tissues, organs and systems (Lisingnoli *et al*., 2001; Pitaru *et al*., 1993; Torregianni *et al*., 2012). They could also contribute for different clinical goals, as transplantation or regeneration procedures (Beyer and da Silva, 2006). Undifferentiated tissue stem/progenitor cells could also circulate in the body and contribute to repair if needed (Bradley *et al*., 2002; Kashofer and Bonnet, 2005; Molofsky *et al*., 2004). As a particular aim has been found that is the possibility for use of hematopoietic stem cells (HSCs) (Zhang *et al*., 2005) and/or of mesenchymal stem cells (MSCs) (Beyer and da Silva, 2006; Jones and Yang, 2011) in various forms of cellular therapies, as well as genetic tools that can be used for a better understanding of the mechanisms leading to repair and regeneration of damaged and/or transformed cells.

The influence of different growth factors and cytokines, their mechanisms of action, as well as signaling cascade pathways, have suggested probability for existing of novel therapeutic targets, including not only those molecules, but also their inhibitors and/or antagonists, which could influence their function both directly or indirectly, by targeting specific step of respective signaling mechanisms (Hughes *et al*., 2006; Zhang *et al*., 2005).

In this connection, the main goal of the present study was to investigate the direction of differentiation of adult stem/progenitor cells, in dependence of the concrete tools and incubation conditions.

Materials and Methods

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

Isolated cells and tissue explants from human ocular cornea limbus and oral mucosa were incubated in different combinations of the growth media Dulbecco's 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, 10 ng/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. The so prepared cultures of cells and tissue explants were cultivated at 37^0C, in incubator with 5% CO_2 and 95% air humidity, and observed by inverted light microscope (Leica), supplied with mega-pixel CCD-camera.

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

Isolated cells and tissue explants from human ocular cornea limbus and 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), 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 feeder cells (fibroblasts from embryos of Balb/c experimental mice), were added. The prepared cultures of cells and tissue explants were incubated at 37^0C, in 5% CO_2-incubator with 95% air humidity, and observed by inverted light microscope (Leica), supplied with mega-pixel CCD-camera.

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

After isolation of normal mesenchymal stem cells (MSCs) from human bone marrow stroma, by Ficoll-Hypaque density gradient separation (Torreggiani *et al*., 2012), Their phenotypic characteristics were proved by flow-cytometry assay: negative on markers CD31, CD34 and CD45, but positive on markers CD90, CD105 and CD106. Subsequently, the so derived MSCs were counted by Eosin Dye for determination of cell viability, and seeded in initial density 3 x 10^4 cells/ml, in basic α-MEM (Minimum Essential Medium, alpha modification), supplemented with 15% heat-inactivated Fetal Bovine Serum (FBS), 50μg/ml freshly prepared Ascorbic acid (Vitamin C) and antibiotics (100μg/ml Penicillin G).

Derivation of osteoblasts from laboratory-incubated human bone-marrow MSCs

For further cell differentiation in osteoblasts, 24 hours after seeding the MSCs, isolated from human bone-marrow material, the cultural fluid, eventually containing the non-adherent cells, should be discarded. The received monolayer of adherent cells was washed twice with PBS, after which cultural fluid, previously prepared by addition of 10^{-8} M Dexamethazone and 10 mM β-Glycerophosphate to the described above basic α-MEM, was added. The media were changed twice a week, and during this time the cell growth and proliferation is followed. When the formation of confluent cell monolayer was observed, the cells were trypsinised (by treatment with trypsin/EDTA solution), tested for viability by Eosin staining, and pre-seeded for cultivation in appropriate conditions, described above. Osteoblast phenotypic characteristics

were observed by Alizarin Dye staining. The cultural fluids were changed twice a week, and the cells were observed on each 24 hours.

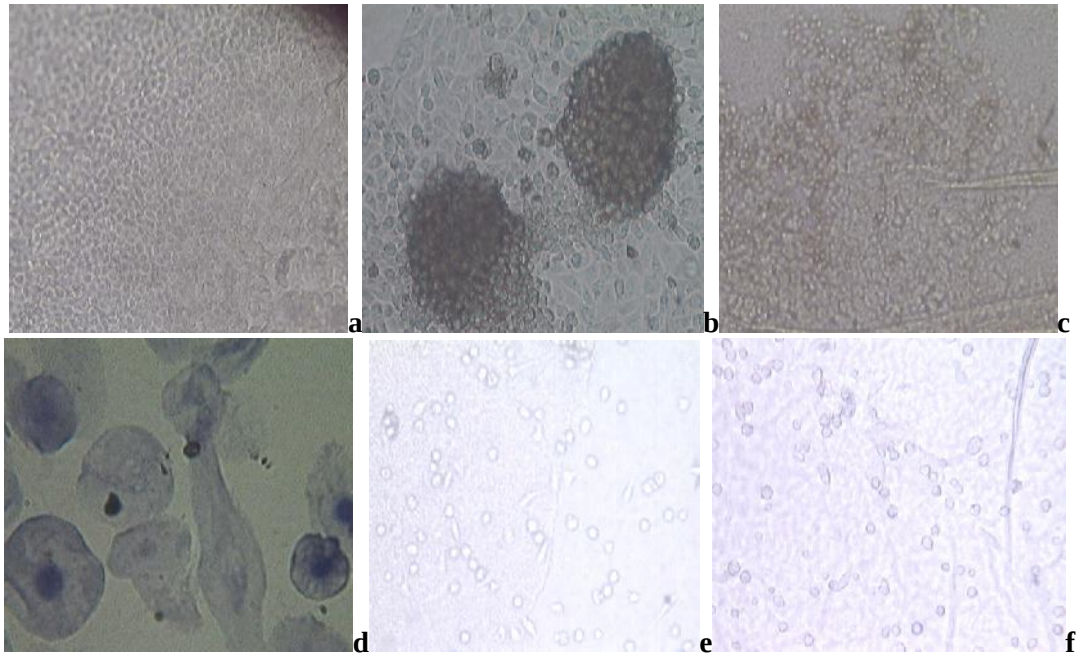
Derivation of osteoclasts from laboratory-incubated human bone-marrow MSCs

For osteoclast differentiation, method, successfully applied by Susa *et al*. (2004), was used. Briefly, after 24-48 hours cultivation in basic α-MEM, separate cell sub-populations were pre-cultured in respective volumes of supplemented α-MEM, to which 25 ng/ml M-CSF and 50 ng/ml RANKL (receptor activator of NF-κB ligand) were added. The media were changed twice a week, and the cells were observed on each 24 hours. The appearance of osteoclasts was observed around the 7^{-th}-10^{-th} day after seeding in medium, supplemented with factors M-CSF and RANKL, by staining with Hematoxylin and TRAP technique. In both cases, appearance of many multi-nuclear cells could be seen, mainly with giant sizes.

Results

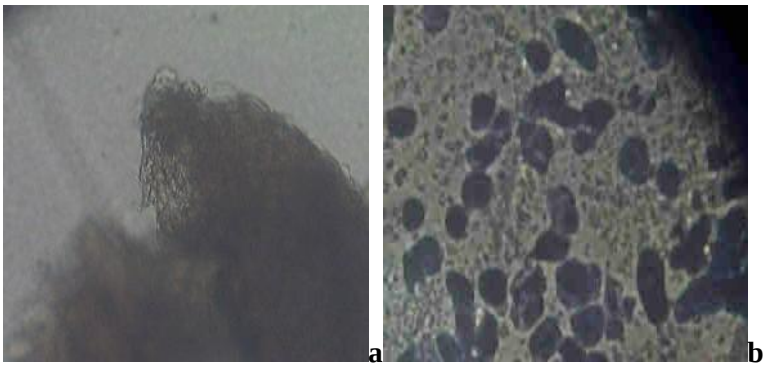
Corneal epithelial cells and tissue explants were *in vitro*-incubated on feeder cell monolayer of 3T3 mouse embryonic fibroblasts; in growth medium with cultural fluid of the same feeder cells, as well as on chemically-modified vitelline membrane, respectively (Fig. 1). In all cases, cells with different 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.

Fig. 1 Epithelial stem/progenitor cells from human ocular limbus, incubated in different laboratory conditions: in growth medium, supplemented with cultural fluid from previously cultivated 3T3 mouse embryonic fibroblasts (feeder cells) in it (a, d); on confluent monolayer of 3T3 cells in their role of feeder cells, after previous stop of further proliferation by Mytomicin-C (b, e); on vitelline membrane, previously treated by Glutamine-Glutaraldehyde (c, f); a-c: Native light microscopy preparations, magnification: x100; d-f: fixed Giemsa-stained light microscopy preparations, magnification: x250 (d); x100 (e, f).



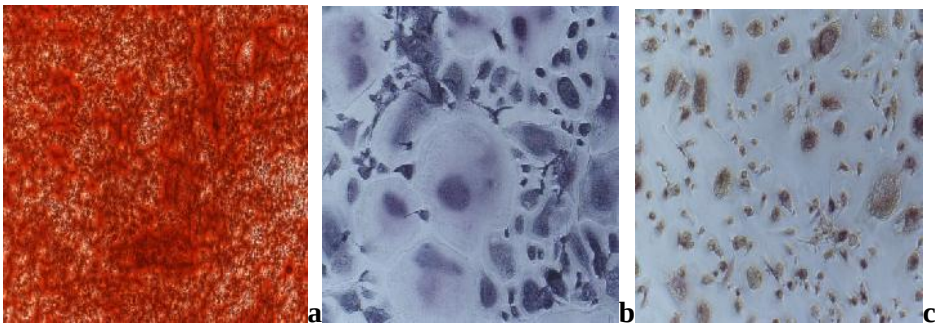
Similar characteristics were observed in *in vitro*-incubation of cells and tissue explants from human oral mucosa epithelium, in the presence only of cultural fluid from embryonic 3T3 cells (Fig. 2).

Fig. 2 *In vitro*-incubated biological material from human oral mucosa epithelium, in the presence of growth medium, supplemented with cultural fluid from previously cultivated 3T3 fibroblasts (feeder cells) in it: a) native light-microscopy preparation of tissue explant (magnification: x100); b) fixed Giemsa-stained preparation of epithelial stem/progenitor cells (magnification: x150).



Analogically, in laboratory incubation of MSCs from human bone-marrow stroma, formation of normal mature osteoblasts and osteoclasts was observed (Fig. 3).

Fig. 3 Different types normal mature cells, derived from human bone-marrow MSCs progenitors, *in vitro*-incubated in different laboratory conditions: a) osteoblasts (fixed light microscopy preparation, stained by Alizarin Red dye, magnification: x100); b) osteoclasts (fixed light microscopy preparation, stained by Hematoxylin, magnification: x100); c) osteoclasts (fixed light microscopy preparation, stained by TRAP-technique, magnification: x100).



Discussion

The results confirmed the literature findings about the role of bio-membranes, but also of the feeder cells, 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; Sudha *et al*., 2006). Also, the data obtained were in agreement with the proved expression from oral mucosa epithelial stem/progenitor cells of some markers, established as characteristic for limbal epithelial stem/progenitor cells (Grueterich *et al*., 2002; Pellegrini *et al*., 1999).

These data proposed any role of the feeder cells metabolites in support of stem cell growth and proliferation. The understanding of molecular events, gene expression mechanisms and signalling pathways, which underline in the processes of growth, proliferation, development and differentiation of stem/progenitor cells, has been characterized to be of a great importance, because it could allow derivation of different mature cell types from common stem/progenitor cells, as well as any mature cell lineage from different cell progenitors, by application of appropriate gene or differentiation strategies. The influence of FGFs and their receptors has also been discussed (Jacob *et al*., 2006; Jones and Yang, 2011; Pitaru *et al*., 1993), as well as of other components of the extracellular matrix (ECM), as metalloproteinases (MMPs), microtubule-activated protein-kinases (MAPKs), Nuclear Factor kappaB (NF-κB), as well as nuclear factor-induced kinases (NIKs) and IκB kinases (IKKs) (Cloutier *et al*., 2007).

More detailed investigations on the direct and/or indirect intra-molecular interactions, which determine the differentiation of cell progenitors to one or another direction in respective incubation conditions, are necessary.

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