Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 2 (9) August 2013: 43-47

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com

ORIGINAL ARTICLE

Development of Novel *In Vitro*-Cultivation Techniques for Derivation of Epithelial Cell Progenitors from Human Cornea and Oral Mucosa for Therapy of Limbal Stem Cells Deficiency

Ilina Valkova, Iskra Sainova, Ani Georgieva, Anatoli Neronov, Marin Bratanov, Elena Nikolova
Institute of Experimental Morphology, Pathology and Anthropology with Museum to Bulgarian Academy of Sciences – "Acad. G. Bonchev" Street 1113 Sofia

ABSTRACT
Novel techniques for in vitro-cultivation and incubation of limbal and oral epithelial stem cells were developed. All cells were characterized on the basis of their morphological characteristics: shape, appearance, presence of mitotic figures, as well as confluence and adherence of substrate. The isolated from human cadaver limbus tissue explants were subsequently cultivated in Petri-dishes, containing glass cover-slips, over-laid with stretched vitelline membranes from fresh hen's eggs. During the period of in vitro-cultivation, formation of non-adherent cell sheets, as well as of adherent to the membrane cell layers, composed by differentiated corneal epithelial cells, were noticed. Chemically-modified by treatment with gelatine-glutharaldehyde vitelline membrane indicated characteristics as a suitable substrate, able to maintain the growth of epithelial stem cells with limbal origin, which proved the abilities about its application in construction of implants from "cell-membrane" type for the needs of reparative ophthalmology. In contrast, the non-modified vitelline membrane didn't show these properties. Epithelial stem cells and tissue explants from human oral mucosa were in vitro-cultivated and incubated in similar laboratory conditions, because they were also proved to induce expression of limbal epithelial stem cell markers. Techniques for substrate adhesion of the isolated tissue explants from human oral mucosa on glass and plastic lamella, previously treated with poly-L-Lysine, with gelatine and with FCS, respectively, were tested. Formation of both adherent and non-adherent cell sheets, consisting of cells with different morphology and maturation degree, was observed. On the other hand, in application of cold trypsinization laboratory technique, primary cultures of human oral mucosa epithelial stem cells were derived, which showed signs of early cell differentiation. Future experiments in this direction are necessary, which should be connected mainly with proof of limbal stem cell markers in the so cultivated cells and tissue explants, as well as cultivation of oral mucosa tissue explants, and epithelial cells on a membrane in its role of biological substrate and in vivo-test with experimental animals for final confirmation of the eventual therapeutic success.
Key words: *epithelial stem cells; vitelline membrane; tissue explants; in vitro-cultivation.*

Received 10.05.2013 Accepted 12.07.2013

INTRODUCTION

The normal ocular surface has been characterized as covered of highly specialized corneal, limbal and conjunctival epithelial cells, which, together with the tear film form its anatomic entireness [1, 2]. The conjunctival epithelial tissue is supplied with blood vessels and it is built of several layers of cells, forming mosaic structure. In contrast, the corneal epithelial tissue, has been found to be built of flat squamous epithelial cells, settled on highly-organized collagen fiber stroma, under which endothelial cell monolayer, composed of prolonged epithelial cells, but also other cellular types, surrounded by blood capillary net. As the richest of blood vessels has been characterized the transitional region between the corneal and the conjunctiva, possessing ring form. In this zone, named ***limbus*** (***Limbus corneae***), localization of limbal stem cells populations has been established [3-6].

Specialized type of cells, possessing stem cell characteristics (*corneal epithelial stem cells - CESCs*), giving all other cornel epithelial cell types, have been indicated [7, 8]. Despite the increasing number of findings, supporting this attitude, the term "*proposed stem cells*" has still been used by some authors, in particular about the basal cells in the limbal region, characterized with poor cytoplasmic amount and hyperchromic dark-stained nucleus. According the finding of Lemp and Mathers (1989), during the regeneration of human ocular surface lesions, active migration of cells from the limbal to the central corneal region has been observed [9]. In this process, the cells have been found to loose some typical markers (as for

example, Keratin 3), which have been established on the surface of the highly differentiated epithelial cells in the central corneal region [9-11].

The concept of *limbal stem cells* has been imposed from the combined presence in them of markers for cell differentiation (as Keratins K3 and K12, Connexin Cx43, etc.), as well as of stem cell markers (Keratin K19, ABCG2, protein p63, Vimentin, Nestin, Integrins 1 and α9, Enolase) [6, 8, 12, 13]. According many literature data, the established stem cell phenotype of those cells could be determined by the positive expression of the last, on the one hand, but also by the negative expression of other molecule markers as Keratins K3 and K12, E-Cadherin, Konnexin 43 and Involucrin, on the other [14].

Because of the established similar success end result from studies, in which auto- and allografts have been used, despite the necessity of different techniques for cell cultivation and transplantation, it has been difficult to be defined the most appropriate method for this goal in each concrete case [15, 16]. Despite that, improvement and application of these therapeutic procedures is necessary, including in the treatment of the so called *Cases of Limbal and Corneal Stem Cells Deficiency* [16-18]. As a potentially hopeful method in this aspect the application of *oral mucosa* epithelium, as a source of epithelial stem cells, has been discussed [3, 16-18].

In this aspect, the main idea was connected with the development of novel methods for laboratory cultivation of tissue explants and cells from both limbal region and oral mucosa, for eventual effective and safe treatment in different cases of *Limbal Stem Cells Deficiency*.

MATERIALS AND METHODS

Different combinations of the growth media DMEM and Ham's or of DMEM and F12, were used. These growth media were additionally supplemented with 10% Fetal Bovine Serum (FBS), antibiotic mixture (100 UI/ml Penicillin, 0.25 mg/ml Streptomycin and 0.25 mg/ml Amphotericin-B), 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 mice). All cells and tissue explants were incubated at 37^{o}C, in incubator with 5% CO_2 and 95% air humidity.

For determination of the phenotype characteristics of the so cultivated cells, fixed light microscopy smears were prepared, which were the stained by Giemsa, and, respectively, subjected on indirect immuno-fluorescence technique with monoclonal antibodies to protein p63 and vimentin. For this aim, the fixed in cold Methanol and subsequently dried preparations were washed with Phosphate Buffered Solution (PBS - Sigma-Aldrich) and treated with monoclonal mouse antibody against human p53 protein and with monoclonal mouse antibody against bovine vimentin, respectively. After incubation in moist camera for 2 hours at room temperature, all preparations were washed three times with PBS and subsequently incubated with FITC-conjugated goat anti-mouse serum at room temperature for 1 hour. After washing with PBS several times, the so prepared smears were included in Glycerol-PBS cover medium and observed by fluorescent microscope Opton, supplied with 35 mm Photo-camera.

After cutting of the isolated from fresh hen's eggs vitelline membrane to small pieces, they were treated with PBS at room temperature, with constant shaking for the full separation from the egg vitelline yolk. The so washed membrane pieces were kept in PBS till the moment of their using. For chemical modification of the vitelline membrane, two methods were applied: one-step and two-step procedure, respectively. In the first technique, after incubation of the pieces in a vessel with 30 ml 5% solution of Gelatine in PBS for 1 hour with shaking, 30 ml 5% solution of Glutharaldehyde in PBS was added gradually on drops. In the other two-steps technique, after incubation of the membrane for 1 hour in a vessel with 30 ml 2.5% solution of Glutharaldehyde in PBS, after subsequent several times washing with PBS, the so treated pieces were replaced in a vessel with 30 ml 5% solution of Gelatine in PBS.

As an alternative source for derivation of epithelial cell progenitors, human oral mucosa was used. For this goal, technique of tissue explants cultivation, as well as cold tripsinization, were applied. In the first case, the isolated tissue explants were seeded on lamella, previously treated with poly-L-Lysine, with Gelatine and with a drop of FCS, respectively. After heating at 37^{o}C, the trypsin action was blocked by addition of FCS and the explant was mechanically resuspended. After centrifugation, of the so obtained cell suspension and subsequent resuspendation of the pellet, the cells from the last were seeded in the described conditions of cultivation.

RESULTS AND DISCUSSION

In *in vitro*-cultivation of corneal epithelial cells on feeder cell monolayer from 3T3 mouse embryonic fibroblasts, cells with different morphology and in different phases of differentiation (early epithelial cell progenitors with round shape, oval nucleus and poor cytoplasmic contention, as well as polygonal-shaped mature epithelial cells), were established (Figure 1).

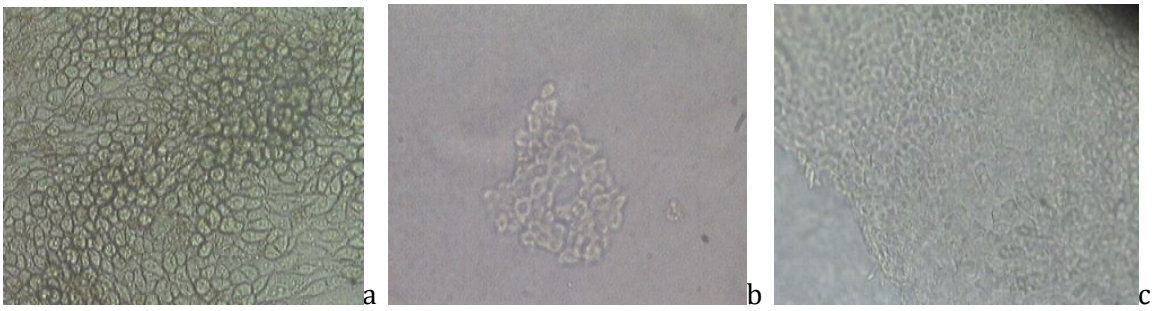

Fig. 1: Human limbal epithelial stem cells, cultivated on monolayer of feeder cells (3T3 Balb/c mouse embryonic fibroblasts), in particular with round and oval shape, which are signs for early stages of differentiation: a) colonies of proliferating cells; b) group of cells; c) confluent monolayer of proliferating cells, deriving from tissue explant (Native preparations)

The data of the performed immuno-fluorescent assay indicated actively expression of markers p63 and Vimentin (Figure 2).

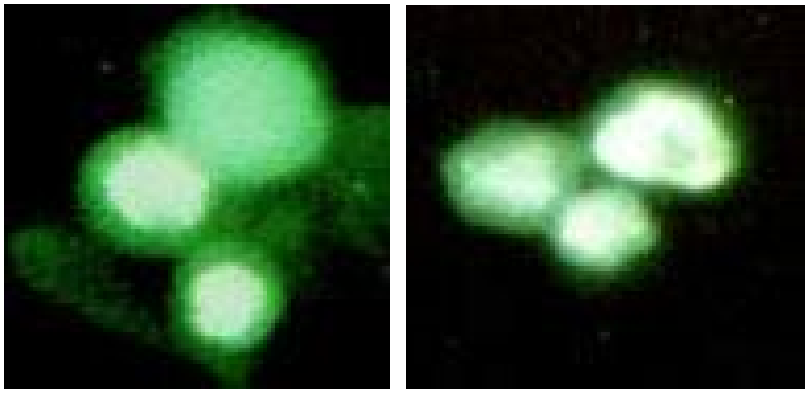

Fig. 2: Positive immunofluorescence of limbal epithelial stem cells for markers p63 and Vimentin (Fixed preparations, treated with appropriate antibodies, conjugated with FITC)

In *in vitro*-cultivation on modified by Gelatine-Glutharaldehyde vitelline membrane, single migrating from the limbal explant round-shaped cells, but also a gradual formation of adherent cell monolayers on the membrane, represented mainly of dense-placed polygonal and highly differentiated squamous cells, were observed (Figure 3).

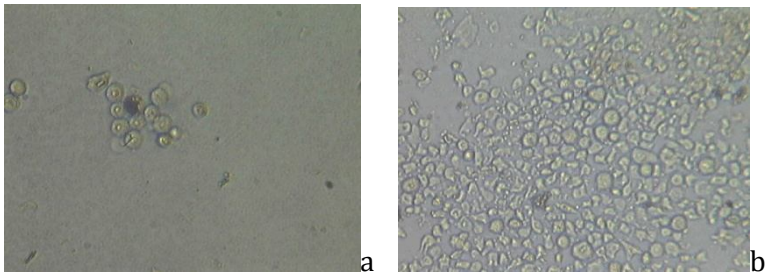

Fig. 3: Human limbal epithelial stem cells, cultivated on vitelline membrane, chemically-modified by treatment with Gelatine-Glutharaldehyde: a) early limbal epithelial stem cell progenitors, a proof of which is the observed round shape, round nucleus and poor cytoplasmic amount; b) actively proliferating early limbal epithelial cells. Despite the observed predominating round cells, oval-shaped and low amount of polygonal cells could also be noticed (Native preparations).

In this way, the results obtained proved the usability of the chemically-modified by treatment with Gelatine-Glutharaldehyde vitelline membrane as appropriate substrate, able to support the growth of limbal epithelial stem cells, which, on the other hand, could suppose eventual abilities for its application in

construction of "cell-membrane" tissue implants for the needs of the reparative ophthalmology. In contrast, these characteristics weren't established for chemically-unchanged vitelline membrane. Similar data have been obtained by use of amniotic membranes, 3T3 fibroblasts from Balb/c mouse embryos, as well as combined membrane-cell substrates [15, 19, 20-25].

Because of the proved expression of some markers, also proved in limbal stem cells [12, 18, 26-28], epithelial cells from oral mucosa were analogically *in vitro*-cultivated and incubated. In this case, cells with different morphology and in different stages of differentiation, were also observed, which could be proved by the established changes in their shape – round, oval or polygonal, respectively (Figure 4). These differences could be seen, when the tissue explant from oral mucosa is cultivated on lamella, previously treated with poly-L-Lysine (Figure 4 - a), with Gelatine (Figure 4 - b) and with FBS (Figure 4 - c), but also in seeding of separate cells, obtained after application of cold trypsinization (Figure 4 - d), respectively.

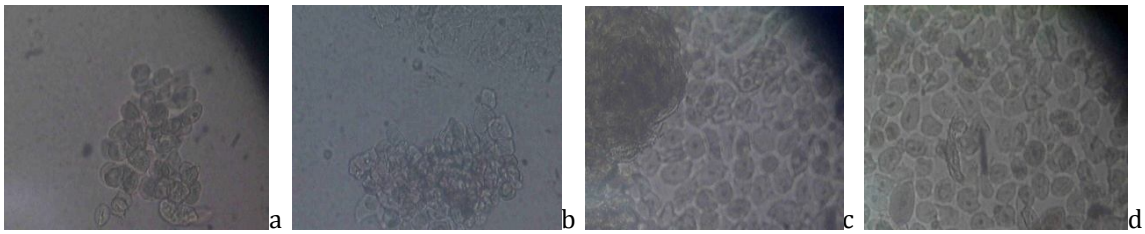

Fig. 4: Human oral mucosa epithelial cell progenitors, in particular with round and oval shape, which are signs for early stages of differentiation: a) group of epithelial cell progenitors in early stages of differentiation, adhered on treated with poly-L-Lysine lamella; b) group of epithelial cells, most of them in early stages of differentiation, adhered on treated with Gelatin lamella; c) early epithelial stem cells, deriving from oral mucosa tissue explant, adhered on lamella, treated with drop of FCS; d) monolayer of early epithelial stem cell progenitors, derived by application of cold tripsinization technique on the isolated oral mucosa tissue explant (Native preparations)

CONCLUSIONS

Novel technology for construction of "cell-membrane" tissue implants by laboratory cultivation of limbal cells and explants from cadaver cornea on chemically-modified by treatment with Gelatine-Glutharaldehyde vitelline membrane, was developed. Ability for application of this chemically-modified vitelline membrane as an appropriate substrate for cultivation and propagation of different cell types was proposed. Methods for differentiation of laboratory-cultivated epithelial stem cells from oral mucosa in cells, expressing some markers, proved in limbal epithelial cells.

Future studies, connected particularly with proof of limbal stem cell markers in the so cultivated tissue explants and epithelial cells from oral mucosa, but also laboratory cultivation of oral mucosa tissue explants and epithelial cells on a membrane in its role of biological substrate, as well as *in vivo*-experiments on experimental animals for final confirmation of the eventual therapeutic success, are necessary.

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How to cite this article

Ilina Valkova, Iskra Sainova, Ani Georgieva, Anatoli Neronov, Marin Bratanov, Elena Nikolova. Development of Novel *In Vitro*-Cultivation Techniques for Derivation of Epithelial Cell Progenitors from Human Cornea and Oral Mucosa for Therapy of Limbal Stem Cells Deficiency. Bull. Env. Pharmacol. Life Sci., Vol 2 (9) August 2013: 43-47