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In vitro-cultivation of human oral mucosa epithelial cells and tissue explants as a modern method for applications in therapy of limbal stem cell deficiency. A pilot study

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Possibilities for application of oral mucosa epithelial cells and tissue explants for development of novel therapeutic strategies in ocular limbal stem cell deficiency were examined. For this goal, because of the proved expression of limbal epithelial stem cell markers, epithelial stem cells and tissue explants from human oral mucosa were *in vitro*-cultivated in appropriate laboratory conditions. Techniques for substrate adhesion of the isolated cells and tissue explants from human oral mucosa on glass or plastic lamella, previously treated with poly-L-Lysine, with gelatine and with Fetal Calf Serum (FCS), respectively, were tested. All cells were characterized on the basis of their morphological characteristics: shape, presence or appearance of mitotic figures, as well as confluence and adherence on the substrate used. Formation of both adherent and non-adherent cell sheets, consisting of cells with different morphology and maturation degree, was observed. Future experiments in this direction should be connected mainly with cultivation of oral mucosa tissue explants and epithelial cells, on a bio-membrane in its role of appropriate biological substrates, as well as with proof of specific markers in them, about eventual possibilities for future applications in construction of implants for the needs of reparative ophthalmology.

Key words: human oral mucosa, epithelial stem/progenitor cells, tissue explants, *in vitro*-cultivation.

Introduction

The concept of limbal stem cells (LSCs) has been imposed from the combined presence in them of markers for cell differentiation (as Keratins K3, K12, Connexin Cx43, etc.), as well as of stem cell markers (Keratin K19, ABCG2, protein p63, Vimentin, Nestin, Integrins 1 and α9, Enolase) [6, 16-18]. Those cells are localized in the so named ***limbus*** (***Limbus corneae***). In the normal ocular surface it has been characterized as covered of highly specialized cells [2-4, 7, 9, 15, 16]. Respectively, the improvement and development of novel therapeutic strategies is necessary in the treatment of limbal stem cell deficiency (LSCD) [8, 13-17], which could be a result of *Stevens-Johnson* syndrome (SJS), ocular cicatricial pemphigoid, as well as different types of mechanical, physical

and/or chemical injury [11]. As a potentially hopeful method in this aspect, the application of oral mucosa epithelium as a source of epithelial stem cells, has been discussed [2, 8, 13-17].

Cultivated autologous oral mucosal epithelial transplantation has been characterized as successful tissue-engineering technique for generation of autologous epithelial cells and/or tissue explants for therapeutic practice, and, in particular, in reconstructing the ocular surface in different cases of LSCD [11, 14, 20]. Analogically to the normal ocular surface, the normal oral cavity has been found to contain several different types of stratified squamous epithelia, including as nonkeratinized, parakeratinized and orthokeratinized [10, 20]. The longevity of epithelial cell cultures, derived from normal, nonpathologic oral mucosa, has been described as dependent of the length of time in culture or of the number of passages and population doublings [10].

In this direction, the main idea was connected with initial studies on development of novel methods for laboratory cultivation of tissue explants and cells from human oral mucosa, for eventual effective and safe treatment in different cases of LSCD.

Materials and Methods

Different combinations of the growth media Dulbecco's Modified Minimal Essential Medium (DMEM) and Ham's or of DMEM and F12 were used. 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 feeder cells (fibroblasts from embryos of Balb/c experimental mice), were added. The isolated cells and tissue explants from human oral mucosa were seeded directly on plastic or glass lamella, previously treated with poly-L-Lysine, Gelatine and/or FCS, respectively, which were put in appropriate dishes for cultivation with liquid growth media, and incubated at 37°C, in incubator with 5% CO_2 and 95% air humidity. The so prepared cultures of cells and tissue explants were observed as native preparations by inverted light microscope (Leica), supplied with mega-pixel CCD-camera.

Results and Discussion

Because of the proved expression of some markers, also indicated in limbal stem cells [3, 6, 10, 11, 14, 15, 19, 20], epithelial cells from oral mucosa were analogically *in vitro*-cultivated.

Cells with different morphology and in different stages of proliferation and maturation were observed, which could be confirmed by the established changes in their shape – round, oval or polygonal, respectively (Fig. 1). Probably, those signs depend on the stage of cell differentiation: round and/or oval cells (Fig. 1A, B), cell sheets, composed mainly of undifferentiated cells with round and/or oval shape (Fig. 1B) and clusters, composed mainly of more differentiated polygonal cells and small amounts of early epithelial progenitors (Fig. 1D, E). These features were observed in use of the three different types of substrates for seeding of the cells.

In seeding of tissue explants from human oral mucosa, gradual separation of smaller tissue fragments, composed mainly of undifferentiated cells with round and/or oval form, could be seen (Fig. 2). Here again these characteristics were present inde-

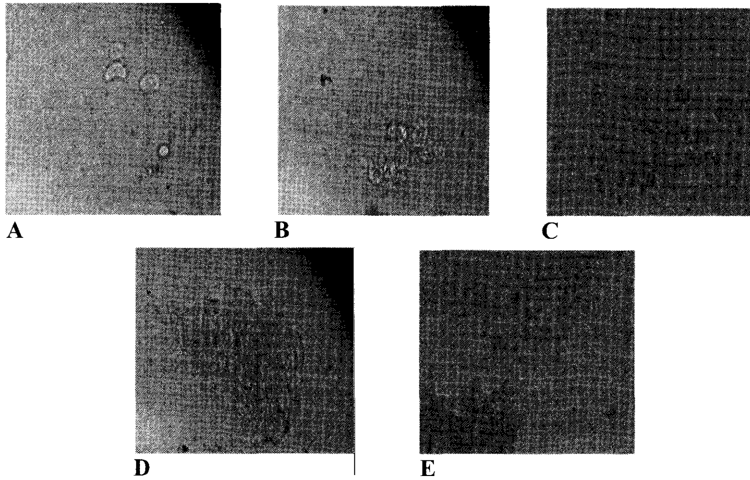

Fig. 1. Human oral mucosa epithelial cells in different phases of proliferation and differentiation: in early phases of differentiation, characterizing with round and oval shape (A) and (B); cell sheets, composed of many actively proliferating early cell progenitors in different sub-stages, characterizing with round and oval shape, but only few amounts of more differentiated cells with polygonal shape could be seen (C); cell sheets, composed mainly of mature epithelial cells with polygonal form (D) and (E) (Native preparations)

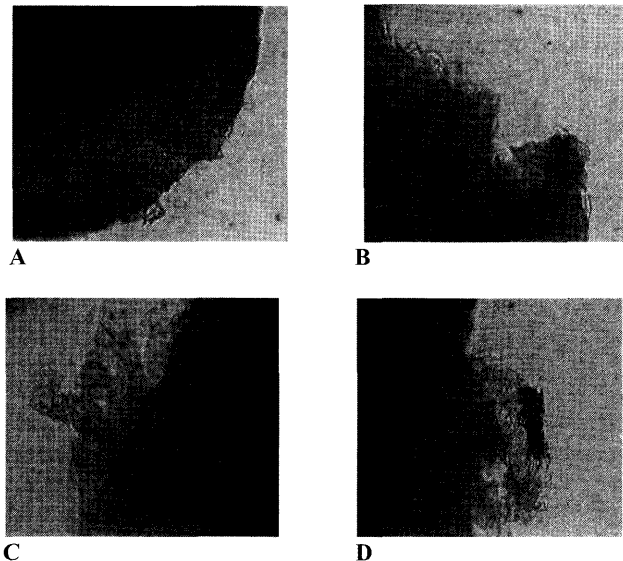

Fig. 2. Tissue explants from human oral mucosa epithelium on different hours from their direct seeding in liquid growth medium: Gradual separation of smaller tissue fragments, composed mainly of undifferentiated cell progenitors cells with round and oval form, could be seen (Native preparations)

pendently of the used substrate. Those our results were in agreement with the literature findings about the proved different types of stratified epithelia in the oral mucosa zone (Fig. 2A) [10]. The noticed increase in the sub-populations from the separate cells in the tissue explants in the time was accepted as a proof for their strong proliferation capacity (Fig. 2B-D).

Conclusion

In *in vitro*-incubation of cells and tissue explants from human oral mucosa, cells with different shape and morphology, in different stages of proliferation and differentiation were noted. A proof for their strong proliferation capacity was the observed increase in the cell sub-populations.

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 of techniques for their laboratory cultivation on appropriate substrates for the needs of reparative ophthalmology, are necessary.

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