Limbal stem cell deficiency (LSCD) has been increasingly implicated as a cause of ocular surface diseases including pterygium. Limbal stem cells act as a physiological barrier to the ingress of conjunctival cells across the cornea\(^1\). Deficiency leads to conjunctivalisation of the cornea, surface irregularity, and vascularisation. Limbal stem cells may be depleted by a wide range of pathological processes affecting the ocular surface including topical medications, ultraviolet, and ionizing radiation. Limbal cells can be absent because of chronic conditions like radiation, keratitis, drug toxicity and ocular cicatrical pemphigoid\(^2\). One of the most serious pathological conditions in which limbal epithelial stem cells are deficient is Stevens-Johnson Syndrome also known as erythema multiforme.
Bilateral LSCD requires allogeneous limbal tissue as a source of limbal stem cells, and this necessitates long-term use of systemic immunosuppressants to avoid graft rejection\textsuperscript{12}. Therefore, sources of autologous tissue that can functionally replace the corneal epithelium have been considered as an alternative to allogeneous limbal transplants. Since the corneal epithelium is of the stratified squamous type, autologous epithelial cells such as oral, conjunctival, nasal and oesophageal epithelium, all having a similar morphology, could be considered as an alternative to allogeneous limbal transplants.

Extensive studies have been performed to investigate the feasibility of oral mucosal epithelium for this purpose, as it is easily available and can be harvested without invasive surgery\textsuperscript{5}. Keratin 3 (K3) is a reliable marker for corneal type differentiation and it is positive for epithelial cells of oral mucosa. Oral epithelial cells are considered as an ideal substitute for corneal epithelial cells for use in ocular surface reconstruction\textsuperscript{6}.

The wet-surfaced epithelia, produces a group of highly glycosylated protective membrane glycoproteins termed mucins – MUC\textsuperscript{7}. Mucin lubricates the apical surface of epithelium and provides a barrier against pathogen. Although the function of these mucins in the oral cavity remains to be elucidated, it is possible that these contribute to the epithelial protective mucin layer and act as receptors initiating one or more intracellular signal transduction pathways\textsuperscript{8}. At the ocular surface, at least three membrane-associated mucins (MUC1, 4, and 16) and two secreted mucins (MUC 5 and 7) are expressed\textsuperscript{9}.

Oral and corneal epithelium also plays a critical role as a microbial barrier\textsuperscript{10}. Various antimicrobial peptides (AMP) are known to be present on the epithelial cells of ocular and oral surface. The AMP produced by oral epithelial cells may act to control many commensal and pathogenic bacteria in oral cavity and play a critical role as a microbial barrier\textsuperscript{10}. The human β-defensins (hBD) are peptides expressed by epithelia throughout the body including epithelia of oral cavity. There are now 28 known β-defensin genes found in human, however; expression of hBD1, 2, and 3 has been most investigated\textsuperscript{11}.

Here, we report the explant culture method of cultivating oral mucosal epithelial cells and their characteristics in comparison to the limbal explant culture.

**Material & Methods**

**Chemicals & reagents:** Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F12 (HF12), Hanks balanced salt solution (HBSS), phosphate buffer saline (PBS), Trypan blue dye, antibiotics solution, foetal bovine serum (FBS), Haematoxylin and Eosin (H&E) staining solutions were purchased from Hi-Media, Mumbai, India. Streptomycin, amphotericin, mouse epidermal growth factor (EGF), transferrin, selenium, keratinocyte growth supplement and hydrocortisone were purchased from Invitrogen CA, USA. The tissue culture plastic plates were from Becton Dickinson, USA. RNA extraction and cDNA conversion (sensiscrypt reverse transcriptase) kit was obtained from Qiagen, Germany. The BrdU assay kit was purchased from Roche Applied Sciences, Germany. The specific primer sequences were obtained from Sigma Chemicals Co., USA.

**Collection of oral mucosal and corneal limbal tissue:** Institutional ethics committee board approved the study protocol. Oral tissues (n=6) were collected from (n=6) patients who underwent oral mucosal graft for bilateral LSCD at Sankara Nethralaya eye hospital, Chennai during 2006-07. After obtaining written consent from each patient, oral cavity was sterilized with topical povidone-iodine; 3x3mm specimen of mucosal tissue was surgically excised from the interior buccal mucosal epithelium under local anaesthesia. The tissue was excised carefully to exclude underlying submucosal connective or fat tissue and collected for further processing in DMEM with 3 per cent FBS and antibiotics as the transport medium.

Corneal limbal tissue (n=6) of 2 mm\textsuperscript{3} from the cadaveric donor eye from the C U Shah eye bank of Medical Research Foundation, Sankara Nethralaya, Chennai, with the consent of donor or donor family was collected in the transport medium for further processing. The donor blood samples were screened for human immunodeficiency virus (HIV) type 1 and 2, hepatitis B virus (HBV), hepatitis C virus (HCV) and Treponema pallidum infections. Data were collected on parameters; like age, sex, cause of death, time of death, time of donation and time of biopsy collection.

**Explant culture of oral mucosal and limbal epithelial tissue:** Both the tissues were processed by same methodology separately. The tissues were washed thrice with PBS containing double strength antibiotics and the epithelial tissues were cut into multiple bits using sterile hard-parker blade and were placed on the centre of the wells (plastic) using a sterile needle. The plates
were incubated at 37°C and 5 per cent CO₂ for 5 min for adhesion. Two ml of the culture medium [equal volume of DMEM and F12 with 50 ng/ml of streptomycin, 1.25 ng/ml of amphotericin B 2 ng/ml of mouse epidermal growth factor (EGF), 5 ng/ml of insulin, 5 ng/ml of transferrin, 5 ng/ml of selenium, 5 mg of keratinocyte growth supplement, 0.5 mg/ml of hydrocortisone] supplemented with 10 per cent FBS was added to each well. The plates were incubated at 37°C and 5 per cent CO₂ for 21 days. The medium was changed once in two days until they reached confluence and cell growth was monitored daily for three weeks with an inverted phase contrast microscope (Nikon, Japan). Confluent cells were collected for further characterization.

Morphology and viability of cultivated cells: Cultures were monitored under an inverted phase contrast microscope (Nikon, Japan). The viability of cultivated cells was determined by staining with trypan blue. The cells were harvested, washed twice with PBS and 0.5 per cent trypan blue solution in PBS was added to the cell pellet and incubated at room temperature. About 10 µl of the sample was loaded on hemocytometer chamber and numbers of viable and nonviable cells were counted.

Hematoxylin and eosin (H&E) staining: The cultures were fixed in 10 per cent neutral buffered formalin, processed and embedded in paraffin wax. The paraffin sections were generated, deparaffinized and stained with H & E stain. Sections were observed under a light microscope.

BrdU retention assay: Cell proliferation was assessed by measuring 5-bromo-2-deoxyuridine incorporation during DNA synthesis in proliferating cultured cells on the sixth day of passage. The detection of BrdU was performed according to manufacturer’s instruction and chased for 1-21 days. The BrdU labelling indices were assessed by counting the nuclei through a microscope using 40X objective. The labelling index was expressed<sup>12</sup> as number of positively labelled nuclei/total number of nuclei X 100 per cent.

RNA isolation and RT-PCR analysis: At the end of 21<sup>st</sup> day limbal and oral mucosal cells were treated with 0.02 per cent trypsin and harvested in RNase free vial to extract total RNA. Semiquantitative RT-PCR was performed using sensiscript reverse transcriptases to study the expression of different putative stem cell markers designed<sup>9,13,14</sup> from published human gene sequences (Table I) along with respective positive control and housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control in eppendorf PCR systems. PCR products were fractionated by electrophoresis using 2 per cent agarose gel containing 0.5 per cent ethidium bromide with molecular marker 

Statistical analysis: All experiments were performed in triplicate. Summary data were reported as the mean ± SD. Student’s t test was used for comparison between the two cultures.

Results

Morphological characterization of limbal and oral epithelial cells: Epithelial migration from both limbal and oral mucosal biopsies was noted at the end of 48 h (Fig. 1a, b). The cells further multiplied with outgrowth forming monolayer. Oral epithelial cells cultured under these conditions resembled corneal epithelium. The optical transparency of harvested cell sheets was equal to that of corneal epithelial- cell sheets originating from limbal stem cell. By the end of fifteenth day 90-100 per cent confluent growth, was seen and cells were harvested (Fig. 1c, d) which is shown by H & E staining (Fig. 1e, f). A slight elevated growth was seen in oral mucosal epithelial cells comparatively to limbal culture. Viable count, as estimated by trypan blue exclusion test, ranged from 95–98 per cent approximate yield of 4x10<sup>4</sup> cells per mm<sup>2</sup> of the plate in both the cultures. There was rapid incorporation of BrdU at the end of 24 h in both the cultures. The cultures were chased continuously for 7, 14 and 21 days and the labelling index were calculated to plot a graph (Fig. 2). There were no statistically significant differences between two cultures.

Phenotypic characterization of limbal and oral epithelial cells: Molecular markers expression in the cultures of oral epithelial cells was compared with limbal epithelial cells (Table II). The mRNA expression of ABCG2, K3, p63, delta Np63 and isoforms of p63, (Fig. 3), MUC (Fig. 4) and AMP (Fig. 5) in both limbus and oral mucosal culture were studied with respective positive and internal controls. All the corneal/limbal phenotype stem cell markers were found to be expressed in oral mucosal epithelial cells. The isoforms of p63 were found to be expressed in oral mucosal cells though a few were absent in limbal cells. The oral cells did not express K12 gene. The MUC 1, 4 and 16 were expressed in oral mucosal culture whereas MUC 16...
### Table I. Primer sequences and reaction conditions for the RT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer sequence - 3’-5’</th>
<th>Annealing temperature (°C)</th>
<th>Base pair size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG2</td>
<td>FP: AGTTCATTGCACTGAGGCTAT&lt;TAGGAGG &lt;TAGGAGG</td>
<td>62</td>
<td>379</td>
</tr>
<tr>
<td>Keratin 3</td>
<td>FP: GGCGAGGCTGAGGTTCTC &lt;TAGGAGG</td>
<td>64</td>
<td>145</td>
</tr>
<tr>
<td>Keratin 12</td>
<td>FP: CATGAAGAGAAGACCAGAGGATG</td>
<td>63</td>
<td>150</td>
</tr>
<tr>
<td>∆Np63</td>
<td>FP: CAGACTCAAATATAGGAGAAGG</td>
<td>54</td>
<td>440</td>
</tr>
<tr>
<td>P63α</td>
<td>FP: AGGGGCTGACCACCATCTAT</td>
<td>64</td>
<td>196</td>
</tr>
<tr>
<td>P63β</td>
<td>FP: CCACCAGGTGCAGCTGAGCTATCT</td>
<td>64</td>
<td>304,210</td>
</tr>
<tr>
<td>P63γ</td>
<td>FP: CCGGAGAGAAACCTCAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAp63α</td>
<td>FP: ATCGCATGCAATGCTGAGG</td>
<td>64</td>
<td>211</td>
</tr>
<tr>
<td>TAp63β</td>
<td>FP: ATCGCATGCAATGCTGAGG</td>
<td>64</td>
<td>1436</td>
</tr>
<tr>
<td>TAp63γ</td>
<td>FP: ATCGCATGCAATGCTGAGG</td>
<td>64</td>
<td>1547</td>
</tr>
<tr>
<td>MUC1</td>
<td>FP: GCATCAGGCTCAGCTTCTACT</td>
<td>64</td>
<td>321</td>
</tr>
<tr>
<td>MUC4</td>
<td>FP: GCATCAGGCTCAGCTTCTACT</td>
<td>64</td>
<td>243</td>
</tr>
<tr>
<td>MUC16</td>
<td>FP: GCGCTTACTTTAACGGTTAACATGAGA &lt;TAGGAGG</td>
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<td>114</td>
</tr>
<tr>
<td>hBD-1</td>
<td>FP: GCCTCCAAAAGGAGCCAGCTAGT</td>
<td>54</td>
<td>287</td>
</tr>
<tr>
<td>hBD-2</td>
<td>FP: CAGCCATCAGCCATAGG</td>
<td>55</td>
<td>204</td>
</tr>
<tr>
<td>hBD-3</td>
<td>FP: AGCTCTGACTGAGGATGTC</td>
<td>61</td>
<td>205</td>
</tr>
<tr>
<td>LL37</td>
<td>FP: CAGCAAGAGACACAGTCACAG</td>
<td>54</td>
<td>145</td>
</tr>
<tr>
<td>GAPDH</td>
<td>FP: GCACCACACTGACATCGAG</td>
<td>63</td>
<td>498</td>
</tr>
</tbody>
</table>

bp, Base pair; FP, Forward primer; RP, Reverse primer. Glyceroldehyde-3-phosphate dehydrogenase (GAPDH) is an internal control.

was absent in limbus. There was complete absence of AMP in limbal cells. hBD 1, 2 and 3 were expressed in oral mucosal epithelial cells. However, the expression of cathelicidin (LL37) was absent in both limbal and oral epithelial cells.

**Discussion**

Studies have shown that oral epithelial cells can be cultured and used as an alternative for allogenous limbal transplants in case of bilateral LSCD. ABCG2 is considered as a side population stem cell marker. In our study, the presence of this side population marker on cultured oral epithelial cells and limbal epithelial cells confirmed the presence of stem cell population. K3, the marker of corneal epithelial cell was found to be expressed by cultured oral epithelial cells, whereas K12 marker was not expressed. In this study we chose to investigate the expression of p63 among the novel
Fig. 1. Epithelial cell migration from the limbal (a) and oral tissues (b) at the end of 48 h. 90-100 per cent confluent culture of limbus (c) and oral mucosal (d) epithelial cells cultures at the end of 21 days. Haematoxylin & Eosin staining of limbus (e) and oral mucosal (f) epithelial confluent cultures.

markers that might be used to validate the phenotype of cultivated oral epithelial cells. Pellegrini et al.\textsuperscript{16} suggested that p63 is the first gene product that distinguishes stem cells from their transient amplified progeny in stratified squamous epithelia. The finding that p63 is specifically expressed by stem cells of human epidermis, limbal and oral epithelia and not by transient cells strongly suggests that p63 can be recognized as a stem cell marker\textsuperscript{17}. Both cultured oral and limbal epithelium expressed all the isoforms of p63 (α,β,γ). However, tranactivating (TA) domain variants were expressed only in the cultured oral epithelium and not in the limbal epithelium. Both oral mucosal and limbal epithelial sheets expressed transcripts for MUC1 and 4. MUC 16 was expressed...
Table II. mRNA expression on the cultured cells harvested at the end of 21 days

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Limbus</th>
<th>Oral mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Keratin 3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Keratin 12</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*Isoforms of p63*

- ΔNp63: +
- p63α: +
- p63αβ: +
- p63γ: +
- TAp63α: -
- TAp63β: -
- TAp63γ: +

*Membrane protein – mucin*

- MUC1: +
- MUC4: +
- MUC16: -

*Antimicrobial peptide*

- hBD-1: -
- hBD-2: -
- hBD-3: -
- LL37: -
- GAPDH: +

GAPDH is an internal control. (+ Positive; - Negative)

Fig. 3. ABCG2, K3 and isoforms of p63 were expressed in oral mucosa though few were absent in limbal cells. Lane 1: Negative control; Lane 2: Limbus; Lane 3: Oral mucosa and Lane 4: 100bp ladder; bp-base pair. GAPDH is an internal control.

Fig. 4. Expression of mucin gene – MUC1, 4 and 16 were seen in oral mucosal epithelial cells whereas MUC 16 was absent in limbal epithelial cells. Lane 1: Negative control; Lane 2: Limbus; Lane 3: Oral mucosa, Lane 4: Positive control – corneal endothelial cell line; Lane 5: 100bp ladder; bp-base pair. GAPDH is an internal control.

Fig. 5. The β-defensin class gene of AMP - hBD1, 2 and 3 were expressed only in oral mucosa and absent in limbal epithelial cells. The Cathelicidin class gene LL37 was not expressed in both the cultures. Lane 1: Negative control; Lane 2: Limbus; Lane 3: Oral mucosa, Lane 4: Positive control – corneal endothelial cell line; Lane 5: 100bp ladder; bp-base pair. GAPDH is an internal control.

only in oral epithelial cells and was absent in limbal cells. Several studies have shown alterations in the expression of carbohydrates and mucin-associated molecules on the ocular surface of patients with ocular cicatricial pemphigoid or other ocular surface diseases. The fact that similar expression pattern of membrane-associated mucins occurs in cultivated oral mucosal epithelial sheets and in limbal epithelium may be one of the reasons for the efficient of ectopic transplantation of a cultivated oral mucosal epithelial sheet on the cornea of patients with ocular surface diseases. Hence these expressions of mucin gene may contribute to the maintenance of a wet and healthy ocular surface after
transplantation. In this study, we also investigated the expression of the AMP, hBD 1, 2 and 3 in limbal and oral mucosa epithelial cells. Despite constant threat from pathogenic microbes in the air and foreign objects, the incidence of ocular surface infection is amazingly low\textsuperscript{20}. The limbal epithelium failed to express any of AMPs which may be due to the presence of antibiotics in culture. Inspite of same culture condition, there is presence of AMP’s in oral epithelial cells which can be explained due to the source of environment. In the oral cavity the buccal epithelium is regularly perturbed by mechanical forces in mastication, acids in food or those produced by bacteria, proteases in saliva, toothpaste, alcohol, thermal insult, \textit{etc.} and responds effectively to bacteria by producing antimicrobial peptides. The oral mucosal model is differentiated, expresses all three defensin group AMPs and has an intact sterile surface with a functional antimicrobial barrier. However, the expression of LL37 was absent in both oral and limbal epithelial cells. This model can serve as a useful basic tool for the study of tissue innate immune responses as a purely epithelial model.

In conclusion, findings of this study, showed that oral epithelial cells can be cultured \textit{in vitro} by explant culture method similar to limbal epithelial cells. Oral epithelial cultures have similar morphological features and express markers resembling limbal epithelial cells. Therefore in future, the feasibility of oral epithelial cells in clinical use should be evaluated for allogenous limbal transplants.

\textbf{Acknowledgment}

Authors acknowledge the Indian Council of Medical Research, New Delhi, for financial support.

\textbf{References}


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