Phenotypes and transdifferentiation of transplanted oral mucosal epithelial cells for limbal stem cell deficiency

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Abstract

Background: To observe the epithelial phenotypes after autologous cultivated oral mucosal epithelial transplantation (COMET), assess the changes in the oral mucosal epithelium after transplantation on the ocular surface, and identify possible influencing factors. Methods: We performed lamellar keratoplasty 6 months after initial COMET procedure on 2 patients with total limbal stem cell deficiency (LSCD) to further visual rehabilitation. The corneal buttons obtained during lamellar keratoplasty were examined by using immunohistochemistry and transmission electron microscopy (TEM). Results: Cell morphology of both excised corneal tissues resembled that of the normal cornea. They were positive for Cytokeratin3, Cytokeratin13, p63 and Zonula occludens-1. Expression of Cytokeratin12 was noted in the corneal epithelium of patient 1 but not found in patient 2. Conclusions: The oral epithelium transplanted onto the ocular surface could express corneal phenotypes after COMET, which might be related to the corneal microenvironment.

Background

Autologous cultivated oral mucosal epithelial transplantation has been reported to be successful in the reconstructing corneas of patients with severe limbal stem cell deficiency up to date. 1 Researchers from Japan firstly reported the use of autologous cultivated oral mucosal epithelial cells for reconstructing the ocular surface patients with total limbal stem cell deficiency in 2004.2,3 However, oral mucosal epithelium is not identical to corneal epithelium although long-term clinical assessments of autologous cultivated oral mucosal epithelial transplantation have yielded favorable results from the perspective of ocular surface stabilization.4,5 There are striking differences between cultivated oral mucosal epithelial transplantation and cultivated limbal epithelial transplantation in terms of complications, including a
substantially higher incidence of corneal epithelial defects and corneal stromal melting in cultivated oral mucosal epithelial transplantation. The anti-angiogenic activity of cultivated oral mucosal epithelial transplantation was inferior to that of cultivated limbal epithelial transplantation, with more prominent corneal neovascularization. Better understanding of cell phenotypes post-cultivated oral mucosal epithelial transplantation is required, which will likely allow for the development of more successful transplantation.

Few studies have reported the cell phenotypes on the human ocular surface after cultivated oral mucosal epithelial transplantation, as it is difficult to clinically determine the changes in ocular surface phenotypes. In this study, 2 patients underwent cultivated oral mucosal epithelial transplantation, then we performed lamellar keratoplasty 6 months post- cultivated oral mucosal epithelial transplantation to further visual rehabilitation. We retrieved the corneal buttons during lamellar keratoplasty to evaluate the epithelial phenotypes present on the ocular surface post- cultivated oral mucosal epithelial transplantation.

Methods

Subjects

This study adhered to the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of the Eye and ENT Hospital of Fudan University.

This study comprised 2 patients with late-stage alkali and thermo burning, respectively. Prior to the transplantation, the ocular surface was covered by fibrovascular membranes in both patients. The cultivated oral mucosal epithelial transplantation procedure was performed as previously reported. Lamella keratoplasty was performed 6 months after initial cultivated oral mucosal epithelial transplantation procedure in both cases. Corneal buttons surgically removed from during lamellar keratoplasty were divided into 3
parts. One half was processed for immunofluorescence, a quarter of the corneal tissue for histopathology, and the remainder was observed via transmission electron microscopy. Oral mucosal tissue was obtained from the patient; normal cornea was donated. Cultivated oral mucosal epithelial cells sheets were from cell sheet for checking previously.

**Histopathology and Immunohistochemistry**

Samples for histopathology were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature prior to embedding in paraffin for sectioning (10-um-thick) with a microtome. The sections were stained with hematoxylin-eosin.

We performed immunofluorescence on normal corneal, oral mucosa, cultivated oral mucosal epithelial cell sheet and two corneal buttons removed during lamellar keratoplasty. Phenotypes of the oral mucosa and cornea, putative stem cell markers, and the cell junction were evaluated.

Tissues for immunohistochemistry were embedded in optimal cutting temperature compound and frozen to a temperature of –80°C. Frozen sections (7-um-thick) were blocked at room temperature with 3% bovine serum albumin for 30 min, then, incubated at 4°C overnight with the primary antibodies diluted in phosphate-buttered saline (PBS). Primary antibodies included mouse monoclonal Cytokeratin3 (1:100; clone AE5; Abcam; USA), rabbit monoclonal antibodies against Cytokeratin12 (1:100; Abcam; USA), Cytokeratin13 (1:100; Abcam; USA), p63 (1:100; Abcam; USA) and Zonula occludens-1 (1:400; CST; USA). After washing 3 times with Tris-buffered saline containing 0.5% Tween 20, the sections were incubated at room temperature for 1 hour with appropriate secondary antibodies including fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (immunoglobulin G) (1:100; Jackson; USA) or Alexa Fluor 594-labeled goat anti-mouse IgG (1:100; Jackson; USA). Sections incubated with FITC-conjugated goat anti-rabbit IgG (1:100; Jackson; USA) and Alexa Fluor 594-labeled goat anti-mouse IgG (1:100; Jackson; USA).
Jackson; USA) were used as negative controls. All sections were washed 3 times in Tris-buffered saline with 0.5% Tween 20 and counterstained with 4’,6-diamidino-2-phenylindole, then were observed with a confocal microscopy (Leica, Germany).

Transmission Electron Microscopy

Corneal tissues for transmission electron microscopy were fixed using 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 hours at 4°C, then fixed in 1% osmium tetroxide for 3 hours. The samples were scraped and pelleted, dehydrated in a graded series of ethanol baths, infiltrated, and embedded in Epon resin. Ultrathin sections (70-nm-thick) were obtained using a Leica Ultracut Microtome, stained with uranyl acetate for 3 minutes, and examined using transmission electron microscopy (Philips CM120, USA).

Results

Clinical Results

The thick fibrovascular membrane of patient 1 (Figure.1A) could easily be removed from the underlining corneal stroma during surgery. The remaining corneal stroma had a smooth surface with localized inferonasal opacity(Figure.1C), indicating the Bowman’s membrane was still intact after alkali burning. In patient 2, the proliferated fibrovascular membrane (Figure.1B) was closely adhered to the stroma closely, making it difficult to remove; the remaining corneal stroma was rough (Figure.1D), implying the Bowman’s layer had been destroyed. After cutivated oral mucosal epithelial transplantation, the corneal surface was stable in both patients. Corneal neovascularization in patient 1 was limited to the peripheral area (Figure.1E). In patient 2, the new vessels invaded to the entire cornea, including the area surrounding the (Figure.1F).

Histopathological and Transmission Electron Microscopy Analysis

Under the light microscope, hematoxylin-eosin staining of the corneal button showed 3 to
6 layers of epithelial cells in patient 1; the amniotic membrane substrate was seen in most areas of the corneal button, as was the Bowman's layer (Figure.2A). In patient 2, 6 to 8 layers of epithelial cells were observed; neither the amniotic membrane nor Bowman’s membrane were observed (Figure.2B). Morphologically, the epithelial cells were identified as two types: 1) small, round-shaped cells with comparatively high nuclei: cytoplasm ratios at the basal layer; and 2) differentiated, flat, squamous epithelial cells with low cell nuclei: cytoplasm ratios in the apical surface. Both types of cell were detected in both patient 1 and patient 2. Inflammatory cells were scattered over the corneal stroma, but this was more prominent in patient 2.

Epithelial structures observed using transmission electron microscopy were consistent with those seen with hematoxylin-eosin staining. High-magnification views showed that epithelial cells formed desmosomes with each other in both patients. (Figure.2C, D). These desmosomes resembled normal corneal epithelial intercellular connections.

**Immunofluorescent Analysis**

Immunofluorescent examination of the two corneal buttons revealed that Cytokeratin3 was present within the cytoplasm in the apical layers (Figure.3a, b), and Cytokeratin13 was present throughout the epithelial layers—especially surface layers (Figure.3k, l) of both tissues. The p63 antibody was present within the nucleus of both post-COMET corneal tissues (Figure.3p, q), particularly in the basal cells. Zonula occludens-1 was expressed in all layers of both corneal buttons (Figure.3u, v), similar to that in normal cornea. However, expression of Cytokeratin12 was detected in patient 1 (Figure.3f), but not in patient 2 (Figure.3g).

Normal corneal epithelium, cultivated oral mucosal epithelial cell sheets, and oral mucosal epithelium shared expression of epithelial differentiation marker Cytokeratin3 in the superficial layers (Figure.3c, d, e). While the Cytokeratin12, corneal epithelial-specific
marker, was expressed in normal corneal epithelium (Figure 3h), it was not seen in cultivated oral mucosal epithelial cell sheets or oral epithelium (Figure 3i, j). Oral mucosal marker Cytokeratin13 was observed in the cytoplasm of cultivated oral mucosal epithelial cell sheets and oral mucosal epithelium (Figure 3n, o) but not in normal corneal epithelium (Figure 3m). The p63 antibody, a putative epithelial stem cell marker, was found in the papilla and ridge of the oral mucosa (Figure 3t), the basal layer of the normal cornea (Figure 3r), and in the basal layer of cultivated oral mucosal epithelial cell sheets (Figure 3s). Epithelial cells of the native cornea and cultivated oral mucosal epithelial cell sheets stained positive for zonula occludens-1 (Figure 3w, x)—a marker for cell tight junctions, in all layers of the cytomembrane.

Discussion

Stem cells are critical for supplying and maintaining homeostasis within the ocular surface to promote epithelial regeneration. Some studies have confirmed the existence of progenitor cells in reconstructed cornea. Soma et al. investigated the maintenance and distribution of epithelial stem progenitor cells after cultivated oral mucosal epithelial transplantation in the rat model and found that the peripheral cornea could maintain more progenitor cells than the central cornea after corneal reconstruction using cultivated oral mucosal epithelial transplantation. The p63 is a transcription factor that is expressed in the nucleus of the basal limbal epithelial cells and is imperative for the regenerative capacity of human keratinocyte stem cells. In our study, basal cells of both corneal buttons were positive for p63, reflecting the potential that cells that can survive over at least 6 months—a finding that was consistent with that of former studies. The corneal epithelium derives from the ectoderm under the influence of pax6, a major regulator in eye development. Differentiation into the epithelial lineage is orchestrated by
p63 and is associated with a switch of Cytokeratin3 and Cytokeratin 12 expression. Oral mucosal epithelium contains heterogeneous populations of progenitor cells and matured epithelial cells and appears very similar to normal in vivo corneal epithelium, making it feasible as a functional ocular surface epithelium. Oral mucosal epithelium differs from corneal epithelium in that it expresses Cytokeratin3 but Cytokeratin12.

Immunofluorescence analysis of both cornea buttons in our study showed that the expression of oral-specific keratins Cytokeratin13 was consistent with that seen in normal oral epithelial cells. Both ocular surface stained positive for Cytokeratin3, a marker for epithelial differentiated cells, but only at the apical side of flat epithelial cells, confirming a higher differentiation of transplanted cultivated oral mucosal epithelial cell sheets. The most obvious distinction of phenotypic expression between the two corneal buttons was in Cytokeratin12, the corneal epithelial specific marker. For patient 1, both Cytokeratin13 and Cytokeratin12 was seen in almost all layers of the surgically removed corneal button, implying the ocular surface was covered with Cytokeratin13 positive oral epithelial cells and Cytokeratin12 positive corneal epithelial cells. Cytokeratin12 staining was not observed in patient 2, which suggests the ocular surface was covered with oral epithelial cells.

When then tried to determine the source of the corneal epithelial-like cells. There are two possibilities: 1) the transplanted oral epithelial cells survived and transdifferentiated into corneal-specific epithelial cells for a long period of time; and/or 2) the transplanted cells revitalized the limbal stem cells of the patient. Sugiyama et al. suggested that the transplanted cultivated oral mucosal epithelial cell sheets supported the proliferation of remaining native limbal stem cells in rabbit limbal stem cell deficiency models, but that cultivated oral mucosal epithelial cell sheets transdifferentiation was not excluded.
The microenvironment of the recipient can regulate the transplanted cell behavior and decide their fate of those cells. Over past 10 years, a number of other autologous cell types have been investigated for treating limbal stem cell deficiency. Mesenchymal stem cells, embryonic stem cells and induced pluripotent stem cells have aroused a great interest in the field of corneal epithelial reconstruction. Several groups have differentiated ESCs or iPSCs into corneal epithelial-like cells using a medium of conditioned limbal fibroblasts or corneal fibroblasts in vitro.\(^{15,16}\) Shalom-Feuerstein et al. established a protocol to produce corneal epithelial cells from human induced pluripotent stem cells and found that differentiated cells were able to stratify and form a corneal-like epithelium on a stroma equivalent.\(^{17}\) Yu et al. differentiated murine iPSCs into corneal epithelium-like cells co-culturing them with corneal limbal stroma using transwell, revealing the importance of paracrine interactions as well.\(^{18}\) Blazejewska et al. showed hair follicle epithelial cells in vitro were capable of differentiating into corneal epithelial cells when exposed to a limbus-specific microenvironment.\(^{19}\) Nieto-Miguel et al. found corneal epithelial-like cells can be induced from extraocular human adipose tissue-derived mesenchymal stem cells by subjecting them to an in vitro microenvironment containing conditioning signals derived from differentiated human corneal epithelial cells.\(^{20}\) Recently, Gopakumar et al. reported that skin keratinocytes were transdifferentiated into the corneal epithelial phenotype using human limbal fibroblast conditioned media.\(^{21}\) All these studies have shown that appropriate cornea specific signals can be activated in vitro to direct transdifferentiation into corneal epithelial cells.

In the case of the corneal epithelium in vivo, the epithelial phenotype is thought to be strongly affected by the subepithelial environment. Proliferation and differentiation of epithelial cells is regulated by stromal-epithelial interaction with keratocytes.\(^{22}\)
Chemokines and growth factors secreted by keratocytes are involved in proliferation and differentiation of the overlying epithelium. The microenvironment is greatly involved in the success of stem cell transplant and the long-term survival of the graft in ocular surface therapy. In cultivated limbal epithelial transplantation, Tsai et al. found that the intact amniotic membrane could provide the niche environment for cultured limbal stem cells and maintain the limbal-like environment, which cell survival related to the severity of the disease. In general, the microenvironment has a central role in the survival and renewal of limbal stem cells.

It was likely that the transformation of transplanted oral epithelial cells is dependent upon the corneal microenvironment also. In our cases, the corneal stroma of patient 1 was almost normal except for localized opacity. Transplanted cultivated oral mucosal epithelial cell sheets might be regulated by some signals in healthy stroma and differentiate toward the corneal phenotype, as shown by the appearance of the mature corneal marker Cytokeratin12. In patient 2, although transplanted cultivated oral mucosal epithelial cell sheet survived, signals that induced cell differentiation to corneal characteristics might be missing or disturbed, given its poor stromal microenvironment in contrast with that of patient 1.

Inconsistent results have been reported regarding the the epithelial phenotypes post-cultivated oral mucosal epithelial transplantation. Given that corneal buttons were derived from patients post-cultivated oral mucosal epithelial transplantation, it was difficult to distinguish the fate of transplanted cells on the ocular surface. One defect in former research on epithelial cell phenotypes post-cultivated oral mucosal epithelial transplantation is that it did not classify the severity of corneal stromal damage, especially the damage thickness. One cannot make the inference that cultivated oral
mucosal epithelial cell sheets suppress Cytokeratin12 expression without knowing the microenvironmental conditions of the previous studies.

Conclusions

In conclusion, our study observed the phenotypes of corneal epithelium after cultivated oral mucosal epithelial transplantation. To our knowledge, it is the first time researchers have seen that different phenotypes post- cultivated oral mucosal epithelial transplantation are related to the condition of the host cornea stroma. In addition, we confirmed that transplanted cultivated oral mucosal epithelial cell sheets can survive and maintain ocular surface integrity. We speculate that the oral cells responded to the signals from the local corneal microenvironment and acquired the corneal phenotype, although they maintained the oral phenotype at the same time. Our findings have valuable basic and clinical implications and provide useful insights into the mechanisms of both graft failure and graft survival after cultivated oral mucosal epithelial transplantation. The key factors for oral cells acquiring the corneal phenotype still required further research. Corneal microenvironments should be paid more attention in order to improve clinical cell therapy.

Abbreviations

COMET: cultivated oral mucosal epithelial transplantation
LSCD: limbal stem cell deficiency
TEM: transmission electron microscopy
PFA: paraformaldehyde
PBS: phosphate-buttered saline
IgG: immunoglobulin G
FITC: fluorescein isothiocyanate
Declarations

Ethics approval and consent to participate

This study adhered to the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of the Eye and ENT Hospital of Fudan University. Written informed consent was obtained from individual participants.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding authors upon reasonable request.

Competing interests

None of the authors has any conflicts of interest to disclose.

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Authors’ contributions

Study concept and design: ZCR; Data collection: ZCR, HFF and QJN; Interpretation and analysis of data: QJN and HFF; Technical and material support: XQ and CRR. All authors have read and approved the manuscript. Jini Qiu and Feifei Huang contributed equally to the work presented here, and should therefore be regarded as equivalent authors.

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Figures
Figure 1

Representative photographs pre-, intra- and post- cultivated oral mucosal epithelial transplantation of patient 1 and patient 2. A Patient 1 showed total limbal stem cell deficiency, ocular surface was covered by fibrovascular proliferation preoperatively. B Fibrovascular proliferation invaded whole cornea in patient 2. C Superficial fibrovascular tissue was removed prior to cell sheet grafting, remained corneal tissue smooth and transparent expect for the inferionasal quadrant. D remained cornea was rough porcelain white. E At 6
months post- cultivated oral mucosal epithelial transplantation, the ocular surface was stable, neovascularization was limited to the periphery and limbal. F 6 months after cultivated oral mucosal epithelial transplantation, there was dense neovascularization in the reconstructed ocular surface.

Figure 2
Representative hematoxylin-eosin staining and transmission electron microscopy of cornea buttons from patient 1 and patient 2. A 3-6 layer epithelial cells and an acellular membrane between epithelium and stroma in patient 1 (arrow). B 6-8
layer epithelial cells without the acellular membrane in reconstructed epithelium of patient 2. C Desmosomes between epithelial cells in patient 1 were seen under higher magnification of transmission electron microscopy (arrow). D Desmosomes between epithelial cells in patient 2 were seen under higher magnification of transmission electron microscopy (arrow).

Figure 3

Immunostaining of corneal tissues from patient 1, patient 2, normal cornea, cultivated oral epithelial cell sheet and normal oral epithelium. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). a-e Cytokeratin3 was positive in the superficial layer of corneal buttons from patient 1, patient 2, normal corneal epithelium, cultivated oral mucosal epithelial cells and oral mucosal epithelium. f Cytokeratin12 staining was positive in whole layer of
specimen from patient 1. g There was no Cytokeratin12 staining in specimen from patient 2. h Normal corneal epithelium expressed its specific marker Cytokeratin12. i-j Both cultivated oral mucosal epithelial cell sheet and oral mucosal was negative in Cytokeratin12. k-l Cytokeratin13 expressed in both corneal buttons. m Normal corneal epithelium did not express Cytokeratin13. n-o cultivated oral mucosal epithelial cell sheet and oral mucosal epithelial were positive for Cytokeratin13. p-t p63 expression in basal layers of specimens from two patients, normal corneal epithelium, cultivated oral mucosal epithelial cell sheet and oral mucosal papilla and ridge. u-x There was Zonula occludens-1 expression in all epithelial layers of two corneal buttons, normal cornea epithelium and cultivated oral mucosal epithelial cell sheet. y Oral mucosal epithelium expressed no Zonula occludens-1.