Characterization and Safety Assessment of Bioengineered Limbal Epithelium

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SUMMARY
Transplantation of cultivated limbal epithelium on substrates such as amniotic membrane is an established treatment for severe ocular surface disease with limbal stem cell deficiency. In this study, we adapted an established method to generate sheets of limbal epithelium on amniotic membrane and characterized the cells contained in these sheets and tested them for safety with regard to microbial contamination. Human limbal biopsies were cultivated on denuded amniotic membranes. After three weeks of culture, the phenotypes of cultivated cells were analyzed by immunohistochemistry and real-time RT-PCR for the expression of a panel of specific markers. Cultivated limbal epithelial cell sheets were also analyzed by scanning (SEM) and transmission (TEM) electron microscopy. Sterility tests and mycoplasma assays were conducted for the safety of product. A confluent layer of polygonal cells was formed in 2 weeks and 1-3 stratified layer of cells were observed after three weeks of culture. Cultivated cells were positive for p63, K3, K19, and involucrin but negative for K14, integrin α9 and ABCG2 when analyzed by immunohistochemistry. Expression of molecular markers was detectable with real-time RT-PCR. SEM showed multilayer of flat squamous polygonal epithelial cells. Desmosomal and hemidesmosomal attachments were evident. Our study showed that cultivated limbal epithelium consists of limbal progenitors as well as differentiated corneal epithelial cells. SEM and TEM analysis showed cultivated cells demonstrated typical features of corneal epithelium. The risk of contamination is low and can be prevented by culturing the cells in a clean room facility complying to Good Manufacturing Practice standard.

KEY WORDS:
Amniotic membrane, Corneal epithelial stem cells, Limbus, Tissue engineering, Stem cell transplantation

INTRODUCTION
Clinical and laboratory evidence has indicated that the human corneo-scleral limbus is a repository for corneal epithelial stem cells, also referred to as limbal stem cells (LSC). The limbus is a very specialized zone around the circumference of the cornea. LSC are believed to be located in the palisades of Vogt and more specifically, concentrated in the limbal epithelial crypts1-3. Conventionally it is accepted that the maintenance of the corneal epithelial cell mass is achieved by the proliferation, migration and differentiation of LSC and their progeny, under both normal conditions as well as following injury. Recent evidence however suggests that physiological homeostasis of the corneal epithelium can be maintained by the central corneal epithelial cells4.

In severe ocular surface diseases, such as Steven-Johnson syndrome and chemical burns, partial or total damage to the limbus can have severe consequences for corneal wound healing and ocular surface integrity secondary to limbal stem cell deficiency (LSCD)4-6. LSCD is characterized by conjunctivalisation of the cornea, vascularization, chronic inflammation, recurrent erosions and persistent ulcers, destruction of the basement membrane; and fibrous tissue ingrowth leading to severe functional impairment of the cornea7-9.

An important treatment modality for LSCD involves the ex vivo expansion and transplantation of cultivated corneal epithelial cells. This approach for reconstructing damaged ocular surfaces was first reported in 1997 by Pellegrini et al10. Ex vivo expansion of human limbal epithelial cells on amniotic membrane or other substrates for the purpose of transplantation10-17 has been reported. Although these techniques have been adopted into clinical practice, the question whether expanded limbal epithelial cells maintain their undifferentiated nature under culture condition still remains unanswered17. Furthermore, the safety and efficacy of the bioengineered ocular surface tissue still remains an issue. Risk factor which caused graft failure such as microbial contaminations from the use of material from animal origin is the major concern related to the use of this bioengineered tissue for ocular surface reconstruction18.

In this country, the study of limbal stem cells and the use of bioengineered ocular surface tissue for transplantation is still in its infancy stage. In this study we bioengineered the ocular tissue in our centre with the aim of introducing it into clinical practice. We cultivated limbal epithelial cells on amniotic membrane (AM) and characterized the cell sheets with a panel of surface and molecular markers considered to be indicative of stemness of limbal epithelial cells21. We also conducted sterility tests and mycoplasma assay of the cultivated cells to evaluate the safety of the cell sheets for human transplantation.

MATERIALS AND METHODS
Limbal Epithelial Cells Culture
The method for limbal epithelial cells culture was adapted,
with minor modifications from the explants method reported by the LV Prasad Eye Institute, Hyderabad, India. Human limbal biopsies and AM were handled according to the procedures approved by (excluded for identification). Informed consent was obtained prior to the collection of tissues from all donors. A total of 35 limbal biopsies, of 2x1 mm² in size, were obtained from patients who underwent the procedure of pterygium excision. Amniotic membranes were procured as per protocol from donated placenta after screening the donor for the human immunodeficiency virus (HIV), hepatitis B surface antigen (HBs Ag) and syphilis (venereal disease research laboratory [VDRL] test). Briefly, the limbal explants were placed on de-epithelialized amniotic membranes (3 x 3 cm²). After de-epithelialization, the AM was spread in a petri dish and 1 ml trypsin-EDTA was added on the surface and incubated for 25 min at 37 °C. The trypsin solution was then discarded and 2 ml of human corneal epithelium medium (HCEM) which contained Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 (Invitrogen Corporation, Carlsbad, CA, USA), epidermal growth factor, insulin, hydrocortisone (both from Sigma-Aldrich Chemie, Steinheim, Germany), antibiotics-antimycotics and 10% fetal bovine serum (both from Invitrogen Corporation, Carlsbad, CA, USA) was added to the AM. The AM was mechanically scraped with cell scraper for complete de-epithelialization and washed three times with phosphate buffered saline (PBS). After washing, the AM was spread taut on a sterile grass slide (2.5 x 2.5 cm²) and carefully placed in a 35 mm culture dish. Fresh limbal biopsy tissue that was held in HCEM was minced into 4-6 tiny bits and explanted onto two denuded AM. The explants were allowed to adhere to the AM for 30 min before 4 ml of HCEM was added to the culture dishes. The medium was changed every alternate day and growth of the cells was monitored under phase contrast microscope. Cells were cultured for 2-3 weeks before being subjected to various tests. Cultivated limbal epithelial sheets were either whole mounted or paraffin sectioned before staining with hematoxylin and eosin (H&E) for morphological evaluation.

**Immunohistochemical Staining**

The IHC Select® Immunoperoxidase Secondary Detection System (Millipore, Temecula, CA, USA) was used to stain paraffin sections of cultivated limbal epithelium. Briefly, 4 µm of paraffin sections were deparaffinized and antigens retrieval were carried out with either proteinase K or Dako retrieval solution (all from DakoCytomation Inc., Carpinteria, California, USA) as per the manufacturer’s instructions. The sections were washed with PBS and then covered with 3% hydrogen peroxide for 10 min. Sections were rinsed with rinsing buffer and incubated with blocking reagent for 5 min. Prediluted (1:100) primary monoclonal antibodies of ABCG2, cytokeratins (K) 3 (Millipore, Temecula, CA, USA); K19, p63, involucrin, integrin α9 (DakoCytomation Inc., Carpinteria, California, USA); K14 (GeneTex Inc., Irvine, California, USA) were added separately to each section and incubated for 10 min. Primary mAb was excluded for negative control. After rinsing, secondary antibodies were added followed by streptavidin HRP. Chromogen reagent, 3, 3’ diaminobenzidine (DAB substrate) was added for 10 min and counterstained with hematoxylin. Finally, slides were mounted with aqueous-based mounting media with cover slips and examined with a Nikon microscope.

**Real time RT-PCR**

Total RNA was extracted from 3-week cultivated limbal epithelial cells. First strand cDNA was synthesised with Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Nonnenwald, Penzberg, Germany) as per protocol. Real time polymerase chain reaction (RT-PCR) was carried out using a LightCycler instrument (Roche Diagnostics, Nonnenwald, Penzberg, Germany). The oligonucleotide primers used for a panel of genes i.e. ABCG2, ΔNp63, K3, K12, Connexin 43, integrin α9 and GAPDH are listed in Table 1. Amplification products were detected via intercalation of the fluorescent dye SYBR green from LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics, Nonnenwald, Penzberg, Germany). Cycling conditions for amplification were as follows: initial enzyme activation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, 58 °C for 5 s and 72 °C for 20 s. All cycling reactions were performed in the presence of 4 mM MgCl₂. Gene specific products were confirmed by melting curve analysis. Expression of the genes were normalised by the expression of GAPDH. Expression ratio of genes from cultivated limbal epithelial cells as compared to limbal biopsy was calculated by REST software. One µL of the gene products was loaded on 1.5% agarose gel for visualization.

Sterility Check for Amniotic membrane, HCEM and Cultures Amniotic membrane, HCEM and cultivated limbal epithelium were randomly subjected to laboratory culture for...
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Table I: Human primer sequences used for real time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>PCR Product</th>
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<td>ΔNp63</td>
<td>XM_036421</td>
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<tr>
<td>K12</td>
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<td>ACATGAAAGAAGAAACCACAGGAT</td>
<td>TCTGTCGAGGATGGTTCA</td>
<td>150 bp</td>
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<tr>
<td>Connexin 43</td>
<td>M65188</td>
<td>CCTCCCTGCTGAGTGCACGTTAC</td>
<td>ACCAAGGGACACACACGGAAT</td>
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<tr>
<td>Integrin α9</td>
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<tr>
<td>GAPDH</td>
<td>M33197</td>
<td>GCCAGGTCATCACATGCGAAC</td>
<td>GTCACCACCCCTGTGGTA</td>
<td>498 bp</td>
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RESULTS

Morphological Evaluation

Sixty four successful cultures were established from 32 limbal biopsies. Clusters of round cells were seen on day 2-5 at the edge of the explants. A closely packed compact monolayer of polygonal cells with a growing age was seen on subsequent days (Figure 1A, B). These cells then expanded to confluence covering the entire AM within a period of 2-3 weeks. Polygonal cells were clearly visible with hematoxylin and eosin staining in all 10 whole mounts thus stained. The nucleus was vesicular with two-three nucleoli (Figure 1C). Frequent mitotic figures were noted. One-three layers of stratified limbal epithelium were observed (Figure 1 D).

Ultrastructure of cultivated limbal epithelium

Examination of the apical surface of the cultivated epithelium by scanning electron microscope showed a multilayer of flat polygonal epithelial cells. Desquamating cells were observed on the surface of the epithelium (Figure 2A). When analyzed by transmission electron microscope, basement membrane material from AM was evident, and the basal cells were attached to the denuded AM by hemidesmosomes. Desmosomal junctions were seen between neighboring basal cells. A prominent nucleolus was seen in the nucleus (Figure 2B).

Immunohistochemistry and immunofluorescence analysis

Immunohistochemistry using a panel of antibodies showed that the 3-week cultivated limbal epithelial cells were strongly positive for the corneal epithelial differentiation marker K3 (Figure 3A). The cultivated cells were also partially positive for involucrin, K19 and p63 in the basal layer (Figure 3B-D). Cells were totally negative for K14, Integrin α9 and ABCG2 (Figure 3E-G). Immunofluorescence staining with anti-K3 antibody was especially positive at the edge of the cell sheet (Figure 3F).

Relative gene expression of limbal epithelial cells from culture and limbal biopsy

The panel of genes comprised of ABCG2, ΔNp63, K3, K12, connexin 43, integrin α9 and GAPDH was successfully amplified from cultivated cells. However, fresh limbal biopsy cells, ABCG2 and integrin α9 were not amplified. Gene expression of p63, from cultivated limbal epithelial cells were upregulated (p=0.01); whereas K12, K3 and connexin 3 were down regulated (p=0.01) as compared to limbal biopsy cells (Figure 4).

Sterility of Medium, AM and Cultures

Freshly prepared AM and HCEM did not show any contamination. Similarly, samples of cultivated limbal epithelium were also clear of any contamination. Out of 70 cultures, six cultures (8.6%) were discarded when the culture medium turned turbid and yellowish in colour after two weeks of culture. Mycoplasma (M. arginini, M. hyorhinis, A. laidlawii, M. orale) contaminating mammalian cell cultures (n=5). Briefly, coating antibodies of four types of mycoplasma species were added to a 96-well microplate. The plate was incubated for 2 h at 37 °C. The antibody solution was removed and the coated wells were then incubated with blocking solution for 30 min at 37 °C. After washing, an aliquot (0.2 mL) of medium from limbal epithelium culture dish, negative control medium and positive control medium provided with the kit were added to the wells. The microplate was covered tightly and incubated overnight at 4 °C. The wells were then washed and incubated with detection antibodies for 2 h at 37 °C. Final incubation with streptavidin-AP-solution was carried out for 1 h at 37 °C and substrate solution was added for 30 min. Wells that developed a yellow colour were considered as positive for mycoplasma.

DISCUSSION

Clinical application of cultivated limbal epithelial cells on amniotic membrane has been reported from several centres in the world. This treatment has produced encouraging results for patients with severe ocular surface diseases11-19. However, this treatment is currently not available in this country yet. In this study, we adapted methods established by the LV Prasad Eye Institute, Hyderabad, India in the preparation of cultivated limbal epithelial sheets. Our results showed that multilayered cultivated limbal epithelial sheets were successfully obtained. The polygonal morphology of the cells was similar to that reported by others20. This submerged culture system as introduced by Sangwan et al. is only able to produce 1-3 layers of epithelial cells as compared to air lifted methods used by Koizumi et al.21,22 which produced 5-6 layers of stratified cells. In this study, when we air lifted the cultivated cells after 2-weeks of submerged culture, the number of layers could be increased up to five (data not shown). The advantage of this culture system is that an
Fig. 1: Morphological evaluation of cultivated limbal epithelial cells. (A) Cluster of round cells were observed under phase contrast microscope on day 2 at the edge of the explants. Background seen was amniotic membrane, AM; (B) A closely packed compact monolayer of polygonal cells with a growing age (arrows) was seen on day 7; (C) Hematoxylin & eosin (H&E) whole mount staining of a confluent layer of expanded limbal epithelial cells at day 14; all magnification 100 x. (D) Paraffin section (4µm) of 3-week cultivated limbal epithelium was stained with H&E. One-three layers of stratified limbal epithelium were observed (magnification 400 x).

Fig. 2: Ultra structure of cultivated limbal epithelium. (A) Scanning electron micrograph showed multilayer of flat polygonal epithelial cells of the cultivated epithelium. Desquamating cells were observed on the surface of epithelium. (B) When analyzed by transmission electron microscope, basement membrane material was evident, and the basal cells were attached to the denuded AM by hemidesmosomes (black arrows). Desmosomal (dotted arrows) junctions were seen between neighboring basal cells. Nucleolus (N) was seen in the nucleus.
Fig. 3: Immunohistochemical staining of paraffin sections of 3-week cultivated limbal epithelial cells using a panel of antibodies against K3, K14, K19, p63, ABCG2 and integrin α9. (A) Cells were strongly positive for cornea differentiation marker i.e. K3 (magnification 200 x). (B-D) The cultivated cells were also partially positive for involucrin, K19 (basal layer only) and p63 (basal layer only) (magnification 200 x). (E-G) Cells were totally negative for K14 (magnification 400 x), integrin α9 and ABCG2 (magnification 200 x). (H) Immunofluorescence staining with anti-K3 showed positive result especially at the edge of cells growth (magnification 100 x).
animal feeder cell layer is not required, which makes this method simpler and safer compared to others. Besides, this method is relatively cheaper as cell culture inserts are not required. Mitotic figures and multiple nucleoli were often seen indicating that the cells were actively proliferating. Moreover, electron microscopy confirmed the formation of normal looking desmosomes and hemidesmosomes. From the cell morphology and nuclear cytoplasmic ration it would appear that most of the cells in the sheets were differentiating cells rather than stem cells. From published studies, it appears that this does not make a difference to the clinical outcome. It is also not clear whether 5-6 layers of stratified epithelium confers any advantage over sheets with 2-3 layers of cells.

To date, no single specific limbal stem cells marker has been identified. However, a panel of markers has been proposed, which may help to identify a sub-population of cells that may contain limbal stem cells. We studied our cultivated cells using such a panel of markers, which comprised of differentiation-associated markers (K3, K12, connexin 43 and involucrin) and putative stem cell-associated markers (p63, K19, K14 ABCG2 and integrin α9). While most putative SC markers label the majority of limbal basal cells and, therefore, may not distinguish SC from progenitor cells; only ABCG2 was strictly confined to small clusters of basal cells in the limbal epithelium. ABCG2 therefore appears to be the most useful cell surface marker for the identification and isolation of limbal SC. Our results showed that the cultivated cells were positive for K3, both by PCR and immunostaining. The cultivated cells were also partially positive for involucrin, K19 and p63 (only basal layer). However the cells were totally negative for K14, integrin α9 and ABCG2. These results revealed a mixture of progenitor cells or known as transit amplifying cells and differentiated limbal epithelial cells being expanded during the culture. Since ABCG2 and integrin α9 were not detected by immunohistochemical staining but detected by real-time RT-PCR method, we conclude that the cultivated cells may be expressing very low levels of ABCG2 and integrin α9. Our real-time RT-PCR results showed that all the genes were expressed by the cultivated cells. Up regulation of p63, compared to expression in limbal epithelium, was noted in the cultivated cells where as gene expression of K3 and K12 was down regulated. These results were similar to that reported by Liu et al.

Six culture showed sign of contamination when the medium changed to yellowish and turbid. The contamination probably occurred from the culture environment as the media and amniotic membranes, when individually tested, were free from contamination. Most of the contaminations can be avoided by proper handling of the cultures in a clean room facility which conforms to Good Manufacturing Practice (GMP) standards. The study emphasizes the risk of contaminated cultured products and all protocols must include evaluation for contamination prior to release for clinical use. Mycoplasm contamination was not noted in any culture. This may relate to the fact that only primary cultures were studied. Multiple passages could increase the risk of mycoplasm contamination. As we did not use 3T3 murine fibroblasts, or any other cell line in our culture system, a major risk associated with the use of animal cells was avoided. We feel that our protocol may be the way forward with ex vivo explants, given its reduced risks. It is suggested that fetal bovine serum used for clinical trials should be sourced from Australia or New Zealand herds, a practice that is consistent with guidelines issued by European Medicines Evaluation agency (EMEA) and Therapeutic Goods Administration (Australia), for managing the risk of transmissible spongiform encephalopathy (TSE) from bovine products. However, use of autologous serum from patient or serum free media for culturing limbal epithelium has been reported as well. Cultivated limbal epithelial cells transplantations have been conducted since 1997 by Pellegrini et al. In all the clinical trials that had been followed up for more than one year, tumour formation has
not been reported\(^1\). Therefore, the findings indicate that there is no risk of tumour formation with transplanted ex vivo expanded limbal epithelium.

There is no universally accepted standard protocol for the production of cultivated limbal epithelium. Each centre has its own protocol with subtle or significant variations from others. Different culture methods and media might produce limbal epithelial cells at different stages of maturation and phenotype which could determine clinical outcomes. Future research will help to establish the best culture method and media for producing the highest quality of limbal epithelium for transplantation. A clean room facility which conforms to GMP standards is a prerequisite. Use of media free of animal derivative is also desirable.

**CONCLUSION**

Our results showed that the limbal epithelial cells sheets were successfully generated by using the adapted method. The expanded limbal epithelial cells possessed the characteristic of progenitor and mature phenotypes. The bioengineered cell sheets were reasonably safe and have the potential to be used in future clinical trial study.

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**REFERENCES**