CHAPTER 6: IN-VIVO SURVIVAL ANALYSIS OF TRANSPLANTED LIMBAL EPITHELIAL CELLS

6.1. Introduction

Regenerative medicine involving cell-based therapies is proving to be a boon for management for several human diseases. One of the most successful stories of the present day regenerative medicine is the ocular surface reconstruction using CLET for LSCD. Globally many centers including ours have successfully applied the technique in clinics. As presented in the thesis world’s largest clinical trial of ocular surface reconstruction using cultivated limbal epithelial cells was conducted at our center (Sangwan et al. 2006). Albeit the above mentioned method being well established now, the long term survival and phenotypic characteristics of transplanted cultivated epithelium has not been well documented. The in-vivo status of the cells has been demonstrated in case of direct limbal transplantations and in isolated case reports for CLET, requiring a need for a study on larger number of cases/series of human samples so as to ensure the success of the therapy in restoration of the corneal epithelium. Since we happen to be the first centre with world’s largest clinical trial of CLET, we have an access to a significant number of corneal tissues obtained after CLET (Sangwan et al. 2005), which are very precious in terms that they allow us to study the status of the corneal epithelium following CLET. The study presented over here aims at providing the proof of restoration of corneal epithelium from the corneal buttons of patients who underwent penetrating keratoplasty following transplantation of cultured limbal epithelial cells grown on HAM which could further help in understanding the molecular mechanisms involved in ocular surface reconstruction following chemical injuries.
The following flow-chart (6.1) depicts the methods followed for in-vivo survival analysis. However till date there are essentially three methods followed for tracking the in-vivo survival of the transplanted limbal epithelial cells at the laboratory level.

I] Phenotypic characterization of the corneal epithelium generated after CLET

II] Ultrastructural studies of the corneal epithelium following CLET

III] Detection of survival of the donor derived cells in allogenic CLET by DNA fingerprinting methods

a] Phenotypic characterization of the corneal epithelium generated after transplantation of cultivated limbal epithelial

Many patients who undergo a successful CLET later require a standard corneal transplantation (Penetrating Keratoplasty) for persistent stromal scarring or endothelial failure. The corneal button that is removed at the time of transplantation consists of the epithelium of the central and peripheral portions of the cornea. For the phenotypic study, the epithelia from these excised corneal buttons are analyzed to investigate the in-vivo survival of the transplanted epithelial cells.

Stoiber et al first reported the phenotypic characterization of the corneal epithelium followed by direct limbal epithelial transplantation (Stoiber J et al 2002). This group analyzed four patients of LSCD, who underwent direct limbal transplantations coupled with HAM transplantations. From their study Stoiber et al, concluded that the HAM transplantation coupled with direct limbal transplantation would be a useful technique to bring about rapid re-epithelialization of the corneal surface in LSCD. Espana et al reported formation of normal stratified corneal epithelium with well-formed basement membrane in a single case of LSCD receiving keratolimbal allograft along with HAM (Espana et al 2003).
Chapter 6: In-Vivo Survival Studies

Flow-Chart 6.1. Methods of investigation of \textit{in-vivo} survival of transplanted limbal epithelial cells

Transplantation of cultivated limbal epithelial sheets in patients with LSCD

Clinical FU of patients post transplantations

Clinical methods of evaluation of the ocular surface and assessment of visual acuity in-patient following transplantations

Fluorophotometry

Impression Cytology

Attempt to evaluate the duration of survival of allogenic cells \textit{in-situ} by DNA fingerprinting

Basic methods of evaluation and assessment of ocular surface in-patient following transplantations

PKP in patients with severe ocular surface disorders following CLET for vision rehabilitation

In-vivo confocal microscopy

Fluorescein

Corneal buttons obtained used for evaluation of \textit{in-vivo} survival of cultivated epithelium in autologous and allogenic patients

Phenotypic characterization of corneal epithelium generated by transplantation of cultivated limbal epithelium

Immunophenotyping

Histopathology

Transmission Electron Microscopy

Ex-Vivo Proliferation and Characterization of Adult Limbal Stem Cells and their Applications in Treating Ocular Surface Disorders Caused by Limbal Stem Cell Deficiency
Ti et al for the first time reported the status of the corneal epithelium following the CLET, in rabbits. The investigation was an animal study with one year FU (Ti et al 2004). They provided evidence of clinical success of the CLET, correlated well with the restoration of a non-keratinized stratified epithelium without goblet cells, and a recovery of corneal epithelial phenotype based on the expression of the corneal epithelium specific cytokeratin K3 and lack of expression of conjunctival goblet cell specific MUC5AC. The authors speculate that the residual corneal epithelial cells could represent a smaller portion of limbal stem cells or simply transient amplifying cells. Thus the authors conclude from the above study that the CLET could bring about restoration of the corneal epithelium.

The study at LVPEI (unpublished data) is the first to report the restoration of the corneal epithelium following CLET in humans.

b] Ultrastructural studies of the corneal epithelium following CLET

The ultrastructural studies of the corneal epithelium following CLET could provide a useful method of knowing the \textit{in-vivo} functioning of the cells following transplantations. Stoiber et al. performed ultrastructural studies by TEM in corneas receiving direct limbal allograft transplantations (Stoiber et al 2002). They found that the corneal epithelium showed vital cells and a continuous basement membrane with lamina lucida and lamina densa.

c] Detection of survival of the donor derived cells in allogenic CLET by DNA fingerprinting methods

The bilateral LSCD require the use of donor derived tissue both in direct and CLET. The donor tissue could be either from the living related donors or cadavers. The cornea of such patients undergoing allogenic limbal transplantations provide a source of tissue to investigate the existence of donor-derived cells following transplantations.
Donor derived cells were detected using DNA polymorphisms with polymerase chain reaction (PCR); in the central cornea 12 weeks after limbal transplantations, but donor cells were no longer detectable at 20 weeks. The DNA polymorphism technique has been used along with impression cytology samples, however the impression cytology samples were highly susceptible to contaminations and had limited sensitivity. And could thus result in misdiagnosis and has a poor reliability (Harper and Handyside 1994). An alternative method was the use of sex-specific molecular probes such as Y-specific probes, to identify Y-chromosomes (Henderson et al 1997). Although sex specific molecular probes allow greater sensitivity, there are several disadvantages: the method is useful when the host and donor are of different sexes and has a much higher risk of contamination. New advances in DNA fingerprinting (Findlay et al 1997) recently have allowed the origin of single cells to be accurately determined using forensic techniques. This extremely powerful DNA fingerprinting method (short tandem repeat [STR] profiling) is used worldwide for forensic identification because of its high specificity; sex of cell is simultaneously detected.

Henderson et al conducted a study to determine whether these DNA fingerprinting techniques could be coupled with impression cytology (Henderson et al 1997). They reported a patient with aniridia (LSCD) after a living related limbal allograft. At 2.5 years only 1% of the sampled epithelial cells were found to be donor derived, whereas at five years donor cells were no longer detectable. Henderson et al reported 5 patients 3-5 years after they had undergone limbal transplantation. Using DNA microsatellites on multiple impression cytology samples, they could find no surviving donor cells (Henderson et al 2001 a, Henderson et al 2001 b).
Several animal studies have examined the survival of the donor derived cells following, limbal transplantation. Swift et al. used the absence of barr bodies and presence of a cell tracer (PHK-26) to identify male donor cell in recipient rabbits (Swift et al 1996).

Li et al showed the survival of the donor derived cells in rabbits receiving CLET using DNA microsatellites (Li et al 2001)

The results at LVPEI (unpublished) using DNA fingerprinting show the survival of donor cells in 3 of the 5 eyes evaluated and absence of donor derived cells in 1 eye, and mixed (both donor and recipient) profile in one eye.

However the limitation of the technique of DNA fingerprinting using impression cytology, is its high susceptibility to contamination. Djalilian et al reported the technique of Laser Capture Microdissection for isolation of cells from allogenic recipients of limbal transplantations. Microsatellite analysis was performed on single cells isolated from various parts of the cornea (Djalilian et al 2005).

6.2. Hypothesis

The monolayer of limbal epithelial cells cultivated *ex-vivo*, facilitates the formation of normal stratified corneal epithelium in patients with LSCD probably due to the corneal niche and inherent nature of these epithelial cells to stratify by proliferation and differentiation to form normal corneal phenotype.

6.3. Aims

To evaluate the corneal epithelium generated after CLET.

6.4. Material and Methods

The study was approved by the Institutional Review Board, L.V. Prasad Eye Institute. The patients enrolled in the study were diagnosed for LSCD by presence of
conjunctivalisation and vascularization of corneal epithelium, and also by impression cytology (Puangsricharen et al 1995, Shridhar et al 2001). All the patients underwent cultivated limbal or limbal and conjunctival epithelial transplantations for ocular surface reconstruction. At different time points these patients underwent penetrating keratoplasty (PK) for visual rehabilitation.

6.4.1. Procedures

The culture and surgical techniques were described in previous chapters.

6.4.1.1. Histopathological Evaluation

Corneal buttons obtained from PK were washed in PBS (1X), processed for the following. Section of it was fixed in OCT to obtain cryosections for immunophenotyping, section embedded in wax to obtain paraffin sections for routine histopathology evaluation, section of corneal button fixed in, formalin:gluteraldehyde (1:1), for transmission electron microscopy.

Corneal buttons of patients who underwent PKP after CLET were reviewed for epithelial changes, stromal changes, inflammation and vascularisation, and for the remnants of HAM present if any. Paraffin embedded sections (3-4μ thick) of above mentioned corneal buttons were stained with H&E, PAS stain and observed under microscope (10X 10, Olympus) to evaluate morphological changes present if any.

6.4.1.2. Immunophenotyping

Immunofluorescence was performed on cryosections of OCT embedded corneal buttons. In brief the cryosections were fixed in cold acetone, air-dried and rehydrated using milli Q followed by PBS. The samples were then incubated with monoclonal antibodies for cytokeratin K3, collagen IV after blocking for non-specific binding with 2.5% BSA. The
corneal buttons that showed presence of goblet cells in the PAS stained histopathological sections were stained with MUC5AC monoclonal antibody against goblet cell mucin. The detection system was FITC conjugated secondary antibody. All the stained samples were screened for positivity under Laser Scanning Confocal Microscope (Carl Zeiss 510 Meta LSM).

Table 6.1. List of antibodies and dilutions used

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Antibody</th>
<th>Dilutions</th>
<th>Company</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>K3</td>
<td>1:500</td>
<td>ICON Biosystems</td>
</tr>
<tr>
<td>2</td>
<td>Collagen IV</td>
<td>1:100</td>
<td>DAKO Cytomation</td>
</tr>
<tr>
<td>3</td>
<td>MUC5AC</td>
<td>1:100</td>
<td>Chemicon Biosystems</td>
</tr>
<tr>
<td>4</td>
<td>FITC- Conjugated 2° Antibody</td>
<td>1:250</td>
<td>DAKO Cytomation</td>
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</table>

6.4.1.3. Transmission Electron Microscopy

Samples for TEM were fixed in 2.5 % gluteraldehyde for 3 hours and washed in PBS. The samples were then post fixed in 1% osmium tetraoxide followed by dehydration and infiltration through propylene resin mix. Embedding was done in pure resin followed by curing for 72 hrs at 60°C. Semithin sections were obtained after proper trimming and cutting. The area for ultrathin sections was selected after evaluation of toluidine blue stained semithin sections. The ultrathin sections were stained with 2% uranyl acetate and lead citrate before scanning with TEM at 80KV.

6.4.1.3. Laser Capture Microdissection

The corneal buttons obtained from patients undergoing CLET followed by PK were archived in OCT. The LCM system used in the study (P.A.L.M. Micro Beam System, Zeiss Instruments, Bernreid, Germany) obviated the need for tissue dehydration prior to microdissection. This enabled isolation of corneal epithelial basal cells directly from 6μ
frozen sections of corneal buttons obtained. Frozen sections, mounted on special membrane-coated slides (P.A.L.M. Microbeam), which facilitated the capture of cells, were briefly (1 min) stained with hematoxylin (HistoGeneTM; Arcturus, Mountain View, CA). In addition, this staining procedure also removed the OCT mounting medium.

6.5. Results

A total of 29 corneal buttons from 29 patients were reviewed. The mean age of the patients being 18 (3-42) years. All these patients presented with the primary etiology of chemical burns and were diagnosed for LSCD. Depending on the severity of burns 23 eyes received pure limbal stem cell culture on HAM, 6 received a composite of limbal and conjunctival cultures. As given in the table 6.2, all the cultures showed good growth, after 10-14 days of ex-vivo culturing. PKP was done for visual rehabilitation with a mean follow up of 5.7 (2-16) months post transplantation.

Histopathological Evaluation

Histopathology of corneal buttons obtained after PKP revealed a stable, well-formed, intact, multilayered (3-4) epithelium (Figure 6.1a & b) resembling the normal epithelium in 86% cases, with a well formed basement membrane. Edema was noted in 10% cases. Goblet cells were seen in 10% cases, indicating the recurrence of LSCD. Calcium deposits in bowmans were seen in 3.4% cases (Figure 6.2a). Corneal stromal scarring was seen in 100%, inflammation in 44.8% and vascularization in 68.9% cases (Figure 6.2b). Remnants of HAM were detected in 17% cases (Figure 6.2c) Table 6.2.
Table 6.2. Histopathological Details of Corneal Buttons

<table>
<thead>
<tr>
<th>HP Findings</th>
<th>Intact MLE</th>
<th>Edema</th>
<th>Goblet Cells</th>
<th>Calcium Deposits</th>
<th>HAM Remanants</th>
<th>Corneal Stromal Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLE</td>
<td>- Multilayered epithelium</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Scr</td>
<td>- Scarring</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Inf</td>
<td>- Inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vas</td>
<td>- Vascularization</td>
<td></td>
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Figure 6.1. Paraffin sections of corneal buttons obtained from PKP in-patients following CLET, showing multilayered epithelium. (H&E, PAS staining respectively, x200)

Figure 6.2. Paraffin sections of the corneal buttons showing the stromal changes – inflammation A, calcium deposits B, remnants of HAM C. (arrows, H & E x100)
Immunophenotyping

Immunophenotyping of cells in corneal buttons using monoclonal antibodies for cornea specific cytokeratin K3 revealed presence of corneal lineage cells in 100% (29/29) cases (Figure 6.3 A-H). In these corneal buttons the suprabasal layers of cells showed positivity while the basal layers did not stain. This staining pattern for K3 exactly mimics the expression of K3 in normal corneal epithelium. The corneal buttons that showed presence of PAS positive goblet cells were also positive for K3 indicating partial recurrence of LSCD. Formation of basement membrane was seen in 26/29 cases (89.6%) (Figure 6.4 A-H). MUC5AC positive goblet cells were seen in the 3 corneal buttons that showed presence of PAS positive goblet cells (Figure 6.5 A-B).

Transmission Electron Microscopy

The transmission electron microscopy results in five corneal buttons further confirmed presence of multilayered corneal epithelium (Figure 6.6A) with formation of basement membrane in each of these tissues. A typical architecture of the corneal epithelium was seen in corneal buttons obtained after CLET. Similar to a natural corneal epithelium pyknotic nuclei of exfoliating surface cells was seen along with the basal columnar cells and central wide wing cells. Cells contained abundant tonofilaments. Tortuous, parallel intercellular borders similar to normal corneal epithelial cells were joined in places by desmosomes (Figure 6.6B). Superficial cells showed conspicuous finger-like projections of microvilli as seen in normal corneal epithelium (Figure 6.6C). Attachment of the basal cells to the basement membrane was mediated by adhesion complex (lamina-lucida, lamina-densa and hemidesmosomes), the hemidesmosomes brushes were clearly visible (Figure 6.6 D).
Figure 6.3. LSCM images of spectrum of corneal buttons showing positivity for cytokeratin K3 (arrows) following CLET, confirming the corneal lineage of the cells (x200).

Figure 6.4. LSCM of spectrum of corneal buttons showing positivity for collagen IV (arrows) following CLET, indicating the formation of basement membrane. (x200)
Figure 6.5. Paraffin sections of corneal buttons obtained from PKP in-patients following CLET, showing the presence of PAS positive goblet cells (arrow) A, Cryosection of the same showing MUC5AC positive (arrow) goblet cell B.

Figure 6.6. TEM picture showing the multilayered corneal epithelium A. Tortuous, parallel intercellular borders joined in places by desmosomes (arrows) B. Attachment of the basal cells to the basement membrane mediated by adhesion complex (lamina-lucida, lamina- densa and hemidesmosomes - arrows C). Superficial cells of the corneal epithelium showing conspicuous finger-like projections of microvilli D.)
Laser Capture Microdissection

Stained sections were viewed with a Zeiss Axiovert 200/200 M inverted light microscope (Zeiss Instruments) using a x40 objective (Figure 6.7A). Basal cells to be isolated were outlined with a “light” pen or cursor on a monitor screen using PALMRoboSoftware (Figure 6.7B). Such an outline defined the area that would be cut (Figure 6.7C) and catapulted (Figure 6.7D) intact into a microtube cap-containing adhesive material for RNA extraction and further downstream processing for transcription profiling.

Figure 6.7 Micrographs of frozen section of corneal button as observed under LCM (x40, H&E) A. Basal cells of corneal button marked with ‘light pen’ B, Marked area cut C, and catapulted D.
6.6. Discussion

The NIH guidelines for any cell based therapy states the following prerequisite for the success of cell therapy. Firstly the cells following cell therapy should survive in the host; secondly they should network/integrate into the host tissue for its proper functioning. Among various forms of cell-based therapies carried these days eye is one such organ, which provides a unique opportunity to document the success of any such therapy.

Replacing the diseased ocular surface with ex-vivo cultured limbal stem cells has now become a well-established treatment modality for LSCD resulting from ocular surface injuries. There are very few reports (Stoiber J et al 2002), which probed the fate of these cells after transplantation. Stoiber et al reported a series of 4 cases of direct limbal transplantation. From their study they concluded that the procedure of direct limbal transplantation was effective in bringing in the ocular surface reconstruction. There are very few reports of isolated cases showing the similar facts about CLET. However there has been detailed evaluation of corneal epithelium in animals following CLET. The study presented here with 29-patients provides an insight into the condition of these cells after transplantation in humans. In cases of severe ocular surface disorders resultant of chemical injuries, the treatment procedure is a three step strategy performing amniotic membrane transplantation, limbal transplantation and PK simultaneously (Sangwan et al 2005). We routinely prefer a two-step procedure performing PK after several months following amniotic membrane transplantation with or without cultured limbal stem cell transplantation – to reduce high risk of corneal graft rejection. The histopathologic studies on corneal buttons revealed an intact, multilayered epithelium in 86% cases, which shows that the cultured monolayer is getting stratified in-vivo and hence mimicking the natural corneal epithelium. The remaining 14% of
the cases showed 2-3 layers of cells – we think in these cases the complete multilayering might not have occurred due to insufficient support from the underlying stromal bed. However from the above results we bring into notice that, a monolayer of cells is enough to bring about ocular surface reconstruction in the most natural form obviating the need for use of any methods of stratification during culturing of the cells (Fatima et al 2006). In this study we provide evidence for the recovery of corneal epithelial phenotype following transplantation. The positive results of immunophenotyping of the samples (100%) with monoclonal antibody for K3 provide evidence of expression of K3. This might have probably occurred due to differentiation of cultured limbal stem cells into cells of corneal lineage, or resurgence/stimulation of residual limbal stem cells in the recipient limbus, which must have further followed the natural course to form a multilayered corneal epithelium. Thus the ex-vivo cultured limbal epithelial cells prove to be suitable candidates for functioning in a similar way to the natural corneal epithelium. Thus the hypothesis holds true in that the limbal epithelium cultivated ex-vivo successfully forms the corneal epithelium in patients with LSCD.

The present series also noted clinical failure in terms of expression of MUC5AC a conjunctival goblet cell component in 10% cases. All these corneal buttons had a mixed phenotype in terms of K3 and MUC5AC expression, indicating that these clinical failures were not associated with complete loss of corneal epithelial phenotype.

The positivity of the corneal buttons for collagen IV a basement membrane protein in HAM shows the presence of intact basement membrane underlying the corneal epithelium. Previous studies concluded that this was probably the HAM basement membrane, however the recent report has shown the presence of collagen IV in corneal basement membrane also.
Hence we hypothesize that following transplantation the corneal epithelial cells form their own basement membrane. The HAM is reported to degenerate within a period of six weeks, and we think that it occurred in our cases as well. The formation of intact basement membrane possibly from the overlying corneal epithelial cells is also confirmed by our transmission electron microscopy results, but the limitations of this finding is that TEM was only on 5 samples and the results cannot be extrapolated to rest of the samples. However we cannot completely rule out the presence of remnants of HAM, as it was histopathologically noted in 17% of our cases that were also positive for collagen IV. Three of our cases were negative for collagen IV, indicating absence of basement membrane. Thus further study into the in-vivo functioning of these cells is warranted, so as to know the source of basement membrane underlying the epithelial cells. However TEM showed that the cells contained a cytoplasmic meshwork of electron dense tonofibrils (intermediate filaments), similar to those found in other epithelia composed of cytokeratins (Sun and Vidrich 1981). Adhesion was seen to be achieved by numerous dense (Fawcett 1966) structures called desmosomes. These structures are presumably sufficiently labile to allow movement over a period of time as cells migrate to the surface as in case of natural corneal epithelium. The basal cells were connected to the underlying basement membrane by adhesion complex (lamina densa, lamina lucida, hemidesmosomes). The superficial cells exhibited surface microvilli or microplicae (Pfister, 1973) sometimes regarded as an exaggeration of plasma membrane infoldings, which exist between all contiguous epithelial cells. Microvilli are likely to serve a physical function in stabilizing the deep precorneal tear film, involved in secretion of the glycoprotein coating the cell surface.
Transcriptional profiling of enriched populations of putative epithelial stem cells has been used to search for epithelial stem cell specific markers. The transcriptional profiling of the limbal epithelial cells has been reported recently. Zhou M et al showed that the various methods used for putative stem cell identification and isolation (FACS analysis, time of binding of trypsinized cells to collagen, making of transgenic mouse etc.) did yield cells for subsequent transcriptional profiling, but the cells were subjected to a variety of manipulations prior to analysis, including proteolytic tissue dissociation, cell culturing, and FACS sorting. These manipulations could potentially perturb the observed genetic profiles. Hence they used laser capture microdissection to isolate the quiescent limbal basal cells and further transcriptional profiling (Zhou M et al 2006). In this study the corneal epithelial basal cells were successfully captured using laser capture microdissection. However further RNA extraction and microarray analysis is required to obtain transcription profiling of the cells following transplantation.

In conclusion, a monolayer of cultured limbal epithelial cells is capable of forming in-vivo multilayered epithelium and serves the very purpose of the corneal epithelium. The need for ex-vivo stratification by airlifting technique is ruled out. Moreover the corneal phenotype, presence of intact basement membrane of these cells contributes to the possible normal in-vivo functioning of corneal epithelium. Thus the CLET successfully restores a normal corneal epithelium and hence is a highly rewarding management strategy for the patients of LSCD. Looking into the comparative transcriptional profiling of the corneal epithelial basal cells would provide an insight into the fate of the cells following CLET, studies for these investigations are highly recommended.