3.1. Introduction

Biologists have had a deep interest in optimizing in-vitro cell culture systems since cell culture plays a central role in much of their research. The sensitivity of in-vitro culture of cells (any type) by and large depends on the simulation of the in-vivo physiological conditions. Thus the cell biology research is focused on simulation of the in-vivo conditions to grow cells in-vitro, so as to study their behavior, function, and interaction linear to their dwelling. To meet this very need there came to existence the “co-culture” systems, which are proving to be an essential tool to study many cell-cell interactions that control cell growth and differentiation. It is also helping in understanding the “disturbances” to living cells that are believed to be a critical reason for many diseases, including the deadly cancer. Moreover the repair or replacement of damaged tissues using in-vitro strategies is possible only on manipulation of the cell environment by modulation of cell-extracellular matrix interactions, cell-cell interactions, or soluble stimuli. These facilities can be obtained in a defined culture system, which has maximum simulation of in-vivo conditions pertaining to specific cell type. Thus by definition “A co-culture is essentially a mixture of two or more different kinds of cells that are grown together”. This technique of culturing mixed cell types in-vitro allows us to study their synergistic or antagonistic interactions, such as CELL DIFFERENTIATION or APOPTOSIS. Co-culture can be of different types of cells, tissues, or organs from normal or disease states.

Co-Culture System of the Eye

The ocular surface of the eye comprising of the corneal, limbal, and conjunctival epithelium, gives a remarkable example of existence of three different cell types in harmony. With the increasing knowledge of pathophysiology of the ocular surface and the
role of limbal stem cells as explained in the introductory chapters, it is now easy to understand the various clinical conditions that result in ocular surface damage (Davenger et al 1971, Thoft et al 1983, Schermer et al 1986). But it is important to clinically distinguish the diseases affecting the limbus alone from those affecting both limbus and conjunctiva, because the treatment modalities would involve replacing the respective tissue from either the fellow eye in unilateral cases or the allograft (living related or cadaveric) in bilateral cases. The options for conjunctival replacement surgeries include autologous conjunctival transplantation from the fellow eye, allografts from living related or cadaveric source, and oral mucosa transplantation (Thoft 1982, Vastine et al 1982, Kwitco et al 1995, Forbes et al 1998).

Severe forms of ocular surface diseases like Stevens-Johnsons syndrome, severe chemical burns and ocular cicatrizing pemphigoid result in damage to limbus and conjunctiva, (Tsubota et al 1996, Dua and Azura-Blanco 2000, Dua et al 2001) posing a challenge to the surgeons. As described earlier in the study a technique for culturing limbal epithelial cells over HAM was developed (Vemuganti and Balasubramanian 2002, Sangwan et al 2003a, Sangwan et al 2003b) and applied to reconstruct the ocular surface in cases of LSCD. Some of the patients of severe ocular surface were benefited by the additional transplantation of conjunctival tissues. Encouraged by the clinical results of combined CLET and conjunctival autograft in severe ocular surface diseases, a culture technique of “composite ocular surface epithelium with the central corneal cells and peripheral conjunctival cells” on single HAM was attempted in the laboratory.

The in-vitro proliferative capacity of the conjunctival cells was demonstrated by many groups (Ang and Tan 2004, Pellegrini et al 1999). Thus a study was attempted at developing the co-culture system of the limbal and conjunctival cells, which could possibly be used for ocular surface reconstruction in patients of LSCD with conjunctival...
involved. The conjunctival tissue used was the support tissue obtained during the limbal biopsy procedure. The conjunctival cells in the co-culture system were characterized with lineage specific cytokeratin K19 and presence of goblet cells (Jonathan et al 1999).

3.2. Hypothesis

It is possible to grow a composite culture of limbal and conjunctival epithelium on a single HAM simulating the natural ocular surface *ex-vivo*.

3.3. Aims

To generate a composite culture of limbal (central) and conjunctival epithelial cells on single HAM, with/without use of barriers

3.4. Material and Methods

The protocol was approved by the Institutional Review Board of L. V. Prasad Eye Institute

3.4.1. Procurement of Limbal and Conjunctival Tissues

The limbal and conjunctival tissues were harvested after obtaining informed consent from patients (19) undergoing routine cataract surgery. The tissues were also collected from 7 patients (between 6-14 years) with manifestation of LSCD who were treated with CLET. HCE medium was used to grow co-culture.

3.4.2. Processing of HAM

A detailed procedure for preparation of HAM has been given in chapter 2. In brief, HAM processed and preserved in DMEM at −70°C was obtained from Ramayamma International eye bank. HAM of sizes 2.5cm x 2.5cm and 5cm x 5cm were used for conjunctival cultures and co-cultures respectively. The HAM was thawed, transferred from the nitrocellulose paper to a glass plate of appropriate size and incubated with trypsin (0.25% trypsin and 0.02% EDTA) at 37°C for 30-40 minutes. The epithelial cells were
dislodged from the surface by gentle scraping of the membrane with glass slides. The membrane was thoroughly rinsed with PBS and tucked behind the glass plate to secure it.

3.4.3. Culture Technique

3.4.3.1. Conjunctival Cultures

HAM processed and preserved at -70°C was obtained. The conjunctival cells were grown on de-epithelialized HAM (2.5 x 5 cm). For de-epithelialization, a small piece of glass slide was placed into a 55mm culture plate and processed as explained in chapter 1. The conjunctival tissue collected in the HCE was then shredded into tiny bits using sterile surgical blade (no.21). The tissue bits were then picked up with 24-gauge sterile needle and explanted onto the denuded HAM. After 20 minutes of explantation few drops of HCE medium were added onto the explants, and kept in the CO₂ incubator for allowing the adherence of the tissue bits to the membrane for about 8-10 hours. Finally the culture dish was flooded with 4-ml HCE medium containing 10% serum.

3.4.3.2. Co-Cultures

For establishing co-cultures limbal tissue was fragmented and 3-4 bits were explanted within the central 15mm of the HAM. Likewise, the conjunctival tissue was fragmented and 4-8 bits explanted in a circular manner at the periphery of the membrane. In 11 explants of co-cultures, a ring-shaped barrier made of perspex (specially designed for the study) with an internal diameter of 1.5 cm and 0.8cm (Figure 3.1 Inset) in height was placed at the center of the HAM. The limbal fragments were explanted on the membrane inside the ring and the conjunctival fragments outside the ring barrier, so as to segregate the growth from the two explants (Figure 3.1). The membranes were incubated with the growth medium and observed under the phase contrast microscope (Olympus CK40) for 2-3 weeks.
3.4.4. Culturing of Limbal and Conjunctival Epithelial Cells on HAM

The culture dish with HAM and tissue explants (Limbal/Conjunctival) was flooded with 4-ml HCE medium with 10% FCS (Table 2.1). The medium was changed every alternate day and growth of the cells was monitored under phase contrast microscope.

![Figure 3.1.](image)

**Figure 3.1.** Preparation of a co-culture of conjunctival and limbal tissues on a single HAM in the petriplate with limbal explants inside the ring barrier and the conjunctival explants outside the barrier. Inset: Self-designed ring shaped barrier made of perspex

For generation of cultures no feeder cell system was used. A complete submerged (no air-lift) technique of culturing limbal and conjunctival epithelium was developed which reduces the culture time from 4 weeks to 2 weeks.

3.4.5. Characterization of Cultured Conjunctival Epithelial Cells

3.4.5.1. Histology Evaluation

The histological evaluation of the cultured conjunctival cells was done by staining the whole mount preparations and paraffin sections by hematoxylin-eosin (H&E) and periodic acid schiff’s stain (PAS).
A] Whole Mount Preparations of Cultured Conjunctival Cells

After confirming confluent growth from the conjunctival explants over 2-3 weeks under the phase contrast microscope, the growth was terminated by replacing the media with 10% buffered formalin for a minimum of 4 hours. The HAM along with cultured cells were then air dried, and used to make whole mounts stained with H & E.

B] Paraffin Sections

A part of HAM with conjunctival culture was fixed in formalin and processed for routine histology with paraffin embedding. 4-5μ thick sections were cut using microtome. These sections were then deparaffinized before staining with H&E and PAS.

3.4.5.2. Immunophenotyping

Immunofluorescence was performed on HAM with cultured conjunctival cells. The cells were first fixed in 4% paraformaldehyde, air-dried and rehydrated using milli Q followed by PBS. The samples were then incubated with primary monoclonal antibodies (Table 3.1) for 1 hour after blocking for non-specific binding with 2.5% bovine serum albumin (15 minutes). The antibodies were diluted in 1X PBS. The detection system was FITC conjugated secondary antibody in which the cells were incubated for 30 minutes in dark. The cells were counterstained with propidium iodide. All the stained samples were screened for positivity under Laser Scanning Confocal Microscope (Carl Ziess, LSM 510 Meta).

Table 3.1. Antibodies for Conjunctival Epithelial Cells

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cytokeratin 19 (K19)</td>
<td>1:100</td>
<td>DAKO Cytomation</td>
</tr>
<tr>
<td>2</td>
<td>Cytokeratin 3 (K3)</td>
<td>1:500</td>
<td>ICON Biosystems</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(^{nd}) antibody</td>
<td>1:250</td>
<td>DAKO Cytomation</td>
</tr>
</tbody>
</table>
3.5. Results

Limbal and conjunctival tissues for explant culture were obtained from 15 males and 11 females with a mean age of 49 years (6-80). From these tissues, nine isolated conjunctival cultures and 22 co-cultures were made; 11 of which were studied with the use of barriers. Growth was observed in all the explant cultures within 2-4 days.

3.5.1. Characteristics of Conjunctival Cultures

Clusters of rounded cells were seen at the edge of the explants (Figure 3.2A) by day 2-3, which expanded to form a monolayer of closely, packed cells by day 4-5 (Figure 3.2B). Within 2 weeks, the entire membrane was covered by a monolayer of cultured cells. In the whole mount preparation a disc like growth around the explanted fragments was noted which became confluent in many areas. Under the microscope, the conjunctival cells were seen as a sheet of epithelial cells with a large vesicular nucleus containing prominent 1-3 nucleoli. Frequent mitotic figures were noted (Figure not shown).

![Figure 3.2](image)

**Figure 3.2.** Explant cultures as observed under a phase contrast microscope A) Clusters of cells at the edge of a conjunctival explant (x200). B) Monolayer of conjunctival cells (x200).

3.5.2. Characteristics of Co-cultures

In co-cultures, the appearance of cells was similar to that seen in conjunctival cells. The cells from both explants were seen as round cells around the edge of the explant which expanded in 2 weeks to form a monolayer of closely packed cells around both the
limbal and conjunctival fragments. In co-cultures with barriers, the limbal cells were restricted to the inner region of the ring while the conjunctival cells were seen outside the ring. In co-cultures without barriers, the cultured cells were initially seen distinctly originating from each fragment but with the formation of a monolayer, the cultured cells from all fragments became confluent, and could not be distinguished from each other.

3.5.3. Characteristics of Limbal Cultures in Co-cultures

The limbal cells showed similar growth pattern (Chapter 2) as the conjunctival cells.

3.5.4. Histology Evaluation

A] Wholemount preparations of conjunctival cultures

Under the microscope, the conjunctival cells were seen as a sheet of epithelial cells with a large vesicular nucleus containing prominent 1-3 nucleoli. On naked eye examination of stained whole mount preparation, a clear demarcation was seen between the limbal and conjunctival cells (Figure 3.3.A). Frequent mitotic figures were noted (Figure 3.3.B). Some of the cells showed a large vacuole with a peripheral nucleus giving signet-ring morphology. The mean percentage goblet cell count was 0.46% (range 0-2.3%) These cells were intensely positive with PAS stain (Figure 3.3.C).

B] Histology of cultured conjunctival epithelial cells

The histology of cells in paraffin sections stained for H&E showed presence of a layer or two of cells overlying the HAM. The PAS stained sections showed presence of similar cells with occasional PAS positive goblet cells (Figure 3.3.D).

3.5.5. Immunophenotyping

The conjunctival epithelial cells were positive for conjunctival phenotype specific cytokeratin K19 (Figure 3.4.A, C). MUC5AC positive goblet cells were found in the cultured conjunctival epithelial cells, further confirming the PAS staining of goblet cells (Figure 3.5 A-
C). The cultured cells were negative for corneal phenotype specific cytokeratin K3 (Figure 3.4.B).

Figure 3.3. Cultured conjunctival epithelial cells on HAM. A) Whole mount preparation with barrier (not seen in the picture) showing the growth of limbal cells in the centre and conjunctival cells at the periphery outside the ring barrier and a clear circular zone, devoid of cells. (H&E, x 100). B) Light microscopic appearance of the whole mount preparation Histologic section showing a monolayer of epithelial cells, some of which show the presence of mitotic activity (arrow) (H&E, x 100). C) Whole mount preparation-showing presence of PAS positive goblet cells (arrow) (PAS, x 400). D) Paraffin section showing 1-2 layers of epithelial cells (PAS, x 200) Inset: Magnified image of the same (x 400).
3.6. Discussion

This study describes a novel technique of culturing an entire ocular surface epithelium consisting of central limbal and peripheral conjunctival cultures, simulating that seen in nature. The conjunctival cells have been cultured, characterized and studied for the location of their stem cells, goblet cell differentiation, and keratin profile (Wei et al 1993, Wirtschafter et al 1999, Pellegrini G et al 1999, Shatos et al 2001, Meller et al 1999a, Diebold et al 1999, Meller et al 2002 b, Marsh et al 2002). The conjunctival cells have been cultured by the explant technique as well as single cells, with or without the
addition of 3T3 feeder cell layer. Conjunctival cultures have also been used to study the effect of clinical conditions and diseases (Rapoza et al 1999, Nowakowski et al 1988). This study describes a novel technique of culturing an entire ocular surface epithelium consisting of central limbal and peripheral conjunctival cultures, simulating that seen in nature. Goblet cells characteristic of the conjunctival epithelium are very essential for the formation and maintenance of the tear film as mucin deficiency can lead to various disorders of the eye. Contrary to the earlier belief that conjunctival keratinocytes and goblet cells have different progenitor cells, it is now believed that they have a common bipotent progenitor cell. According to Pelligrini et al, commitment to a goblet cell occurs relatively late, and the total amount of goblet cells generated by old transient amplifying cell is consistently higher than that generated by young transient amplifying cells (Pelligrini et al 1999).

In this study, conjunctival tissue was harvested from the bulbar region as it obviated the need for an additional incision during limbal biopsy. The choice of substrate for culturing limbal and conjunctival cells was the HAM for its known anti-inflammatory, anti-apoptotic properties and its extensive usage in the treatment of ocular surface disorders. As described in previous chapter HAM is believed to preferentially preserve and expand limbal epithelial stem cells that retain their in-vivo characteristics of differentiation, slow cycling, label retaining and resistance to phorbol ester tumor promoter, (Meller et al 2002a) thus obviating the need for 3T3 feeder layers.

Conjunctival cells like limbal cells could be easily cultured on HAM by the explant technique and grew to confluence within 10-14 days. Morphologically, the two types of cells appeared the same, but were phenotypically distinct. The corneal epithelial cells from limbal explants express cytokeratin K3 while the conjunctival cells were negative or weakly positive for the same marker. Goblet cells were observed in almost all the cultures
(conjunctival as well as co-cultures). This is in contrast to findings reported by Marsh et al. who did not observe goblet cells in the conjunctival epithelial cultures (Marsh et al 2002). They hypothesize that one of the reasons for this could be the source of the conjunctival tissue as it was taken from the bulbar region, which is poor in goblet cells. The tissues used in this study have also been taken from the bulbar region and a mean goblet cell count of 0.46 %, within 10-15 days of growth was found. Owing to the small number of goblet cells seen in this preparation, it is difficult to conclude if they represent the persistence of goblet cells from the original explant or the newly generated cells in the culture. Further studies are required to confirm the clonality and the proliferative potential of the cultured cells.

Generating a “composite cultured epithelium of limbal and conjunctival cells” is a novel technique that would be of immense benefit to the patients suffering from severe ocular surface disorders. The two types of cultured epithelia on two separate membranes could possibly serve the same purpose and also avoid the risk of mixing up of both cell types. But from the surgeon’s point of view suturing two different membranes probably would be tedious and time consuming. Co-culturing of two different types of cells, in principal, has been used to promote the growth or differentiation of either cell type (El-Ghalbzouri et al 2002, Nakamura et al 2002, Dietze et al 2002, Wagner S et al 2003, Oishi Y et al 2004). This study aimed at culturing a single epithelium similar to that produced in nature. The successful culturing of cells from two types of tissues over the same membrane, without hampering or impeding the growth of either cell type, is a proof of the compatibility of both epithelia. The ring barriers were able to restrict the growth of limbal cells to the demarcated region ensuring that there was no intermingling of the two cell types. The use of a ring barrier is crucial in preventing the overgrowth of conjunctival cells into the central region.
In conclusion, this study describes a novel method of culturing a “composite ocular surface epithelium” from limbal and conjunctival tissues using a single HAM. This would greatly facilitate the reconstruction of the entire ocular surface of the severely damaged eyes to a near normal anatomic configuration in a one step surgery. The use of ring barriers to segregate the two types of cells so as to prevent the outgrowth of one type of cell into the zone of the other is recommended.