

Technique of cultivating limbal derived corneal epithelium on human amniotic membrane for clinical transplantation

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ABSTRACT

Background: The technique of transplantation of cultivated limbal epithelium rather than direct limbal tissue is a novel method of "cell therapy" involved in reconstructing the ocular surface in severe limbal stem cell deficiency [LSCD], caused by chemical burns.

Aim: To describe a simple feeder-cell free technique of cultivating limbal epithelium on human amniotic membrane [HAM].

Materials and Methods: The limbal tissues (2 mm) were harvested from patients with LSCD. These tissues were proliferated *in vitro* on HAM supplemented by human corneal epithelial cell medium and autologous serum. Cultures covering more $\geq 50\%$ area of 2.5x5 cm HAM were considered adequate for clinical use. The cultured epithelium was characterized by histopathology and immunophenotyping.

Results: A total of 542 cultures out of 250 limbal tissues were cultivated in the laboratory from January 2001 through July 2005. The culture explants showed that clusters of cells emerging from the edge of the explants in one-three days formed a complete monolayer within 10-14 days. In 86% of cultures (464 of 542), the growth was observed within one-two days. Successful explant cultures were observed in 98.5% (534 of 542 cultures) with 91% explant cultures showing an area of ≥ 6.25 cm² (6.25 - 12.5 cm² range). The cultivated epithelium was terminated between 10-14 days for clinical transplantation. The problems encountered were inadequate growth (2 of 542) and contamination (2 of 542).

Conclusions: We demonstrate a simple technique of generating a sheet of corneal epithelium from a limbal biopsy. This new technique could pave the way for a novel form of cell therapy.

KEY WORDS: Corneal epithelium, limbus corneae, stem cell transplantation

Limbal stem cell deficiency (LSCD) is now a well-established entity, which is caused by damage to limbal stem cells.^[1,2] In this vision-threatening condition, the damaged limbal-corneal epithelium is replaced by overgrowth of conjunctiva and is associated with pain, watering, neovascularization and opacification of the cornea, finally leading to visual loss. Effective therapeutic modalities include reconstructing the ocular surface with human amniotic membrane (HAM), replenishing the stores of limbal stem cells by healthy stem cells from direct limbal tissue transplantation^[3] or by transplantation of cultivated limbal epithelium. The hypothesis underlying the treatment is that, *ex-vivo* cultivated limbal epithelial cultures have stem cells/progenitors cells that after transplantation would help in regenerating the corneal epithelium in patients with LSCD.

Over the last few years, various centers,^[4-8] including our team, have reported the successful use of cultured limbal epithelial

cells (which possibly contain a population of stem cells) for reconstructing the damaged ocular surfaces.^[9] Interestingly, the technique of culturing these cells has varied with different investigators. In this study we report the success of a simple cost-effective technique of *ex vivo* expansion of limbal epithelial cells using HAM as substrate, for clinical transplantations. HAM has been extensively used for ocular surface reconstruction and has properties which facilitate the growth of epithelial cells controlling inflammation and scarring.^[10-14] It also has antibacterial and anti-apoptotic properties. Taking cues from the above we chose HAM as substrate for our cultures. By this technique, we were able to treat the patients suffering from partial or complete limbal stem cell deficiencies giving them good vision rehabilitation.^[9,15,16]

Materials and Methods

The protocol was approved by the Institutional Review Board

at our institute.

We used a modified human corneal epithelial cell (HCE) medium [Table 1] for culturing of limbal tissues. The medium was prepared using 9.7 g/l modified eagle medium with addition of 16.2 g/l Ham's F12 serum, 0.01mg/l epidermal growth factor, 0.25 mg/l insulin, 0.1 mg/l cholera toxin and hydrocortisone. This was supplemented with 10% fetal calf serum at the time of use in the initial cases, later 10% autologous serum was used.

Modified Eagles medium, HamsF12, cholera toxin, epidermal growth factor, insulin, fetal bovine serum, trypsin, ethylenediaminetetraacetic acid, diaminobenzidine were obtained from Sigma-Aldrich Chemie (Steinheim, Germany) and Sigma Chemical Co (St. Louis, USA). 0.22 u filters were from Millipore Corporation (Bedford, MA, USA).

The standard protocol proposed by Kim *et al.*,^[17] was used for the preparation of HAM. In brief, the placenta (which has two layers called amnion and chorion) obtained from the caesarian section deliveries was used to obtain the amniotic membrane (AM), after screening the donor for HIV, HBs Ag and VDRL. The placenta placed in a sterile pan and washed repeatedly (by discarding the water in the sink) with antibiotic containing ringer lactate/normal saline until clear water is obtained. The placenta is then transferred aseptically to another sterile pan and carried to the laminar flow hood, which is precleaned and UV sterilized. Amniotic membrane is peeled, separating amnion and chorion. The stretched membrane is cleaned using cotton swab and intermittent wetting with ringer lactate/normal saline using wash bottle. Once a clean transparent

approximately 2"x2" area (7.5x7.5) is available the nitrocellulose paper is attached on the chorion side keeping the epithelium side up. The AM was cut around the paper while rolling the edges on the other side of the paper. (AM should be stuck to the nitrocellulose paper perfectly without gaps or air-bubbles). The nitrocellulose paper was then cut to get small pieces of membrane as per requirement. The AM pieces (2.5x2.5, 2.5x5, 5x5cm) were then inserted in vials containing Dulbecco's modified eagles medium and stored at "70°C. Just before use, the AM was thawed at 37°C for 30 min.

The limbal tissue was harvested after obtaining informed consent from patients undergoing limbal biopsy for cultivated limbal transplantation. The limbal biopsy was performed on the second group of individuals based on the diagnosis of LSCD. The patient work-up included a detailed clinical examination for presence of the classical features of LSCD, a triad of symptoms - conjunctivalization, neovascularization and chronic inflammation were looked for in all patients.

For harvesting the limbal tissue (biopsy) under local or general anesthesia, the conjunctiva of the eye was incised 3 mm behind the limbus at 12 O'clock position and dissected towards limbus and into the clear cornea up to 1mm, using # 15 blade on Bard Parker handle. Depth of dissection was superficial and followed corneal insertion of the limbal conjunctival. The conjunctiva was then excised at limbus just behind pigmented line (Palisades of Vogts) and limbal tissue with 1 mm clear corneal tissue was excised. The limbal tissue was then collected in the HCE medium in an eppendorf.

HAM processed and preserved in Dulbecco's minimum essential medium at -70°C was obtained from Ramayamma International eye bank. The limbal cells were grown on deepithelialized amniotic membranes (2.5x5 cm). For deepithelialization, 1ml trypsin-EDTA was added onto the surface and incubated for 30 min at 37°C. The membrane was then mechanically scraped for complete deepithelialization, confirmed by observation under the phase contrast microscope. The limbal tissue collected in the human corneal epithelium medium was then shredded into tiny bits and explanted onto the denuded amniotic membrane. After allowing the explants to adhere to the HAM, the culture dish was flooded with 4 ml HCE medium with 10% autologous serum [Table 1]. The medium was changed every alternate day and growth of the cells was monitored under phase contrast microscope. The cultures were observed and studied for three-four weeks, but for clinical transplantation, the epithelium was harvested within 10-14 days.

During the standardization of the technique, the extent of growth was studied by whole mount preparation stained with hematoxylin - eosin. The membranes at various points of time were submitted for histological studies to confirm the stratification of the epithelium. Immunophenotyping was done using monoclonal antibody AE5 for corneal phenotype specific cytokeratin K3, to determine the lineage of cells. The cell kinetic studies were performed with BrdU/Thymidine pulse chase experiments (Data not shown here).

Table 1: Composition of the human corneal epithelium medium

Ingredients	Quantity	Company
Minimal essential medium	1.99 g	Sigma M0643
Nutrient mixture Ham's F-12	3.33 g	Sigma N4388
Penicillin	75 mg	
Streptomycin	50 mg	
Amphotericin	1.25 mg	
Gentamicin	50 µl (4 mg/L)	
Epidermal growth factor	50 µl (0.01 mg/L)	Sigma E4127
Cholera toxin	50 µl (0.1 mg/L)	Sigma C8052
Insulin	2.5-5 mg	Sigma I1882
Sodium bicarbonate	0.690 g	

Method of preparation: Add the first two ingredients in 100 ml of Milli Q water, in a sterile 500 ml flask/beaker, add sodium bicarbonate, dissolve and add the remaining ingredients. Ensure that the pH is 7.0. Make up the medium volume to 412 ml. Sterilize the medium with vacuum filter using Millipore filter membrane (0.22 µ). After sterilization add a few drops of the medium to chocolate agar plates for sterility check. The medium is then stored at 4°C. The shelf life of the medium is about 15 days as after that the pH of the medium increases indicated by change in color (phenol red) towards more pinkish.

Separation of autologous serum: About 10 ml blood is drawn from the patient in nonheparin, 15 ml graduated falcon tubes. The blood is kept at room temperature undisturbed for a couple of hours to allow the settlement of cellular components. It is then centrifuged at 2000 rpm for 10 minutes. The serum collected is pipetted out in a fresh falcon and filter sterilized using 0.22 µ millipore syringe filters and used in the culture medium in 10% concentration.

Results

Successful cultures from limbal explants were established. In all cultures, the growth of the cells was monitored under the phase contrast microscope. On an average on Days 1-3 clusters of round cells were seen at the edge of the explant. Cells further divided to form a monolayer by Days 4-5. A closely packed compact monolayer of cells (honeycomb pattern) with a growing edge was seen on the subsequent days [Figure 1]. These cells then expanded to confluency covering the entire membrane within a period of two-three weeks.

In 25 cultures, whole mounts stained with hematoxylin and eosin were prepared. When observed under a microscope, the stained whole mount preparation of the cultured explants revealed a monolayer of polygonal cells originating from the edge of the explanted tissue [Figure 2a and b]. The nucleus was vesicular with two-three nucleoli in the cells. Frequent

mitotic figures were noted. Some of the cells showed nuclear fragmentation suggestive of apoptosis. Few stellate-shaped pigmented cells suggestive of melanocytes were seen overlying the epithelial cells.

Histology in 25 cultured cells revealed one-two layers of cuboidal cells [Figure 3a and b]. In other areas stratified epithelium resembling corneal epithelium was noted. The cultured epithelial cells were immunopositive for corneal specific cytokeratin K3 antibody, indicating the corneal phenotype of the cultured cells [Figure 4].

For each tissue two cultures were grown parallel for unilateral requirement and four cultures for bilateral requirement. Four hundred and fifty-eight cultures were grown for 229 unilateral requirements and 84 cultures for 21 bilateral requirements. Initial 88 tissues and 21 bilateral tissues were grown in medium with 10% fetal calf serum and 141 tissues were cultured using 10% autologous serum.

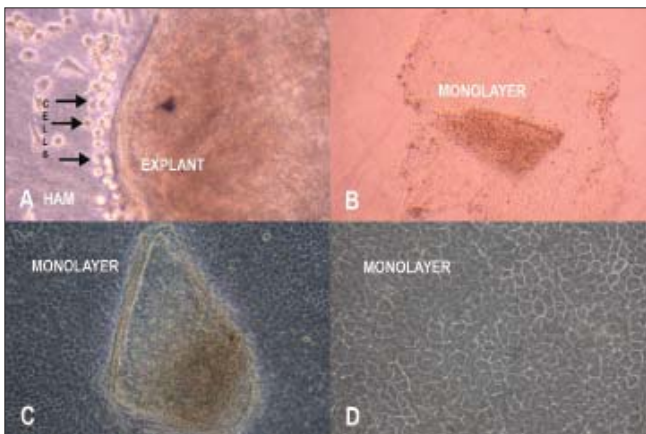


Figure 1: Limbal cultures as seen under phase contrast microscope (Clockwise) - Cells proliferating from the periphery of the explant on days 2-3 (200x) A, Monolayer of limbal cells on days 4-5 (40x) B, extension of monolayer through days 6-10 (40x) C, confluent monolayer on days 10-14 (200x) D, as observed under phase contrast microscope

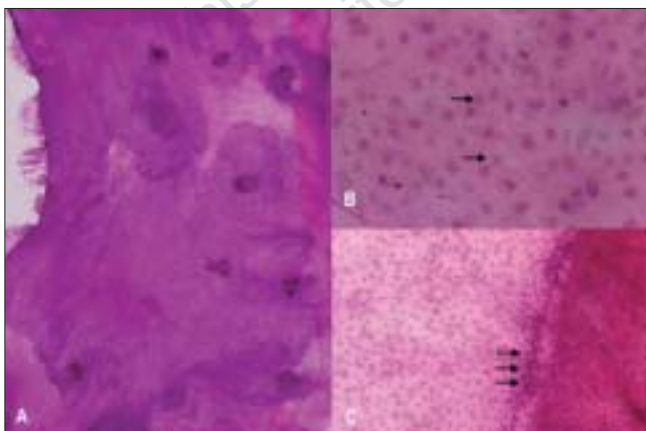


Figure 2: Whole mount preparation of the cultured limbal epithelial cells on naked eye examination revealing rings of pink darkly stained areas around the explants (arrows), H and E, a. Stained whole mounts as observed under microscope revealing a confluent monolayer of polygonal cells (H and E, 400x) b which were seen originating from the edge of the explants (arrows) (H and E, 200x) c.

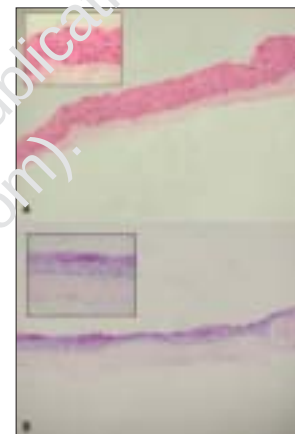


Figure 3: Paraffin sections of HAM with cultured limbal epithelial cells showing 2-3 layered cuboidal cells stained with H and E (3a) and PAS (3b), 100x - Insets: Higher magnifications (200x) of the same

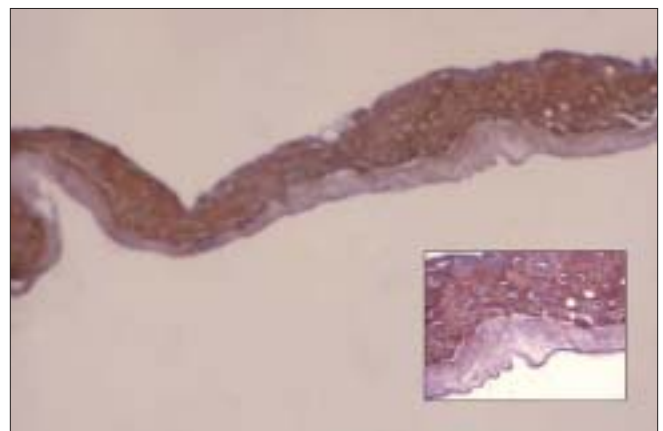


Figure 4: Paraffin section of HAM with cultured limbal epithelial cells showing immunopositivity for K3 (100x) - Inset: higher magnification (200x) of the same

Growth initiation was seen in 232 tissues on day 1, [Figure 1a], in 6 tissues on day 2, in 3 tissues on day 3, 2 tissues each on days 2, 4, 5, 6 and 7 and in 1 tissue on day 10. The total of 246 tissues (464 cultures - 85.6%), which showed growth, had covered more than 50% area of the 2.5x5 cm HAM ≥ 6.25 cm² (6.25-12.5 cm range).

Out of 250 tissues (542 cultures) that were grown in the laboratory during April 2001 to August 2005, a remarkable 98.5%, 246 (534 cultures) tissues showed growth, 2 (4 cultures) were lost to contamination and 2 (4 cultures) did not show any growth for reasons unknown. This projects a very insignificant rate (0.36%) of contamination hence revealing high safety of our culture system.

Discussion

The human ocular surface epithelial cells were grown for the first time on the feeder layer system by Sun *et al.*^[18] and as an explant technique by Ebato *et al.*^[19] A decade later, it was Lindberg *et al.* who made an attempt at transplanting the cultured epithelial sheets over the subdermis of mice.^[20] Subsequently, Pelligrini *et al.*, Tsai *et al.*, Schwab *et al.* and others,^[4-9] have cultured the limbal stem cells as explant cultures using either the HAM or other substrates such as fibrin, extracellular matrix protein (fibronectin, laminin, collagen IV) coated petridishes, corneal stroma and temperature responsive gels to culture the limbal epithelial cells and for their probable application in ocular surface reconstruction.

Taking advantage of the easy and ready availability of processed good quality HAM, we chose to adopt the direct explant culture method of cultivating the corneal epithelium. Human amniotic membrane has been reported to have anti-inflammatory properties due to down-regulation of key pro-inflammatory cytokines such as IL-1 α and IL-2 β and TGF- β .^[21] Shimmura *et al.*^[22] reported the anti-inflammatory effects of AM transplantation in ocular surface disorders. And also interleukins 1a and 1b were found to be down-regulated^[23] in limbal epithelial cells cultured on HAM making it an ideal candidate for use as carrier for cultivated limbal epithelial transplantations.

Varki *et al.*^[24] reported that exposure of embryonic stem cells to serum replacements and nonhuman feeder layers, made the cells deposit nonhuman sialic acid Neu5Gc against which many humans have circulating antibodies. Our group has developed a simple, feeder-cell free method of culturing limbal epithelium. The use of feeder cells and the fetal calf serum is believed to propagate the stemness of the explant culture. However, we made two deviations from the previous publications with the intention of making it xeno-free and due to the non-availability of the culture inserts which could physically separate the feeder cells and the explant cultures. We chose to exclude feeder-cell layer and replace fetal calf serum with autologous serum for clinical transplantation. There was no difference in the growth pattern when we removed the feeder-cell layer from the culture system, which prompted us to continue the feeder-cell free method of cultures. Similarly,

substitution of autologous serum (10%) did not make any difference in growth pattern or nature of cultured cells, hence was used for all autologous limbal cultures. However, for cultivation of allogenic tissues we continued to use fetal calf serum, as was done by others. The reason for this was because we were not sure if we should use the donor or recipients' sera and for the lack of guidelines on the use of allogenic serum in such cases. This problem could possibly be overcome in future, if we could identify an alternate source of autologous tissues which can be used to function like corneal epithelium and thus used for ocular surface reconstruction for LSCD e.g., oral mucosa.^[25-27] Irrespective of the type of sera used, the serum-containing medium is washed out with plain HCE medium for 24 hours before transplantation.

The air-lift technique used by many groups involves the culturing of cells for a period of three weeks and then lowering the amount of medium of the culture so as to provide an air - water interphase for another week which promotes stratification of the epithelial cell cultures and also improves intercellular junctions. However, due to lack of availability of these culture inserts and in a search for a cost-effective system, we cultivated the epithelium in submerged conditions. The histology of epithelium at different points of submerged culture conditions showed varying stratification which led us to believe that there is an inherent property of the cells to stratify, which was further confirmed by the histological studies on the corneal button from patients who underwent penetrating keratoplasty after CLET. This change in culture technique has obviated the need for culture inserts and has cut down the cost. Thus the significant advantages of the technique presented here over the previously reported ones,^[5,7,28] are: a simple feeder cell-free explant culture technique that can generate the epithelium within 10-12 days; which also obviated the need for fetal calf serum and use of culture inserts to promote *ex vivo* stratification as adapted by others.^[5,21]

Estimation of the exact number of stem cells in the cultivated epithelium would have added value to this study. Though generation of a monolayered epithelium with variable expression of cytokeratin K3 was used to characterize the cultivated epithelium in this series, there is a need to conduct more studies in this direction. Since the initiation of this study in 2000, various groups have proposed specific markers for limbal stem cells that include p63,^[29,30] ABCG2 and SP population.^[31] To the best of our knowledge and literature search, this is the first series using this novel technique in a developing country and also the largest series in the world, using cultivated limbal epithelium for clinical transplantation. Our technique is highly reproducible, cost-effective and easily adaptable and having immense clinical significance as is evident from our clinical results.^[15,16] This technique we believe could be considered as a successful model of cell therapy, which could be extrapolated to other systems as well.

Conclusion

We demonstrate in this study that using a simple explant

culture technique, it is possible to generate a sheet of corneal epithelium from limbal tissue with stem cells. Experiments on further characterization of these cells both *in vitro* and *in vivo* are in progress.

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Announcement

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The entire editorial team of the journal thanks the contributors and the reviewers of the journal who are an integral part of the journal's success.