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Successful Reconstruction of Damaged Ocular Outer Surface in Humans using Limbal and Conjunctival Stem Cell Culture Methods

Virender S. Sangwan,¹ Geeta Kashyap Vemuganti,¹ Shashi Singh,² and D. Balasubramanian¹

When the ocular outer surface is badly damaged, subsequent corneal transplantation fails due to the absence of basal cells that are needed to support the graft. With the realization that the limbus and the conjunctiva have adult stem cells that can be cultured, it has been possible for us to explant culture these on de-epithelized human amniotic membrane, and to graft the resulting viable and transparent epithelium to 125 needy human patients with success. Ultrastructural, histological, biochemical and immunological assays establish the identity of the cells and the tissue formed.

KEY WORDS: Adult stem cells; cornea; limbus; conjunctiva; ocular outer surface; human amniotic membrane explant culture.

INTRODUCTION

An important cause of avoidable blindness the world over is the damage that occurs to the outer surface of the eye through unresolved infection, persistent acid/alkali, or thermal burns, natural disasters, and acts of violence. Restoration of sight to the unfortunate victim requires sufficient endogenous levels of cells of the constituent conjunctiva, limbus and cornea, which help to repair the surface and are vital to ensure the success of the inevitable corneal transplant from a donor eye. None of these is always guaranteed in many cases. An initial useful step, introduced recently [1–3], has been to graft the damaged surface with processed human amniotic membrane obtained from maternity clinics, and that has been successfully tried in our clinic to manage acute chemical and thermal injury [4]. Since the ocular surface tissue undergoes rapid and steady cell turnover, such grafting is not sufficient; (a continuous supply of the various constituent cells is required). With the realization that stem cells are present in, and can be harvested from the conjunctiva and limbus, attempts have been made with some degree of success to isolate and cultivate these stem cells in culture and transplant them on the needy ocular surface [5, 6]. We report here our experiments on combining these individually successful leads to

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introduce a simple and high throughput method (particularly needed in high incidence situations), which has proven useful in treating 125 patients with varying degrees of uni- and bilateral ocular surface damage. In many of these cases, the success rate of eventual corneal graft (penetrating keratoplasty) was remarkably improved because of such initial reconstruction.

MATERIALS AND METHODS

The general principles of culturing the cells by explant culture technique involve the following steps: harvesting the limbal tissue; selecting the appropriate carrier: a sheet of multi-layered epithelium, human amniotic membrane, collagen shields or contact lens; preparation of human corneal epithelial medium; and explant cultures. Confirmation of growth can be done by various methods including-direct observation, whole mount stained preparation, histopathology, immunohistochemistry, thymidine incorporation and by flow cytometry using markers for cell cycle. Details of a few of the procedures can be found in an earlier publication [7].

Limbal Cell Culture

Limbal Biopsy. After satisfying the Institutional Review Board and obtaining informed consent from the patients who donate the limbal tissue, about 1–2 mm of limbal tissue is harvested approximately 1 mm on either side of the corneo-conjunctival junction.

Human Amniotic Membrane Preparation. The standard protocol proposed by Kim *et al.* [2] was used. The placenta (which has two layers called amnion and chorion) obtained from the caesarian section deliveries was used to obtain the amniotic membrane, after screening the donor for HIV, HBs Ag and VDRL, washed with Ringer solution containing antibiotics and the amnion is separated from chorion. The amniotic membrane thus separated was spread on top of a nitrocellulose paper with the epithelial side up, and the paper along with the amniotic membrane was cut into the required dimensions and stored in DMEM (Dulbecco's Modified Eagles Medium) vials at -70°C . Just before use, the amniotic membrane was thawed at 37°C for 30 min. and placed on a sterile cut glass slide. The amniotic epithelium was removed by digestion with 0.1% trypsin-EDTA at 37°C , followed by scraping. Under sterile conditions, the membrane was inspected under the microscope for complete removal of cells. The de-epithelized membranes were then spread on a slide (used as culture inserts) in a Petri plate tucking the edges for a uniform surface.

Human Corneal Epithelial Cell Growth Medium. We used a modified Hormone Corneal Epithelial Cell (HCE) culture medium, prepared using 9.7 g/l Modified Eagle Medium (MEM) 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 (FCS) at the time of use.

Explant Culture Technique. Limbal tissues, cut into 4–6 fragments, were placed on the de-epithelized membranes separately and incubated with HCE medium with 10% FCS, and allowed to settle down by overnight incubation at 37°C with 5% CO

2. The following day, 2.5 ml of HCE with 10% FCS was added to the plates and incubated for 10–12 days, changing the medium on alternate days. In four days polygonal cells could be seen growing from the edges of the explant (Figure 1A).

Whole mount preparation: After confirming confluent growth from the explanted tissue over 1–2 weeks by examining under a phase contrast microscope, the growth was terminated by replacing the medium with 10% buffered formalin. The whole mount preparation, stained with hematoxylin and eosin, could be seen as bluish rings of stained areas around the original explanted tissue. When observed under the microscope, the cultured cells appear as a monolayer of large polygonal cells with an epithelial appearance (Fig. 1B). The membrane with the cultured limbal tissue and the cultured cells is fixed in formalin and processed for routine histopathology with paraffin embedding. The sections are cut at 4–5 μm and after deparaffinization, stained by hematoxylin and eosin stain and periodic acid schiffs stain. In contrast to the cuboidal epithelium of the normal amniotic membrane the cultured cells form an epithelium of 1–2 layered cells over the amniotic membrane. Immunostaining on the formalin-fixed, paraffin embedded sections can be done using prediluted antibodies to cytokeratin 3 (K3) and cytokeratin 19 (K19) from DAKO, Denmark, Germany, to confirm the corneal phenotype of the cultured cells [7].

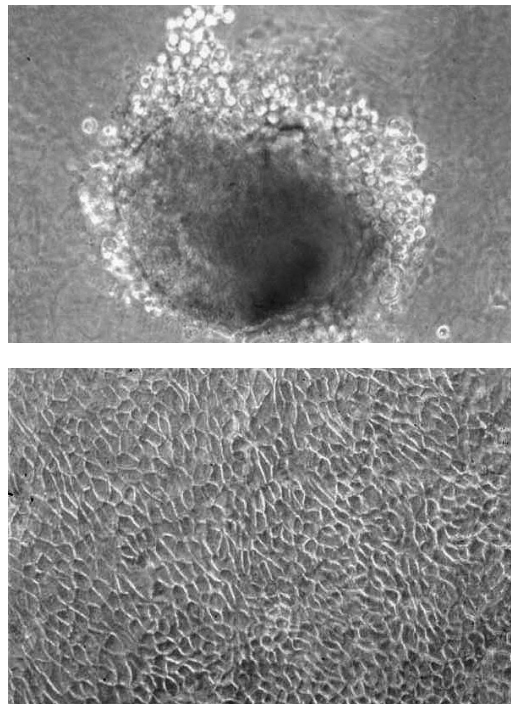


Fig. 1. *Panel A:* Clusters of rounded cells are seen around at the edge of the explant on day four (magnification: $\times 450$). *Panel B:* The cultured cells are seen as a monolayer of polygonal cells on day nine (magnification: $\times 400$).

Conjunctival Cell Culture

Under local or general anesthesia, the conjunctiva of the eye was incised 3 mm behind the limbus at the 12 o'clock position and dissected towards the limbus and into the clear cornea upto 1 mm. The conjunctiva was then excised at the limbus just behind the pigmented line (Palisades of Vogt), and the limbal tissue with 1 mm clear corneal tissue was excised. About 2 × 3 mm of conjunctival tissue was taken from the bulbar region randomly from the inferior and superior quadrants. The details of conjunctival culture and co-culturing conjunctival and limbal cells are presented below.

Conjunctival Cultures. The conjunctival tissue was cultured in a manner described above for culturing the limbal tissue. In brief, the technique involved fragmenting of the conjunctival tissue using a sterile surgical blade (size 21) into small bits and placing 4–6 bits on the denuded amniotic membrane. The membrane was flooded with HCE medium supplemented with 10% fetal bovine serum and incubated overnight at 37°C in 5% CO₂. 3–4 ml of medium was added to the culture the following day. The medium was changed every alternate day and the cultures were incubated for 2–3 weeks during which they were observed daily under the inverted microscope.

Co-culturing of Limbal and Conjunctival Cells

For establishing co-cultures, a ring barrier made of Perspex (specially designed for the study) with an internal diameter of 1.5 and 0.8 cm in height was placed at the center of the substrate amniotic membrane. Limbal tissue was fragmented and 3–4 bits were explanted within the central 15 mm region of the amniotic membrane. Likewise, the conjunctival tissue was fragmented and 4–8 bits explanted in a circular manner at the periphery of the membrane. In other words, the limbal fragments were explanted on the membrane inside the ring and the conjunctival fragments around the ring barrier, so as to demarcate the growth of the two cell types. As mentioned previously, the membranes were incubated with HCE medium and growth was observed under the inverted microscope for 2–3 weeks.

Characterization of Growth. After 2–3 weeks of growth, the material was fixed with 10% formalin, the ring barrier removed and assayed using microscopy of the whole mount preparation, periodic acid Schiff's (PAS) reagent for staining the goblet cells of the conjunctiva, and immunophenotyping of the cells using the specific antibody AE5. A brief report of the clinical use of such cultures was presented at the meeting of Association of Research in Vision and Ophthalmology (ARVO) [8].

RESULTS AND DISCUSSION

Taking advantage of the easy and ready availability of processed good quality human amniotic membranes, we chose to adopt the direct explant culture method of cultivating the corneal epithelium, rather than subculturing from trypsinized cells. The cultivated cells appeared as clusters of round cells (Fig. 1A) which subsequently

formed a monolayer of cells covering the whole membrane (Fig. 1B). With the success of generating a corneal epithelium, we co-cultured limbal and conjunctival epithelium on the same membrane so as to create a continuous surface of conjunctiva and cornea which produced rewarding results in some patients with severe ocular surface diseases. The nature of the cultivated cells was established by histopathology, immunohistochemistry and thymidine uptake studies. After standardizing the process, the clinical phase was initiated for patients suffering from severe limbal stem cell deficiency. In all cases, the cultivated epithelium along with amniotic membrane was transplanted on to the damaged ocular surface, after removal of the fibrovascular pannus. Stable ocular surface was noted in all patients within 2 weeks of transplantation. Over 15 of these patients have undergone subsequent penetrating keratoplasty for visual rehabilitation. Panels A, B and C of Fig. 2 show, respectively, the eye of one such patient immediately upon arrival for treatment, 12 weeks after autologous limbal stem cell treatment, and after the subsequent corneal graft (penetrating keratoplasty). The excised corneal button in all of these cases showed a normal looking stratified corneal epithelium (Fig. 2D), indicating the inherent property of the cultivated monolayer to stratify *in vivo*. The successful *in vivo* stratification is a very encouraging result observed in our series, and can replace or bypass

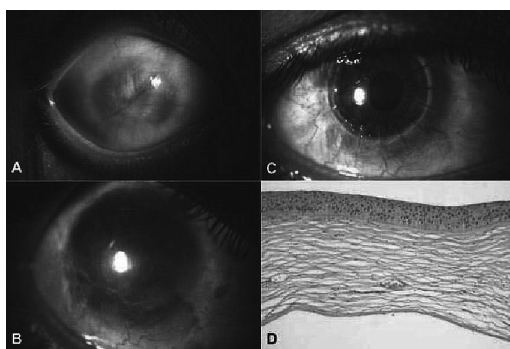


Fig. 2. *Panel A:* Slit lamp photograph of right eye of a patient with total stem cell deficiency with conjunctivalization of cornea following acid injury three years prior to presentation at our institute. The visual acuity was “counting fingers close to face”. *Panel B:* Slit lamp photograph of the same eye three month after autologous cultivated limbal epithelial transplantation. The cornea can be seen to be clear with no conjunctivalization. Visual acuity improved to “counting fingers at 2 meters”. *Panel C:* Slit lamp photograph of the same eye five months after a corneal graft (penetrating keratoplasty). The graft is clear with all sutures intact. The best corrected visual acuity improved to 20/40. *Panel D:* Photomicrograph of the excised corneal button (removed at the time of keratoplasty) shows a lining epithelium of 3–4 layers resembling the normal corneal epithelium. The underlying stroma shows irregular collagen lamellae and vasularization (hematoxylin and eosin staining, magnification: $\times 250$).

the cumbersome process of *ex vivo* stratification by creating an air-water interface as adapted by others [9–11]. This method also proves the efficacy of cultivating the corneal and conjunctival epithelium (Fig. 1) in a feeder (fibroblast) cell-free system within a span of 10–12 days.

In 15 patients with severe bilateral limbal deficiency we were successful in cultivating limbal tissue harvested from one quadrant of apparently normal looking limbus. The cultivated epithelium generated from this tissue was used to reconstruct the ocular surface of eyes, thereby avoiding allograft, and the obligate immunosuppression with its attendant cost and potential side effects. Similar results were achieved in a few monocular patients with areas of normal looking limbus.

The significant advantages of the technique presented here over the previously reported ones [6, 9–11] are: (a) a simple feeder cell-free explant culture technique that can generate the epithelium within 10–12 days; (b) *In vivo* stratification of a cultured monolayer of epithelium; and (c) generation of a composite culture of inner corneal and outer conjunctival epithelium to reconstruct the ocular surface in severe surface disorders.

ACKNOWLEDGMENTS

This work has been supported by a grant from the Department of Biotechnology, India (grant # BT/PR2305/Med/09/336/2001).

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