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Graziella Pellegrini Carlo E Traverso Adriano Tito Franzi Mario Zingirian
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Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium

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Summary

Background Complete loss of the corneal-limbal epithelium leads to re-epithelialisation by bulbar conjunctival cells. Since conjunctival and corneal-limbal epithelial cells represent two different cell lines, this conjunctival healing of the cornea is followed by stromal scarring, decreased visual acuity, and severe discomfort. Unilateral corneal-limbal epithelial defects can be resolved by the transplantation of limbal grafts taken from the uninjured eye. However, this procedure requires a large limbal graft to be taken from the healthy eye, and is not possible for bilateral lesions. We investigated the possibility of restoring the human corneal surface with autologous corneal epithelial sheets generated by serial cultivation of limbal cells.

Methods Cells were cultivated from a 1 mm² biopsy sample taken from the limbus of the healthy eye of two patients with severe alkali burns, and thus complete loss of the corneal-limbal surface, of one eye. Normal corneal differentiation was tested with a specific biochemical marker. Autologous cultured corneal sheets were then grafted onto the damaged eyes of the two patients. The patients were followed up at more than 2 years after grafting.

Findings We have shown that corneal progenitor cells are localised in the limbus, that cultured limbal cells generate cohesive sheets of authentic corneal epithelium, and that autologous cultured corneal epithelium restored the corneal surface of two patients with complete loss of the corneal-limbus epithelium. Long-term follow-up showed the stability of regenerated corneal epithelium and the striking improvement in patients' comfort and visual acuity.

Interpretation The cultivation of corneal epithelium might offer an alternative to patients with unilateral lesions and a therapeutic chance to patients with severe bilateral corneal-limbal epithelial defects. Our findings give a new perspective on the treatment of ocular disorders characterised by stem-cell deficiency.

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Istituto Dermopatico dell'Immacolata, Roma (G Pellegrini PhD, M De Luca MD); **Istituto Nazionale per la Ricerca sul Cancro, Genova** (G Pellegrini, R Cancedda MD, M De Luca); **and Clinica Oculistica** (C E Traverso MD, M Zingirian MD) **and Istituto di Anatomia Umana Normale, Università di Genova, Genova, Italy** (A T Franzi MD)

Correspondence to: Dr Michele De Luca, Laboratory of Tissue Engineering, Istituto Dermopatico dell'Immacolata, Via dei Castelli Romani 83/85, 00040 Pomezia, Rome, Italy

Introduction

The anterior ocular surface is covered with the highly specialised conjunctival and corneal epithelia, which are formed by two phenotypically different cell types.[1-4] The conjunctival epithelium is well vascularised and consists of loosely organised cell layers populated by mucin-secreting goblet cells, which contribute to the maintenance of a tear film on the ocular surface.[1,2] The corneal epithelium is a stratified squamous epithelium,

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devoid of goblet cells, as well as of other cell types, with a cuboid basal layer lying on the avascular corneal stroma by the Bowman's layer.

Visual acuity is dependent on the corneal epithelium, the integrity of which is maintained by the centripetal migration of stem-cell-derived transient amplifying cells.[7,8] The corneal (but not the conjunctival[9]) stem cell is thought to be located in the limbus,[7,10] which is the narrow transitional zone of the ocular surface located between the cornea and the bulbar conjunctiva.[1,2] The limbus consists of several layers of epithelial cells devoid of goblet cells and populated by Langerhans cells and melanocytes.

Complete loss of the corneal-limbal epithelium leads to re-epithelialisation by bulbar conjunctival cells.[3,4,11] Since the conjunctival and the corneal-limbal cells form two distinct cell lines,[1-6] corneal repair of the conjunctival epithelium is followed by neovascularisation, chronic inflammation, recurrent epithelial defects, and stromal scarring; it causes a pronounced decrease in visual acuity and severe discomfort.[3,4,11] Unilateral defects of the corneal-limbal epithelium can be restored by the transplantation of limbal grafts taken from the uninjured eye.[12] This procedure, however, requires a large limbal withdrawal from the healthy eye, and is not possible for severe bilateral lesions. These drawbacks prompted us to investigate whether the human corneal surface could be restored by autologous sheets of corneal epithelium generated by serial cultivation of limbal cells.

Methods

For preliminary experiments, epithelial cell cultures[13-16] were established from 1–2 mm² full-thickness biopsy samples taken from the ocular surface (bulbar conjunctiva, cornea, and limbus) of donors (patients admitted for keratoplasty) or cadavers. All patients provided informed consent. Briefly, biopsy samples were minced and treated with trypsin (0·05% trypsin and 0·91% edetic acid) at 37°C for 3 h. Cells were plated on lethally irradiated 3T3-J2 cells (2·4×10⁴/cm²) and cultured in 5% carbon dioxide in: Dulbecco-Vogt Eagle's and Ham's F12 media (3:1 mixture) containing fetal bovine serum (10%), insulin (5 mg/mL), transferrin (5 mg/mL), adenine (0·18 mmol/L), hydrocortisone (0·4 mg/mL), cholera toxin (0·1 nmol/L), triiodothyronine (2 nmol/L), epidermal growth factor (10 ng/mL), glutamine (4 mmol/L), penicillin-streptomycin (50 IU/mL). Subconfluent primary cultures were passaged at a density of 4×10³ to 1·3×10⁴ cells/cm² and cultured as above. For histological examination, samples were fixed in Susa fixative solution, embedded in paraffin, and stained with: haematoxylin-eosin (figure 1A), the keratin 3 (K3)-specific AE5 monoclonal antibody (figure 1B),[10] and periodic acid/Schiff.

For autologous transplantation, cells were cultivated from a 1 mm² biopsy sample taken, with the aid of a surgical microscope, from the limbus of the uninjured eye of two patients with severe alkali burns. Informed consent from the patients and approval from the local ethics committee were obtained. Biopsy samples were then cultured, as above. Subconfluent primary cultures were treated with trypsin and edetic acid and aliquots were either plated onto six multiwell plates (4×10⁴ cells per well), or frozen and stored. Grafts were prepared (19 days and 16 days after biopsy samples were taken) from confluent secondary cultures, which were released from the plastic dish with the neutral protease Dispase II (Boehringer, Mannheim, Germany; 2·5 mg/mL, 45–60 min at 37°C) and either mounted on a petrolatum gauze[16] about 1·5×1·5 cm or on a soft contact lens (figure 1C), with the basal layer exposed on the concave part of the lens. In both cases, grafts contained about 2·0×10⁶ cells.

The injured eye was prepared for the transplant, draped, and topical anaesthesia with a lid block by 2% plain xylocaine was given. The conjunctival epithelium covering the cornea and limbus was removed with a blunt knife and scissors, and a 360° peritomy, extending for at least 2 mm beyond the limbus, was done. For patient 1, the cultured epithelial graft was placed on the prepared eye mounted on a petrolatum gauze, which was gently removed under a microscope immediately after grafting. A soft therapeutic hydrophilic contact lens was then placed over the graft. For patient 2, the cultured epithelium was placed already mounted on the therapeutic hydrophilic contact lens (figure 1C). The procedure is schematised in figure 1D.

After grafting, the eye was patched tightly for 3 days. Topical prednisolone acetate and chloramphenicol were administered twice a day for 10 days, followed by artificial tears four times a day, and fluorometholone 0·3% twice a day. The therapeutic contact lens was removed 2 weeks after grafting.

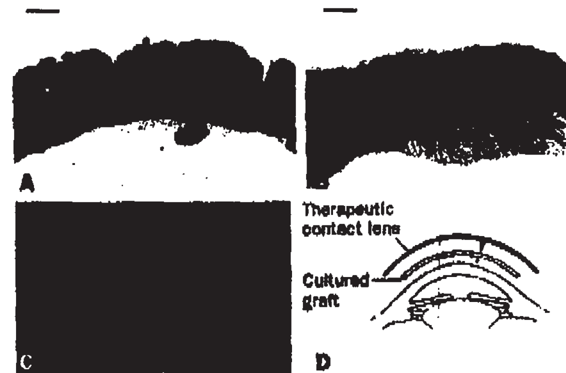

Figure 1: **Cultivation of corneal epithelium**
Sheets of epithelial cells cultivated from limbal biopsy samples released from plastic dish and stained with haematoxylin-eosin (A) and with K3-specific AE5 monoclonal antibody (B). C: epithelial sheet mounted on a soft contact lens and ready for transplantation. D: schematised surgical procedure: small arrowhead indicates prepared corneal wound bed after removal of conjunctival epithelium. Bars= 30 µm.

Results

Preliminary experiments were done on cells isolated from the central cornea, limbus, and bulbar conjunctiva of 20 donors. Colony-forming efficiency and growth rate of limbal cells were similar to those of skin-derived keratinocytes[15-17] and were far superior to similar cultures from bulbar conjunctiva.[15] Indeed, limbal cells generated large colonies (about 1×10³ cells for each colony after 14 days of culture) with a very smooth and regular perimeter and were easily serially cultivated, as for skin keratinocytes.[15-17] In contrast, bulbar conjunctival cells formed irregular colonies composed of larger cells and invariably underwent senescence within 2–3 passages. Cells derived from the central part of the cornea generated mostly terminal colonies and could never be subcultivated more than twice after senescence. In six cases, central corneal cells could not be subcultivated at all. Limbus-derived colonies eventually fused and gave rise to a stratified epithelium devoid of goblet cells (figure 1A). Cytokeratin 3 (K3) is a specific marker of the corneal lineage.[10] Limbus-derived cultures stained positively for K3 (figure 1B), indicating the maintenance of the corneal phenotype. We never observed K3 staining in conjunctival epithelium either in vivo or in vitro.

These data demonstrate that human limbus-derived epithelial cells reconstitute an authentic corneal epithelium in vitro, and suggest that corneal progenitors are concentrated in the limbus, whereas the central cornea is formed by transient amplifying cells.

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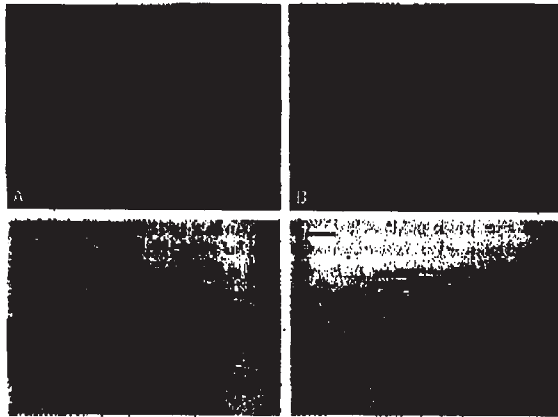

Figure 2: Transplantation and histology of patient 1
A: appearance of injured eye at admission. B: corneal surface about 11 months after grafting (6 months after penetrating keratoplasty). C: regenerated epithelium about 2 years after grafting; haematoxylin and eosin; arrowheads indicate Bowman's layer. D: K3 staining of regenerated epithelium 2 years after grafting. The K3-specific AE5-moAb shows a uniform positive staining.
Bars= 20 µm.

Patient 1 was a man aged 62 years who had had an alkali burn in his right eye 22 years earlier. 2 years after the burn, he had penetrating keratoplasty, which failed and perforated with loss of the lens. At admittance, slit-lamp examination showed extensive scarring of the corneal-limbal surface, iridocorneal synechiae, pannus, and neovascularisation (figure 2A). His visual acuity was reduced such that he could see only hand movements. Histological examination of the removed conjunctival epithelium showed an absence of K3 expression. 2 weeks after grafting of cultures, his cornea was covered with a transparent normal-looking epithelium and fluorescein revealed minimal punctate staining (not shown). 4 months later, the patient had a penetrating keratoplasty combined with pupilloplasty and anterior chamber intraocular lens implantation. Figure 2B shows the appearance of his eye 6 months after the keratoplasty. The cornea was covered by a transparent epithelium with no neovascularisation. The patient recovered a best corrected visual acuity of 0·7. 16 months later (more than 2 years after grafting) no changes were noted in terms of corneal transparency, vascularisation, or visual acuity and the patient gave his permission for a corneal biopsy. Histological examination showed a stratified squamous epithelium with a cuboid basal layer, resembling a normal cornea (figure 2C). The epithelium showed no papillary structures, lay on an avascular stroma with a well-defined Bowman's layer, and was devoid of goblet cells. The specimen was stained with the K3-specific AE5 monoclonal antibody. A uniform positive staining was present (figure 2D), as described for normal corneal epithelium.[10]

Patient 2 was a man aged 39 years who presented with an alkali burn of his left eye, received 10 years earlier. 1 year after the burn, he had a conjunctival graft from the uninjured eye, which was unsuccessful,[18] followed 1 year later, by penetrating keratoplasty, which ulcerated and failed. 2 and 4 years later, he received second and third corneal allografts, which also failed. At admission, he had severe vascularisation, persistent ulceration, tremendous discomfort, stromal melting, and hardly any vision.

After grafting of cultured epithelium, results in terms of reconstitution of a stable and transparent corneal epithelium, absence of vascularisation with negative fluorescein staining, and improvement in comfort were identical to those obtained in patient 1. The patient was satisfied with his clinical results and, with the outcome of the three previous keratoplasties in mind, he refused to undergo additional surgery aimed at improving his visual acuity. 19 months after grafting, histological examination of a corneal biopsy confirmed a uniform positive staining for K3. Vision improved from near blindness to counting fingers at 1 m.

At the last follow-up (at more than 2 years after grafting) both patients were clinically stable. The finding that the 360° peritomy extended far beyond the limbal edge in both patients, without subsequent keratoplasty in patient 2, and the long-term follow-up strongly suggest that re-epithelialisation has been achieved by cultured limbal cells and not by resident or donor cells. The stability of the regenerated epithelium and the long-term maintenance of the proper corneal phenotype strongly suggest that stem cells had engrafted successfully.

Discussion

Coverage of extensive defects of the corneal epithelium is a difficult clinical task. As with allogeneic epidermis,[19] allogeneic limbal epithelium might have a higher rejection rate if immunosuppressive agents are not used.[19] Therefore, even though keratoplasty is crucial for the reconstruction of damaged corneal stroma, it promotes true corneal re-epithelialisation only if residual autologous limbal epithelial stem cells are present in the injured eye.[4,20] Limbal cells could be induced to migrate and proliferate by the stimulatory paracrine effects of donor cells, as happens with cultured skin keratinocytes.[14] In the absence of naturally occurring limbal-corneal epithelial cells[20] (as in our patients), keratoplasty causes corneal re-epithelialisation by conjunctival cells. Since the possibility of conjunctival transdifferentiation into corneal epithelium has been ruled out,[1-4,20-22] the clinical outcome of this aberrant healing process is as described in our patients. In such patients, the autologous transplantation of limbal cells[12] is the only possibility for normal corneal healing. Such a procedure involves transfer of two free limbal grafts (about 60 mm²) from the uninjured to the injured eye.[12,13]

In our study, we show that enough epithelium to cover the entire corneal-limbal epithelial surface can be obtained from a 1 mm² limbal biopsy sample, allowing minimal stem-cell depletion from the healthy eye. Our study also shows that autologous grafts prepared from limbal cultures can generate an authentic corneal epithelium in patients with complete loss of the corneal-limbal epithelial surface. Primary cells can even be frozen and stored, to allow, if needed, additional transplantation.

The cultivation of corneal epithelium might offer an alternative to patients with unilateral lesions and a therapeutic chance to patients with severe bilateral corneal-limbal epithelial loss. Such cultivation also gives new perspectives on the treatment of ocular disorders characterised by stem-cell deficiency.

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References

1 Friend J, Kenyon KR. Physiology of the conjunctiva: metabolism and biochemistry. In: Smolin G, Thoft RA, eds. The cornea. Scientific foundation and clinical practice. Boston: Little, Brown, 1987: 16–38.
2 Thoft RA, Friend J. Biochemical transformation of regenerating ocular surface epithelium. *Invest Ophthalmol Vis Sci* 1977; 16: 14–20.
3 Tsai RJF, Sun TT, Tseng SCG. Comparison of limbal and conjunctival autograft transplantation in corneal surface reconstruction in rabbits. *Ophthalmology* 1990; 97: 446–55.
4 Wei ZG, Wu RL, Lavker LM, Sun TT. In vitro growth and differentiation of rabbit bulbar, fornix and palpebral conjunctival epithelia: implications on conjunctival epithelial transdifferentiation and stem cells. *Invest Ophthalmol Vis Sci* 1993; 34: 1814–28.
5 Chen WY, Mui MM, Kao WW, Liu CY, Tseng SCG. Conjunctival epithelial cells do not transdifferentiate in organotypic cultures: expression of K12 keratin is restricted to corneal epithelium. *Curr Eye Res* 1994; 13: 765–78.
6 Wei ZG, Sun TT, Lavker RM. Rabbit conjunctival and corneal cells belong to two separate lineages. *Invest Ophthalmol Vis Sci* 1996; 37: 523–33.
7 Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell* 1989; 57: 201–09.
8 Dua H, Forrester JV. The corneoscleral limbus in human corneal epithelial wound healing. *Am J Ophthalmol* 1990; 110: 646–56.
9 Wei ZG, Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells are preferentially located in fornical epithelium: implications on conjunctival epithelial homeostasis. *Invest Ophthalmol Vis Sci* 1995; 36: 236–46.
10 Shermer A, Galvin S, Sun TT. Differentiation related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 1986; 103: 49–62.
11 Shapiro MS, Friend J, Thoft RA. Corneal re-epithelialization from the conjunctiva. *Invest Ophthalmol Vis Sci* 1981; 21: 135–42.
12 Kenyon KR, Tseng SCG. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* 1989; 96: 709–23.
13 Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975; 6: 331–44.
14 Mathor MB, Ferrari G, Dellambra E, et al. Clonal analysis of stably transduced human epidermal stem cells in culture. *Proc Natl Acad Sci USA* 1996; 93: 10371–76.
15 Lindberg KL, Brown ME, Chaves HV, Kenyon KR, Rheinwald JG. In vitro propagation of human ocular surface epithelial cells for transplantation. *Invest Ophthalmol Vis Sci* 1993; 34: 2672–79.
16 De Luca M, Albanese E, Bondanza S, et al. A multicentre experience in the treatment of burns with autologous and allogeneic cultured epithelium, fresh or preserved in a frozen state. *Burns* 1989; 15: 303–09.
17 Barrandon Y, Green H. Three clonal types of keratinocytes with different capacities for multiplication. *Proc Natl Acad Sci USA* 1987; 84: 2302–06.
18 Thoft RA. Conjunctival transplantation. *Arch Ophthalmol* 1977; 95: 1425–27.
19 Tsai RJ, Tseng SCG. Human allograft limbal transplantation for corneal surface reconstruction. *Cornea* 1994; 13: 389–400.
20 Kruse FE, Cheng JJY, Tsai RJF, Tseng SCG. Conjunctival transdifferentiation is due to incomplete removal of limbal basal epithelium. *Invest Ophthalmol Vis Sci* 1989; 30: 520 (suppl).
21 De Luca M, Albanese E, Megna M, et al. Evidence that human oral epithelium reconstituted in vitro and transplanted onto patients with defects in the oral mucosa retains properties of the original donor site. *Transplantation* 1990; 50: 454–59.
22 Romagnoli G, De Luca M, Faranda F, et al. Treatment of posterior hypospadias by the autologous graft of cultured urethral epithelium. *N Engl J Med* 1990; 323: 527–30.