Phenotypic Study of a Case with Successful Transplantation of Ex Vivo Expanded Human Limbal Epithelium for Unilateral Total Limbal Stem Cell Deficiency

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Objective: To minimize the risk to the donor eye when a conjunctival limbal autograft is performed for unilateral total limbal stem cell deficiency (LSCD), a new approach has been reported of expanding limbal epithelial progenitor cells from a small limbal biopsy cultured on amniotic membrane (AM). Herein, we present for the first time the morphologic and phenotypic outcome of one such patient.

Design: Interventional case report.

Methods: A 31-year-old male with a severe acid burn to his left eye received AM transplantation at the acute stage and a keratolimbal allograft (KLAL) at the chronic stage for total LSCD. As an alternative to combat the failed KLAL, the above-mentioned new surgical procedure was performed. The corneal button, obtained after a penetrating keratoplasty performed 5.5 months later, and a normal corneal button as a control were submitted to hematoxylin–eosin and immunofluorescence staining for keratin K3, connexin 43, goblet-cell mucin MUC 5AC, laminin 5, and integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$.

Main Outcome Measures: Clinical and immunohistologic features.

Results: The resultant epithelium was stratified with five to six cell layers and anchored to laminin 5 of the amniotic basement membrane via integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$ in a manner similar to the normal corneal epithelium. Intriguingly, the epithelial phenotype was limbal and not corneal, based on the negative expression of keratin K3 and connexin 43 of the basal epithelium.

Conclusions: The technique described ensures the preservation of amniotic basement membrane, which allows formation of adhesion complexes and maintains normal corneal architecture. The preservation of a limbal epithelial phenotype on the reconstructed corneal surface indicates that AM provides a unique stromal environment conducive to the preservation and expansion of limbal epithelial progenitor cells. *Ophthalmology 2002;109: 1547–1552 © 2002 by the American Academy of Ophthalmology.*

Total limbal stem cell deficiency (LSCD), a common feature of a number of corneal diseases, remains one of the most challenging ocular surface problems.[1] Histopathologically, LSCD is characterized by conjunctivalization (i.e., conjunctival ingrowth), destruction of the basement membrane, vascularization, and chronic inflammation of the cornea.[2–7] Conventional corneal transplantation cannot cure LSCD, in part because of a high risk of allograft rejection, but also because the limbal stem cell population is not restored.[1,8]

When total LSCD is limited to one eye, conjunctival limbal autograft is performed to transplant limbal epithelial stem cells from the fellow eye. This was first proposed by Kenyon and Tseng[9]and has since been practiced successfully by many others to treat unilateral LSCD of various causes. One major concern is that two large free grafts, each spanning 5 to 7 mm in limbal arc length, would have to be removed from a healthy eye. Documented complications at the donor eye include localized haze in a patient with contact lens-induced keratopathy,[10] pseudopterygium,[11,12] filamentary keratitis,[13] microperforation during surgery,[14] "abnormal epithelium,"[15] and "corneal depression."[16] Furthermore, experimental animal studies have shown that in eyes with such limbal removal, LSCD can develop when the

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Dr. Tseng has obtained a patent for the preservation and clinical uses of human amniotic membrane.

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remaining corneal epithelium is subsequently challenged by debridement.[2,5]

One attractive option for circumventing the aforementioned concern, is expansion of limbal epithelial progenitor cells from a small biopsy on amniotic membrane (AM) in culture before transplanting them together with AM to treat total LSCD. The success of using such a new surgical approach has been reported in several human studies.[17–20] This report highlights the morphologic and immunohistochemical evidence showing how this new approach can restore a normal limbal epithelial phenotype on the corneal surface in a patient with unilateral total LSCD caused by a chemical burn. To the authors' knowledge, this is the first human case report correlating the clinical outcome with immunohistochemical features of ex vivo expanded limbal epithelium on amniotic membrane.

Case Report

A 31-year-old male first sought treatment at the Emergency Service of the Bascom Palmer Eye Institute with an acute acid burn in his left eye. External examination showed a total corneal and conjunctival epithelial defect sparing the temporal bulbar conjunctiva. There was moderate stromal edema and limbal ischemia. The anterior chamber was deep. His Snellen visual acuity was 20/400 in the left eye. He received intensive irrigation and debridement of necrotic tissue followed by prophylactic topical ofloxacin (Ocuflox; Allergan, Irvine, CA) four times daily and 1% prednisolone (Predforte; Allergan) hourly for the first 3 days. The prednisolone was subsequently replaced with 1% preservative-free methylprednisolone (Bascom Palmer Pharmacy) five times daily to control inflammation. Because there was no appreciable healing after 9 days (Fig 1A), amniotic membrane transplantation was used as a temporary patch in a manner recently reported,[21] resulting in a complete healing of the conjunctival and the corneal surface in 3.5 weeks. Nevertheless, continued photophobia and recurrent epithelial breakdown with superficial corneal neovascularization were noted 3 months later (Fig 1B arrows). The visual acuity remained 20/400 (Fig 1B,C), and the use of a bandage contact lens did not stabilize the epithelium. Total LSCD was diagnosed after impression cytologic analysis revealed conjunctival goblet cells, that is, conjunctivalization, on the corneal surface (Fig 1C, inset).[6] A 360° keratolimbal allograft (KLAL) was performed 7.5 months after the initial insult to reconstruct the corneal epithelial surface. The visual acuity improved to 20/200, limited in part by a corneal stromal scar and cataract formation. Despite systemic immunosuppression with oral cyclosporin A (200 mg twice daily) and topical 1% preservative-free methylprednisolone hourly, an irreversible episode of allograft rejection to KLAL was noted, resulting in the dissolution of one segment of the graft at the temporal portion 1 year after KLAL (Fig 1D, arrows). The visual acuity at that time was counting fingers.

Because all conventional surgical options had been exhausted and the patient did not wish to risk his healthy right eye by undergoing conventional conjunctival–limbal autograft, he gave a compassionate consent for ex vivo expansion of the limbal epithelium on AM using a small limbal biopsy (3×2 mm) from his healthy eye. This was performed 14 months after KLAL (Fig 1E, arrows). The excised limbal tissue was divided in two equal pieces and placed in a sterile tube containing culture medium consisting of an equal volume of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY) containing bicarbonate and Ham's F12 (Gibco, Grand Island, NY), supplemented with 0.5% dimethyl sulfoxide, 2 ng/ml mouse Epidermal growth factor (EGF), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 0.5 μg/ml hydrocortisone, 30 ng/ml cholera toxin A subunit, 5% fetal bovine serum, 50 μg/ml gentamicin, and 1.25 μg/ml amphotericin B. Under sterile conditions, the two pieces were placed in the center of cryopreserved AM (Fig 1E, inset) obtained from Bio-Tissue (Miami, FL), which was fastened to a culture insert (Millipore, Bedford, MA) in a manner recently reported.[22] After 3 weeks of culture, during which time the medium was changed every 2 days, a confluent epithelial layer (approximately 400 mm^2) was obtained (Fig 1E, inset). After superficial keratectomy to remove the rejected KLAL and the corneal pannus, the composite graft consisting of AM and expanded cells was placed over the ocular surface, secured perilimbally with a running suture (10-0 Vicryl) (Ethicon, Somerville, NJ), and fastened to the conjunctiva with a second continuous 10-0 Vicryl suture. The composite graft was secured by a second AM used as a patch to cover the entire ocular surface using a 10-0 nylon purse-string running suture. The composite graft integrated well and became progressively translucent. The ocular surface remained stable and noninflamed, and was completely epithelialized on follow-up (Fig 1F,G). Five and a half months later, a combined penetrating keratoplasty and cataract extraction with intraocular lens implantation was performed to restore visual acuity. Without systemic immunosuppressive therapy, the corneal graft remained clear with a smooth and healthy epithelium for a total follow-up of 21 months (Fig 1H). The visual acuity at that time was 20/50.

Experimental Investigation

The corneal button was cryosectioned and subjected to hematoxylin–eosin and immunofluorescence staining with monoclonal antibodies against keratin K3 (AE-5 1:100; ICN, Aurora, OH), goblet-cell mucin (MUC 5AC 1:100; Jacques Bara, MD, Paris, France), connexin 43 (Cx43 1:200; Chemicon, Temeluca, CA), laminin 5 (1:100; Accurate Chemical, Westbury, NY), and integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$ (1:100; Accurate Chemicals). Nuclear counterstaining with propidium iodide also was performed. For comparison, we used a normal corneal button as a control. The results showed that a stratified epithelium consisting of five to six cell layers was situated on an acellular layer of AM (Fig 2A) with an eosinophilic basement membrane at the interface (asterisks), which yielded a linear staining to laminin 5 (Fig 2A, inset). The expression of keratin K3, a cornea-specific keratin, was found to be negative in the basal layer but positive in all suprabasal cell layers (Fig 2B). This pattern was similar to that of the normal limbal epithelium (Fig 2C) but not to that of the normal corneal epithelium, which was positive throughout all epithelial layers (Fig 2D). The expression of Cx43, a gap-junction protein, was found in the suprabasal and superficial layers of the patient's reconstructed epithelium (Fig 2E). This pattern was similar to that of the normal limbal epithelium (Fig 2F, limbus) but not to that of the normal corneal epithelium (Fig 2F, cornea), which showed intercellular staining in the basal epithelial layer.[23–25] For integrins expressed by basal epithelial cells for the binding to laminin 5, we found that the expression of integrin $\alpha 3\beta 1$ was located predominantly on the surface of the basal and, to a lesser extent, in the more suprabasal layers (Fig 2G), a pattern identical to that of the normal corneal and limbal epithelium (not shown). We also noted that the expression of integrin $\alpha 6\beta 4$, a component of hemidesmosomes, was limited to the basal surface of the basal epithelial layer (Fig 2H), a pattern identical to that of the normal corneal and limbal epithelium (not shown). We did not detect any immunoreactivity to goblet cell mucin (data not shown).

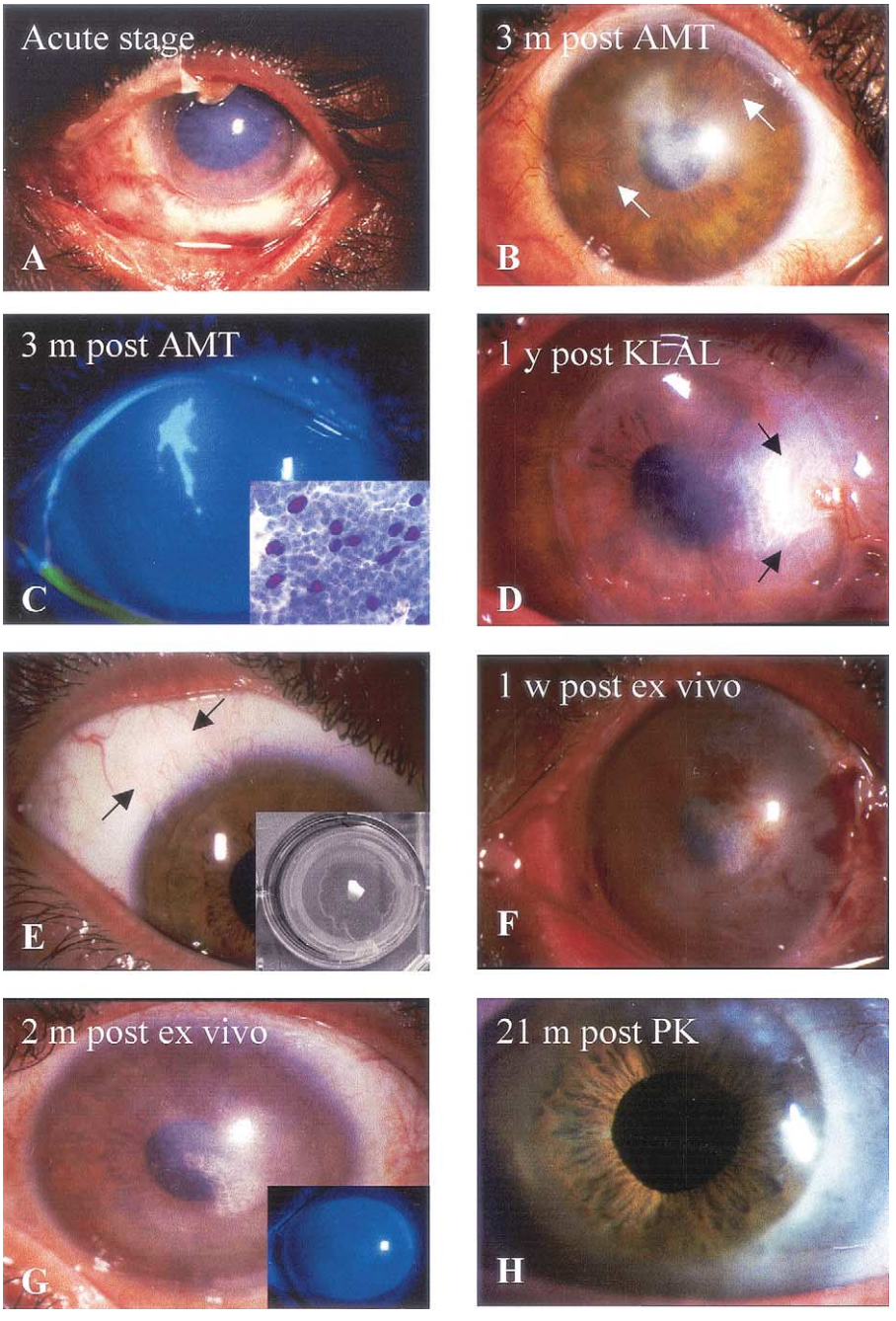

Figure 1. Clinical course. **A,** In the acute stage, the left eye had a total corneal and conjunctival epithelial defect as a patch except at the temporal bulbar conjunctiva, stromal edema, and limbal ischemia. **B,** Three months after amniotic membrane transplantation, a patch resulted in total healing with superficial corneal vascularization (arrows). **C,** Recurrent epithelial breakdown occurred as a result of total limbal stem cell deficiency, which was diagnosed by the presence of conjunctival goblet cells, that is, conjunctivalization of the cornea (**C** inset) using the impression cytologic method. **D,** A keratolimbal allograft (KLAL) was performed 7.5 months after the acute insult. Nevertheless, one segment of the KLAL showed irreversible rejection with dissolution (arrows) despite continuous oral cyclosporin. **E,** A small biopsy measuring 3 × 2 mm was removed from his healthy right eye (arrows) and placed on amniotic membrane (AM) fastened to a culture insert. After 3 weeks of culturing, a confluent layer of approximately 400 mm^2 was obtained (**E** inset). **F,** One week after transplantation of the composite AM graft with expanded limbal epithelium, the ocular surface was smooth and intact, with some blood trapped underneath. **G,** Two months later, the ocular surface remained smooth without any epithelial defect (**G** inset), and the corneal transparency had markedly improved. **H,** The corneal epithelium remained intact without conjunctivalization or epithelial breakdown 21 months after a penetrating keratoplasty.

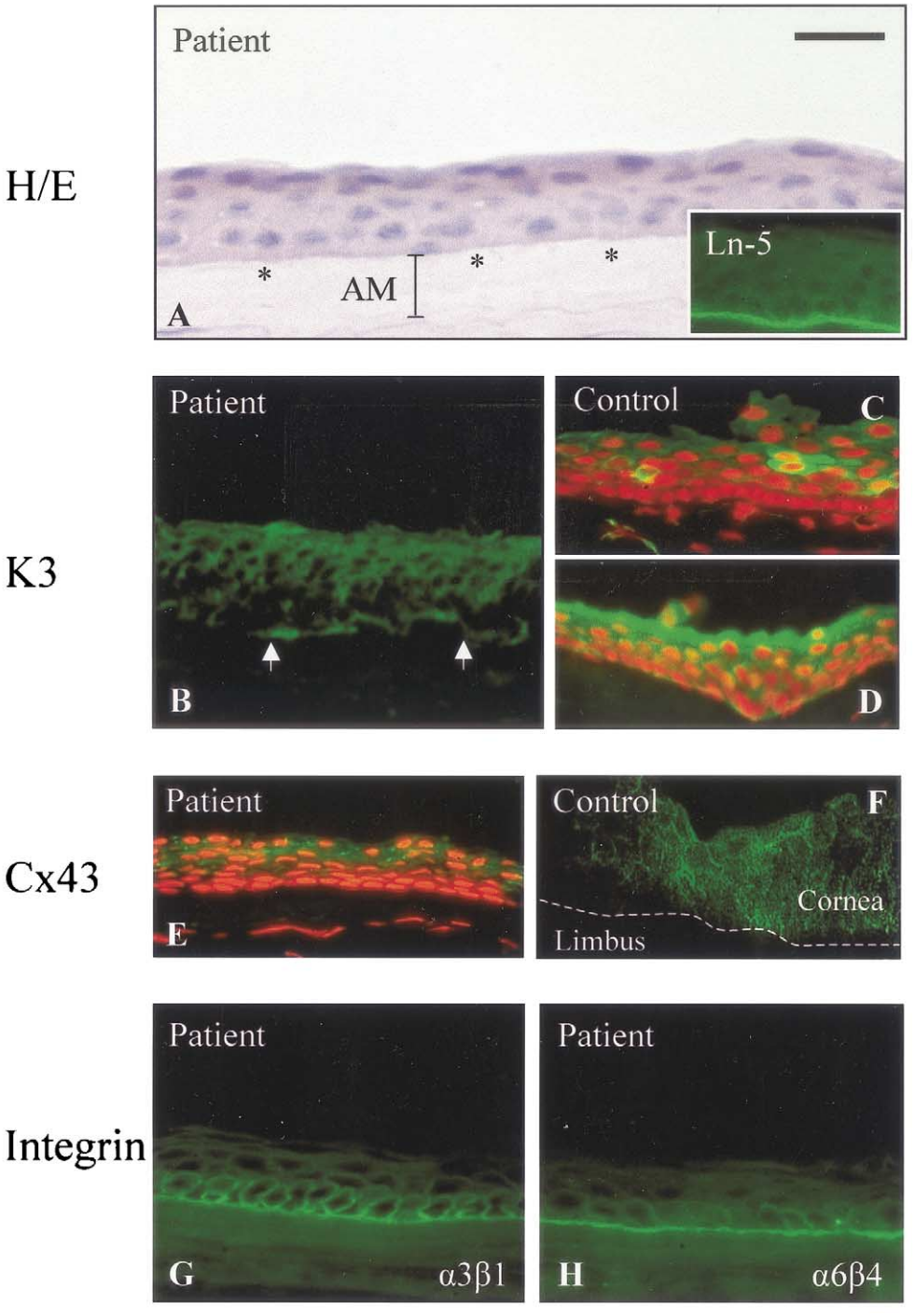

Figure 2. Histopathologic analysis. **A,** Hematoxylin–eosin staining revealed a stratified epithelium consisting of five to six cell layers resting on a thick amniotic basement membrane (asterisks) that was integrated into the stroma (AM). Intense linear immunoreactivity to laminin 5 was present at the basement membrane (**A** inset; bar = 50 μm; original magnification, ×200). **B,** Cornea-specific keratin K3 was found in the suprabasal and superficial layers of the epithelium but not in the basal layer (arrows indicate the basement membrane; original magnification, ×100). **C,** The normal limbal epithelium expressed K3 in the suprabasal and superficial layers but not in the basal layer (red indicates nuclear counter staining with propidium iodide; original magnification, ×100). **D,** In contrast, the corneal epithelium expresses K3 throughout all layers (red indicates nuclear counter staining with propidium iodide; original magnification, ×100). **E,** Cx43 was found in the suprabasal and superficial layers of the restored epithelium but not in the basal layer (red indicates nuclear counter staining with propidium iodide; original magnification, ×100). **F,** In the normal control specimen, the limbal epithelium (left) showed an expression pattern similar to that of **E**, whereas the corneal epithelium (right) showed positive expression of Cx43 in the basal layer (original magnification, ×1000). **G,** Expression of integrin $\alpha 3\beta 1$ was positive in the basal layer and, to a lesser extent, in suprabasal layers (original magnification, ×100). **H,** In contrast, expression of integrin $\alpha 6\beta 4$ was limited to the basal surface of the basal epithelial layer in direct contact with the underlying basement membrane (original magnification, ×100).

Discussion

Five and a half months after transplantation of autologous limbal epithelial cells cultured on AM, an intact, smooth, avascular, and quiescent ocular surface resulted. This successful outcome was in contrast to the inflamed and rejected KLAL despite systemic cyclosporin A administration. Histologically, the clinical success was correlated with the restoration of a nonkeratinized stratified epithelium without goblet cells and the preservation of an amniotic basement membrane (Fig 2A). The preservation of amniotic basement membrane was reflected by a thick eosinophilic layer and the presence of laminin 5 underneath the epithelium (Fig 2, inset). Laminin 5 has been shown to be a major component of the amniotic,[26] corneal, and limbal basement membrane and a major ligand for integrin $\alpha 3\beta 1$ and $\alpha 6\beta 4$, which is involved in epithelial cell adhesion and migration.[27–33] The reconstructed epithelium presented here expressed integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$ in the same pattern as the normal limbal and corneal epithelium (Fig 2G,H). Expression of these integrins reflects the formation of a normal epithelium–basement membrane complex and a normal basal epithelial phenotype.

We further confirmed that the phenotype of the resultant epithelium resembled that of the limbal epithelium and not that of corneal epithelium. This notion was supported by the finding that the basal epithelium did not express K3 keratin (Fig 2B). This pattern of negative K3 keratin expression was first reported by Schermer et al[34] to support the notion that the limbal epithelium contains corneal epithelial stem cells. The absence of Cx43 expression in the basal layers of our patient's specimen (Fig 2E) further supports the fact that reconstructed epithelium exhibited limbal characteristics. The stem cells (SC) containing limbal basal epithelium has been reported to be devoid of Cx43 expression and gap-junction mediated intercellular communication and this has been proposed to be one mechanism how limbal SC are maintained in their distinct niche.[24,25] In contrast, the resultant phenotype was corneal when a KLAL was performed together with AM (Espana et al, manuscript in press, 2001). Collectively, our data would imply that ex vivo expanded limbal epithelium still retained the phenotype of limbal epithelial progenitor cells despite transplantation to an ectopic site, i.e., the cornea, even after 5.5 months.

Further studies are needed to prove that this type of epithelium will have a lifespan longer than the normal corneal epithelium. If this were the case, it would prove that the method of ex vivo expansion of the limbal epithelium by AM is valid and plays a significant role in the field of ocular surface reconstruction. The obvious major advantage is that a much smaller amount of limbal tissue is actually removed, thus avoiding potential complications to a healthy donor eye. This new method may be applied to bilateral LSCD when a less injured eye is to be used as a donor eye. Furthermore, it also implies that AM is a unique substrate to provide a stromal environment conducive to the preservation and expansion of the epithelial stem cell population even at an ectopic site. Investigation into this question will unravel additional new applications in the future.

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