

RECONSTRUCTION OF DAMAGED CORNEAS BY TRANSPLANTATION OF AUTOLOGOUS LIMBAL EPITHELIAL CELLS

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ABSTRACT

Background The Stevens–Johnson syndrome, ocular pemphigoid, and thermal or chemical burns can cause scarring and opacification of the cornea and loss of vision. Transplantation of epithelial cells from the limbus of the contralateral cornea can restore useful vision. However, this procedure requires a large limbal graft from the healthy eye and is not possible in patients who have bilateral lesions.

Methods We took specimens of limbal epithelial cells from the healthy contralateral eyes of six patients with severe unilateral corneal disease. The epithelial cells were cultured and expanded on amniotic membrane. The amniotic membrane, together with the sheet of limbal epithelial cells, was transplanted to the denuded corneal surface of the damaged eye after superficial keratectomy to remove fibrovascular ingrowth. The mean (\pm SD) follow-up period was 15 ± 2 months.

Results Complete reepithelialization of the corneal surface occurred within two to four days of transplantation in all six eyes receiving transplants. By one month, the ocular surface was covered with corneal epithelium, and the clarity of the cornea was improved. In five of the six eyes receiving transplants (83 percent), the mean visual acuity improved from 20/112 to 20/45. In one patient with a chemical burn who had total opacification of the cornea, the acuity improved from the ability to count fingers at 40 cm to 20/200. No patient had recurrent neovascularization or inflammation in the transplanted area during the follow-up period.

Conclusions Transplantation of autologous limbal epithelial cells cultured on amniotic membrane is a simple and effective method of reconstructing the corneal surface and restoring useful vision in patients with unilateral deficiency of limbal epithelial cells. (N Engl J Med 2000;343:86-93.)

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THE normal ocular surface is covered by corneal, limbal, and conjunctival epithelial cells that, together with a stable preocular tear film, maintain its integrity. Severe damage to the limbal epithelial cells from chemical or thermal burns, the Stevens–Johnson syndrome, ocular cicatricial pemphigoid, contact lenses, severe microbial infection, or multiple surgical procedures or cryotherapy in the limbal region may lead to loss of the limbal epithelial cells.¹ Limbal-cell deficiency is usually manifested by vascularization and chronic inflam-

mation of the cornea, ingrowth of fibrous tissue, and corneal opacification.²⁻⁵

Patients with limbal epithelial-cell deficiency in one eye only, or in both eyes but affecting different areas of the limbus in the two eyes, may be treated by transplantation of autologous limbal tissue.⁶⁻⁹ A serious limitation of transplantation of autologous limbal epithelial cells is that one or two limbal-cell grafts, spanning two to three clock hours of the limbus, have to be removed from the healthy contralateral eye. Although no reports have described complications in donor eyes in humans, limbal-cell deficiency can occur in rabbits if the central corneal epithelium is subsequently removed from donor eyes.^{10,11} To avoid this potential risk, limbal epithelial cells from a small limbal-biopsy specimen may be expanded *in vitro*. Transplantation of corneal epithelial cells expanded *in vitro* on 3T3 fibroblasts resulted in reconstruction of the corneal surface in two patients with total loss of limbal epithelial cells.¹²

Transplantation of amniotic membrane to provide a substrate for regenerating epithelial cells has been found to be effective in reconstructing the corneal surface in rabbits.¹³ Transplantation of limbal-tissue allografts,¹⁴ together with amniotic membrane,¹⁵⁻¹⁸ in patients with partial or total limbal deficiency resulted in rapid reepithelialization of the reconstructed corneal surface, but prolonged immunosuppression was required.¹⁴ In this study, we took a small limbal-biopsy specimen from the normal contralateral eyes of six patients with unilateral limbal epithelial-cell deficiency and expanded the specimen on amniotic membrane to form an epithelial-cell sheet. We then transplanted the limbal epithelial-cell sheet, together with the amniotic-membrane substrate, to the damaged eye of the same patient.

METHODS

Subjects

This study was approved by the Ophthalmologic Society and the Department of Health, Executive Yuan, Taiwan, and all the patients gave oral informed consent. All surgical procedures were performed by the same surgeon at Chang Gung Memorial Hospital, Taoyuan, Taiwan.

The study subjects were two men and four women, with a mean (\pm SD) age of 24 ± 9 years. All had partial or total unilateral limbal epithelial-cell deficiency due to chemical burns (Patients 1,

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TABLE 1. CHARACTERISTICS OF THE PATIENTS RECEIVING LIMBAL EPITHELIAL-CELL TRANSPLANTS.*

PATIENT No.	DIAGNOSIS	EYE	TIME TO RE-EPITHELIALIZATION (DAYS)	VISUAL ACUITY IN DAMAGED EYE†		LENGTH OF FOLLOW-UP (MO)	PREVIOUS SURGERY
				PREOPERATIVE	POSTOPERATIVE		
1	Chemical burn with PLD	Right	2	20/60	20/40	18	Autologous conjunctival transplantation from the left to the right eye for correction of the cicatricial change of the lower bulbar conjunctival area in 1992; amniotic-membrane transplantation in 1993
2	Pseudopterygium with PLD	Left	2	20/20	20/20	15	Excision of dermoid cyst in 1998; excision of pseudopterygium and autologous conjunctival transplantation in 1991
3	Congenital pterygium with PLD	Left	2	20/30	20/20	14	Superficial keratectomy and amniotic-membrane transplantation in 1994
4	Chemical burn with PLD	Right	3	20/60	20/50	12	None
5	Phlyctenular disease with TLD	Right	3	20/200	20/50	15	Superficial keratectomy and amniotic-membrane transplantation in 1994
6	Chemical burn with TLD	Left	4	CF at 40 cm	20/200	15	None

*PLD denotes partial limbal deficiency, TLD total limbal deficiency, and CF ability to count fingers.

†An improvement from, for example, 20/25 to 20/20 would be equivalent mathematically to a change from 0.8 to 1.0, or 2 lines on the Snellen visual-acuity scale.

4, and 6), pseudopterygium after excision of a dermoid cyst (Patient 2), congenital pterygium (Patient 3), or recurrent chronic inflammation with phlyctenular degeneration (Patient 5) (Table 1). Before this study, four of the patients had undergone surgery on their damaged eye. Patient 2 had undergone autologous conjunctival transplantation, Patients 3 and 5 had undergone amniotic-membrane transplantation, and Patient 1 had undergone both procedures. All the previous transplantations had failed and had resulted in recurrent fibrovascular ingrowth around the limbal areas, indicating that the remaining limbal epithelial cells were unable to restore the corneal surface. Chemical burns had damaged 270 degrees of the limbus in Patient 4 and 360 degrees of the limbus in Patient 6, resulting in fibrovascular ingrowth around the injured limbal area and recurrent ulceration of the central cornea. Patients 5 and 6 had total deficiency of limbal epithelial cells, as judged by fibrovascular ingrowth on 360 degrees of the limbus.

Preparation of Human Amniotic Membrane

Amniotic-membrane tissue was obtained, processed, and preserved as reported by Lee and Tseng.¹⁹ The amniotic membranes, with the basement-membrane side up, were placed on a culture plate and incubated at 37°C in an atmosphere of 5 percent carbon dioxide and 95 percent air overnight before they were used.

Limbal Biopsy and Culture of Explanted Tissue

Limbal biopsy was performed on the normal contralateral eye (Fig. 1). The eyelid was sterilized with povidone-iodine, and a piece of limbal tissue 1 by 2 mm that contained epithelial cells and part of the corneal stroma was separated from the limbal margin and excised from the superficial corneal stroma by lamellar keratectomy. The tissue was placed in a 35-mm dish containing 1.5 ml of supplemental hormonal epithelial medium in 5 percent fetal-calf serum and immediately sent to the laboratory for culture. The medium consisted of Dulbecco's modified Eagle's medium and Ham's F12 (in a 1:1 ratio), supplemented with 0.5 percent dimethyl sulfoxide, 2 µg of mouse epidermal growth factor per milliliter, 1 µg of bovine insulin per milliliter, and 0.1 µg of cholera toxin per milliliter.

The limbal epithelial cells were cultured as previously described, with some modifications.^{20,21} The limbal epithelial-cell explants were inoculated onto the basement-membrane side of the amniotic membrane. The medium was changed every two days and the culture was maintained for two to three weeks, by which time the epithelial cells had grown and spread to form a cell layer covering an area 2 to 3 cm in diameter (Fig. 1).

Histologic Findings

The limbal epithelial-cell sheets on the amniotic membrane were examined by both light and electron microscopy. Samples were fixed and processed with use of standard histologic procedures. For light microscopy, 4-µm sections were cut and stained with hematoxylin and eosin or periodic acid-Schiff reagent and Alcian blue. For electron microscopy, ultrathin sections were examined under a transmission electron microscope (Jeol-1200 CX, Jeol, Tokyo, Japan) for the presence of characteristic structures of the basement membrane.

Transplantation of Limbal Epithelial Cells Cultured on Amniotic Membrane

After periotomy at the limbus, the perilimbal subconjunctival scar and inflamed tissues were removed to the bare sclera. Corneal fibrovascular tissue was removed by lamellar keratectomy in a manner similar to that described for allograft limbal transplantation.¹⁴ For eyes with limbal and corneal damage but with a normal central cornea, the limbal epithelial cells and the amniotic membrane were used as a sectorial limbal and corneal graft or a limbal equivalent, fashioned according to the size of the recipient eye, and transplanted to the corresponding recipient limbal area (from 90 to 360 degrees) (Fig. 2A). For eyes with damage to the entire limbal and corneal surface, the limbal epithelial cells and the amniotic membrane were transplanted as a sheet to cover the damaged area (Fig. 2B).

In all patients, the entire defect was covered with a sheet of cultured limbal epithelial cells together with the amniotic membrane substrate, with the epithelial side up. The graft was then secured to the corneal side by interrupted 10-0 nylon sutures and to the

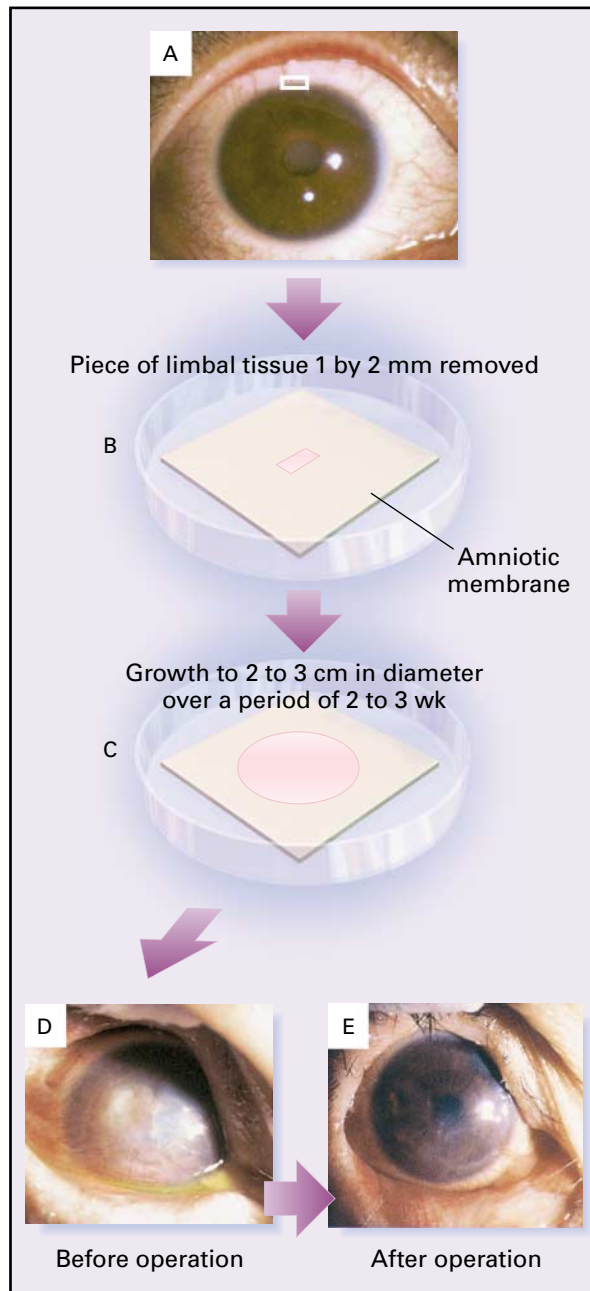


Figure 1. Transplantation of Autologous Limbal Epithelial Cells Cultured on Amniotic Membrane.

Limbal tissue (1 by 2 mm) was removed by lamellar keratectomy from the superior limbus of the healthy contralateral eye (Panel A). The explanted tissue was placed on amniotic membrane in a 35-mm dish containing 1.5 ml of culture medium (Panel B). After two to three weeks, the epithelial cells had grown and spread to form a circular sheet of cells with a diameter of 2 to 3 cm (Panel C). The cultured limbal epithelial cells with amniotic membrane were then transplanted to the diseased eye (Panels D and E).

surrounding conjunctival edge by interrupted 8-0 Vicryl sutures. During the procedure, the cultured limbal epithelium was protected by sodium hyaluronate (Healon, Pharmacia & Upjohn, Uppsala, Sweden). After surgery, the eye was covered overnight with a pressure patch. A therapeutic contact lens was placed on the eye for one week, beginning on the day after surgery. A 1 percent solution of prednisolone acetate was applied topically four times a day for the first week and twice a day for the next two weeks, followed by 0.1 percent fluorometholone twice a day for two to three months, depending on the severity of inflammation and conjunctival congestion around the surgical field.

RESULTS

In two to three weeks, the limbal epithelial cells grew to form a sheet 2 to 3 cm in diameter on the amniotic membrane (Fig. 3A). In flat-mount preparations, the epithelial-cell layer did not stain with periodic acid–Schiff reagent or Alcian blue, and the bare amniotic membrane was stained purple by both reagents (Fig. 3B). On histologic examination, the epithelial sheet was seen to be composed of four to five cell layers at the margin (Fig. 3C) and one to four cell layers in the area between the margin and the original explanted tissue. Ultrastructural examination revealed the presence of a basement-membrane structure, ranging from rudimentary to developed, at the junction of the basal cells and the amniotic membrane (Fig. 3D).

Clinical Results

After a mean follow-up period of 15 ± 2 months, vision had improved in five of the six eyes that received transplants (83 percent). The improvement was by more than 10 lines in one eye, 2 to 3 lines in two eyes, and 1 line in two eyes according to the Snellen visual-acuity scale. In the one eye that did not improve after receiving a transplant, the visual acuity was maintained at 20/20 (Table 1). Complete reepithelialization occurred within 2 to 4 days (mean, 3 ± 1) in all eyes that underwent surgery. Inflammation was reduced and vascularization regressed in the reconstructed corneal surfaces within one to two weeks. By one month after operation, corneal clarity was improved and the surface was smooth and wettable. There has been no recurrent neovascularization or inflammation in the transplanted areas during the follow-up period. A minimal scar formed at the donor site, but there was no neovascular growth into the cornea. Depending on the area of limbal and corneal damage, cultured limbal epithelial cells with an amniotic-membrane substrate can be used as a limbal equivalent or as a sheet covering the entire limbus and cornea.

Transplantation of a Limbal Equivalent

Cultured limbal epithelial cells with an amniotic-membrane substrate were transplanted to cover about 180 degrees of the limbus in Patients 1 and 3 and about 90, 270, and 360 degrees of the limbus in Patients 2, 4, and 5, respectively (Fig. 4).

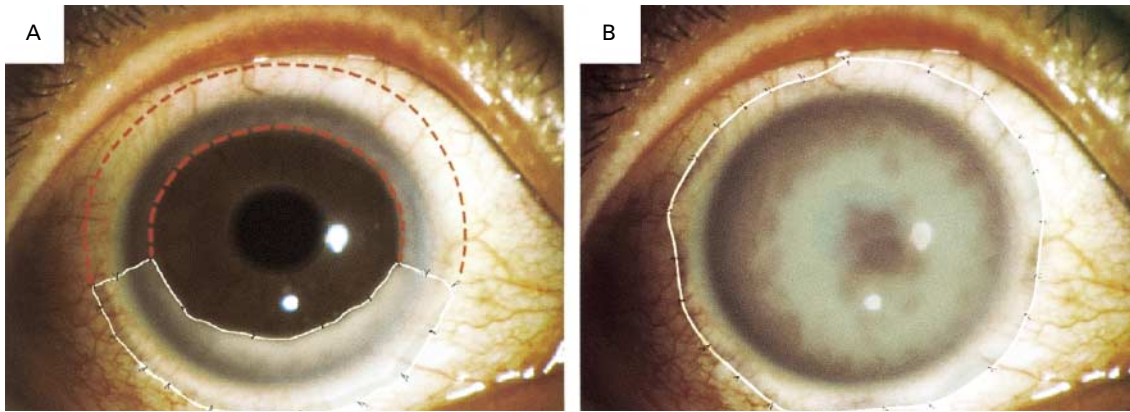


Figure 2. Types of Corneal Transplantations.

For eyes with limbal and corneal damage but with a normal central cornea (Panel A), the cultured limbal epithelial cells, together with the amniotic-membrane substrate, were transplanted to the corneal surface as a sectorial graft (white lines) or a limbal equivalent (red and white lines), fashioned according to the size of the diseased area. For eyes with damage to the entire limbal and corneal surface (Panel B), the limbal epithelial cells with the amniotic membrane were transplanted as a sheet to cover the damaged area.

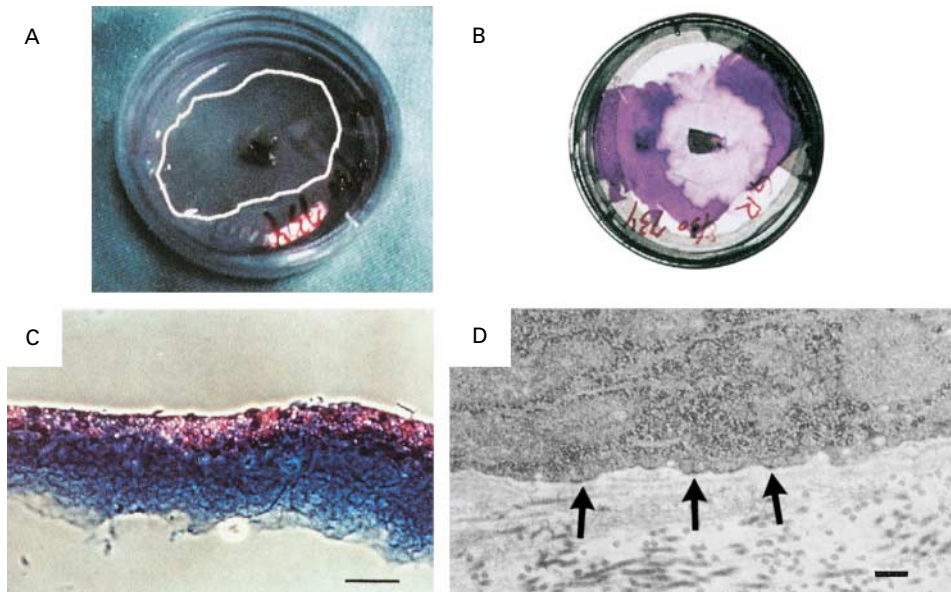


Figure 3. Morphologic Features of Limbal Epithelial Cells Cultured on Amniotic Membrane.

Explanted limbal tissue was placed on amniotic membrane (encircled by the white line in Panel A) in a 35-mm dish containing 1.5 ml of culture medium. A flat-mount preparation of the epithelial-cell sheet (Panel B) showed that the cell layer did not stain with periodic acid–Schiff reagent and Alcian blue, and that the bare amniotic membrane stained purple. Histologic examination showed that the epithelial sheet was composed of four to five layers of cells at the margin (Panel C). The scale bar represents 200 μ m. An electron micrograph shows the presence of a rudimentary-to-developed basement membrane with focal condensation of electron-dense ground substance at the junction between the basal cells and the amniotic membrane (Panel D, arrows). The scale bar represents 200 nm.

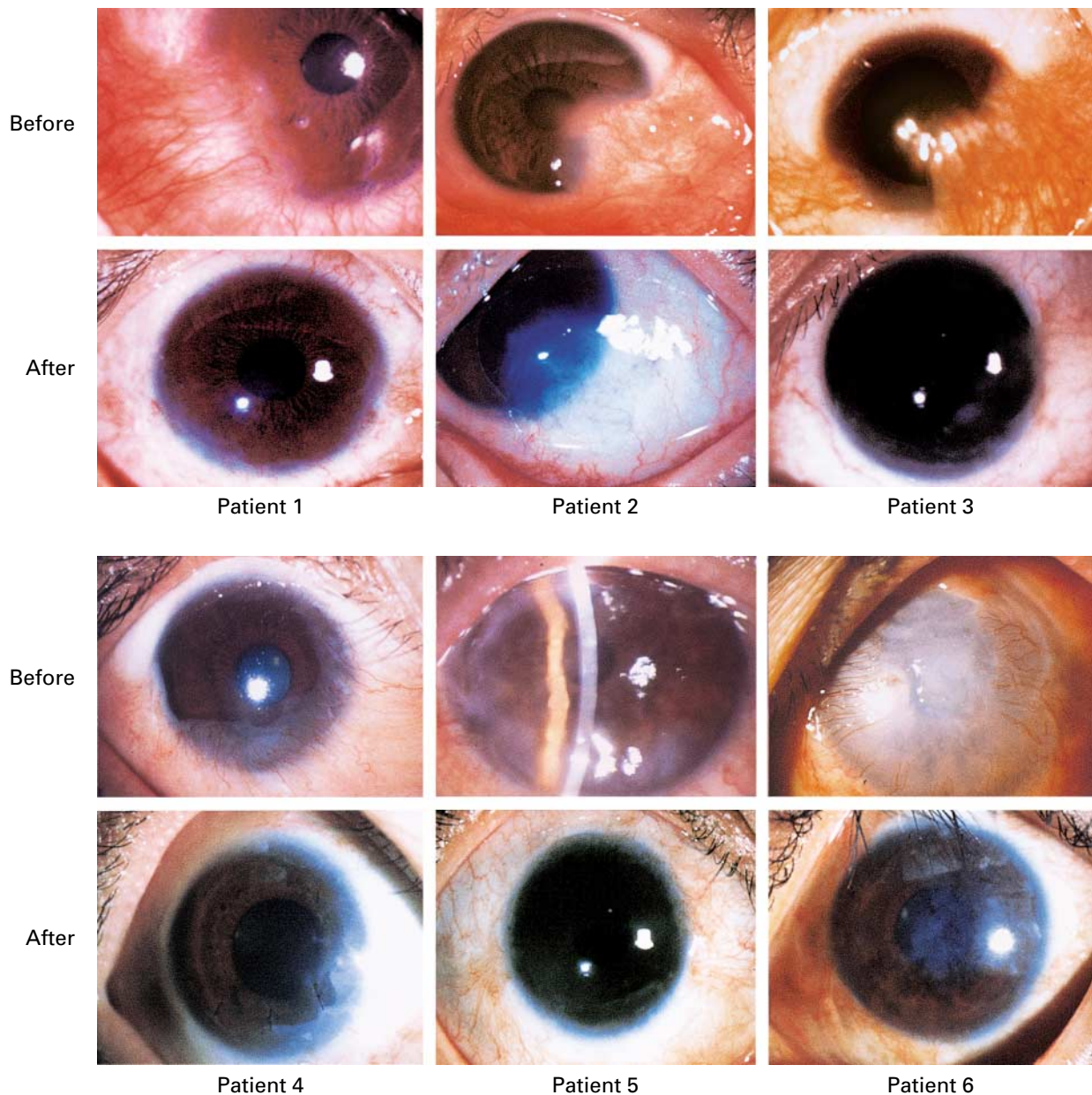


Figure 4. The Eyes before and after They Received Transplants.

The lower photographs were taken after a mean postoperative follow-up of 15 ± 2 months. Patients 1, 2, 3, 4, and 5 received sectorial limbal and corneal grafts. In Patients 1 and 3, the area of transplantation encompassed about 180 degrees of the limbus, and in Patients 2, 4, and 5, it encompassed about 90, 270, and 360 degrees of the limbus, respectively. Patient 6 received a transplant over the whole corneal surface.

Patient 5, a 23-year-old woman, had had recurrent inflammation in both eyes, due to phlyctenular disease, since the age of 10 years. Slit-lamp examination showed hyaline degeneration, and there was fibrovascular growth into the limbal area, which was worse in the right eye than in the left eye. She had undergone several operations in the right eye, including

superficial keratectomy and amniotic-membrane transplantation. Before this study, her visual acuity was 20/200 in the right eye and 20/20 in the left eye. At surgery, the fibrovascular membrane over the entire peripheral cornea of the right eye (about 2 mm from the limbus) and over the limbus was removed by superficial keratectomy, leaving the central cornea

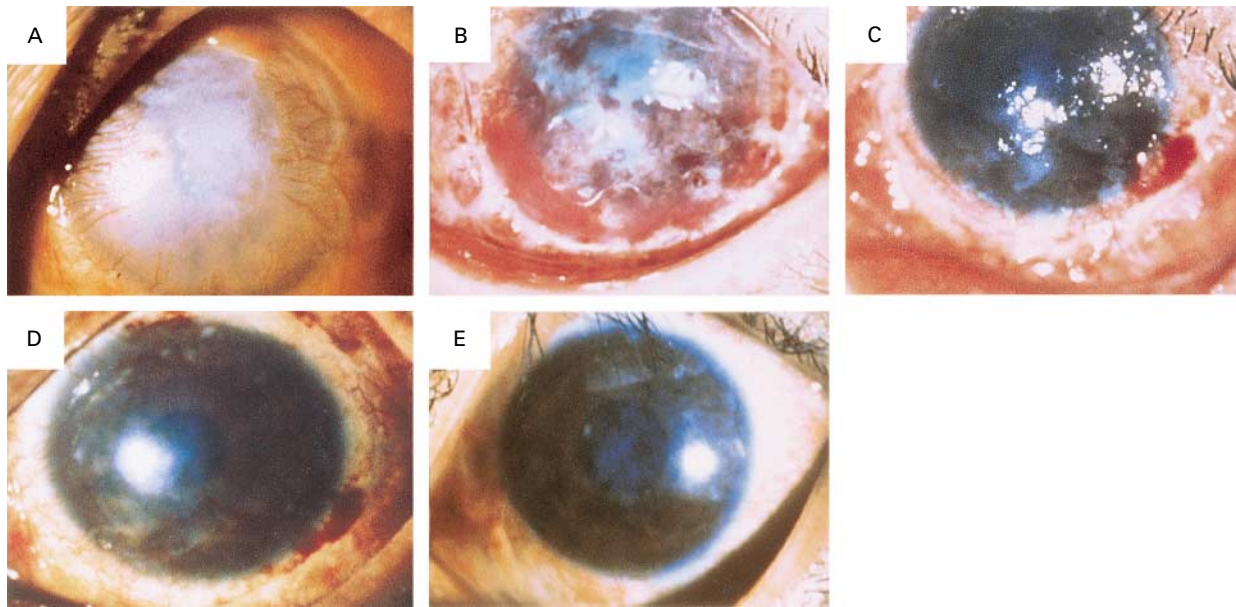


Figure 5. Serial Photographs of the Eye of Patient 6 before and after Transplantation.

Initial examination (Panel A) revealed corneal opacity with central corneal erosion and neovascular growth extending into the entire cornea for 4 to 6 mm. Lamellar keratectomy was performed to remove the entire opacified limbal and corneal area to a thickness of about one third of the corneal layer (Panel B). Limbal epithelial cells with the amniotic-membrane substrate were transplanted onto the denuded limbal and corneal area. Photographs were taken 1 day (Panel B), 7 days (Panel C), 30 days (Panel D), and 450 days (Panel E) after the operation.

untouched. The cultured limbal epithelium with amniotic-membrane substrate was transplanted onto the limbal and peripheral corneal area. Three days after transplantation, the limbal area was covered with transparent, normal-looking epithelium, which did not stain with fluorescein. During the ensuing 12 months, the patient's best corrected visual acuity improved to 20/50, the corneal and limbal epithelium remained stable, and there was no inflammation or neovascularization (Fig. 4).

Transplantation of a Sheet Covering the Whole Limbus and Cornea

Patient 6, a 29-year-old woman, had had a chemical burn on her left eye 18 months previously. After the injury, she received emergency treatment with normal saline irrigation and was referred to us one month later. Slit-lamp examination of her left eye revealed a corneal opacity with a central epithelial defect and neovascular growth extending 4 to 6 mm into the entire cornea (Fig. 5A). Her best corrected visual acuity was counting fingers at 40 cm with the left eye and 20/20 with the right eye. Biopsy of her right upper limbal area (1 by 2 mm) and culture of explanted limbal cells were performed as described above. Three weeks later, the epithelial-cell sheet was

about 2.5 by 3 cm in size. Lamellar keratectomy was performed to remove the entire opacified limbal and corneal area to a thickness of about one third of the corneal layer, and a sheet of limbal epithelial cells with amniotic membrane was transplanted onto the denuded area. The graft was extended to the conjunctival area about 3 mm from the limbus and sutured with 8-0 Vicryl. The corneal portion was secured with interrupted 10-0 nylon mattress sutures onto the peripheral cornea. Total reepithelialization was noted 4 days after surgery, and the cornea started to become clear about 30 days later (Fig. 5D). During a 15-month follow-up period, the patient's best corrected visual acuity improved to 20/200, and the cornea and limbus were covered by a stable epithelium with no signs of inflammation or neovascularization (Fig. 4 and 5).

DISCUSSION

In the past 10 years, therapeutic techniques for reconstruction of the ocular surface have been greatly advanced by the introduction of limbal epithelial-cell transplantation and amniotic-membrane transplantation. The therapeutic effectiveness of these two procedures lies in their ability, respectively, to replenish the limbal epithelial-cell population and to

restore the limbal stroma that supports the epithelial cells.^{1,4,15,22} In this study, we have demonstrated that the combination of both methods — that is, transplantation of limbal epithelial cells cultured on amniotic membrane — offers additional advantages.

In the four patients who had partial limbal deficiency (Patients 1, 2, 3, and 4), this new technique successfully restored the damaged limbus and the adjacent peripheral cornea, resulting in a noninflamed limbus with a stable, nonvascular cornea. Our results suggest that limbal epithelial cells, together with an amniotic-membrane substrate, are sufficient to restore a corneal surface in which previous amniotic-membrane transplantation has failed. Partial deficiency of limbal epithelial cells has been successfully treated by transplanting amniotic membrane, which promotes the growth of residual limbal stem cells.¹⁵ In our study, Patient 1 (with a chemical burn) and Patient 3 (with congenital pterygium) had partial limbal deficiency and had previously undergone amniotic-membrane transplantation, but both patients had persistent inflammation at the margins of the prior surgical field. The inflammation could have been due to incomplete removal of subconjunctival fibrovascular tissue (in Patient 3) or persistent stromal inflammation (in Patient 1). Stromal inflammation has been shown to impede the success of limbal grafting.⁴

In two patients with total limbal deficiency, transplantation of either a 360-degree limbal equivalent (in Patient 5) or the entire limbal and corneal sheet (in Patient 6) successfully restored the damaged corneal and limbal surfaces. In both patients, the reconstructed cornea started to become clear about 30 days after surgery. Monthly follow-up examinations for more than 12 months in both patients revealed no neovascularization or conjunctivalization, indicating that the grafted limbal epithelial cells and their amniotic-membrane substrates were functioning well.

Limbal deficiency of the donor eye due to removal of a relatively large piece of limbus for transplantation has been reported in rabbits.^{10,11} Our method substantially reduces the likelihood of these complications, because only a small piece of limbus is removed. Moreover, this method can also be used when both eyes have limbal deficiency but the location of the deficiency differs in the two eyes. *Ex vivo* expansion of autologous limbal epithelial cells on amniotic membrane provides sufficient limbal epithelial cells for transplantation in two to three weeks. For patients with total bilateral limbal deficiency, use of heterologous limbal grafts from cadavers^{14,18,23-25} or living donors^{23,26-28} has been reported. Our approach is still advantageous when living donors are selected, because the surgical technique is easier and less tissue is removed from the donor.

The use of autologous limbal epithelial cells grown on amniotic membrane for transplantation also has all the benefits of amniotic-membrane transplanta-

tion, including the facilitation of epithelialization,^{19,20} reduction of inflammation and scarring,^{29,30} and replacement of substrate when the underlying stromal tissue is destroyed. This approach differs from that reported by Pellegrini et al.,¹² in that the presence of amniotic membrane simplifies handling and suturing and eliminates the risk of infection associated with the use of mouse cells. Most important, amniotic membrane provides a natural substrate on which limbal epithelial stem cells can survive and proliferate,³¹ forming an autologous cell mass sufficient for corneal reconstruction. Moreover, because only autologous cells are transplanted, immunosuppression is not required.

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