Survival of Donor Epithelial Cells after Limbal Stem Cell Transplantation

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PURPOSE To determine the long-term fate of donor epithelial cells after limbal allograft transplantation.

METHODS. Corneal buttons and peripheral blood leukocytes were obtained at the time of penetrating keratoplasty from three patients who had undergone a successful limbal allograft transplantation. Microdissection was used to remove the corneal epithelial cells from the button. The presence of donor and recipient epithelial cells in each sample was determined by using PCR for DNA microsatellites. Phenotypic analysis of the epithelium was performed by immunohistochemistry.

RESULTS. Various patterns of DNA microsatellites were observed. Nonrecipient cells (presumed to be donor) were consistently detected in all three corneal buttons. In two of the three cases, recipient cells were also detected, whereas in the third case, exclusively donor epithelial cells were found at 3.5 years after limbal allograft transplantation. Mild T-lymphocytes and macrophages were observed in one of the corneal buttons.

CONCLUSIONS. This study provides evidence for the persistence of donor epithelial cells up to 3.5 years after limbal allograft transplantation and supports the use of systemic immunosuppressive therapy. (*Invest Ophthalmol Vis Sci.* 2005;46: 803–807) DOI:10.1167/iovs.04-0575

The outer surface of the cornea is covered by a layer of epithelium that is essential for its integrity and clarity. The corneal epithelial progenitor cells that maintain the epithelium reside in the basal aspect of the limbus. These limbal epithelial "stem cells" can be damaged or destroyed by various mechanisms, such as chemical or thermal injuries, Stevens-Johnson syndrome, and ocular cicatricial pemphigoid.[1] In these situations, the corneal epithelium begins to break down, and the surface of the cornea is invaded by the conjunctiva. Patients with limbal stem cell deficiency have recurrent nonhealing corneal epithelial defects, scarring, and neovascularization, all of which can lead to pain and loss of vision.

Limbal stem cell transplantation has been shown to reverse the signs of deficiency of these cells by restoring a corneal epithelial phenotype.[1] In patients with bilateral limbal deficiency, the donor limbal tissue must be taken from a living relative or a cadaver. In these cases, systemic immunosuppression is necessary to prevent graft rejection. In patients who have a successful clinical outcome, it is difficult to determine clinically whether the corneal epithelium is all donor-derived or if the host cells could have repopulated the surface. This question is important in part because it determines the length of immunosuppressive therapy while providing insight into the longevity of limbal allograft transplantation.

Many patients who have undergone a successful limbal stem cell transplantation may also later require a standard corneal transplantation (penetrating keratoplasty) for persistent stromal scarring or endothelial failure. The corneal button that is removed at the time of corneal transplantation consists of the epithelium of the central and peripheral portions of the cornea. In this study, the epithelia from these excised corneal buttons were analyzed to determine the presence or absence of donor-derived corneal epithelial cells in patients who had undergone limbal allograft transplantation. Microdissection and DNA microsatellites were used to distinguish the donor cells from those of the host.

METHODS

Patients

Case 1. A 67-year-old woman with a history of chemical injury underwent penetrating keratoplasty 4 months after receiving a cadaveric donor limbal allograft. Clinically, at the time of keratoplasty, she had a stable ocular surface and was receiving tacrolimus, mycophenolate, and low-dose prednisone.

Case 2. A 45-year-old man with a history of chemical injury underwent transplantation of a combined cadaveric and living-related limbal allograft, followed 3 months later by a penetrating keratoplasty. Two years later, he required a second penetrating keratoplasty for chronic endothelial decompensation (Fig. 1). Clinically, he maintained a stable corneal epithelium but continued to require systemic immunosuppression consisting of cyclosporine, azathioprine, and low-dose prednisone because of recurrent inflammation on previous attempts to taper the drug therapy.

Case 3. A 70-year-old man with a history of rheumatoid arthritis had severe ocular surface inflammation, causing limbal stem cell deficiency. He underwent limbal allograft transplantation followed by a penetrating keratoplasty 3 months later. He maintained a stable ocular surface and systemic antirejection medications were tapered and discontinued after the first year. He intermittently received 5 mg/d prednisone for rheumatoid arthritis. Three and a half years after his limbal allograft transplant, he required a second penetrating keratoplasty for chronic endothelial decompensation (Table 1).

Tissue Procurement and Processing

After institutional review board approval, three corneal buttons were obtained at the time of penetrating keratoplasty from the three patients. Another cornea from a patient with keratoconus (with no history of limbal transplantation) was used as the control. The research

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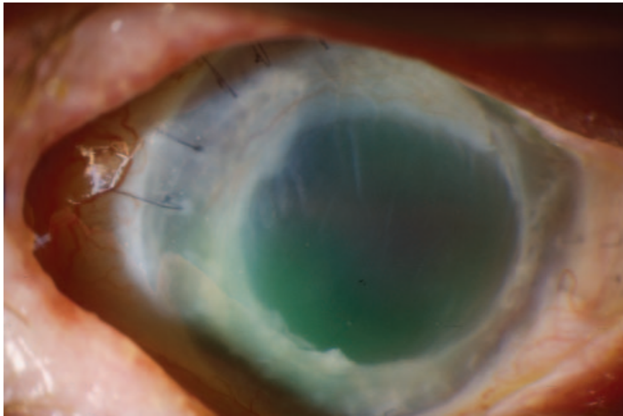

Figure 1. Clinical appearance of patient 2, two years after limbal stem cell transplantation and before undergoing penetrating keratoplasty for endothelial decompensation.

adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients involved.

The buttons were placed in formaldehyde or 80% ethanol until the time of processing. Each cornea was divided into four quadrants and sectioned separately. The specimens were embedded in paraffin and 5-μm sections were cut. Routine histology was performed to evaluate the disease in each quadrant.

The epithelial cells on the deparaffinized corneal sections were carefully microdissected, either manually or by laser capture microdissection (PixCell II; Arcturus, Mountain View, CA). Between 100 and 200 epithelial cells were harvested from the peripheral and central cornea from each section (Fig. 2). Approximately 500 epithelial cells were collected in each portion from two to three sections.

Immunohistochemistry

To determine whether the epithelium was of corneal phenotype, adjacent sections were also evaluated by immunohistochemistry. After deparaffinization, the sections were incubated at room temperature in 0.3% hydrogen peroxide for 10 minutes, 2% goat serum for 1 hour, primary monoclonal antibody AK2 (against cornea-specific K3) or AM3 (against conjunctival goblet cell mucin; both kindly provided by Scheffer Tseng, Ocular Surface Center, Miami, FL) for 1 hour (1:100 dilution) and secondary goat anti-mouse antibody (1:500) for 45 minutes (Vector Laboratories, Burlingame, CA). After incubation with the avidin-biotin-peroxidase complex (Vector Laboratories) for 45 minutes, the sections were developed in 3,3′-diaminobenzidine and counterstained with 1% methyl green.

The presence of inflammatory cells in the cornea was similarly evaluated with primary monoclonal antibodies (Dako, Carpinteria, CA) against CD3 (1:40), CD20 (1:40), and CD68 (1:100) incubated at room temperature for 1 hour. The remainder of the staining procedure was the same as described earlier. A normal human cornea was used as the negative control.

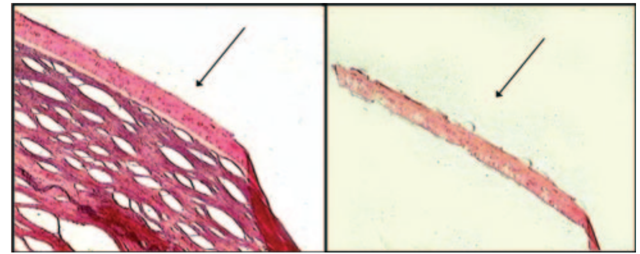

Figure 2. Microphotograph of the corneal epithelium (*arrow*) before (*left*) and after (*right*) removal by laser capture microdissection. Hematoxylin and eosin, ×100.

DNA Extraction

Procured cells were immediately resuspended in 20 μL buffer containing 0.5 mg/mL proteinase K, and were incubated at 37°C overnight. The mixture was boiled for 10 minutes to inactivate the proteinase K, and 2 μL of this solution was used for polymerase chain reaction (PCR) amplification of the DNA. The patient's own DNA (recipient DNA) was isolated from the blood with a DNA purification kit (Promega, Madison, WI).

DNA Microsatellite Markers

Microsatellite markers are short tandem repeats present in noncoding regions throughout the entire genome (e.g., TTATTATTATTA in which TTA is repeated four times). The number of repeats at each locus is highly polymorphic and can be used to distinguish one person from the other.[2] The region containing a microsatellite is first amplified by PCR and then separated based on the length of the fragments. Each person typically has two fragments—one from each allele—unless he or she is homozygous for that particular microsatellite. In this study, the following microsatellite markers were amplified by PCR: 4S1825, 5S820, and 14S306.

PCR Conditions

Each PCR sample contained 2 μL of template DNA as described earlier, 10 pM of each primer (Invitrogen-Research Genetics, Huntsville, AL), 20 nM dNTP, 15 mM $MgCl_2$, 0.1 U *Taq* DNA polymerase, 0.05 μL ^{32}P dCTP (6000 Ci/mmol), and 1 μL 10× buffer in a total volume of 10 μL PCR was performed for 35 cycles: denaturing at 94°C for 1 minute, annealing at 58°C for 1 minute, and extending at 72°C for 1 minute. The final extension was continued for 10 minutes. The PCR products were subjected to 6% polyacrylamide gel electrophoresis. The PCR was repeated three to five times for each patient, to exclude the possibility of contamination.

Results

Case 1

The corneal button showed scarring with mild inflammation in the periphery. The epithelium was of corneal phenotype (pos-

Table 1. Patient Clinical Summary

Patient	Age/Sex	Etiology of Limbal Deficiency	Previous Procedures	Indication for Current PK	Time after Limbal Tx
1	67 F	Chemical injury	KLAL	Stromal Scarring	4 mo
2	45 M	Chemical injury	KLAL/lr-CLAL, PK 3 mo later	Endothelial decompensation	2 y
3	70 M	Severe surface inflammation	KLAL, PK 3 mo later	Endothelial decompensation	3.5 y

PK, penetrating keratoplasty; KLAL, keratolimbal allograft; lr-CLAL, living-related conjunctival allograft; Tx, transplant.

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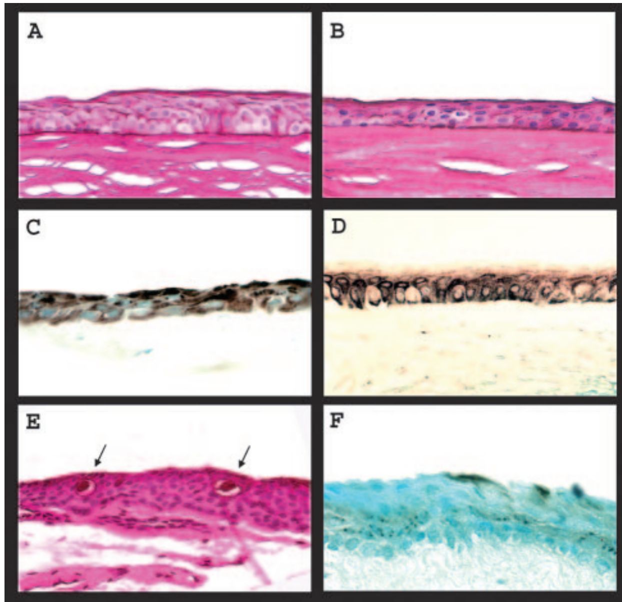

FIGURE 3. The epithelium from the corneal buttons in patients 1 (**A**, **C**) and 2 (**B**, **D**) demonstrating the absence of goblet cells by PAS staining (**A**, **B**) and the presence of corneal-specific keratin 12 by AK2 staining (**C**, **D**). A human conjunctival section is shown as the control, demonstrating goblet cells (*arrows*) by PAS (**E**) and nonspecific (negative) staining by AK2 (**F**).

itive AK2) with no evidence of goblet cells by PAS or AM3 staining (Fig. 3). No T-cells (CD3), B-cells (CD20), or macrophages (CD68) were detected in the cornea. The results of the PCR (5S820 marker) for the analyzed corneal sections are shown (Fig. 4). The analysis indicated the presence of nonrecipient cells (presumed donor cells) in both central and peripheral areas of the quadrants examined. Recipient cells were found mixed with the donor cells in one of the six sections examined. Overall, the surface was interpreted as being predominantly covered by donor cells. The patient subsequently continued systemic immunosuppressive therapy for an additional 20 months and at last follow-up, 24 months after keratoplasty, was found to have retained a stable ocular surface.

Case 2

Histologic examination indicated corneal scarring, neovascularization, and moderate inflammation in the stroma. The epithelium was of corneal phenotype (AK2$^+$) without goblet cells

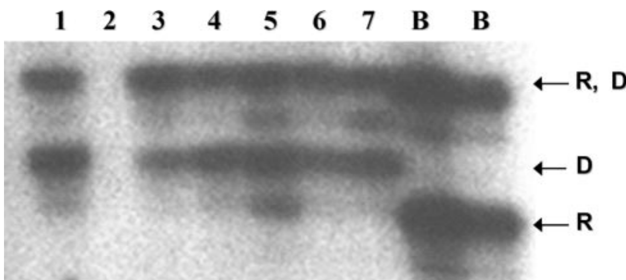

FIGURE 4. Gel electrophoresis after microsatellite amplification in patient 1. In *lanes 1* (central + peripheral epithelium), *3* (peripheral), *4* (central), *6* (peripheral), and *7* (central), only donor cells (D) were detected, whereas in *lane 5* (central+peripheral), both donor and recipient cells (R) were present. Negative control is in *lane 2* and the patient's own blood (B) is shown as the reference in the two *rightmost* lanes.

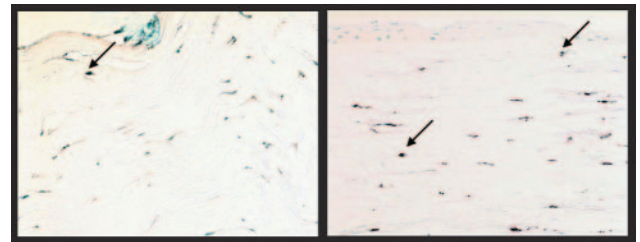

FIGURE 5. Immunostaining for CD3 (*left*) and CD68 (*right*) demonstrating a mild infiltrate of T cells (*arrow*) and macrophages (*arrows*) in the corneal stroma of patient 2.

(Fig. 3). A mild T-cell infiltrate and mild infiltrate of macrophages were detected in the cornea (Fig. 5). No B-cells were detected. The molecular analysis indicated the presence of both recipient and nonrecipient (presumed donor) cells in the corneal epithelium. In particular, two distinct nonrecipient genotypes were consistently detected that were attributed to the two different donors (Fig. 6). The patient has subsequently maintained a stable ocular surface and continues to require low-dose immunosuppression.

Case 3

Histologic examination indicated corneal scarring with minimal inflammation in the periphery. Instead of microdissection, the entire epithelial sheet was removed manually from three of the four quadrants, and the DNA was extracted and analyzed similarly to other microdissection samples to analyze larger samples of the epithelium and compare to the smaller microdissected samples. The results indicated the corneal epithelium to be entirely nonrecipient (presumed donor) cells with no evidence of recipient cells in any parts of the corneal button. At last follow-up, 18 months after keratoplasty, the patient has retained a stable ocular surface.

Control

Microsatellite analysis of the control patient revealed exclusively host cells with no evidence of nonhost polymorphisms (Fig. 7).

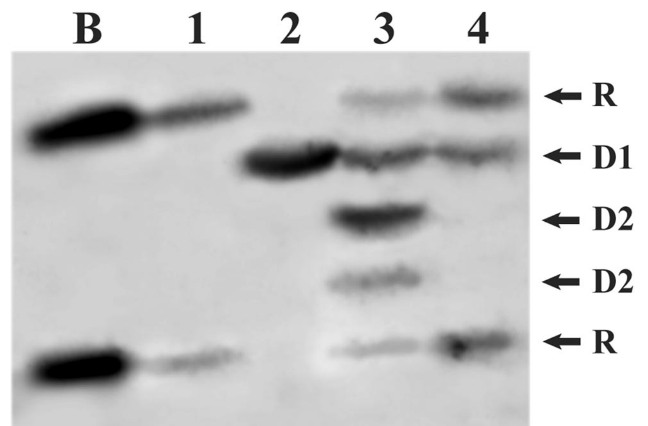

FIGURE 6. A composite of the different polymorphisms detected on the gel electrophoresis in patient 2 after microsatellite amplification. *Lane 1* (central epithelium): only recipient cells (R) were detected, which matches the pattern represented by the patient's own blood (B). *Lane 2* (peripheral): only one type of donor cells (D1) was found (donor was homozygous). *Lane 3* (central): both donor types (D1, D2) and recipient cells. *Lane 4* (peripheral): a mixture of recipient cells and cells of one of the donors (D1).

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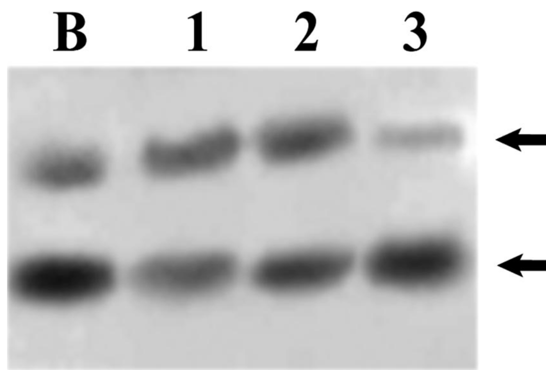

FIGURE 7. The gel electrophoresis from the control patient after microsatellite amplification. The same two polymorphisms (*arrows*) are detected in the blood (B) and in the central (*lanes 1*, *3*) and peripheral (*lane 2*) epithelium.

DISCUSSION

Limbal stem cell transplantation is currently a standard treatment for severe ocular surface disease and limbal stem cell deficiency. Clinically, it is effective in 50%–90% of the cases, depending on the underlying diagnosis and the severity of the disease.[1,3,4] Long-term success with this procedure is highly dependent on the prevention of immunologic rejection—when using allograft tissue—and the maintenance of a healthy ocular tear film. There are currently a number of reports on patients who have continued to maintain a stable ocular surface several years after limbal allograft transplantation.[4,5]

In this study, we analyzed the epithelium from the corneal button of patients who were undergoing a penetrating keratoplasty after a previous limbal allograft transplant. We found that the donor epithelial cells from the limbal graft had survived in all cases. In the first case, predominantly donor cells were found 4 months after keratolimbal allograft. In the second patient, 2 years after surgery, the corneal epithelium was a chimera of two different donor cells along with recipient cells. In the last case, only donor cells were detected on the ocular surface at 3.5 years after limbal allograft transplantation. These results further advance our understanding of limbal stem cell transplantation. In particular, they indicate that the transplanted limbal stem cells from an allodonor do indeed survive and are responsible for long-term maintenance of the corneal surface epithelium. This is in contrast to some previous studies in which investigators had postulated that transplanted limbal stem cells do not survive in the long run and the clinical efficacy of limbal transplantation does not necessarily correlate with the survival of donor cells on the ocular surface.[6]

In most studies that have failed to detect donor cells, either adequate immunosuppression was not used, or patients were included with recurrent limbal deficiency or patients with only partial limbal disease.[6–10] Williams et al. evaluated a patient with contact lens-induced partial limbal deficiency who had received partial limbal allografts.[7] Using DNA polymorphism on impression cytology samples, donor cells were detected only up to 12 weeks. Henderson et al.[8] reported a patient with aniridia who had only received topical steroids after a living related limbal allograft. At 2.5 years, only 1% of the sampled epithelial cells were found to be donor derived, whereas at 5 years, when the clinical picture had further deteriorated, donor cells were no longer detectable.[6] Henderson et al.[9,10] reported five patients 3 to 5 years after they had undergone limbal transplantation. Using DNA microsatellites on multiple impression cytology samples, they could not find any surviving donor cells. In four of the five cases, the patients had not received systemic immunosuppression and had recurrent limbal stem cell deficiency at the time of sampling.

In contrast, long-term donor survival has been reported in cases in which systemic immunosuppression was used. Shimazaki et al.[11] analyzed epithelial samples taken from the paracentral cornea of 10 eyes with a stable ocular surface and at least 300 days of follow-up after limbal transplantation.[11] The systemic immunosuppression consisted of steroids and cyclosporine. They found exclusively donor cells in four eyes, exclusively recipient in two eyes, and mixed in four eyes. Donor cells were detected up to 888 days after surgery. In a similar study, Reinhard et al., [5] found donor cells up to 56 months after penetrating limbokeratoplasty, using cyclosporin and mycophenolate for immunosuppression.

Several animal studies have examined the survival of donor cells after limbal transplantation. Swift et al.[12] used the absence of Barr body and the presence of a cell tracer (PHK-26) to identify male donor cell in recipient rabbits.[12] Using only topical steroids for immunosuppression, they primarily detected recipient (conjunctival) cells on the surface. In another study, using cultured corneal epithelial cells, male donor cells were positively identified in 27 of 32 rabbits by PCR.[13] In a rat model, despite the use of intramuscular cyclosporine and topical steroids, all the limbal allografts were rejected at an average of 6 to 7 days after transplantation and thus, male donor cells no longer detected.[14] In contrast, in the animals that had received syngeneic immune-compatible grafts, donor cells were detectable until the last day of examination (55 days).

This study offers several distinct advantages over previous ones. The use of laser capture microdissection allowed for a clean dissection without involving the stroma or other contaminants. Having a purified sample is particularly important, given the sensitivity of PCR, which can amplify minute contaminants. Henderson et al.[10] reported two contaminants in 14 impression cytology samples obtained as negative controls and four presumed contaminations in 23 samples taken from their patient.[10] This is particularly important in our present study which was inherently limited by not having the donor genotype available. In this study, nonrecipient polymorphisms were presumed to be of the donor type. While having the donor genotype would have strengthened the results, having access to the entire corneal button allowed us to repeat as many sections as necessary to validate our results. Thus, we could distinguish the donor cells from the occasional contaminants that were not reproducible in other sections. This is in contrast to all previous clinical studies that have been limited by a small sample of the epithelium.

A theoretical possibility in patients 2 and 3, who had undergone a prior penetrating keratoplasty, is that donor epithelial cells from the previous keratoplasty graft could have also been present in the cornea. Although studies have rarely shown the donor epithelial cells in a central keratoplasty graft to persist significantly beyond 1 year, it is still possible for a population of donor transient amplifying cells to survive in the graft for a long period.[15,16] This possibility cannot be completely excluded in patient 2; however, it is less likely in patient 3 in whom only a single donor type was found.

Another advantage of this study was the ability to examine the tissue histologically and determine the phenotype. In all three cases, there was no histologic and immunohistochemical evidence of conjunctival cells on the surface. As discussed earlier, conjunctival cells can be misinterpreted as recipient corneal epithelial cells, when genotypic analysis is used without phenotypic evaluation. The presence of host conjunctival cells in our samples cannot be completely excluded, because the phenotype was verified immunohistochemically on adjacent sections (5 μm apart) and not on the same sections that were used for the DNA analysis. Therefore, in patients 1 and 2,

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the recipient cells could have still represented the host conjunctiva. Another possible source of recipient cells, although minute, is the host Langerhans cells, which are known to be present in the corneal epithelium.[17]

Finally, a remarkable finding in this study is the long-term survival of donor cells in patient 3, nearly 2 years after discontinuing major immunosuppressive therapy. Although the patient was intermittently receiving low-dose prednisone for rheumatoid arthritis, the absence of any rejection reactions toward the limbal allograft suggests that a state of immunologic tolerance may have been reached. The degree of inflammatory cell infiltrate in patient 3 was likewise minimal. In contrast, in patient 2 who was clinically dependent on immunosuppression, a mild infiltrate consisting of T-cells and macrophages was demonstrated. The significance of these findings is difficult to determine, given the small and heterogeneous sample size; nonetheless, it suggests a possible correlation between the absence of inflammation and the persistence of donor cells. Examination of previously failed limbal grafts has revealed a significant infiltrate of T cells and macrophages (data not shown). In most of our patients, the immunosuppressive therapy can be tapered after the first 18 months; however, it is continued for longer periods in patients who demonstrate persistent signs of inflammation.[1,4] Our current regimen involves a combination of prednisone, tacrolimus, and mycophenolate.[4]

In summary, in this study we used microsatellite analysis to demonstrate the survival of donor epithelial stem cells up to 3.5 years after limbal transplantation. More important, we demonstrated that the surviving donor stem cells were the primary source of epithelium on the cornea. These findings along with other reports indicate that the long-term success of limbal allograft transplantation is dependent on the survival of the donor stem cells, which in turn requires adequate immunosuppression to prevent rejection.

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