Factors Affecting Outcome Following Transplantation of Ex vivo Expanded Limbal Epithelium on Amniotic Membrane for Total Limbal Deficiency in Rabbits

Seng-Ei Ti, 1,2 David Anderson, 1 Amel Touhami, 1 Charles Kim, 1 and Scheffer C. G. Tseng 1,3

PURPOSE. To determine factors affecting the outcome of corneal surface reconstruction in rabbits with total limbal stem cell deficiency (LSCD), by using autologous limbal epithelial stem cells (LSC) ex vivo, expanded on rabbit amniotic membrane (AM).

METHODS. Left eyes of 52 rabbits were rendered totally limbal stem cell deficient by n-heptanol debridement of the entire corneal epithelium followed by surgical removal of 360° of limbal rim. After cytologic verification of LSCD, the fibrovascular pannus of each cornea was removed. Group I (n = 10) received a rabbit AM transplant, whereas groups II, III, and IV (n = 42) underwent transplantation of LSCs cultured on rabbit AM (LSC-AM graft) derived from a small limbal biopsy specimen from the right eye. Clinical outcome was graded as a success if a smooth, avascular corneal surface was restored, a partial success if more than two quadrants of corneal surface were smooth, or a failure if the corneal surface was revascularized and irregular.

RESULTS. A long-term follow-up of more than 1 year was achieved. Compared with the 100% failure rate in group I, inclusion of expanded LSCs resulted in variable success rates in groups II, III, and IV (all P < 0.001). Kaplan-Meier survival analysis showed that different suturing techniques, subconjunctival injection of long-acting steroid, and tarsorrhaphy used in groups II (n = 17) and III (n = 13) did not significantly alter the outcome (P = 0.89). However, the use of a larger graft and human AM as a temporary patch with the explant retained for 1 week in group IV (n = 12) significantly improved the success rate to 83% (P = 0.002). Among eyes showing clinical failure, there was a significant correlation between the logarithm of the first day when an epithelial defect was noted and the time of graft failure (r² = 0.60, P < 0.001). Furthermore, the presence of severe lid deformity was borderline significant when correlated with failure cases in all four groups (P = 0.069).

CONCLUSIONS. Ex vivo expansion of LSCs can be achieved by using rabbit AM culture. Such expanded LSCs can successfully reconstruct corneal surfaces affected by total LSCD. This animal model is useful to investigate culturing variables affecting epithelial stenosis so that surgical reconstruction of corneas with total LSCD can be successfully performed. Furthermore, this model can be used to test the feasibility of gene therapies targeting LSCD in the future. (Invest Ophthalmol Vis Sci. 2002; 43:2584–2592)

Epithelial stem cells located at the limbus represent the ultimate source for corneal epithelial renewal. 1,2 When these limbal stem cells (LSCs) are dysfunctional or deficient, a disease state termed limbal stem cell deficiency (LSCD) develops. LSCD may arise from a variety of congenital or acquired causes and has been acknowledged as the underlying pathogenesis of severe ocular surface disorders, such as Stevens-Johnson syndrome, ocular cicatricial pemphigoid, chemical injuries, and extensive recurrent pterygium. The clinical hallmark of LSCD is conjunctivalization of the corneal surface, by conjunctiva-derived cells including goblet cells, 3 accompanied by corneal scarring secondary to destruction of the basement membrane, corneal neovascularization, chronic inflammation, and fibrous ingrowth. 4 As conventional corneal transplantation cannot satisfactorily reconstruct corneal surfaces with extensive LSCD, therapeutic strategies are directed at replacing LSCs derived from an autologous (autograft) or allogeneic source (allograft) for reviews see2,4.

Unilateral total LSCD may be effectively managed by limbal conjunctival autograft, a procedure popularized by Kenyon and Tseng. 5 This involves transplantation of two large free conjunctival limbal grafts, each spanning approximately 6 to 7 mm in limbal arc length, to be harvested from the healthy fellow eye. Despite its successful application for treating unilateral LSCD of various causes, 5–10 one major concern is that LSCD may develop in eyes with such limbal removal. This potential risk was demonstrated when the remaining corneal epithelium was subsequently removed in experimental rabbits. 11,12 Basti and Mathur 13 reported pseudo-PTG formation at the donor site in three human eyes, and Jenkins et al. 6 noted that rapid decompensation can ensue in donor eyes with subclinical LSCD.

The latest approach to LSC transplantation is based on the tenet that under suitable laboratory conditions, a small population of LSCs may be expanded for subsequent reconstruction of the ocular surface. Because LSCs are derived from a very small limbal biopsy specimen, this new approach does not compromise the donor eye. The technique was first reported by Pellegrini et al., 14 who used a culture system with 3T3 fibroblast feeder layers. Subsequently, the role of preserved human amniotic membrane (AM) as a native culture substrate and carrier of expanded LSCs was recognized, and clinical successes using cultivated LSC-AM grafts have been reported in patients with unilateral partial or total LSCD 15,16 and in those with bilateral total LSCD. 17,18 These favorable results in humans suggest that cultivated limbal transplantation is a viable

From the 1Department of Ophthalmology, Bascom Palmer Eye Institute, and the 2Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida; and the 3Singapore National Eye Centre, Singapore.

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Corresponding author: Scheffer C. G. Tseng, Ocular Surface Center and Ocular Surface Research and Education Foundation, 8780 92nd Street, Suite 205, Miami, FL 33176; tseng@ocularsurface.com.

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alternative to conjunctival limbal autograft or keratolimbal allograft. To further prove and evaluate its clinical efficacy in a controlled manner, it becomes necessary to establish an animal model that facilitates assessment of tissue cultivating and surgical variables. In this regard, Koizumi et al.25 have reported a short-term rabbit study showing successful epithelialization for up to 5 days after cultivated limbal transplantation, presumably limited by their use of human AM as the carrier substrate on rabbit eyes, which could incite a xenograft reaction.26,27

In this article, we report that a long-term study for up to 1 year can be achieved in rabbits by using rabbit AM. In this model, we also investigated various surgical factors that could affect clinical outcome after transplantation of ex vivo expanded LSCs. The clinical relevance of our findings is also discussed.

**Materials and Methods**

**Animals**

Sixty-five New Zealand White rabbits, each weighing between 2 and 2.5 kg, were subjected to a protocol approved by the Animal Research Committee of the University of Miami (protocols 98-267 and 00-127) in a manner consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research during the period from October 1999 through November 2001. They were housed in filter-covered cages under temperature-, humidity-, and light- (12-hour light cycle; lights on at 7 AM) controlled conditions and consumed standard chow and water ad libitum. Before surgery, all rabbits received intramuscular injections of 35 mg/kg ketamine, 5 mg/kg xylazine, and 0.75 mg/kg acepromazine, and all eyes were prepared with 0.5% povidone iodine.

After 15 rabbits were excluded because of inadvertent contamination of the cultured epithelial sheet (n = 1), inadvertent corneal perforation during transplantation (n = 6), more than two limbal biopsies performed (n = 4), and early postoperative bacterial infection (n = 2), the remaining 52 rabbits were subdivided into four surgical groups.

**Materials**

 Dulbecco’s modified Eagle’s medium (DMEM), F-12 nutrient mixture, Hanks’ balanced salt solution (HBBS), fetal bovine serum (FBS), HEPES-buffer, penicillin, streptomycin, neomycin, amphotericin B, gentamycin, and dispase II were purchased from Gibco-BRL (Grand Island, NY). All other reagents and chemicals including 1-n-heptanol, mouse-derived epidermal growth factor, cholera-toxin (subunit A), dimethyl sulfoxide, hydrocortisone, and insulin-transferrin-sodium selenite (ITS) medium supplement were obtained from Sigma Chemical Co. (St. Louis, MO). Plastic cell culture dishes (35- and 60-mm), tissue culture plastic plates (six-well), and 15- and 50-mL sterile centrifuge tubes were obtained from Becton Dickinson (Lincoln Park, NJ). Thirty-millimeter-diameter culture plate inserts (0.4 μm; Milllicell-PC) and polycarbonate filters were from Millipore (Bedford, MA). All sutures including 6-0 Vicryl, 9-0 Vicryl, 10-0 nylon, and 9-0 nylon were obtained from Ethicon (Piscataway, NJ).

**Creation of Total LSCD Model**

Total LSCD was created in the left eye of all rabbits by a method previously described.26,27 After anesthesia, the corneal epithelium was removed by topical application of 1-n-heptanol and by mechanical debridement, followed by 360° surgical removal of a lamellar limbal ring, defined as a 1.5-mm segment on either side of the anatomic junction between the cornea and the conjunctiva. After surgery, the rabbits received neomycin-polymyxin B-dexamethasone ointment (Maxitrol; Alcon Laboratory, Inc., Fort Worth, TX) twice daily for 1 week and were followed up at the first postoperative day and weekly thereafter until the clinical signs of total LSCD were evident: corneal haze, vascularization, and epithelial irregularity (for examples, see Figs. 1A, 1B). Impression cytology was performed as reported,7 to verify the establishment of total LSCD based on the presence of conjunctival goblet cells on the corneal surface.

**Pretransplantation Cicatricial Lid Abnormality**

After the creation of the LSCD model, all rabbits had varying extents of cicatricial lid deformity. This ranged from mild lid notching due to focal cicatriz to severe ectropion due to scarring and contracture of the entire tarsal plate. The cicatricial lid deformity was graded as severe if it led to lagophthalmos, poor lid–globe apposition, or exposure keratopathy (Figs. 1C, 1D).

**Preparation of Rabbit AM**

Rabbit AM was harvested from 28-day pregnant does under sterile conditions, by a method previously described.26 In brief, the placenta was rinsed of blood clots in a sterile phosphate-buffered saline solution containing 50 μg/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin, and 2.5 μg/mL amphotericin B. The amnion was separated from the chorion by blunt dissection and flattened, with the stromal side on a nitrocellulose paper (Bio-Rad, Gainesville, FL). The paper with adherent AM was stored in DMEM with HEPES buffer and the listed antibiotics at –80°C before use.

Typically, each pregnant rabbit had four to eight fetuses, and the AM obtained from each rabbit was enough for approximately three or four (30-mm diameter) culture inserts. Some membrane was lost, because rabbit AM is thinner and more delicate than human AM and consequently, tears more easily. Also, rabbit AM was discarded if there was evidence of meconium contamination. For this study, a total of 36 pregnant rabbits were used.

**Preparation of Rabbit AM Inserts, Limbal Biopsy Specimens, and Culturing**

After thawing and rinsing in HBSS, AM was fastened onto inserts, as reported.32 A limbal biopsy was performed in the right eye at the 12 or 6 o’clock position of each rabbit in groups II, III, and IV. The exact biopsy site was recorded. The limbal segment (~4 × 2 mm) was dissected to include 1.0 to 1.5 mm peripheral cornea and 1.0 to 1.5 mm beyond the anatomic limbus and placed in supplemental hormonal epithelial medium (SHEM), made of an equal volume of HEPES-buffered DMEM containing bicarbonate and Ham’s F12 and supplemented with 0.5% dimethyl sulfoxide, 2 mg/mL mouse epidermal growth factor, 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% FBS, 50 μg/mL gentamycin, and 1.25 μg/mL amphotericin B. The specimens were immediately sent to the laboratory, treated with 2 to 3 mL neutral protease (Dispase II; 1:2 U/mL in Mg2+– and Ca2+–free HBSS; Roche Molecular Biochemicals, Indianapolis, IN), and placed in the incubator at 37°C under humidified 5% CO2 for 5 to 10 minutes. The purpose of using neutral protease was to facilitate separation of limbal epithelial cells from the underlying stromal tissue, so as to promote epithelial outgrowth onto the AM. The digestive action of neutral protease was stopped by dilution with SHEM. A single, long limbal segment (~4 × 2 mm) was placed on the center of each AM culture insert in groups II and III, whereas the segments in group IV were cut into two small segments (~2 × 2 mm each) and placed midperipherally. A few drops of FBS were added, and the insert was placed in a six-well dish and incubated at 37°C under humidified 5% CO2. SHEM was added to keep the AM moist for 24 hours, when 2 to 3 mL SHEM was added to submerge the AM, and the medium was changed every 2 to 3 days.

**Monitoring Epithelial Outgrowth**

The extent of epithelial outgrowth was monitored and photographed with phase-contrast microscopy. Growth patterns were classified as one of three patterns: (1) compact and homogenous epithelial growth in a continuous sheet reaching the edge of the insert; (2) more sparsely distributed epithelial growth emerging like chains from the biopsy specimen, with large vacuolated areas; and a mixed pattern of (1) and (2).
The number of repeated biopsies needed to obtain tissue to generate sufficient outgrowth was also recorded. For this study, we excluded those rabbits that had undergone two limbal biopsies.

Transplantation of Expanded Limbal Epithelial Cells on AM

After the creation of total LSCD, transplantation was performed at approximately 2 to 2.5 months in groups I, II, and IV ($n = 39$), but was delayed until 2 to 5 months ($n = 9$) or 8 to 12 months ($n = 4$) in group III ($n = 13$; see Table 1 for more details).

The left eye of each rabbit in all groups underwent 360° conjunctival peritomy followed by removal of the fibrovascular pannus. Group I ($n = 10$) underwent rabbit AM transplantation alone without expanded LSCs. The thawed AM was removed from the nitrocellulose paper, applied as a graft with the basement membrane side up, and secured by interrupted episcleral 10-0 nylon monofilament sutures through the edge of the membrane to the conjunctiva.

Groups II, III,

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**Figure 1.** Creation of a total LSCD model. (A, B) Two representative examples of total LSCD are shown with irregular surface, diffuse vascularization, and pannus on the entire cornea. (C) An example of severe cicatricial lid deformity is noted in the upper lid (thin arrow) and ectropion in the lower lid (thick arrow). The lower lid appears in its natural position, and the exposure was not created by the examination. As a result, the initial smooth and avascular success (C) led to a final outcome of failure of this graft, with lid deformity (D).

**Figure 2.** Definition of clinical outcome. Failure was defined as the return of a vascularized and irregular corneal surface (A); partial success as the partial return of vascularized and irregular corneal surface that comprised less than two quadrants of the cornea (B); and success as the restoration of a smooth, avascular, and clear corneal surface (C).
<table>
<thead>
<tr>
<th>Groups</th>
<th>Time Interval from LSCD Induction to Transplantation (mo)</th>
<th>Grafts</th>
<th>Sutures</th>
<th>Subconjunctival Steroid Injection</th>
<th>Tarsorrhaphy</th>
<th>Human AM as a patch over LSC-AM Graft</th>
<th>Early Failures* (n)</th>
<th>Total Follow-up after Transplantation (mo)</th>
<th>Final Clinical Outcome Assessed at the Last Follow-up Date (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (n = 10)</td>
<td>1.8 ± 0.3</td>
<td>AM only</td>
<td>Continuous 10-0 nylon</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>4</td>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td>II (n = 17)</td>
<td>2.5 ± 0.8</td>
<td>Small LSCAM trimmed to fit cornea; explant removed</td>
<td>Continuous 10-0 nylon (n = 10), interrupted 9-0 nylon (n = 7), Interrupted 9-0 nylon</td>
<td>No</td>
<td>Yes, small and removed at 1 week</td>
<td>No</td>
<td>6</td>
<td>7</td>
<td>11.6 ± 2.8</td>
</tr>
<tr>
<td>III (n = 13)</td>
<td>5.9 ± 2.6</td>
<td>Small LSC-AM trimmed to fit cornea; explant removed</td>
<td>Interrupted 9-0 nylon</td>
<td>Yes</td>
<td>Yes, subtotal and removed at 1 week</td>
<td>No</td>
<td>9</td>
<td>10</td>
<td>7.3 ± 2.3</td>
</tr>
<tr>
<td>IV (n = 12)</td>
<td>2.2 ± 0.4</td>
<td>Large LSC-AM covering beyond limbus; one explant retained</td>
<td>Interrupted 9-0 nylon</td>
<td>Yes</td>
<td>No</td>
<td>Yes, removed at 7-14 days together with the explant</td>
<td>2</td>
<td>3</td>
<td>8.0 ± 2.5</td>
</tr>
</tbody>
</table>

* Defined as the occurrence of non-healing epithelial defect, increased inflammation and recurrent vascularization.
† Due to uncorrected severe lid deformity leading to exposure keratopathy.
IV (n = 42) underwent transplantation of an LSC-AM graft generated as described by removing it from the culture insert and attaching it in a manner similar to that used in group I. Sodium hyaluronate (Healon; Phar- macia, Kalamazoo, MI) was used to protect the expanded LSCs during transplantation.

Key Surgical Variables Tested in Groups II, III, and IV
In group II (n = 17), the LSC-AM graft was trimmed to the cornea’s size and secured with interrupted (n = 10) and continuous (n = 7) episcleral 10-0 nylon sutures placed 1 mm outside the limbus. The explant was removed from the LSC-AM graft. A small temporal tarsorrhaphy was performed with 60 Vicryl sutures and left in place for 1 week. In group III (n = 13), the LSC-AM graft was also trimmed to the cornea size, the explant was detached from the graft, and the graft was secured with interrupted 9-0 nylon sutures, just beyond the limbus. Subconjunctival injection of 40 mg triamcinolone (Kenalog, Apothecon, Princeton, NJ) was administered to superior and inferior fornices. The subtotal tarsorrhaphy was maintained for 1 week. In group IV (n = 12), the LSC-AM graft, with one explant removed while the other remained undetached, was secured with interrupted 9-0 nylon sutures placed circumferential to the sclera at 3 to 4 mm from the limbus. An additional cryopreserved human AM (Bio-Tissue, Miami, FL) was placed as a temporary patch, with the basement membrane side down to protect the cultivated limbal epithelial sheet, and was secured to the sclera with interrupted 9-0 nylon sutures. Subconjunctival injection of 40 mg triamcinolone was administered, but no tarsorrhaphy was performed.

All rabbits received topical neomycin-polymixin B sulfate-dexa- methasone ointment (Maxitrol; Alcon Laboratories) once daily for 7 days and topical prednisolone acetate 1% (Pred Forte; Allergan Inc., Irvine, CA) twice daily for 4 to 6 weeks.

Follow-up and Clinical Outcome Analysis
After transplantation, each eye underwent slit lamp examination, fluo- orescein staining, and photography at the first postoperative week, the first month, and monthly thereafter to document reepithelialization of the corneal surface. The tarsorrhaphy was removed at 1 week in groups II and III, and the human AM was removed 7 to 14 days after surgery in group IV.

The clinical outcome was evaluated by a masked investigator, by comparing preoperative and postoperative photographs. The graft was designated a failure if the cornea was covered by progressive subepithelial scarring, or progression of corneal vascularization, or if the graft was noted immediately after transplantation, or if the graft was not revascularized. The procedure was graded as a partial success if the cornea showed partial revascularization with a smooth and avascular surface in two or more quadrants (Fig. 2B). Graft survival time for partial successes was defined from the time of transplantation to the time when diagnosis of partial success was first noted. The graft was graded a success if the cornea regained its smoothness, avascularity, and clarity (Fig. 2C). Rabbits with procedures deemed a success were followed up for as long as 1 year.

Statistical Analysis
Statistical analysis was performed by the Department of Biostatistics at the Bascom Palmer Eye Institute (Miami, FL). Kaplan-Meier survival curves were constructed to analyze the differences in the time to transplant failure between surgical groups. Statistical significance was assessed with the log rank test. To evaluate whether significant cica-trical lid abnormality influenced the clinical outcome, a Cox survival regression analysis was conducted on data in the four groups. Pearson correlation analysis was used to investigate the strength of association between the postoperative day in which the epithelial defect was first noted and the time to transplant failure.

Results
Rabbit Model of Total LSCD
Animals began to show signs of total LSCD 4 to 6 weeks after surgical removal of the complete limbal lamellar tissue and debridement with 1-n-heptanol of the entire corneal epithelium.30,32 The corneal surface became progressively irregular with stromal opacity. Total LSCD became manifest as new blood vessels sprouted from the limbal area in all four quadrants and moved toward the central cornea. This neovascularization developed into a thick corneal pannus, as fibrous and granulation tissues also invaded (Figs. 1A, 1B). Such corneal surfaces stained unevenly with fluorescein, revealing a mixture of superficial punctate and whorled patterns. The fluorescein staining pattern denoted a poor epithelial barrier caused by conjunctivalization.33-34 A hallmark of LSCD. Based on the natural course reported previously and verified in this study, we routinely allowed at least 6 weeks to elapse, so that a full-blown picture of total LSCD would be manifest before transplantation. To ensure that total LSCD had been induced, impression cytology35 was performed to confirm the presence of conjunctival goblet cells on the corneal surface.3 Nearly all rabbits had some degree of cicatricial lid deformity, and 30% of animals in all four groups showed severe lid deformity, which led to lagophthal- mos and exposure keratopathy (Figs. 1C, 1D).

Ex Vivo Expansion of Autologous Limbal Epithelial Cells
Outgrowth from explants derived from the limbal biopsy speci-men was noted during days 4 to 7 after culturing. The outgrowth reached the limit of the 30-mm culture well inserts after a mean of 18.2 days (range, 9–34) in groups I, II, and III, in which one large limbal explant was used per AM insert. In group IV, such confluent growth was attained much quicker with a mean of 11.2 days (range, 10–14), because two small limbal explants were seeded on one AM insert. Under phase-contrast microscopy, resultant epithelial cells were mostly uni-formly small and compact, with a nucleus-to-cytoplasm ratio of approximately 1:1, a pattern resembling that reported in hu-man conjunctival31 and limbal36 cultures on human AM. App-roximately 30% of outgrowths showed some vacuoles be-tween cells, and some showed evidence of stratification to two or three layers. We did not notice any correlation between these outgrowth patterns and clinical outcome (to be described later). We noted that more than one limbal biopsy was necessary in some animals because of failure of outgrowth after the first biopsy. The frequency of second biopsies was 4 of 17 rabbits in group II, 10 of 13 rabbits in group III, and 3 of 12 rabbits in group IV. There was no correlation between the success and the number of biopsies performed. Table 1 summarizes the key surgical variables and clinical outcome achieved in all four groups.

Healing Response
In group I, which an AM transplant alone, no epithelialization was noted immediately afterward, and all eyes remained in-flamed. The ocular surface was rapidly invaded by the conjunc-tival epithelium. All corneas became completely vascularized—that is, the grafts were graded as failures (for example, Fig. 2A), with the median graft survival time of 20 ± 2 days (Fig. 3, group I).

In group II, the transplanted epithelium was intact in 70% of the eyes immediately after transplantation, based on fluorescein staining. In the remaining 30% of the eyes, epithelial defects were attributed in part to the removal of the limbal explant at the end of transplantation, which inadvertently
detached some cells. From days 1 to 4, most eyes were only mildly inflamed without graft detachment. Two eyes showed fresh hemorrhage below the LSC-AM graft (for example, Fig. 4A). From day 5 onward, epithelial defects (Fig. 4B) verified by fluorescein staining began to develop in 12 of 17 eyes, and these corneas invariably manifested progressive vascularization and an outcome of failure (Fig. 4C). Vessels were initially associated with the episcleral sutures but then spread radially toward the center of the graft. In 17 rabbits, there were 2 graft successes, 3 partial successes, and 12 failures (Table 1, Fig. 2). The median graft survival time was 48 ± 16 days (Fig. 3, group II). The eyes with grafts graded a success (n = 5) were followed up for 11.6 ± 2.8 months.

In group III, the clinical outcome was similar to that in group II. The occurrence of an early epithelial defect also signaled that graft failure would ensue (Figs. 4B, 4C, 5). The median graft survival time was 48 ± 11 days (Fig. 3, group III). In 13 rabbits, there were 2 graft successes (15.3%), 2 partial successes (15.3%), and 9 failures (69.4%; Table 1). The successful cases were followed up for 7.3 ± 2.3 months.

In group IV, the use of a human AM as a temporary patch promoted rapid suppression of ocular surface inflammation, as evidenced by the eyes’ needing fewer injections than those in groups I, II, and III during the same time interval (i.e., 1–3 weeks). In addition, the transplanted epithelium was more stable during the early postoperative phase, as shown by the absence of fluorescein staining in 10 of 12 eyes up to 2 weeks after surgery. The explant spontaneously detached, as human AM started to loosen between the first and second postoperative weeks. However, in two cases, the explant remained on the peripheral cornea and later formed a small granuloma (Fig. 4D). Grafts in 9 (75%) of 12 corneas were rated a success as the explants became clear, smooth, and nonstaining at the end of 4 weeks of follow-up. The two eyes that showed early failure with an epithelial defect and increased inflammation at 1 week, the results were attributed to exposure keratopathy secondary to severe upper and lower lid cicatrical ectropion (Figs. 1C, 1D) and premature detachment of human AM within the first postoperative week. Another graft failure in 1 month was also attributed to chronic exposure arising from a lower lid irregularity noted beginning in the second postoperative week. A tarsorrhaphy performed at 1 month did not alter the clinical outcome in any of these three eyes. The successful outcome in all eight remaining eyes continued during the follow-up of 8.0 ± 2.5 months.

**Figure 3.** Kaplan-Meier survival curves. The survival time from the time of transplantation to failure was plotted for grafts in all four groups. Graft survival time in group I differed significantly from that in groups II, III, and IV (all P < 0.001) and survival in group IV from that in groups II and III (all P = 0.002). Survival time in group II did not differ from that in group III (P = 0.89).

**Figure 4.** Examples of clinical outcome. Fresh blood developed below the LSC-AM graft immediately after transplantation in one eye (A). An early epithelial defect verified by fluorescein staining began to develop in 12 of 17 eyes, and these corneas invariably manifested progressive vascularization and an outcome of failure (Fig. 4C). Vessels were initially associated with the episcleral sutures but then spread radially toward the center of the graft. In 17 rabbits, there were 2 graft successes, 3 partial successes, and 12 failures (Table 1, Fig. 2). The median graft survival time was 48 ± 16 days (Fig. 3, group II). The eyes with grafts graded a success (n = 5) were followed up for 11.6 ± 2.8 months.

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Statistical Results

Grafts in all group I rabbits failed within 30 days (Fig. 3). At 180 days after transplantation, the cumulative percentage of graft survival in eyes retaining clear, smooth, and avascular corneas was 28% in group II, 26% in group III, and 87% in group IV, respectively (Fig. 3). The log rank test showed that the group I failure rate was significantly different from failure rates in groups II, III, and IV (all P < 0.001). Grafts in group IV had a significantly longer survival time than those in each of the other 3 groups (all P = 0.002). Survival in group II did not differ from that in group III (P = 0.89). The long-term study also showed that eyes with clinically successful grafts maintained a stable surface when the follow-up extended beyond 90 days. The results also indicate that different surgical techniques had a significant impact on the outcome and that the surgical technique used in group IV was overwhelmingly superior to that used in the other three groups. Among eyes showing clinical failure, there was a statistically significant correlation between the logarithm on the first day, when an epithelial defect was noted, and that on the day when the graft failed (r² = 0.60, P < 0.001, Fig. 5). Furthermore, the presence of severe lid deformity was borderline significant when correlated with graft failures in all four groups (P = 0.069).

Complications

Thirteen rabbits were excluded from the final analysis for the following reasons: They had received more than two limbal biopsies (n = 4), experienced inadvertent contamination of the cultured epithelial sheet (n = 1), and showed evidence of inadvertent corneal perforation during transplantation (n = 6) and early postoperative bacterial infection (n = 2). In the remaining 52 rabbits, surgical transplantation was uneventful. After transplantation, we noted the following complications: early postoperative hemorrhage below the graft (n = 10); intense inflammatory reaction leading to hypopyon (n = 3), which resolved with intensive topical steroids; and conjunctival inclusion cyst (n = 5). These complications occurred with approximate similar frequencies in all three groups.

Discussion

This is the first study illustrating that a long-term follow-up of more than 1 year can be achieved in rabbits, after transplantation of autologous, grafts of ex vivo LSCs expanded on AM. We attribute the success of this model to the use of rabbit rather than human AM as a culture substrate and as a carrier for transplantation. As a result, there was no inflammation caused by xenograft reactions. This rabbit model thus resembles that of human patients and permits meaningful long-term follow-up and analysis.

We noted that grafts in all 10 eyes (100%) in group I failed after transplantation of rabbit AM alone. Such a failure rate was higher than the 60% reported in an earlier study in which human AM was transplanted to treat total LSCD in a similar rabbit model. Because human AM may elicit xenograft reactions in rabbits, the earlier study should thus have been complicated by unwanted inflammation, which should have diminished the success rate. Therefore, the unexpected lower failure rate in the earlier study can be explained in part by the possibility that human AM differs from rabbit AM in having an as yet unknown action. An alternative explanation is that the surgical removal of the limbal epithelium may have been incomplete in the earlier study, leading to a state of partial LSCD. It has now been recognized that transplantation of human AM alone is effective in reconstructing corneal surfaces with partial LSCD in human patients. The state of total LSCD was confirmed by impression cytology in each eye before transplantation in the present study, the potential artifact of incomplete removal of the limbal epithelium was eliminated. We thus conclude that transplantation of AM without LSCs cannot be used to treat eyes with total LSCD, a notion asserted also in reports of human patients.

In contrast to the 100% failure rate noted in group I, a range of success rates were observed in groups II, III, and IV (Table 1, Fig. 3), indicating that inclusion of ex vivo expanded LSCs was indeed useful for treatment of total LSCD. However, the clinical outcome was affected by different surgical techniques. The low clinical success rates in groups II and III indicated that such surgical methods as suturing techniques, small or subtotal tarsorrhaphy, or subconjunctival injection of steroids did not play major roles in affecting clinical outcome. That subconjunctival steroid injections in group III failed to improve postoperative outcome was a surprising result, because groups II and III also differed in the time interval from induction of LSCD to transplantation, and chronic inflammation may have been prolonged in group III. Nevertheless, we did not note a significant difference in the extent of inflammation between groups II and III. In all animals, inflammation was most intense in the initial 2 to 3 weeks after induction of LSCD. Subsequently, it was observed that with conjunctivalization of the ocular surface, the inflammation became less marked. Cicatrization reached a plateau at approximately 8 weeks. Contour lid defects were noted from 4 weeks onward.

In groups II and III, sutures were placed near the limbus to secure a graft trimmed to fit the corneal surface, and this may have incited inflammation and vascularization. Of note is that subconjunctival injections of steroids did not significantly improve the outcome, suggesting that suture-induced inflammation was not suppressed by steroid injections. Compared with the procedures used in groups II and III, the surgical technique used in group IV significantly improved the clinical success rate. Such differences in success rates correlated well with the graft survival time shown by Kaplan-Meier analysis (Fig. 3). Because the graft survival time also correlated well with the first appearance of an epithelial defect on the LSC-AM graft (r² = 0.60, P < 0.001, Fig. 5), we thus speculate that clinical success depends on the use of surgical techniques that help maintain the integrity of the ex vivo expanded LSCs after transplantation.

In group IV, the key variables were the use of a larger graft to cover the sclera (3 to 4 mm from the limbus), the retention...
of one limbal explant on the LSC-AM graft at the time of transplantation, and the use of human AM as a temporary patch over the composite graft. The use of a larger graft not only delivered more LSCs, but also permitted the placement of sutures away from the limbus, which avoided inciting inflammation and vascularization. It has previously been reported that chronic inflammation is deleterious to the effectiveness of limbal conjunctival autograft in rabbits with total LSCD.49 Therefore, this maneuver may indirectly help the survival of LSCs in the graft. The retention of one limbal explant at the time of transplantation was meant to avoid the detachment of adjacent sheets of LSCs from the AM, a phenomenon noted in groups II and III. It can be argued that the retained limbal explant may aid additional in vivo expansion of LSCs during the early postoperative period. However, because the explant spontaneously detached or was removed within 1 to 2 weeks after transplantation, we believe that the delayed removal probably did not significantly contribute to long-term epithelialization and survival of the expanded LSCs. It should be noted that the explant is routinely removed at the time of transplantation in human patients.21 It should also be noted that a granuloma gradually developed in the explant that was left behind, with vascularization into the adjacent cornea in two eyes in group IV (Fig. 4D). Therefore, it is advised that the explant be removed at the time of surgery or in the early postoperative period.

The use of human AM as a temporary patch was the most important variable in group IV in helping maintain the transplanted LSCs that were expanded on rabbit AM. We chose to use human and not rabbit AM as a temporary patch, because of the ready availability of the former and the scarcity of the latter. Moreover, we theorized that human AM would function similarly but would not elicit unwanted xenograft reaction if it was used as a temporary patch for less than 2 weeks and not as a permanent graft. Because graft failure correlated well with early epithelial defect and early epithelial defect somewhat correlated with pretransplant lid deformity (borderline significant, \( P = 0.069; \) Fig. 5), we thus believe that the first therapeutic effect of using human AM as a temporary patch was to protect the graft from exposure. The deformity in the lid was caused in part by the speculum inserted for a prolonged period during the induction of LSCD and transplantation—no doubt worsened by the intrinsically weak tarsus in rabbits and by a cicatrinal reaction created during the establishment of the LSCD model. Of note also is that rabbit eyes differ from humans in that they have a slower blink rate, and the presence of the nictitating membrane exerts additional friction across the ocular surface, which probably explains the observed higher incidence of reformation of pannus in the nasal quadrant compared with formation of pannus elsewhere.

Regarding protective function, we were surprised to find that both small and subtotal tarsorrhaphies were not effective. We did not examine the protective role of a bandage contact lens, which was routinely applied by Tsai et al.21 in his human studies, because of the difficulty of maintaining it in rabbit eyes for a prolonged period. The successful application of a bandage contact lens rather than an AM patch in human patients for protection of the epithelial sheet21 also implies that exposure-related problems are less pronounced in human patients than in rabbits. We also believe that the role of the human AM patch extends beyond that of merely providing protection from exposure. This theory is supported by the findings that human AM used as a temporary patch is effective in suppressing inflammation and scarring and promoting epithelial healing in acute chemical burns in humans46 and rabbits,41 in excimer laser keratectomy in rabbits,42–45 and in herpes simplex virus-induced keratitis in rats.47 Because exposure due to severe lid deformity invariably leads to failure, we also conclude that prompt attention and surgical correction of this abnormality is a prerequisite for the success of corneal surface reconstruction.

Our laboratory data support that the use of intact AM (with the devitalized amniotic epithelium) in the culture system described herein preserved and expanded the LSCs. This notion was supported by the experiments using this cultivating technique to help expand human LSCs and the subsequent xenotransplantation of the resultant grafts in nude mice. We have demonstrated that such expanded LSCs carried slow-cycling and labeling-retaining characteristics and did not express K3 and K12 keratins46–47 and connexin 43,48 features resembling those found in the SC-containing limbal basal epithelium in vivo. This cultivating system is identical with that used by Tsai et al.21 in a human study. Although clinically successful transplantations in group IV are still being monitored to complete a 1-year follow-up before the animals are killed, our preliminary phenotypic studies, in which immunohistochemical response to various epithelial markers has been recorded, have shown that grafts with clinical failure exhibited a conjunctival epithelial phenotype and the dissolution of the rabbit AM. In contrast, eyes with clinically successful grafts in groups II and III showed the restoration of a normal corneal epithelial phenotype and preservation of rabbit AM (Ti et al., manuscript in preparation).

It is known that the LSCs expanded on intact AM used in our culturing system do not form adhesion complexes as strong as those expanded on epithelially denuded AM (treated with EDTA to remove the amniotic epithelium and expose the amniotic basement membrane).49 This finding explains why expanded LSCs in this study were susceptible to such trauma as exposure, lid and nictitating membrane blinking, and removal of limbal explants at the time of transplantation. To overcome this problem, Koizumi et al.25–29 used denuded AM to promote epithelial growth and adhesion46–48 and practiced air-lifting to promote epithelial stratification. In addition, they included 3T3 fibroblast feeder layers in their cultivating system. The establishment of the rabbit model reported herein will allow investigators to examine these cultivation variables in the future so that an ideal system can be developed to preferentially maintain and expand LSCs. The establishment of such an effective and reproducible cultivation system for ex vivo expansion of LSCs is the first step in development of gene therapies targeted at the LSCs.

References
