Glaucoma Filtration Surgery using Amniotic Membrane Transplantation

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PURPOSE. To investigate the potential use of amniotic membrane transplantation (AMT) in the construction of glaucoma filtering blebs.

METHODS. Twenty-four albino rabbits underwent glaucoma filtration surgery in one eye. In alternate cases, the conjunctival flap was replaced with AMT. Postoperative examination data were grouped into three time points. Six animals with AMT and six filtration surgery-controls were euthanatized at each of two postoperative time points, and tissue was obtained for histologic examination. Conjunctival biopsies were explanted for estimation of fibroblast outgrowth.

RESULTS. Bleb formation was observed in all eyes, and amniotic membranes were epithelialized after 11.2 ± 2.48 (mean ± SD) days. Throughout the study IOPs were significantly lower in operated than unoperated fellow eyes. Between postoperative days 11 and 16 (the middle time point), the percentage IOP reduction in AMT eyes was significantly greater than in filtration surgery controls (P = 0.014), though not at other time points. Filtration surgery survival was significantly longer in the AMT group (22.3 ± 3.8 days; mean ± SE) than in “No AMT” controls (14.0 ± 1.6 days; P = 0.035). In tissue culture, significantly less fibroblast outgrowth occurred from AMT explants when compared with unoperated conjunctiva (P = 0.01) between postoperative days 3 and 9 (the early time point). Amniotic membrane transplants were intact on histologic examination after 14 days but were associated with considerable granulomatous inflammation. After 56 days, the ocular surfaces remained clinically intact, but lysis of AMT was noted histologically.

CONCLUSIONS. AMT exhibits potential as an alternative tissue to conjunctiva in the construction of glaucoma filtration blebs. The healing response as demonstrated by fibroblast outgrowth is retarded when compared with conventional conjunctival closure. The improvement in bleb survival must be weighed against the potential for complications related to delayed healing. In rabbits, human amniotic membrane elicited a late xeno-graft reaction, leading to granulomatous inflammation and dissolution of the membrane. (Invest Ophthalmol Vis Sci. 2001; 42:1762–1768)

The formation of an adequate bleb after filtration surgery may be compromised by subconjunctival fibrosis, which obstructs aqueous outflow from the operation site. To combat this effect, antifibrotic drugs such as 5-fluorouracil1–9 and mitomycin c10–17 are commonly used at the time of filtration surgery. However, these drugs may also influence the integrity of the conjunctival barrier, resulting in a thin-walled avascular drainage bleb.18–21 The end result is often poor epithelialization and increased susceptibility to leakage and hypotony.22–24 or infection,25–28 sometimes months or years after surgery. Conversely, when adjunctive antifibrotics are not used, drainage surgery in certain patient groups, such as those who have had previous conjunctival surgery, is more likely to result in failure than success.7 Replacement of conjunctiva over the filtration surgery site with a tissue that heals less aggressively is an alternative approach to the reduction of subconjunctival healing in glaucoma filtration surgery. Amniotic membrane transplantation (AMT) has been used recently in the reconstruction of the ocular surface after pterygium excision,29 in nonhealing corneal epithelial defects,30–32 in cicatrizing conjunctival disease,33–34 and to retard corneal neovascularization in patients with limbal stem cell deficiency.35 Amniotic membrane exhibits a number of characteristics that might be of benefit in filtering bleb construction, that is, good epithelialization, good integration with surrounding tissue, a low-healing response, suppression of TGF-β activity,56 poor immunogenicity, and yet a high hydraulic conductivity.57 The purpose of this study was to investigate the potential usefulness of AMT in the construction of glaucoma filtering blebs in an adapted animal filtration surgery model.

METHODS

Approval for the study was obtained from the Animal Care and Use Committee of the University of Miami. Experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animals

Twenty-four female New Zealand White rabbits, weighing 2 to 4 kg, were used.

Anesthesia for Surgery

Animals were anesthetized with an intramuscular (dorsal surface of thigh muscle) injection of xylazine (7 mg/kg) and ketamine (14 mg/kg). The anesthesia level was monitored by blink reflex and toe withdrawal reflex.

Surgery

After adequate anesthesia, the left eye of each animal was prepared and draped in a sterile manner. No traction suture was used. A temporal...
approach was made in each case for ease of access. The nictitating membrane was not removed.

Twenty-four animals underwent filtration surgery using a trans-scleral cannula to maintain patency of the sclerostomy. Animals were allocated a study number from 1 to 24 according to chronological order of surgery. The filtration surgery procedure is described below.

The two study groups differed in the method of conjunctival closure. In odd-numbered animals a 5-mm fornix-based conjunctival flap was prepared. At the conclusion of the procedure, conjunctival closure was obtained by reposition of the conjunctival flap to the limbus using one 10/0 monofilament nylon suture at each corner and one mattress suture in the center of the limbal inter-suture along the conjunctival interface with interrupted sutures at each limbus-based conjunctival flap was prepared and excised before performing the filtration surgery. At the conclusion of the procedure, closure was achieved using AMT cut to fit the conjunctival defect. The method of AMT procurement, preparation, and storage is outlined below. AMT was secured using a 10/0 monofilament nylon continuous suture along the conjunctival interface with interrupted sutures at each limbal corner and a mattress suture in the center of the limbal interface.

**Filtration Surgery Model**

The adapted filtration surgery model used was as originally reported by Cordeiro, using a trans-scleral cannula to maintain a patent sclerostomy and prevent scleral healing. Briefly, the conjunctival flap was prepared as described above. A short tunnel was then fashioned from sclera into the anterior chamber just behind the corneo-scleral limbus using a 23-gauge hypodermic needle. A 22-gauge intravenous cannula (Abbocath, Sligo, Republic of Ireland) was then introduced via the same track, and the trocar was removed. The tube was placed in the anterior chamber with the internal opening close to the visual axis to avoid plugging of the tube with iris, while also avoiding corneal touch. The tube was then fixed to sclera using a single 9/0 nylon interrupted suture (Ethicon, Somerville, NJ). At this point the ocular surface was closed using either the AMT or fornix-based conjunctival flap as described above.

An anterior chamber paracentesis was performed with a 30-gauge hypodermic needle, and the anterior chamber was reformed with balanced salt solution to confirm the presence of a patent sclerostomy and inflate the bleb. At the conclusion of the procedure dexamethasone ophthalmic ointment was applied to the eye. Figures 1A through 1E demonstrate the stages of the surgical procedure.

**Amniotic Membrane**

Preserved human amniotic membrane was obtained, preserved, and stored as previously described. Briefly, human placenta was obtained shortly after elective Cesarean section when human immunodeficiency virus, hepatitis B and C virus, and syphilis had been excluded by serologic testing. Under a lamellar flow hood, the placenta was cleaned of clots with sterile Earle’s balanced salt solution (Life Technologies Inc., Gaithersburg, MD) containing 50 mg/ml penicillin, 50 mg/ml streptomycin, 100 mg/ml neomycin, and 2.5 mg/ml amphotericin B (Life Technologies Inc.). The amnion was separated from chorion by blunt dissection. The isolated amnion was then flat ted onto nitrocellulose paper (Bio-Rad, Gainesville, FL) with epithelium/basement membrane surface up. The nitrocellulose combined with amniotic membrane was then cut into 3 × 4 cm² rectangles and stored at −80°C in a sterile vial containing Dulbecco’s modified Eagle medium (Life Technologies Inc.) and glycerol (Baxter Health Care Corp., Stone Mountain, GA) at the ratio of 1:1 (vol/vol) before transplantation.

**Postoperative Examinations**

Postoperatively, each eye received dexamethasone ointment daily for 3 days. Postoperative examinations were carried out twice weekly under general anesthesia (xylazine/ketamine as above). The IOP was measured in each eye on each occasion using a Tonopen XL (Mentor, Norwell, MA) with readings repeated to obtain a <5% disparity between the highest and lowest readings. The frequency of aqueous leakage from conjunctiva/AMT after surgery, and the degree of AMT epithelialization was examined by staining the bleb surface with 2% fluorescein drops (Chauvin Pharmaceuticals Ltd., Romford, United Kingdom). The stained surface was observed for the presence of aqueous leakage and an epithelial defect during each examination.

**Euthanasia and Tissue Harvesting**

Animals 1 to 12 were euthanatized 14 days after surgery (early time point) and 13 to 24 at approximately 30 days (late time point). Euthanasia was performed by initially anesthetizing the animals using xylazine/ketamine as described above. This was followed by intravenous overdose of pentabarbital sodium (Euthasol; Delmarva Laboratories Inc., Midlothian, VA) while the animals were anesthetized.

The left eyes were removed immediately after death, preserving the AMT and bulbar conjunctiva. The enucleated eyes were placed in a 10-cm Petri dish under sterile conditions, and biopsies of conjunctiva/tenons and AMT taken using a 1.5-mm-diameter trephine as indicated in Figure 2. Four zones were identified for biopsy purposes: (1) unop-
Conjunctival Biopsy Sites

A. AMT Eyes

1. Unoperated conjunctiva (180° from filtration surgery site)
2. Peri-AMT conjunctiva
3. AMT
4. Conjunctiva from the bleb area in control operations

B. Control Eyes

1. Unoperated conjunctiva
2. Conjunctiva adjacent to amniotic membrane
3. AMT
4. Conjunctiva from the bleb area in control operations

FIGURE 2. Diagram illustrating biopsy sites. (A) AMT eyes and (B) control eyes. (1) unoperated conjunctiva (180° from filtration surgery site), (2) peri-AMT conjunctiva, (3) AMT, and (4) conjunctival bleb biopsy from control filtration surgery.

Conjunctival biopsy specimens were explanted immediately into tissue culture as described below. The remaining amniotic membranes/conjunctival flaps were then excised with underlying sclera and surrounding conjunctiva attached and placed in 10% formal saline for histologic examination.

Conjunctival/Amniotic Membrane Explants

Conjunctival and amniotic membrane biopsies were explanted individually in each well of 12-well tissue culture plates (Costar, Cambridge, MA) in tissue culture medium containing Dulbecco's modified Eagles's medium/F-12/M-199/Hepes buffer (Gibco, Grand Island, NY) with 20% fetal calf serum (Gibco) as previously described. Each explant was incubated initially in one drop of tissue culture medium at 37°C in a humidified atmosphere containing 5% CO₂. After approximately 1 hour, 1 ml of tissue culture medium was gently added to each well so as not to disturb the explants.

Explants were examined daily for fibroblast and epithelial cell outgrowth and photographed using a microscope-mounted 35-mm camera. After 14 days the monolayers were fixed in 100% methanol and stained with Gram Crystal Violet (Becton, Difco Laboratories, Detroit, MI). After fixation, wells were individually photographed, and a series of 5 x 7-inch black and white prints were produced, each representing one well. The area of fibroblast outgrowth from each explant was assessed from its respective photograph by planimetry using a Kurta 1212 Summsketch Tablet Board and Sigmascan software (SPSS Inc., Chicago, IL). The magnification factor was calculated from the ratio of the diameter of a well on photographic print to the actual well diameter and the actual area of outgrowth expressed in cm².

Histology

Samples taken for histology were mounted in paraffin, sectioned, mounted, stained with either periodic acid-Schiff (PAS) or Masson-Trichrome, and examined by light microscopy.

PAS-stained sections were examined by a masked observer (consultant ophthalmic pathologist) for the presence and state of amniotic membrane (whether intact or fragmented), presence of histologically complete epithelialization, and degree of underlying inflammation.

Outcome Measures

Primary outcome measures included bleb formation, rate of epithelialization, frequency of aqueous leakage, degree and duration of IOP lowering, and the frequency of postoperative complications. The rate of epithelialization of AMT, as judged by lack of fluorescein staining by unepithelialized membrane, was calculated as the time (days) from surgery to complete epithelialization.

IOP lowering in the operated eye was calculated as the percentage lowering in comparison with the unoperated eye. The time to filtration surgery failure was defined as the time to normalization of the IOP when compared with the unoperated eye. Postoperative complications such as bleb infection, hypotony, and intraocular hemorrhage were also recorded.

Secondary outcome measures included quantification of fibroblast outgrowth from AMT biopsies versus conjunctival biopsies in tissue culture, the qualitative histologic state of the amniotic membrane postoperatively, and the pattern of subconjunctival/submembrane inflammatory response.

Statistical Measurements

Statistical calculations were performed using SPSS for Windows release 8.0 (SPSS Inc.). Means were compared using an unpaired two-tailed Student’s t test. Filtration surgery survival analysis was performed using Kaplan–Meier survival analysis and the log rank test.

RESULTS

Primary Outcome Measures

Bleb Formation, Epithelialization of Amniotic Membranes, and Aqueous Leakage from Blebs. In both groups, bleb formation was observed clinically in all eyes at the conclusion of surgery (Figs. 1F through 1H). No epithelial defects were noted on the bleb surface of filtration surgery control eyes on the first postoperative day. In the AMT group, all blebs were noted to have epithelial defects over the entire AMT at the first postoperative examination. Clinical epithelialization of amniotic membranes had occurred (as judged by absence of staining of membrane with 2% fluorescein) by 11.2 ± 2.5 days (mean ± SD), excluding one case that was not fully epithelialized at harvesting on day 14. In this and one other AMT case, persistent aqueous leakage was noted at the limbal edge of the membrane. This was rectified in later cases by a change in suturing technique (i.e., the addition of extra horizontal mattress sutures at the limbus to prevent leakage).

Epithelialization was observed to occur in all AMT cases by creeping ingrowth from both limbal and conjunctival interfaces (Fig. 3), except in the case that had failed to epithelialize by day 14. In that particular case, epithelialization occurred from the conjunctival interface but not from the leaking limbal edge. Similarly slow vascularization of membranes occurred over the study period by ingrowth from all sides (Fig. 3).

Lowering of IOP Relative to Unoperated Eye and Filtration Surgery Survival. The IOP-lowering effect of surgery was quantified by measuring the percentage reduction in IOP in the operated eye when compared with the unoperated eye. This method was used to minimize the influence of anesthesia on the IOP reduction. IOPs were measured during nine postoperative examinations throughout the study, and results averaged for each animal to give three broad time points; days 3 to 9 (early), days 11 to 16 (middle), and days 17 to 38 (late).
Values for percentage reduction and actual reduction in terms of mm Hg are presented for comparison (Table 1).

Throughout the study IOPs were significantly lower in all operated eyes than in unoperated eyes. In the early postoperative period (days 1–10), there was no significant difference in IOP-lowering between the two study groups (20.7% ± 5.5% for AMT, 17.7% ± 5.8% for filtration surgery controls). From days 11 to 16 the percentage IOP reduction in AMT eyes was significantly greater than in filtration surgery controls (21.7% ± 6.0% for AMT, 2.0% ± 4.3% for controls; \( P = 0.014 \)). Thereafter (days 17–38) there was no significant difference in IOP reduction between groups (8.4% ± 3.5% for AMT, 10.1% ± 4.4% for controls), by which time 50% of the animals had already been euthanatized, and therefore remaining numbers were smaller (Table 1).

The survival time of each filtration procedure was defined as the time (days) from operation to normalization of the IOP (i.e., IOP in operated eye under anesthesia equal to that in unoperated eye) on repeated measurement at one time point.

The filtration surgery survival time for the AMT group was 22.3 ± 3.8 days (mean ± SEM), compared with 14.0 ± 1.6 days in controls (\( P = 0.035 \), log-rank test; Fig. 4).

Complications. Ocular hypotony was defined as an IOP < 5 mm Hg. In one eye hypotony was documented (one of the two leaking eyes) with an IOP of 3 mm Hg, but on only one examination. No episodes of severe intraocular hemorrhage or postoperative intraocular infection were documented.

Secondary Outcome Measures

Fibroblast Outgrowth from AMT Biopsies versus Conjunctival Biopsies in Tissue Culture. In tissue culture, no fibroblast outgrowth had occurred from AMT explants harvested at the early time point after 14 days in tissue culture (Table 2). This was significantly less than in conjunctiva taken 180° from the site of surgery (\( P = 0.003 \)). At the late time point, some fibroblast outgrowth was documented from AMT explants. This was not significantly different from unoperated conjunctiva 180° from the site of surgery. Fibroblast outgrowth at the late time point was significantly higher from the peri-AMT biopsies than from unoperated conjunctiva (\( P = 0.032 \)).

Histologic State of Amniotic Membrane. AMTs remained intact on histologic examination after 14 days but were associated with marked granulomatous inflammation, with giant cell formation. After 36 days, the ocular surfaces remained clinically intact, but beneath the epithelium, lysis of AMTs was noted histologically (Fig. 5).

### Table 1. Percentage versus Absolute Reduction in IOP

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Absolute Reduction in IOP Relative to Unoperated Eye (mmHg)</th>
<th>Reduction in IOP Relative to Unoperated Eye (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>AMT</td>
</tr>
<tr>
<td>Days 3–9</td>
<td>2.32 ± 0.74</td>
<td>2.47 ± 1.10</td>
</tr>
<tr>
<td>Days 11–16</td>
<td>0.25 ± 0.62</td>
<td>2.20 ± 0.50</td>
</tr>
<tr>
<td>Days 17–38</td>
<td>2.01 ± 0.62</td>
<td>1.29 ± 0.71</td>
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Values are mean ± SE. NS, not significant.
DISCUSSION

The success of glaucoma filtration surgery is often hampered by excessive fibrosis, which obliterates the subconjunctival space in the vicinity of the filtration site reducing the area available for aqueous drainage and absorption. 40–42 Antifibrotic drugs inhibit this activity, thereby improving surgical success, but there is widespread concern that these drugs result in an increase incidence of postoperative complications.28

A different approach to the reduction of postoperative scarring after glaucoma filtration surgery would be to replace conjunctiva and tenon’s capsule in the vicinity of the filtration site with a tissue that has a lower propensity to scar. Suitable candidates should be biocompatible and non-immunogenic, as well as semipermeable to water. These requirements are similar to the biological characteristics of amniotic membrane. Placental membranes were first reported to assist healing of exposed body surfaces in 1912.43 When amniotic membrane without chorion is used, healing occurs with less scarring than with primary healing alone. A number of studies have reported similar findings when amniotic membrane is used in ocular surface reconstruction.29,30,33,35,44 These effects are mediated via an influence on conjunctival epithelial and subconjunctival fibroblast function, promoting epithelial maturation, 45,46 and down-regulating fibrogenic TGF-β signaling and myofibroblast differentiation. 36,47 Finally, amniotic membrane is also relatively permeable to water. 37

In this study, a trans-scleral cannula was used to prevent scleral healing. This extra step prevents scleral closure, which would otherwise occur at an early stage in the rabbit model. In the absence of scleral closure as a confounding factor, bleb failure results largely from subconjunctival fibrosis and bleb survival time can be used as an index of the subconjunctival healing response.

AMT was observed to form satisfactory drainage blebs. The time to complete epithelialization of the AMT bleb (11.2 ± 2.5 days, mean ± SD) was longer than would normally be expected for conjunctiva, but this did not appear to be of clinical importance and was also comparable to epithelialization times reported with other uses of AMT. The two cases of early leakage, both from the limbal edge, highlight a potential problem when attempting to achieve a watertight seal from suturing avascular tissue. This was corrected in later cases by advancing the AMT edge over the limbus onto peripheral cornea
and adding extra sutures. There were no episodes of bleb-related infection, intraocular hemorrhage, or sequelae of hypotony observed during the study period. One eye had a low IOP at the time of harvesting. This was attributable to a leak as discussed above.

AMT filtration procedures were shown to have longer survival than control procedures in terms of IOP reduction (Fig. 4). This achieved statistical significance at the middle time point (Table 1). Exclusion of the two cases with persistent bleb leakage from the analysis increased the P value slightly (P = 0.027 for absolute reduction and P = 0.018 for percentage reduction) but did not affect significance at the 95% confidence level.

It is interesting that the IOP reduction in the control filtration group was lower in the middle time point when compared with the previous and subsequent time points. However, closer examination of the data revealed that by chance a disproportionate number of early failures had been harvested at day 14, thereby influencing the IOP level at the middle (days 21–30) but not the late (days 28–58) time point. There was no hypertensive phase noted in these animals.

The observation of significantly lower fibroblast outgrowth in tissue culture from explanted AMT biopsies is a likely explanation for the improved duration of survival. Previous studies have shown that AMT has the ability to downregulate fibroblast activity chiefly through an effect on TGF-β function. We consider this model to represent, as closely as possible, the effects of subconjunctival fibrosis on filtration function rather than scleral closure because of prevention of the latter by indwelling trans-scleral cannula. Scleral closure would otherwise occur early in the rabbit.

Histologically, amniotic membranes were found to be intact after 2 postoperative weeks, whereas those examined at the end of the study period showed significant granulomatous inflammation with giant cell formation. This finding was also reflected in the significantly higher fibroblast outgrowth in tissue culture at the late time point from peri-AMT conjunctival biopsies. We presume the observed inflammatory response was because of the xenogenic nature of the human AMT used in rabbits. Similar findings have not been reported in previous studies in which human to human transplants have been used.

There are two potential areas where we consider that an alternative low-healing tissue might of use in filtration surgery. In certain first trabeculectomies where there is deemed to be a high risk of failure or in repeat trabeculectomies, a low-healing tissue might be useful where a muted wound healing response might be a higher risk of leakage after surgery.

References


