

# Transplantation of Human Limbal Epithelium Cultivated on Amniotic Membrane for the Treatment of Severe Ocular Surface Disorders

Jun Shimazaki, MD, Masayo Aiba, BS, Eiki Goto, MD, Naoko Kato, MD, Shigeto Shimmura, MD, Kazuo Tsubota, MD

**Purpose:** To study the short-term clinical results of transplanting of cultivated corneal/limbal epithelial cells on human amniotic membrane (AM) for limbal deficiency.

**Design:** Noncomparative, retrospective interventional case series.

**Participants:** Thirteen eyes of 13 patients with severe limbal deficiency (Stevens-Johnson syndrome in eight eyes, ocular cicatricial pemphigoid in three eyes, and chemical burns in two eyes) were treated at the department of Ophthalmology, Tokyo Dental College, Japan.

**Intervention:** Cultivated allo-limbal epithelium was transplanted onto the ocular surface of patients with severe limbal deficiency.

**Main Outcome Measures:** Ocular surface reconstruction with corneal epithelialization, changes in visual acuity, and postoperative complications were studied. Histologic examinations were also performed on cultivated epithelium.

**Results:** Cultivated corneal epithelium on AM formed two to three layers with the formation of basement membrane-like structures. After the surgery, the epithelium regenerated and covered the ocular surface in eight eyes (61.5%). However, three of the eight eyes developed partial conjunctival invasion, and two eyes later developed epithelial defects. At last examination, corneal epithelialization was achieved in six eyes (46.2%). Five eyes had conjunctivalization, one eye had dermal epithelialization, and one eye was not epithelialized. Complications were corneal perforation in four eyes and infectious keratitis in two eyes.

**Conclusions:** This study demonstrates that the success rate for transplanting cultivated allo-limbal epithelium on the AM is not different from the conventional limbal and AM transplantation for the treatment of severe limbal stem cell dysfunction. *Ophthalmology* 2002;109:1285-1290 © 2002 by the American Academy of Ophthalmology.

Treatment of cicatricial keratoconjunctivitis has been challenging, because the underlying disease is often complicated by multiple disorders, including decreased tear secretion, abnormalities in lids and meibomian glands, subconjunctival fibrosis, and limbal stem cell dysfunction. Transplantation of limbal stem cells using human amniotic membrane (AM) grafting is a recently developed surgical method for the treatment of such diseases.<sup>1-5</sup> After surgery, the corneal epithelial cells derived from transplanted limbal

tissue regenerate over the ocular surface. Although this approach has substantially improved the surgical prognosis of patients with Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid, and chemical and thermal burns of the cornea, the long-term success of the procedure remains approximately 50% according to the recent study by our group.<sup>5</sup> The most common cause of failure is lack of proper regeneration of the corneal epithelial cells on AM. Decreased tear secretion was also shown to be a significant risk factor of surgical failure in SJS.<sup>6</sup>

Transplantation of cultivated corneal or limbal epithelial cells to the ocular surface is used to overcome this difficulty. Initial reports using patients' own limbal tissue (auto-transplantation) were encouraging.<sup>7,8</sup> In addition, results of an animal experiment and clinical trial using allografts obtained either from cadaver eyes or from patients' living relatives have also been reported.<sup>9-11</sup> This study was conducted to examine the efficacy and limitation of transplanting cultivated epithelium derived from allo-limbal tissues. Histologic examinations and clinical outcomes are described.

Originally received: February 15, 2001.

Accepted: February 8, 2002.

Manuscript no. 210105.

From the Department of Ophthalmology, Tokyo Dental College, Chiba, Japan.

Supported in part by a grant of the Ministry of Health and Welfare, Japan (H12-Saisei-012).

The authors do not have any proprietary interest in the products mentioned or used in this study.

Reprint requests to Jun Shimazaki, MD, Department of Ophthalmology, Tokyo Dental College, 5-11-13 Sugano, Ichikawa-shi, Chiba, 272-8513, Japan.

Table 1. Patients' Demographic Profile

Case	Age/Gender	Disease	Preoperative Epithelium	Symblepharon	Previous Keratoplasty	Preoperative Glaucoma	Schirmer Values (mm)	Donor (% HLA Matching)
1	68/M	SJS	C	+	0		4	allo
2	61/M	Burn	C		PKP×6		35	allo
3	44/M	Burn	C		0		5	allo
4	68/F	OCP	C	+	LKP×1		0	allo
5	75/F	OCP	C	+	PKP×2		6	allo
6	66/M	SJS	C	+	PKP×1		5	allo
7	32/M	SJS	C	+	0	+	0	allo
8	18/F	SJS	C	+	0	+	3	sister (100%)
9	23/F	SJS	CK		0		5	father (50%)
10	70/F	OCP	CK	+	0	+	0	son (50%)
11	72/M	SJS	D	+	0		0	sister (50%)
12	48/F	SJS	D			+	0	sister (100%)
13	27/F	SJS	C	+	LKP×1		0	mother (50%)

Burn = chemical or thermal burn of the cornea; C = conjunctival; CK = conjunctival with keratinization; D = dermal; LKP = lamellar keratoplasty; OCP = ocular cicatricial pemphigoid; PKP = penetrating keratoplasty; SJS = Stevens-Johnson syndrome.

## Material and Methods

### Cultivation of the Corneal Epithelium

Limbal tissue samples of approximately  $2 \times 2$  mm in size were obtained from donor eyes. Written informed consent was obtained from patients and living donors after the purpose and potential risks of the procedure were explained. The limbal tissue of patients' relatives was excised using a surgical blade, with the donors under topical anesthesia. Excised tissues were washed, cut into small pieces, and placed in culture dishes 35 mm in diameter. Human AM was obtained at the time of elective cesarean section and processed as previously reported.<sup>2,4,5</sup> The epithelium of the AM was removed by chemical processing with 10% ammonium for 15 minutes followed by gentle scraping with surgical blades. The method enabled almost complete removal of AM epithelium, but left the basement membrane structure intact (data not shown). The AM was placed at the bottom of culture dishes, and the donor epithelium was then placed on the basement membrane side of the AM. Supplemental Hormonal Epithelial Medium (SHEM) culture medium consisted of Dulbecco's modified Eagle's medium/F12 (Gibco BRL, Grand Island, NY), 2.5 g/l NaHCO<sub>3</sub>, 5 μg/ml insulin (Sigma, St. Louis, MO), 10 μg/ml human recombinant epidermal growth factor (Gibco BRL), 1 μg/ml cholera toxin (Gibco BRL), 0.5% dimethyl sulfoxide (Sigma, St. Louis, MO), antibiotics (benzylpenicillin and streptomycin), and 15% patients serum. The medium was used until the cells became approximately 70% confluent, then exchanged with medium 165 (Kurabo, Osaka, Japan) plus 10% patient serum. Cells reached confluency after approximately 2 weeks in this culture condition. Some of the tissues were subjected to histologic examination using hematoxylin-eosin and periodic acid-Schiff staining. Some tissues were examined by transmission electron microscopy to study the attachment structure of the epithelium to AM.

### Patients

Thirteen eyes of 13 patients with severe cicatricial keratoconjunctivitis associated with total limbal deficiency underwent transplantation of cultivated limbal epithelial cells on AM. These patients

received a full explanation of the surgical procedure, including a comparison of the conventional approach consisting of limbal allograft transplantation and AM transplantation. Serologic testing of human leukocyte antigen was performed on both patients and family members. The patients and family then chose whether the donor cells would be obtained from cadaver eyes or from family members.

Profiles of the patients are shown in Table 1. There were six males and seven females, with the mean age of  $51.7 \pm 20.7$  years. Underlying conditions were SJS (eight eyes), ocular cicatricial pemphigoid (three eyes), and chemical burns (two eyes). The corneal surface was totally covered by either dermal epithelium (2 eyes) or conjunctival epithelium (11 eyes). Symblepharon was noted in nine eyes. Five eyes received either lamellar or penetrating keratoplasty. Preoperative visual acuity ranged from light perception to 20/2000. Only five eyes had Schirmer values  $\geq 5$  mm. The values of Schirmer test with nasal stimulation, which represents reflex tearing, were decreased (less than 5 mm) in six of seven eyes examined.

### Surgical Methods

Six patients underwent living-related transplantation (LR group) with tissue obtained from their parents (two eyes), brothers or sisters (three eyes), or a son (one eye). In two of these six eyes, there was a 100% match of human leukocyte antigen between recipients and donors. The other seven patients had allo-transplantation from cadaver eyes (Allo group).

All surgical procedures were performed with the patient under retrobulbar anesthesia, using 2% lidocaine (Xylocaine, Fujisawa Pharmaceutical Co., Osaka, Japan). First, all scar tissues on the ocular surface were removed using surgical scissors. All functioning lacrimal punctum were occluded by heat coagulation combined with 10-0 nylon sutures. Human AM with cultivated limbal stem cells was placed on the bare sclera and corneal stroma, epithelial side upward and sutured with 8-0 Vicryl. Hyaluronic acid (Healon, Pharmacia Upjohn, Tokyo, Japan) was placed on the AM so as not to damage the cultivated cells. At the end of surgery, cultivated cells on AM were further covered by another sheet of AM or by therapeutic soft contact lenses for protection. For the former method, AM was placed on the cultivated epithelium with the basement membrane side facing up.

Table 2. Surgical Methods and Outcome

Case	Surgical Procedure	Systemic Cyclosporin A	Time until Corneal Epithelialization (days)	Conjunctivalization	Persistent Epithelial Defect	Complications			Final Epithelial Status	Corrected Visual Acuity	
						Neovascularization	Symblepharon	Ulcer Perforation		Preoperative	Postoperative
1	CET	+	28				+		K	HM	20/200
2	CET	+	16						K	HM	20/50
3	CET	+	22						K	HM	20/60
4	CET	+	26	+			+		C	HM	20/200
5	CET	+	15		+			+	K	HM	HM
6	CET	+	N/A		+			+	C	LP	HM
7	CET	+	N/A		+		+		C	HM	20/600
8	CET	-	17		+			+	C	HM	20/2000
9	CET	+	12	+			+		K	20/2000	20/600
10	CET, MMC	+	23	+			+		K	HM	20/500
11	CET, MMC	+	N/A					+	ED	HM	20/2000
12	CET	+	N/A		+			+	D	LP	LP
13	CET, lens asp	+	N/A		+			+	C	HM	HM

C = conjunctival; CET = cultivated epithelial transplantation; ED = epithelial defect; HM = hand motions; K = corneal; MMC = mitomycin-C; N/A = not applicable; LP = light perception.

## Postoperative Management

After surgery, hyaluronic acid eyedrops (Hyalein Mini, Santen Pharmaceutical Co., Osaka, Japan), autologous serum eyedrops, and preservative-free steroid eyedrops (Solu-Medrol, Upjohn) were used for intensive epithelial maintenance. Antibiotics (Tarivid, Santen, Japan) were given five times a day.

All but one patient had systemic immunosuppression using cyclosporin A (Sandimmune, Novartis Pharma, Tokyo, Japan) with blood trough levels maintained at approximately 100 to 150 ng/ml unless abnormalities of renal and liver function developed. Topical cyclosporin A dissolved in  $\alpha$ -cyclodextrin was used five times a day in seven patients.

## Results

### Histology of the Cultivated Tissue

Cultivated corneal epithelium proliferated on culture plates and became confluent. The mean time required was 13.9 days (range, 12–20 days). Under phase microscopy, the cells were small round and they formed two to three layers (Fig 1). These cells were firmly attached to the AM, and basement membrane-like structures were observed between the epithelium and AM (Fig 2).

### Surgical Outcome

After surgery, the corneal epithelium regenerated and covered the ocular surface in eight eyes (61.5%), with mean period of 19.9 days (range, 12–28 days). Three of these eyes maintained corneal epithelialization, and two had subsequent penetrating keratoplasty, resulting in good visual rehabilitation (Fig 3). One cornea (case 2) was subjected to histologic examination; but, the transplanted AM was not found. Three of the eight eyes developed partial conjunctival invasion. Two other eyes later developed epithelial defects. Despite intensive epithelial management, both of these eyes developed corneal perforation (Fig 4).

One of five eyes (three in the Allo group and two in the LR group), that were not epithelialized underwent repeated surgery.

One eye developed descemetocoele, two developed corneal perforation, and one developed corneal ulcer. Four of the five eyes (80.0%) that were not epithelialized had Schirmer test values of <5mm.

Five eyes (38.5%, four in the LR group and one in the Allo group) required additional surgeries for ocular surface reconstruction. At last examination, corneal epithelialization was achieved in six eyes (46.2%), although three of these had partial conjunctival invasion. Five eyes had conjunctivalization, one eye had dermal epithelialization, and one eye was not epithelialized (Table 2).

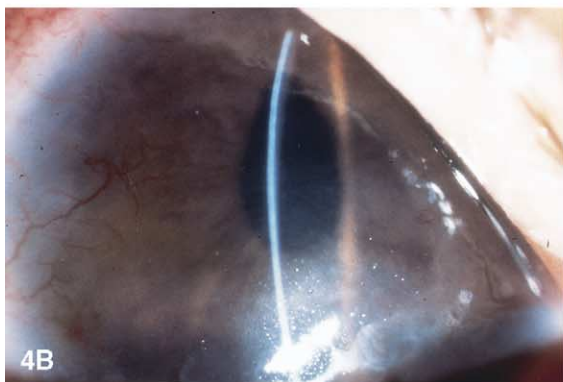
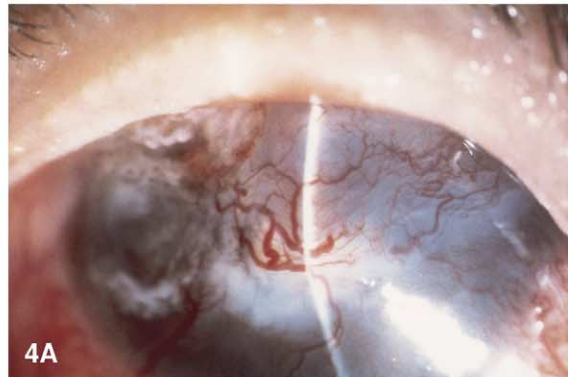
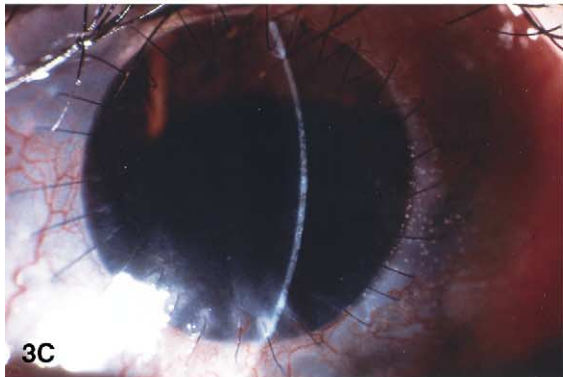
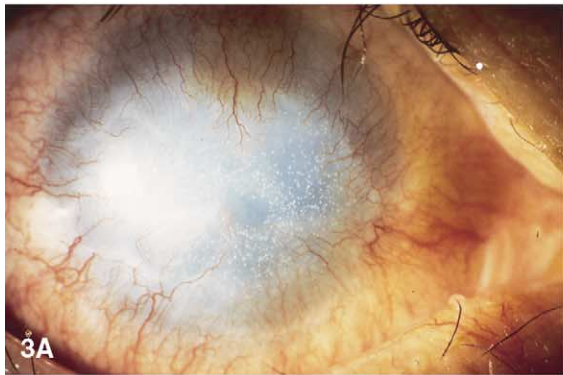
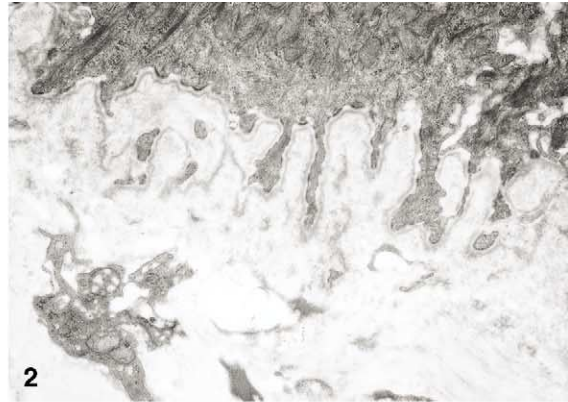
Initial corneal epithelialization was achieved in five and three eyes in the Allo and LR groups, respectively. Corneal epithelialization was observed in four eyes in the Allo group and two eyes in the LR group, although two eyes in the LR group showed partial conjunctivalization.

### Complications

Corneal perforation developed in four eyes, and infectious keratitis in two eyes. The causative organism was isolated in two eyes (fungus plus *Flavobacterium* and methicillin-resistant *Staphylococcus aureus*). Recurrence of neovascularization and symblepharon was observed in two and three eyes, respectively. One eye developed glaucoma that did not respond to medical treatment, and trabeculotomy was performed.

## Discussion

Human AM transplantation has been used for a variety of ocular surface diseases, including chemical or thermal burns, SJS, ocular cicatricial pemphigoid, pterygium, conjunctival defect, persistent epithelial defect of the cornea, and corneal ulcer.<sup>1–5,12–20</sup> Application of this technique for the treatment of cicatricial keratoconjunctivitis has been especially welcome because of the lack of effective treatment modalities. Cicatricial keratoconjunctivitis accompanied by limbal stem cell deficiency had been considered a contraindication for surgical treatment; however, encourag-



ing clinical successes have been reported using AM with limbal stem cell transplantation. Despite the current enthusiasm, a study from our department of the long-term prognosis of AM transplantation plus stem cell transplantation using allograft demonstrated that long-lasting ocular surface reconstruction was achieved in less than half of the cases.<sup>5</sup> Persistent epithelial defect of the cornea was the most common postoperative complication developing in 60% of cases despite meticulous postoperative management of the epithelium.

To overcome this difficulty, *in vitro* cultivation of the corneal epithelium on the AM was introduced. When cultivated corneal epithelium is transferred with AM, the corneal surface is expected to epithelialize instantly. An outstanding clinical report was made recently by Tsai et al,<sup>8</sup> who used limbal tissue from the patient's contralateral eye as a source of stem cells (autograft). They applied the method to six eyes with various ocular surface abnormalities and achieved corneal epithelialization with visual recovery in all cases. Similar encouraging results were also reported by other groups.<sup>21,22</sup> Being encouraged by this report, we started using a similar approach. However, the results of this study were not impressive. Although initial epithelialization was obtained in eight eyes (61.5%), the final success rate was limited to 46.2%. This success rate is no better than our previous approach using AM transplantation and stem cell transplantation.<sup>5</sup> In addition, corneal perforation, the most severe complication, developed in four eyes (30.8%).

There were several differences between our study and Tsai's report. First, they used autograft as a source of corneal stem cells, whereas we used allografts, either from cadaver eyes or from living relatives. It is obvious that the use of autograft is advantageous over allograft, because the former never develops immunologic rejection. Second, preoperative conditions of the ocular surface seemed much worse in our series compared with Tsai's. All eyes in our series had total limbal dysfunction, whereas some of the eyes in Tsai's series seemed to have partially viable limbal stem cells. Moreover, most of our cases were complicated by decreased tear secretion and surface cicatrization. It was reported that preoperative tear function was a strong determinant for successful ocular surface reconstruction in SJS patients.<sup>6</sup> It is likely that these environmental factors affected the prognosis. It

is intriguing that in some cases the transplanted corneal epithelium first covered the entire surface but later developed recurrent small epithelial defects in many different areas of the cornea. We did not see any rejection line such as we find in cases of epithelial rejection after keratoplasty. The observation leads us to assume that the cultivated epithelium underwent desquamation because of biologic rather than immunologic attack.

Comparing our results using limbal tissue from cadavers and living relatives as a source of stem cells, we did not find significant differences. In fact, corneal ulcer and/or perforation tended to be more common in the LR group than in the Allo group (50.0% vs. 28.6%). If the complications are mediated by immunologic reactions, cadaver eyes are likely to cause more complications than those of living relatives. It is noteworthy that the number of limbal cells used may be different between these two groups. In cadaver eyes, we obtained two pieces of limbal tissue (about 1×3 mm each), and the tissues were transferred to the epithelial side of AM. After the limbal epithelium proliferated on the AM, the limbal tissue and surrounding epithelium with AM was transplanted on patients' eyes. The size of the excised limbal tissues was much smaller in relatives' eyes. It is conceivable that the transferred limbal allo-tissues might exert a continuous source of corneal epithelium in cadaver eyes. More experimental and clinical work is needed to explain the biologic and immunologic changes in transplanted epithelium.

The question of whether the prognosis for this method would be improved by using a different cultivation protocol is intriguing. Although multilayered epithelium with a basement membrane-like structure was obtained by the current cultivation method, other techniques, such as the use of feeder fibroblasts or airlifting, might improve structural integrity. We observed that some cultivated epithelium sloughed off immediately after surgery, suggesting that adhesion of cells to the AM was not firm enough. Further studies are needed to achieve optimal culture conditions.

In summary, our short-term clinical experience suggests that the use of cultivated allo-limbal epithelium on AM did not seem to improve the surgical prognosis for severe limbal stem cell dysfunction. More improvement in postoperative management and refinements in the cultivation system may be needed.

**Figure 1.** Histologic examination of cultivated limbal epithelium on amniotic membrane (AM) reveals stratified epithelial cells that are well attached on the AM (stain, hematoxylin–eosin; original magnification, ×200).

**Figure 2.** Transmission electron microscopy reveals the basement membrane-like structure between the basal epithelial cells and the amniotic membrane (original magnification, ×10,000).

**Figure 3.** Clinical course of case 3. **A**, Preoperatively, the cornea is opaque and covered by vascularized conjunctiva. Vision is hand motions. **B**, After transplantation of the cultivated limbal epithelium, the ocular surface is less vascularized. Arrows indicate limbal tissues on the amniotic membrane (AM). **C**, Penetrating keratoplasty and cataract surgery are performed 6 and 11 months after epithelial transplantation. The corneal graft remains clear and covered by the corneal epithelium. Visual acuity is 20/200.

**Figure 4.** Clinical course of case 8. **A**, Preoperatively, severe fibrosis covers the cornea. Vision is hand motions. **B**, After cultivated epithelial transplantation, the corneal surface is well reconstructed, and visual acuity recovers to 20/100. **C**, Six months later, recurrent epithelial erosion develops that leads to invasion of keratinized epithelium from the temporal side. Note that the cornea is thinned and protruded. Later the cornea is perforated, and tectonic penetrating keratoplasty is needed.

## References

---

1. Kim JC, Tseng SCG. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. *Cornea* 1995;14:473–84.
2. Tsubota K, Satake Y, Ohyama M, et al. Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome. *Am J Ophthalmol* 1996;122:38–52.
3. Tseng SCG, Prabhasawat P, Barton K, et al. Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. *Arch Ophthalmol* 1998;116:431–41.
4. Shimazaki J, Yang H-Y, Tsubota K. Amniotic membrane transplantation for ocular surface reconstruction in patients with chemical and thermal burns. *Ophthalmology* 1997;104:2068–76.
5. Tsubota K, Satake Y, Kaido M, et al. Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation. *N Engl J Med* 1999;340:1697–703.
6. Shimazaki J, Shimmura S, Fujishima H, Tsubota K. Association of preoperative tear function with surgical outcome in severe Stevens-Johnson syndrome. *Ophthalmology* 2000;107:1518–23.
7. Pellegrini G, Traverso CE, Franzi AT, et al. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 1997;349:990–3.
8. Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med* 2000;343:86–93.
9. Koizumi N, Inatomi T, Quantock AJ, et al. Amniotic membrane as a substrate for cultivating limbal corneal epithelial cells for autologous transplantation in rabbits. *Cornea* 2000;19:65–71.
10. Schwab IR. Cultured corneal epithelia for ocular surface disease. *Trans Am Ophthalmol Soc* 1999;97:891–986.
11. He YG, Alizadeh H, Kinoshita K, McCulley JP. Experimental transplantation of cultured limbal and amniotic epithelial cells onto the corneal surface. *Cornea* 1999;18:570–9.
12. Tseng SCG, Prabhasawat P, Lee S-H. Amniotic membrane transplantation for conjunctival surface reconstruction. *Am J Ophthalmol* 1997;124:765–74.
13. Kruse FE, Rohrschneider K, Völcker HE. Multilayer amniotic membrane transplantation for reconstruction of deep corneal ulcers. *Ophthalmology* 1999;106:1504–11.
14. Lee S-H, Tseng SCG. Amniotic membrane transplantation for persistent epithelial defects with ulceration. *Am J Ophthalmol* 1997;123:303–12.
15. Prabhasawat P, Barton K, Burkett G, et al. Comparison of conjunctival autografts, amniotic membrane grafts, and primary closure for pterygium excision. *Ophthalmology* 1997;104:974–85.
16. Fujishima H, Shimasaki J, Shinozaki N, Tsubota K. Trabeculectomy with the use of amniotic membrane for uncontrollable glaucoma. *Ophthalmic Surg and Lasers* 1998;29:428–31.
17. Shimazaki J, Shinozaki N, Tsubota K. Transplantation of amniotic membrane and limbal autograft for patients with recurrent pterygium associated with symblepharon. *Br J Ophthalmol* 1998;82:235–40.
18. Meller D, Pires RTF, Mack RJS, et al. Amniotic membrane transplantation for acute chemical or thermal burns. *Ophthalmology* 2000;107:980–90.
19. Pires RTF, Tseng SCG, Prabhasawat P, et al. Amniotic membrane transplantation for symptomatic bullous keratopathy. *Arch Ophthalmol* 1998;116:431–41.
20. Ma DH, See LC, Liao SB, Tsai RJ. Amniotic membrane graft for primary pterygium: comparison with conjunctival autograft and topical mitomycin C treatment. *Br J Ophthalmol* 2000;84:973–8.
21. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. *Cornea* 2000;19:421–6.
22. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. *Ophthalmology* 2001;108:1569–74.