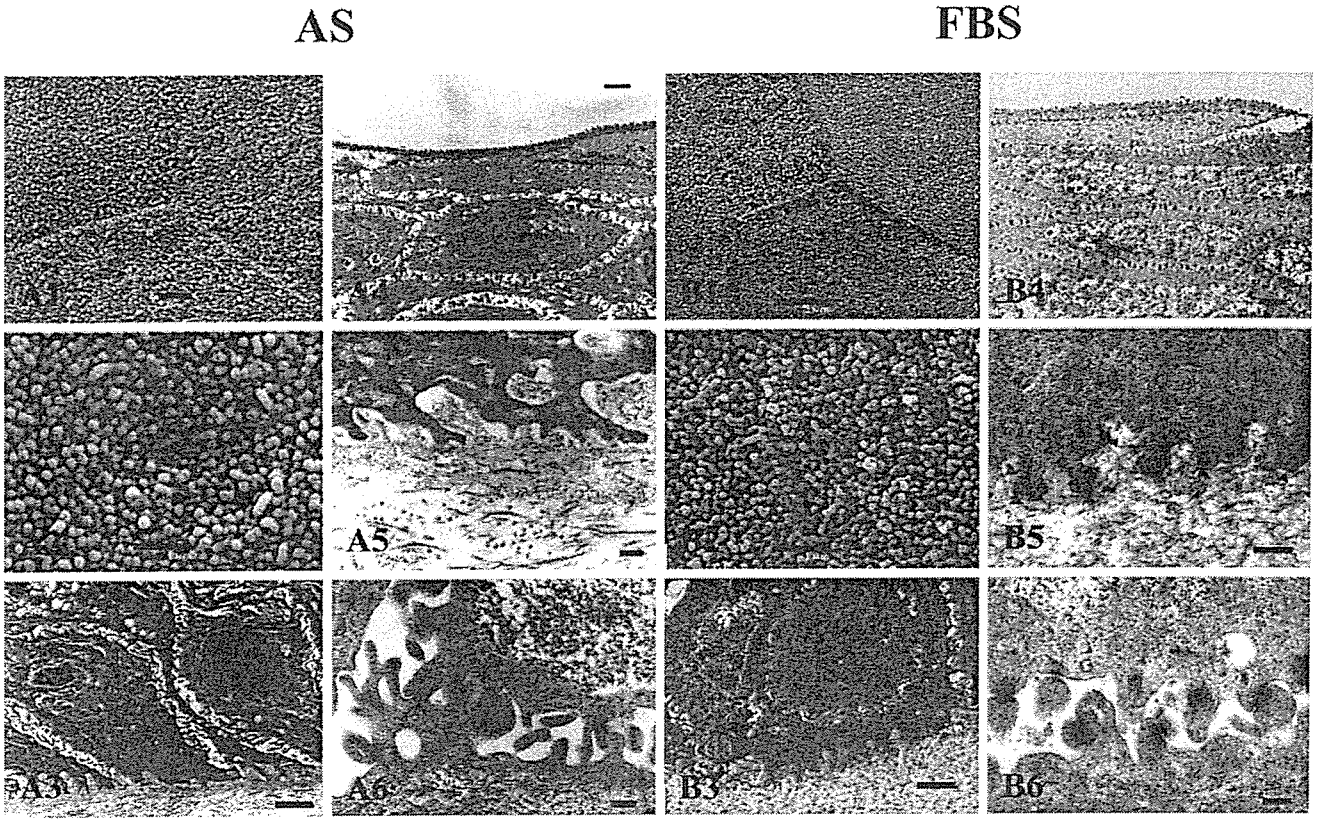


FIGURE 7. Scanning electron microscopic examination showed a continuous layer of flat squamous polygonal epithelial cells in the cultivated corneal epithelial cells when using AS (A1) and FBS (B1). These cells were closely attached to each other with tightly opposed cell junctions and distinct cell boundaries. The apical surfaces of the AS- and FBS-derived epithelial sheets were covered with numerous microvilli (A2, B2). Transmission electron microscopic examination of the corneal epithelial culture sheet showed that the cells appeared healthy and were differentiated into basal columnar cells, suprabasal cuboid wing cells, and flat squamous superficial cells (A3, A4, B3, B4). The basal epithelial cells adhered well to the AM substrate with hemidesmosomal attachments and produced basement membrane material (A5, B5). In all cell layers, the epithelial cells were comparatively closely attached to neighboring cells by numerous desmosomal junctions (A6, B6). The morphologic appearance was very similar in the AS- and FBS-supplemented culture systems. Scale bars: (B1) 5 μm; (A1, A3, A4, B3, B4) 2 μm; (A2, B2) 1 μm; (A5, B5) 500 nm; (A6, B6) 200 nm.

Normal epithelial cells have specialized junctions on their cell surfaces to ensure firm adhesion to neighboring cells and the extracellular matrix below.[26-28] Desmosomal junctions are present between the cell-to-cell surfaces and give the cell-sheet structural integrity, while hemidesmosomes present on the basal-cell surfaces serve to attach the basal cells to the basement membrane.[26-28] In both AS- and FBS-supplemented cultures, TEM demonstrated the presence of a basal lamina with hemidesmosomal attachments at the basal-cell–substrate junctional zone. Immunohistochemistry confirmed the expression of $\alpha 6$ and $\beta 4$ integrins, which are associated with hemidesmosomes, as well as the presence of collagen IV and laminin 5. These are essential for cell-to-substrate adhesion and the maintenance of tissue integrity. Adjacent cells in the cultivated sheet were also joined by numerous desmosomal junctions. These findings are important in ensuring graft integrity during surgical manipulation, as well as after transplantation.

In summary, our study is the first to demonstrate the effective use of AS from patients with SJS in supporting the ex vivo expansion of corneal and oral epithelial cells. The elimination of animal and nonhuman material from the culture system offers significant advantages over existing bovine serum–supplemented culture procedures, because it reduces the risk of transmission of zoonotic infection. These findings bring us one step closer to the development of a safe and effective xenobiotic-free bioengineered tissue equivalent for clinical transplantation. This has significant clinical implications, because these cultivated ocular surface epithelial equivalents may potentially be used in the treatment of patients with severe OSD.

Acknowledgments

The authors thank Narisato Kanamura and Takashi Amemiya for performing the oral biopsies, Hideo Honjyo for providing AM, and Hisayo Sogabe and Tomoko Horikiri for assisting with the culture procedures.

References

1. Shapiro MS, Friend J, Thoft RA. Corneal re-epithelialization from the conjunctiva. *Invest Ophthalmol Vis Sci.* 1981;21:135–142.
2. Dua H, Forrester JV. The corneoscleral limbus in human corneal epithelial wound healing. *Am J Ophthalmol.* 1990;110:646–656.
3. Tsai RJF, Sun TT, Tseng SCG. Comparison of limbal and conjunctival autograft transplantation in corneal surface reconstruction in rabbits. *Ophthalmology.* 1990;97:446–455.
4. Thoft RA. Keratoepithelioplasty. *Am J Ophthalmol.* 1984;97:1–6.
5. Kenyon KR, Tseng SCG. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology.* 1989;96:709–723.
6. Pellegrini G, Traverso CE, Franzi AT, et al. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet.* 1997;349:990–993.
7. 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.

8. Koizumi N, Inatomi T, Suzuki T, et al. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. *Ophthalmology*. 2001;108:1569–1574.
9. Shimazaki J, Aiba M, Goto E, Kato N, Shimmura S, Tsubota K. Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders. *Ophthalmology*. 2002;109:1285–1290.
10. Nakamura T, Inatomi T, Sotozono C, et al. Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. *Br J Ophthalmol*. 2004;88:1280–1284.
11. Nishida K, Yamamoto M, Hayashida Y, et al. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med*. 2004;351:1187–1196.
12. Boyce ST, Ham RG. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J Invest Dermatol*. 1983;81:33s–40s.
13. Wille JJ Jr, Pittelkow MR, Shipley GD, Scott RE. Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum-free medium: clonal analyses, growth kinetics, and cell cycle studies. *J Cell Physiol*. 1984;121:31–44.
14. Kruse FE, Tseng SC. Growth factors modulate clonal growth and differentiation of cultured rabbit limbal and corneal epithelium. *Invest Ophthalmol Vis Sci*. 1993;34:1963–1976.
15. Ang LP, Tan DT, Seah CJ, Beuerman RW. The use of human serum in supporting the in vitro and in vivo proliferation of human conjunctival epithelial cells. *Br J Ophthalmol*. 2005;89:748–752.
16. Koizumi N, Cooper L, Fullwood NJ, et al. An evaluation of cultivated corneal limbal epithelial cells using cell suspension culture. *Invest Ophthalmol Vis Sci*. 2002;43:2114–2121.
17. Nakamura T, Endo K, Cooper LJ, et al. The successful culture and autologous transplantation of rabbit oral mucosal epithelial cells on amniotic membrane. *Invest Ophthalmol Vis Sci*. 2003;44:106–116.
18. Nakamura T, Inatomi T, Sotozono C, et al. Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. *Acta Ophthalmol Scand*. 2004;82:468–471.
19. Ang LP, Tan DT, Beuerman RW, Lavker RM. Development of a conjunctival epithelial equivalent with improved proliferative properties using a multistep serum-free culture system. *Invest Ophthalmol Vis Sci*. 2004;45:1789–1795.
20. Nakamura T, Nishida K, Dota A, et al. Elevated expression of transglutaminase 1 and keratinization-related proteins in conjunctiva in severe ocular surface disease. *Invest Ophthalmol Vis Sci*. 2001;42:549–556.
21. Nakamura T, Nishida K, Dota A, et al. Changes in conjunctival clustering expression in severe ocular surface disease. *Invest Ophthalmol Vis Sci*. 2002;43:1702–1707.
22. Tsai RJ, Tseng SC. Substrate modulation of cultured rabbit conjunctival epithelial cell differentiation and morphology. *Invest Ophthalmol Vis Sci*. 1988;29:1565–1576.
23. Lindberg K, Brown ME, Chaves HV, Kenyon KR, Rheinwald JG. In vitro propagation of human ocular surface epithelial cells for transplantation. *Invest Ophthalmol Vis Sci*. 1993;34:2672–2679.
24. Zieske JD, Mason VS, Wasson ME, et al. Basement membrane assembly and differentiation of cultured corneal cells: importance of culture environment and endothelial cell interaction. *Exp Cell Res*. 1994;214:621–633.
25. Ang LPK, Tan DTH, Phan TT, Li J, Beuerman R, Lavker RM. The in vitro and in vivo proliferative capacity of serum-free cultivated human conjunctival epithelial cells. *Curr Eye Res*. 2004;28:307–317.
26. Garrod DR. Desmosomes and hemidesmosomes. *Curr Opin Cell Biol*. 1993;5:30–40.
27. Gipson IK, Sugrue SP. Cell biology of the corneal epithelium. In: Albert DM, Jakobiec FA, eds. *Principles and Practice of Ophthalmology*. Philadelphia: WB Saunders; 1994:2–16.
28. Green KJ, Jones JCR. Desmosomes and hemidesmosomes: structure and function of molecular components. *FASEB J*. 1996;10:871–881.

Novel clinical application of sterilized, freeze-dried amniotic membrane to treat patients with pterygium

Takahiro Nakamura,[1,2] Tsutomu Inatomi,[1] Eiichi Sekiyama,[1] Leonard P. K. Ang,[1] Norihiko Yokoi[1] and Shigeru Kinoshita[1]

[1]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Graduate School of Medicine, Kyoto, Japan
[2]Research Centre for Regenerative Medicine, Doshisha University, Kyoto, Japan

ABSTRACT.

Purpose: **To evaluate the use of sterilized, freeze-dried amniotic membrane (FD-AM) transplantation for pterygium surgery.**

Methods: **This study involved a prospective, non-comparative, interventional case series. Thirteen eyes of 13 patients with primary (eight eyes) or recurrent (five eyes) pterygium were studied. After excision of the pterygium fibrous tissues and application of intraoperative use of mitomycin-C, sterilized FD-AM was sutured over the bare scleral defect. The integrity of the FD-AM graft, epithelialization over the FD-AM, pterygium recurrence and postoperative complications were evaluated.**

Results: **Postoperatively, the FD-AM was well retained in all patients, and complete epithelialization over the transplanted membrane was achieved within 1–2 weeks. All patients demonstrated early resolution of ocular inflammation and there was no recurrence of pterygium in any of the treated patients during the mean follow-up of 13.9 ± 6.0 months. No ocular complications were noted following transplantation.**

Conclusion: **Sterilized FD-AM showed excellent biocompatibility on the human ocular surface. This novel and promising biomaterial may be a useful alternative to conjunctival grafting in the treatment of pterygium.**

Key words: amniotic membrane – freeze-dry – pterygium – biomaterial – ocular surface

Acta Ophthalmol. Scand. 2006: 84: 401–405

doi: 10.1111/j.1600-0420.2006.00667.x

Introduction

The fresh human amniotic membrane (AM), the innermost layer of the placental membrane, is a thin, semitransparent tissue comprised of a single layer of ectodermally derived columnar cells, a thick, continuous basement membrane, and subjacent avascular stroma (van Herendael et al. 1978). Cryopreserved AM has been shown to possess anti-inflammatory (Kim et al. 2000; Solomon et al. 2001a), antifibroblastic activity (Tseng et al. 1999) and antiangiogenic properties, as well having limited immunogenicity (Akle et al. 1981; Hao et al. 2000). Because of these desirable characteristics, cryopreserved AM has been used in a wide variety of ocular surface reconstructive procedures.

Pterygium is a common external ocular disease characterized by fibrovascular overgrowth of degenerative conjunctiva onto the cornea, together with associated ocular inflammation. A wide variety of surgical methods have been employed in its treatment, with the aims of ensuring a good cosmetic result, reducing the risk of recurrence, and minimizing complications. These include bare sclera excision, with or without the adjunctive use of mitomycin-C or, β-irradiation (Manning et al. 1997; Lam et al. 1998), or wound closure using a conjunctival autograft (Lewallen 1989), conjunctival limbal autograft (Mutlu et al. 1999), cryopreserved human amniotic membrane (Prabhasawat et al. 1997; Solomon et al. 2001b), or more recently, autologous cultivated conjunctival transplantation (Ang et al. 2005). Amniotic membrane transplantation, used alone, or in combination with conjunctival autograft, limbal autograft or mitomycin-C (MMC) has been shown to be an effective method for treating pterygium, although its reported clinical efficacy has varied between reports (Prabhasawat et al. 1997; Shimazaki

et al. 1998; Ma et al. 2000; Solomon et al. 2001b; Shimazaki et al. 2003).

The anti-inflammatory, antifibrotic and antiangiogenic properties of cryopreserved AM, as well as its ability to promote epithelialization and maintain the epithelial stem cell niche make it useful in the treatment of a variety of ocular surface diseases, including pterygium. However, safety and logistical issues pose potential problems with its use. Preparation of AMs for clinical use usually entails several washings with buffered saline containing antibiotics. However, complete disinfection is not always guaranteed. Human AMs, being biological tissues, pose the potential risk of transmission of infection from the donor, or from contamination or inadequate disinfection during its processing and preservation (Adds et al. 2001; von Versen-Hoynck et al. 2004). As such, proper and reliable sterilization of AM is vital to render it completely safe for clinical applications. Furthermore, cryopreservation of AM requires the use of expensive, space-consuming freezers. These are hindrances to the widespread availability and usage of AM in many parts of the world, particularly in less developed countries. In order for AM to be ideally suited for widespread clinical use, it needs to be sterile, free of contamination, easily obtainable, and its transport and prolonged storage at room temperature should not have any adverse effects on its quality.

Our group previously reported that sterilized, freeze-dried amniotic membrane (FD-AM) produced by our unique protocol retained much of the physical, biological and morphological characteristics of cryopreserved AM (Nakamura et al. 2004a). Freeze-dried amniotic membrane is wafer-thin, type 4 collagen-rich substrate. Although its flexibility upon hydration is slightly less than that of cryopreserved AM, we found it was a useful substrate for ocular surface reconstruction in a rabbit model (Nakamura et al. 2004a). In the current study we evaluated the biocompatibility of FD-AM on the human ocular surface. We successfully applied this novel, safe and convenient biomaterial to the eyes of 13 patients with pterygium. This report is the first step in assessing the feasibility of using FD-AM for pterygium surgery and ocular surface transplantation.

Materials and Methods

Subjects

The study included 13 eyes of 13 consecutive patients with pterygium who underwent FD-AM transplantation at our hospital between July 2003 and December 2004. All patients were treated by the same two surgeons (SK and TI). All patients were followed-up for more than 6 months after transplantation. The Human Studies Committee of Kyoto Prefectural University of Medicine approved the transplantation of FD-AM and we obtained prior oral and written informed consent from all patients.

Preparation of sterilized, freeze-dried AM

The FD-AM was prepared according to our previously reported method (Nakamura et al. 2004a). Briefly, after obtaining proper informed consent in accordance with the tenets of the Declaration of Helsinki for research involving human subjects, and with approval by the Institutional Review Board of Kyoto Prefectural University of Medicine, human AM was obtained from donors undergoing elective caesarean section. The AM was washed with sterile phosphate buffered saline containing antibiotics and antimycotics. Amniotic epithelial cells were removed by incubation with 0.02% ethylene diamine tetra-acetic acid. Denuded AM was freeze-dried under vacuum conditions, vacuum-packed at room temperature, and sterilized by gamma-irradiation (25 kGy). The resultant material was wafer-like, very lightweight and thin, easy to handle, and could be sutured without tearing.

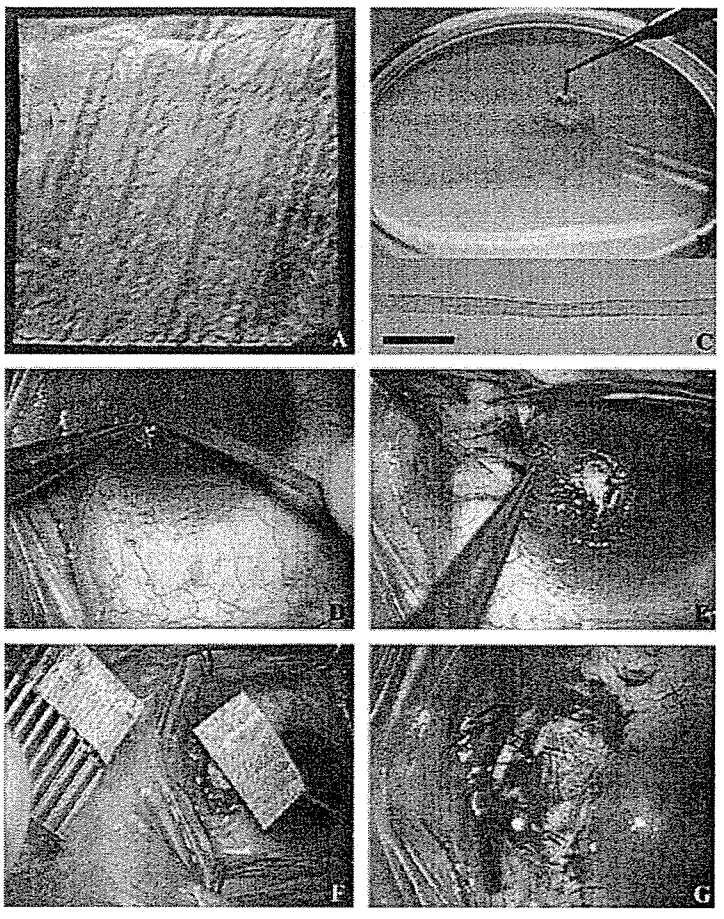


Fig. 1. (A) The sterilized, freeze-dried AM was wafer-like and thin. (B) It became smooth and relatively flexible on hydration. (C) It is a well compacted material. (Haematoxylin and eosin staining; scale bar 20 μm.) (D) In surgery, the head of the pterygium is carefully excised with forceps as bluntly as possible. (E) Subconjunctival fibrotic tissue is then excised as much as possible and the sclera is exposed. (F) FD-AM, trimmed to the appropriate size to fit the bare sclera, is then placed on the sclera. (G) The FD-AM is secured with 10–0 nylon sutures.

Table 1. Characteristics of cases and clinical outcomes.

Case	Age (years)	Gender	Diagnosis	Eye	Epithelialization at 2 weeks	Recurrence	Complications	Visual acuity		
								Preop	Postop	Follow-up (months)
1	76	M	PP	R	+	–	–	20/50	20/40	24
2	70	M	PP	R	+	–	–	20/60	20/60	20
3	58	M	PP	L	+	–	–	20/15	20/15	20
4	75	M	PP	R	+	–	–	20/25	20/25	20
5	62	M	RP	R	+	–	–	20/15	20/15	18
6	65	M	PP	R	+	–	–	20/40	20/40	16
7	55	F	PP	R	+	–	–	20/15	20/15	14
8	71	M	RP	L	+	–	–	4/100	6/100	10
9	77	F	RP	R	+	–	–	30/50	30/50	9
10	44	M	PP	R	+	–	–	20/20	20/20	9
11	67	F	RP	R	+	–	–	20/50	20/50	8
12	68	M	RP	L	+	–	–	20/20	20/20	7
13	62	F	PP	R	+	–	–	30/50	20/20	6

M = male; F = female; PP = primary pterygium; RP = recurrent pterygium.

It could be preserved at room temperature and became relatively smooth and flexible upon hydration (Fig. 1A, B). Haematoxylin and eosin staining showed it to be a compact, collagen-rich substrate (Fig. 1C).

Surgical procedures

Topical anaesthesia with 4% lignocaine was used in most patients. The head of the pterygium was first removed with blunt dissection (Fig. 1D). After extensive dissection of subconjunctival fibrotic tissue with surgical scissors to expose the sclera (Fig. 1E), the residual subconjunctival tissue was treated for 5 mins with 0.04% MMC followed by vigorous repeated washing with saline. The surrounding conjunctiva was secured onto the bare sclera with 6–0 silk sutures. Using 10–0 nylon sutures, the FD-AM was then transplanted over the bare scleral defect with the epithelial basement membrane side facing up (Fig. 1F, G). The free edge of the FD-AM was imbricated under the host conjunctiva to allow the outgrowth of conjunctival epithelium onto the FD-AM. Finally, the area of transplantation was covered with a therapeutic soft contact lens. Patients were evaluated on postoperative days 1, 3, 7, 14 and 30 and then every 3 months. The sutures were removed 2 weeks after the operation. Postoperatively, 0.3% ofloxacin and 0.1% dexamethasone were instilled four times a day for 2–3 months; this treatment was subsequently replaced with the administration of fluorometholone three to four times a day for the next few months. Recurrence was defined as any fibrovascular growth beyond the limbus onto the cornea.

Results

Our subjects included nine men and four women; their mean age was 65.4 ± 9.33 years. Of the 13 eyes, eight had primary pterygium and five had recurrent pterygium. The mean follow-up period was 13.9 ± 6.0 months. The clinical data for the 13 patients are presented in Table 1.

The FD-AM was easy to handle and could be easily sutured. The membrane's flexibility increased following hydration. Postoperatively, all transplanted FD-AMs maintained their graft integrity and were well retained. In all 13 eyes, complete conjunctival epithelialization over the transplanted FD-AM was achieved within 1–2 weeks (Fig. 2). The surrounding conjunctival inflammation began to gradually subside and slit-lamp examination confirmed that conjunctival fibrosis was successfully suppressed in all patients. We encountered no significant intra- or postoperative complications at the transplantation site and none of the 13 patients suffered loss of visual acuity. There was no recurrence of the pterygium during the follow-up period (Fig. 3). At the last follow-up visit, all transplanted FD-AMs continued to show excellent biocompatibility with the ocular surface. All treated eyes

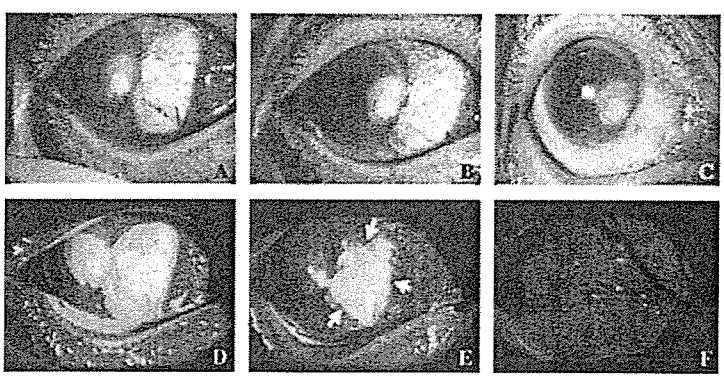


Fig. 2. Representative primary time-course slit-lamp photographs of pterygium eyes after FD-AM transplantation (case 5), taken with and without fluorescein. (A, D) At 24 hours after transplantation, the eye manifested epithelial defects at the transplanted site and host cornea. (B, E) At 72 hours post-transplantation, surrounding conjunctival epithelium gradually migrated onto the FD-AM. (C, F) At 1 week, the ocular surface including the transplanted AM was completely epithelialized and stable without defects.

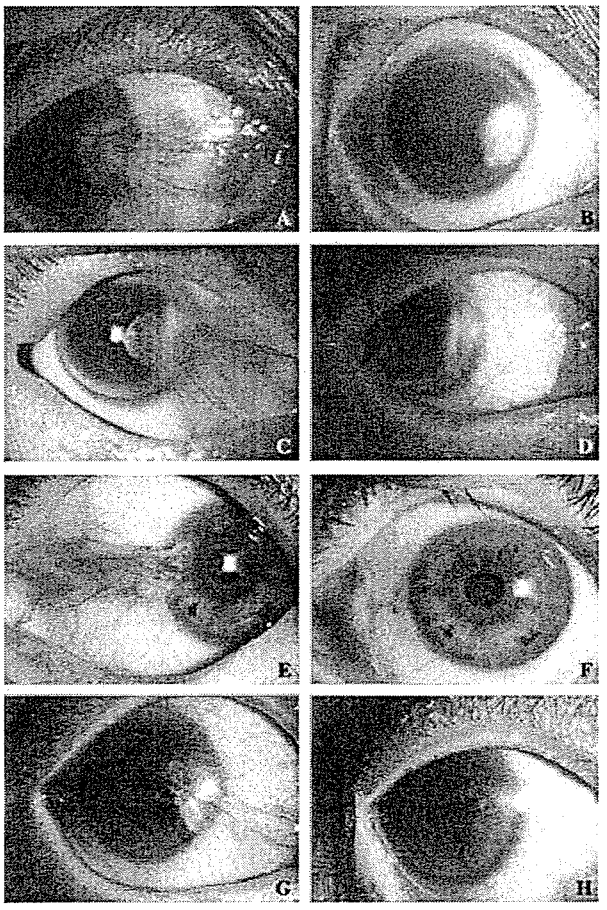


Fig. 3. Representative slit-lamp photographs taken before transplantation (A) case 1, (C) case 2, (E) case 3, (G) case 6; and at the last follow-up visit (B) case 1, (D) case 2, (F) case 3, (H) case 6. Before transplantation, the eyes manifested fibrovascular overgrowth of degenerative conjunctiva onto the cornea. At the last follow-up visit, fibrosis was markedly suppressed and the conjunctival surface was stable without inflammation or rejection.

remained stable without ocular infection or graft rejection.

Discussion

As AM possesses several beneficial characteristics such as the promotion of epithelialization and inhibition of fibrosis (Tseng et al. 1997; Meller & Tseng 1999; Lee et al. 2000), it has been successfully used in the treatment of patients with pterygium (Prabhasawat et al. 1997; Shimazaki et al. 1998; Ma et al. 2000; Shimazaki et al. 2003). Complete sterilization of the AM is of utmost importance to render it safe for clinical use. In the evaluation of FD-AM as an effective biomaterial, its biocompatibility on the human ocular surface must first be established. We previously reported that transplanted FD-AM adapted well in the corneal stroma and bare sclera of rabbits and that there was no evidence of subepithelial cell infiltration, stromal oedema, neovascularization, inflammation, or rejection (Nakamura et al. 2004a). All transplanted FD-AMs in our current clinical study also adapted well to the bare host sclera without dissolution following pterygium excision. There was no immunological rejection of the transplanted FD-AM in any of our 13 patients. These results indicate that the FD-AM we produced exhibited excellent biocompatibility with the human ocular surface and suggests that FD-AM may be used for the treatment of pterygium.

Conjunctival autografting is safe and effective. However, the procedure is more time-consuming and technically demanding to perform compared with other procedures, such as bare sclera excision. We must also point out that a wide variation in recurrence rates exists because of the differences in surgical procedure and surgeons' experience. As the superior bulbar conjunctiva is the preferred site for autograft harvesting, subsequent glaucoma surgery in these patients is associated with a high risk of failure. As such, the use of FD-AM may therefore be a useful alternative to conjunctival autograft surgery as it eliminates the need for harvesting autografts and causing iatrogenic injury to the donor site, which is particularly important when dealing with more extensive pterygium. Further evaluation of the relative efficacy of conjunctival autografts and FD-AMs, with or without adjunctive MMC, would therefore provide valuable information regarding pterygium management.

The coverage of a large area by the AM following the excision of pterygium may promote the proliferation and differentiation of the remaining conjunctival epithelium. This may have an inhibitory effect on postoperative inflammation and may reduce the risk of complications such as postoperative infections and scleral melting. The suppression of postoperative inflammation is essential for achieving successful results following pterygium surgery (Prabhasawat et al. 1997; Shimazaki et al. 1998). Complete epithelialization over the FD-AM was achieved within 2 weeks, which was similar to our previous experience with cryopreserved AM, suggesting that FD-AM may be a useful substrate for epithelium migration and proliferation. Furthermore, the large size of AM allows the extensive excision of fibrotic scar tissue. These attributes may contribute to favourable clinical results in the treatment of pterygium. In addition, from a cosmetic point of view, the outcome of this transplantation procedure was highly satisfactory.

Also important for the achievement of satisfactory clinical results in patients treated for pterygium is the suppression of postoperative fibrosis. Amniotic membrane exerts antiscarring effects via the transforming growth factor-β pathway (Tseng et al. 1999). Although FD-AM may lose several characteristics during the freeze-drying process, FD-AM combined with MMC treatment strongly suppressed postoperative fibrosis in the transplantation area. In our current study, we

were unable to determine the exact efficacy of FD-AM in prevention of inflammation and pterygium recurrence. Treatment with MMC alone is effective in reducing pterygium recurrence and postoperative topical steroids would also reduce postoperative inflammation and scarring. In view of these findings, further studies comparing the use of FD-AM alone, FD-AM with MMC and cryopreserved AM, with or without MMC, are needed to clarify these points.

We found that, in our patients, the transplanted area had minimal inflammation and scarring, suggesting that our procedures can be used in patients with severe cicatricial diseases requiring conjunctival reconstruction, such as Stevens–Johnson syndrome or chemical injury. We are in the process of adapting our technique to treat patients with ocular surface disease with severe symblepharon that requires fornix reconstruction. In addition, we are also using this biomaterial as a substrate for cultivated corneal and oral epithelial sheets for ocular surface reconstruction (Koizumi et al. 2001; Nakamura et al. 2004b).

In conclusion, ours is the first demonstration of the excellent biocompatibility of FD-AM for clinical use. Freeze-dried AM offers important advantages over cryopreserved AM because it is free of contamination, may be easily transported and stored, and can be used readily with minimal preparation at the time of surgery. Although the longterm and comparative clinical results of these grafts await confirmation, this modality of treatment may be a useful alternative to conjunctival graft or cryopreserved AM surgery for the treatment of pterygium.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labour and Welfare (H16-Saisei-007) and the Japanese Ministry of Education, Culture, Sports, Science and Technology 14657450, a research grant from the Kyoto Foundation for the Promotion of Medical Science, and the Intramural Research Fund of Kyoto Prefectural University of Medicine.

References

Adds PJ, Hunt C & Hartley S (2001): Bacterial contamination of amniotic membrane. Br J Ophthalmol **85**: 228–230.

Akle CA, Adinolfi M, Welsh KI, Leibowitz S & McColl I (1981): Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. Lancet **2**: 1003–1005.

Ang LPK, Tan DTH, Cajucom-Uy H, Phan TT, Beurman R & Lavker R (2005): Autologous cultivated conjunctival transplantation for pterygium surgery. Am J Ophthalmol **139**: 611–619.

Hao Y, Ma DH, Hwang DG, Kim WS & Zhang F (2000): Identification of antiangiogenic and anti-inflammatory proteins in human amniotic membrane. Cornea **19**: 348–352.

van Herendael BJ, Oberti C & Brosens I (1978): Microanatomy of the human amniotic membrane: a light microscopic, transmission and scanning microscopic study. Am J Obstet Gynecol **83**: 470–477.

Kim JS, Kim JC, Na BK, Jeong JM & Song CY (2000): Amniotic membrane patching promotes healing and inhibits proteinase activity on wound healing following acute corneal alkali burn. Exp Eye Res **70**: 329–337.

Koizumi N, Inatomi T, Suzuki T, Sotozono C & Kinoshita S (2001): Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. Ophthalmology **108**: 1569–1574.

Lam DS, Wong AK, Fan DS et al. (1998): Intraoperative mitomycin C, to prevent recurrence of pterygium after excision: a 30-month follow-up study. Ophthalmology **105**: 901–904.

Lee SB, Li DQ, Tan DT, Meller DC & Tseng SC (2000): Suppression of TGF-â signalling in both normal conjunctival fibroblasts and pterygial body fibroblasts by amniotic membrane. Curr Eye Res **20**: 325–334.

Lewallen S (1989): A randomized trial of conjunctival autografting for pterygium in the tropics. Ophthalmology **96**: 1612–1624.

Ma DH, See LC, Liau SB & Tsai RJ (2000): Amniotic membrane graft for primary pterygium: comparison with conjunctival autograft and topical mitomycin C treatment. Br J Ophthalmol **84**: 973–978.

Manning CA, Kloess PM, Diaz MD & Yee RW (1997): Intraoperative mitomycin in primary pterygium excision. A prospective randomized trial. Ophthalmology **104**: 844–848.

Meller D & Tseng SC (1999): Conjunctival epithelial cell differentiation on amniotic membrane. Invest Ophthalmol Vis Sci **40**: 878–886.

Mutlu FM, Sobaci G, Tatar T & Yildirim E (1999): A comparative study of recurrent pterygium surgery: limbal conjunctival autograft transplantation versus mitomycin C with conjunctival fiap. Opthalmology **106**: 817–821.

Nakamura T, Inatomi T, Sotozono C, Amemiya T, Kanamura N & Kinoshita S (2004b): Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. Br J Ophthalmol **88**: 1280–1284.

Nakamura T, Yoshitani M, Rigby H et al. (2004a): Sterilized, freeze-dried amniotic membrane: a useful substrate for ocular surface reconstruction. Invest Ophthalmol Vis Sci **45**: 93–99.

Prabhasawat P, Barton K, Burkett G & Tseng SC (1997): Comparison of conjunctival autografts, amniotic membrane grafts, and primary closure for pterygium excision. Ophthalmology **104**: 974–985.

Shimazaki J, Kosaka K, Shimmura S & Tsubota K (2003): Amniotic membrane transplantation with conjunctival autograft for recurrent pterygium. Ophthalmology **110**: 119–124.

Shimazaki J, Shinozaki N & Tsubota K (1998): Transplantation of amniotic membrane and limbal autograft for patients with recurrent pterygium associated with symblepharon. Br J Ophthalmol **82**: 235–240.

Solomon A, Pires RT & Tseng SC (2001b): Amniotic membrane transplantation after extensive removal of primary and recurrent pterygia. Ophthalmology **108**: 449–460.

Solomon A, Rosenblatt M, Monroy D, Ji Z, Pflugfelder SC & Tseng SC (2001a): Suppression of interleukin-1â and interleukin-1â in human limbal epithelial cells cultured on the amniotic membrane stromal matrix. Br J Ophthalmol **85**: 444–449.

Tseng SC, Li DQ & Ma X (1999): Suppression of transforming growth factor-â isoforms, TGF-â receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. J Cell Physiol **179**: 325–335.

Tseng SC, Prabhasawat P & Lee SH (1997): Amniotic membrane trasplatation for conjuntival surface reconstruction. Am J Opthalmol **124**: 765–774.

von Versen-Hoynck F, Syring C, Bachmann S & Moller DE (2004): The influence of different preservation and sterilization steps on the histological properties of amnion allografts – light and scanning electron microscopic studies. Cell Tissue Bank **5**: 45–56.

Received on June 8th, 2005.
Accepted on December 21st, 2005.

Correspondence:
Takahiro Nakamura MD, PhD
Department of Ophthalmology
Kyoto Prefectural University of Medicine
Kawaramachi Hirokoji
Kamigyo-ku
Kyoto 602-0841
Japan
Tel: + 81 75 251 5578
Fax: + 81 75 251 5663
Email: tnakamur@ophth.kpu-m.ac.jp

Transplantation of Autologous Serum-Derived Cultivated Corneal Epithelial Equivalents for the Treatment of Severe Ocular Surface Disease

Takahiro Nakamura, MD, PhD,[1,2] *Tsutomu Inatomi, MD, PhD,*[1] *Chie Sotozono, MD, PhD,*[1] *Leonard P. K. Ang, MD,*[1] *Noriko Koizumi, MD, PhD,*[1,2] *Norihiko Yokoi, MD, PhD,*[1] *Shigeru Kinoshita, MD, PhD*[1]

Purpose: To evaluate the use of autologous serum (AS)-derived cultivated corneal epithelial transplantation for the treatment of severe ocular surface disease.

Design: Retrospective noncomparative case series.

Participants: Nine eyes from 9 patients with total limbal stem cell deficiency were studied. These consisted of 2 eyes with Stevens–Johnson syndrome, 1 with chemical injury, 1 with ocular cicatricial pemphigoid, 1 with Salzmann corneal dystrophy, 1 with aniridia, 1 with graft-versus-host disease, and 2 with idiopathic ocular surface disease.

Methods: Autologous serum obtained from patients was used for cultivating corneal epithelial cells on an amniotic membrane substrate. These AS-derived corneal epithelial equivalents were compared with those derived from fetal bovine serum (FBS)-supplemented medium. At the time of surgery, complete removal of the corneal pannus and conjunctiva up to 3 mm from the limbus was performed. Allogeneic (7 cases) and autologous (2 cases) AS-derived cultivated corneal epithelial equivalents were transplanted onto the ocular surface. Postoperative follow-up included serial slit-lamp examinations with fluorescein staining, as well as photographic documentation.

Main Outcome Measures: Ocular surface reconstruction with corneal epithelialization, graft integrity, visual acuity, and postoperative complications.

Results: The corneal epithelial sheets cultivated in AS- and FBS-supplemented media were morphologically similar, and demonstrated the normal expression of tissue-specific keratins and junctional specialization assembly proteins. After transplantation, complete corneal epithelialization was achieved within 2 to 5 days. All eyes demonstrated an improvement in visual acuity by ≥2 lines. During the follow-up period of 14.6±4.36 months, the corneal surface of all patients remained stable and transparent, without significant complications.

Conclusions: Transplantation of AS-derived cultivated corneal epithelial equivalents was shown to be a feasible method of treating patients with severe ocular surface disease. The use of AS is of clinical importance in the development of autologous xenobiotic-free bioengineered ocular surface equivalents for clinical transplantation. *Ophthalmology 2006;113:1765–1772 © 2006 by the American Academy of Ophthalmology.*

Severe ocular surface disease, such as Stevens–Johnson syndrome, ocular cicatricial pemphigoid and, chemical injury, are devastating conditions that represent a major clinical challenge. In such cases, the corneal epithelial stem cells in the corneal limbus are destroyed, and invasion of the corneal surface by the neighboring conjunctival epithelium results in neovascularization, chronic inflammation, ingrowth of fibrous tissue, and stromal scarring.[1–3] Patients with these conditions do poorly with conventional corneal transplantation. Ocular surface reconstruction and corneal epithelial replacement by means of keratoepithelioplasty and limbal transplantation, often combined with amniotic

Originally received: October 12, 2005.
Accepted: April 24, 2006. Manuscript no. 2005-982.

[1] Department of Ophthalmology, Kyoto Prefectural University of Medicine, Graduate School of Medicine, Kyoto, Japan.

[2] Research Center for Regenerative Medicine, Doshisha University, Kyoto, Japan.

Supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labor, and Welfare, Tokyo, Japan (no.: H16-Saisei-007), and Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan (Kobe Translational Research Cluster); a research grant from the Kyoto Foundation for the Promotion of Medical Science, Kyoto, Japan; and the Intramural Research Fund of Kyoto Prefectural University of Medicine.

The authors have no financial interest in this work.

Correspondence to Shigeru Kinoshita, MD, PhD, Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kamigyo-ku, Kyoto 602-0841 Japan. E-mail: shigeruk@ophth.kpu-m.ac.jp.

ISSN 0161-6420/06/$–see front matter
doi:10.1016/j.ophtha.2006.04.030

Table 1. Characteristics of Cases

Age/Gender	Disease	Eye	Donor	Days to Reach Confluence	Quality of Culture Sheet	Previous Surgery
33/F	SJS	Right	Allo	6	Excellent	–
27/M	SJS	Left	Allo	7	Excellent	COET
81/F	Idiopathic	Right	Allo	8	Good	–
49/M	Chemical	Right	Auto	6	Excellent	–
70/M	Idiopathic	Right	Auto	9	Excellent	Ptosis
42/F	Salzmann	Left	Allo	6	Excellent	–
71/M	OCP	Left	Allo	8	Excellent	–
41/F	Aniridia	Right	Allo	7	Excellent	–
47/M	GVHD	Right	Allo	7	Excellent	–

ALKP = automated lamellar keratoplasty; Allo = allologenic cultivated corneal epithelial transplantation; AMT = amniotic membrane transplantation; defect; F = female; GVHD = graft-versus-host disease; HM = hand motion; IOL = intraocular lens; M = male; OCP = ocular cicatricial pemphigoid;

membrane (AM) transplantation, have been effectively used to treat these severely damaged eyes.[4–8]

More recently, cultivated corneal epithelial stem cell transplantation has been shown to be a promising treatment modality in the management of severe limbal stem cell deficiency.[9–15] The use of fetal bovine serum (FBS)-supplemented media remains the media of choice for the ex vivo expansion of cells in corneal epithelial equivalents. The use of bovine material for cultivating epithelial cells for clinical use is an important health and safety issue; its use is associated with the risk of transmission of zoonotic infection (e.g., bovine spongiform encephalitis) and other unknown pathogens. Because bovine spongiform encephalitis cannot be detected by any known in vitro assay, the use of FBS in the culture medium is a major concern worldwide. The use of human autologous serum (AS) as an alternative to FBS is therefore significantly advantageous because it eliminates the need for bovine material in the culture process and reduces the risk of transmission of disease.

We previously showed that human serum was able to support the in vitro and in vivo proliferation of epithelial cells.[16] In our study, we evaluated the use of AS in developing cultivated corneal epithelial equivalents and compared it with conventional FBS supplemented cultures. These AS-derived cultivated epithelial equivalents were used in the treatment of patients with severe limbal stem cell deficiency. To our knowledge, a study of this nature has not been previously reported. This study has important clinical implications and provides the basis for developing safer autologous bioengineered tissues for clinical transplantation.

Materials and Methods

Subjects

All experimental procedures and clinical applications introduced here were approved by the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine; prior informed consent was obtained from all patients in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

The study included 9 eyes from 9 consecutive patients with severe ocular surface disease that underwent ocular surface reconstruction with AS-derived cultivated corneal epithelial equivalents between February 2004 and April 2005. Seven eyes underwent allogeneic transplantation and 2 eyes underwent autologous transplantation. There 5 male and 4 female patients; their ages ranged from 27 to 81 years (mean 51.2±18.6 years). All were diagnosed as totally stem cell–deficient on the basis of complete disappearance of the palisade of Vogt and 360 degrees of conjunctivalization. The preoperative diagnosis was Stevens–Johnson syndrome (n = 2), ocular cicatricial pemphigoid (n = 1), chemical injury (n = 1), Salzmann corneal dystrophy (n = 1), aniridia (n = 1), graft-versus-host disease (n = 1), and idiopathic ocular surface disorder of unknown etiology (n = 2). Preoperatively, all 9 eyes manifested severe destruction of the ocular surface with total limbal stem cell deficiency. Tear production was diminished but not absent in all patients, as evidenced by the presence of a tear meniscus level with diminished tear film breakup time. All patients were followed for a minimum of 6 months.

Cultivation of Corneal Epithelial Sheet

Harvesting and Preparation of Autologous Serum. Under aseptic conditions, AS was obtained from each patient by venesection at the antecubital fossa. Thirty milliliters of blood were collected into a sterile container, centrifuged, filtered, and the resultant serum (approximately 10 ml) was purified. Each patient's serum was stored in sterile tubes at −30° C before use.

Preparation of Cultivated Corneal Epithelial Equivalent. We cultured human corneal epithelial cells using a previously reported system.[12,17–21] For allogeneic corneal epithelial cultures, human corneal limbal tissues from Northwest Lion Eye Bank (Seattle, WA) were incubated at 37° C for 1 hour with 1.2 IU neutral protease (Dispase, Basel, Switzerland) to separate the epithelial cells. The resultant corneal epithelial cells (1×10^5 cells/well) were then seeded onto denuded AM spread on the bottom of culture inserts, and cocultured with mitomycin C–inactivated 3T3 fibroblasts (2×10^4 cells/cm^2). For autologous corneal epithelial cultures, a corneal limbal epithelial explant from a 3-mm^2 biopsy of the uninjured eye was placed directly on a denuded AM carrier. The culture medium consisted of a proprietary defined keratinocyte growth medium (kindly provided by ArBlast Co. Ltd., Kobe, Japan), which included several growth factors and hormones, without any animal-derived material, supplemented with 5% AS. The cultured cells were submerged in medium for 2 weeks and then exposed to air by lowering the medium level (airlifting) for 1 to 2 days. Cultures were incubated at 37° C in a 5% CO_2–95% air incubator, with medium change carried out every day. The AS-derived corneal epithelial equivalents were compared with corre-

and Clinical Outcome

Combined Surgery	Epithelialization (2–5 days)	Visual Acuity		Complication	Follow-up (mos)
		Before	*After*		
AMT	+	20/100	20/32		20
–	+	HM	20/320	ED	19
AMT	+	HM	20/100		18
–	+	CF	20/125		16
–	+	CF	20/630		14
–	+	20/2000	20/125	ED	14
PEA + IOL	+	20/63	20/25		13
ALKP + PEA + IOL	+	CF	20/200		11
AMT + PEA + IOL	+	HM	20/32		6

Auto = autologous cultivated corneal epithelial transplantation; CF = count finger; COET = cultivated oral epithelial transplantation; ED = epithelial PEA = phacoemulsification and aspiration; SJS = Stevens–Johnson syndrome.

sponding corneal epithelial equivalents prepared in FBS-supplemented medium.

Morphologic Examination of Cornea Epithelial Equivalents

The morphologic examination of the cultivated epithelial equivalents was performed on additional epithelial sheets that were cultivated in AS- and FBS-supplemented media. Based on the histologic appearance of the culture sheet, we graded the quality of the cultivated epithelial sheet as follows: excellent = entire sheet demonstrated normal cell differentiation and stratification, maintenance of the cuboidal basal layer, and good adhesion to the underlying AM substrate; good = >80% of the epithelial sheet demonstrated normal cell differentiation and stratification, with some areas that were less adherent; or average = between 50% and 80% of the epithelial sheet demonstrated normal cell differentiation and stratification, with basal cells adopting a more columnar or flattened appearance, and areas that were detached from the AM substrate.

Cryostat sections (7 μm thick) were placed on gelatin-coated slides, air dried, then rehydrated in phosphate-buffered saline at room temperature for 15 minutes. Morphologic analysis was performed by staining with hematoxylin and eosin. Immunohistochemical studies of cell junction specialization–related proteins and tissue-specific keratins in corneal epithelial sheets cultivated using AS or FBS were carried out after our previously described method.[21,22] To block nonspecific binding, the tissues were incubated with 2% bovine serum albumin at room temperature for 30 minutes. Subsequently, the sections were incubated at room temperature for 1 hour with primary antibodies to keratin 12 (Transgenic, Kumamoto, Japan), ZO-1 (Zymed, San Francisco, CA), desmoplakin (Progen, Heidelberg, Germany), integrin α6 (Chemicon, Temecula, CA), and collagen 7 (Chemicon). Control incubations were with the appropriate normal mouse and rabbit IgG (Dako, Kyoto, Japan) at the same concentration as the primary antibody, and omission of the primary antibody for the respective specimen. After staining with the primary antibody, the sections were incubated at room temperature for 1 hour with appropriate secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). The sections were coverslipped using antifading mounting medium containing propidium iodide (Vectashield; Vector, Burlingame, CA) and examined by confocal microscopy (Olympus Fluoview, Tokyo, Japan).

Surgical Procedure

We performed a 360-degree conjunctival peritomy 3 mm from the limbus, and removed all perilimbal scarred or inflamed subconjunctival tissue down to bare sclera. The corneal pannus was completely removed by blunt dissection or superficial keratectomy using surgical scissors or a blade. We then treated the residual subconjunctival tissue with 0.04% mitomycin C for 5 minutes, followed by vigorous repeated washing with saline.[12] The cultivated corneal epithelial sheet was placed over the corneal surface and secured in place with 10-0 nylon sutures at the limbus. The integrity of the cultivated epithelium was confirmed by fluorescein staining at the end of surgery, and the ocular surface was protected with a medical-use bandage contact lens.

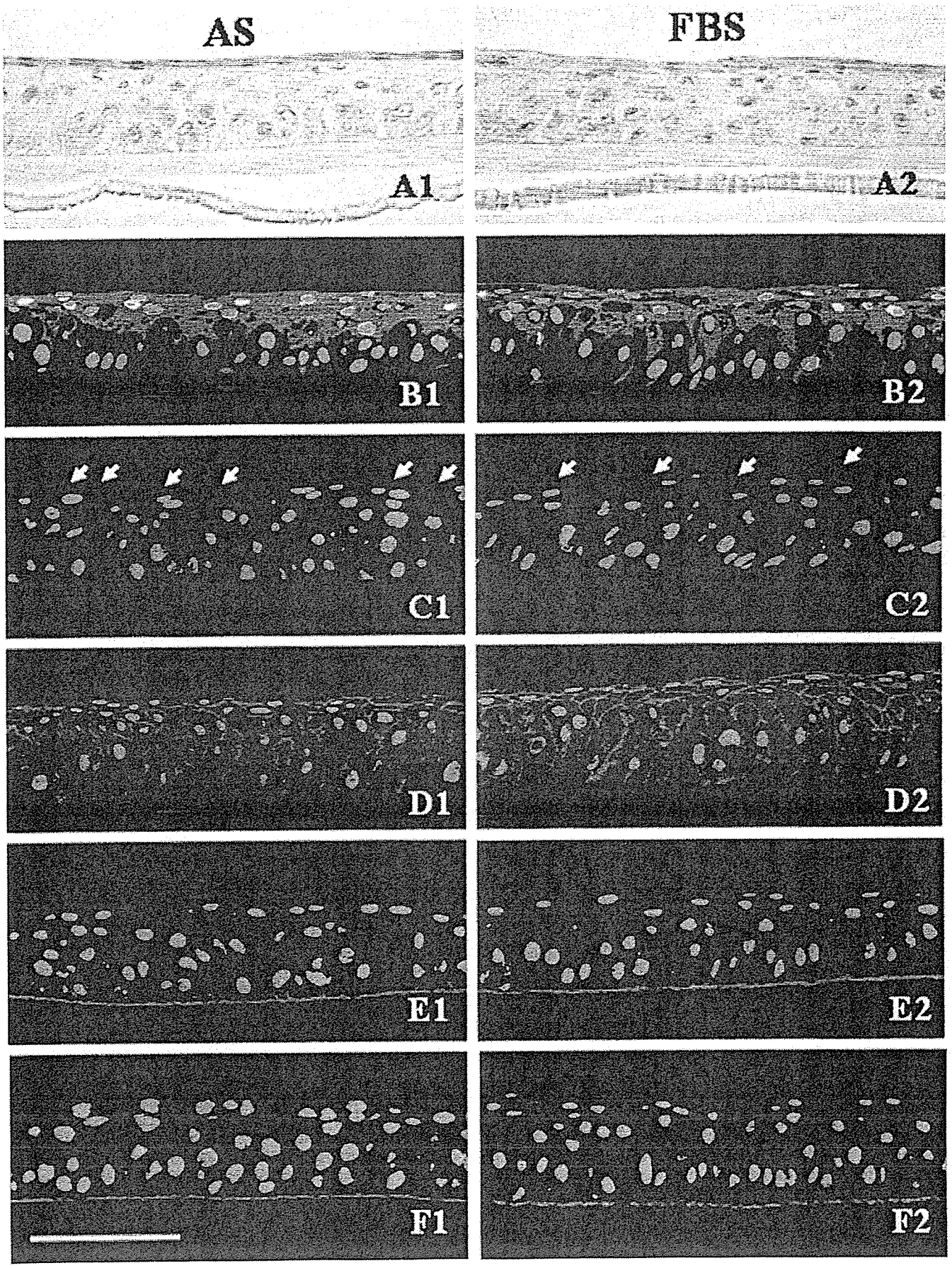
Postoperatively, 0.3% ofloxacin and 0.1% dexamethasone eye drops were instilled 4 times a day. The doses were tapered to a maintenance dose of 2 to 3 times a day after 2 to 3 months, depending on the severity of inflammation. For allogeneic transplantation, systemic betamethasone (1 mg/day), cyclophosphamide (50 mg/day), and cyclosporine (100 mg/day) were administered orally to reduce postoperative inflammation, scarring, and allograft rejection. Renal and liver functions were monitored periodically. Cyclophosphamide and cyclosporine were discontinued between 1 and 3 months after surgery.

Results

Morphology of Cultivated Corneal Epithelial Equivalent

The baseline data regarding the corneal epithelial culture are summarized in Table 1. Within 5 to 8 days in culture, a confluent sheet of corneal epithelial cells was established on the AM substrate. After 2 weeks, the cultivated corneal epithelium consisted of 4 to 6 cell layers and was composed of a well-conserved basal layer formed by cuboidal cells and several suprabasal wing and flat layers. In 8 of 9 instances, the quality of the cultivated epithelial sheets was excellent. Hematoxylin and eosin staining showed that the histology of corneal epithelial cells cultivated in AS- and FBS-supplemented media were comparatively similar (Fig 1A1, A2).

The expression patterns of tissue-specific keratins and cell junctional specialization-related proteins in cultivated corneal epithelium were similar between AS- and FBS-derived corneal epithelial equivalents. In the AS-cultivated corneal epithelial sheet, the cornea-specific keratin 12 was expressed in the superficial and intermediate layers, with less discernible immunostaining in the basal cell layers (Fig 1B1, B2). ZO-1, which is a tight junction-related component, was expressed in the apical surface of cultivated epithelial sheet (Fig 1C1, C2). Desmoplakin, which is a cell–cell junction component, was expressed in the cell membrane of epithelial cells (Fig 1D1, D2). The basement mem-


AS
FBS
A1
A2
B1
B2
C1
C2
D1
D2
E1
E2
F1
F2

brane assembly proteins, integrin α6 and collagen 7, showed linear positive staining on the basement membrane side of basal cells (Fig 1E1–F2).

Clinical Results

The clinical data and surgical outcomes of the 9 patients are also summarized in Table 1. Before transplantation, all eyes manifested severe destruction of the ocular surface, with severe limbal stem cell deficiency (Fig 2, left column). Two to 5 days after transplantation, the corneal surface in all treated eyes was clear and smooth, and fluorescein staining confirmed that the entire corneal surface was covered by the cultivated corneal epithelium (Fig 2, middle column). The transplanted epithelial sheet was surrounded 360 degrees by conjunctival epithelial defects, indicating no contamination of the host conjunctival epithelium. Within a short time after surgery, the conjunctival inflammation was found to have rapidly subsided in all patients.

Patients were followed for 14.6±4.4 months, with the longest follow-up being 20 months. Successful engraftment was achieved in all patients with none of the grafts sloughing off. Slit-lamp examination showed that conjunctival fibrosis was successfully suppressed in all patients, with no conjunctival invasion onto the corneal surface throughout the follow-up period (Fig 2, right column). Visual acuity improved in all eyes and all were restored to good vision (postoperative visual acuity improved by ≥2 lines) at the last follow-up visit. During the follow-up period, 2 patients (cases 2 and 6) developed a small paracentral and peripheral epithelial defect, measuring approximately 2×2 mm, with mild cellular infiltration at the edge and base of the defect. This was treated as a low-grade bacterial infection with topical antibiotic medication (ofloxacin and cefmenoxime eye drops), and the epithelial defect and infiltrate gradually resolved within 2 to 3 weeks.

At the last follow-up visit, the ocular surface of the eyes with surviving transplanted cultivated epithelium remained stable without any epithelial defects. There were no significant postoperative complications and none of the patients developed graft rejection.

Discussion

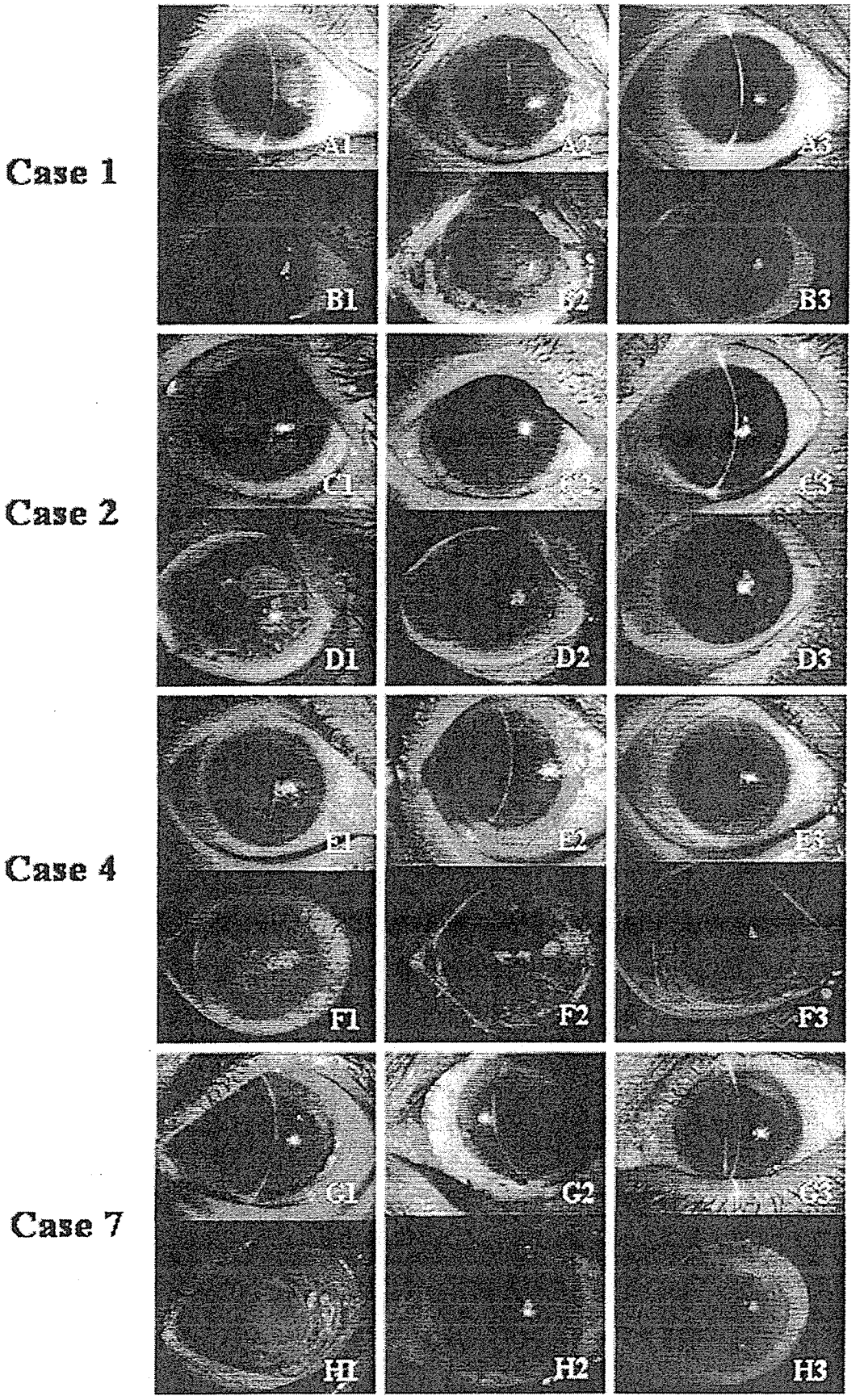
Cultivated corneal epithelial transplantation has been shown to be effective in the treatment of severe ocular surface disease. Successful epithelial cell propagation, using FBS-supplemented medium, was first described by Rheinwald and Green.[23] Since then, the use of FBS-supplemented media remains the most widely used method for cultivating ocular surface epithelial cells.[9–14,24,25] In establishing tissue equivalents for transplantation, the ideal culture condition is one that is safe from disease transmission, as well as being able to recapitulate the tissue of origin. We evaluated the biological and clinical aspects of these AS-derived corneal epithelial equivalents, and showed that they were comparable to current established methods using FBS-supplemented cultures. We describe for the first time the effective use of AS-derived cultivated corneal epithelial transplantation for the treatment of severe ocular surface disease. The novel approach of using AS as an alternative to FBS in the culture process represents a significant advancement in the development of safer bioengineered ocular surface tissues for clinical use.

A critical issue in the derivation of tissue equivalents is the ability to recapitulate a differentiated epithelial equivalent bearing the morphologic characteristics of the original tissue. Previous investigators have almost exclusively relied on FBS-supplemented medium for culturing corneal epithelial cells and developing corneal epithelial equivalents that may be used for clinical transplantation.[9–14,24,25] It is particularly important for AS-derived corneal epithelial equivalents to be able to achieve comparable results to that of FBS-derived tissues. In our study, we demonstrated that the histologic appearance of AS-derived cultivated corneal epithelium was similar to that of normal in vivo cornea as well as FBS-derived cultures in terms of cellular morphology, degree of stratification, number of cell layers, and the presence of a cuboidal basal cell layer. Immunohistochemical analyses also demonstrated the normal expression pattern of the differentiation-related proteins.

The ocular surface is exposed to significant shearing stresses from blinking and eye movement. Maintaining graft integrity is a critical factor for ensuring successful engraftment after transplantation. The AS-derived corneal epithelial equivalents not only demonstrated the presence of cell-to-cell junction–related proteins (ZO1, desmoplakin), but also the formation of basement membrane–related proteins (integrin α6 and collagen 7). These are important for ensuring graft integrity during surgical manipulation and after transplantation. These findings were similar to FBS-supplemented cultures, confirming that AS supported normal epithelial cell proliferation and differentiation and could be used safely and effectively in the development of corneal epithelial equivalents. In our study, the cultivated corneal epithelial sheet demonstrated good cell-to-substrate adhesion; successful engraftment was achieved in all patients.

The ability of transplanted corneal equivalents to continue to regenerate and replenish the corneal epithelial surface is of utmost importance when evaluating its use for clinical transplantation. Our previous experience with cultivated corneal epithelial transplantation has confirmed the long-term efficacy of this procedure for the treatment of severe ocular surface disease.[12,17–20] These cultivated corneal epithelial sheets were obtained with FBS-supplemented cultures. After transplantation, complete epithelialization

Figure 1. Representative light micrographs showed that the cultivated corneal epithelial sheet using autologous serum (AS)– and fetal bovine serum (FBS)–supplemented media had 4 to 6 layers of stratified, well-differentiated cells and the histology of these sheets were comparatively similar (A1, A2). Representative immunohistochemical results showed that cornea-specific keratins 12 (B1, B2) were expressed in the superficial and intermediate layers, with less discernible immunostaining in the basal cell layers. Cell-to-cell junctional assemblies, such as ZO1 (C1, C2) and desmoplakin (D1, D2), were expressed at the apical surface and cell membrane of cultivated corneal epithelia sheets, respectively. We observed the linearly positive staining of integrin α6 (E1, E2) and collagen 7 (F1, F2) on the basement membrane side of corneal epithelial cells. Expression pattern of these proteins were similar between cultivated epithelial sheet using AS- and FBS-supplemented culture system. Scale bar, 100 μm.


Case 1
Case 2
Case 4
Case 7
B1
B2
B3
D1
D2
D3
F1
F2
F3
H1
H2
H3

could be achieved within 2 to 5 days. In this study, we performed ocular surface reconstruction by transplanting allogeneic or autologous AS-derived cultivated corneal epithelial cells to evaluate their efficacy in the treatment of severe ocular surface disease. We found that epithelial cells cultivated using AS could survive and completely epithelialize the cornea within 2 to 5 days after transplantation. The corneal surface of all eyes in the current study remained reasonably clear and smooth, and the entire corneal surface was covered with transplanted epithelium at the last follow-up visit. Although this was a noncomparative clinical study, based on our previous clinical experience, we could find no significant differences between the clinical results of AS- and FBS-derived corneal epithelial transplantation, suggesting that the AS culture system is a safe and effective procedure for ocular surface reconstruction in patients with severe ocular surface disease.

To increase the likelihood of long-term tissue regeneration, it is important that stem or progenitor cells are retained in the cultivated corneal epithelial sheet, as these cells have greater proliferative potential. There is currently no available direct method for determining the exact proportion of stem cells in transplanted tissue equivalents. The ability of transplanted cells to continue to regenerate the ocular surface after prolonged periods is an indirect indicator of the long-term proliferative potential of transplanted cells. Based on the positive clinical findings and the stability of the ocular surface in our patients, up to the longest follow-up period of 20 months, it may be reasonable to assume that cells possessing long-term regenerative potential were present in the original transplanted corneal epithelial sheet. This would suggest that AS was able to maintain and possibly even support the proliferation of stem or progenitor cells in the culture process. This is an important issue that is the subject of ongoing research.

In conclusion, this is the first study that demonstrates the use of AS-derived cultivated corneal epithelial transplantation for ocular surface reconstruction in the treatment of severe limbal stem cell deficiency. The use of AS as an alternative to FBS offers significant advantages because it helps to eliminate the use of animal material from the culture process, thereby reducing the risk of transmission of zoonotic infection. These findings bring us one step closer to the development of a safe and effective xenobiotic-free bioengineered tissue equivalent for clinical transplantation. Although the long-term survival of these grafts has yet to be determined, these findings are an important advancement in the field of ocular surface bioengineering for the treatment of patients with severe ocular surface disease.

References

1. Shapiro MS, Friend J, Thoft RA. Corneal re-epithelialization from the conjunctiva. Invest Ophthalmol Vis Sci 1981;21: 135–42.
2. Dua H, Forrester JV. The corneoscleral limbus in human corneal epithelial wound healing. Am J Ophthalmol 1990;110:646–56.
3. Tsai RJF, Sun TT, Tseng SCG. Comparison of limbal and conjunctival autograft transplantation in corneal surface reconstruction in rabbits. Ophthalmology 1990;97:446–55.
4. Thoft RA. Keratoepithelioplasty. Am J Ophthalmol 1984;97: 1–6.
5. Kenyon KR, Tseng SCG. Limbal autograft transplantation for ocular surface disorders. Ophthalmology 1989;96:709–22; discussion 722–3.
6. 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.
7. Shimazaki J, Yang HY, Tsubota K. Amniotic membrane transplantation for ocular surface reconstruction in patients with chemical and thermal burns. Ophthalmology 1997;104: 2068–76.
8. 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.
9. 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.
10. 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.
11. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. Cornea 2000;19:421–6.
12. Koizumi N, Inatomi T, Suzuki T, et al. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. Ophthalmology 2001;108:1569–74.
13. Rama P, Bonini S, Lambiase A, et al. Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. Transplantation 2001;72:1478–85.
14. Grueterich M, Espana EM, Touhami A, et al. Phenotypic study of a case with successful transplantation of ex vivo expanded human limbal epithelium for unilateral total limbal stem cell deficiency. Ophthalmology 2002;109:1547–52.
15. Shimazaki J, Aiba M, Goto E, et al. Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders. Ophthalmology 2002;109:1285–90.
16. Ang LPK, Tan DT, Seah CJ, Beuerman RW. The use of human serum in supporting the in vitro and in vivo proliferation of human conjunctival epithelial cells. Br J Ophthalmol 2005;89:748–52.

Figure 2. Before transplantation, all eyes manifested severe destruction of the ocular surface with limbal stem cell deficiency (**left column**). Two to 4 days after transplantation, the corneal surface of all treated eyes was clear and smooth; fluorescein staining confirmed that the entire corneal surface was covered by the cultivated epithelium (**middle column**). The transplanted epithelial sheet was surrounded 360 degrees by conjunctival epithelial defects, indicating that there was no contamination of the host conjunctival epithelium. Slit-lamp examination showed that conjunctival fibrosis was successfully suppressed in all patients and the ocular surface of the eyes with surviving transplanted cultivated epithelium remained stable without any epithelial defects at the last follow-up visit (**right column**).

17. Koizumi N, Inatomi T, Suzuki T, et al. Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome. Arch Ophthalmol 2001;119:298–300.
18. Koizumi N, Cooper LJ, Fullwood NJ, et al. An evaluation of cultivated corneal limbal epithelial cells using cell suspension culture. Invest Ophthalmol Vis Sci 2002;43:2114–21.
19. Nakamura T, Koizumi N, Tsuzuki M, et al. Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease. Cornea 2003;22:70–1.
20. Nakamura T, Inatomi T, Sotozono C, et al. Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. Acta Ophthalmol Scand 2004;82;468–71.
21. Nakamura T, Endo K, Cooper LJ, et al. The successful culture and autologous transplantation of rabbit oral mucosal epithelial cells on amniotic membrane. Invest Ophthalmol Vis Sci 2003;44:106–16.
22. Nakamura T, Nishida K, Dota A, et al. Elevated expression of transglutaminase 1 and keratinization-related proteins in conjunctiva in severe ocular surface disease. Invest Ophthalmol Vis Sci 2001;42:549–56.
23. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 1975;6:331–43.
24. Nishida K, Yamato M, Hayashida Y, et al. Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. Transplantation 2004;77:379–85.
25. Sangwan VS, Matalia HP, Vemuganti GK, et al. Early results of penetrating keratoplasty after cultivated limbal epithelium transplantation. Arch Ophthalmol 2005;123:334–40.

Autologous Serum–Derived Cultivated Oral Epithelial Transplants for Severe Ocular Surface Disease

Leonard P. K. Ang, FRCS, MRCOphth; Takahiro Nakamura, MD, PhD; Tsutomu Inatomi, MD, PhD; Chie Sotozono, MD, PhD; Noriko Koizumi, MD, PhD; Norihiko Yokoi, MD, PhD; Shigeru Kinoshita, MD, PhD

Objective: To evaluate the use of autologous serum (AS)–derived cultivated oral epithelial transplants for the treatment of severe ocular surface disease.

Methods: We used AS from 10 patients with severe ocular surface disease and total limbal stem cell deficiency to develop autologous cultivated oral epithelial equivalents. These were compared with epithelial equivalents derived from conventional fetal bovine serum–supplemented medium. Surgery involved removal of the corneal pannus and surrounding diseased tissue and transplantation of the AS-derived epithelial equivalents. The oral equivalents were analyzed by review of histologic and immunohistochemical findings.

Results: Oral epithelial sheets cultivated in AS- and fetal bovine serum–supplemented media were similar in morphology, and both formed basement membrane assembly proteins important for maintaining graft integrity. Complete corneal epithelialization was achieved within 2 to 5 days postoperatively. The ocular surface remained stable without major complications in all eyes during a mean±SD follow-up of 12.6±3.9 months. The visual acuity improved by more than 2 lines in 9 of 10 eyes, with transplanted oral epithelium surviving up to 19 months.

Conclusion: The successful use of an AS-derived oral epithelial equivalent to treat severe ocular surface disease represents an important advance in the pursuit of completely autologous xenobiotic-free bioengineered ocular equivalents for clinical transplantation.

Arch Ophthalmol. 2006;124:1543-1551

SEVERE OCULAR SURFACE DISEASE (OSD) arising from conditions such as Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid, and chemical injury is a potentially serious blinding condition that represents a major clinical challenge. In such cases, destruction of the corneal epithelial stem cells located at the limbus results in conjunctival invasion, corneal neovascularization, chronic inflammation, and stromal scarring.[1-3] These conditions respond poorly to conventional corneal transplantation. Corneal epithelial replacement by means of keratoepithelioplasty and limbal transplantation has been used to treat these severely damaged eyes.[4-6] However, a significant proportion of these allografts ultimately fail, resulting in visual loss.[7-9] In recent years, bioengineered corneal epithelial equivalents, developed from the ex vivo expansion of limbal stem cells, have been used to treat severe limbal stem cell deficiency, with promising results.[10-17]

Most of the previous reports on cultivated corneal epithelial transplantation used allogeneic tissue because many of these severe conditions have bilateral eye involvement.[10,12-17] Fetal bovine serum (FBS)–supplemented medium remains the medium of choice in the culture process.[11-20] We previously demonstrated that autologous oral epithelial transplantation for treating severe OSD is particularly useful in bilateral disease where healthy tissue is lacking.[18,19] However, the use of FBS may be associated with the risk of transmission of zoonotic infection (eg, bovine spongiform encephalitis) and other unknown pathogens. Because bovine spongiform encephalitis cannot be detected by any known in vitro methods, this use of bovine products is a major health concern in many parts of the world. A group from our institution previously showed that human serum was able to support epithelial cell proliferation,[21] which raises the possibility of using the patient's own serum as an alternative to FBS in the culture process. The use of autolo-

CME course available at www.archophthalmol.com

Author Affiliations: Department of Ophthalmology, Kyoto Prefectural University of Medicine (Drs Ang, Nakamura, Inatomi, Sotozono, Koizumi, Yokoi, and Kinoshita), and Research Center for Regenerative Medicine, Doshisha University (Drs Nakamura and Koizumi), Kyoto, Japan; and Singapore National Eye Centre (Dr Ang).

gous serum (AS) is advantageous because it eliminates the need for bovine material and reduces the risk of disease transmission.

In this study, we compared the efficacy of AS supplementation with that of conventional FBS supplementation in developing cultivated oral epithelial equivalents and evaluated the use of AS-derived cultivated oral epithelial transplantation for the treatment of severe limbal stem cell deficiency. We describe the successful clinical use of bioengineered ocular surface equivalents that are derived almost entirely from autologous tissue and material. This study has important clinical implications and represents an important advance in the pursuit of completely autologous xenobiotic-free bioengineered ocular equivalents for clinical transplantation because it reduces the risks of transmitted infection and allograft rejection, as well as the need for long-term immunosuppression.

METHODS

SUBJECTS

All experimental procedures and clinical applications were approved by the institutional review board for human studies of the Kyoto Prefectural University of Medicine. Prior informed consent was obtained from all patients in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

The study included 10 eyes from 10 patients with severe OSD who underwent autologous cultivated oral epithelial transplantation at our hospital between April 1, 2004, and May 30, 2005. The patients consisted of 7 men and 3 women; their ages ranged from 19 to 75 years (mean±SD age, 57.1±18.9 years). The preoperative diagnosis was SJS in 7 patients, thermal injury in 1, chemical injury in 1, and ocular cicatricial pemphigoid in 1. Preoperatively, all 10 eyes manifested severe destruction of the ocular surface with total limbal stem cell deficiency. These patients demonstrated a reasonable reflex tear function and tear meniscus level. All patients were followed up for a minimum of 6 months after transplantation, with the longest follow-up being 19 months.

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR SOLUBLE Fas LIGAND

Previous reports have shown that a high concentration of soluble Fas ligand (sFasL) at the onset of SJS may play a crucial role in keratinocyte apoptosis.[22] We analyzed the serum sFasL levels of the patients with severe OSD to determine whether AS could be used safely as a cell culture supplement. We determined the concentrations of sFasL by means of an sFasL enzyme-linked immunosorbent assay kit (Medical & Biological Laboratories Co, Ltd, Nagoya, Japan), following the manufacturer's protocol, with the reaction measured at 450 nm. The limit of detection was 100 pg/mL. Each individual sample was analyzed in duplicate. Human serum samples from healthy age-matched volunteers were used as control samples.

CULTIVATION OF ORAL EPITHELIAL SHEET

Under aseptic conditions, AS was obtained from each patient by venesection at the antecubital fossa. We collected 30 mL of blood in a sterile container and centrifuged and filtered the sample, yielding a purified serum sample of approximately 10 mL. Each patient's serum sample was stored in sterile tubes at −30°C before use.

PREPARATION OF AUTOLOGOUS CULTIVATED ORAL EPITHELIAL EQUIVALENT

The presence of healthy oral mucosa was first confirmed by a dentist before biopsy. An oral mucosal biopsy specimen, 2 to 3 mm^2, was obtained with the patient under local anesthesia. The submucosal connective tissue was first carefully removed with scissors. The oral epithelium was then incubated at 37°C for 1 hour with 1.2-IU dispase, followed by treatment with a solution of 0.05% trypsin and EDTA for 10 minutes to separate the cells. The resultant oral epithelial cells (1×10^5/mL) were then seeded onto denuded amniotic membranes spread on the bottom of culture inserts and cocultured with mitomycin-inactivated 3T3 fibroblasts.[18,19] The culture medium consisted of defined keratinocyte growth medium (ArBlast Co Ltd, Kobe, Japan) supplemented with 5% AS and insulin (5 μg/mL), cholera toxin (0.1 nmol/L), human recombinant epidermal growth factor (10 ng/mL), and a mixture of penicillin and streptomycin (50 IU/mL).[17]

The cultured cells were submerged in the medium for 2 weeks and then airlifted for 1 to 2 days by lowering the medium level. Cultures were incubated at 37°C with 5% carbon dioxide and 95% air, with the medium changed every day. To compare the use of the AS-supplemented medium with that of the conventional FBS-supplemented medium, we also cultivated the patient's oral epithelial cells in FBS-supplemented medium. We compared the morphological and immunohistochemical results of the oral epithelial equivalents prepared in AS-supplemented medium with the corresponding oral epithelial equivalents prepared in FBS-supplemented medium.

SURGICAL PROCEDURE

We performed a 360° conjunctival peritomy 3 mm from the limbus, and removed all perilimbal scarred or inflamed subconjunctival tissue to the bare sclera. The corneal pannus was completely removed by blunt dissection or superficial keratectomy using surgical scissors or a blade. We then treated the residual subconjunctival tissue with 0.04% mitomycin for 5 minutes, followed by vigorous repeated washing with isotonic sodium chloride solution.[13,14] The cultivated autologous oral epithelial sheet was cut from the culture insert using a 19-mm diameter trephine, transferred over the corneal surface, and secured in place with 10-0 nylon sutures at the limbus. In patients with more extensive disease such as symblepharon formation or fornical shortening, ocular surface reconstruction was performed by transplanting an additional amniotic membrane over the surrounding scleral surface or fornix, securing it in place with 10-0 nylon sutures, and reconstructing the rest of the ocular surface. The integrity of the cultivated epithelium was confirmed by fluorescein staining results at the end of surgery, and the ocular surface was protected with a medical-use bandage contact lens.

Postoperatively, eyedrops consisting of 0.3% ofloxacin and 0.1% dexamethasone sodium phosphate were instilled 4 times a day. The eyedrop therapy was tapered to a maintenance dosage of 2 to 3 times a day after 2 to 3 months, depending on the severity of inflammation. Oral betamethasone sodium phosphate (1 mg/d) and cyclosporine (100 mg/d) were administered to reduce inflammation, and dosages were tapered and stopped 1 month postoperatively. Patients were followed up with slitlamp examination, fluorescein staining, and photographic documentation.

MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL EXAMINATION

A representative piece of oral epithelial equivalent was sent for histological and immunohistochemical analyses. Cryostat sections (7-μm thick) were placed on gelatin-coated slides and air-dried. Morphological analysis was performed by staining with hematoxylin-eosin. Immunohistochemical studies of tissue-specific keratins and cell junction specialization-related proteins were performed as previously described.[23,24] Tissue sections were incubated for 1 hour with primary antibodies to keratin 3 (Progen Biotechnik GmbH, Heidelberg, Germany), keratin 4 (Novocastra, Newcastle, England), keratin 13 (Novocastra), ZO-1 (Zymed Laboratories, Inc, South San Francisco, Calif), desmoplakin (Progen), integrin α6 (Chemicon International, Temecula, Calif), laminin 5 (Chemicon International), and collagen IV (MP Biomedicals, Irvine, Calif). This was followed by incubation with the appropriate secondary antibodies, fluorescein isothiocyanate–conjugated donkey anti–mouse IgG and fluorescein isothiocyanate–conjugated goat anti–rabbit IgG (Molecular Probes; Eugene, Ore). The sections were were covered with antifading mounting medium containing propidium iodide (Vectashield; Vector Laboratories, Burlingame, Calif) and were examined by confocal microscopy (Fluoview; Olympus Corp, Tokyo, Japan).

RESULTS

SERUM sFasL LEVEL IN SEVERE OSD

The serum levels of sFasL were undetectable in the 10 patients with severe OSD. These results were similar to the control group of 10 healthy volunteers.

MORPHOLOGY OF CULTIVATED ORAL EPITHELIAL EQUIVALENT

Cultivated oral epithelial cells proliferated on the denuded amniotic membranes and formed a confluent sheet of epithelial cells within 5 to 8 days. After 2 weeks, the cultivated oral epithelium consisted of 4 to 6 layers of cells, with a well-conserved basal layer consisting of cuboidal cells and progressively flattened cells superficially. Results of the histological examination showed that the structure and organization of the oral epithelial sheets cultivated in AS- and FBS-supplemented media were similar (**Figure 1**A).

The expression patterns of tissue-specific keratins and cell junction specialization-related proteins were similar in AS- and FBS-derived oral epithelial equivalents. In the AS- and FBS-supplemented cultures, keratin 4 was expressed in the superficial and upper half of the intermediate layers (Figure 1B). Keratin 13 was expressed throughout the epithelium (Figure 1C). Keratin 3, a cornea-associated differentiation marker, was expressed in all cell layers (Figure 1D). The tight junction–related protein ZO-1 was expressed in the apical surfaces of cultivated epithelium (**Figure 2**A). Desmoplakin, a cell-to-cell junctional component, was expressed in the cell membranes of epithelial cells (Figure 2B). Basement membrane assembly proteins, such as integrin α6, laminin 5, and collagen IV, showed linear positive staining on the basement membrane side of the epithelium (Figure 2C-E). The expression patterns of all of these proteins were similar in oral epithelial sheets cultivated in the AS- and FBS-supplemented culture media.

CLINICAL RESULTS

The clinical data and surgical outcomes of the 10 patients are summarized in the **Table**. The mean±SD follow-up period was 12.6±3.9 months, with the longest follow-up being 19 months. Before transplantation, all eyes manifested severe destruction of the ocular surface with total limbal stem cell deficiency. Two to 5 days after transplantation, the corneal surfaces of all treated eyes were clear and smooth, and fluorescein staining confirmed that they were entirely covered by the cultivated oral epithelium. The presence of an initial intervening nonepithelialized area between the host conjunctiva and the transplanted oral epithelium confirmed that epithelialization did not arise from the adjacent host conjunctiva.

Successful engraftment was achieved in all patients, with no sloughing off of any of the grafts. Ocular inflammation was found to subside rapidly after surgery in all patients. Slitlamp examination showed that conjunctival fibrosis was successfully suppressed in all patients, with no conjunctival invasion on the corneal surface throughout the follow-up. At the last follow-up visit, the ocular surface of the eyes with surviving transplanted cultivated epithelium remained stable without any epithelial defects. The clinical progress of 2 representative patients with total limbal stem cell deficiency arising from SJS (patient 3) and ocular cicatricial pemphigoid (patient 7) is shown in **Figure 3** and **Figure 4**.

Nine (90%) of the 10 eyes were restored to good vision, with visual acuity improving by 2 lines or more at the last follow-up visit. The ocular surface in the right eye of patient 5 was successfully reconstructed and remained stable; however, residual corneal stromal scarring precluded good vision in this eye. All patients experienced a significant improvement in symptoms and a reduction in ocular inflammation compared with their preoperative condition.

There were no significant postoperative complications, and none of the patients developed graft rejection. The right eye of patient 3 developed a small epithelial defect with mild cellular infiltration, suggestive of a low-grade bacterial infection. This was resolved promptly after administration of antibiotic eyedrops consisting of ofloxacin and cefmenoxime hydrochloride. During the follow-up period, 4 eyes developed small epithelial defects that eventually healed over from the adjacent oral mucosal epithelium (**Figure 5**). All of the eyes demonstrated some degree of superficial peripheral corneal neovascularization. This gradually abated with time and did not interfere with vision or cause any postoperative complications.

COMMENT

Cultivated epithelial equivalents have been used for corneal epithelial replacement and regeneration in severe

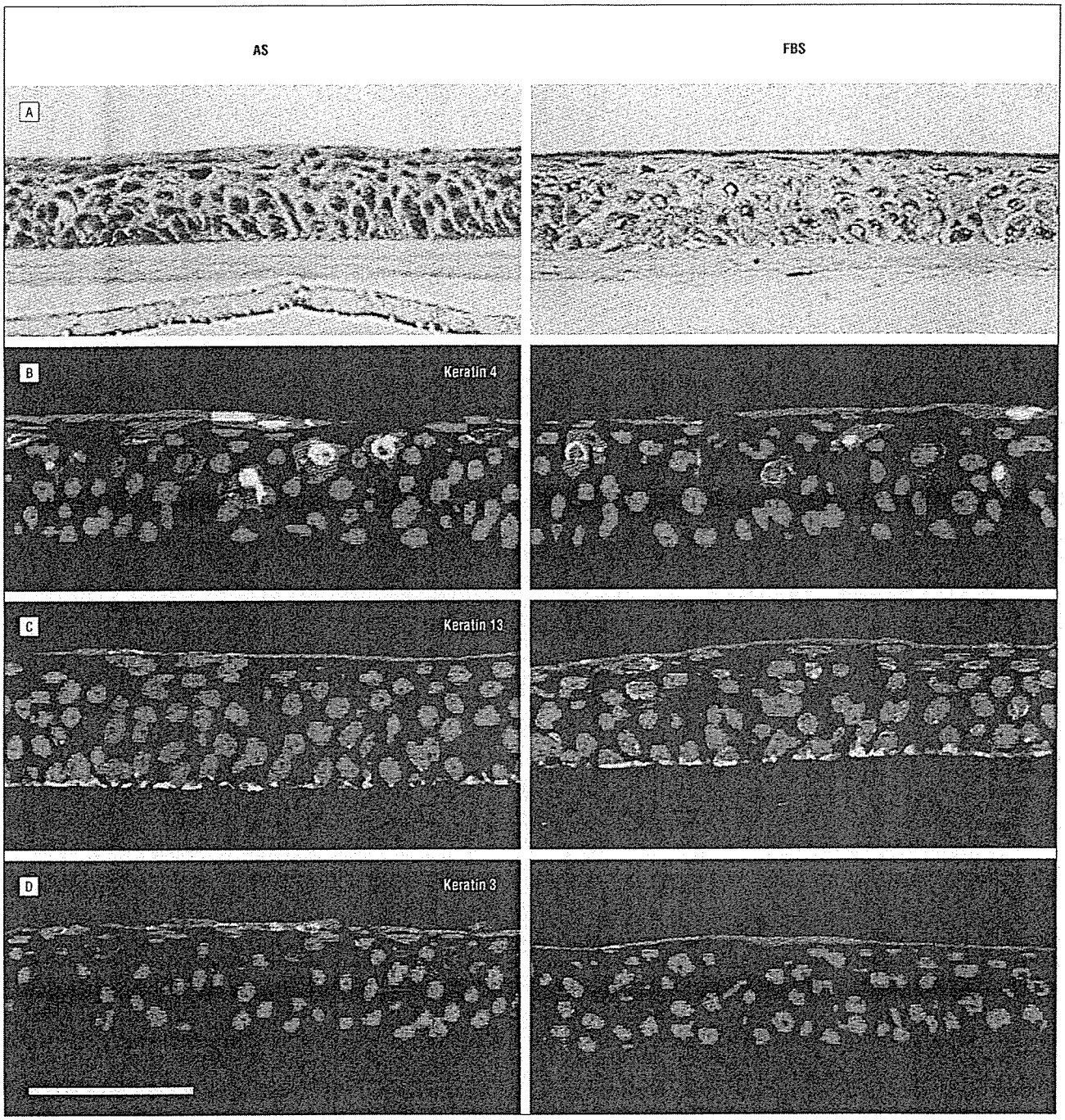


Figure 1. Light microscopy (A) and immunohistochemical findings (B-D) of autologous cultivated oral epithelial sheets. Oral epithelial sheets cultivated in autologous serum–supplemented and fetal bovine serum–supplemented media (left and right sides, respectively) demonstrated a similar histological appearance, with 4 to 6 layers of stratified, well-differentiated cells (A). In both culture systems, the mucosal-specific keratin 4 was expressed in the superficial and upper half of the intermediate layers (B). Keratin 13 was expressed throughout the epithelium (C). Keratin 3 was also expressed in all epithelial cell layers (D). Scale bar indicates 100 μm.

OSD. Fetal bovine serum–supplemented medium remains the most widely used culture system for ocular surface epithelial cell propagation.[11-20,25-29] The ideal culture system for developing transplantable tissue equivalents is one that is safe from disease transmission and maintains the properties of the tissue of origin. We have demonstrated that AS-derived oral epithelial equivalents were similar in structure and organization to those derived from conventional FBS-supplemented cultures. We describe herein the effective use of AS-derived cultivated oral epithelial transplantation for the treatment of severe OSD. The novel approach of using AS as an alternative to FBS in the culture process represents a significant advance in the development of safer, completely autologous bioengineered tissue equivalents for clinical transplantation.

Transplanting autologous eye tissues is possible only if there is sufficient healthy tissue available from the contralateral eye. In most cases of severe OSD, ocular involvement is bilateral. As such, most of the previous

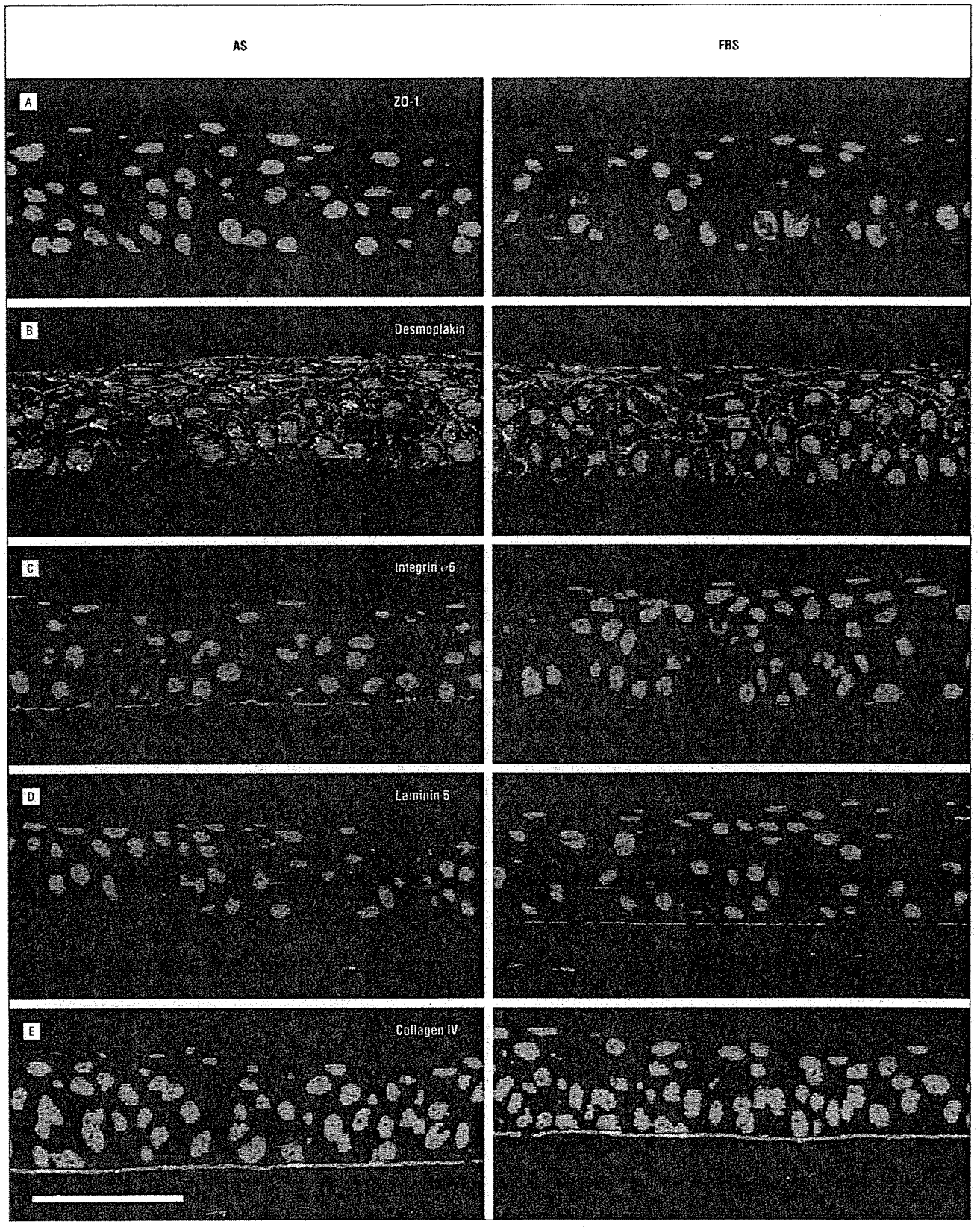


Figure 2. Immunohistochemical findings of cell-to-cell and basement membrane junctional assembly proteins in autologous serum (AS)–supplemented and fetal bovine serum (FBS)–supplemented culture systems (left and right sides, respectively). The ZO-1 protein was expressed at the apical surfaces of the cultivated oral epithelial sheets (A). Desmoplakin, a cell-to-cell junctional component, was expressed in the cell membranes of epithelial cells (B). We noted linear positive staining of integrin α6 (C), laminin 5 (D), and collagen IV (E) on the basement membrane side of the cultivated oral epithelial sheet. The expression patterns of these proteins were similar in cultivated epithelial sheets derived from AS-supplemented and FBS-supplemented culture systems. Scale bar indicates 100 μm.