

Novel Sutureless Transplantation of Bioadhesive-Coated, Freeze-Dried Amniotic Membrane for Ocular Surface Reconstruction

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PURPOSE. To evaluate the efficacy and safety of a novel sutureless transplantation of bioadhesive-coated, sterilized, freeze-dried amniotic membrane (FD-AM) for ocular surface reconstruction.

METHODS. A bioadhesive-coated, freeze-dried amniotic membrane was made by freeze drying the denuded AM in a vacuum, applying the minimum amount of fibrin glue (mixture of fibrinogen and thrombin) necessary to retain adhesion on the chorionic side, and sterilizing it by γ -radiation. The resultant AM was characterized for its biological and morphologic properties by immunohistochemical and electron microscopic examination. In addition, fibrin glue-coated, freeze-dried (FCFD) AM was transplanted onto a rabbit scleral surface without sutures, to examine its biocompatibility.

RESULTS. Immunohistochemistry of the FCFD-AM revealed that fibrinogen existed on its chorionic side, and the process of applying fibrin glue did not affect its biological and morphologic properties. Moreover, electron microscopic examination of the chorionic side of the FCFD-AM revealed tiny microfibrils (which are probably fibrinogen protofibrils), and showed that the epithelial surface of FCFD-AM consisted of intact basal lamina similar to that of FD-AM. FCFD-AM transplantation was very easily performed, and the graft adhered to the bare sclera immediately. Though the fibrinogen naturally biodegraded within 2 weeks, the FCFD-AM remained for at least 12 weeks after transplantation. Epithelialization on the FCFD-AM was achieved within 2 weeks, as was the case with FD-AM transplantation. The conjunctival epithelium on the FCFD-AM was well stratified and not keratinized, suggesting that FCFD-AM supports normal cell differentiation.

CONCLUSIONS. The FCFD-AM retained most of the biological characteristics of FD-AM. Consequently, this sutureless method of transplantation of FCFD-AM is safe, simple, and useful for ocular surface reconstruction. (*Invest Ophthalmol Vis Sci* 2007;48:1528-1534) DOI:10.1167/iovs.06-1104

Fresh human amniotic membrane (AM) has been shown to possess anti-inflammatory,^{1,2} antifibroblastic activity³ and antiangiogenic properties⁴ and has been used as a basement membrane substitute for several decades.⁵ Because of these desirable characteristics, AM has been used in a wide variety of ocular surface reconstructive procedures: corneal re-epithelialization in chemical burns,⁶ persistent corneal epithelial defects,^{7,8} symblepharon,^{9,10} pterygium,^{2,11} and stem cell deficiency.^{6,10,12,13} However, some biological and logistical problems have remained: deficiency of proper sterilization and difficulties in transport and storage. Recently, we have resolved such problems by making a sterilized freeze-dried (FD) AM and have successfully used it for clinical treatment.^{14,15}

For FD-AM to be ideally suited for widespread clinical use, application of a sutureless transplantation technique is desirable. The most current method of attaching AMs is by means of suturing. The use of sutures requires a high degree of surgical skill and is associated with several disadvantages, including prolonged operating time and suture-related complications, such as suture abscesses, granuloma formation, and tissue necrosis.¹⁶⁻²³ Moreover, postoperative pain and discomfort due to foreign body sensation and tearing with ocular surface reconstruction is a significant problem for patients.²⁴ Recently, there have been some reports on sutureless techniques for ocular surface reconstruction. Szurman et al.²⁵ showed the feasibility of a reproducible, sutureless method of attaching AM to the corneal surface by using a drop of fibrin glue, and Harvey et al.²⁴ showed the efficacy of fibrin glue for attaching conjunctival autografts in patients undergoing pterygium excision. Fibrin glue, a mixture of fibrinogen and thrombin, is a sealant that imitates the final step of coagulation. Briefly, the thrombin converts the fibrinogen to fibrin by enzymatic action, and factor XIII (present in the fibrinogen component of the glue) cross-links and stabilizes the fibrin monomers. Usually, fibrin glue is applied directly to the ocular surface or graft tissues. This procedure is time consuming and results in the unnecessary application of excess glue to healthy areas. Considering the inflammatory effect of fibrinogen,²⁶⁻²⁹ it is best to use fibrin glue minimally. Therefore, a process in which only the appropriate amount of fibrin glue is preapplied on the AM would be extremely beneficial. To the best of our knowledge, this type of biotool has not been developed.

In this study, we developed a novel sutureless technique for AM transplantation in which we utilized the minimum amount of fibrin glue necessary to retain adhesion by making a fibrin glue-coated FD-AM (FCFD-AM). To the best of our knowledge, there have been no reports assessing the feasibility of using sterilized, bioadhesive-coated FD-AM for ocular surface transplantation. In addition, we found that FCFD-AM retained most

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Supported in part by Grants-in-Aid for scientific research from the Japanese Ministry of Health, Labor, and Welfare (H16-Saisei-007), and the Japanese Ministry of Education, Culture, Sports, Science, and Technology (Kobe Translational Research Cluster), a research grant from the Kyoto Foundation for the Promotion of Medical Science, and the Intramural Research Fund of Kyoto Prefectural University of Medicine.

Submitted for publication September 14, 2006; revised November 1, 2006; accepted January 23, 2007.

Disclosure: E. Sekiyama, None; T. Nakamura, None; E. Kuribara, None; L.J. Cooper, None; N.J. Fullwood, None; M. Takaoka, None; J. Hamuro, None; S. Kinoshita, None

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of the biological and morphologic properties and supported normal epithelial cell differentiation.

MATERIALS AND METHODS

Preparation of FCFD-AM

The FD-AM was prepared according to our previously reported method.¹⁴ Briefly, after obtaining proper informed consent in accordance with the tenets of the Declaration of Helsinki and with approval by the Institutional Review Board of Kyoto Prefectural University of Medicine, human AM was obtained from seronegative donors undergoing elective caesarean section. The AM was washed with sterile phosphate buffered saline (PBS; Nissui, Tokyo, Japan) containing antibiotics and antimycotics. The chorion was peeled off manually and epithelial cells were removed by incubation with 0.02% ethylene diamine tetra-acetic acid (EDTA; Nacalai, Kyoto, Japan) at 37°C for 2 hours. Denuded AM was freeze-dried in a vacuum.

A commercially available fibrin glue (Tisseel; Baxter AG, Vienna, Austria) imitates the final step of coagulation. It contains the powders fibrinogen and thrombin and aprotinin solution. Each powder was dissolved separately into 100 μ L of 99.5% ethanol (Nacalai, Kyoto, Japan), and the aprotinin solution was added only to the thrombin solution. These two solutions were mixed immediately before use and stirred carefully. The minimum amount of this mixture necessary to retain adhesion was determined by performing dose-dependent examinations (data not shown). The appropriate amount of adhesive solution (10 μ L/cm²) was applied to the entire chorionic surface of the FD-AM, which was then placed horizontally at room temperature for 30 minutes. The FD-AM with adhesive solution was then dried under reduced pressure for 30 minutes and vacuum-packed at room temperature. Finally, γ -radiation (15 kGy) was used to sterilize the resultant FCFD-AM.

Immunohistochemistry for Extracellular Matrix Molecules

Immunohistochemical studies on the FCFD-AM were performed using our previously described method.¹⁴ FD-AM was also examined for the purpose of comparison. Briefly, semithin (8 μ m) cryostat sections were obtained from unfixed tissue embedded in OCT compound (Tissue-Tek; Miles, Elkhart, IN). After they were fixed with cold ethanol for 10 minutes, the sections were incubated with 3% bovine serum albumin for 15 minutes. Subsequently, the sections were incubated at room temperature for 1 hour with the primary antibody (Table 1) and washed three times in PBS for 15 minutes. In control experiments, we replaced the primary antibody with identical concentrations of the appropriate nonspecific normal mouse and rabbit IgG (Dako, Kyoto,

Japan). In the negative controls we replaced the primary antibody with the appropriate nonimmune IgG. After incubation with 3% bovine serum albumin for 15 minutes, the sections were then incubated at room temperature for 1 hour with appropriate secondary antibodies; Alexa Fluor 488-conjugated anti-mouse and rabbit IgG antibody (Invitrogen- Molecular Probes Inc., Eugene). After several washings with PBS, the sections were coverslipped with antifade mounting medium containing propidium iodide (Vectashield; Vector, Burlingame, CA) and examined by fluorescence microscopy (Leica, Tokyo, Japan).

Electron Microscopic Examination of FCFD-AM

The samples were initially fixed in 2.5% glutaraldehyde in PBS buffer. They were divided into two groups so that both the upper and lower sides of the AM could be examined. The specimens were then washed in PBS for 15 minutes, followed by postfixing in 2% osmium tetroxide for 2 hours. They were washed again in PBS before being dehydrated through a graded ethanol series (50%, 70%, 80%, 90%, and 100%) for 20 minutes at each concentration. After two 20-minute changes of 100% ethanol, the samples were then transferred to hexamethyldisilazane for 20 minutes and air dried. The samples were then mounted on aluminum specimen stubs and sputter-coated with gold before being examined on a scanning electron microscope (JSM 5600; JEOL, Tokyo, Japan).

Sutureless Transplantation of FCFD-AMs

To investigate the biocompatibility of FCFD-AM with the ocular surface, we transplanted it onto the bare sclera of a rabbit. FD-AM transplantations with suture and simple recession of the conjunctiva without AM transplantation were also performed for the purpose of comparison. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the experimental procedure approved by the Committee for Animal Research at Kyoto Prefectural University of Medicine.

The transplantation of FCFD-AM was performed by the following method. First, we removed the rabbit conjunctiva (15 \times 10 mm) with surgical scissors. The remaining, severed edge of the conjunctiva was secured to the sclera with 10-0 nylon and, after the scleral surface was wiped with a microsponge, the FCFD-AM was then simply applied with the epithelial basement membrane side facing up. After surgery, a topical antibiotic ointment (0.3% ofloxacin) was administered.

At 2, 4, 8, and 12 weeks after transplantation, we examined the epithelialization and hyperemia of the surgical area by slit lamp microscopy ($n = 5$). For the degree of hyperemia, each slit lamp photograph was qualitatively and independently scored (3, intense; 2, moderate; 1, faint; and 0, negligible) by four examiners in a masked fashion. We took the average of the points scored by the examiners for each sample and assessed statistical significance by Mann-Whitney test. At 0,

TABLE 1. First Antibodies and Sources Used in Our Study

Specificity	Immunized	Dilution	Sources	Cat. Number
Collagen I	Rabbit, pAb	$\times 200$	LSL*	LB1190
Collagen III	Mouse, mAb	$\times 100$	Chemicon†	MAB1343
Collagen IV	Mouse, mAb	$\times 100$	MP Biomedicals‡	63173
Collagen V	Rabbit, pAb	$\times 200$	LSL	LB1581
Collagen VII	Mouse, mAb	$\times 100$	Chemicon	MAB1345
Laminin-5	Mouse, mAb	$\times 100$	Chemicon	MAB19562
Fibronectin	Mouse, mAb	$\times 100$	Novocastra§	568
Fibrinogen	Mouse, mAb	$\times 100$	Chemicon	MAB1901
Cytokeratin 1	Mouse, mAb	$\times 20$	Novocastra	NCL-CK1
Cytokeratin 4	Mouse, mAb	$\times 200$	Novocastra	NCL-CK4
Cytokeratin 10	Mouse, mAb	$\times 50$	Novocastra	NCL-CK10
Cytokeratin 13	Mouse, mAb	$\times 200$	Novocastra	NCL-CK13

* Tokyo, Japan.

† Temecula, CA.

‡ Costa Mesa, CA.

§ Newcastle-upon-Tyne, UK.

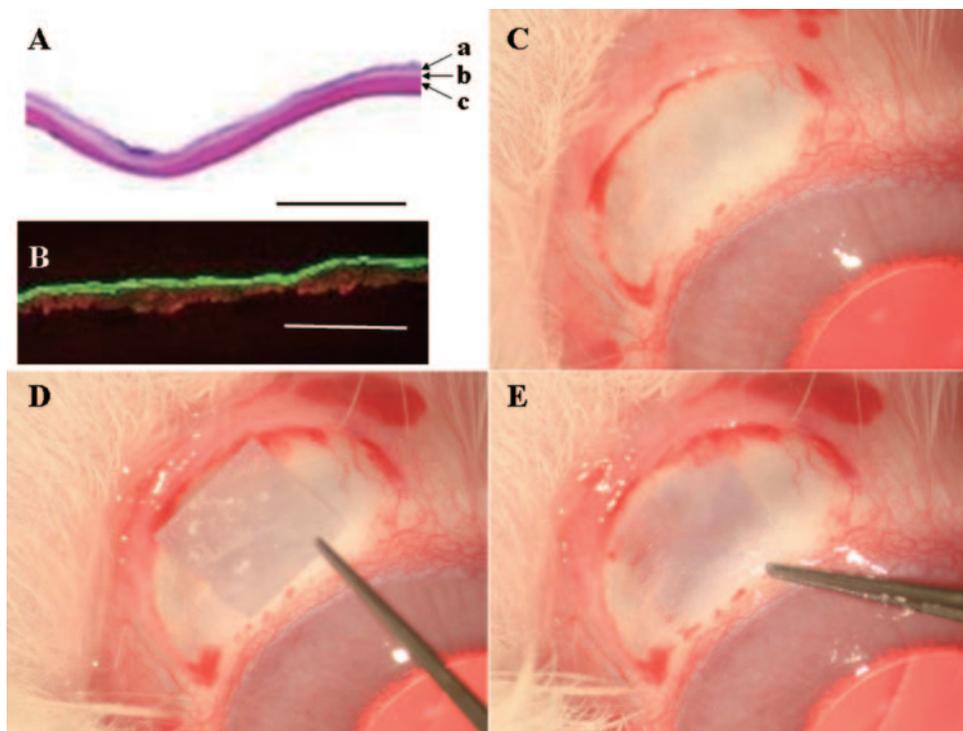


FIGURE 1. Representative HE and immunohistochemical staining of FCFD-AM. HE staining revealed the removal of amniotic epithelial cells from their substrate and the existence of the fibrinogen layer stained with eosin on its chorionic side: from the epithelial side (A), basal membrane (a), compact layer (b), and fibrinogen layer (c). Immunoreactivity of fibrinogen was also detected on the chorionic side of FCFD-AM (B). Scale bar, 200 μ m. (C-E) Representative photographs of FCFD-AM transplantation. FCFD-AM adhered immediately after transplantation onto the bare sclera: bare sclera (C), sclera before FCFD-AM transplantation (D), and sclera after FCFD-AM transplantation (E).

1, 2, and 4 weeks after transplantation, rabbits were euthanized by phlebotomy of 1 mL pentobarbital sodium, and the transplanted sclera was embedded in OCT compound and frozen with liquid nitrogen. We also checked the immunohistochemical staining of fibrinogen at the FCFD-AM transplanted area, and the staining of cytokeratin (CK)-1, -4, -10, and -13 in the epithelial cells of the FCFD-AM ($n = 3$). Normal rabbit conjunctiva was also examined for the purpose of comparison.

RESULTS

Examination of FCFD-AM

Hematoxylin-eosin (HE) staining of the FCFD-AM revealed that the amniotic epithelial cells were completely removed. Eosin staining showed the existence of the fibrinogen layer on the

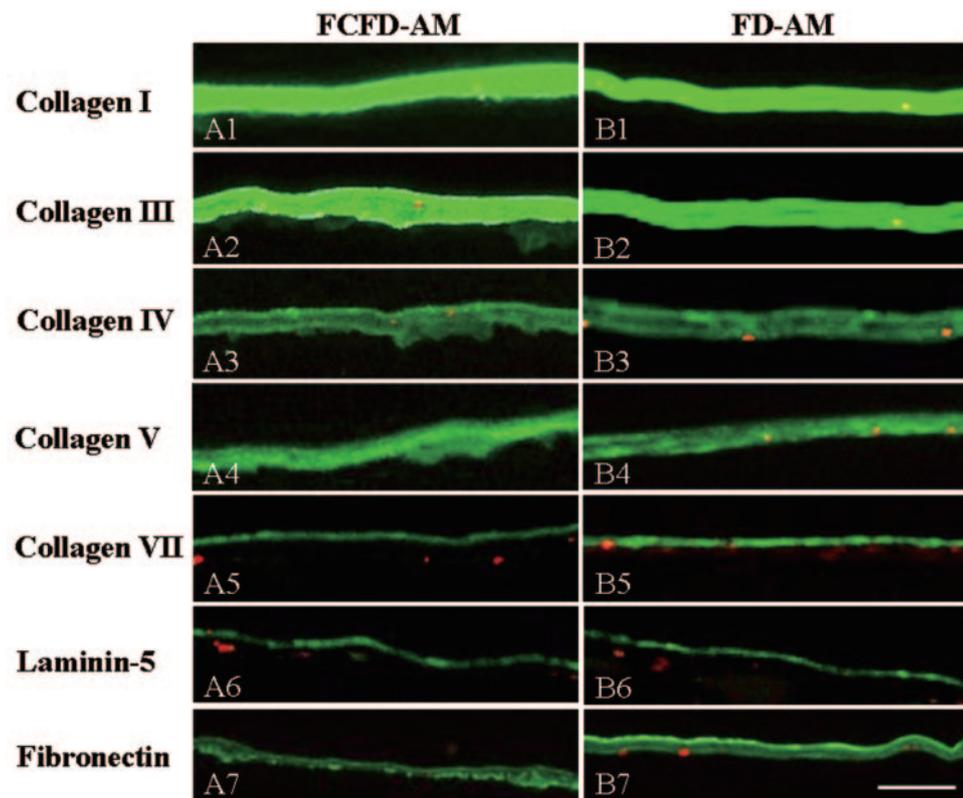


FIGURE 2. Representative immunohistochemical staining in the FCFD-AM (A) and FD-AM (B) of collagen I (1), collagen III (2), collagen IV (3), collagen V (4), collagen VII (5), laminin-5 (6), and fibronectin (7). Collagen I, III, IV, and V and fibronectin were expressed in the entire AM. In contrast, collagen VII and laminin-5 were expressed in the basement membrane side of the AM. Nuclei were stained with propidium iodide (red). Scale bar, 100 μ m.

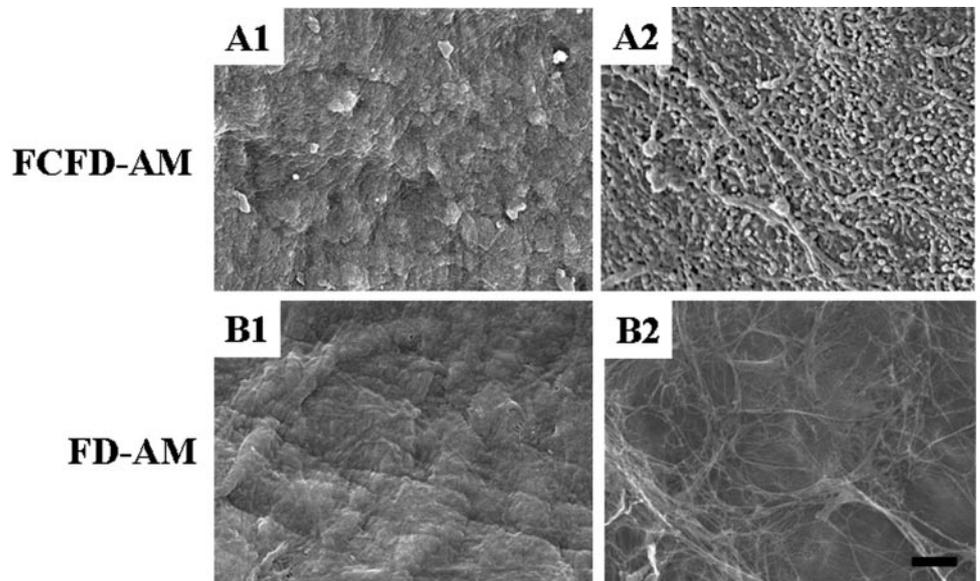


FIGURE 3. Scanning electron micrographs of the FCFD-AM (A) and FD-AM (B) on the epithelial (1) and chorionic (2) sides. The uncoated surface consisted of the intact basal lamina (A1 and B1). On the chorionic side of the untreated FD-AM (B2) a meshwork of collagen fibers was evident. In contrast the chorionic side of the FCFD-AM was covered with microfibrils that appeared to form small spheres, and a few irregularly polymerized fibrils of fibrin were also present (A2). Scale bar, 10 μ m.

chorionic side of the FCFD-AM (Fig. 1A, c). Immunohistochemistry for fibrinogen also showed its existence on the chorionic side of the FCFD-AM (Fig. 1B). Though we also performed immunohistochemical examination for thrombin, we were unable to detect it because amount present was minute.

FCFD-AM was easily removed from its packaging by forceps without any damage (Fig. 1C). Immediately after transplantation, the FCFD-AM adhered in such a way that it strongly resisted dislocation when lateral pressure was applied with forceps (Figs. 1D, 1E), yet it could still be peeled away quite easily. A few minutes after transplantation, the FCFD-AM became increasingly more difficult to remove, with considerable effort needed to peel it away from the scleral surface.

Immunohistochemistry of FCFD-AM

The patterns of extracellular matrix molecule expression in the FCFD-AM samples were investigated with immunohistochemistry. Negative control sections, one incubated with normal mouse and rabbit IgG and the other with primary antibodies omitted, exhibited no discernible specific immunoreactivity over the entire regions. The immunoreactivity observed in each specimen was compared with that in FD-AM samples. In FCFD-AM, collagen I, III, IV, and V and fibronectin were expressed throughout the interstitial tissue, and collagen VII and laminin V were expressed on the basement membrane (Figs. 2A1–2A7). As previously reported, the immunoreactivity was similar to those in FD-AM (Figs. 2B1–2B7).

Electron Microscopic Examination of FCFD-AM

Examination of FCFD-AM and FD-AM showed that the epithelial, uncoated surface consisted of the intact basal lamina (Figs. 3A1, B1). The chorionic side of untreated FD-AM showed a typical appearance of a network of collagen fibers (Fig. 3B2). In contrast, examination of the fibrin glue treated chorionic side of FD-AM showed that the tiny microfibrils formed an even layer on the surface. A few large-diameter irregularly polymerized fibrils were also visible (Fig. 3A2).

Sutureless Transplantation

We transplanted FCFD-AM on the bare rabbit sclera without sutures, as shown in Figures 1C–E. FD-AM transplantations with suture and simple recession of the conjunctiva without AM transplantation were also performed for the purpose of

comparison. We examined the epithelialization and hyperemia of the surgical area by slit lamp microscopy, with and without fluorescein.

Though the bare sclera without AM transplantation did not epithelialize within 2 weeks (Fig. 4C1, inset), the surface of the FCFD-AM was covered with conjunctival epithelium at 2 weeks after transplantation, equal to that of FD-AM-sutured eyes (Figs. 4A1, 4B1, insets). With regard to the hyperemia of the ocular surface epithelium, there was no significant difference between suture models and nonsuture models (Figs. 4A1–4A4, 4B1–4B4).

Biodegradability of Fibrinogen

We examined the residual fibrin glue and AM at 0, 1, and 2 weeks after transplantation by immunohistochemistry for fibrinogen. To examine the distribution of AM we also performed immunohistochemistry for human collagen III. Significant staining for both fibrinogen and collagen III was detected on the scleral surface during the first week (Figs. 5A1, B1). Though significant staining for collagen III was detected under the epithelial cell layer at 1 and 2 weeks after transplantation (Figs. 5A2, 5A3), very limited staining for fibrinogen was detected at 1 week, and little or no staining was detected at 2 weeks (Figs. 5B2, 5B3) and also at 4 weeks (data not shown). The fibrinogen was fully biodegraded within 2 weeks after transplantation. No staining for fibrinogen or collagen III was detected in the normal rabbit conjunctiva and sclera (Figs. 5A4, 5B4).

Characterization of the Epithelial Cells on FCFD-AM

We compared the characterization of the epithelial cells on FCFD-AM and on FD-AM by immunohistochemistry for CK1, -4, -10, and -13. The staining for CK4 and -13 was detected in the epithelial cell layer of both FCFD-AM and FD-AM (Figs. 6A1, 6A2, 6B1, 6B2). We were unable to detect any staining for CK1 and 10 (Figs. 6A3, 6A4, 6B3, 6B4). These staining patterns were similar to that of normal rabbit conjunctiva (Figs. 6C1–6C4).

DISCUSSION

Most ophthalmologists currently perform AM transplantation by means of suturing that is time-consuming¹⁶ and is associated with some disadvantages.^{17–25} To overcome these problems,

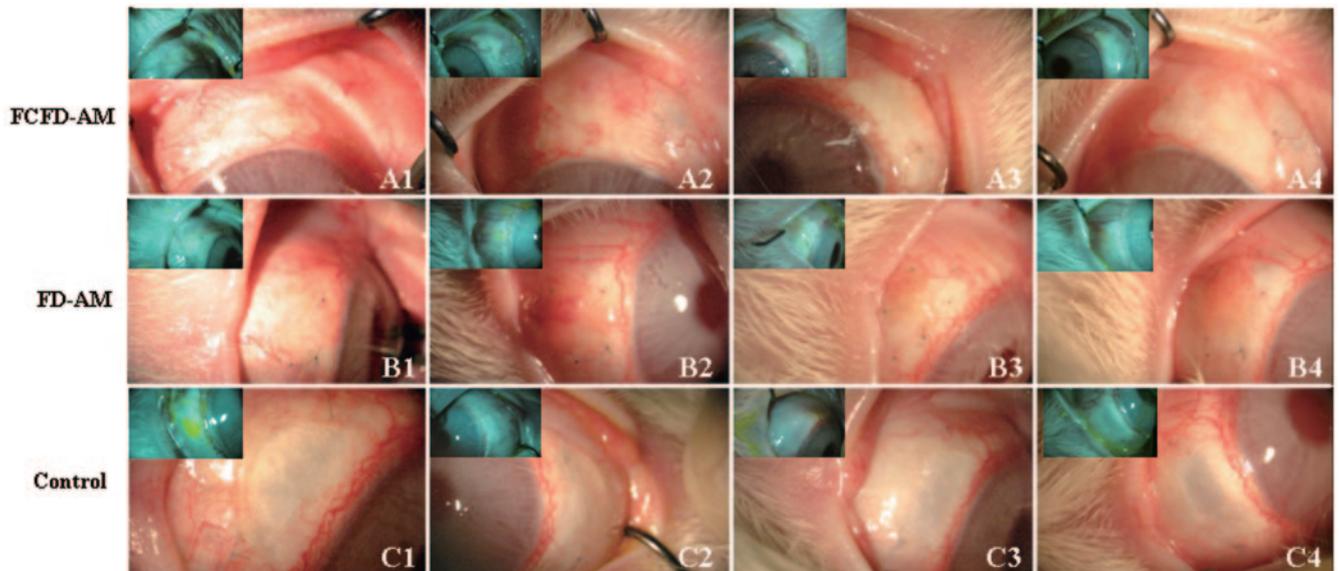


FIGURE 4. Representative slit lamp photographs of rabbit eyes taken 2 (1), 4 (2), 8 (3), and 12 (4) weeks after transplantation with FCFD-AM (A) and FD-AM (B) and no graft (C; $n = 5$). *Insets:* representative fluorescein images. Though the bare sclera without AM transplantation failed to epithelialize within 2 weeks (C1, *inset*), the surface of the FCFD-AM (A1, *inset*) and FD-AM (B1, *inset*) were completely covered with epithelium at 2 weeks after transplantation. The epithelial coverings on the FCFD-AM and FD-AM were stable (A2–A4, *inset* and B2–B4, *inset*), and there was no significant difference between the FCFD-AM and FD-AM grafted eyes in regard to hyperemia (A2–A4, B2–B4).

we devised a novel sutureless transplantation technique using FD-AM precoated with the appropriate amount of fibrin glue on its chorionic side.

The main components of fibrin glue are fibrinogen and factor XIII on the one hand, and thrombin, calcium chloride, and an antifibrinolytic agent such as aprotinin on the other. It mimics the final steps of the coagulation cascade. In the presence of thrombin, fibrinogen is converted to fibrin. Thrombin also activates factor XIII, which stabilizes the clot by promoting cross-linking of the fibrin chains to long fibrin strands. This process occurs in the presence of calcium ions. Basically, fibrinogen and thrombin are mixed immediately before use, and it has been reported that the product had to be used within 4 hours once the two were mixed.²⁶ However, we confirmed that

FCFD-AM precoated with the mixture of fibrinogen and thrombin can still retain its adhesion qualities for a period of at least 6 months when stored at room temperature (data not shown).

Fibrinogen is reported to play a vital role in the process of inflammation.^{27–30} Fibrin glue on the chorionic surface may cause inflammation on the ocular surface and lead to delay in wound healing; however, regarding the period of epithelialization and degree of hyperemia observed in our method, FCFD-AM-transplanted eyes showed a course similar to that of FD-AM-sutured eyes, and both methods revealed prompt epithelialization when compared to non-grafted eyes. These facts indicate that the amount of fibrin glue that we incorporate in our FCFD-AM is appropriate for clinical use.

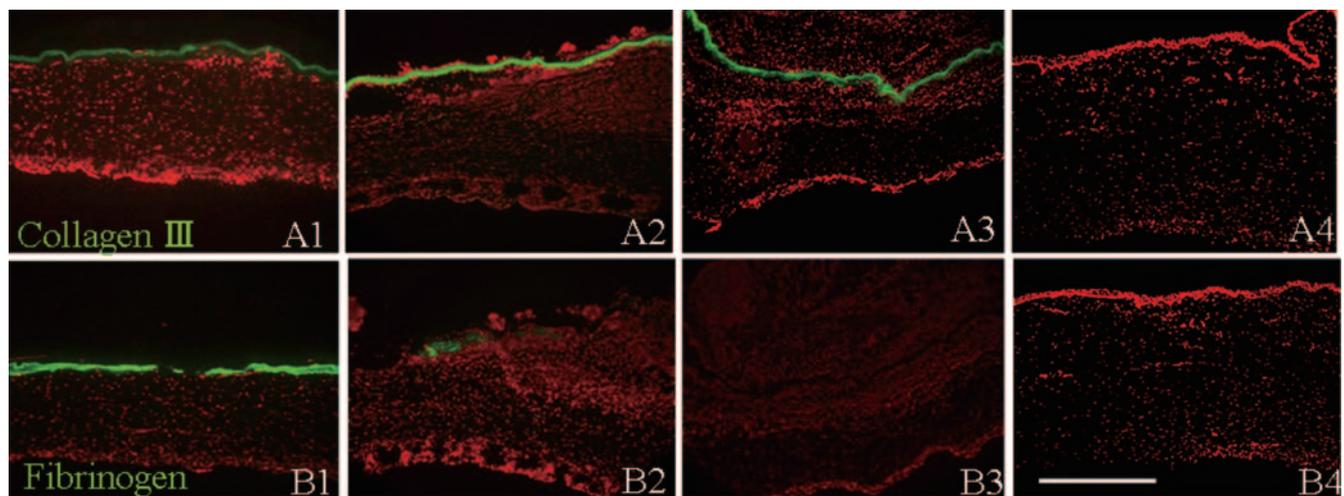


FIGURE 5. Representative immunohistochemical staining of collagen III (A1–A3) and fibrinogen (B1–B3) in cryosections of FCFD-AM-grafted sclera at 0, 1, and 2 weeks after surgery ($n = 3$). Significant staining for collagen III was detected under the epithelial cell layer at 1 and 2 weeks after transplantation (A2–A3). However, only slight staining for fibrinogen was detected at 1 week (B2), and little or no staining was detected at 2 weeks (B3). Fibrinogen was biodegraded within 2 weeks after transplantation. No staining for fibrinogen or collagen III was detected in the normal rabbit conjunctiva and sclera (A4, B4). Scale bar, 100 μ m.

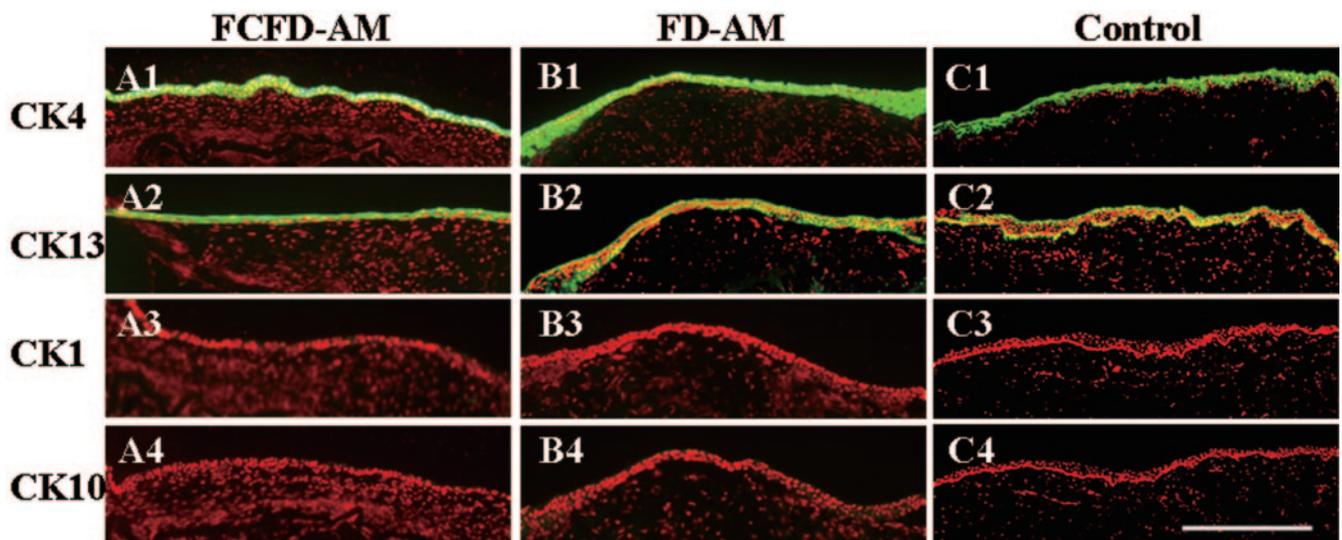


FIGURE 6. Representative immunohistochemical staining in FCFD-AM (A) and FD-AM (B) of CK-4 (1), -13 (2), -1 (3), and -10 (4), at 4 weeks after transplantation. CK4 and CK13 were expressed at the epithelial cell layer of FCFD-AM and FD-AM. In contrast, CK1 and -10 were not expressed in any of FCFD-AM or FD-AM. These staining patterns were similar to that of normal rabbit conjunctiva (C1, C4). Scale bar, 50 μ m.

Immunohistochemical and HE staining revealed that fibrinogen existed on the chorionic side of the FCFD-AM, and electron microscopy showed tiny microfibrils (which are probably fibrinogen protofibrils) approximately 7 to 10 nm in diameter.³¹ We also demonstrated that collagen types I, III, IV, and V and fibronectin were expressed throughout the FCFD-AM, whereas laminin-5 and collagen VII were observed in the basement membrane side of the FCFD-AM. These results were similar to those with the FD-AM. Moreover, our electron microscopic results showed a continuous flat layer of smooth basal lamina. Therefore, we postulate that fibrin glue does not affect the organization of the extracellular matrix macromolecules that play an important role in the physical and biological properties of AM. Moreover, we examined the expression of CK1, -4, -10, and -13 in the transplanted FCFD-AM covered with conjunctival epithelium. The epithelium covering expressed the conjunctival-epithelium-specific proteins CK4 and -13, and did not express the keratinization marker CK1 or -10, indicating that FCFD-AM does not affect the differentiation of the conjunctival epithelium.

When we use fibrin glue for ocular surgery, we usually drop the fibrinogen and thrombin solutions directly onto the tissues.^{16,24,25} When using this method, at least 2 to 3 drops of fibrinogen solution (containing 3.6–5.4 mg of fibrinogen) was needed to glue the FD-AM onto the bare rabbit sclera (data not shown). On the other hand, FCFD-AM contains only 0.125 mg of fibrinogen in 0.5 cm². This lesser amount of fibrinogen is important, as fibrinogen is reported to play a vital role in the process of inflammation.^{27–30} Moreover, it may lessen the risk of infection as some unknown and untargeted infectious agents may survive the commercial sterilization process for fibrinogen. With that in mind, using FCFD-AM with the optimal amount of fibrinogen preapplied offers great advantages over the usual technique of fibrin glue application. Moreover, the fibrinogen on FCFD-AM biodegraded within 2 weeks, whereas the transplanted AM remained for at least 12 weeks (data not shown). So our study shows that FCFD-AM transplantation is a safe and useful technique for clinical use, providing a distinct advantage over directly applied fibrin glue.

In conclusion, our study is the first to demonstrate a safe and simple technique for sutureless AM transplantation, on the basis of several experiments evaluating the biological properties of AM and the optimal amount of fibrin glue. We have

shown that the FCFD-AM we produced retains the characteristics of FD-AM and promotes rapid adhesion onto the sclera in vivo without the need for suturing. On the basis of these results, we are now in the process of using this bioadhesive material for ocular surface reconstruction in patients with symblepharon and pterygium and of making FCFD-AM using patient-derived fibrinogen to reduce the risk of inflammation.

Acknowledgments

The authors thank Hisayo Sogabe and Tomoko Horikiri for assisting with the experiments and John Bush for editing our manuscript.

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