Sterilized, Freeze-Dried Amniotic Membrane: A Useful Substrate for Ocular Surface Reconstruction

Takahiro Nakamura,[1] Makoto Yoshitani,[2] Helen Rigby,[3] Nigel J. Fullwood,[3] Wakana Ito,[1] Tsutomu Inatomi,[1] Chie Sotozono,[1] Tatsuo Nakamura,[2] Yasuhiko Shimizu,[2] and Shigeru Kinoshita[1]

Purpose. To examine the feasibility of using sterilized, freeze-dried amniotic membrane (FD-AM) as a substrate for cultivating autologous corneal epithelial cells for ocular surface reconstruction.

Methods. Human AM deprived of amniotic epithelial cells by incubation with EDTA was freeze dried, vacuum packed, and sterilized with γ-irradiation. The resultant FD-AM was characterized for its physical, biological, and morphologic properties by stretch stress tests, immunohistochemistry, electron microscopy, and cell culture. In addition, 3 weeks after an ocular surface injury, the conjunctivalized corneal surfaces of eyes in eight rabbits were surgically reconstructed by transplantation of autologous cultivated corneal epithelial cells on FD-AM.

Results. A stretch stress test revealed no significant differences between sterilized FD-AM and cryopreserved AM. Immunohistochemistry for several extracellular matrix molecules and electron microscopic analysis of FD-AM revealed that the process of drying and irradiation did not affect its biological and morphologic properties. The corneal epithelial cells cultivated on FD-AM had four to five stratified, well-differentiated cell layers. Corneas that were grafted with the cultivated corneal epithelial cells on FD-AM were clear and were all epithelialized at 10 days after surgery.

Conclusions. The sterilized, freeze-dried AM retained most of the physical, biological, and morphologic characteristics of cryopreserved AM; consequently, it is a useful biomaterial for ocular surface reconstruction. (*Invest Ophthalmol Vis Sci.* 2004;45:93–99) DOI:10.1167/iovs.03-0752

The amniotic membrane (AM), the innermost layer of the placental membrane, has been used as surgical material in a variety of fields.[1–7] In ophthalmic applications, several researchers have reported limited success in the use of AM as a conjunctival graft in symblepharon in a variety of ocular surface disorders.[8,9] In 1995, Kim and Tseng[10] reported the transplantation of preserved human AM for corneal surface reconstruction in a rabbit model. These reports encouraged the use of preserved human AM for ocular surface reconstruction in patients with severe ocular surface diseases.[11–13]

A variety of characteristics make AM ideally suited for use in ocular surface reconstruction. It has an anti-inflammatory effect,[14,15] antifibroblastic activity,[16] antimicrobial[17] and antiangiogenic[18] properties, and very limited immunogenicity.[19–20] In addition, it provides a healthy new substrate suitable for reepithelialization by the corneal epithelium.[21] Recently, particular attention has been focused on the ex vivo expansion of corneal and oral epithelial cells on various substrates, including preserved human AM.[22,23] Our group has developed both cultivated corneal and oral epithelial transplantation using preserved AM as a carrier and has successfully achieved ocular surface reconstruction with this technique.[24–26]

Thus, AM has unique properties that can be helpful in treating a variety of ocular surface diseases; however, some biological and logistic problems remain. First, human AMs are obtained at the time of elective cesarean section and cryopreserved at −80°C under sterile conditions, using our previously reported protocol. However, this procedure does not guarantee a completely sterile AM because of its biological origins. In view of the attention focused on various pathogenic organisms in recent years, proper sterilization of the AM is vital. Second, cryopreservation of AM requires an expensive and bulky −80°C deep freezer. These problems are a barrier to the wider use of AM, particularly in developing countries. Ideally, for clinical use, AM should be sterile and free of contamination. It should also be easily to obtain, transport, and store for long periods without deterioration.

Therefore, in this study we investigated the possibility of producing AM that can be sterilized and preserved at room temperature. To the best of our knowledge, there have been no papers reporting the effectiveness of sterilized, freeze-dried amniotic membrane (FD-AM) for ocular surface reconstruction, although dehydrated AM has recently become commercially available in the United States. We have produced sterilized, FD-AM using our unique protocol and have successfully used this biomaterial as a substrate in ocular surface reconstruction.

Materials and Methods

Preparation of FD-AM

Human AM was prepared according to our previously reported standard method.[24–26] With proper informed consent in accordance with the tenets of the Declaration of Helsinki for research involving human subjects and on approval by the Institutional Review Board of Kyoto Prefectural University of Medicine, human AMs were obtained at the time of elective cesarean section in volunteers who were seronegative for human immunodeficiency virus, human hepatitis B and C, and syphilis. Under sterile conditions, the AM was washed with sterile

From the [1]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan; the [2]Department of Bioartificial Organs, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan; and the [3]Institute of Environmental and Natural Sciences, Lancaster University, Lancaster, United Kingdom.

Supported in part by Grants-in-Aid for scientific Research from the Japanese Ministry of Health, Labour and Welfare, a research grant from Kyoto Foundation for the Promotion of Medical Science, the Intramural Research Fund of Kyoto Prefectural University of Medicine, the Wellcome Trust and the TFC Frost Trust.

Submitted for publication July 17, 2003; revised August 25, 2003; accepted August 29, 2003.

Disclosure: **T. Nakamura**, None; **M. Yoshitani**, None; **H. Rigby**, None; **N.J. Fullwood**, None; **W. Ito**, None; **T. Inatomi**, None; **C. Sotozono**, None; **T. Nakamura**, None; **Y. Shimizu**, None; **S. Kinoshita**, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Takahiro Nakamura, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Hiroko-ji, Kawaramachi, Kamigyo-Ku, Kyoto, Japan;
tnakamur@ophth.kpu-m.ac.jp.

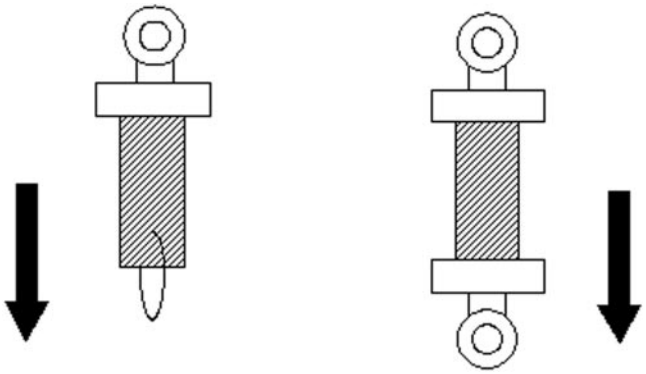

FIGURE 1. Diagrams illustrating the PSS test (*left*), and SS test (*right*). Each sample (*hatched area*) is held with a single clip and a 10-0 nylon suture (PSS) or with two clips (SS). Each sample was pulled vertically until it broke, to determine the maximum tear resistance.

phosphate-buffered saline (PBS) containing antibiotic-antimycotic liquid (penicillin, 10,000 U/mL; streptomycin, 10,000 μg/mL; and amphotericin B, 25 μg/mL), and cut into approximately 4 × 4-cm pieces. The AM was then deprived of amniotic epithelial cells by incubation with 0.02% ethylene diamine tetraacetic acid (EDTA; Nacalai Tesqu Co., Kyoto, Japan) at 37°C for 2 hours. Denuded AM was freeze dried under vacuum conditions and vacuum packed at room temperature as soon as possible. Finally, γ-irradiation (25 kGy) was used to sterilize the resultant FD-AM. Bacteriologic tests were performed on the cultures to confirm sterilization.

Physical Characteristics of FD-AM

To investigate the physical characteristics of FD-AM, one-point suspension stretch stress (PSS) tests and stretch stress (SS) tests were performed on 10 × 30-mm samples, using our previous protocol.[27] Samples examined were cryopreserved AM, FD-AM with γ-irradiation, and FD-AM without γ-irradiation ($n = 6$). The PSS and SS tests are outlined in Figure 1. In the PSS test, one end of each sample is held with a clip and the other end with a 10-0 nylon suture. In the SS test, each end is held with a clip. Each sample is then pulled vertically with a uniaxial stretching device. Cross-head speed was set at 10 mm/min.

Immunohistochemistry for Extracellular Matrix Molecules

Immunohistochemical studies of several extracellular matrix molecules in the FD-AM were performed, using our previously described method.[28,29] Cryopreserved AM was also examined for comparison. Briefly, semithin (6 mm) cryostat sections were obtained from unfixed tissue embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles, Inc., Elkhart, IN). After fixation with cold acetone for 20 minutes, the sections were incubated with 10% goat serum for 30 minutes. Subsequently, the sections were incubated at room temperature for 1 hour with the primary antibody (Table 1) and then washed three times in PBS containing 0.15% TritonX-100 (PBST) for 15 minutes. The controls consisted of replacing the primary antibody with the appropriate nonspecific normal mouse and rabbit IgG (Dako, Kyoto, Japan) at the same concentration. As an additional control, the primary antibody was omitted. After incubation with the primary antibody, 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 (Molecular Probes Inc., Eugene, OR). After several washings with PBS, the sections were coverslipped using antifade mounting medium containing propidium iodide (Vectashield; Vector, Burlingame, CA) and examined by confocal microscopy (Fluoview; Olympus, Tokyo, Japan).

Intracorneal Transplantation of FD-AM

To investigate the compatibility of FD-AM with corneal tissue, we transplanted it into the intracorneal stroma ($n = 4$). This was performed by marking the rabbit cornea 3.0 mm inside the limbus, after which a semilayer incision of the corneal stroma was performed with Vannas scissors. The FD-AM was then inserted into the intrastromal layer. One suture of 10-0 nylon was placed around the corneal wound. After surgery, a topical antibiotic (revofloxacin) was applied three times daily. Corneal transparency and neovascularization were assessed by slit lamp microscopy. The transplanted cornea was stained with hematoxylin and eosin (HE).

Primary Culture of Rabbit Corneal Epithelial Cells on FD-AM

We cultured rabbit corneal epithelial cells using a previously reported system.[24–26] Briefly, confluent 3T3 fibroblasts were incubated with 4 μg/mL of mitomycin C (MMC) for 2 hours at 37°C under 5% CO_2 to inactivate proliferation. They were then rinsed with PBS, trypsinized, and plated onto plastic dishes at a density of 2×10^4 cells/cm^2. The FD-AM was spread, epithelial basement membrane-side up, on the bottom of culture plate inserts (Corning Inc., Corning, NY) placed in dishes containing treated 3T3 fibroblasts. We also used an air-lifting technique to promote epithelial differentiation and epithelial barrier function. Limbal biopsy specimens, each 4 mm^2 in size, were taken from eight adult albino rabbits (2–2.5 kg) anesthetized by intramuscular injection of xylazine hydrochloride (5 mg/mL) and ketamine hydrochloride (50 mg/mL). 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. Corneal endothelium and half the corneal stroma were removed with scissors to the extent possible, and the resultant samples were then incubated at 37°C for 1 hour with 1.2 IU dispase. The medium consisted of DMEM and Ham's F12 (1:1 mixture) with 10% fetal bovine serum (FBS), insulin (5 μg/mL), cholera toxin (0.1 nmol/L), human-recombinant epidermal growth factor (10 ng/mL), and penicillin-streptomycin (50 IU/mL). This was centrifuged twice for 5 minutes at 1000 rpm, and the resultant cell pellet was resuspended in culture medium. The suspension of limbal epithelial cells was then seeded onto FD-AM spread on the bottom of culture inserts, and cocultured with MMC-inactivated 3T3 fibroblasts. The culture was submerged in medium for 2 weeks and then exposed to air by lowering the medium level (air-lifting) for 1 week. Cultures were incubated at 37°C in a 5% CO_2-95% air incubator for up to 21 days, with the medium changed

TABLE 1. Primary Antibodies and Sources

Antibodies	Category	Dilution	Source
Collagen 1	Rabbit polyclonal	×300	LSL, Tokyo Japan
Collagen 3	Rabbit polyclonal	×300	LSL
Collagen 4	Rabbit polyclonal	×300	LSL
Collagen 5	Rabbit polyclonal	×300	LSL
Collagen 7	Mouse monoclonal	×100	Chemicon, Temecula, CA
Fibronectin	Mouse monoclonal	×100	Novocastra, Newcastle-upon-Tyne, UK
Laminin 5	Mouse monoclonal	×100	Chemicon

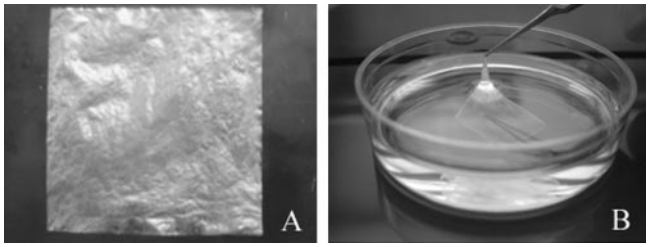

FIGURE 2. The sterilized, freeze-dried amniotic membrane was waferlike, very light and thin (**A**). It became smooth and flexible on hydration, similar to cryopreserved AM (**B**).

every day. We also checked the immunohistochemical staining of cornea-specific keratin-3 and -12 in the cultivated corneal epithelial sheet on FD-AM, using our previously reported method.[28,29]

Electron Microscopy

Both FD-AM and rabbit corneal epithelial cells cultured on FD-AM were examined by scanning (SEM) and transmission (TEM) microscopy. Specimens were fixed in 4% glutaraldehyde in 0.1 M PBS, washed three times for 15 minutes each in PBS, and postfixed for 2 hours in 2% aqueous osmium tetroxide. They were washed three more times in PBS before being passed through a graded ethanol series. For SEM preparation, specimens were transferred to hexamethyldisilazane (TAAB Laboratories Equipment Ltd., Aldermastron, UK) for 10 minutes and allowed to air dry. When dry, specimens were mounted on aluminum stubs and sputter coated with gold before examination in a digital scanning electron microscope (JSM 5600; JEOL, Tokyo, Japan). For TEM preparation, the specimens were embedded in agar 100-epoxy resin (Agar Scientific, Stansted, UK). Ultrathin (70 nm) sections were collected on copper grids and stained for 1 hour each with uranyl acetate and 1% phosphotungstic acid and then for 20 minutes with Reynolds lead citrate before examination on a transmission electron microscope (JEM 1010; JEOL).

Autologous Transplantation of Cultivated Corneal Epithelial Cells on FD-AM

To simulate the conditions found in stem cell deficiencies,[24,26] an ocular-surface injury was created in one eye of each of eight adult albino rabbits by excising all the conjunctival tissue within 5 mm of the limbus and performing a superficial keratectomy of the entire corneal surface, including the limbal epithelial cells. Antibiotic eye drops (revofloxacin) and intramuscular gentamicin (1 mg/kg) were administered after surgery. At 3 to 4 weeks after the ocular surface injury, the conjunctivalized ocular surfaces of all eight rabbits were surgically reconstructed by transplanting autologous corneal epithelial cells cultivated on FD-AM. In all cases, the damaged corneal surface, including the 5-mm zone of adjacent conjunctival tissue, was carefully excised in animals under anesthesia. All animals, whose corneal epithelial cells had been placed in culture 3 weeks earlier, received autologous cultivated corneal epithelial cells on FD-AM. The sheets were sutured to the keratectomized corneal surface with 10-0 nylon sutures and covered with a therapeutic soft contact lens. After surgery, topical antibiotics (revofloxacin) and steroids (betamethasone) were applied three times daily. For experimental controls, four eyes received FD-AM only on keratectomized corneas.

RESULTS

Appearance and Morphologic Features of FD-AM

The FD-AM used in our study was waferlike, very light and thin (Fig. 2A), easy to handle, and suturable without tearing. It became smooth and flexible on hydration, similar to preserved AM (Fig. 2B). The results of the bacteriology tests performed were all negative.

Examination of the FD-AM by SEM revealed a continuous flat layer of smooth basement membrane (Fig. 3A). The basal lamina was clearly present and intact, forming a continuous flat and generally smooth layer above the fibrous collagen stroma. The AM epithelial cells had been successfully removed. Examination of the FD-AM by TEM also confirmed that the AM was well preserved and there were no cells remaining on the surface. The basal lamina was also clearly visible (Fig. 3B). Figure 3C shows that the stroma of the FD-AM also appeared normal and the collagen fibers making up the AM stroma are well preserved by the freeze-drying technique.

Physical Strength of FD-AM

The one-point suspension PSS and SS tests were performed on 10 × 30-mm samples to determine the maximum tear resistance of all membranes. Under wet conditions, preserved AM (control) showed an average tearing strength of 14.3 gram-force (gf) (PSS) and 257.7 gf (SS). FD-AM without γ-irradiation showed an average tearing strength of 15.4 gf (PSS) and 286.7 gf (SS). Finally, FD-AM with γ-irradiation showed an average tearing strength of 11.7 gf (PSS) and 221.9 gf (SS) (Figs. 4A, 4B). There were no statistically significant differences in the physical strength between cryopreserved AM, FD-AM without γ-irradiation, and FD-AM with γ-irradiation (*t*-test, $n = 6$).

Immunohistochemistry of Extracellular Matrix Molecules

Six individual cryopreserved AM and FD-AM samples were examined. The patterns of extracellular matrix molecule expression in the samples were investigated with immunohistochemistry. Negative control sections, incubated with normal mouse and rabbit IgG, and without primary antibody, exhib-

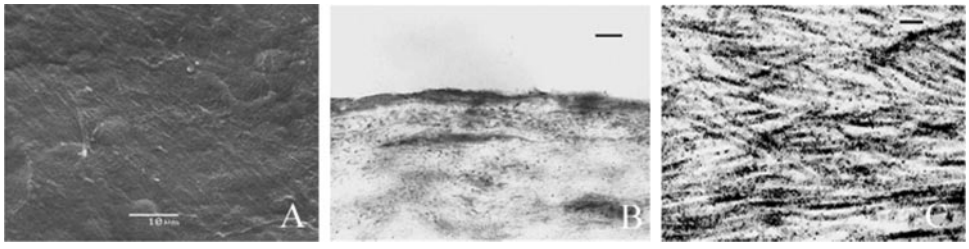

FIGURE 3. Scanning and transmission electron micrographs of the sterilized, freeze-dried amniotic membrane. SEM micrograph showing a continuous flat layer of smooth basement membrane (**A**). TEM micrograph showing that the amniotic membrane was denuded of cells and that apically the basal lamina remained intact (**B**). TEM micrograph showing that the collagen fibrils making up the stromal matrix appeared to be well preserved by the freeze-drying process (**C**). Scale bar: (**A**) 10 μm; (**B**) 200 nm; (**C**) 100 nm.

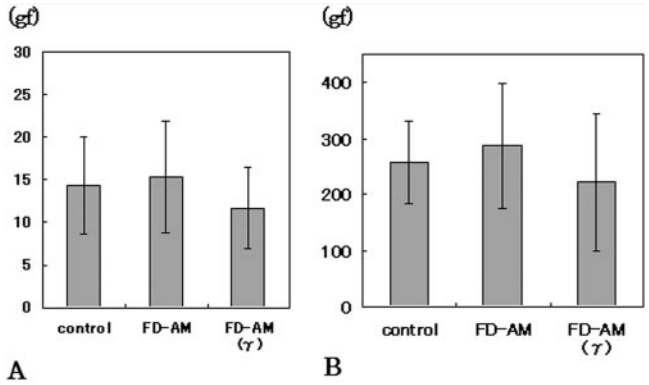

FIGURE 4. The graphs show the results of the PSS test (**A**) and SS test (**B**). In both tests, there were no statistically significant differences in physical strength between control freeze-dried amniotic membrane without γ-irradiation and freeze-dried amniotic membrane with γ-irradiation ($n = 6$, *t*-test).

ited no discernible specific immunoreactivity over the entire region. The immunoreactivity observed in each specimen was compared with these controls. Immunohistochemistry showed the presence of collagen (types I, III, IV, and V) and fibronectin throughout the whole FD-AM. In contrast, collagen VII and laminin-5 were expressed on the basement membrane side of FD-AM (Figs. 5A1–A7). As previously reported, these immunoreactivities were similar to those in cryopreserved AM (Figs. 5B1–B7).[30,31]

Intracorneal Transplantation

One month after intracorneal FD-AM transplantation, we observed the transplanted rabbit corneal surface by slit lamp microscopy. All the transplanted membranes adapted well to the host corneal stroma, with no evidence of subepithelial cell infiltration or stromal edema (Fig. 6). There was no neovascularization on the corneal surface. The FD-AM clarity was also markedly improved (Fig. 6B) and some host keratocytes appeared inside the matrix of the transplanted FD-AM (Fig. 6C).

Cultivated Corneal Epithelial Sheet

Corneal epithelial cells began to form colonies on the FD-AM within 3 days. After 7 days in culture, a confluent primary culture of corneal epithelial cells had been established that covered the entire FD-AM (Fig. 7A). At 3 weeks, the cultivated corneal epithelial cells showed four to five layers of stratification, were well differentiated, and appeared very similar to normal corneal epithelium (Fig. 7B). These sheets showed immunoreactivity for cornea-specific keratin-3 and -12 (Figs. 7C, 7D).

SEM examination of the cultivated corneal epithelial cells revealed a continuous layer of flat squamous polygonal epithelial cells. These cells appeared healthy and well formed, and the apical surfaces were covered in short, regular microvilli (Fig. 8A). TEM examination of the corneal epithelial culture sheet showed that the cells produced five to six layers of well-stratified epithelium, appeared healthy, and were differentiated into basal columnar cells, suprabasal cuboid wing cells, and flat squamous superficial cells (Fig. 8B). The epithelial cells in the basal cell layers were well attached to the FD-AM substrate with hemidesmosome attachments, and produced basement membrane material (Fig. 8C). In all cell layers the epithelial cells were closely attached to neighboring cells by numerous desmosomal junctions (Fig. 8D).

Autologous Transplanted Epithelium

At 3 to 4 weeks after the ocular surface injury, conjunctival epithelium completely covered the damaged corneal surface in all eight albino rabbits, with considerable neovascularization and subconjunctival inflammation evident (Figs. 9A1–D1). The extent of injury was similar in all animals. After the conjunctivalized tissue was removed, we then reconstructed the ocular surface with a cultivated corneal epithelial sheet on 12-mm diameter discs of FD-AM (day 0). No signs of infection, bleeding, or sheet detachment were observed. At an early stage (day 2) after transplantation, the eight eyes that had received autologous cultivated corneal epithelial cells on FD-AM all possessed an epithelialized area (Figs. 9A2–D2), most of which was not stained by fluorescein and was separated from the outer rim of healing conjunctiva by an annular epithelial defect, which stained with fluorescein. At 10 days after transplantation, the area covered by the epithelium had expanded outward and was connected with healing conjunctival epithelium in some areas (Figs. 9A3–D3). Moreover, the corneal surfaces of all eyes were clear and smooth, and the entire corneal surfaces were completely covered with transplanted autologous corneal epithelium (Figs. 9A4–D4). The control animals, which had received FD-AM only, showed no evidence of epithelialization at day 10 (Figs. 9E1–E4).

Discussion

Most ophthalmologists currently use cryopreserved AM under conditions that are as sterile as possible; however, complete sterilization cannot be achieved with present procedures. We believe that complete sterilization of AM is very important and would make for safer and more frequent use of AM. Previously, several methods have been used to preserve AM, including hypothermic storage and freezing. However, these methods require expensive and bulky equipment such as low-temperature freezers. If AM could be preserved at room temperature, it would be extremely convenient, especially for people in developing countries. We attempted to achieve this by preserving AM in the dry state and using γ-irradiation for sterilization.

Our examinations of the physical properties were of particular interest. PSS and SS tests disclosed no significant differences in the mechanical properties of cryopreserved AM, FD-AM without γ-irradiation, and FD-AM with γ-irradiation. Our system for producing FD-AM is unique in several important respects. We subjected the AM to freeze drying under vacuum conditions. Under these conditions, AM can maintain its flexibility and strength. If the AM is dried under ambient conditions, it loses both its smoothness and flexibility and is quite different from cryopreserved AM. Our group previously reported that AM dried under ambient conditions had an average tearing strength of 4.5 gf (PSS) and 48.7 gf (SS), much weaker than the FD-AM reported herein.[27] After freeze-drying the AM, we vacuum-packed it as soon as possible to prevent oxidation. FD-AM kept in ambient conditions invariably became biodegraded. These findings are all consistent with a previous report.[27]

The organization of the extracellular matrix macromolecules plays an important role in the physical and biological properties of AM. We used immunohistochemistry in this study to demonstrate that collagen (types I, III, IV, and V) and fibronectin are expressed in the whole FD-AM, whereas collagen-VIII and laminin-5 were observed in the basement membrane side of FD-AM. These results are similar to those of cryopreserved AM.[30,31] Moreover, our electron microscopic results for FD-AM showed that a continuous flat layer of smooth basement membrane and basal lamina was clearly present and intact, indicating that AM was well preserved by

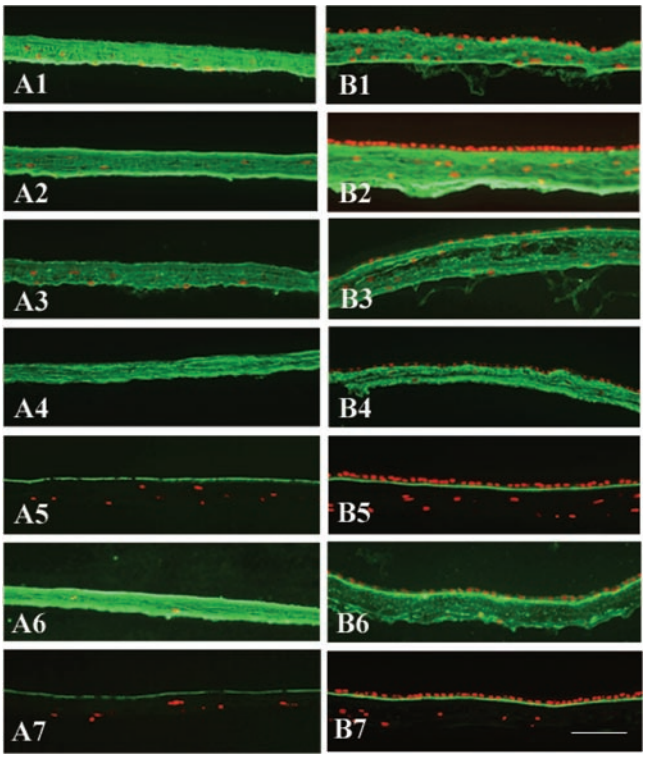

FIGURE 5. Representative immunohistochemical staining of collagen I (**A1**, **B1**), collagen III (**A2**, **B2**), collagen IV (**A3**, **B3**), collagen V (**A4**, **B4**), collagen VII (**A5**, **B5**), fibronectin (**A6**, **B6**), and laminin-5 (**A7**, **B7**) in the sterilized, freeze-dried amniotic membrane (**A**) and cryopreserved intact AM (**B**). Collagen (types I, III, IV, and V) and fibronectin were expressed in the whole AM. In contrast, collagen VII and laminin-5 were expressed in the basement membrane side of AM. Nuclei were stained with propidium iodide (*red*). Scale bar, 200 μm.

the freeze-drying technique. On the basis of these physical, immunohistochemical, and microscopic examinations, we strongly believe that the process of drying and irradiation does not affect the physical or biological properties of AM.

To use FD-AM as a biomaterial, it is important to examine its biocompatibility. We did this by intracorneal transplantation. All the transplanted FD-AM examined in this study adapted well in the host corneal stroma, with no evidence of subepithelial cell infiltration, stromal edema, or neovascularization. Nor was there evidence of infection or rejection on the corneal surface. These results are consistent with the previous report regarding cryopreserved human AM.[32] From these results, we are confident that the FD-AM we produced has excellent biocompatibility with ocular surface tissues.

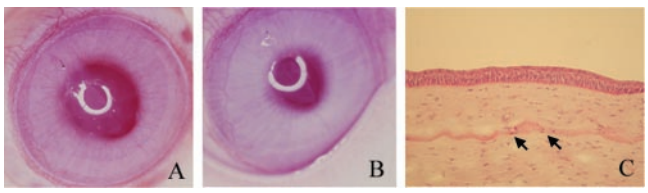

FIGURE 6. Representative slit-lamp photographs of one rabbit taken just after transplantation (**A**) and 1 month after transplantation (**B**) of freeze-dried amniotic membrane (FD-AM). A cross-section of the cornea 1 month after FD-AM transplantation (**C**). One month after intracorneal FD-AM transplantation, there was no evidence of neovascularization or stromal edema on the corneal surface (**B**) and the clarity of FD-AM was markedly improved. (**C**) The membrane adapted well to the corneal stroma and some host keratocytes appeared inside the matrix of the transplanted FD-AM (*arrows*). Original magnification: ×200.

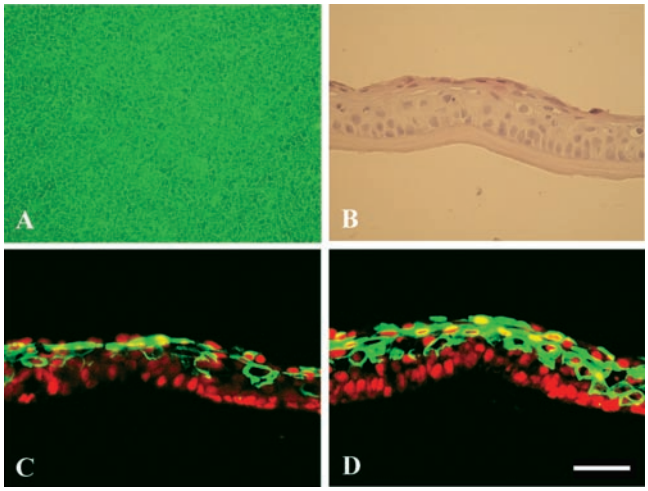

FIGURE 7. Shows a confluent primary culture of corneal epithelial cells on freeze-dried amniotic membrane (**A**). Light micrograph showing a cross-section of the cultivated corneal epithelial cells on freeze-dried amniotic membrane stained with hematoxylin and eosin (**B**). The cultivated corneal epithelial sheet had four to five layers of stratified, well-differentiated cells and appeared very similar to in vivo normal corneal epithelium (**A**, **B**). Light micrographs showing immunohistochemical staining for keratin-3 (**C**) and -12 (**D**). Keratin-3 and -12 were expressed in the superficial and intermediate layers of the cultivated corneal epithelial sheet (**C**, **D**). Original magnification, (**A**) ×100. Scale bar: (**B**, **C**, **D**) 50 μm.

Recently, preserved AM has been widely used as a substrate for cultivating corneal, conjunctival, and oral mucosal epithe-

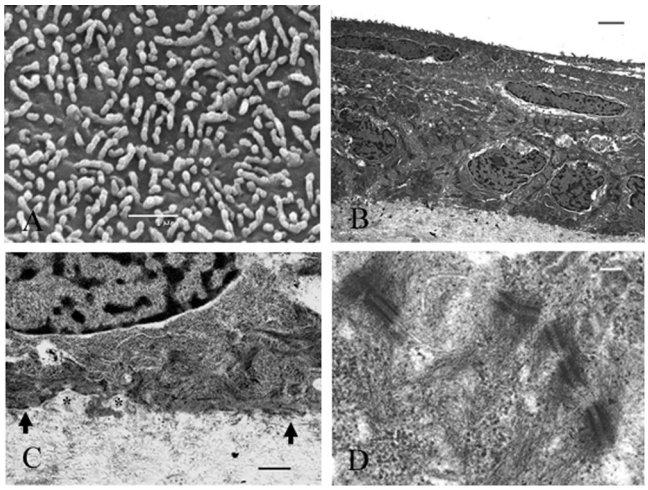

FIGURE 8. Scanning (**A**) and transmission (**B**, **C**, **D**) electron micrographs of rabbit cultivated corneal epithelial cells on freeze-dried amniotic membrane. The cultivated corneal epithelial sheet appeared healthy and well-formed and the apical surface of the cells was covered in short, regular microvilli (**A**). TEM examination showed that the cells produced four to five layers of well-stratified epithelium, appeared healthy, and were differentiated into basal columnar cells, suprabasal cuboid wing cells, and flat squamous superficial cells (**B**). The epithelial cells in the basal cell layers were columnar, well joined to the FD-AM substrate with hemidesmosome attachments (*arrows*), and produced basement membrane material (**C**, ✻). In all cell layers, the epithelial cells were closely attached to neighboring cells by numerous desmosomal junctions (**D**). Scale bar: (**A**) 1 μm; (**B**) 2 μm; (**C**) 500 nm; (**D**) 100 nm.

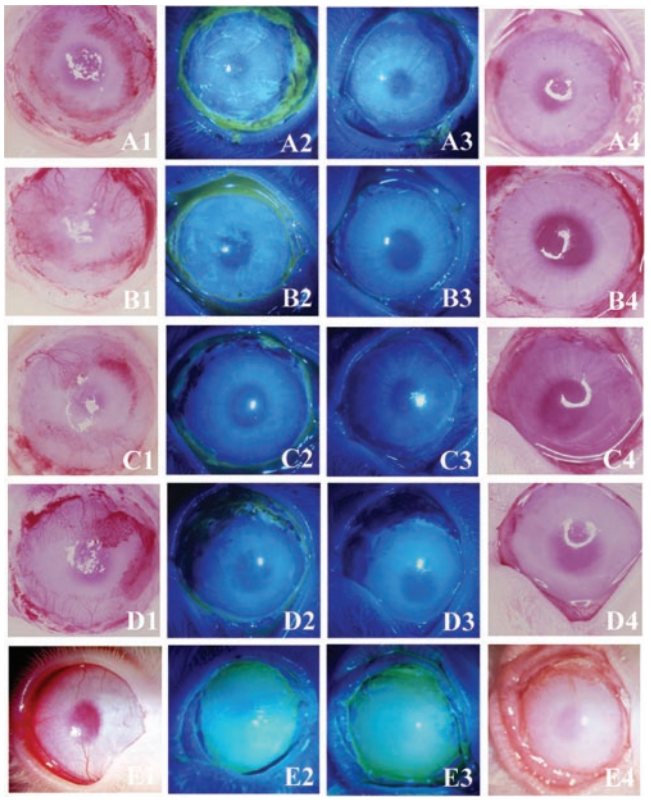

FIGURE 9. Representative slit-lamp photographs of four rabbits taken before transplantation (**A1–D1**), 48 hours after transplantation with fluorescein (**A2–D2**), and 10 days after transplantation with (**A3–D3**) and without (**A4–D4**) fluorescein. Before transplantation, all cases showed total limbal stem cell destruction (**A1–D1**). Forty-eight hours after surgery, most of the corneal surfaces were covered with transplanted cultivated corneal epithelial cells and so showed no fluorescein staining (**A2–D2**). The epithelialized, non–fluorescein-stained area was clearly separated from the surrounding conjunctival epithelium by an annular epithelial defect. At 10 days after surgery, the central epithelialized area had spread outward (**A3–D3**). The epithelium at this time covered almost the entire corneal surface, and in some areas was in contact with the inner part of the healing conjunctival epithelium. At this time, the corneal surface of all rabbits was covered with clear corneal epithelium (**A4–D4**). Representative slit-lamp photographs of a control rabbit that received a freeze-dried AM transplant only (**E1– E4**) onto its keratectomized cornea. The time course of these photographs is the same as detailed above.

lium.[22–26] After successfully producing FD-AM, we tried to cultivate rabbit corneal epithelial cells on it to investigate its effectiveness as a substrate. Fortunately, we could use the experience we gained from cultivating corneal and oral epithelial cells on cryopreserved AM in rabbits and humans.[24–26] We adopted this system for culturing rabbit corneal epithelial cells (with some modifications) and successfully generated confluent cultures of cells on FD-AM. At 3 weeks, the cultivated corneal epithelial cells showed four to five layers of stratification, were well differentiated, and demonstrated immunoreactivity for cornea-specific keratin-3 and -12, indicating that FD-AM supported normal corneal differentiation.

Important points regarding corneal epithelial cell growth on FD-AM include how the basal cells attach to the underlying FD-AM and how the most superficial cells contact the tear–ocular surface interface. We believe these to be key factors in the successful transplantation of cultivated corneal epithelial sheet on FD-AM. Our TEM results showed that cultivated corneal epithelium was firmly attached to the basement membrane with hemidesmosomal junctions. Adjacent cells in the cultivated corneal epithelium were also joined by numerous desmosomal junctions. Examination by SEM also revealed that the apical surface of the cultivated corneal epithelial cells was covered with numerous microvilli, almost identical with those on in vivo corneal epithelial cells. From these results, we believe that corneal epithelial cells cultivated on FD-AM have morphologic properties similar to those of in vivo corneal epithelial cells.

Using FD-AM as a culture substrate, we reconstructed damaged corneal surfaces by transplanting autologous cultivated corneal epithelial cells. Two days after transplantation, most of the corneal surfaces on which cultivated corneal epithelium had been placed were intact without epithelial defects, and were surrounded by a conjunctival epithelial defect at 360°, suggesting no contamination of host conjunctival epithelium. There were no biological immunoresponses to the transplanted FD-AM. Shortly after the transplantation procedure, all conjunctival inflammation in the rabbits rapidly subsided. At 10 days after transplantation, the area covered by the cultivated corneal epithelium had expanded outward and was in contact with healing conjunctival epithelium in some areas. We examined sections of these transplanted corneas using the periodic acid-Schiff reaction and confirmed that there was no contamination of the host conjunctival epithelium (data not shown). From our experiments, we conclude that corneal epithelial cells cultivated on FD-AM can survive and spread onto the adjacent keratectomized corneal surface.

In conclusion, our study is the first to demonstrate the usefulness of sterilized FD-AM for ocular surface reconstruction, on the basis of several experiments evaluating physical, morphologic, and biological properties. We have shown that the sterilized, FD-AM we produced retains the characteristics of cryopreserved AM. On the basis of these results, we are in the process of using this biomaterial for ocular surface reconstruction in patients with severe ocular surface diseases and are carefully evaluating the long-term clinical usefulness of FD-AM.

Acknowledgments

The authors thank Kenichi Endo, Eiichi Sekiyama, and Hisayo Sogabe for assisting in the immunohistochemistry and culture procedure.

References

1. van Herendael, Oberti C, Brosens I. Microanatomy of the human amniotic membrane: a light microscopic, transmission and scanning microscopic study. *Am J Obstet Gynecol*. 1978;83:470–477.
2. Davis JW. Skin transplantation with a review of 550 cases at the Johns Hopkins Hospital. *Johns Hopkins Med J*. 1910;15:307.
3. Stern W. The grafting of preserved amniotic membrane to burned and ulcerated skin surfaces substituting skin grafts. *JAMA*. 1913; 13:973–974.
4. Troensagaard-Hansen E. Amniotic grafts in chronic skin ulceration. *Lancet*. 1950;1:859–860.
5. Trelford JD, Trelford-Saunder M. The amnion in surgery, past and present. *Am J Obstet Gynecol*. 1979;134:844–845.
6. Dhall K. Amniotic graft for treatment of congenital absence of the vagina. *Br J Obstet Gynaecol*. 1984;91:279–282.
7. Rennekampff H-O, Dohrman P, Fory R, et al. Evaluation of amniotic membrane as adhesion prophylaxis in a novel surgical gastroschisis model. *Invest Surg*. 1994;7:187–193.
8. de Roth A. Plastic repair of conjunctival defects with fetal membrane. *Arch Ophthalmol*. 1940;23:522–525.
9. Sorsby A, Symmons HM. Amniotic membrane grafts in caustic burns of the eye. *Br J Ophthalmol*. 1946;30:337–345.
10. Kim JC, Tseng SC. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. *Cornea*. 1995;14:473–484.
11. 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.

12. Shimazaki J, Yang HY, Tsubota K. Amniotic membrane transplantation for ocular surface reconstruction in patients with chemical and thermal burns. *Ophthalmology*. 1997;104:2068–2076.
13. Tseng SC, Prabhasawat P, Barton K, Gray T, Meller D. Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. *Arch Ophthalmol*. 1998;116:431–441.
14. Kim JS, Kim JC, Na BK, et al. Amniotic membrane patching promotes healing and inhibits proteinase activity on wound healing following acute corneal alkali burn. *Exp Eye Res*. 2000;70:329–337.
15. Solomon A, Rosenblatt M, Monroy D, et al. Suppression of interleukin 1alpha and interleukin 1beta in human limbal epithelial cells cultured on the amniotic membrane stromal matrix. *Br J Ophthalmol*. 2001;85:444–449.
16. Tseng SC, Li DQ, Ma X. Suppression of transforming growth factor-beta isoforms, TGF-beta receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. *J Cell Physiol*. 1999;179:325–335.
17. Talmi YP, Sigler L, Inge E. Antibacterial properties of human amniotic membranes. *Placenta*. 1991;12:285–288.
18. Hao Y, Ma DH, Hwang DG, Kim WS, Zhang F. Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. *Cornea*. 2000;19:348–352.
19. Akle CA, Adinofli M, Welsh KI, et al. Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. *Lancet*. 1981;2:1003–1005.
20. Adinofli M, Akle CA, McColl I, et al. expression of HLA antigens, beta 2-microglobulin and enzymes by human amniotic epithelial cells. *Nature*. 1982;295:325–327.
21. Tsubota K, Satake Y, Kaido M, et al. Treatment of severe ocular surface disorders with corneal epithelial stem-cell transplantation. *N Engl J Med*. 1999;340:1697–1703.
22. Tsai RJF, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med*. 2000;343:86–93.
23. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. *Cornea*. 2000;19:421–426.
24. Koizumi N, Inatomi T, Quantock AJ, et al. Amniotic membrane as a substrate for cultivating limbal corneal epithelial cells for autologous transplantation in rabbits. *Cornea*. 2000;19:65–71.
25. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. *Ophthalmology*. 2001;108:1569–1574.
26. Nakamura T, Endo K, Cooper L, 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.
27. Yamamoto Y, Ueda H, Matsumoto K, et al. Production of acellular matrix membranes from human amnion for medical application. *Jpn J Artif Organs*. 1997;26:219–224.
28. 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.
29. Nakamura T, Nishida K, Dota A, Kinoshita S. Changes in conjunctival clusterin expression in severe ocular surface disease. *Invest Ophthalmol Vis Sci*. 2002;43:1702–1707.
30. Malak TM, Ockleford CD, Bell SC, et al. Confocal immunofluorescence localization of collagen types I, III, IV, V and VI and their ultrastructural organization in term human fetal membranes. *Placenta*. 1993;14:385–406.
31. Fukuda K, Chikama T, Nakamura M, Nishida K. Differential distribution of subchains of the basement membrane components type IV collagen and laminin among the amniotic membrane, cornea and conjunctiva. *Cornea*. 1999;18:73–79.
32. Kubo M, Sonoda Y, Muramatsu R, Usui M. Immunogenicity of human amniotic membrane in experimental xenotransplantation. *Invest Ophthalmol Vis Sci*. 2001;42:1539–1546.