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

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**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 Tseng10 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.11–15

A variety of characteristics make AM ideally suited for use in ocular surface reconstruction. It has an anti-inflammatory effect,13–15 antifibroblastic activity,16 antimicrobial17 and antiangiogenic18 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

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physical characteristics of FD-AM.
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., Aldermaston, 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 with 20 minutes at 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, 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 (revoloxacin) and intramuscular gentamicin (1 mg/kg) were administered after surgery. At 5 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 (revoloxacin) 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 sutureable 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).

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-
Corneal epithelial cells began to form colonies on the FD-AM within 3 days. After 7 days in culture, a confluence of cells was observed, and by 3 weeks, the cultured corneal epithelial sheet showed differentiation into basal columnar cells, suprabasal cuboid wing cells, and flat squamous superficial cells. The cells appeared healthy and well differentiated, 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 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 the 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 well-preserved (Fig. 7A). The control animals, which had received FD-AM only, showed no evidence of epithelialization at day 10 (Figs. 9E1–E4).

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 the 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 FD-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.47

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...
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 to the host corneal stroma, with no evidence of subepithelial inflammation or 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. From these results, we are confident that the FD-AM we produced has excellent biocompatibility with ocular surface tissues.

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

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.

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.
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 immunoreponses 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 keratotomized 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.

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**References**


