Biological and Ultrastructural Properties of Acelagraft, a Freeze-Dried $\gamma$-Irradiated Human Amniotic Membrane

Laurence S. Lim, MRCSEd; Rebekah W. Y. Poh, BSc; Andri K. Riau, BSc; Roger W. Beuerman, PhD; Donald Tan, FRCOphth; Jod S. Mehta, MBBS, MD

Objectives: To compare the biological and ultrastructural properties of a commercially available decellularized and dehydrated human amniotic membrane (DDHAM) product with cryopreserved human amniotic membrane (CHAM) and to demonstrate the feasibility of DDHAM transplant in a case of chronic ocular surface disease.

Methods: Histologic examination, immunohistochemical examination for extracellular matrix molecules and growth factors, transmission and scanning electron microscopy, and atomic force microscopy were performed on both DDHAM and CHAM specimens. A DDHAM transplant was performed in a patient with chronic bulous keratopathy and ocular surface instability.

Results: Histologic examination and transmission electron microscopy revealed the disruption of the trilaminar structure of the basement membrane compared with CHAM, and immunohistochemical examination confirmed the loss of collagen IV and VII, laminin, and fibronectin in DDHAM. Lower levels of several growth factors were also seen in DDHAM compared with CHAM. Clinical transplant of DDHAM was, however, successful, with rapid reepithelialization.

Conclusion: Significant differences in composition and ultrastructure exist between DDHAM and CHAM but do not appear to compromise cell survival in vivo.

Clinical Relevance: The ease of storage and handling of DDHAM make it potentially valuable in ocular surface surgery, but its biological properties have not been well characterized, and there are also few data on its clinical application.


Human amniotic membrane (HAM) has been used successfully in ocular surface reconstruction as a biological bandage and as a substrate for stem cell expansion. The combination of anti-inflammatory, antimicrobial and antiviral, antifibrotic, and antiangiogenic properties of HAM provides a favorable environment for cellular attachment and expansion in both in vivo and in vitro settings. Commercially available HAM products, such as AmnioGraft and ProKera (Bio-Tissue, Inc, Miami, Florida), are currently treated with cryopreservation at $-80^\circ$C following harvesting to render amniotic epithelial cells nonviable and the tissue nonimmunogenic. This approach has 2 main drawbacks, namely incomplete sterilization with the possibility of disease transmission and the need for an expensive and cumbersome deep freezer.

Acelagraft (Acelagraft Cellular Therapeutics, Cedar Knolls, New Jersey) is a commercially available decellularized and dehydrated HAM (DDHAM) product. The tissue is sterilized by $\gamma$-irradiation and is acellular and freeze dried. It can thus be stored and shipped at room temperature, making handling much easier. Bhatia et al have previously reported that Acelagraft is a primarily collagenous extracellular matrix–like material and have demonstrated that it is able to support dermal fibroblast assembly and secretion of key wound healing cytokines.

The aim of this study was to compare the biological and ultrastructural properties of DDHAM with conventional cryopreserved HAM (CHAM) with respect to cellular components, biochemical composition, and membrane surface characteristics and to demonstrate its feasibility in a case of ocular surface disease requiring resurfacing with HAM.

Methods

PROCUREMENT AND PREPARATION OF HAM

The CHAM was prepared from human placentas obtained from mothers who had undergone cesarean sections. The membranes were...
washed with phosphate-buffered saline (PBS) to remove the blood clots. The HAM was peeled away from the chorion and flattened onto a sterilized nitrocellulose filter paper (Millipore, Bedford, Massachusetts). The CHAM was then stored in 50% Dulbecco modified Eagle medium and 50% glycerol (Invitrogen Gibco, Carlsbad, California) at −80°C. In preparation for its use, the CHAM was thawed and rinsed with PBS.

The DDHAM (Acelagraft) was prepared using proprietary methods that have been described previously. Briefly, the amniotic membrane was excised from qualified term placentas and washed and scraped to remove extraneous tissue and cells. This was then followed by decellularization of the tissue using deoxycholic acid and drying of the tissue using a gel dryer. Sterilization of the product was achieved with electron-beam irradiation, and the recommended storage temperature for the product is between 15°C to 30°C.

**IMMUNOHISTOCHEMICAL EXAMINATION FOR EXTRACELLULAR MATRIX MOLECULES AND GROWTH FACTORS**

Tissues were embedded in optimal cutting temperature freezng compound (Leica, Nussloch, Germany). Six-micromole-thick sections were cut and positioned on polylysine-coated glass slides and then air dried for 20 minutes. They were then subjected to hematoxylin-eosin staining or indirect immunostaining analysis. The tissues were fixed in −20°C acetone for 15 minutes, followed by washing 3 times for 5 minutes in PBS Triton, 0.15%. The samples were then immersed for 1 hour in blocking solution (bovine serum albumin, 4%, in 0.01M PBS) for 1 hour at room temperature in a humid chamber. After washing for 5 minutes in PBS Triton, 0.15%, they were incubated overnight, at 4°C, with primary antibody solutions (Table). Each sample (CHAM or DDHAM [Acelagraft]) was tested in quadruplicate. Epithelialized CHAM sections and normal mouse/goat/rabbit immunoglobulin were used as positive and negative controls, respectively. The tissues were then washed 3 times for 5 minutes in PBS (0.01M) and were subsequently incubated with fluororescent (fluorescent isothiocyanate conjugated) secondary antibody (Alexa fluor 488-labeled antimouse IgG, antirabbit IgG, and antigoat IgG; Invitrogen) at a dilution of 1:200. After 1 hour, the tissues were washed 3 times for 5 minutes in PBS (0.01M), in the dark. Slides were then mounted with Fluorsave with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, California). They were subsequently examined with a Zeiss Axioplan 2 fluorescence microscope (Zeiss, Oberkochen, Germany).

**WESTERN BLOTTING**

The CHAM was homogenized individually into 50mM TRIS-hydrochloride buffer containing protease inhibitors. Insoluble materials were removed by 15 minutes of centrifugation (10,000g at 4°C). An equal volume of 4% sodium dodecyl sulfate loading buffer was added to each sample, which was then subjected to boiling for 5 minutes at 100°C and centrifugation for 5 minutes (10,000g at 4°C). For DDHAM (Acelagraft), the membranes were divided into small pieces, then incubated in ristocetin-induced platelet agglutination lysis buffer at room temperature for 15 minutes. The samples were then sonicated.
for 15 minutes and heated at 100°C for 15 minutes. Following this, they were cooled to room temperature and centrifuged at 12,000g at 4°C for 10 minutes. The sample was then left on ice for 10 minutes and stored at −20°C until reduction on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (gradient, 6%–15%). The amounts of protein applied ranged from 30 to 60 μg. Resolved proteins were electrically transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California) and blocked in PBS containing 5% nonfat milk, followed by 2-hour incubation with the primary antibody (Table) at room temperature with agitation. Samples were then washed vigorously 3 times each for 5 minutes in PBS polysorbate 20, 0.05%. The appropriate secondary reagent was then applied, ie, goat-antimouse horseradish peroxidase, goat-antirabbit horseradish peroxidase, or rabbit-antigoat horseradish peroxidase (Santa Cruz Biototechnology Inc, Santa Cruz, California) at a dilution of 1:2000. Immunoreactivity was visualized with a chemiluminescence reagent (SuperSignal West Pico; Pierce Biotechnology, Rockford, Illinois).

**ELECTRON MICROSCOPY**

**Transmission Electron Microscopy**

The CHAM and DDHAM tissues were fixed with cold paraformaldehyde, 2%, and glutaraldehyde, 2%, in 0.1M sodium cacodylate buffer (pH 7.4) (Electron Microscopy Sciences, Hatfield, Pennsylvania) at 4°C overnight. Tissues were washed in sodium cacodylate buffer, rinsed with distilled water, and trimmed into smaller pieces. Tissues were then postfixed in osmium tetroxide, 1%, and potassium ferrocyanide (Electron Microscopy Sciences) to enhance membrane contrast. After extensive rinsing with distilled water, tissues were dehydrated in a graded series of ethanol and embedded in Araldite (Electron Microscopy Sciences). Semithin sections of 0.5 to 1 µm thickness were cut with Reichert-Jung Ultratcut E Ultramicrotome (C. Reichert Optische Werke AG, Vienna, Austria), counterstained with toluidine blue/basic fuchsian stain, and examined using a Zeiss Ultramicroscope (Zeiss). Ultrathin sections of 60 to 80 nm were collected on copper grids, double stained with uranyl acetate and lead citrate for 8 minutes each, and then viewed and photographed on a JEOL 1220 electron microscope (JEOL, Tokyo, Japan) at 100 kV.

**Scanning Electron Microscopy**

Specimens were immersed in a fixative containing glutaraldehyde, 2.0%; paraformaldehyde, 2.0%; and 0.1M sodium cacodylate (pH 7.4) overnight at 4°C. They were then transferred and stored in sodium cacodylate buffer (Electron Microscopy Sciences). Before processing, the specimens were washed twice in distilled water for 10 minutes. They were then secondary fixed in osmium tetroxide, 1% (FMB, Singapore), for 2 hours at room temperature. Following this, they were dehydrated in a graded ethanol series of 25%, 50%, 75%, 95%, and 100% each for 10 minutes, with repetition in 100% ethanol 3 times. The samples were then dried in a critical point dryer (Bal-Tec, Liechtenstein, Germany) and mounted on scanning electron microscopy (SEM) stubs using carbon adhesive tabs and sputter coated with a thin layer of gold (15 nm) (Bal-Tec). A scanning electron microscope (JSM-5600LV; JEOL) at an accelerating voltage of 20 kV was used for imaging.

**Atomic Force Microscopy**

The DDHAM tissues were stored in PBS at 4°C. Tissues of about 1 cm × 0.5 cm were rinsed with deionized water, mounted on mica sheets, and left to dry overnight in a desiccator.

Atomic force microscopy imaging in air was conducted using a Nanoscope IV Multimode (Veeco Digital Instruments, Santa Barbara, California). Images were captured in tapping mode using TappingMode etched silicon probes (RTESP; Veeco Digital Instruments) with resonant frequency about 300 kHz, spring constant of around 40 N/m, and a tip radius of less than 10 nm. All images were acquired with a 256 × 256 point resolution at a scan rate of 3 Hz.

Ten reference areas were analyzed from each tissue. Each area of 625 μm² was scanned twice to ensure that the force exerted was not sufficient to damage the sample surface and cause artifacts. Areas scanned were selected based on a clock-face distribution.

Height, amplitude, and phase images, as well as 3-dimensional images, were simultaneously acquired. Surface-roughness values were calculated from height-mode images using Nanoscope 6.11 software (Veeco Digital Instruments). The mean root-mean-square (RMS) values were calculated by collecting 10 RMS values over 10 areas of 625 μm² on each tissue and calculating the mean. The RMS values reflecting fluctuations of surface heights were calculated according to the following equation:

$$R_{rms} = \sqrt{\frac{\sum (h_i - \bar{h})^2}{n}}$$

(where n is the number of data points) and taking the mean. All images used to gather RMS values were 25 × 23 μm in scan size to provide roughness measurements, and all tips were from the same wafer batch to alleviate variations in tip geometry. Skewness, representing the symmetry of surface data about a mean data profile, was calculated from the following equation with $R_q$ as the RMS roughness:

$$Skewness = \frac{1}{R_q^3} \sum_{j=1}^{N} Z_j^3$$

**RESULTS**

**HISTOLOGIC EXAMINATION**

Histologic examination of a CHAM specimen stained with hematoxylin-eosin revealed a single layer of cuboidal epithelial cells on a densely eosinophilic basement membrane (BM) overlying a loosely arrayed basophilic stroma. In contrast, hematoxylin-eosin staining of a DDHAM specimen was notable for the lack of cellular structures or a prominent BM. The specimen showed a homogeneous pale basophilia (Figure 1).

**IMMUNOHISTOCHEMICAL EXAMINATION OF EXTRACELLULAR MATRIX MOLECULES**

Normal sections incubated with mouse, goat, and rabbit immunoglobulins and without primary antibody demonstrated no immunoreactivity. Compared with CHAM, DDHAM expression of collagen I, II, and VI; elastin; tenascin; and thrombospondin were comparable. The DDHAM immunoreactivity to fibronectin was less intense than CHAM and negative for collagen IV and VII and laminin 5. Expression of all growth factors evaluated (epidermal growth factor receptor, fibroblast growth factor 2, insulinlike growth factor 1, platelet-derived growth factor A, platelet-derived
growth factor B, transforming growth factor (TGF) β1, TGF-α, TGF-β2 receptor, and vascular endothelial growth factor) was markedly lower in DDHAM compared with CHAM (Figure 2). Western blot analyses for fibronectin, collagens IV and VI, and elastin confirmed the differential expression of these extracellular matrix components in CHAM and DDHAM (Figure 3).

The BM of CHAM supports the overlaying epithelium and is composed of a 3-layered basal lamina and lamina fi-
broreticularis. Lamina rara externa (lucida) is an electron-lucent zone directly bordering the adjacent cell that makes up the upper portion of the basal lamina. Lamina densa is an electron-dense zone that appears somewhat amorphous and granular and constitutes the intermediate part of the basal lamina. Lamina rara interna comprises the basal portion of the basal lamina. The 3 layers of basal lamina sit on top of the lamina fibroreticularis, which is synthesized by cells from underlying connective tissue and contains fibrillar structures, namely anchoring fibrils, elastic fibrils, and microfibril bundles (Figure 4A).

In the transmission electron micrographs of DDHAM, the absence of epithelial cells was demonstrated and there was no lamina lucida. The lamina densa was present and approximately the same thickness as that of CHAM (Figure 4B).

The smoothness of DDHAM was evaluated by SEM and by atomic force microscopy (Figure 5). Surface structure morphology of DDHAM was cauterized using both SEM and atomic force microscopy. Although these 2 techniques provide complementary images, different information on the surface structure can be derived to give a more complete examination. The SEM micrographs at low magnification (Figure 5A) demonstrated a large area view of DDHAM showing the distinctive grids. At higher magnification (Figure 5B), it is clearly devoid of cellular structures and the surface appears smooth. On a smooth surface, SEM has difficulty resolving features because of the subtle variations in height. Moreover, tissues are subjected to shrinkage owing to chemical processing and the mandatory gold coating might conceal fine structures. To address these setbacks, atomic force microscopy was further used to image the samples at nanoscale resolution. Mean RMS and skewness were calculated using height data (Figure 5C). Mean RMS indicates height deviations and was calculated at 113 nm while skewness at 0.184 nm indicated a relatively even distribution of height on a flat plane. Height data show dark areas representing depressions and brighter areas representing protrusions on the surface. Amplitude data (Figure 5D) correlate height data and give information on the surface texture. Phase data (Figure 5E) depict hardness variations where bright areas represent hard regions whereas dark areas represent soft regions. The 3-dimensional (Figure 5F) reconstruction presented a topographical map on the imaged area.

**CLINICAL CASE REPORT**

A 40-year-old man with a history of irido-corneal-endothelial syndrome, advanced chronic glaucoma, and an Ahmed glaucoma drainage device implantation was referred for bullous keratopathy. Penetrating keratoplasty was performed but the patient experienced endothelial rejection with an edematous graft, poor ocular surface, and severe pain (Figure 6A). The patient was keen to avoid enucleation, and conjunctival scarring from the Ahmed glaucoma drainage device implantation precluded a Gundersen flap. A DDHAM transplant was thus performed for ocular surface stabilization. The DDHAM was punched with an 8.25-mm trephine and rehydrated for 10 minutes prior to use. The recipient epithelium was debrided and the prepared DDHAM was sutured with 11-0 nylon sutures and protected with a hydrophilic bandage contact lens. The postoperative regimen included topical preservative-free levofloxacin and dexamethasone eyedrops applied with a gradually tapering frequency.

On the first postoperative day, epithelialization had already begun with an epithelial defect over approximately 80% of the corneal surface (Figure 6B). Further recovery was uneventful, with only a small epithelial defect remaining at 17 days after surgery (Figure 6C) and restitution of a completely epithelialized ocular surface by 24 days.
The treatment of ocular surface disease is challenging, and current treatment strategies are resource intensive and inaccessible to underdeveloped countries. In this context, the ease of handling of DDHAM presents notable advantages over the use of conventional CHAM because it can be transported at room temperature and simply rehydrated before use. Absolute microbial and viral sterility, which are of particular importance in a compromised ocular surface, are also assured. Extensive laboratory and clinical data on DDHAM are, however, limited. Bhatia et al. have reported on the composition of DDHAM (Acelagraft) as well as the biology of fibroblasts grown on DDHAM, while Nakamura et al. have described the physical characteristics and biocompatibility of DDHAM.

**Figure 5.** Smoothness of decellularized and dehydrated human amniotic membrane (DDHAM) evaluated by scanning electron microscopy (A and B) and by atomic force microscopy (C-F). A, Low magnification (original magnification ×18; bar=1 mm) of Acelagraft (Acelagraft Cellular Therapeutics, Cedar Knolls, New Jersey). B, High magnification (original magnification ×500; bar=50 µm) showing smoothness of DDHAM surface. C, Height data of DDHAM where dark areas represent depressions and brighter areas represent protrusions on the surface. D, Amplitude data. E, Phase data depicting hardness variations. Bright areas represent hard regions whereas dark areas represent soft regions on the surface. F, 3-Dimensional presentation of topographical map. Image scale: 50×50 µm.
ility of a noncommercial DDHAM in a rabbit model of ocular surface disease. To our knowledge, there are no reports of detailed analyses of Acelagraft or its use in human ocular surface disease.

In our study, a prominent feature of the transmission electron micrographs of DDHAM was the disruption of the trilaminar structure of the BM compared with CHAM. Immunohistochemical staining for BM components likewise revealed the absence of several characteristic BM components, including collagens IV and VII and laminin, and reduced expression of fibronectin compared with CHAM. These findings were confirmed with Western blot. Solubilization of DDHAM and analysis of its components has shown that it is composed mainly of collagen types I and III (44% dry weight) with only minimal amounts of collagen IV, laminin, and fibronectin. Nakamura et al. were, however, able to demonstrate collagen IV, laminin, and fibronectin expression similar to CHAM in their preparation of DDHAM. These differences may be attributable to different preparation methods, because deoxycholic acid is used in the decellularization of Acelagraft prior to drying whereas EDTA was used by Nakamura et al. Laminin is localized primarily to the lamina lucida, which was disrupted in transmission electron microscopy images in the DDHAM in our study but well preserved in the freeze-dried HAM produced by Nakamura et al. The role of the BM in HAM as a substrate for cell growth, and particularly its collagen IV component, is presently unclear, but several authors have reported that HAM may serve as a more specific substrate for human conjunctival cells rather than corneal epithelial cells. Interestingly, the DDHAM used in our study, devoid of collagen IV and a normal BM, was able to support the growth of cells with conjunctival morphology in our case of human transplant, whereas Nakamura et al were able to cultivate rabbit corneal epithelial cells on their DDHAM preparation with an intact BM. It has been shown that limbal epithelial cells grown on HAM cause dissolution of the native BM prior to secret ing a new BM. An intact BM hence may not be essential for cell attachment and expansion.

Several growth factors have been investigated for their roles in promoting epithelialization on HAM. High levels of epidermal growth factor, keratinocyte growth factor, hepatocyte growth factor, TGF-β1, fibroblast growth factor, and several other growth factors have been identified in HAM, most of which—particularly epidermal growth factor, TGF-β1, and fibroblast growth factor—have well-documented roles in the epithelial-mesenchymal cell interactions involved in corneal wound healing. Significantly higher levels of growth factors are seen in HAM bearing epithelium compared with denuded HAM, suggesting that the amniotic epithelium actively secretes growth factors to support epithelial growth. Bhatia et al., using guanidinium-hydrochloride extraction methods to solubilize DDHAM before enzyme-linked immunosorbent assay analysis, were unable to detect vascular endothelial growth factor, fibroblast growth factor, TGF-β1, platelet-derived growth factor A and B, or pigment epithelium-derived factor in DDHAM. Using immunohistochemical examination, we were able to detect the presence of fibroblast growth factor 2, plate-
kines involved in chemotaxis and wound healing, including interferons, tumor necrosis factor α, and macrophage inflammatory protein. The interaction between fibronectin, which is relatively well conserved in DDHAM, and fibroblasts, which are abundant in scar tissue, may thus be a key mediator of wound healing augmentation by DDHAM.

In conclusion, our study has demonstrated the feasibility of a sterile, dehydrated HAM product for ocular surface reconstruction in a human subject. Decellularized and dehydrated HAM may be a more cost-effective solution for ocular surface rehabilitation in smaller centers with lower patient numbers because the cost of purchasing and maintaining cryopreservation facilities are considerable. Larger tertiary centers for which the amortized costs of CHAM are lower may, however, still find DDHAM useful in situations of donor shortage. Significant differences in composition and ultrastructure exist between DDHAM and CHAM but do not appear to compromise cell survival in vivo. Further in vitro studies to demonstrate the viability of corneal, limbal, and conjunctival explants would help to establish the veracity of our findings, and more clinical trials evaluating the efficacy of DDHAM as an alternative to CHAM for a variety of indications may also expand the role of DDHAM in ocular surface reconstruction.

Submitted for Publication: October 7, 2009; final revision received February 4, 2010; accepted February 18, 2010.

Correspondence: Jod S. Mehta, MBBS, MD, Singapore National Eye Centre, 11 Third Hospital Ave, Singapore 168751 (jodmehta@gmail.com).

Author Contributions: Dr Mehta had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: None reported.

Funding/Support: Dr Mehta is partially funded by a Peel Travel Grant Award 2006 from the Peel Medical Research Trust, London, England.

REFERENCES

3. Tsu-bota K, Satake Y, Ohyama M, et al. Surgical reconstruction of the ocular sur-

4. Tseng SC, Prabhasawat P, Barton K, Gray T, Meller D. Amniotic membrane transplanta-

tion with or without limbal allografts for corneal surface reconstruction in pa-

5. Solomon A, Rosenblatt M, Monroy D, Ji Z, Pflugfelder SC, Tseng SC. Suppres-

6. Tseng SC, Li DQ, Ma X. Suppression of transforming growth factor-beta iso-

7. Talma YP, Sigler L, Inge E, Finkelstein Y, Zohar Y. Antibacterial properties of hu-

10. Adinolfi M, Akle CA, McColl I, et al. Expression of HLA antigens, beta 2-micro-

11. Fernandes M, Sridhar MS, Sangwan VS, Rao GN. Amniotic membrane trans-

12. Bhatia M, Pereira M, Rana H, et al. The mechanism of cell interaction and re-


14. Fukuda K, Chikama T, Nakamura M, Nishida T. Differential distribution of sub-

18. Li W, He H, Kuo CL, Gao Y, Kawakita T, Tseng SC. Basement membrane disso-

22. Kruse FE, Rohrschneider K, Völcker HE. Multilayer amniotic membrane trans-

23. Letko E, Stechschulte SU, Kenyon KR, et al. Amniotic membrane inlay and over-