The Effect of Amniotic Membrane De-Epithelialization Method on its Biological Properties and Ability to Promote Limbal Epithelial Cell Culture

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Purpose. We characterized the de-epithelialized human amniotic membrane (HAM), and compared cell attachment and proliferation efficiencies.

Methods. HAM was de-epithelialized by 20% ethanol (AHAM), 1.2 U/mL Dispase (DHAM), 0.02% EDTA (EHAM), 0.25% trypsin-EDTA (THAM), and 5 M urea (UHAM), respectively, followed by gentle scraping with a #15 blade. Surface topology, extracellular matrix (ECM), and growth factor content were characterized and compared to intact HAM by electron microscopies (EM), atomic force microscopy (AFM), immunohistochemistry, and Western blotting. Primary human limbal epithelial cells (LEC) attachment and proliferation efficiencies were assayed. Statistical significance was calculated by SPSS and Fisher's least significant difference test.

Results. EHAM, THAM, and UHAM had intact basal lamina and smooth basement membrane surface shown under transmission and scanning EM, and AFM. Cell remnants stayed on AHAM. Disrupted basement membrane and stroma was found in DHAM. Immunostaining intensity quantification and hierarchical clustering revealed that ECM composition of EHAM and UHAM resembled intact HAM. In contrast, DHAM and THAM had drastic loss of ECM and growth factor content. LEC attachment efficiency at 24 hours after seeding was the highest in EHAM (51% as on conventional culture surface), followed by UHAM and AHAM. However, cell proliferation indices at day 10 of culture were similar among different HAM substrates, suggesting repair of ECM and basement membrane by growing epithelial cells.

Conclusions. Urea denudation preserved the basement membrane integrity, ECM, and growth factor composition, and had higher cell attachment and proliferation efficiencies. With its short processing time, urea treatment offers a novel alternative for HAM de-epithelialization.

Keywords: amniotic membrane, preparation, cell proliferation, growth factor, basement membrane

Maintaining the integrity of the corneal epithelium is essential for corneal clarity and transparency. Limbal epithelial stem cells, which are located at the palisades of Vogt, have an important role in replenishing the corneal epithelial cells due to their rapid turnover and in vivo regeneration after an injury.[1,2] Deficiency of the limbal epithelial stem cells can be congenital, for example, in aniridia; or acquired in cases of Stevens Johnson syndrome, ocular cicatricial pemphigoid, contact lens-induced keratopathy, or caused by acid or alkali burn injuries.[3] Such patients normally present with neovascularization, chronic inflammation, persistent and recurrent epithelial defects, and conjunctivalization, resulting in decreased visual acuity, increased tearing, recurrent pain, photophobia, blepharospasm, and symblepharon with redness.[4–6]

Cultivated limbal cell transplantation is a surgical technique for the treatment of total limbal stem cell deficiency. It was described first by Pelligrini et al. in 1997 to reconstruct the defective ocular surface in patients with severe alkali burn.[7] Subsequently, much effort has been spent on searching for a suitable substrate for ex vivo expansion of limbal epithelial cells (LEC) and delivery for transplantation.[8–11] To date, human amniotic membrane (HAM) is the most widely used biological substrate for the cultivated LEC expansion due to its inherent ability to promote epithelialization.[12] Its low immunogenicity, antimicrobial, antiviral, antifibrotic, and antiangiogenic properties also support its use in ocular surface reconstruction.[13,14] Furthermore, HAM also is recruited for the ex vivo expansion of oral mucosal and conjunctival epithelial cells.[15,16]

Retrieved from donor placenta to transplantation, HAM must undergo a series of preoperative preparation.[8,17,18] One important step is the de-epithelialization to uncover the basement membrane for epithelial cell attachment and proliferation. Prior studies have shown that the denuded HAM promotes a better growth and expansion of isolated LEC, while intact HAM is more suitable for limbal explant; however, this

discrepancy is yet to be explained.[8,14] Until now, there have been several published protocols for HAM denudation used in clinical and animal studies. The three more commonly used methods are treatments with 0.02% EDTA for 2 hours at room temperature,[19,20] 1.2 U/mL Dispase II for 2 hours at 37°C,[21,22] and 0.25% trypsin-EDTA for 30 minutes at 37°C.[23] These methods involve a long exposure to enzymatic solution and/or metal chelator, which potentially affect the cell binding property of the remaining basement membrane components. Chelating calcium by EDTA can prevent cadherin binding between cells or detach cells from aggregates.[24,25] It also is known to inhibit various metallopeptidases[26,27]; hence, it might affect MMP activity in cell proliferation and migration. To have a complete removal of nonviable amniotic epithelial cells, mechanical scraping is performed routinely after these treatments. This would physically damage the basement membrane structure and composition, and affect the subsequent LEC attachment and proliferation. Thus, search of alternative method for effective cell removal and better preservation of basement membrane would be necessary for ex vivo LEC expansion and clinical transplantation to reconstruct the defective ocular surface.

In our study, we characterized the denuded HAM prepared by the aforementioned chemicals, and studied the expression of extracellular matrix (ECM) components and growth factors, ultrastructure, and surface topography. We designed two new methods, treatment with 20% ethanol for 30 seconds at room temperature and treatment with ice-cold 5 M urea for 5 minutes at room temperature (Mehta JS, et al. *IOVS* 2007;48:ARVO E-Abstract 453). We also examined the efficiency of LEC attachment and proliferation on HAM denuded by different treatments.

Materials and Methods

The experimental procedures conformed to the guidelines of the Declaration of Helsinki for biomedical research involving human subjects, and were approved by the institution review board of Singapore National Eye Centre and Singapore Eye Research Institute, Singapore.

Specimens

All tissues for experiments were free of infection with human immunodeficiency virus (HIV), human T-lymphotrophic virus (HTLV), hepatitis B and C, and syphilis according to guidelines. Cadaveric human cornea rims (Table 1) and HAM were obtained from the Singapore Eye Bank with written informed consent. Corneal rims were transported in Optisol GS (Chiron Ophthalmics, Irvine, CA) at 4°C to the laboratory. Human placenta was obtained from a 38-year-old donor who underwent cesarean section. After washes with PBS (0.01 M) to remove blood clots, the amnion was peeled from the chorion, flattened onto sterilized nitrocellulose paper (Millipore, Bedford, MA), cut to pieces (5×5 cm^2), and stored in Dulbecco's modified Eagle's medium (DMEM)/glycerol (50%:50%) at –80°C.

Human Limbal Epithelial Cell Isolation and Culture

Human corneal rims were washed in DMEM/PBS (1:1, vol/vol) with penicillin S-streptomycin sulfate and amphotericin B (Invitrogen, Carlsbad, CA). After clearing of other tissues, peripheral-limbal cornea was treated with Dispase-TryPLE method for single cell isolation.[28] After passing through a cell strainer (70 μm pore size; Becton Dickinson, Franklin Lakes, NJ), LEC were collected and cultured in CnT50 medium (CELLnTEC, Basel, Switzerland).

HAM Denudation

HAM was cut to 1×2 cm^2 strips. Each strip was treated with 20% ethanol in PBS (vol/vol, absolute ethanol; Merck, Darmstadt, Germany) for 30 seconds at room temperature (AHAM), Dispase II (1.2 U/mL; Roche, Indianapolis, IN) in PBS for 2 hours at room temperature (DHAM), EDTA (0.02% wt/vol; Sigma-Aldrich, St. Louis, MO) at pH 8.0 for 2 hours at room temperature (EHAM), trypsin-EDTA (0.25%/0.02%, wt/vol; Invitrogen) in PBS for 30 minutes at 37°C (THAM), and ice-cold 5 M urea (Sigma-Aldrich) in PBS for 5 minutes at room temperature (UHAM), respectively. All HAM samples were scraped gently with a #15 blade (Heinz Herenz Medizinalbedarf, Hamburg, Germany) to remove remaining epithelial cells. The scraping was done by a trained person (AKR) masked of treatment details. It was performed under a dissecting microscope to ascertain a complete removal of epithelial cells. The HAM substrates then were washed thoroughly and ready for experiments.

Morphologic Examination

Samples were embedded in OCT compound (Leica Microsystems, Nussloch, Germany). Cryosections (6 μm thick) were processed for hematoxylin-eosin (H&E) histochemistry and visualized under light microscopy (LM; Zeiss Axioplan 2; Carl Zeiss, Oberkochen, Germany). Alternatively, samples were fixed in freshly prepared neutral buffered 2% paraformaldehyde (Sigma-Aldrich) and 2% glutaraldehyde (EM Sciences, Hatfield, PA) in 0.1 M sodium cacodylate buffer (pH 7.4) for overnight at 4°C, followed by aqueous solution of 1% osmium tetroxide (FMB Industries, Singapore). After washes and dehydration, they were critical point dried, sputter-coated with gold-aluminum alloy, and examined under scanning electron microscopy (SEM, JEOL JSM-5600LV; JEOL, Tokyo, Japan) at an accelerating voltage of 20 kV. Also, the dehydrated samples were infiltrated and embedded with araldite resin (EM Sciences). Ultrathin sections (70–80 nm thick) were contrast-stained and examined under transmission electron microscopy (TEM, JEOL 1220) at 100 kV.

Atomic Force Microscopy (AFM)

Glutaraldehyde-fixed samples were rinsed with deionized water, mounted on mica sheet and dried completely. They were examined using a Nanoscope IV Multimode microscope (Veeco Digital Instruments, Santa Barbara, CA). Images were obtained with a rotating tapping mode at resonant frequency of approximately 300 kHz and spring constant of approximately 40 N/m. Height, amplitude, and phase images were acquired simultaneously. Three-dimensional images were generated and surface-roughness value was obtained from height-mode using Nanoscope 6.11 software (Nanoscope Services, Bristol, UK). The height and amplitude images were presented with colored pixels, relating to the height level. This estimated the height (Z value) measurement from images. Measurement of surface roughness by means of root mean square (RMS) value of the surface within a given area was used to quantify the unevenness of specimen surface created by projecting structures. The RMS value was calculated in three randomly chosen areas (each 25 μm^2 in size). Surface roughness was defined as the standard deviation of elevation (Z values) within the given area and was calculated using the formula:

Table 1. Corneal Donor Information

Donor	Age	Sex	Days to Culture	Cause of Death
1	3	M	6	Pneumonia
2	16	M	7	MVA
3	23	F	12	Traumatic injury
4	63	F	6	Ischemia cardiomyopathy
5	43	M	10	Gunshot wound

$$\text{RMS value} = \sqrt{\sum_{i=1}^{N} \frac{(Z_i - Z_{avg})^2}{N}}$$

where Z_{avg} was the average of Z values within the given area, Z_i was the Z value for a given point, and N was the number of points within the given area.

Immunofluorescence

Samples were embedded in OCT compound and cryosections fixed in ice-cold acetone for 15 minutes. After blocking with 4% BSA (Sigma-Aldrich) in PBS, they were incubated with primary antibodies (see Supplementary Table S1) for overnight at 4°C, followed by Alexa Fluor 488-conjugated secondary antibody, and nuclei stained with 4′6-dimidino-2-phenylindole (DAPI; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Result was visualized under fluorescence microscopy (Zeiss Axioplan 2; Carl Zeiss). Control incubation had the primary antibody step replaced by normal mouse/goat/rabbit immunoglobulin. Signal quantification was performed by Quantity One Image Analysis software (BioRad, Hercules, CA). Using DAPI intensity as internal reference, the staining signal intensity was measured in a minimum of 10 randomly chosen regions. After normalization, the mean intensity and SD were calculated and compared.

Western Blotting

HAM sample was homogenized in RIPA buffer containing protease inhibitors (cOmplete; Roche). RIPA soluble fraction was denatured with 2% SDS (BioRad), resolved with 6%–15% gradient SDS-PAGE, blotted and immunolabeled with primary antibodies (Supplementary Table S1), followed by appropriate horseradish peroxidase-conjugated immunoglobulin secondary antibodies (Santa Cruz Biotechnology, Inc.). Signal was detected by enhanced chemiluminescence (Super Signal West Pico, Pierce, Rockford, IL) and quantified.

Cell Attachment Assay

HAM was mounted with basement membrane side up in a well (7 mm diameter) with CnT50 medium on both sides. LEC at passage 2 were labeled with Cell Tracker Green CMFDA (Invitrogen) and plated on HAM surface at a density of 5000 cells/cm^2. At 24 hours, nonattached cells were removed and cells on HAM were imaged under fluorescence microscopy. The adherent cells on HAM were quantified and calculated at a percentage to the seeding density.

Cell Proliferation Assay

Cells on HAM were maintained for 10 days with fresh medium change every 3 days. They were incubated in bromodeoxyuridine (BrdU, 10 μM; Sigma-Aldrich) for overnight. After fixation with acid ethanol (90% ethanol and 5% acetic acid) for 30 minutes, treatment with hydrochloric acid (2 N) for 30 minutes and neutralization with 0.1 M borate buffer, they were incubated with mouse monoclonal anti-BrdU antibody (Sigma-Aldrich) for 2 hours, followed by Alexa Fluor 488 conjugated secondary antibody (Invitrogen) and nuclei stained with Hoechst 33342 (Sigma-Aldrich). Result was visualized under fluorescence microscopy. Percentage of BrdU-positive cells was determined in 5 randomly selected fields (×10 objective). The assay was triplicated for each treatment mode.

Statistical Analysis

Atomic Force Microscopy. One-way ANOVA was used to test for the roughness differences among treatment groups. Difference between mean was compared using post hoc Tukey HSD test. All analyses were performed using SPSS Version 17 (SPSS, Chicago, IL). The significance level was set at $P < 0.05$.

BrdU Proliferation Assay. Data were expressed as mean ± SEM. The percentages of BrdU-positive cells were compared among treatment groups using two-way ANOVA. Donors were modeled as a random effect and treatment groups as the fixed effect. The normality assumption for residuals was verified graphically. In addition to the standard *F*-test for differences among treatment groups, Fisher's least significant difference (LSD) was performed on all pair-wise comparisons among treatment groups. Statistical significant level was set as $P < 0.05$. Calculation was performed using SPSS version 17.

Results

Characterization of Human Amniotic Membrane Denuded by Different Methods

Cryosections of denuded HAM were stained with H&E solutions, and viewed under light microscopy. Alternatively, same samples were processed for TEM. Amniotic epithelial cells remained on AHAM sample (Fig. 1A), of which the basal lamina was shown clearly under TEM (Fig. 1B). Nearly no cell was found on EHAM, THAM, and UHAM samples (Figs. 1E, 1G, 1I). Under TEM, intact basal lamina could be seen in EHAM and UHAM (Figs. 1F, 1J). On urea-treated HAM, the loosened cells even were removed efficiently by a cotton stick, which resulted in intact basement membrane (Supplementary Fig. S1A). THAM had electron-dense basal lamina, but the underlying stromal matrix appeared loosened and disrupted (Fig. 1H). In contrast, DHAM had an abrupt destruction of membrane structure. We observed a complete absence of basal lamina and much loosened stromal matrix (Figs. 1C, 1D).

The morphologic changes were characterized further by AFM and SEM assays. In the DHAM sample, the stromal collagen fibers were exposed to the surface due to the absence of basement membrane (Fig. 2, second panel from top). A less defined fibrillar structure was observed on AHAM surface (Fig. 2, first panel from top). Cell fragments also were detected on the membrane, which agreed with our LM observation. In contrast, a smooth surface was observed in EHAM, THAM, and UHAM (Fig. 2, third to bottom panel), suggesting that the basement membrane still existed after the denudation methods. Similarly, smooth basement membrane surface was found for urea-treated HAM with cell removal by a cotton stick (Supplementary Figs. S1B–S1D). From AFM measurement, we calculated the surface roughness in term of mean RMS values. They were increased in the order of EHAM (21.83 ± 2.64), UHAM (24.33 ± 1.29), THAM (26.53 ± 2.52), AHAM (26.57 ± 1.17), and finally DHAM (30 ± 1.31). Using one-way ANOVA, the surface roughness among the five treatment groups was significantly different ($P = 0.004$). Post hoc comparison using

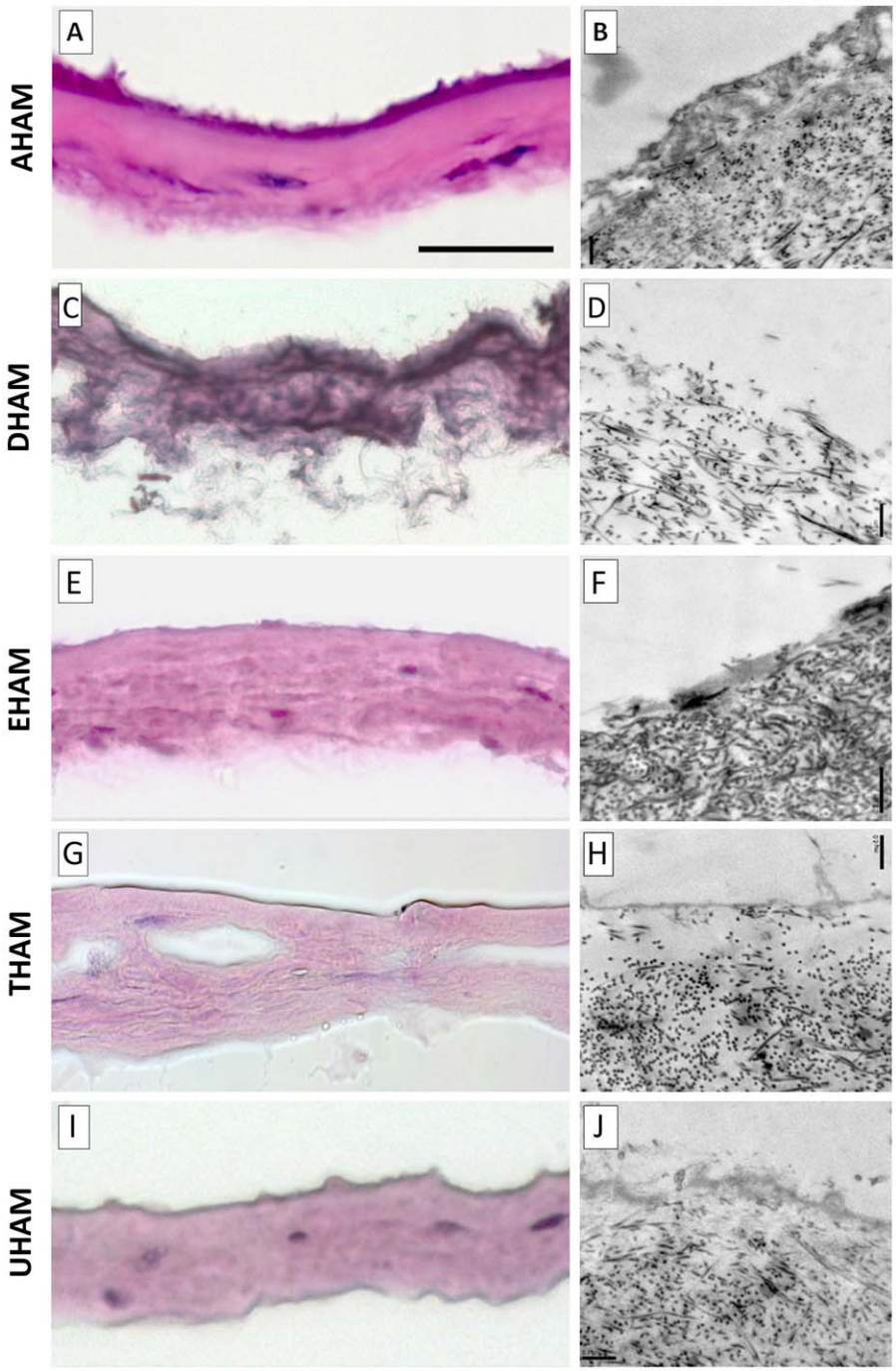

FIGURE 1. HAM morphology after various treatments. Micrographs of H&E histochemistry (**A**, **C**, **E**, **G**, **I**) and TEM (**B**, **D**, **F**, **H**, **J**) of membrane after treatment with (**A**, **B**) 20% ethanol for 30 seconds (AHAM), (**C**, **D**) Dispase II (1.2 U/mL) for 2 hours (DHAM), (**E**, **F**) 0.02% EDTA for 2 hours (EHAM), (**G**, **H**) 0.25% trypsin-EDTA for 30 minutes at 37°C (THAM), and (**I**, **J**) ice-cold 5M urea for 5 minutes (UHAM). Note the significant depletion of basement membrane in DHAM compared to other samples. *Scale bars*: 50 μm (H&E), 2 μm (TEM).

Tukey HSD test indicated that statistically significant difference was between DHAM and EHAM ($P = 0.003$), and between DHAM and UHAM ($P = 0.028$), respectively. Mean RMS values among AHAM, EHAM, THAM, and UHAM had no statistically significant difference.

We characterized the expression and distribution of various ECM molecules in the denuded HAM. Before treatment, intact HAM had distinct expression of collagen I, II, IV, and VI, as well as fibronectin and thrombospondin in the basement membrane and stromal region (Fig. 3A). Besides, collagen VII and laminin were detected predominantly in the basement membrane, whereas elastin stayed in the surface epithelium. For denuded HAM, we observed that AHAM, EHAM, and UHAM had ECM an expression profile similar to intact HAM (Figs. 3B, 3D, 3F). Signal quantification analysis showed the comparable intensity levels (Fig. 4A). However, DHAM and THAM revealed more drastic ECM changes. THAM had much lower expression of elastin, fibronectin, and thrombospondin than did intact HAM (Figs. 3E, 4A). In DHAM, except for collagen types I and II, all ECM molecules were not detectable (Figs. 3C, 4A). The

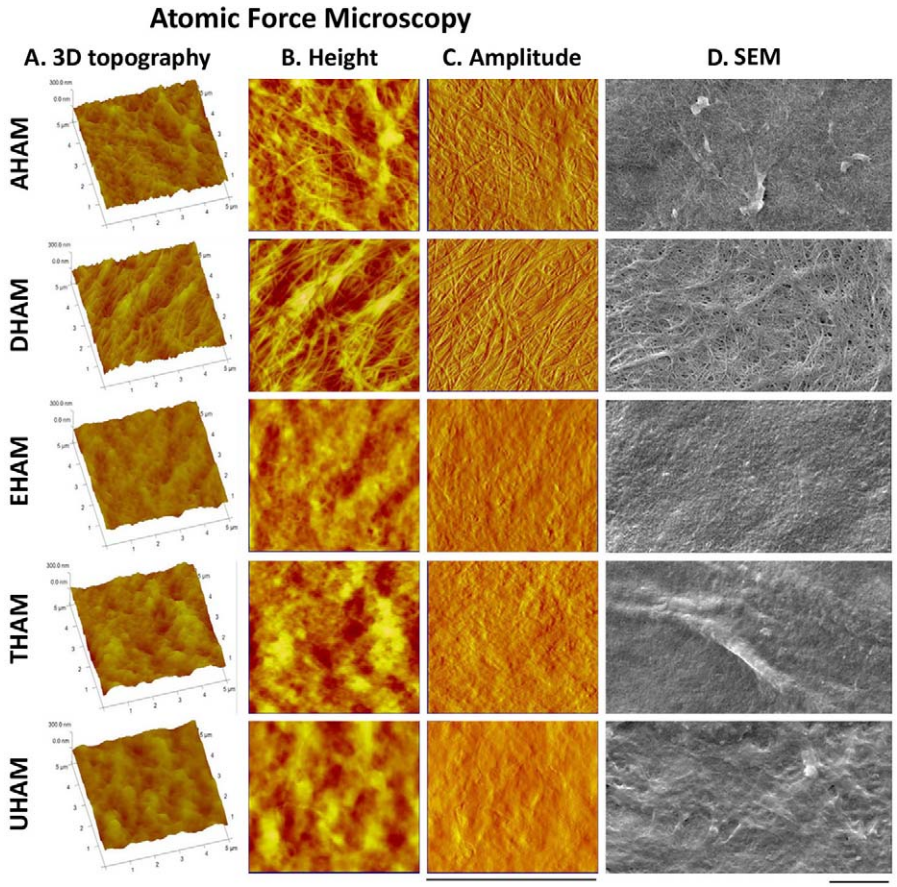

FIGURE 2. Surface analysis of denuded HAM evaluated by AFM and SEM. Sets of high resolution topography (*image scale*: 5 × 5 μm) are depicted in (**A**) three-dimensional presentation, (**B**) height where dark areas represented depressions and brighter areas were protrusions on the surface, and (**C**) amplitude showing the deflection generated by cantilever tip when it encountered the sample topography. (**D**) SEMs showed the complementary surface characteristics under high magnification. Note the exposed stromal fibers in DHAM, compared to other samples. *Scale bars*: 5 μm.

quantified intensity data were scrutinized further by the hierarchic clustering analysis (GeneSpring GX11.0; Agilent Technologies, Santa Clara, CA) to elucidate which denudated HAM had ECM protein expression closer to the intact HAM. Results confirmed that EHAM and UHAM belonged to one cluster, and they were more similar to intact HAM than other treated HAM samples (Fig. 4B). DHAM showed the most drastic difference due to the intense loss of ECM molecules.

By Western blotting, a variety of cytokines (bFGF, KGF, TGFα, and TGFβ1) were expressed simultaneously in AHAM, EHAM, and UHAM (Figs. 5A, 5B). The relative expression was similar among these samples. TGFβ1 was the most expressed, followed by moderate levels of bFGF, KGF, and TGFα. All tested growth factors expressed at lesser amount in DHAM and were almost absent in THAM sample.

Residual Urea in Treated HAM

We were concerned with the effect of residual denudation agent in HAM substrate on subsequent LEC growth. Ethanol, EDTA.Na_2, trypsin, and Dispase are water-soluble and can be cleared easily by buffer washes. However, urea is partially soluble in water and the solubility is temperature-dependent, and is highly penetrative in tissues. Though, in this study, the urea treatment lasted for 5 minutes only and samples were washed extensively afterwards, it might still remain in the denuded AM basement membrane and stromal matrix. To examine the residual urea content in UHAM, we carried out urea assay by LC-MS/MS (see Supplementary Method). Only a negligible amount of urea was detected, which ranged from 59 to 70 nM ($n = 2$, which was more than 50,000 times below the normal blood urea level, 4 mM). The untreated HAM had 31 nM. Supplementary Figure S2 showed the chromatogram of urea detection peak in untreated and urea-treated HAM.

Human LEC Attachment on Denuded HAM

Primary human LEC were plated on HAM without or with epithelium removed by different methods. At 24 hours, amounts of adherent cells were quantified and expressed as the percentage to the seeding density (attachment efficiency). Compared to plating on a regular culture dish (which had almost 100% efficiency, Fig. 6G), much reduced attachment was observed for cells on different HAM substrates (Figs. 6A–F). Among them, EHAM showed the highest efficiency (51% as on culture dish, Fig. 6H). AHAM and UHAM had moderate efficiency (AHAM 15.2%, UHAM 20%). Very few cells attached on DHAM (6% efficiency) and THAM (10.2% efficiency).

Human LEC Proliferation on Denuded HAM

Primary LEC were cultivated on denuded HAM for 10 days. BrdU incorporation in dividing cells was performed and the cell proliferation index represented by the percentage of BrdU positive cells was calculated. For each sample, five nonoverlapping and random regions were selected for quantification of

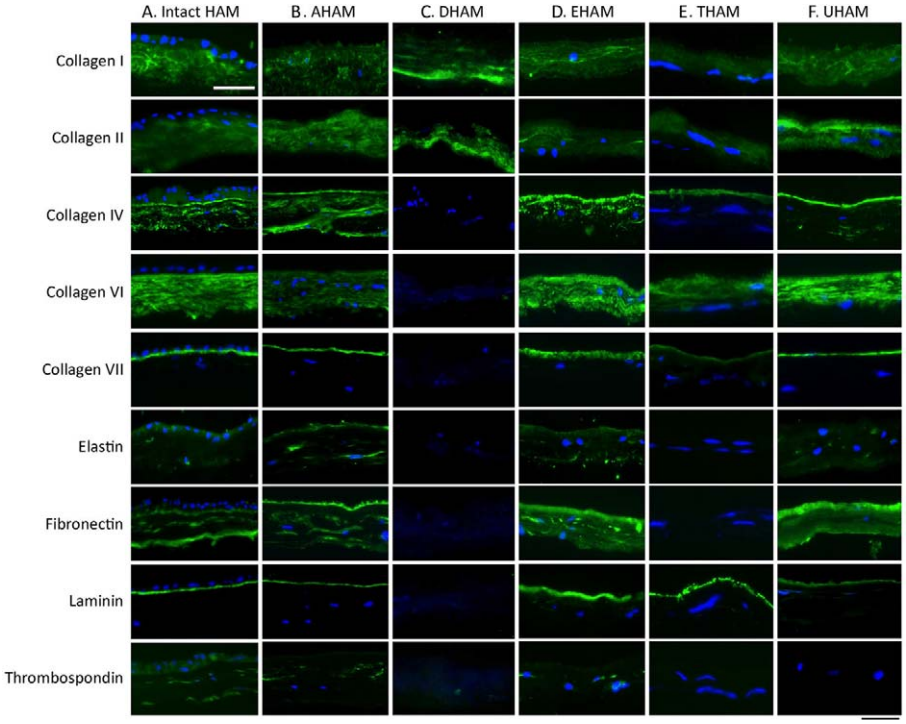

Figure 3. Expression of ECM in HAM denuded by various methods. Immunofluorescence of collagen types I, II, IV, VI, and VII, elastin, fibronectin, laminin, and thrombospondin in (**A**) intact cryopreserved HAM, and HAM denuded by (**B**) 20% ethanol for 30 seconds (AHAM), (**C**) 1.2 U/mL Dispase II for 2 hours (DHAM), (**D**) 0.02% EDTA for 2 hours (EHAM), (**E**) 0.25% trypsin-EDTA for 30 minutes at 37°C (THAM), and (**F**) ice-cold 5M urea for 5 minutes (UHAM). *Scale bar*: 50 μm.

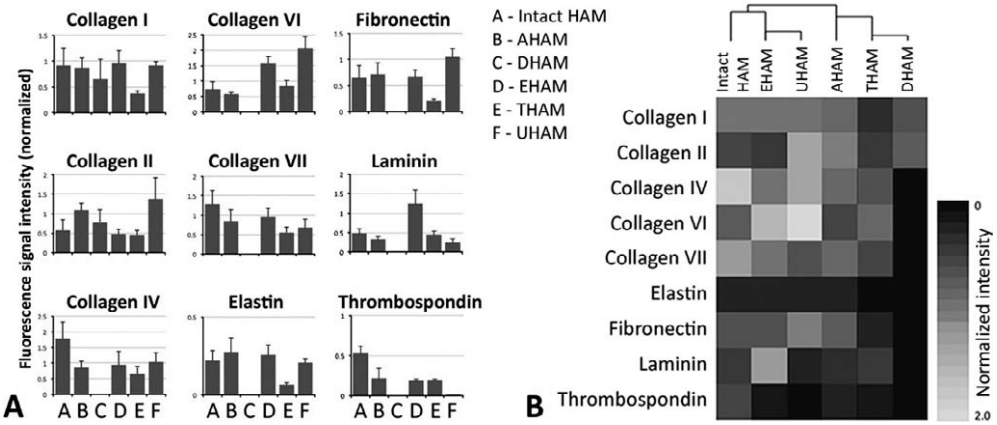

Figure 4. (**A**) Quantification analysis of staining intensities of various ECM components. (**B**) Clustering analysis of differential ECM protein expression in various HAM samples. EHAM and UHAM were more similar to intact HAM, whereas DHAM displayed the most drastic difference.

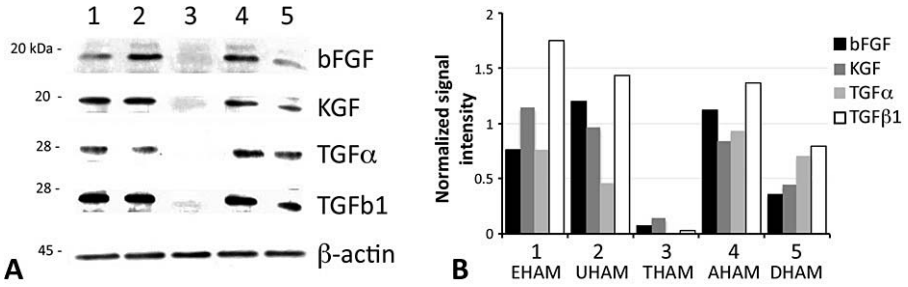

Figure 5. (**A**) Western blotting of bFGF, KGF, TGFα, TGFβ1, and β-actin in the HAM denuded by various methods. (**B**) Band densitometry and comparison. Note the reduced expression of studied cytokines in HAM after treatment by trypsin and Dispase, respectively.

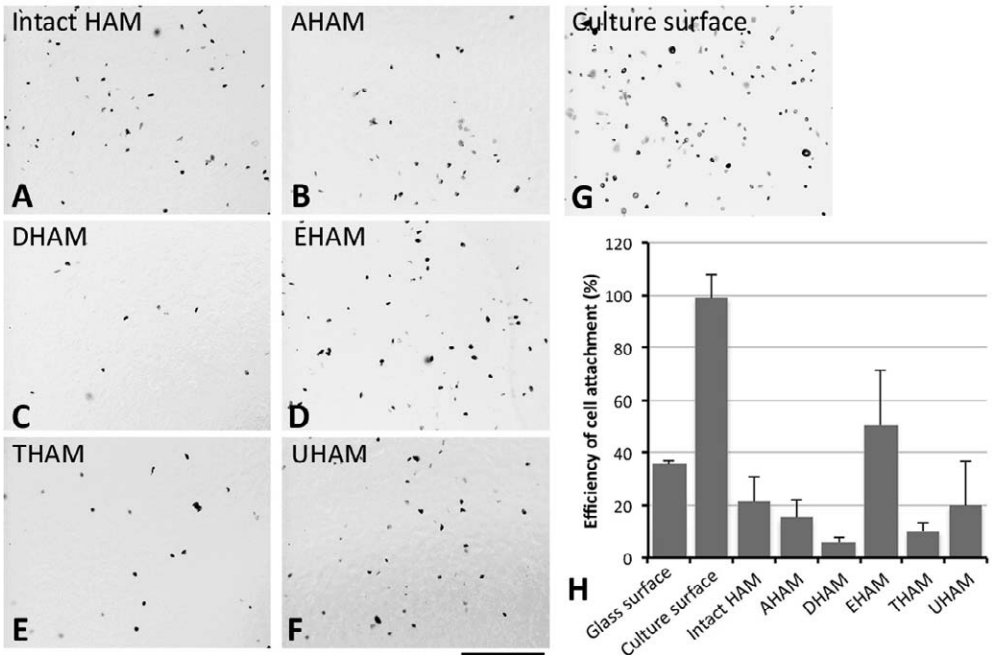

Figure 6. Human LEC attachment on HAM. (**A–G**) Grey scale image of CMFDA-labeled HLEC attached on different substrates. (**A**) Intact HAM. (**B**) AHAM. (**C**) DHAM. (**D**) EHAM. (**E**) THAM. (**F**) UHAM. (**G**) Culture surface. *Scale bar*: 0.5 mm. (**H**) Percentage of cell attachment to different substrate.

total and BrdU positive cells (Fig. 7). Table 2 illustrates the cell percentage with regards to corneal rim tissue from different donors. LEC cultured on EHAM, THAM, and UHAM showed higher percentages of BrdU-positive cells (EHAM 75.6 ± 3.4%, THAM 77.2 ± 5.2%, and UHAM 78.1 ± 4.7%) (Fig. 7G). AHAM and DHAM had lower cell proliferation rates (AHAM 62.2 ± 6.7% and DHAM 66.0 ± 7.3%). All indices were greater than that of cells cultured on an uncoated glass surface (53.7 ± 4.8%). Owing to the sample size and in consideration of type II error (false negative), Fisher's LSD pair-wise comparison was performed on the mean index values of the treatment groups. Statistical significance was found between AHAM and UHAM ($P = 0.039$), and between AHAM and THAM ($P = 0.041$). There was no significant difference among other treatment groups.

Extracellular Matrix Protein Expression After LEC Culture on Treated HAM

Immunofluorescence showed that laminin and collagen IV were detected in basement membrane of DHAM and THAM after LEC culture (Fig. 8). In addition, the staining of laminin and collagen IV was found in LEC cytoplasm, indicating the cellular production. The primary LEC had positive expression of basal epithelial proteins, cytokeratin 5 and 14.

Discussion

Two goals were achieved in this study. Firstly, we characterized the HAM after denudation by five different methods, namely 20% ethanol for 30 seconds, ice-cold 5 M urea for 5 minutes, 0.25% trypsin-EDTA.Na_2 for 30 minutes at 37°C, 1.2 U/mL Dispase II for 2 hours, and 0.02% EDTA.Na_2 for 2 hours. We then identified that urea treatment for 5 minutes had a relatively more effective removal of nonviable amniotic epithelial cells, and better preservation of basement membrane structure and ECM composition. This resulted in higher attachment and proliferation rates of primary human LEC that were plated afterwards.

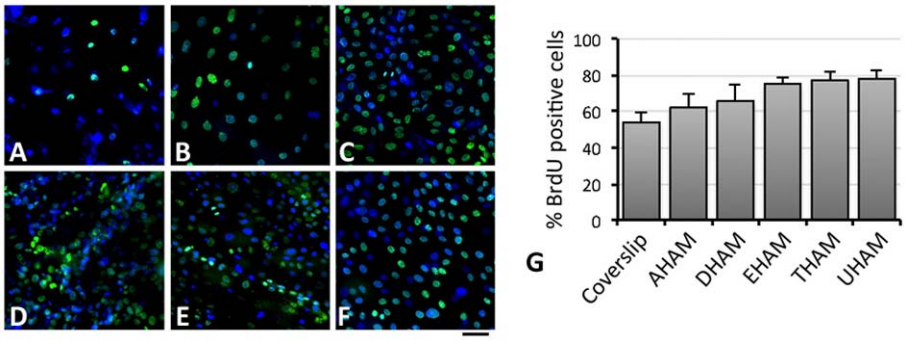

Figure 7. Proliferative index of human limbal epithelial cells on HAM denuded by various methods. (**A–F**) Immunofluorescence of BrdU (*green color*) in LEC after 10 days of culture on different substrates. (**A**) Uncoated coverslip. (**B**) AHAM. (**C**) DHAM. (**D**) EHAM. (**E**) THAM. (**F**) UHAM. Nuclei were stained with DAPI. *Scale bar*: 50 μm. (**G**) Percentage of BrdU-positive cells on different substrates. Each column represents mean ± SE.

Table 2. Percentages of Brdu-Positive Cells in LEC Culture on HAM at Day 10

	HAM Pretreatment	Donor 1	2	3	4	5	Mean %	SE
1	Ethanol	74.6	62.6	39.7	65.2	68.9	62.2	6.7
2	Dispase	68.4	70	45.6	60.7	86.3	66	7.4
3	EDTA	65.5	74.9	77.9	84.6	75.2	75.6	3.4
4	Trypsin-EDTA	70.5	62.6	88.2	82.1	82.7	77.2	5.2
5	Urea	83.4	62.7	76	82.1	86.4	78.1	4.7
6	Coverslip	58.9	58.3	43.4	64.2	43.5	53.7	4.8

In our study, we introduced two new reagents, ethanol and urea, for HAM de-epithelialization. In clinical practice, ethanol delamination is performed before photorefractive keratectomy to remove epithelium, and currently it is used to detach corneal epithelial cells from the Bowman's membrane to create an epithelial flap.[29,30] A brief treatment with 20% ethanol resulted in a smooth cleavage at the level of hemi-desmosome attachment at basal lamina, giving viable stromal cells.[31] Urea, a protein denaturant, can solubilize proteins and, hence, cell detachment.[32] It also acts as an osmolyte to cause cell shape changes, and loosen cell-cell and cell-extracellular matrix contacts. It has been proposed to remove cells to prevent posterior capsular opacification (US patent US008114432B2, 2012, available in the public domain at http://www.patentstorm.us/patents/8114432.html). The reagent availability and short incubation time are the primary advantages of these two techniques. Our morphology study showed that AHAM had a relatively uneven surface due to the cell remnants even after scraping. On the other hand, UHAM had a complete removal of epithelial cells, and the basal lamina was smooth and intact, and ECM components were well preserved. In addition, we could even remove the amniotic epithelial cells completely by using a cotton stick with much gentle dislodging force. Similar observation was noted for EHAM. In contrast, enzymatic treatment with Dispase or trypsin resulted in an easy removal of epithelium; however, a defective structure and chemistry of basement membrane. We quantified the roughness value generated by AFM measurement and compared it among all HAM samples. DHAM had the highest roughness score, and this could be due to the harsh enzymatic action to destroy the basal lamina and loosen the organized stromal collagen matrix. Similarly, trypsin action on HAM (THAM) also induced a high RMS score through its enzymatic digestion to destroy the basement membrane molecules,[33] which then secondarily altered the phenotypic appearance of basement membrane surface or disrupted the stromal collagen as seen under TEM. AHAM also presented with significant roughness because of the presence of epithelial cell remnants. Smooth surface with well-embedded stromal collagen fibrils was seen for UHAM and EHAM (lower RMS values).

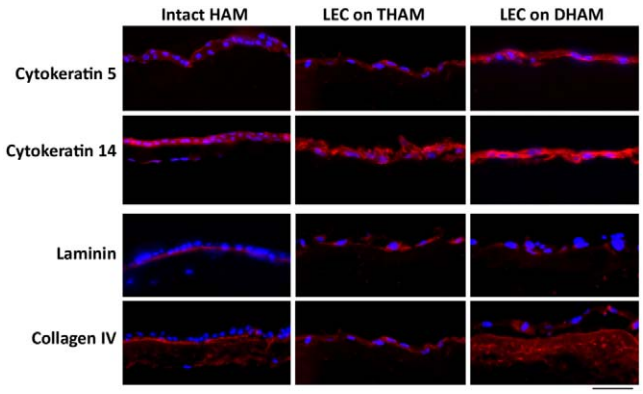

Figure 8. Deposition of ECM protein expression in treated HAM upon LEC culture. Immunofluorescence showed the similar expression pattern of cytokeratin 5 and 14, laminin, and collagen IV (*red fluorescence*) in primary human LEC culture on THAM and DHAM when compared to intact HAM. Nuclei were stained with DAPI. *Scale bar*: 50 μm.

Synergistic action of ECM molecules and growth factors is essential in maintaining corneal integrity and, hence, transparency.[34,35] The preservation of ECM content and growth factors of HAM could be crucial in facilitating the corneal reepithelialization, and reducing scarring and inflammation after HAM transplantation into eyes with limbal stem cell deficiency. We identified that treatments with Dispase and trypsin-EDTA resulted in a substantial loss of ECM proteins and growth factor expression. In AHAM, EHAM, and UHAM, these molecules were preserved and indifferent to the intact HAM. Such preservation of growth factors with their anti-inflammatory property may further aid the clinical success of ocular surface reconstruction in vivo.

With this experimental evidence, we propose that urea (a protein denaturant) and EDTA (a cationic chelator) are the better reagents for HAM denudation than enzymatic treatments. The treatments provided smooth and intact basement membrane, with well-preserved content of ECM proteins and growth factors, and these were beneficial for LEC attachment and proliferation. In general, basement membrane serves as a biological scaffold to support the attachment of epithelial cells, and promote their proliferation and differentiation.[36–38] Therefore, treated HAMs with intact basement membrane without considerable loss of ECM should offer a suitable environment for LEC proliferation. However, results of BrdU proliferation assay seemed to suggest otherwise. At day 10 of culture, excluding AHAM, there was no significant difference in the proliferation rate of LEC among the remaining four treatment groups. This highly suggested a repair of basal lamina environment by epithelial cells. Crosstalk between epithelium and basement membrane has been proposed in various epithelial cell systems, in particular in precancerous situations.[36,39–41] While the epithelial cells contribute significantly to the formation of basement membrane, this underlying structure in return regulates epithelial cell polarity and differentiation. Loss of these functions could contribute to induction and/or progression of epithelial cancer.[42] In our system, we proposed that once LEC attach and proliferate, they synthesize and deposit ECM proteins to the defective basement membrane, as shown by the cellular expression of laminin and collagen IV in Figure 8. This modifies the basement membrane environment, so that LEC can continue to grow into monolayer. Hence, the cell proliferation rates were similar at a later time point among the treated HAM samples. Our result showed that basement membrane defect caused by denudation

(loss of basement membrane, and ECM and growth factor molecules) is crucial for LEC attachment and initial growth. After sufficient time, cultured epithelial cells deposited basement membrane proteins. In a previous report of HAM denudation by EDTA treatment, basement membrane components initially were degraded and were reassembled in subsequent cell culture.[43] The repair process started with the secretion of laminin 5 at the first week of culture, followed by collagen IV and VII, and finally perlecan. Shortt et al. similarly reported that, albeit with different denudation methods (hypotonic buffer, SDS, or nuclease), there was no significant difference between the confluence rates of LEC.[44] Significant difference existed only between the denuded and intact HAM, and this also was shown in our study.

We observed significant differences between ethanol and urea, and between ethanol and trypsin-EDTA with respect to the proliferation rates. A postulated biological explanation for such differences observed for ethanol could be due to the epithelial remnants on the membrane even after scraping, and this might result in an irregular surface that is less favorable for LEC to adhere.[45] In addition, ethanol could inhibit adhesion molecules, affecting cell attachment and proliferation.[46]

Our study was limited by the sample size. Multiple comparisons of BrdU staining were significant for cells on AHAM versus UHAM, and cells on AHAM versus THAM, respectively, but the overall P value was insignificant. With more samples, a potentially significant result could be unmasked. Another potential limitation lies in the inherent variability of donor HAM tissue. We used the HAM region distal to the placenta for the experiments. In addition, the mechanical scraping was required to remove the loosened amniotic epithelial cells for a complete HAM denudation. We had minimized the variability by having a well-trained person, who was masked of the treatment details, to perform the operation. Under a dissecting microscope, we could distinguish the less translucent region, which indicated the presence of attached epithelium, from the more translucent part representing the denuded HAM area. Indeed, this mechanical scraping has been frequently reported by different studies to remove the amniotic epithelium completely.[8,19,20,43] Hopkinson et al. revealed that most enzymatic treatments were ineffective in releasing the nonviable amniotic epithelial cells without mechanical scraping.[17] A treatment that does not require epithelial cell scraping, thus, would be ideal to standardize the HAM denudation method. With urea treatment, the amniotic epithelial cells were dislodged easily, even by a gentle brushing with a cotton stick. This was beneficial to preserve the underlying basement membrane structure and integrity, which was evidenced by better cell attachment efficiency. Toxicity of residual urea in treated AM to LEC culture should not be overlooked. By LC-MS/MS, we detected an average of 65 nM (range 59–70 nM, $n = 2$) urea remained in the UHAM after PBS washes thrice. This was less than 1/50,000 of the normal blood urea level (4 mM).[47] Further supported by our cell culture data, this urea denudation of amniotic epithelial cells was safe for the subsequent LEC growth.

In conclusion, we suggested that urea treatment could result in a complete amniotic epithelial cell removal with a better preservation of basement membrane integrity, and ECM and growth factor composition, similar to the conventional EDTA treatment. It shows effective cell attachment and proliferating abilities. Other denudation methods can disrupt the basement membrane structure and ECM environment. Hence, we propose a new method of HAM denudation by ice-cold 5 M urea, which has a shorter processing time (5 minutes versus 2 hours for EDTA treatment) as a viable alternative for HAM denudation for cultivating LEC for transplantation.

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