Lowered Humidity Produces Human Epidermal Equivalents with Enhanced Barrier Properties

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Multilayered human keratinocyte cultures increasingly are used to model human epidermis. Until now, studies utilizing human epidermal equivalents (HEEs) have been limited because previous preparations do not establish a normal epidermal permeability barrier. In this report, we show that reducing environmental humidity to 50% relative humidity yields HEEs that closely match human postnatal epidermis and have enhanced repair of the permeability barrier. These cultures display low transepidermal water loss and possess a calcium and pH gradient that resembles those seen in human epidermis. These cultures upregulate glucosylceramide synthase and make normal-appearing lipid lamellar bilayers. The epidermal permeability barrier of these cultures can be perturbed, using the identical tools previously described for human skin, and recover in the same time course seen during *in vivo* barrier recovery. These cultures will be useful for basic and applied studies on epidermal barrier function.

Introduction

SKIN SERVES AS the protective interface between an organism and its external environment. Mammalian skin is composed of two layers: the epidermis and dermis. The outermost layer of the epidermis, the stratum corneum (SC), serves as the protective barrier against dehydration and penetration of exogenous agents. Many of these barrier properties can be attributed to the programmed expression of intercellular junctions and lipid-related proteins during epidermal development.^{1–4} In addition, the presence of ionic epidermal gradients (calcium [Ca^{2+}] and pH) is crucial for a fully functional permeability barrier.^{5,6} In normal skin, there is a characteristic intraepidermal calcium gradient, with peak concentrations in the stratum granulosum and lowest levels in the stratum basale.^{7–9} Ca^{2+} plays an important role in keratinocyte differentiation¹⁰ and in regulation of lamellar body secretion during barrier recovery.¹¹ Normal skin also has a pH gradient where the SC has pH 4.5–5.5 and the viable epidermis displays neutral pH.¹² This acidic milieu is required for processing precursor lipids into mature barrier-forming lipids, where key lipid-processing enzymes (e.g., β -glucocerebrosidase and acidic sphingomyelinase) exhibit their optimal activities.^{13,14}

In vitro human epidermal equivalents (HEEs) that parallel epidermal properties are essential for studying barrier development and function. Although previous HEEs have shown morphological and biochemical similarities to native human skin, their barrier function is reduced compared to *in vivo* skin,^{15–21} necessitating the addition of fibroblasts or de-epidermized dermis (DED) can result in batch-to-batch variability and increased lead time to developing functionally optimal HEEs. Several parameters have been identified that enhance barrier formation and homeostasis in human and animal models. Experimental models of fetal rat skin development and human keratinocyte cultures demonstrated that air exposure alone or treatment with exogenous Ca^{2+} , estrogen, thyroid hormone, or corticosteroids accelerated epidermal barrier development.^{22–24} The link between air exposure and barrier maturation extends to humans, shown by the fact that lower ambient humidity (50% vs. 75% relative humidity [RH]) accelerates barrier development and protects premature infants from infections.^{25,26} Many attempts have been made to improve the quality of barrier function in HEEs by supplementing the medium with vitamins,^{16,27} essential fatty acids,^{28,29} the use of serum-free medium,³⁰ or adopting culture conditions that reflect the *in vivo* skin environment.^{16,31} Building upon

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these results, we demonstrate that a reduction of environmental humidity to 50% RH can improve barrier function in HEEs without the use of fibroblasts or DED.

Materials and Methods

Cell culture

Primary human keratinocytes were isolated from a single neonatal foreskin and grown in 0.07 mM Ca^{2+} 154CF medium (Life Technologies) supplemented with human keratinocyte growth supplement. A suspension of first-passage keratinocytes ($\sim 2.21 \times 10^5/\text{cm}^2$ insert) was seeded on Cellstart CTS (Life Technologies)-coated PET, 0.4- μm inserts (EMD Millipore) in CnT-07 media (CELLnTEC) according to manufacturer's protocol. Day 3 (D3) after seeding, the media were switched to CnT-02-3D (CELLnTEC). On day 4, the HEEs were air exposed by feeding the bottom of the insert with CnT-02-3D. From day 4 onward, HEEs were fed daily with CnT-02-3D until harvested. HEEs were grown in a humid ($\sim 100\%$ RH) or dry incubator ($\sim 50\%$ RH) at 37°C and $5\% \text{CO}_2$. A dial hydrometer (Fisher Scientific) was used to measure incubator humidity. Low incubator humidity was maintained by removal of water pan. To control for possible changes in osmolarity, media were refreshed daily. Significant changes in osmolarity were not detected using this protocol, as measured by a Micro Osmometer (Precision Systems). Twelve-well inserts were used for transepithelial electrical resistance (TEER) measurements, light microscopy, and electron microscopy, while six-well inserts were used for transepidermal water loss (TEWL) measurements and immunoblotting.

Light and electron microscopy

HEEs were harvested and halved for light and electron microscopy. Samples for light microscopy were fixed in Formalde-Fresh (Fisher) and paraffin embedded, and 5- μm sections were stained by hematoxylin/eosin.

Transmission electron microscopy (TEM) was performed using the methods published previously.³² Briefly, HEEs were fixed in modified Karnovsky's solution. Paired samples were prefixed in half-strength Karnovsky's fixative, followed by postfixation in $1\% \text{OsO}_4$, to assess morphology. After postfixation, all samples were dehydrated in a graded ethanol/propylene oxide series, and embedded in an Epon-epoxy mixture. Ultrathin sections were collected and assessed either unstained or counterstained with lead citrate. Samples were visualized with a Zeiss 10A (Carl Zeiss) electron microscope operated at 60 kV.

SC thickness. The number of cell layers in the SC was counted in at least two low-power ($\times 3000$) electron micrographs from each HEE ($n = 10/\text{condition}$).

Cornified envelope and corneocyte thickness. Cornified envelope (CE) and corneocyte (CC) thickness in the lower SC of HEEs were measured in randomized, coded electron micrographs using Gatan software. At least 30 measurements were taken from each HEE ($n = 10/\text{condition}$).

Corneodesmosome/CC length. The ratio between the total length of intact corneodesmosomes (CDs) to total length of CCs was determined in the first two layers above the SC-

stratum granulosum junction by planimetry. At least 10 measurements were taken from each HEE ($n = 10/\text{condition}$).

TEWL measurements

HEEs ($n = 3-4/\text{condition}$) were excised and placed on top of a bead of media over parafilm. TEWL was measured with the Tewameter (Courage + Khazaka, TM300). The negative control was CTS-coated inserts not seeded with cells. Healthy normal humans (23-62 years, mean age = 36.4 years, $n = 5$) had their TEWL measured from their volar forearms at three separate sites. Participants gave their written informed consent.

TEER measurements

TEER measurements ($n = 12/\text{HEE condition}$) were recorded with EVOM (World Precision Instruments) according to manufacturer's instructions. Measurements were performed using fresh CnT-02-3D: 0.5 mL on top of the Transwell and 1 mL below the Transwell. After the measurement is completed, the apical media were aspirated.

TEER barrier recovery

TEER basal measurements were recorded on day 10 as listed previously. Afterward, HEEs ($n = 12/\text{condition}$) were left for 2 h in their respective incubators to allow the residual apical media to evaporate to ensure proper tape adhesion to HEE SC. A precut D-squame D100 (CuDerm) was applied on top of HEE with consistent and constant pressure and was stripped away from HEE. A single tape-strip typically yielded a 50% decrease in TEER relative to its TEER basal reading. HEE whose TEER reading was $< 500 \Omega$ was discarded from the study. TEER measurements were recorded 0, 6, and 24 h after tape stripping. Control HEEs were not tape stripped. Barrier recovery data were calculated by dividing the TEER at a specific time point with its corresponding TEER basal measurement.

Western blot

HEEs were lysed with RIPA buffer (Sigma-Aldrich) containing protease inhibitors (Roche) and centrifuged to remove cell debris. Protein quantification was determined by BCA assay (Thermo Fisher). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer were conducted using NuPAGE Novex 4-12% Bis-Tris gels and nitrocellulose membranes according to the manufacturer's protocol (Life Technologies). Membranes were blocked with 5% milk/PBST and incubated with rabbit polyclonal anti-GCS antibody (ab124296, 1:1000; Abcam). Mouse monoclonal β -actin (A3854, 1:30,000; Sigma-Aldrich) was used as a loading control. Secondary antibodies were anti-mouse IgG (A9044, 1:2000; Sigma-Aldrich) and anti-rabbit IgG (No. 7074, 1:2000; Cell Signaling). Proteins were detected with ECL western blotting substrate (Thermo Fisher) and imaged and quantified with LAS-3000 (Fujifilm).

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed as described previously.³³ Briefly, transcribed cDNA ($n = 3/\text{HEE condition}$) was amplified using SYBR green/ROX qPCR master mix (Bioline). Thermal cycling

was initiated by denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s. mRNA expression was normalized to levels of GAPDH. The following human primer sets were used: filaggrin (*FLG*) 5'-CCATCATGGATCTGCGTGG-3' and 5'-CACGAGAGG AAGTCTCTGCGT-3'; taurine transporter (*SLC6A6* or *TauT*) 5'-AGGGAAGTCTGAGGTGCAGAGA-3' and 5'-CTGGAAGG AGAGCATCCAAG-3'; betaine/GABA transporter (*SLC6A12* or *BGT-1*) 5'-CATGTCCTGTGTGGGCTATG-3' and 5'-CGC AAAGATGAGTGTGTCAGGA-3'; glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) 5'-GGAGTCAACGGATTGGTTC GTA-3' and 5'-GCAACAATATCCACTTTACCAGAGT TAA-3'.

Ca^{2+} gradient measurements

Human skin explants were obtained from the dermatology surgical unit under protocols approved by the University of California, San Francisco, and San Francisco Veterans Affairs Medical Center and in accordance with the principles expressed in the Declaration of Helsinki. Explants were incubated overnight with 40 μM Calcium Green 5N (CG5N; Life Technologies) in CnT-07 such that the dermal side was in contact with the medium. Day-11 HEEs ($n=3\text{--}5/\text{condition}$) were incubated overnight with 20 μM CG5N in CnT-02-3D and then excised for measurement. Samples were placed SC down on a coverglass slide and secured to an Axiovert 200 510 Meta confocal microscope (Carl Zeiss) coupled with a mode-locked Ti:Saph Mira 900 laser (Coherent). A TCSPC SPC-830 (Becker and Hickl) module was used for time-resolved FLIM imaging. Z-stacks were acquired scanning the sample at 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, and 50 μm depth. Only one location/sample was measured at its center. Phasor analysis of the FLIM data and conversion into calcium concentrations was performed using SimFCS (Laboratory for Fluorescence Dynamics) as previously described.³⁴ All epidermal layers were identified by their morphology and all images from the same epidermal layers were averaged together.

pH gradient measurements

Day-11 HEEs ($n=5/\text{condition}$) were incubated overnight with 40 μM SNARF-4F (Life Technologies) in CnT-02-3D, excised and placed SC down on a coverglass slide, and secured to an Axiovert 200 510 Meta confocal microscope (Carl Zeiss). Z-stacks were acquired scanning the sample every 2 μm with a 543-nm HeNe laser. Fluorescence was collected in two spectrally separated channels using a 510 Meta detector (Carl Zeiss). The two channels were set from 570 to 612 nm (channel 1) and from 634 to 719 nm (channel 2). The normalized ratio of the fluorescence intensities collected in the two channels was calculated using SimFCS (Laboratory for Fluorescence Dynamics), and converted into pH values using a calibration curve. The calibration curve was obtained from imaging 20 μM solutions of SNARF-4F in buffered solutions at pH 4.0, 5.0, 6.0, 7.4, and 8.0.

Statistical analysis

Paired and unpaired *t*-tests were calculated for statistical analysis according to experimental conditions. Error bars in graphs represent standard deviation calculated from three or more replicates. Asterisks indicate statistical significance of *p*-value < 0.05.

Results

Lowered environmental humidity alters SC ultrastructure

HEEs developed under humid (100% RH) and dry (50% RH) environments showed no differences in epidermal thickness and number of cell layers of the viable epidermis by light microscopy (Fig. 1A). However, a significant difference in SC ultrastructure was visualized by TEM (Fig. 1B). Dry HEEs have an increased density of CD present in the SC compactum (24.7% vs. 3.51%), more SC layers (17.8 vs. 11.7), thicker CCs (1.74 vs. 0.55 μm), and thinner CE (8.6 vs. 11.1 nm) than humid HEEs as summarized in

FIG. 1. Morphology of day-12 HEEs generated under 100% (humid) and 50% (dry) relative humidity conditions. (A) Hematoxylin and eosin staining of HEEs. White arrows indicate the SC compactum. The areas denoted by yellow squares were examined in further detail with transmission electron microscopy in (B, C). (B) Ultrastructure of the SC compactum of HEEs. (C) Ultrastructure highlighting processed lipid-enriched lamellar bilayers in SC compactum of HEEs. Black arrows indicate corneodesmosomes. HEE, human epidermal equivalent; LB, lipid-enriched lamellar bilayers; SB, stratum basale; SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; TM, transwell membrane. Color images available online at www.liebertpub.com/tec

TABLE 1. AN OVERVIEW OF THE STRATUM CORNEUM BARRIER MEASUREMENTS OF DAY-12 HUMID AND DRY HUMAN EPIDERMAL EQUIVALENT IS SHOWN IN THE TABLE

	No. of SC layers	CC thickness (μm)	CE thickness (nm)	CD/CC length in lower SC (%)
Humid HEEs	11.70 ± 1.20	0.55 ± 0.15	11.10 ± 1.10	3.51 ± 1.57
Dry HEEs	17.80 ± 1.00	1.74 ± 0.20	8.60 ± 0.70	24.7 ± 6.10
p-Value	$3.00\text{E-}10$	$1.48\text{E-}12$	$1.71\text{E-}05$	$4.36\text{E-}10$

The data represent the mean and standard deviation. Measured number of CD is reflected in the ratio of CD/CC.

CC, corneocyte; CDs, corneodesmosomes; CE, cornified envelope; HEE, human epidermal equivalent; SC, stratum corneum.

Table 1. The lowered environmental humidity normalized lipid lamellar bilayer formation (Fig. 1C).

Functional barrier assessments show that HEEs can reproduce the baseline and recovery parameters seen in human epidermis

To assess the barrier function of humid and dry HEEs, three tests commonly used in investigative dermatology were performed: TEWL, TEER, and barrier recovery from tape stripping. TEWL is a clinically relevant measure of

barrier function since it directly measures water loss across the skin. Humid and dry HEEs progressively develop TEWL values comparable to normal adult forearm skin³⁵; however, there was no significant difference in TEWL values between the two culture conditions (Fig. 2A). TEER measures the ability of water-soluble ions to traverse an epidermis; it is a sensitive measure of paracellular and transcellular water flux in HEEs.^{2,36} Significant TEER differences were observed 4 and 8 days after exposing the cultures to the air-liquid interface (day 8 and 12 of culture, respectively); the TEER was higher in the dry HEEs (Fig. 2B). Epidermal barrier

FIG. 2. Functional assessment of HEE barrier properties and their recovery from perturbation. (A) TEWL measurements of developing humid HEE and dry HEE. The negative control was an unseeded Transwell membrane. (B) TEER measurements of developing humid and dry HEEs. (C) Curve comparing barrier recovery of a single tape-stripped humid versus dry cultures. D4, day 4; D8, day 8; D12, day 12. The data represent the mean and standard deviation. Asterisks indicate $p < 0.05$. TEER, transepithelial electrical resistance; TEWL, transepidermal water loss.

recovery is commonly measured as the TEWL recovery profile following barrier disruption by tape-stripping off SC layers. This “concept” was adapted to make amenable the study of HEE barrier recovery by measuring the TEER barrier recovery. A single tape-strip was sufficient to produce a significant TEER decrease in HEEs (Fig. 2C) without perturbing the viable layers (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tec). Dry HEEs were able to recover to their basal TEER values prior to tape stripping after 24 h, while humid HEEs lagged in their recovery (100% vs. 68% recovery). TEER values of nontape-stripped controls were stable across the experiment (data not shown).

Differential gene expression in HEEs cultured under reduced humidity

The improved barrier quality of the dry HEEs may be due to differential gene expression during their development. A broad panel of proteins associated with barrier function, including markers relating to terminal differentiation; lipids; adhesion; antimicrobial defense; and osmolarity sensing were investigated. Glucosylceramide synthase (*UGCS* or *GCS*) expression did not change in the humid cultures, but progressively increased in the dry cultures (Fig. 3A). Similarly filaggrin expression was upregulated as both humid and dry HEEs developed (data not shown); however, its synthesis was preferentially increased 2.5-fold in the dry HEEs (Fig. 3B). The reduction in environmental humidity stimulated expression of two osmolyte regulators: taurine transporter (*SLC6A6* or *TauT*) and betaine/GABA transporter (*SLC6A12* or *BGT-1*) (Fig. 3C). Markers commonly associated with adhesion and differentiation, such as *KRT1*, *KRT10*, *DSP*, *DSG1*,

DSG3, *DSC1*, *IVL*, *LOR*, *CDH1*, *CLDN1*, *OCNL*, and *TJPI1*, were not significantly different between HEEs developed under humid and dry conditions (data not shown). Also unchanged were lipid-related proteins (*ABCA12*, *STS*, and *GBA*), antimicrobial peptides (*CAMP* and *DEFB103A*), and an osmolyte regulator (*SLC5A3*) (data not shown).

Epidermal ionic gradients are formed in HEEs developed under reduced humidity

The difference in Ca^{2+} distribution between HEEs cultured in a high-to-low humidity environment and a permanently high humidity environment is marked (Fig. 4A). Dry HEEs have a comparable Ca^{2+} gradient to *ex vivo* skin; Ca^{2+} levels peak in the stratum granulosum and gradually decrease toward the lower viable epidermal layers. In contrast, Ca^{2+} levels remained consistently low in all epidermal layers of the humid HEEs ($\sim 2 \mu\text{M}$ Ca^{2+}). While the Ca^{2+} gradient is present across the epidermis, the pH gradient is confined to the SC; there is an acidic SC (pH 4.5–5.5) where the pH increases toward the neutral layers of the viable epidermis. We have tested a commercially available model (MatTek Epiderm) and did not observe a Ca^{2+} gradient (data not shown). The dry HEEs displayed a pH gradient matching human epidermis *ex vivo*, with lowest pH at the SC surface (pH 5.8) that gradually increases with depth into the viable layers (Fig. 4B). Conversely, the humid HEEs have a constant pH throughout their SC (pH 6.7).

Discussion

The ultimate goal in the development of HEEs is to generate an epidermal equivalent with the same barrier

FIG. 3. Differential expression of glucosylceramide synthase, filaggrin, taurine transporter, and betaine/GABA transporter in HEEs developed under humid and dry environments. (A) Western blot of glucosylceramide synthase (*UGCS*) and β -actin (loading control). Graph represents densitometric analysis of *UGCS* in (A) normalized against the β -actin. (B) qPCR analysis measuring the terminal differentiation marker filaggrin (*FLG*). (C) qPCR analysis measuring the osmolyte transporters: taurine transporter (*SLC6A6* or *TauT*) and betaine/GABA transporter (*SLC6A12* or *BGT-1*). Data were normalized to the housekeeping gene *GAPDH*. Data are presented as the relative expression between humid and dry HEEs at a given time point. The data represent the mean and standard deviation. Asterisks indicate $p < 0.05$. qPCR, quantitative polymerase chain reaction.

FIG. 4. Epidermal ionic gradients formed in day-12 dry HEEs. **(A)** Calcium gradient distribution in humid and dry HEEs compared with *ex vivo* human skin. **(B)** pH gradient distribution in the SC of humid (white squares) and dry (black diamonds) HEEs. X-axis designates the depth in microns. All data represent the mean and standard deviation.

properties found in native human skin. While humid and dry HEEs show similar morphology at the light microscopy level, dry HEEs display a marked increase in the CD density in the SC compactum, as visualized by its ultrastructure. Since there is a direct relationship between number of CD and the strength of CC cohesion,^{37–39} the increased CD density in dry HEEs likely reflects a more cohesive SC. It is interesting to note that the CC density in the dry HEEs approaches the density found in normal human SC (24.7% vs. ~20%).⁴⁰

The actual status of skin (or HEEs) is best assessed under dynamic rather than static conditions. In a previous report,⁴¹ it was noted that a reduction in environmental humidity increased SC hydration levels in HEEs. Yet TEWL rates were not altered by differences in SC hydration properties. A similar finding was observed in aged skin barrier,⁴² where basal barrier function assessed by TEWL does not reveal a defect, but barrier recovery rates (the “cutaneous treadmill exam” test)⁴³ demonstrated clear abnormalities. The same test performed on dry HEEs showed that they have a faster TEER recovery than the humid HEEs (100% vs. 78%, 24 h after tape strip).

Previous reports show subtle defects in epidermal barrier function in HEEs cultured in a permanently high humidity environment.^{15–21} The enhanced barrier of the dry HEEs might be due to altered gene expression. The upregulation of GCS in dry cultures may reflect an increased synthesis of glucosylceramides, a major source of SC lipids, which are known to be important in barrier homeostasis.^{44–46} Additionally, the induction of osmolyte transporters TauT and BGT-1 at reduced humidity could reflect the stratum granulosum keratinocytes sensing the desiccating environment.⁴⁷ Yet, it is important to note that there were no significant differences in medium osmolality between humid and dry HEEs. This implies that there are negligible alterations in supplement concentrations, which could have affected HEE development. The increased TEER in dry HEEs likely reflects enhanced lipid production, as tight junction (TJ) protein expression remained unchanged between humid and dry HEEs. However, whether keratinocyte TJ function adapts to alterations in the environmental humidity is an open question.

Exposure to 50% RH during HEE development allowed for the establishment of key epidermal ionic gradients (Ca^{2+} and pH) that are critical for the function of native human skin. The Ca^{2+} and pH gradients are involved in maintaining epidermal permeability homeostasis.^{48–50} There is a link between ambient humidity and enhanced barrier function that has been described in both animals and humans,^{25,51} and recapitulated *in vitro* with neonatal human keratinocytes,^{41,52,53} although the exact molecular mechanisms remain unknown.

Conclusions

The experiments described in the previous paragraphs model normal skin development when the normal epidermis transitions from an aqueous to a terrestrial environment. We believe the Transwell pore size, the extracellular matrix used, the medium shift from low to high Ca^{2+} , the exposure to the air–liquid interface, the role of medium supplements, and the decrease in environmental humidity collectively added to HEE barrier properties; none of these alone would be sufficient in enhancing barrier function. HEEs generated in a high-to-low humidity environment had enhanced barrier properties and normal ionic gradients, and can be used to track barrier recovery. These HEEs might be useful in many areas, including testing agents that improve skin barrier function and as a model of prenatal or perinatal skin barrier development.

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Disclosure Statement

No competing financial interests exist.

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