Incubation of cultured skin substitutes in reduced humidity promotes cornification in vitro and stable engraftment in athymic mice

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Cultured skin substitutes have been used successfully for adjunctive treatment of excised burns and chronic skin wounds. However, limitations inherent to all models of cultured skin include deficient barrier function in vitro, and delayed keratinization after grafting in comparison to native skin autografts. Experimental conditions for incubation of skin substitutes were tested to stimulate barrier development before grafting, and measure responses in function and stability after grafting. Cultured skin substitutes consisted of human keratinocytes and fibroblasts attached to collagen-glycosaminoglycan biopolymer substrates. Parallel cultured skin substitutes were incubated at the air–liquid interface in ambient (48–61%) or saturated (79–91%) relative humidity, and grafted to athymic mice on culture day 14. Additional cultured skin substitutes were incubated in the experimental conditions for a total of 28 days. Cadaveric human skin and acellular biopolymer substrates served as controls. Epidermal barrier was evaluated as the change in surface hydration by surface electrical capacitance with the NOVA™ Dermal Phase Meter. Cultured skin substitutes and cadaveric skin incubated in ambient humidity had lower baseline surface electrical capacitance and less change in surface electrical capacitance than parallel samples incubated in saturated humidity at all time points in vitro. Data from healing cultured skin substitutes at 2, 4, 8 and 12 weeks after grafting showed an earlier return to hydration levels comparable to native human skin, and more stable engraftment for skin substitutes from ambient humidity. The data indicate that cultured skin substitutes in ambient humidity have lower surface electrical capacitance and greater stability in vitro, and that they reform epidermal barrier more rapidly after grafting than cultured skin substitutes in saturated humidity. These results suggest that restoration of functional epidermis by cultured skin substitutes is stimulated by incubation in reduced humidity in vitro. **(WOUND REP REG 1999;7:226–237)**

The restoration of the protective barrier against fluid loss and microbial invasion[1] provided by human epidermis is a definitive requirement for the healing of skin wounds.[2,3] Virtually all of the barrier function of human skin is attributed to the stratum corneum of the epidermis, and specifically to the intercellular lipid complex deposited during keratinocyte differentiation.[4] The use of cultured skin substitutes (CSS) to treat large skin wounds is an experimental alternative that provides definitive wound closure by restoration of epidermal barrier. Previous studies have

CHS	Cadaveric human skin
CSS	Cultured skin substitutes
CSS_{AMB}	Cultured skin substitutes at ambient RH
CSS_{SAT}	Cultured skin substitutes at saturated RH
%OWA	Percent original wound area
pF	picoFarad
RH	Relative humidity
SEC	Surface electrical capacitance
SEC_{cont}	SEC at 10 seconds
SEC_{Δ}	Initial SEC change
SEC_{inst}	Instantaneous capacitance
TEWL	Transepidermal water loss

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reported CSS[5] that engraft clinically to excised burns and chronic wounds[6–10] and preclinically to surgical wounds in athymic mice.[11–13] However, limitations of all cultured skin models include deficient barrier function at the time of grafting[14] and slower keratinization after grafting as compared to skin autografts. Barrier function can be improved by altering the in vitro conditions used during incubation of cultured cells or skin substitutes. Incubation of cultured epidermal cells[15,16] or skin substitutes[17,18] at the air–liquid interface[19] has been reported to stimulate epidermal stratification and differentiation and to improve barrier function. Additional improvement in barrier function of keratinocytes cultured in reduced humidity over those cultured in saturated humidity has been reported.[16]

Wound healing requires restoration of epidermal barrier function comparable to that of native human skin.[2] Biophysical measurements of hydration at the epidermal surface have been performed by Trans-Epidermal Water Loss (TEWL),[20–24] and by Surface Electrical Capacitance (SEC).[20,25–28] TEWL measures the flux rate of water vapor emanating from the epidermis, and SEC measures the hydration of the stratum corneum. Although TEWL and SEC measure different parameters of epidermal hydration, vapor flux and hydration of the stratum corneum have been found to correlate sufficiently well[20,28,29] that either method is considered valid for assessment of epidermal barrier in healthy skin. However, measurement of SEC does not require controlled atmospheric conditions that make in situ measurement of TEWL impractical in humidified incubators, biosafety cabinets or rooms of hospitalized patients.[21–24] Therefore, SEC is a more practical alternative than TEWL for valid assessment of epidermal barrier in skin substitutes that contain cultured cells.

Biophysical analyses of human skin have identified relationships between epidermal surface hydration, and electrical impedance and capacitance.[30,31] Biophysical assessment by noninvasive techniques is well-documented for studying the effects of physical and/or chemical disturbances of the epidermal barrier of human skin. These methods are based on the inverse relationship between skin hydration and electrical impedance, and the direct relationship between surface hydration and electrical conductance and capacitance.[30,31] As surface hydration increases, SEC also increases. SEC has been used to evaluate chemically or physically induced changes in the hydration state and barrier properties of native human skin.[25,32] The rate at which SEC increases during a period of occluded sampling is directly related to the rate of water accumulation and retention in the stratum corneum. The rate of change in surface hydration (and subsequently the rate of increase in capacitance) is inversely related to the degree of barrier against water movement into and through the stratum corneum of the skin.[26,27] Therefore, repeated sampling of CSS at various time points during in vitro or in vivo maturation provides a quantitative index of the kinetics of epidermal barrier development. SEC has been used to assess epidermal barrier development in neonatal rat[20] and human[28] skin. SEC has also provided a noninvasive, noninjurious method for evaluation of epidermal barrier development in CSS in vitro and after grafting to athymic mice[26] or pediatric patients.[27]

This study investigates whether reduced relative humidity levels during in vitro incubation of CSS affect stratification and cornification prior to grafting, and barrier development and stability of healed skin after grafting to athymic mice. Changes in surface hydration, as measured by SEC, were used to provide an assessment of cornification and barrier properties in CSS in vitro and in vivo. Physiologic and anatomic stability of CSS were evaluated in vitro by SEC and histology, and in vivo by SEC, wound area measurements and photography. This report presents quantitative data indicating stimulation of barrier formation and physiologic stability in CSS before and after grafting as a function of the relative humidity levels during in vitro incubation.

MATERIALS AND METHODS

CSS were incubated in vitro in identical conditions with relative humidity (RH) of the incubation chamber as the dependent variable. Conditions of saturated RH (range = 79–91%, mean ± SEM = 84 ± 0.43%) and ambient RH (range = 48–61%, mean ± SEM = 52 ± 0.44%) were compared. RH levels were determined by a microcomputer based humidity sensor in the tissue culture incubator (NU-2700, NuAire™, Plymouth, MN), and recorded daily before the incubation chambers were opened for data collection.

Preparation of Skin Substitutes

CSS were prepared by sequential inoculation of separate cultures of human fibroblasts and keratinocytes onto collagen-glycosaminoglycan biopolymer substrates.[8] Inoculation of fibroblasts preceded inoculation of keratinocytes by one day, and the latter is defined as day 0 of in vitro incubation. On culture day 3 CSS (~70 cm^2), were bisected and lifted to the

air–liquid interface[12] on cotton filter-pads (#15250, Schleicher & Schuell, Keene, NH) supported by stainless steel mesh. One half of each CSS was placed in a tissue culture incubator with ambient RH (CSS_{AMB}), and the other half was placed in an incubation chamber with saturated RH (CSS_{SAT}). Both chambers were maintained at 37° C and 5% CO_2 during the in vitro maturation of the CSS. CSS were incubated with daily changes of a lipid-supplemented nutrient medium[14] during the 28 day in vitro evaluation period (n = 5/RH level) or until grafted to athymic mice on culture day 14.

Grafting of CSS to Athymic Mice

All animal studies were conducted with the approval of the University of Cincinnati Institutional Animal Care and Use Committee, and according to the principles of laboratory animal care established by the NIH. On culture day 14, parallel CSS_{AMB} and CSS_{SAT} (n = 5) were grafted orthotopically to 2×2 cm full-thickness skin wounds prepared surgically in athymic mice.[11] The mice (female, nu/nu; Harlan, Indianapolis, IN) were housed in micro-isolator cages and nourished with autoclaved water and standard autoclavable chow for the duration of the in vivo study. CSS (~4 cm^2) were sutured in place and the wounds were dressed as previously described.[12] The grafts were irrigated daily for two weeks with 1 cc/graft of culture medium containing nutrients and antimicrobials[13] to nourish the cells in CSS during vascularization of the grafts. On day 14 after surgery, dressings and stent sutures were removed from all animals. The mice were re-bandaged from days 14–20 with a dressing consisting of N-Terface (Winfield Laboratories, Richardson, TX), Xeroform (Sherwood Laboratories, St. Louis, MO), cotton gauze, and Coban (3M Medical Division, St. Paul, MN). On day 21, dressings were removed and replaced with N-Terface, cotton gauze and Coban. All dressings were removed on day 28 for the remainder of the in vivo evaluation period.

Experimental Controls

Fresh, split-thickness (~0.012 inch) human skin (n = 4) was obtained from a local skin bank. Each sample (~40 cm^2) was bisected and lifted to the air–liquid interface as described above. One half of each cadaveric human skin (CHS) sample was incubated in ambient RH (CHS_{AMB}) and the reciprocal half was incubated in saturated RH (CHS_{SAT}). The same culture conditions (37° C, 5% CO_2) and nutrient medium described above for the CSS were used to maintain the CHS throughout the 21 day in vitro evaluation period. The four CHS samples were obtained from male donors 51 ± 2.5 years old (mean ± SEM), range = 46–59 years of age. The evaluation period began 6.25 ± 1.2 days postmortem, with the overall range = 4–10 days.

Acellular biopolymer substrates (n = 2) were bisected and maintained at the air–liquid interface in either ambient or saturated RH as described above. Daily changes of the same nutrient medium used for the CSS were made for the duration of the analysis. The acellular substrates were monitored for 28 days.

Native human skin on the volar forearm of healthy volunteers (n = 10) was sampled as an in vivo control. Data collection was conducted in the same laminar flow hood used for evaluation of athymic mice to match the environmental conditions of humidity, temperature and convective air flow.

Surface Electrical Capacitance Measurements

Hydration of the skin surface is directly proportional to the retention of electrical charge, or capacitance, according to the relationship:

$$Z = [R_x^2 + (\tfrac{1}{2} \pi f C_x)^2]^{\tfrac{1}{2}}$$

where resistance (R_x), and capacitative reactance (C_x) contribute to impedance (Z) as a function of the frequency (f) of an applied alternating current.[31] Measurements of SEC were collected with an impedance-based NOVA™ Dermal Phase Meter (DPM 9003; NOVA Technology Corp., Gloucester, MA) connected to a portable computer that recorded 10 serial readings at 1-second intervals. The capacitance probe (model 9105) has a spring-loaded head with a flat conductance surface (5-mm diameter) containing two concentric brass electrodes separated by a nonconducting resin. Direct contact of the probe to the surface being evaluated creates an occluded sampling site. SEC is directly proportional to surface hydration, and is detected by a phase shift in the current applied across the skin between the two electrodes. The magnitude of the phase shift increases as water accumulates between the skin surface and the probe during an occluded sampling period.

SEC readings were collected in situ at one second intervals from CSS on culture days 7, 10, 12, 14, 17, 19, 21, 26 and 28 during the in vitro evaluation period. Six SEC values were collected from both CSS_{AMB} and CSS_{SAT} to yield 30 values/condition at each time point. Healing CSS on athymic mice were monitored for 12 weeks after surgery. SEC data were collected from

CSS grafts on athymic mice at 2, 4, 8, and 12 weeks after grafting. Triplicate SEC readings were obtained from each animal to generate 15 values/time point for CSS_{AMB} and CSS_{SAT} grafts. Six SEC readings were collected from parallel CHS samples in vitro on days 1, 3, 5, 7, 10, 12, 14, 17, 19 and 21 to produce 24 values/condition/time point. Six SEC readings were collected from parallel acellular biopolymer substrates incubated as described above in ambient or saturated RH on culture days 7, 10, 12, 14, 17, 19, 21, 26 and 28 to serve as negative controls. Native human skin values were calculated from triplicate readings collected from healthy subjects to provide reference data from uninjured human skin.

SEC values generated by the DPM-9003 represent an aggregate value collected during a controlled scan of applied frequencies up to 1 MHz, and were converted from arbitrary units to picoFarads (pF) as described in earlier studies.[26,32] Instantaneous capacitance (SEC_{inst}) is defined as the initial SEC value recorded after one second of direct contact of the capacitance probe to the surface of the sample being evaluated. This value indicates the baseline hydration state of the sample's surface, where lower SEC_{inst} values reflect a drier epidermal surface indicative of increased cornification. The initial change value (SEC_{Δ}) is defined as the average amount of change in SEC between one and five seconds of the occluded sampling period. SEC_{Δ} is calculated using the following equation: $(SEC_{5sec} - SEC_{1sec}) / (5sec - 1sec)$. SEC_{Δ} reflects the rate of water accumulation at the sample's surface, and is inversely related to the restriction of water movement through the skin. This value is relevant because SEC values generate an asymptotic function (during occluded data collection) with the greatest and most rapid changes occurring in the earliest portion of the sampling period for all experimental groups (data not shown).

In an effort to focus on the most dynamic portion of the sampling period and avoid the influence of water accumulation on the surface, the SEC data collected in this study were subjected to regression analysis (described below) to establish the predictive validity of SEC_{Δ}, and the correlation between SEC_{Δ} and SEC_{cont} values. For all relationships analyzed, SEC_{Δ} was found to be a significant predictor of SEC_{cont} values and the two variables were significantly correlated (Table 1); therefore, only SEC_{Δ} data are presented in this report. SEC_{Δ} is expressed as pF/sec for CSS and CHS samples only.

Histological Evaluation

Biopsies were collected from CSS_{AMB}, CSS_{SAT}, CHS_{AMB}, and CHS_{SAT} and prepared for microscopic analysis to monitor tissue morphology during the in vitro incubation period. CSS_{AMB} and CSS_{SAT} specimens were collected on culture days 10, 12, 14, 17, 21 and 28. CHS samples from both RH levels were obtained on days 3, 7, 10, 14, 17, and 21 of incubation. All biopsies were fixed with 2% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate (pH = 7.4). Samples were postfixed in 1% osmium tetroxide and embedded in glycol-methacrylate resin. Thick sections (~6 μm) were stained with toluidine blue and examined/photographed using a Nikon Microphot-FXA microscope (Nikon, Inc., Instrument Group, Melville, NY).

CSS engraftment was determined by immunofluorescence staining of frozen sections of wound biopsies collected at 12 weeks after grafting CSS_{AMB} or CSS_{SAT} to athymic mice. Biopsies were frozen in OCT embedding compound, sectioned on a cryostat, and prepared for immunohistochemistry using standard techniques. Direct immunofluorescence staining using a monoclonal antibody against a common hapten of the HLA-ABC histocompatibility antigens[33,34] was performed to verify that wounds in both groups were healed with human keratinocytes.

Measurement of Wound Contraction

Direct tracings of wound perimeters onto frosted mylar sheets were made from healing CSS_{AMB} and CSS_{SAT} grafts at 2, 3, 4, 8 and 12 weeks after surgery.

Table 1. Linear regression analysis of data for SEC_{Δ} as a predictor of SEC_{cont} values

	Ambient RH		Saturated RH	
Experimental group	***p*-value[a]**	**r[b]**	***p*-value**	**r**
CSS – in vitro	0.0001	0.9645	0.0176	0.7982
CHS – in vitro	0.0007	0.9324	0.0047	0.8721
CSS – in vivo	0.0001	0.9999	0.0015	0.9985

Statistical results based on linear regression analysis of SEC_{Δ} and SEC_{cont} values in each experimental group.

[a] Significance of the predictor variable

[b] Pearson Product-moment correlation coefficient

Wound area was determined by image analysis of the tracings of the wound perimeters using the Image-1 system (Universal Imaging Corp.; Media, PA). Data for wound contraction are expressed as a percentage of the original wound area (%OWA) at the time of grafting,[11,12,35] calculated independently for each animal at each time point as follows:

$$\%\text{OWA} = [(\text{temporal wound area}) \div (\text{original wound area})] \times 100$$

Photographs were taken at 2 and 8 weeks after grafting to document healing and re-epithelialization of the wounds.

Statistical Analysis

SEC and %OWA data are expressed as mean ± SEM. Data from each experimental series were first subjected to one-between, one-within repeated measures analyses of variance (RM-ANOVA). If RM-ANOVA indicated an overall significant group (ambient vs. saturated) effect, pairwise comparisons were performed using Tukey's Studentized Range Test, and significance was determined at the 95% confidence level (*, $p < 0.05$). SEC data from CSS and CHS evaluations were subjected to simple linear regression to determine the p-value for the regression model and for the significance of the predictor variable SEC_{Δ}. If significance ($p < 0.05$) was determined, then the Pearson Product-Moment Correlation Coefficient (r) was calculated to reflect the magnitude of relationship between SEC_{Δ} and SEC_{cont} values.

RESULTS

The comparative time course of epithelial development and stability during in vitro incubation of CSS_{AMB} or CSS_{SAT} is represented in Figure 1. By 12 days of incubation (9 days of air exposure) human keratinocytes have developed a stratified and differentiated

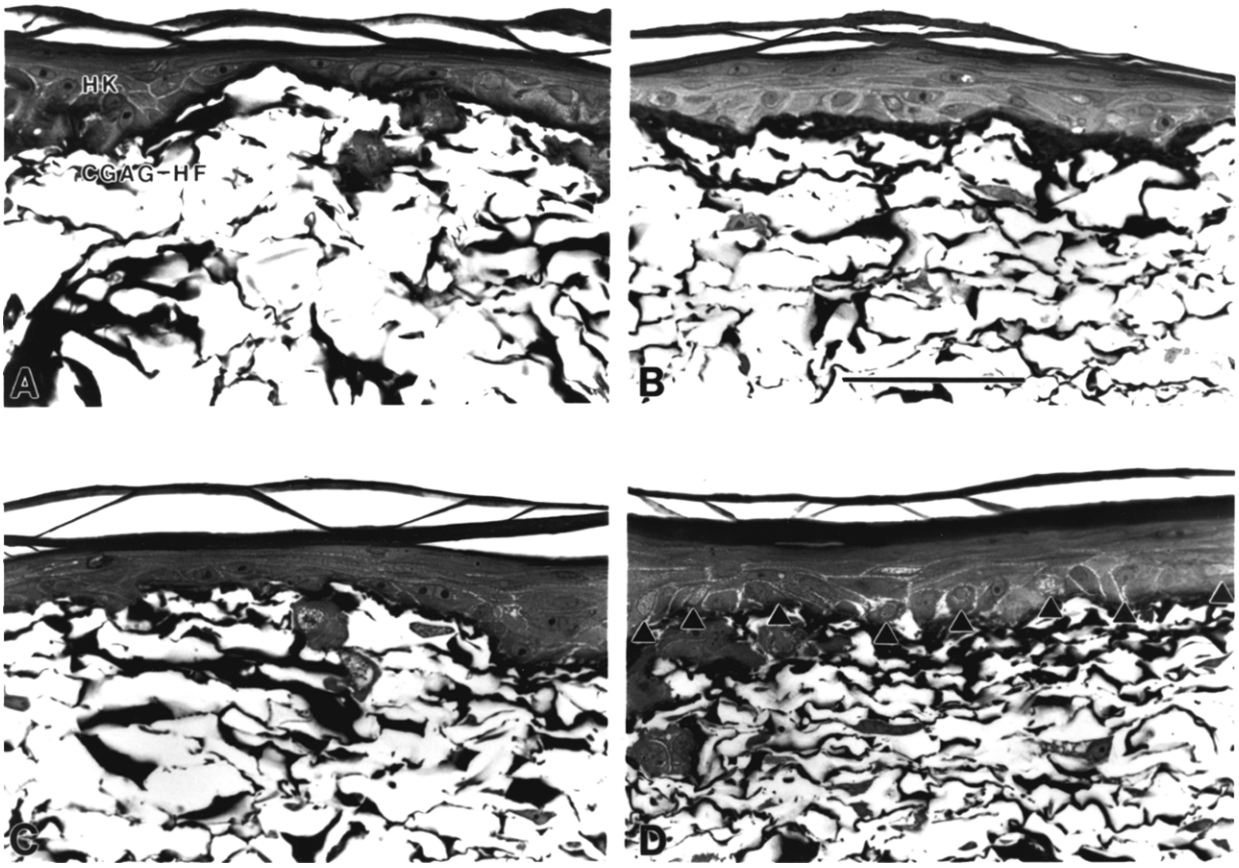

Figure 1. Increased epidermal stability in vitro in CSS_{AMB}. Human keratinocytes (HK) develop a stratified and differentiated epithelium attached to biopolymer substrates populated with human fibroblasts (CGAG-HF) by culture day 12 in CSS_{AMB} (A) and CSS_{SAT} (B). At culture day 21 CSS_{AMB} (C) exhibit a cornified and differentiated epithelium with nucleated HK in the lower layers. CSS_{SAT} (D) exhibit increased swelling and vacuolization of basal HK (arrowheads), fewer nucleated cells and a deteriorated morphology. Scale bar = 0.1 mm (B), for all four panels.

epithelium in both CSS_{AMB} (Figure 1A) and CSS_{SAT} (Figure 1B). At culture day 21 CSS_{AMB} (Figure 1C) still exhibit a differentiated morphology with an increased number of cornified layers and many nucleated keratinocytes present in the lower layers of the epidermis. However, CSS_{SAT} (Figure 1D) exhibit increased swelling and vacuolization of basal keratinocytes, fewer nucleated cells and a deteriorated morphology.

In Vitro Assessment of Surface Hydration in CSS.

CSS incubated in vitro in either ambient or saturated RH exhibit a time-dependent development of epidermal barrier properties. As shown in Figure 2, CSS_{AMB} have lower absolute values of SEC_{inst} (drier epidermal surface) throughout the 28 day evaluation period. Both CSS_{AMB} and CSS_{SAT} reach a minimal SEC_{inst} value by day 12 of incubation (264 ± 133 pF, and 413 ± 201 pF, respectively). However, CSS_{SAT} samples exhibit a steady and rapid increase in surface hydration through the remainder of the study, approaching initial levels. CSS_{AMB} maintain SEC_{inst} values near minimal levels through day 19 (398 ± 183 pF) before increasing away from the range of native human skin. SEC_{Δ} values presented in Figure 2 indicate that CSS_{AMB} and CSS_{SAT} exhibit maximal barrier function (lowest SEC_{Δ}) at day 12 of incubation (43 ± 9 pF/sec, and 60 ± 16 pF/sec, respectively). CSS_{AMB} maintained SEC_{Δ} values near the day 12 minimum through culture day 19 (63 ± 14 pF/sec) while CSS_{SAT} values increased rapidly after day 14 (93 ± 27 pF/sec). CSS_{AMB} had lower SEC_{Δ} as compared to CSS_{SAT} through day 26, with significantly lower values on days 7, 10 and 19.

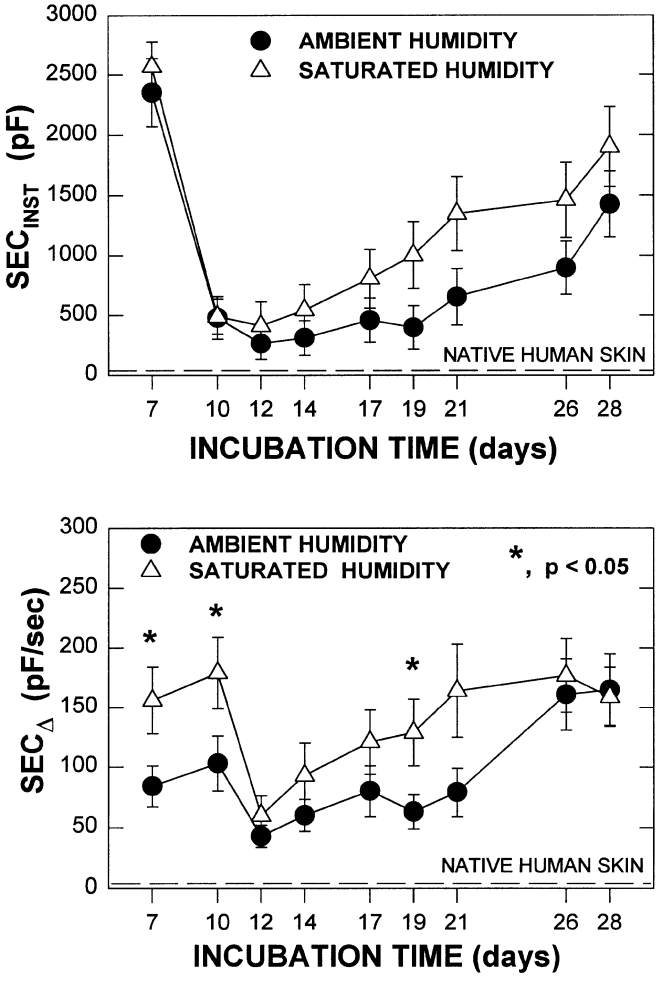

Figure 2. Improved in vitro epidermal development in CSS_{AMB}. Instantaneous capacitance (top) and initial change (bottom) values for cultured skin substitutes incubated for four weeks in ambient (48–61%) or saturated (79–91%) relative humidity. Significant differences are noted (*), and dashed reference lines indicate values for native human skin (SEC_{inst} = 13.63 ± 2.7 pF, SEC_{Δ} = 2.73 ± 0.4 pF/sec). Values indicate mean ± SEM.

Morphologic Stability and Functional Evaluation of CHS in Vitro

A comparison of the morphologic stability of cadaveric human skin during in vitro incubation of CHS_{AMB} or CHS_{SAT} humidity is presented in Figure 3. Both CHS_{AMB} (Figure 3A) and CHS_{SAT} (Figure 3B) exhibit normal morphology for human skin on day 3 of incubation (3 days of air exposure). However, by day 17 of incubation noticeable changes have taken place in the structure of both samples. CHS_{AMB} (Figure 3C) maintain a stratified and differentiated morphology, but the number of viable cell layers in the epithelium decreased and the amount of cornified layers increased from the day 3 sample. CHS_{SAT} (Figure 3D) samples exhibit increased hydration (swelling) and vacuolization of the basal keratinocytes and a deteriorated epidermal morphology compared to CHS_{AMB}.

Data shown in Figure 4 present SEC_{inst} and SEC_{Δ} values collected from human skin samples maintained in ambient or saturated humidity. SEC_{inst} data show that CHS_{AMB} had lower SEC_{inst} and less change in SEC_{inst} values than CHS_{SAT} throughout the three weeks of incubation. In addition, CHS_{AMB} had reduced absolute values and a smaller range of SEC_{Δ} values (11.0–33.4 pF/sec) compared to CHS_{SAT} (33.0–78.3 pF/sec). Both SEC_{inst} and SEC_{Δ} values recorded for CHS_{AMB} were significantly lower than those for CHS_{SAT} through the final 10 days of evaluation.

Acellular Substrates as Experimental Controls

To determine whether changes in SEC values observed in CSS during in vitro incubation were simply a function of desiccation or environmental conditions,

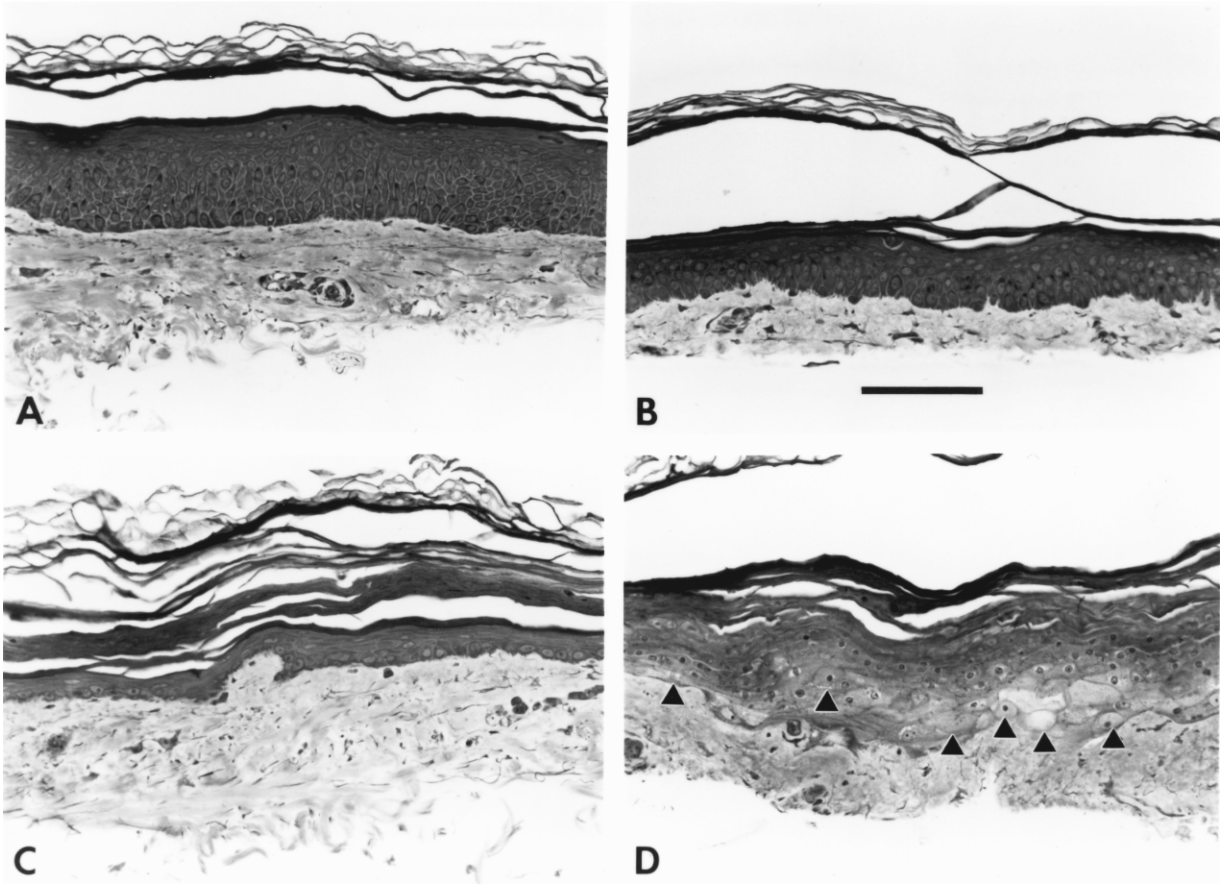

Figure 3. Improved morphologic stability of CHS_{AMB}. CHS exhibit a normal morphology after 3 days of incubation in either ambient (A) or saturated (B) RH. By culture day 17 CHS_{AMB} (C) still have a stratified and differentiated morphology, and the relative amount of cornified layers is increased from day 3. CHS_{SAT} (D) exhibit increased swelling and vacuolization of the basal HK (arrowheads) and a deteriorated epidermal morphology compared to CHS_{AMB}. Scale bar = 0.1 mm (B), for all four panels.

acellular substrates were monitored for four weeks. Figure 5 presents SEC_{inst} data collected from substrates incubated in ambient or saturated RH. SEC_{inst} values for the acellular substrates were stable, but very elevated compared to CSS and CHS. The range of SEC_{inst} values (3592 ± 25–4288 ± 16 pF) remained at levels near those for saturated cotton filter-pads (4388 ± 22 pF) during the 28 day incubation with no consistent or significant differences between time points or the two conditions. No SEC_{Δ} values were calculated for the acellular substrates because the samples were very wet (high SEC_{inst}) and at or above the upper level of sensitivity for the instrument,[15] which would result in artifactually low SEC_{Δ} values.

Healing and Wound Contraction of CSS on Athymic Mice

Figure 6 shows photographs of healing CSS at 2 and 8 weeks after surgery from representative animals grafted with CSS_{SAT} or CSS_{AMB}. In both conditions the grafts were well adhered to the woundbed by two weeks, with well-defined graft perimeters (arrows at corners). However, the CSS_{SAT} grafts (upper left) had nonuniform epithelial surfaces and areas along the graft perimeter that were not fused with the wound margin (arrowheads), which were not observed in the CSS_{AMB} grafts (upper right) at the 2 week time point. By 8 weeks after surgery all grafts were fully fused with the wound margin, but the healed grafts were smaller and more irregular for the CSS_{SAT} grafts (lower left) than the CSS_{AMB} grafts (lower right). Quantitative assessment of the differences in wound contraction between CSS_{AMB} and CSS_{SAT} grafts is presented below. Areas of hyperpigmentation produced by passenger melanocytes were evident in only the ambient grafts by 8 weeks after surgery.

The relative engraftment of CSS_{AMB} and CSS_{SAT} in athymic mice was determined by immunofluorescence labeling of human keratinocytes in the epithelium of healed wounds. Histologic evaluation of biopsies collected at 12 weeks indicated the presence of human keratinocytes (HLA-ABC positive) in all wounds, rep-

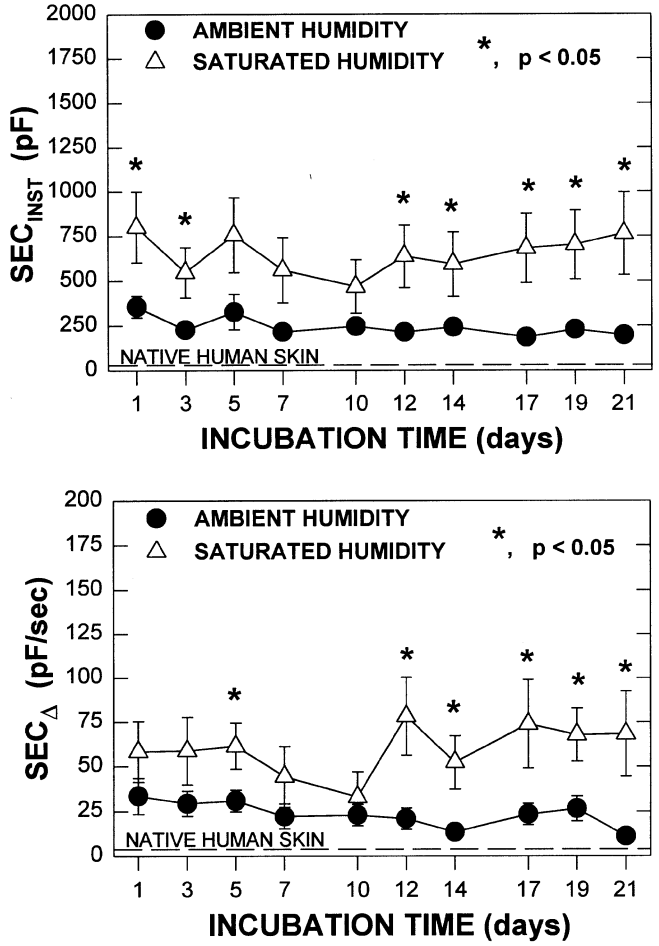

Figure 4. Increased epidermal stability in CHS_{AMB}. Instantaneous capacitance (top) and initial change (bottom) values for CHS incubated for three weeks in ambient (48–61%) or saturated (79–91%) relative humidity. Significant differences are noted (*), and the dashed reference lines indicate values for native human skin (SEC_{inst} = 13.63 ± 2.7 pF, SEC_{Δ} = 2.73 ± 0.4 pF/sec). Values represent mean ± SEM.

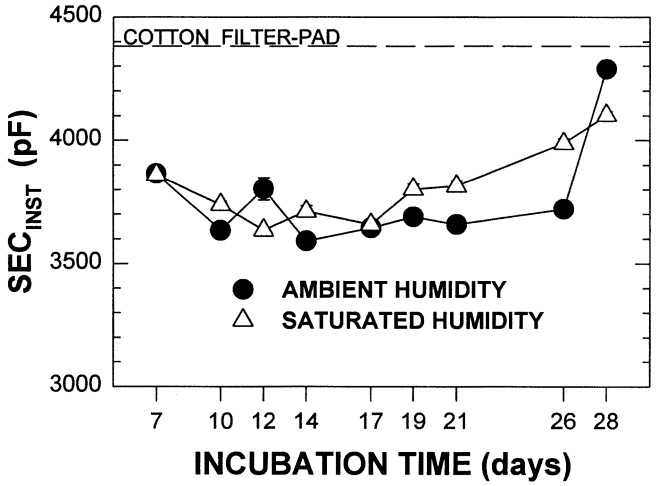

Figure 5. Acellular substrates do not desiccate. Instantaneous capacitance values for acellular biopolymer substrates incubated for four weeks in ambient (48–61%) or saturated (79–91%) relative humidity. The dashed reference line indicates SEC_{inst} (4388 ± 22 pF) value for a saturated cotton filter-pad in both conditions. Values represent mean ± SEM.

resentative of epidermal engraftment in all animals from both experimental groups (data not shown). The %OWA data were similar for the two groups at 2, 3 or 4 weeks, while the animals were still in dressings. The %OWA values for CSS_{AMB} grafts were significantly greater than the values for CSS_{SAT} grafts at 8 weeks (30.4 ± 4.05% and 14.7 ± 2.58%, respectively) and 12 weeks (26.6 ± 3.02% and 9.66 ± 0.71%, respectively) after surgery.

Restoration of Barrier Function by CSS

The kinetics of epidermal barrier restoration by CSS after grafting to athymic mice are shown in Figure 7, which presents SEC_{inst} and SEC_{Δ} data collected from healing CSS at 2, 4, 8 and 12 weeks after surgery. SEC_{inst} data indicate that CSS_{AMB} grafts have reduced surface hydration (lower SEC_{inst}) compared to CSS_{SAT} grafts throughout the in vivo study period. SEC_{inst} values for CSS_{AMB} (4 weeks = 1.73 ± 0.0 pF) reach levels at or below those for native human skin (13.63 ± 2.7 pF) earlier, than values for CSS_{SAT} (8 weeks = 6.25 ± 2.2 pF). The SEC_{inst} values for the CSS_{AMB} group have a smaller range of SEM than the CSS_{SAT} group (0.0 – 26.1 pF and 2.2 – 144.7 pF, respectively). SEC_{Δ} data show that CSS_{AMB} grafts (4 weeks = 0.16 ± 0.1 pF/sec) reach values near those for native human skin (2.73 ± 0.4 pF/sec) more rapidly after grafting to mice than CSS_{SAT} grafts (8 weeks = 3.11 ± 1.6 pF/sec).

DISCUSSION

The SEC data collected in vitro show lower instantaneous capacitance and initial change values for CSS incubated in ambient humidity. Because basic diffusion principles would predict increased water movement from a liquid reservoir (nutrient medium below a cotton filter-pad) in a drier environment, lower SEC_{Δ} for CSS_{AMB} suggests greater restriction to water movement through those samples. This indicates improved epidermal barrier development in CSS_{AMB}. Lower SEC_{inst} values for CSS_{AMB} reflect a lower baseline hydration state of the epithelial surface, indicative of increased cornification as compared to CSS_{SAT}. Both sample types reach their lowest SEC_{inst} and SEC_{Δ} values on culture day 12, but CSS_{AMB} maintain minimal values through day 19 (as compared to day 14

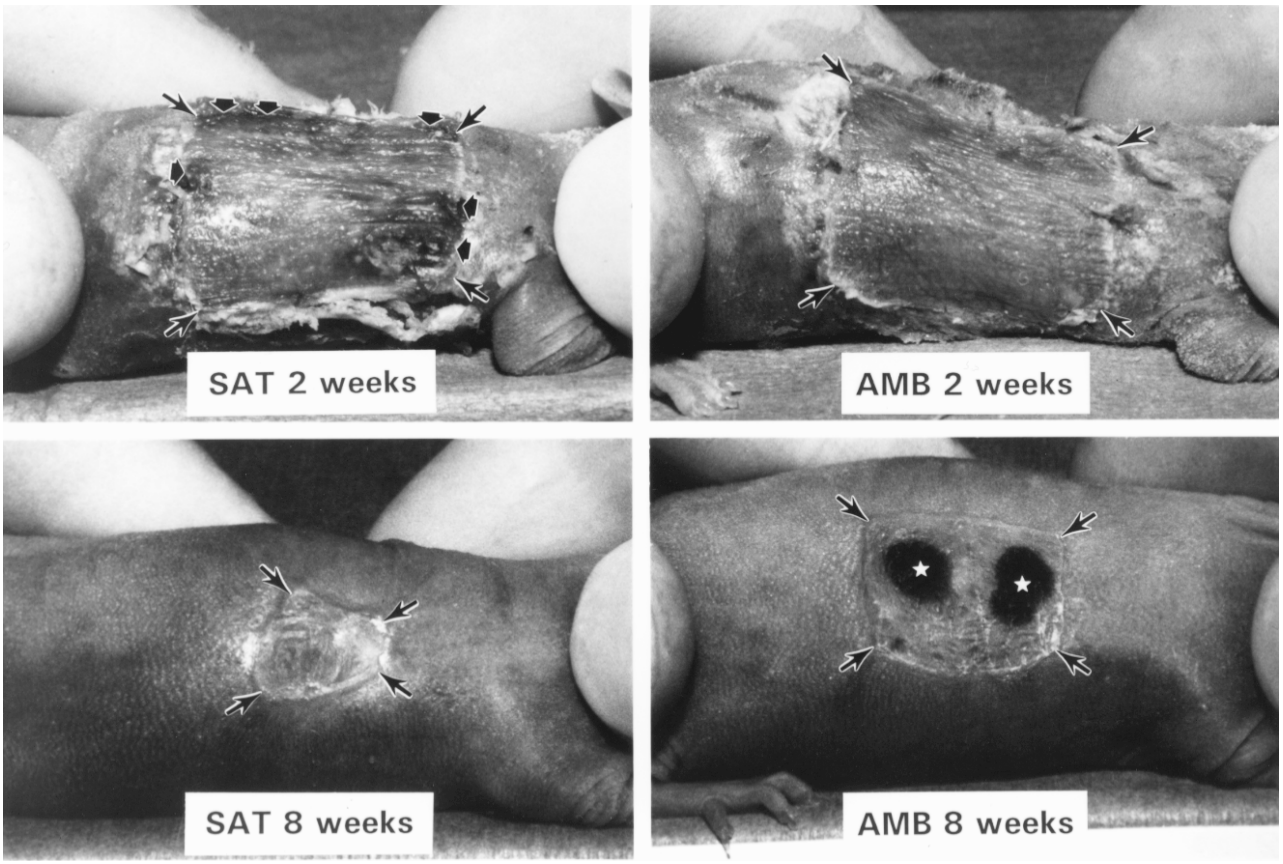

Figure 6. Closure of skin wounds in athymic mice following engraftment of CSS. Time course photographs show re-epithelialization and engraftment of cultured skin substitutes from saturated (*SAT*) or ambient (*AMB*) humidity. The perimeters of the healing grafts are marked at the corners (*arrows*) in all photographs. At 2 weeks, saturated grafts (*upper left*) are nonuniformly epithelialized and have areas along the perimeter which are not completely fused with the wound margin (*arrowheads*) compared to ambient grafts (*upper right*). By 8 weeks the wound perimeter is more uniform and stable in ambient grafts (lower right) than in saturated grafts (lower left). Foci of hyperpigmentation (☆) from passenger melanocytes are evident in ambient grafts by 8 weeks after grafting.

for CSS_{SAT}) before increasing. The rate of increase in both SEC_{inst} and SEC_{Δ} values between culture day 12 and 28 is faster for CSS_{SAT} than for CSS_{AMB} which suggests greater stability of epidermal barrier in the latter group. SEC_{inst} and SEC_{Δ} values for CSS_{AMB} had smaller standard error of the mean throughout the in vitro evaluation, indicative of less intra- and inter-sample variability in this group. Less variability shows more uniform surface hydration and epidermal barrier formation in skin substitutes incubated in reduced humidity. The SEC_{inst} data collected from the acellular substrates show no distinguishable variation based on incubation time or RH level, suggesting the changes in SEC values for CSS and CHS observed during in vitro incubation are related to biological processes not just environmental conditions. The time course histologies of CSS_{AMB} and CSS_{SAT} in vitro support the quantitative data, and suggest that cornification increases and epidermal stability can be improved in CSS by incubation in ambient humidity. These results indicate that incubation of CSS in reduced RH stimulates development of stable epidermal barrier in vitro.

The in vivo data are consistent with the conclusions discussed above regarding the effects of in vitro humidity levels on epidermal development in cultured skin substitutes. Although both CSS_{AMB} and CSS_{SAT} grafts exhibit a return to normal SEC values, the CSS_{AMB} group reach native human skin levels faster than the CSS_{SAT} group. Lower SEC_{inst} and SEC_{Δ} values for ambient grafts at 2 and 4 weeks after surgery indicate greater cornification and more rapid barrier development in these grafts. Smaller variability for CSS_{AMB} grafts at all time points may represent development of a more uniform and consistent epithelium by CSS_{AMB}, as was indicated in vitro. SEC_{inst} and SEC_{Δ} values below those for native human skin may reflect hyperkeratosis of CSS grafted to athymic mice.[11] The photographs and wound contraction data collected from healing grafts support the

interpretation that CSS_{AMB} exhibit more stable engraftment than CSS_{SAT}. The presence of foci of hyperpigmentation in engrafted CSS_{AMB} is consistent with previous reports describing hyperpigmentation in human xenografts on athymic mice[36] and in CSS grafts on pediatric patients.[9] Previous studies from this laboratory have shown the ability to regulate pigmentation in healing CSS on athymic mice.[12,37]

Cadaveric human skin served as a biological control and provided a point of comparison for analyzing the epidermal characteristics of cultured skin substitutes in vitro. Lower SEC_{inst} and SEC_{Δ} values with less change throughout the evaluation period indicate a drier epidermal surface, improved preservation of barrier function and better in vitro stability in CHS_{AMB}. Microscopic evaluation of biopsies collected from CHS_{AMB} and CHS_{SAT} during the 3 week study period indicates that the in vitro morphologic stability of CHS can be improved by incubation in ambient RH at the air–liquid interface. The apparent increase in the amount of cornified cell layers in both CSS_{AMB} and CHS_{AMB} after extended incubation, as compared to parallel samples maintained in saturated humidity, is consistent with a recent study describing increased stratum corneum thickness in hairless mouse skin following prolonged exposure to low humidity.[38] These results suggest that cadaveric human skin incubated in ambient humidity exhibits more stable (less change in SEC values) and uniform (smaller variability) surface hydration and barrier function than samples in saturated humidity. These preliminary findings may suggest improved methods for maintaining human skin used for surgical applications or in vitro testing of topically applied pharmaceutical or cosmetic compounds.

Although TEWL is a common method for measuring water flux through the stratum corneum of human skin, that sampling method has several limitations which include susceptibility to environmental conditions such as temperature, relative humidity levels and convective air flow.[21–24] Therefore, to assess the effects of humidity level on epidermal development in CSS before and after grafting to mice it was necessary to use a method for barrier evaluation that was not significantly affected by the environmental conditions. SEC data collection is not significantly affected by humidity levels or convective air flow.[24,26,28] Because SEC evaluation is noninvasive and noninjurious, the technique permits repeated sampling of CSS in vitro and in vivo to gain information about the kinetics of epidermal development and restoration of barrier function. Although

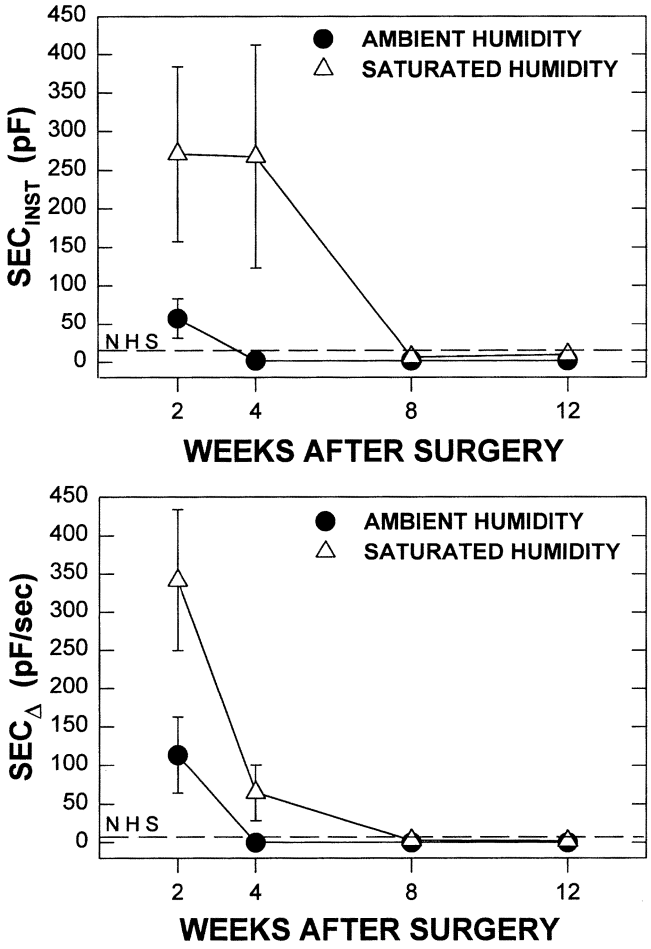

Figure 7. Earlier barrier restoration in athymic mice by CSS_{AMB}. Instantaneous capacitance (top) and initial change (bottom) values from full-thickness wounds healed with cultured skin substitutes incubated in ambient (48–61%) or saturated (79–91%) RH in vitro. Dashed reference lines indicate values for native human skin (NHS) (SEC_{inst} = 13.63 ± 2.7 pF, SEC_{Δ} = 2.73 ± 0.4 pF/sec). Values represent mean ± SEM.

SEC is a beneficial method for epidermal assessment, it also has limitations in range of detection that preclude collection of data from samples that are very dry, or very wet on the surface.

Importantly, SEC_{Δ} values are shown here to predict values for SEC_{cont}. In systems where restriction to water movement is present or developing, lower SEC_{cont} values have been reported to be indicative of improved epidermal barrier function.[20,26,27] The slope of SEC values vs. time, collected during an occluded sampling period, has been shown to correlate to TEWL values and provide an assessment of transepidermal water movement.[20,28,29] The SEC slope is similar to the "initial slope value"[27] previously re-

ported to provide a quantitative measure of barrier function in healing CSS, and both concepts are analogous to the SEC_{Δ} values calculated in this study. Although previous reports have described the use of biophysical methods such as SEC to assess epidermal development in CSS,[26,27] this study shows the use of SEC to quantitatively differentiate the effects of experimental in vitro conditions on in vitro and in vivo barrier formation. The ability of SEC_{Δ} data to distinguish improved in vitro barrier function in ambient CSS, despite environmental factors that would favor increased water movement, verifies the usefulness of SEC as a reliable, predictive index.

The results of this study indicate the ability to regulate the regeneration of functional epithelium with CSS by adjustment of the environmental conditions used for in vitro incubation. This is expected to contribute to improvement of skin substitutes that better satisfy the requirements for permanent wound healing.[2] Further improvements in nutrient medium formulations, biopolymer substrate compositions and/or in vitro incubation conditions are expected to result in cultured skin substitutes that more closely resemble human skin in expression of anatomic and physiologic phenotypes, and efficacy for clinical wound healing.

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REFERENCES

1. Kligman AM. The biology of the stratum corneum. In: Montagna W, Lobitz WC, editors. The epidermis. New York: Academic Press, 1964: 387–433.
2. Lazarus GS, Cooper DM, Knighton DR, Margolis DJ, Pecarro RE, Rodeheaver G, Robson MC. Definitions and guidelines for assessment of wounds and evaluation of healing. Arch Dermatol 1994;130:489–93.
3. Clark RAF. Cutaneous wound repair. In: Goldsmith LA, editor. Physiology, biochemistry and molecular biology of the skin. New York: Oxford University Press, 1991: 576–601.
4. Elias PM. Epidermal lipids, barrier function, and desquamation. J Invest Dermatol 1983;80:44S–49S.
5. Boyce ST, Hansbrough JF. Biologic attachment, growth, and differentiation of cultured human epidermal keratinocytes on a graftable collagen and chondroitin-6-sulfate substrate. Surgery 1988;103:421–31.
6. Boyce ST, Glatter R, Kitzmiller WJ. Treatment of chronic wounds with cultured cells and biopolymers. Wounds 1995;7:24–9.
7. Boyce ST, Greenhalgh DG, Housinger TA, Kagan RJ, Rieman M, Childress CP, Warden GD. Skin anatomy and antigen expression after burn wound closure with composite grafts of cultured skin cells and biopolymers. Plast Reconstr Surg 1993;91:632–41.
8. Hansbrough JF, Boyce ST, Cooper ML, Foreman TJ. Burn wound closure with cultured autologous keratinocytes and fibroblasts attached to a collagen-glycosaminoglycan substrate. JAMA 1989;262:2125–30.
9. Harriger MD, Warden GD, Greenhalgh DG, Kagan RJ, Boyce ST. Pigmentation and microanatomy of skin regenerated from composite grafts of cultured cells and biopolymers applied to full-thickness burn wounds. Transplantation 1995;59:702–7.
10. Boyce ST, Goretsky MJ, Greenhalgh DG, Kagan RJ, Rieman MT, Warden GD. Comparative assessment of cultured skin substitutes and native skin autograft for treatment of full-thickness burns. Ann Surg 1995;222:743–52.
11. Boyce ST, Foreman TJ, English KB, Stayner N, Cooper ML, Sakabu S, Hansbrough JF. Skin wound closure in athymic mice with cultured human cells, biopolymers, and growth factors. Surgery 1991;110:866–76.
12. Boyce ST, Medrano EE, Abdel-Malek ZA, Supp AP, Dodick JM, Nordlund JJ, Warden GD. Pigmentation and inhibition of wound contraction by cultured skin substitutes with adult melanocytes after transplantation to athymic mice. J Invest Dermatol 1993;100:360–5.
13. Boyce ST, Supp AP, Harriger MD, Greenhalgh DG, Warden GD. Topical nutrients promote engraftment and inhibit wound contraction of cultured skin substitutes in athymic mice. J Invest Dermatol 1995;104:345–9.
14. Boyce ST, Williams ML. Lipid supplemented medium induces lamellar bodies and precursors of barrier lipids in cultured analogues of human skin. J Invest Dermatol 1993;101:180–4.
15. Bernstam LI, Vaughan FL, Bernstein IA. Keratinocytes grown at the air–liquid interface. In Vitro Cell Dev Biol 1986;22:695–705.
16. Mak VHW, Cumpstone MB, Kennedy AH, Harmon CS, Guy RH, Potts RO. Barrier function of human keratinocyte cultures at the air–liquid interface. J Invest Dermatol 1991;96:323–7.
17. Harriger MD, Hull BE. Cornification and basement membrane formation in a bilayered human skin equivalent maintained at an air–liquid interface. J Burn Care Rehabil 1994;13:187–93.
18. Higounenc I, Demarchez M, Regnier M, Schmidt R, Ponec M, Shroot B. Improvement of epidermal differentiation and barrier function in reconstructed human skin after grafting onto athymic nude mice. Arch Dermatol Res 1994;286:107–14.
19. Prunieras M, Regnier M, Woodley DT. Methods for cultivation of keratinocytes at the air–liquid interface. J Invest Dermatol 1983;81:28s–33s.
20. Wickett RR, Nath V, Tanaka R, Hoath SB. Use of continuous electrical capacitance and transepidermal water loss measurements for assessing barrier function in neonatal rat skin. Skin Pharmacol 1995;8:179–85.
21. Grice K, Sharratt SM, Baker H. Skin temperature and transepidermal water loss. J Invest Dermatol 1971;57:108–10.
22. Mathias CG, Wilson DM, Maibach HI. Transepidermal water loss as a function of skin surface temperature. J Invest Dermatol 1981;77:219–20.
23. Pinnagoda J. Hardware and measuring principles: The evaporimeter. In: Elsner P, Berardesca E, Maibach HI, editors. Bioengineering of the skin: water and the stratum corneum. Boca Raton: CRC Press, 1994: 51–8.
24. Pinnagoda J, Tupker R, Agner T, Serup J. Guidelines for transepidermal water loss (TEWL) measurement. Contact Dermatitis 1990;22:164–78.
25. Gabard B, Treffel P. Hardware and measuring principle: the Nova DPM 9003. In: Elsner P, Berardesca E, Maibach HI, editors. Bioengineering of the skin: water and the stratum corneum. Boca Raton: CRC Press, 1994: 177–95.

26. Boyce ST, Supp AP, Harriger MD, Pickens WL, Wickett RR, Hoath SB. Surface electrical capacitance as a non-invasive index of epidermal barrier in cultured skin substitutes in athymic mice. J Invest Dermatol 1996;107:82–7.
27. Goretsky MJ, Supp AP, Greenhalgh DG, Warden GD, Boyce ST. Surface electrical capacitance as an index of epidermal barrier properties of composite skin substitutes and skin autografts. Wound Rep Reg 1995;3:419–25.
28. Okah FA, Wickett RR, Pickens WL, Hoath SB. Surface electrical capacitance as a noninvasive bedside measure of epidermal barrier maturation in the newborn infant. Pediatrics 1995;96:688–92.
29. Okah FA, Pickens WL, Hoath SB. Effect of prenatal steriods on skin surface hydrophobicity in the premature rat. Pediatr Res 1995;37:402–8.
30. Tagami H. Impedance measurement for evaluation of the hydration state of the skin surface. In: Leveque JL, editor. Cutaneous investigation in health and disease. New York: Marcel Dekker Inc., 1989: 79–111.
31. Tagami H, Masatoshi O, Keiji I, Kanamura Y, Yamada M, Ichijo B. Evaluation of the skin surface hydration in vivo by electrical measurement. J Invest Dermatol 1980;75:500–7.
32. Okah FA, Wickett RR, Pompa K, Hoath SB. Human newborn skin: the effect of isopropanol on skin surface hydrophobicity. Pediatr Res 1994;35:443–6.
33. Briggaman RA. Human skin grafts-nude mouse model: techniques and application. In: Skerrow D, Skerrow CJ, editors. Methods in skin research. New York: John Wiley and Sons, 1985: 251–76.
34. Demarchez M, Sengel P, Prunieras M. Wound healing of human skin transplanted onto the nude mouse. Dev Biol 1986;113:90–6.
35. Harriger MD, Supp AP, Warden GD, Boyce ST. Glutaraldehyde cross linking of collagen implants in vitro inhibits degradation in cultured skin substitutes grafted to athymic mice. J Biomed Mater Res 1997;35:137–45.
36. Farooqui JZ, Auclair BW, Robb E, Sarkisian E, Cooper C, Alexander JW, Warden G, Boissy RE, Nordlund J. Histological, biochemical, and ultrastructural studies on hyperpigmented human skin xenografts. Pigment Cell Res 1993;6:226–33.
37. Swope VB, Supp AP, Cornelius JR, Babcock GF, Boyce ST. Regulation of pigmentation in cultured skin substitutes by cytometric sorting of melanocytes and keratinocytes. J Invest Dermatol 1997;109:289–95.
38. Denda M, Sato J, Masuda Y, Tsuchiya T, Koyama J, Kuramoto M, Elias PM, Feingold KR. Exposure to a dry environment enhances epidermal permeability barrier function. J Invest Dermatol 1998;111:858–63.