Surface Electrical Capacitance as a Noninvasive Index of Epidermal Barrier in Cultured Skin Substitutes in Athymic Mice

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Restoration of an epidermal barrier is a definitive requirement for wound closure. To determine formation of an epidermal barrier as a function of hydration of the stratum corneum, we measured surface electrical capacitance (SEC) of the epidermis in cultured skin substitutes (CSS) *in vitro* and after grafting to athymic mice. CSS were prepared from human keratinocytes and fibroblasts attached to collagen-glycosaminoglycan substrates. On culture days 3, 7, 14, 17, and 21, SEC was measured *in situ*. CSS (n = 18; mean ± SEM) showed a time-dependent decrease of SEC (picoFarads, "pF") from 4721 ± 28 pF on day 3 to 394 ± 117 pF on day 14, and subsequent increase to 1677 ± 325 pF on day 21. After 14-d incubation, parallel CSS samples (n = 5) or murine autografts (n = 5) were grafted orthotopically to athymic mice. After grafting, CSS showed decreases in SEC from 910 ± 315 pF at 2 wk to 40 ± 10 pF at 4 wk with no significant decreases thereafter. Control values for murine autograft were 870 ± 245 pF at 2 wk, and 87 ± 30 pF at 4 wk. SEC values for native murine skin (n = 10) were 91 ± 18 pF, and for native human skin (n = 10) were 32 ± 5 pF. The data demonstrate that SEC decreases with time in culture and that healed or intact skin has approximately 10- to 100-fold lower SEC than CSS *in vitro*. This noninvasive technique provides a quantitative index of epidermal barrier in CSS *in vitro* and demonstrates the development of functional epidermal barrier during healing of wounds treated with cultured skin substitutes. ***Key words: biophysical instrumentation/wound healing. J Invest Dermatol 107:82–87, 1996***

Cultured epidermal keratinocytes provide an abundant supply of biologic material for long-term closure of acute and chronic wounds. Skin substitutes that contain cultured cells have been demonstrated to serve as adjunctive therapies in treatment of full-thickness skin wounds. Cultured epithelial autografts have been used to close burns and giant nevi (Gallico *et al*, 1984; Gallico *et al*, 1989), and cultured allografts (Phillips *et al*, 1989) have served to facilitate closure of cutaneous ulcers caused by diabetes, pressure, or venous stasis (Carver and Leigh, 1991). Although these progressive studies have made important contributions to development of alternative therapies for wound treatment, it is recognized that repair of both epidermal and dermal tissues is needed to restore optimal function and cosmesis. Dermal replacements have included cadaveric allodermis (Cuono *et al*, 1987), collagen-based implants (Bell *et al*, 1981; Boyce and Hansbrough, 1988), and synthetic biopolymers (Hansbrough *et al*, 1992) to stimulate repair of connective tissue, but none of the alternative materials has yet been validated for routine clinical use.

Assessment of safety and efficacy of alternative biomaterials for wound closure depends on development of uniform standards for measurement of properties in wounds before and after treatment. Standards for assessment of chronic wounds have recently been established by application of semiquantitative indexes of color, vascularity, odor, and pain (Lazarus *et al*, 1994). Evaluation of scar formation after skin grafting has also been described by semiquantitative analysis of multiple parameters of closed wounds (Sullivan *et al*, 1990). Although ordinal scores of wound properties provide useful relative indexes of healing wounds, the inherently subjective factors in these approaches limit the uniformity of interpretation of data collected by investigators in clinical studies. Conversely, quantitative and objective measures of skin properties have been described by noninvasive, biophysical instruments including: planimetry, volumetric castings, transcutaneous gases (O_2, CO_2), laser Doppler blood flow, transepidermal water loss, mechanical properties, skin color, and surface hydration (Serup and Jemec, 1995).

Previous studies from this laboratory have described the combination of cultured epidermal keratinocytes and fibroblasts with a collagen-glycosaminoglycan (GAG) sponge to prepare a cultured skin substitute for grafting to excised full-thickness wounds in a single procedure (Boyce *et al*, 1988; Hansbrough *et al*, 1989). Reports of preclinical studies have demonstrated modulation of synthesis of barrier lipids by medium formulation (Boyce and

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Abbreviations: CSS, cultured skin substitute; pF, picoFarads; SEC, surface electrical capacitance; GAG, glycosaminoglycan.

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Williams, 1993), regulation of skin pigmentation by addition of cultured epidermal melanocytes (Boyce *et al*, 1993b), and inhibition of wound contraction by irrigation of healing wounds with topical nutrients (Boyce *et al*, 1995c). Clinical studies have demonstrated closure of burn wounds (Boyce *et al*, 1993a, 1995b) and chronic wounds (Boyce *et al*, 1995a), expression of skin antigens during healing (Boyce *et al*, 1993a), repigmentation of skin by passenger melanocytes (Harriger *et al*, 1995), quantitative measurement of surface hydration of burn wounds (Goretsky *et al*, 1995), and semiquantitative assessment of efficacy compared to split-thickness skin grafts (Boyce *et al*, 1995b). The objective of the study was to apply biophysical instrumentation for noninvasive evaluation of skin properties to healing of skin substitutes in wound treatment. This report presents quantitative data for assessment of wound healing by surface electrical capacitance after grafting of full-thickness wounds with cultured skin substitutes.

MATERIALS AND METHODS

Cultured Skin Substitutes (CSS) Cultured skin substitutes were prepared as described in previous studies (Boyce *et al*, 1988, 1991, 1993a, 1993b, 1995c; Boyce and Williams, 1993) from collagen-glycosaminoglycan substrates populated sequentially with human dermal fibroblasts and epidermal keratinocytes. Beginning with inoculation of cultured keratinocytes as day 0 of incubation, cultured cell-biopolymer composites were incubated 2 d submerged in MCDB 153 medium (Boyce and Ham, 1983) containing 0.2 mM calcium. On culture day 2, medium was changed to replace bovine pituitary extract with a lipid supplement plus carnitine, increased serine (Boyce and Williams, 1993), and calcium 0.5 mM. On culture day 3, constructs were lifted to the air–liquid interface on cotton filter pads supported by stainless steel mesh, epidermal growth factor was decreased to 1 ng/ml, and calcium was increased to 1.0 mM. On culture day 4, epidermal growth factor was removed, calcium was increased to 1.5 mM, and grafts were incubated in these conditions with daily medium changes until day 21.

Grafting to Athymic Mice and Irrigation All animal studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Human skin substitutes or autologous murine skin were grafted to athymic mice as described in previous studies (Boyce *et al*, 1991, 1993b, 1995c). The entire wound area was covered with a semipermeable adhesive film (OpSite; Smith & Nephew United, Largo, FL). This produced a liquid-tight compartment over the wound into which 1.5 cc of modified keratinocyte growth medium containing antimicrobials (20 μg of Norfloxacin per ml and 100 U of Nystatin per ml) was injected through the OpSite into the cotton gauze immediately following surgery. Constant conditions for irrigation media included 1.5 mM calcium, 10 ng of epidermal growth factor per ml, 5.0 μg of bovine insulin per ml, and 0.5 μg of hydrocortisone per ml. Dressed grafts were then covered with a self-adherent bandage (Coban; 3M Medical Division; St. Paul, MN) to protect treated sites from mechanical disturbance. Dressings covering treated sites were injected with 1.0 cc/day of irrigants described above for 13-d postgrafting. On day 14 after grafting, dressings and stent sutures were removed from all animals. Mice were rebandaged on day 14 and day 21 after grafting. On day 28, all animals were removed from their dressings. At 12 wk, animals were sacrificed, and tissue samples from wounds were collected for microscopy.

Measurement of Surface Electrical Capacitance (SEC) Hydration of the skin surface is directly proportional to retention of electrical charge, or capacitance (Tagami *et al*, 1980), according to the relationship:

$$Z = [R_x^2 + (1/2\pi fC_x)^2]^{1/2}$$

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. Measurements of SEC were collected with an impedance-based NOVA™ Dermal Phase Meter (DPM 9003; NOVA Technology Corporation, Gloucester, MA) connected to a portable computer that recorded 10 serial readings at 1-s 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. Continuous measurement of electrical capacitance under occlusion by the probe has been shown to correlate with transepidermal water loss and development of barrier function *in vivo* (Okah *et al*, 1995; Wickett *et al*, 1995).

SEC measurements were recorded *in vitro* on days 3, 7, 14, 17, and 21 after inoculation of epidermal keratinocytes onto collagen-GAG substrates. After surgery, SEC recordings were collected in triplicate at postoperative weeks 2, 4, 8, and 12.

Data Collection and Analysis SEC data are expressed as mean ± SEM in picoFarads (pF). The initial reading (t = 1 s) is defined as instantaneous capacitance. Subsequent readings (t = 10 s) after occlusion of the skin by the probe are defined as continuous capacitance. The impedance-based data measured by the DPM 9003 represent aggregate values collected at defined intervals during a frequency scan up to 1 MHz. Instrument readings (C) were converted to picoFarads by the formula (Okah *et al*, 1994):

$$pF = (8.38 \times 10^{-6} \times C^3) - (4.0 \times 10^{-3} \times C^2) + 1.668C - 122.1$$

that was determined from a calibration curve supplied by the manufacturer (Gabard and Treffel, 1994). Wound areas were determined by direct tracings of wounds at weeks 2, 3, 4, 8, and 12 after grafting. Computer-assisted image analysis was performed to quantify wound areas. Treated wounds were photographed at weeks 2, 4, 8, and 12 after grafting. Data were subjected to repeated measures analysis of variance ($p < 0.0001$), and Tukey's Studentized Range Test with significance accepted at the 95% confidence level ($p < 0.05$).

RESULTS

Epithelial Morphogenesis of CSS Epithelial morphogenesis during incubation of CSS *in vitro* is represented in **Fig 1**. Three days after inoculation, multiple layers of undifferentiated keratinocytes cover the surface of the collagen-GAG substrate. By 7 days incubation (4-d air exposure), cornified strata have begun to develop. Cornified strata accumulate by day 14 to form an analog of stratum corneum that remains well established at day 21 of incubation.

Temporal Formation of Epidermal Barrier *in Vitro* After exposure of cultured epithelium to the air-liquid interface, capacitance values dropped rapidly. As shown in **Fig 2**, partial reformation of the epidermal barrier occurs *in vitro*. The magnitude of the change between t = 1 and t = 10 s values is directly proportional to the amount of hydration that accumulated on the CSS surface during the interval of occluded sampling. During a fixed sampling interval (10 s), the magnitude of change in SEC decreases as a function of decrease in rate of moisture flux through the CSS. Rate of moisture flux through the epidermal analog is inversely proportional to barrier formation in the developing stratum corneum. Therefore, the magnitude of the differences between instantaneous and continuous SEC values provides an indirect index of epidermal barrier function. Continuous capacitance decreases to approximately 400 pF by day 14 of incubation and increases subsequently to approximately 1700 pF by incubation day 21. SEC values at day 3 were at maximum limits of instrument sensitivity and showed no differences between instantaneous and continuous readings; however, each reading at day 3 was significantly greater than subsequent respective values of instantaneous or continuous capacitance. Analysis of variance showed significantly greater values for continuous capacitance at days 7 and 21 of incubation. Minimal SEC values at day 14 demonstrate maximal epidermal barrier *in vitro* under these experimental conditions.

Wound Closure on Athymic Mice Photographs of athymic mice grafted with cultured skin substitutes **(Fig 3)** show complete epithelialization of the wound at 2 wk, development of a hyperkeratotic surface at 4 wk, and formation of stable human epidermis at 8 and 12 wk. Also noteworthy is repigmentation of these grafts by passenger melanocytes.

Formation of Permanent Epidermal Barrier *in Vivo* Kinetics of reformation of the epidermal barrier *in vivo* is presented in **Fig 4**. Continuous capacitance drops rapidly after wet dressings are stopped at 2 wk after grafting, and SEC values decrease to the range of normal human skin at 4 wk and later. Control values (mean ± SEM) for murine autograft, native murine skin, and native skin of human volunteers (n = 10) are shown in **Table I**. At 4 wk after grafting, values of continuous SEC from cultured skin substitutes (40 ± 10 pF) were not statistically different from those from 10 human volunteers (32 ± 5 pF), and values for murine autografts (87

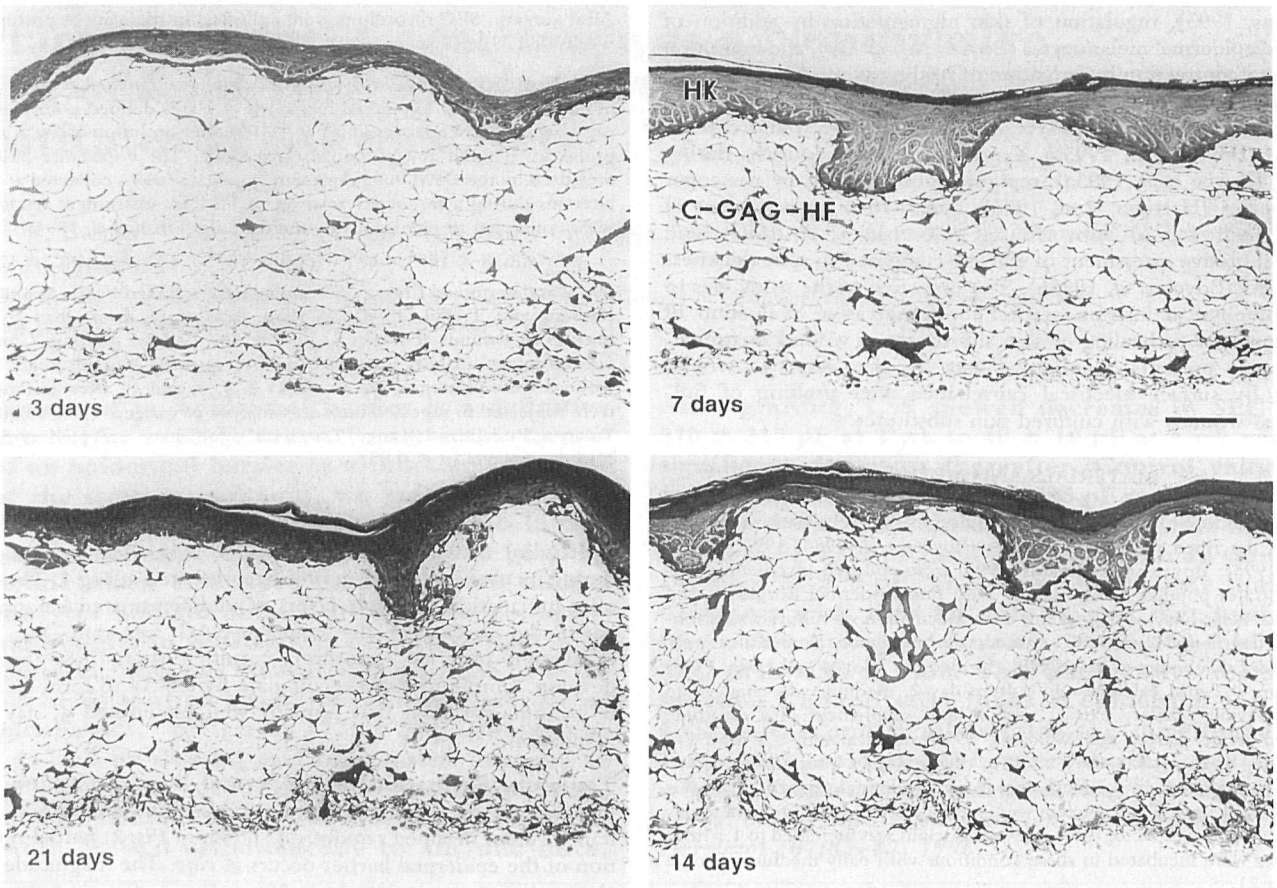

Figure 1. Morphogenesis of cultured skin substitutes *in vitro*. No corneocytes have developed by 3 d (*upper left*) after inoculation of keratinocytes (*HK*) onto collagen-glycosaminoglycan substrates populated with fibroblasts (*C-GAG-HF*), and incubation submerged in medium. By day 7 (*upper right*), cornified strata have begun to develop. At 14 d (*lower right*) and 21 d (*lower left*), a morphologic analog of stratum corneum has resulted from culture of keratinocytes with air exposure in a lipid-supplemented medium. *Scale bar* = 0.1 mm.

± 30 pF) were not statistically different from those from uninjured murine skin (91 ± 18 pF).

Grafting of CSS Stimulates Formation of Permanent Epidermal Barrier Development of a normal epidermal barrier from cultured skin substitutes is represented by combined plot of *in vitro* and *in vivo* continuous capacitance as shown in **Fig 5**. After a partial barrier forms *in vitro*, the skin substitute is grafted and maintained in wet dressings for 2 wk. Simultaneously, capacitance values for skin substitutes maintained in cell culture begin to increase, demonstrating the relative instability of tissue analogs *in vitro*, but parallel samples grafted to animals become vascularized and reform stable skin tissue with a fully functional epidermal barrier. Therefore, skin substitutes are capable of forming a stable barrier *in vivo*, but these culture conditions do not yet support expression of this definitive phenotype of human skin *in vitro*.

Wound Areas of Human CSS Are Not Different From Murine Autografts Measurement of wound areas after treatment with skin substitutes or skin autograft is presented in **Fig 6**. Healed skin substitutes were not statistically different in area from murine skin autografts during 12 wk of observation, and by then wound areas were relatively stable.

DISCUSSION

Data presented in this report demonstrate a reliable, convenient, consistent, and valid technique for kinetic evaluation of reformation of the epidermal barrier in healing wounds. Although the barrier formed rapidly after grafting, the use of wet dressings through day 14 artifactually maintained high SEC, but irrigation of skin substitutes with nutrients and antimicrobial agents after surgery

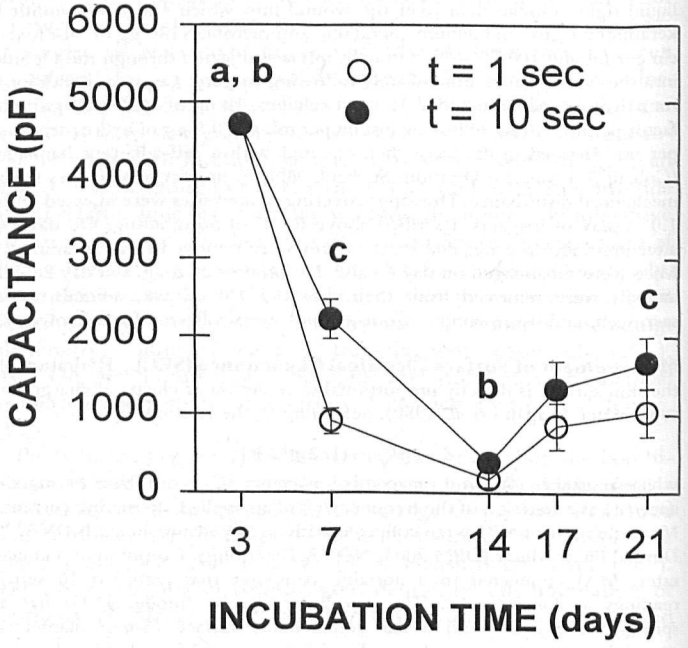

Figure 2. Instability of surface electrical capacitance of cultured skin substitutes *in vitro*. Instantaneous (t = 1 s) and continuous (t = 10 s) capacitance decrease to a minimum value by day 14, and then increase through day 21. Significant differences are noted ($p < 0.05$, repeated measures ANOVA; *a*, t = 1-s plot, all time points; *b*, t = 10-s plot, all time points; *c*, t = 1-s *vs.* t = 10-s plots, single time points). *Error bars*, SEM.

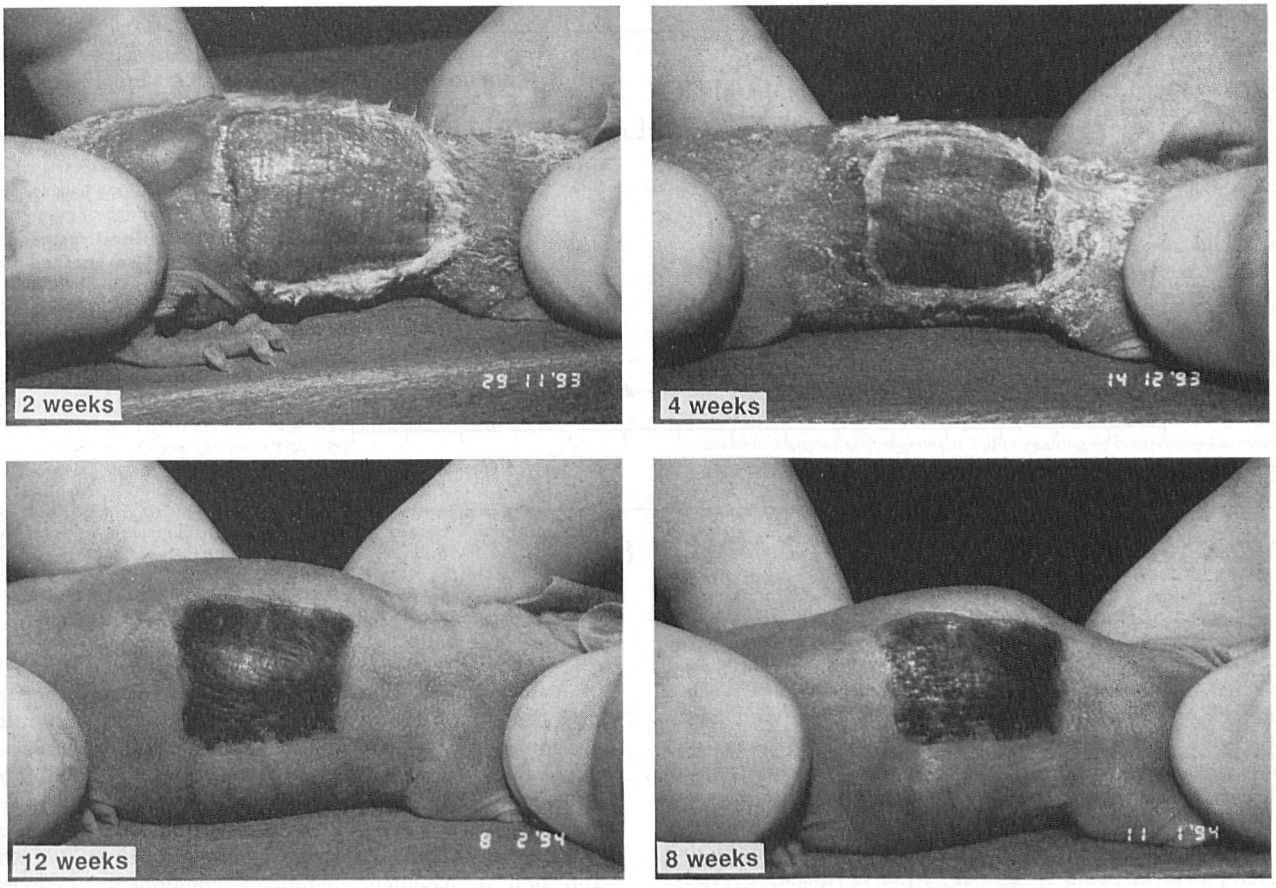

Figure 3. Closure of skin wounds on athymic mice after grafting with cultured skin substitutes. Progressive epithelialization with hyperkeratosis in healing skin proceeds from 2–4 wk after grafting (*upper left* and *right*), followed by development of stable skin tissue by 8–12 wk (*lower right* and *left*).

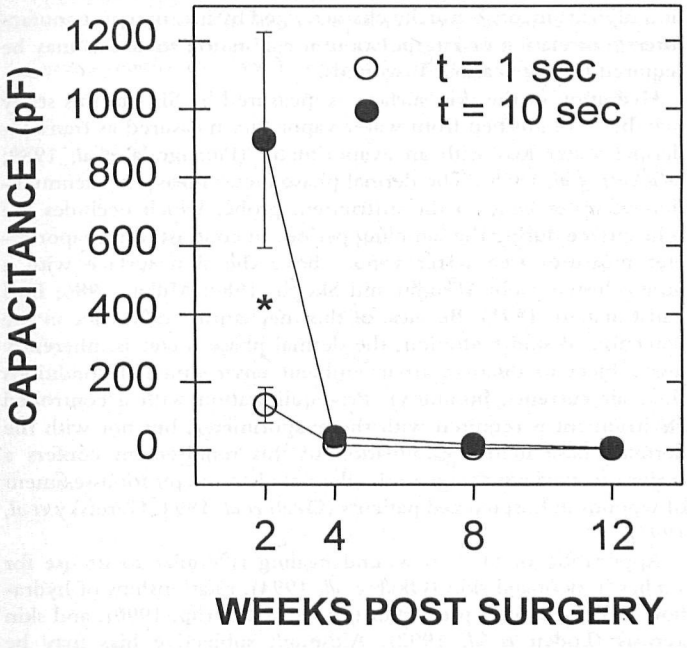

Figure 4. Stability of surface electrical capacitance after grafting of cultured skin substitutes to athymic mice. Instantaneous (t = 1 s) and continuous (t = 10 s) capacitance decrease to values of normal human skin by 4 wk, and remain stable throughout the remainder of the observation period. (*, $p < 0.05$; t = 1-s *vs.* t = 10-s plots). *Error bars*, SEM.

promoted engraftment and inhibited wound contraction[1] (Boyce *et al*, 1995c). Therefore, delay of formation of an epidermal barrier due to administration of irrigations after grafting of skin substitutes is not considered a disadvantage to wound closure.

Skin substitutes *in vitro* develop a transient epidermal barrier, as is

[1] Boyce ST, Supp AP, Harriger MD, Swope VB, Warden GD. Effective management of microbial contamination of cultured skin substitutes on athymic mice with formulations of non-cytotoxic antimicrobial agents. *Wound Repair Regen* 3:83, 1995 (abstr.)

Table I. SEC Values[a] for Murine and Human Controls

Control Condition		n	Instantaneous (t = 1 s)	Continuous (t = 10 s)
MAG[b]	2 wk[e]	5	563 ± 224[f]	870 ± 245[f]
	4 wk	5	26 ± 6	87 ± 30
	8 wk	5	6 ± 1	19 ± 3
	12 wk	5	15 ± 3	41 ± 8
NMS[c]	2 wk	10	35 ± 5	57 ± 10
	4 wk	10	37 ± 6	91 ± 18
	8 wk	10	8 ± 1	22 ± 4
	12 wk	10	9 ± 2	20 ± 3
NHS[d]		10	14 ± 3	32 ± 5

[a] Mean ± SEM.
[b] Murine autograft.
[c] Native murine skin.
[d] Native human skin.
[e] Number of weeks postsurgery.
[f] Mean ± SEM.

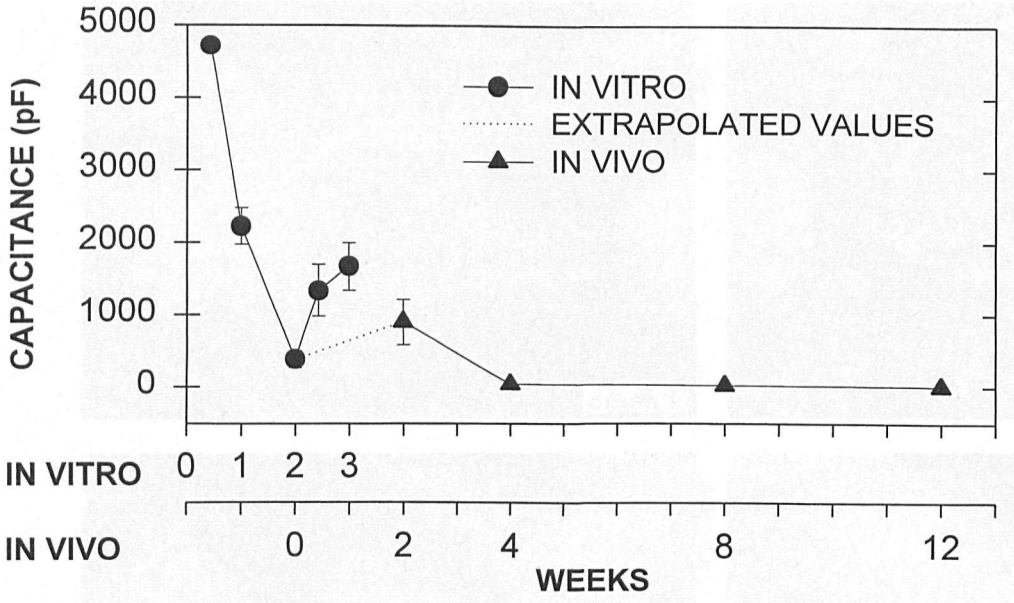

Figure 5. Combined assessment of continuous (t = 10 s) surface electrical capacitance *in vitro* and *in vivo*. Surface hydration decreases from maximal values during incubation, and subsequently normalizes completely after grafting and healing on athymic mice. *Error bars*, SEM.

demonstrated by SEC in this study. Capacitance values reach a minimum at day 14 of incubation in this experiment, with subsequent increases until day 21. Although minimum values for skin substitutes (~400 pF) approach those for uninjured human skin (~40 pF), increases in capacitance at later times *in vitro* show the biologic instability of skin substitutes. Instability of SEC values in CSS may be explained by deficiencies in culture conditions that limit development of stable epidermal barrier *in vitro*. Conversely, after grafting to athymic mice, skin substitute capacitance decreases to values of native skin and remains stable for the 12-wk observation period of this study. Furthermore, a subset of animals from this study were maintained for more than 1 yr and showed a stable epidermal barrier. Transplantation of CSS to athymic mice provides a valuable model for identification of factors that contribute to normalization of skin functions after grafting. Identification of nutritional, cellular, and humoral factors in wounds that promote skin healing with CSS is expected to result in modification of culture conditions to improve expression of skin phenotypes by CSS *in vitro*. Because most skin functions are restored after grafting of CSS to athymic mice, this model may also be useful in studies of expression of basement membrane components, cytokine networks, and pigmentation during healing of human skin.

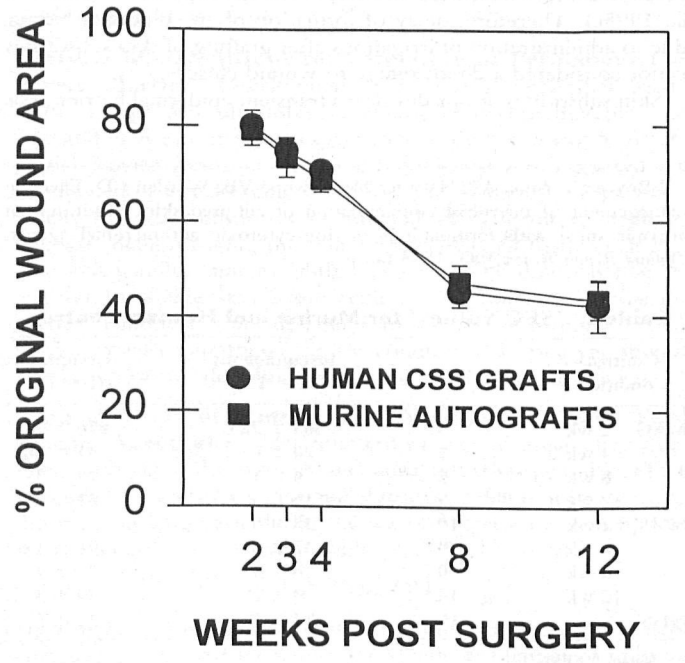

Figure 6. Wound areas after grafting of cultured skin substitutes and murine skin autograft. No statistically significant differences in wound areas were detected during 12 wk of observation. Healed skin of both graft types was fully stable by 8 wk after surgery. *Error bars*, SEM.

Quantitative assessments of wounds has multiple advantages over qualitative assessments and semiquantitative scales (Sullivan *et al*, 1990). Subjective interpretations are greatly reduced, accuracy may be gained, sensitivity of detection may be increased, reproducibility of data collection is greater, and data may be recorded directly into computer files for analysis. Although standardization and calibration of instruments is usually characterized by instrument manufacturers, correlation or interpolation of calibration standards may be required for application to wounds.

Hydration of the skin surface as measured by SEC in this study may be distinguished from water vapor flux measured as transepidermal water loss with an evaporimeter (Pinnagoda *et al*, 1989; Wickett *et al*, 1995). The dermal phase meter measures accumulation of water beneath the instrument probe, which occludes the skin surface during the sampling period. In contrast, the evaporimeter measures free water vapor above the skin surface with a nonocclusive probe (Frodin and Skogh, 1984; Miller, 1986; Kajs and Garstein, 1991). Because of this mechanistic difference in the detection of skin hydration, the dermal phase meter is inherently less subject to fluctuations in ambient environmental conditions (i.e., air currents, humidity). Pre-equilibration with a controlled environment is required with the evaporimeter, but not with the dermal phase meter. Elimination of this requirement confers a major practical advantage to the dermal phase meter for assessment of wounds in hospitalized patients (Okah *et al*, 1994; Goretsky *et al*, 1995).

Application of SEC to wound healing is similar to its use for studies in neonatal skin (Okah *et al*, 1994), relationships of hydration and mechanical properties (Jemec and Serup, 1990), and skin xerosis (Loden *et al*, 1992). Although subjective bias may be introduced by selection of sites to be measured, this may be reduced by increasing the sample size to include all representative assessment conditions. Application of objective instrumentation will decrease bias in data collection within individual studies and increase consistency among investigations performed at different

facilities. Acceptance and adaptation of quantitative techniques for assessment of wound healing will facilitate validation of experimental alternatives for treatment and closure of skin wounds.

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