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# Vitamin C Regulates Keratinocyte Viability, Epidermal Barrier, and Basement Membrane *In Vitro*, and Reduces Wound Contraction After Grafting of Cultured Skin Substitutes

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Cultured skin substitutes have become useful as adjunctive treatments for excised, full-thickness burns, but no skin substitutes have the anatomy and physiology of native skin. Hypothetically, deficiencies of structure and function may result, in part, from nutritional deficiencies in culture media. To address this hypothesis, vitamin C was titrated at 0.0, 0.01, 0.1, and 1.0 mM in a cultured skin substitute model on filter inserts. Cultured skin substitute inserts were evaluated at 2 and 5 wk for viability by incorporation of 5-bromo-2'-deoxyuridine (BrdU) and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion. Subsequently, cultured skin substitute grafts consisting of cultured human keratinocytes and fibroblasts attached to collagen-glycosaminoglycan substrates were incubated for 5 wk in media containing 0.0 mM or 0.1 mM vitamin C, and then grafted to athymic mice. Cultured skin substitutes ( $n = 3$  per group) were evaluated *in vitro* at 2 wk of incubation for collagen IV, collagen VII, and laminin 5, and through 5 wk for epidermal barrier by surface electrical capacitance. Cultured skin substitutes were grafted to full-thickness wounds in athymic mice ( $n = 8$  per group),

evaluated for surface electrical capacitance through 6 wk, and scored for percentage original wound area through 8 wk and for HLA-ABC-positive wounds at 8 wk after grafting. The data show that incubation of cultured skin substitutes in medium containing vitamin C results in greater viability (higher BrdU and MTT), more complete basement membrane development at 2 wk, and better epidermal barrier (lower surface electrical capacitance) at 5 wk *in vitro*. After grafting, cultured skin substitutes with vitamin C developed functional epidermal barrier earlier, had less wound contraction, and had more HLA-positive wounds at 8 wk than without vitamin C. These results suggest that incubation of cultured skin substitutes in medium containing vitamin C extends cellular viability, promotes formation of epidermal barrier *in vitro*, and promotes engraftment. Improved anatomy and physiology of cultured skin substitutes that result from nutritional factors in culture media may be expected to improve efficacy in treatment of full-thickness skin wounds. **Key words:** ascorbic acid/wound healing/stratum corneum/skin grafts. *J Invest Dermatol* 118:565–572, 2002

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**E**ffective treatment of full-thickness skin wounds requires restoration of a functional and stable epidermal barrier. Many approaches to restoration of epidermal barrier have been described, including skin substitutes prepared from autologous or allogeneic cultured keratinocytes (Green *et al*, 1979; Bell *et al*, 1981; Bell and Rosenberg, 1990; Beumer *et al*, 1993; Boyce, 1996; Berthod and Damour, 1997). A general deficiency of cultured keratinocytes has been failure to form functional epidermal barrier *in vitro* (Mak *et al*, 1991; Ponec and Kempenaar, 1995), and the coincident accumulation of triglycerides (Ponec *et al*, 1995). However, cultured keratinocytes form a

barrier after transplantation, to close wounds (Boyce *et al*, 1996; Goretzky *et al*, 1995). Hypothetically, cultured skin substitutes (CSS) that form *in vitro* an epidermal barrier that is homologous anatomically and physiologically with uninjured human skin may promote more efficacious and more rapid wound closure.

Epidermal barrier results from anabolic metabolism of carbohydrate into a variety of specialized lipids that form a liquid-crystal matrix among corneocytes in the stratum corneum (Wertz and Downing, 1991). Failure to form a barrier may be assumed to be caused by errors in keratinocyte metabolism in the pathways of carbohydrate to lipid, particularly in the formation of complex ceramides. Critical to the formation of barrier lipids is the esterification of ceramides with essential fatty acids in the pathway to synthesis of acyl-glucosyl-ceramides (Wertz and Downing, 1991). The esterification reaction requires hydroxylation of the methyl ends of fatty acids as a precursor to the addition of essential fatty acids. A recent study by Holleran and colleagues (Behne *et al*, 2001) showed that blocking of the hydroxylation reaction during synthesis of fatty acids in keratinocytes blocked the synthesis of the

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Abbreviations: CSS, cultured skin substitutes; GAG, glycosaminoglycan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; SEC, surface electrical capacitance.

specialized lipids that are required for epidermal barrier. Definitive studies by Poniec *et al* (1997) showed that the formation of barrier lipids in CSS is greatly enhanced by the presence of ascorbic acid and linoleic acid in the incubation medium. The enhancement of epidermal barrier by vitamin C agrees very well with the requirement for vitamin C in hydroxylation reactions during collagen synthesis to form connective tissue (Kivirikko and Kivuniemi, 1987; Wenstrup *et al*, 1991). Similarly, vitamin C has been shown to stimulate the growth of cultured rabbit corneal keratinocytes (Saika *et al*, 1991). These examples of the multiple activities of vitamin C represent the importance of this cofactor in both general and specific metabolic pathways.

Anatomic and physiologic deficiencies of cultured cells for transplantation result from deficiencies in culture conditions, perhaps the most important of which is medium formulation. Although vitamins are essential for proper nutrition (Bettger and Ham, 1982; Bettger and McKeehan, 1986), conventional media provide predominantly B vitamins for energy production. Other vitamins that are required for specialized cellular phenotypes, however (e.g., vitamins A, D, K), are usually not components of standard media for reasons of solubility or stability. Rather, some vitamins are derived from tissue extracts, such as sera, which are added to basal media. Consequently, defined media will exaggerate nutritional deficiencies from absence of vitamins from sources such as sera, but also are more responsive to factors that regulate cellular proliferation and differentiation (Ham and McKeehan, 1979). Therefore, the nutritional composition of defined media must provide a full complement of factors that may otherwise be provided by tissue extracts. In addition to growth factors (e.g., epidermal growth factor, Keratinocyte Growth Factor (KGF), insulin-like growth factor 1) that are required to promote mitosis in the absence of tissue extracts, both essential nutrients and vitamins can limit or promote growth in defined media (Ham and McKeehan, 1979; McKeehan, 1982). Historically, media that stimulate rapid proliferation of keratinocytes in serum-free or defined conditions have contained neither essential fatty acids nor vitamin C (Boyce and Ham, 1983; Pittelkow and Scott, 1986; Shipley and Pittelkow, 1987).

Previous studies from this laboratory have described nutritional requirements for cultured keratinocytes (Boyce and Ham, 1985), stimulation of barrier lipids by addition of lipid supplements to the culture medium (Boyce and Williams, 1993), measurement of epidermal barrier with noninvasive biophysical measurements (Boyce *et al*, 1996), regulation of keratinocyte growth and differentiation by essential nutrients (Boyce *et al*, 1993a) and by insulin (Swope *et al*, 2001), and healing of burns and chronic wounds with CSS (Hansbrough *et al*, 1989; Boyce *et al*, 1995a; 1995b; 1999; 2002). This study investigated whether supplementation with vitamin C of media for maturation and differentiation of keratinocytes in CSS affects the viability and stability of the grafts *in vitro*, and their efficacy for closure of full-thickness cutaneous wounds. Hypothetically, an increase of viability and stability *in vitro* may increase the therapeutic efficacy of cultured skin grafts, contribute to more rapid and complete healing of cutaneous wounds with transplanted cells, and extend the incubation time for which cultured skin grafts may be applied with confidence of favorable outcome.

## MATERIALS AND METHODS

**Cell culture** Human keratinocytes and fibroblasts were coisolated from surgical discard tissue using selective growth media and cryopreserved at passage 1 for these experiments (Boyce and Ham, 1983; 1985). Two formats of cell culture were used for two purposes. Cell culture inserts were prepared in six-well dishes to titrate an optimal concentration of vitamin C into the incubation medium. After an optimal concentration of vitamin C was determined, CSS grafts were prepared with and without the optimal concentration of vitamin C for assessment *in vitro* and for grafting to full-thickness surgical wounds.

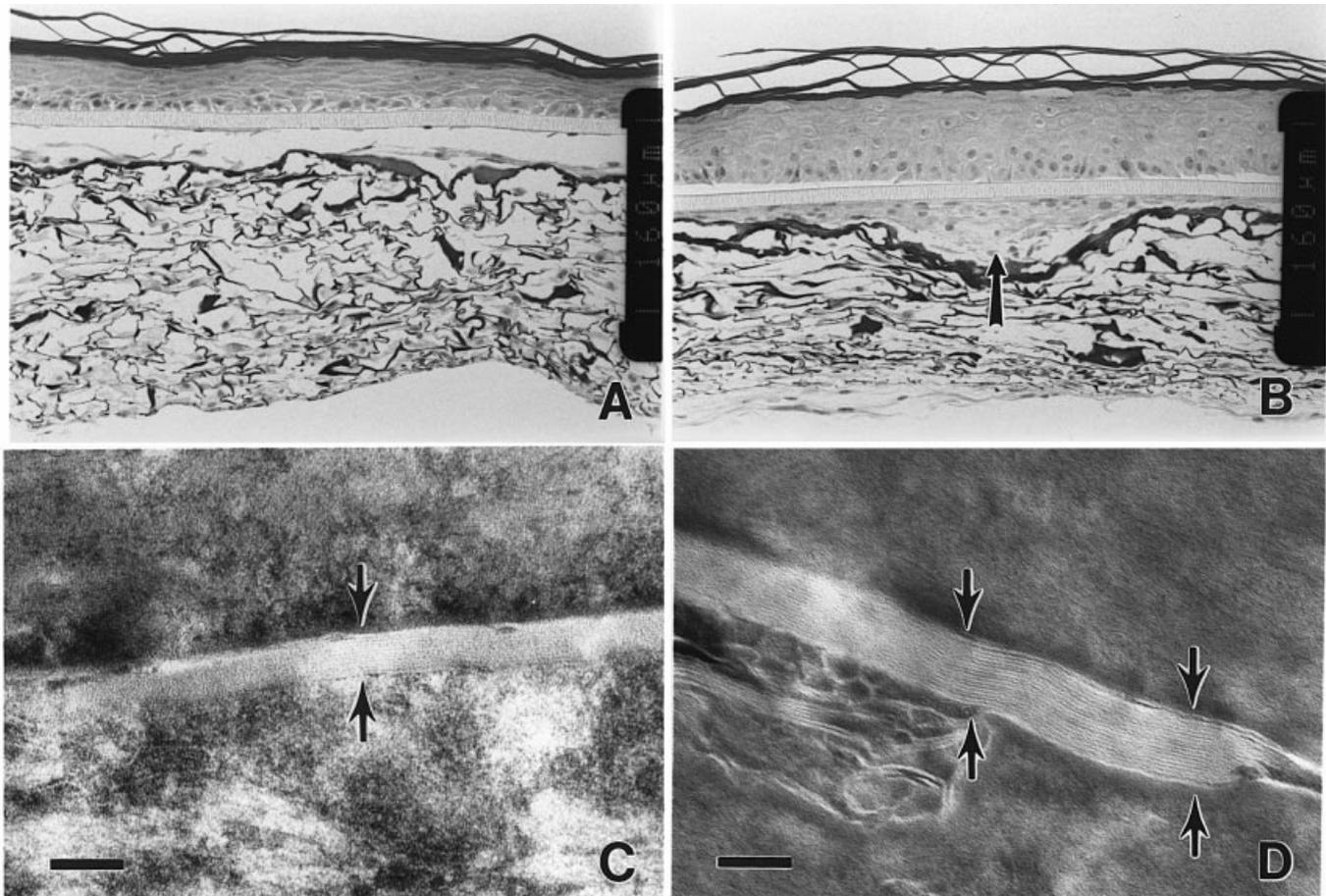
**Cell culture insert preparation** A cultured skin model was developed using a collagen-glycosaminoglycan (GAG) substrate inoculated with human fibroblasts at  $5 \times 10^5$  per  $\text{cm}^2$ . Human keratinocytes were inoculated at  $8.3 \times 10^5$  per  $\text{cm}^2$  into Costar polycarbonate Transwell inserts, and designated as incubation day 0. Dulbecco's modified Eagle's medium (DMEM) (1.8 mM calcium) supplemented as reported by Chen *et al* (1995) with modifications was used beginning on day 0 for the human keratinocyte insert inoculation with the following test conditions: 0.0, 0.01, 0.1, or 1.0 mM vitamin C. On day 1, the insert membrane was removed from the plastic support and combined with the human fibroblast inoculated collagen disks in the DMEM test media. Each human fibroblast collagen disk and human keratinocyte insert membrane was lifted to the air-liquid interface on day 3 as a unit with the insert membrane overlying the human fibroblast collagen disk. The vitamin C (Sigma Chemical, St. Louis, MO) was prepared daily in the test medium. Four paired human fibroblast/human keratinocyte cell strains were inoculated onto two to three collagen-insert units per cell strain ( $n = 10$  per condition). Samples were collected weekly for histologies, and at days 14 and 35 for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and incorporation of 5-bromo-2'-deoxyuridine (BrdU). For light microscopy, biopsies of the insert model were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**CSS graft preparation** CSS ( $n = 3$  per condition) were prepared by sequential inoculation with human fibroblasts ( $5 \times 10^5$  per  $\text{cm}^2$ ) and human keratinocytes ( $1 \times 10^6$  per  $\text{cm}^2$ ) onto acellular collagen-GAG biopolymer substrates as previously described (Boyce *et al*, 1991) with the following modifications. On day 3 after human keratinocyte inoculation, the CSS were lifted and maintained at the air-liquid interface in saturated relative humidity at 37°C and 5% CO<sub>2</sub> (Boyce and Williams, 1993). Vitamin C (0.0 and 0.1 mM) was prepared fresh and replaced daily for 5 wk. Samples were collected at 2 wk for immunohistochemistry, and at 5 wk for light microscopy and assay of MTT conversion.

**CSS grafting to athymic mice** All animal studies were conducted according to the guidelines established by the National Institutes of Health, with the approval of the University of Cincinnati Institutional Animal Care and Use Committee. On culture day 35, CSS incubated in media containing 0.0 mM vitamin C ( $n = 8$ ) or 0.1 mM vitamin C ( $n = 9$ ) were grafted orthotopically to  $2 \times 2$  cm skin wounds prepared surgically (Boyce *et al*, 1991; Boyce, 1998) in athymic mice (nu/nc; Harlan, Indianapolis, IN). Wounds were prepared leaving the panniculus carnosus intact and the CSS ( $\approx 4 \text{ cm}^2$ ) were sutured in place with an overlay of nonadherent dressing (N-Terface; Winfield Laboratories, Richardson, TX). The wounds were dressed as previously described (Supp *et al*, 2000) and left undisturbed for 2 wk. On day 14 after surgery, dressings and suture sutures were removed, data were collected, and mice were re-banded with N-Terface, cotton gauze, and Coban (3M Medical Division, St. Paul, MN). All dressings were removed on day 21 for the remainder of the *in vivo* study period.

**MTT assay** Six millimeter punch biopsies were collected on days 14 and 35 ( $n = 10$  CSS inserts per condition; 24–48 punches per condition) and on day 35 only ( $n = 3$  CSS grafts per condition; 12–18 punches per condition). Biopsies were incubated for 3 h at 37°C with 0.5 mg per ml MTT (Sigma Chemical). The mitochondria of viable cells cleave the tetrazolium salt MTT to formazan (Mosmann, 1983; Swope *et al*, 2001). The MTT-formazan reaction product was released by incubating the biopsies in 2-methoxy-ethanol for 3 h on a rotating platform. The optical density of the MTT-formazan product was read at 590 nm on a microplate reader (Cambridge Technologies, Watertown, MA).

**BRDU incorporation** Biopsies ( $1 \text{ cm}^2$ ) from the collagen-insert unit were incubated for 22 h with 65  $\mu\text{M}$  BrdU (Roche Molecular Biochemicals, Indianapolis, IN) in a lifted format on day 28. The BrdU-labeled tissue was fixed in 10% buffered neutral formalin and processed for paraffin embedding. The tissue sections were deparaffinized and rehydrated in graded alcohols. Three 0.01 M phosphate-buffered saline (PBS) washes were performed between the labeling steps outlined below and all solutions were prepared in the same buffer. The sections were partially digested with 0.025% trypsin for 20 min at 37°C followed by 2% bovine serum albumin to inactivate the trypsin. The cellular DNA was denatured with 1.5 N HCl for 15 min at 37°C and then neutralized with 0.1 M sodium tetraborate. Following a blocking step, the sections were incubated with murine anti-BrdU-FITC (Becton-Dickinson, San Jose, CA) at 4°C overnight, and counterstained with propidium iodide (5  $\mu\text{g}$  per ml). The slides were coverslipped with Fluoromount G



**Figure 1. Vitamin C promotes more complete anatomy and structural assembly of epidermal barrier lipids in a CSS model on filter membranes.** (A), (C) 0.0 mM vitamin C. (B), (D) 0.1 mM vitamin C. At incubation day 14, the epithelial morphology of CSS is well stratified and cornified in conditions with (A) 0.0 mM vitamin C or (B) 0.1 mM vitamin C. Fibroblast density in the dermal compartment, however, is greater in the presence of vitamin C (arrow). TEM of intercorneocyte lipids at incubation day 14 shows poor organization of lamellar structures in (C) 0.0 mM vitamin C, but lipid lamellae characteristic of stratum corneum in (D) 0.1 mM vitamin C. Scale bars: (A), (B) 0.160 mm; (C), (D) 50 nm.

(Southern Biotechnology Associates, Birmingham, AL). The BrdU-FITC-positive and propidium-iodide-labeled nuclei in cells attached to the filter membrane were counted per microscopic field (field length 550  $\mu$ m). Four to six unique sections were counted per sample ( $n = 8$  collagen-insert units per condition) at the day 14 and day 35 time point. The percentage of BrdU-positive human keratinocytes was calculated by dividing the BrdU-FITC-positive human keratinocytes by the propidium-iodide-labeled nuclei  $\times 100$ .

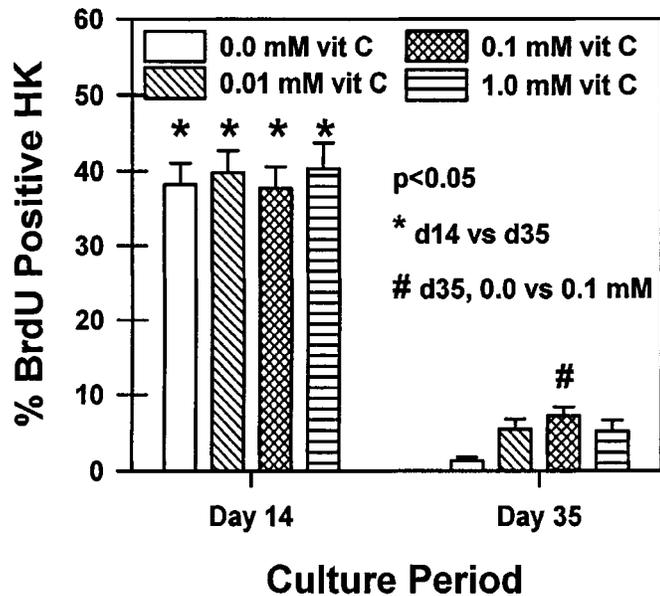
**Transmission electron microscopy (TEM)** TEM was performed to identify multilamellar lipids of the stratum corneum. Stratified epithelia on Millipore inserts were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, followed by postfixation for 2 h in aqueous 1% osmium tetroxide and 0.25% ruthenium tetroxide (Swartzendruber *et al*, 1989). Samples were dehydrated in a graded series of alcohols and embedded in Medcast-Araldite resin. Ultra-thin sections were prepared on a Reichert-Jung Ultracut E, mounted on TEM grids, and examined with a JEOL 100 CX II transmission electron microscope.

**Immunohistochemistry** Biopsies were collected from CSS grafts (0.0 or 0.1 mM vitamin C) on day 14 of *in vitro* incubation, and from healing grafts at 8 wk after surgery. Tissue samples were frozen in M-1 Embedding Matrix (Lipshaw, Pittsburgh, PA) using a two-step process to ensure proper orientation of the specimens. The biopsies were frozen flat initially in a small volume of M-1 in a steel mold contacted to dry ice. The embedded sample was removed from the mold and the block was trimmed with a razorblade to reveal one edge of the tissue. The block was rotated so the trimmed edge was down and held in place with forceps in a steel mold filled with M-1 until freezing was initiated on dry ice. The mold was transferred to a steel plate chilled with liquid nitrogen and a plastic embedding ring was frozen to the mold with an

additional volume of M-1 to facilitate subsequent sectioning of the frozen block. Cryostat sections ( $\approx 15 \mu$ m) were dehydrated in methanol, fixed in acetone, and stored at  $-20^\circ\text{C}$  until rehydration. After warming to room temperature, sections were rehydrated in PBS at pH 7.6.

All washing, blocking, antibody incubation, and staining steps were performed at room temperature. Specific primary antibodies and a nonspecific negative control antibody (see below) were incubated for 30 min on sections from CSS grafts for identification of basement membrane proteins. The antibodies were detected by peroxidase labeling with a Vectastain Universal Elite ABC Kit (#PK-6200; Vector Laboratories, Burlingame, CA) followed by chromogenic visualization with 3,3'-diaminobenzidine (DAB) using a DAB Substrate Kit (#SK-4100; Vector Laboratories). The manufacturer's protocol for frozen sections was followed with minor modifications. To reduce electrostatic charges on the sections a rinse (5 min) with PBS/0.05% Tween 20 (vol/vol, pH = 7.6) was added prior to primary antibody incubation. Endogenous peroxidase activity was quenched with a rinse (30 min) of 0.3% hydrogen peroxide following the biotinylated secondary antibody incubation. All sections were counterstained with filtered Toluidine Blue, rinsed, and coverslipped with Clearslip (#CS-160; IMEB, San Marcos, CA). The specific primary antibodies used were all murine monoclonal antibodies against human basement membrane antigens: collagen type IV (#MAB1910; Chemicon International, Temecula, CA) diluted 1:500, collagen type VII (#MAB1345; Chemicon International) diluted 1:500, laminin 5/kalinin (#MAS160; Harlan Sera-Lab Ltd., Loughborough, U.K.) diluted 1:20. The negative control antibody was a monoclonal mouse IgG<sub>1</sub> isotype control (#MAB002; R&D Systems, Minneapolis, MN) used at a concentration of 2  $\mu$ g per ml.

CSS engraftment to athymic mice was confirmed by direct immunofluorescence staining of frozen sections collected at 8 wk after surgery. A



**Figure 2. CSS with vitamin C synthesize more DNA.** Scoring of BrdU positive keratinocytes at day 14 and day 35 of incubation *in vitro* shows no differences among conditions at incubation day 14. All conditions at day 14 were greater than corresponding conditions at day 35 (\*). At incubation day 35, 0.1 mM vitamin C supports greater incorporation of BrdU than 0.0 mM vitamin C (#).

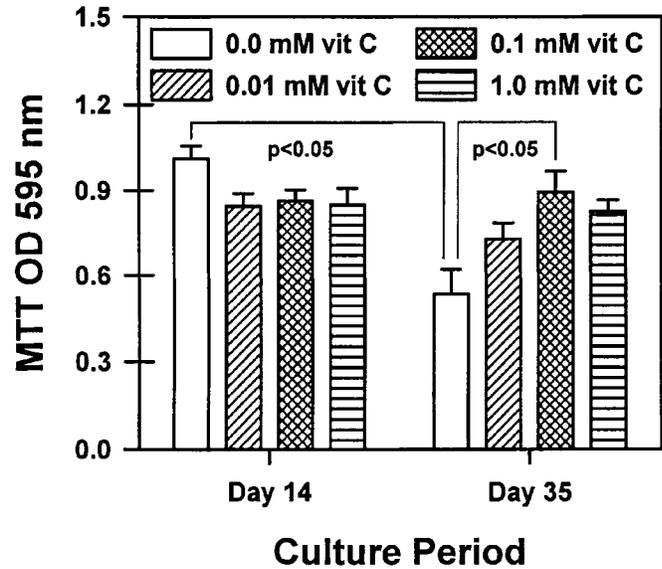
fluorescein-labeled antibody (#MAS1532; Harlan Sera-Lab Ltd.) against a common hapten of the HLA-ABC histocompatibility antigens (Briggaman, 1985; Boyce *et al*, 1991) was used to detect human cells in healed CSS grafts.

**Surface electrical capacitance (SEC)** Capacitance (SEC) represents a measure of skin surface hydration, which is inversely proportional to the electrical impedance (Boyce *et al*, 1996; Supp *et al*, 1999). The NOVA Dermal Phase Meter (DPM 9003; NOVA Technology, Portsmouth, NH) was used to collect SEC data from CSS grafts *in vitro* and from healing CSS on athymic mice. An alternating current applied between two electrodes at the skin surface enables the NOVA meter to detect an electrical phase shift over time. The magnitude of the phase shift increases as water moves through the stratum corneum and accumulates beneath the capacitance probe, with a corresponding increase of the SEC value. Therefore, CSS with a functional barrier yield low SEC values and CSS with a poor barrier result in high SEC readings.

Ten serial readings at 1 s intervals were recorded from each CSS graft *in vitro* ( $n = 3$  per condition) on culture days 7, 12, 14, 19, 21, 26, 28, 32, and 35, and from healing grafts at 2, 3, 4, and 6 wk after surgery. Six SEC measurements were taken from each CSS *in vitro* at each time point (18 SEC values per condition), and three SEC measurements from each healing CSS at each time point (24 or 27 SEC values per group). The SEC data are expressed in picofarads as mean  $\pm$  SEM.

**Measurement of wound contraction** Direct tracings of wound perimeters were made from CSS at the time of grafting (original area) and at 2, 3, 4, 5, 6, and 8 wk after surgery. Image analysis (Image-1; Universal Imaging, Downingtown, PA) of the wound perimeter tracings was performed to determine wound area. Data for wound contraction during CSS healing are expressed as a percentage of the original wound area at the time of grafting as previously described (Boyce *et al*, 1995c, 1997; Supp *et al*, 1999). Data for each group are presented as mean  $\pm$  SEM.

**Statistical analysis** The MTT assay and BrdU incorporation data were analyzed by one-way analysis of variance. The method used for multiple comparisons was Student–Newman–Keuls and significance was established at the 95% confidence level ( $p < 0.05$ ). The SEC and wound contraction data were analyzed using one-between, one-within repeated measures analyses of variance (RM-ANOVA) to determine overall differences between groups. Significant pair-wise differences ( $p < 0.05$ ) were found by univariate ANOVA following RM-ANOVA.



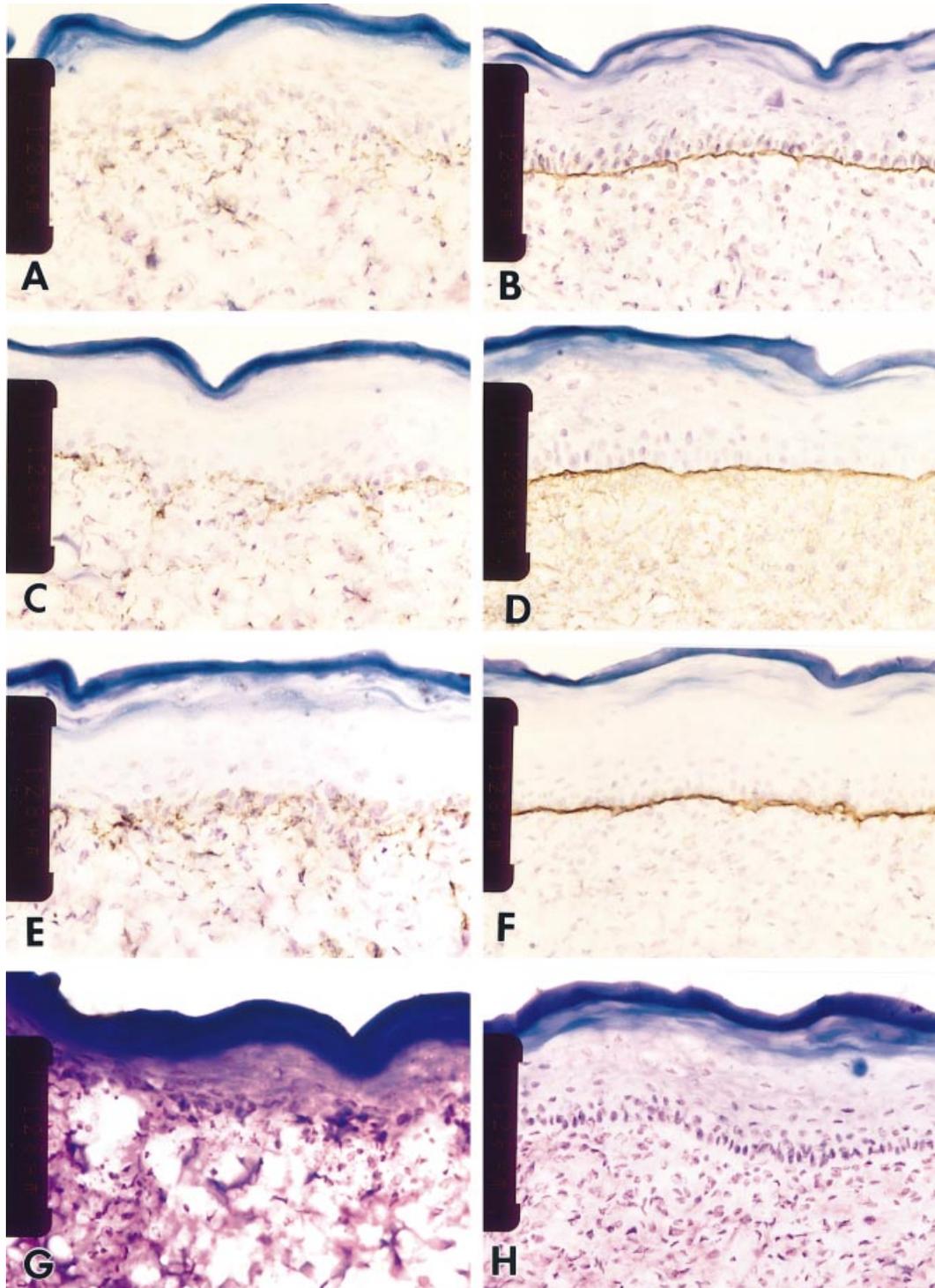
**Figure 3. Vitamin C supports higher levels of MTT conversion in a CSS model on filter membranes at incubation day 35 *in vitro*.** In 0.0 mM vitamin C, a statistically significant decrease of MTT conversion occurs between incubation day 14 and 35, which is reversed by 0.1 mM vitamin C.

## RESULTS

**Vitamin C promotes morphogenesis of epidermal barrier and fibroblast proliferation in CSS** As shown in Fig 1, addition of 0.1 mM vitamin C to serum-free medium stimulates epidermal maturation, and supports fibroblast survival and proliferation. At day 14 of incubation (including 11 d of air exposure), CSS without (Fig 1A) or with (Fig 1B) vitamin C have well-stratified epidermal analogs. The keratinocyte strata are less compacted, however, and the fibroblast population of the dermal substitute is greater in the presence of vitamin C (Fig 1B). Very importantly, TEM of the stratum corneum of the epidermal analog showed sparse and poorly developed lipid lamellae in CSS at day 14 of incubation without vitamin C (Fig 1C), but with vitamin C the characteristic broad-narrow lipid lamellae of epidermal barrier were found (Fig 1D).

**Vitamin C sustains DNA synthesis and mitochondrial metabolism** Measurements of cellular viability *in vitro* demonstrated both vitamin-C-dependent and time-dependent responses of DNA synthesis (BrdU incorporation) and mitochondrial metabolism (MTT conversion). In Fig 2, BrdU-positive keratinocytes in all conditions at incubation day 14 were statistically greater than at day 35, but were not different from each other. Therefore, at incubation day 14, there was no difference in keratinocyte proliferation that was dependent on vitamin C, and a general decrease of DNA synthesis in keratinocytes occurred in all concentrations of vitamin C as a function of time. At incubation day 35, a statistical difference in percentage BrdU-positive keratinocytes was found between 0.0 mM ( $1.34 \pm 0.46$ ) and 0.1 mM ( $7.19 \pm 1.19$ ) vitamin C, but no other significant differences were found. This result demonstrates a concentration-dependent response to vitamin C of DNA synthesis in CSS.

Substantial differences in mitochondrial metabolism were also observed as shown in Fig 3. CSS without vitamin C demonstrated a statistically significant decrease in MTT conversion between day 14 ( $1.01 \pm 0.04$ ) and day 35 ( $0.53 \pm 0.09$ ), and this decrease was reversed by addition of 0.1 mM ( $0.89 \pm 0.07$ ) vitamin C to the medium. No differences were found among any conditions at day 14, or among conditions containing vitamin C at day 35. These results demonstrate that addition of 0.1 mM vitamin C to the

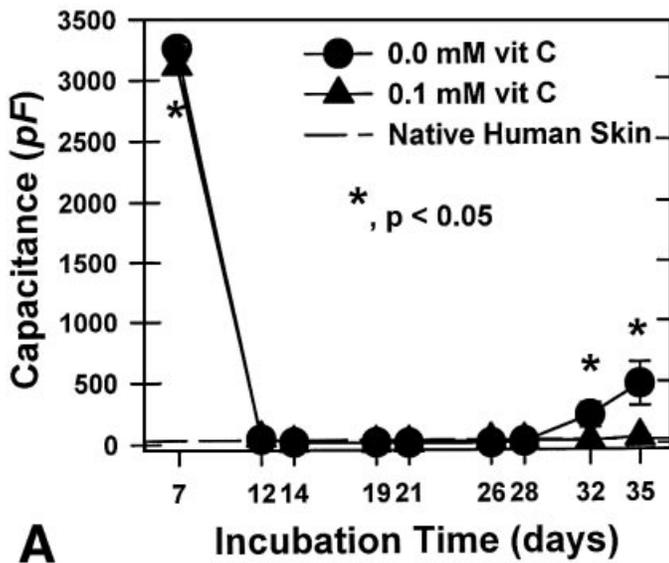


**Figure 4. Basement membrane forms in CSS *in vitro* at incubation day 14.** (A), (C), (E), (G) 0.0 mM vitamin C. (B), (D), (F), (H) 0.1 mM vitamin C. (A), (B) Collagen VII; (C), (D) collagen IV; (E), (F) laminin 5; (G), (H) negative control. Addition of vitamin C to incubation medium supports organization of these basement membrane antigens into linear structures at the dermal-epidermal junction of CSS. Scale bars: 0.128 mm.

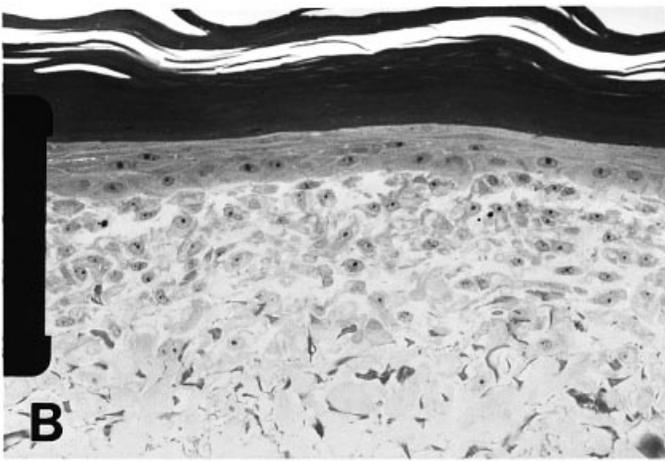
culture medium can maintain basal cellular metabolism for 1 mo or longer.

**Vitamin C promotes development of basement membrane** Expression and organization of basement membrane antigens is greatly stimulated by addition of vitamin C to the medium as shown in Fig 4. In the absence of vitamin C, the same antigens were expressed specifically, but a reticulated pattern of staining extended down from the basement membrane zone into

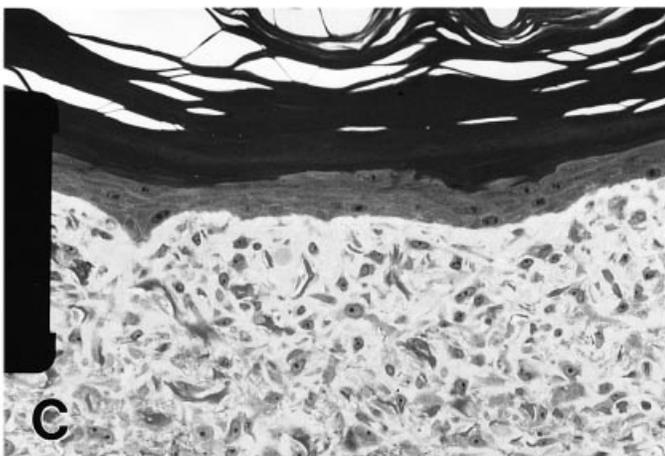
the dermal substitute (Fig 4A, C, E). CSS incubated for 14 d in medium containing 0.1 mM vitamin C showed distinct linear staining of collagen VII (Fig 1B), collagen IV (Fig 1D), and laminin 5 (Fig 1F). No staining was observed in the control samples without (Fig 1G) or with (Fig 1H) vitamin C, which were reacted with a nonspecific monoclonal antibody. Another general observation between the conditions was that addition of vitamin C supported a much denser population of fibroblasts, and a more histiotypic epithelium.



A



B



C

**Figure 5. SEC and microscopic anatomy demonstrate epidermal barrier formation.** (A) Expressed as picofarads (pF), CSS demonstrate statistically lower SEC after incubation in 0.1 mM than in 0.0 mM vitamin C at incubation days 7, 32, and 35 *in vitro*. At day 35 of incubation, MTT was statistically greater in CSS incubated with vitamin C ( $OD_{590} 0.90 \pm 0.06$ ) than without vitamin C ( $OD_{590} 0.46 \pm 0.02$ ). Microscopic anatomy of CSS showed no obvious differences between incubation in vitamin C at 0.0 mM (B) or 0.1 mM (C) concentrations. Scale bars: 0.128 mm.

### Vitamin C maintains epidermal barrier formation and mitochondrial metabolism *in vitro*

Measurement of SEC before grafting is shown in Fig 5(A). Statistically lower SEC was found for 0.1 mM *vs* 0.0 mM vitamin C, and was observed at incubation days 7, 32, and 35 (Fig 5A). These results indicate that vitamin C in the culture medium stimulates more rapid formation of epidermal barrier, and maintains the barrier for a longer period of time. At day 35 of incubation, MTT was statistically greater in CSS incubated with 0.1 mM vitamin C ( $OD_{590} 0.90 \pm 0.06$ ) than without vitamin C ( $OD_{590} 0.46 \pm 0.02$ ). Differences in cellular morphology were more subtle at incubation day 35. In the absence of vitamin C, an analog of stratum corneum forms, and nucleated keratinocytes are attached to the dermal substitute (Fig 5B), but the staining of the samples with Toluidine Blue is pale in comparison to equivalent sections from CSS incubated in vitamin C (Fig 5C). There was also a subjective difference that CSS have greater fibroblast density with vitamin C than without vitamin C.

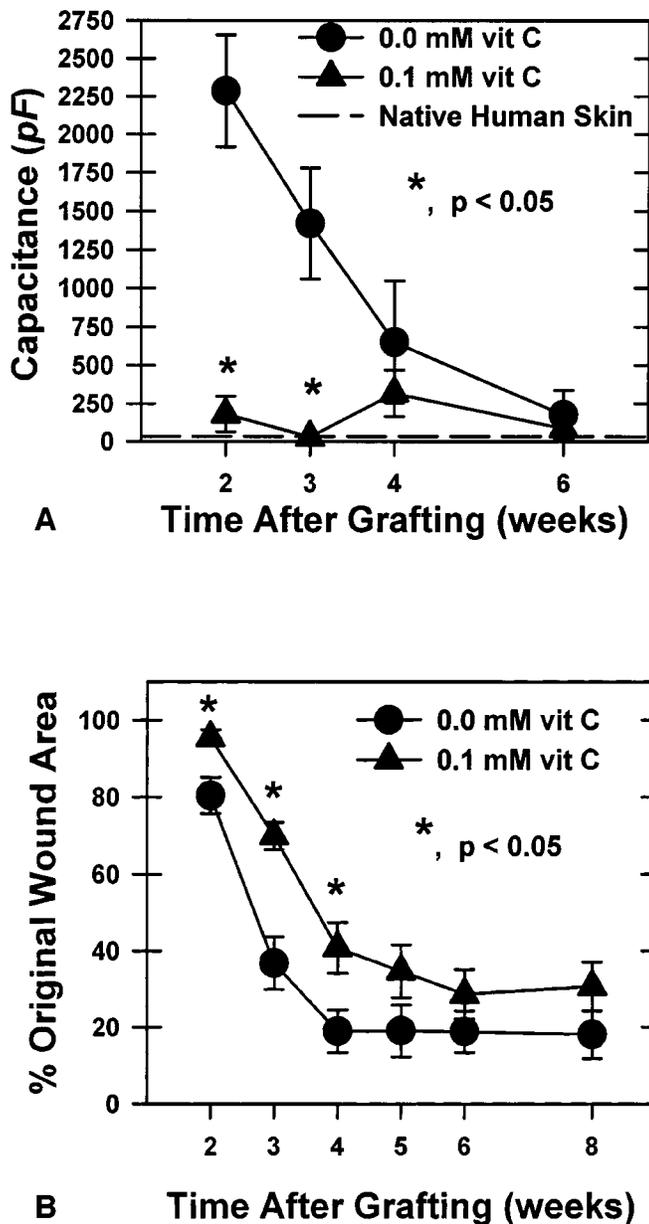
### Vitamin C promotes wound closure and reduces wound contraction

CSS incubated in vitamin C stimulated more rapid restoration of epidermal barrier at 2 or 3 wk after grafting to athymic mice (Fig 6A). At these time points, grafted CSS incubated *in vitro* with vitamin C had SEC values that were not different statistically from uninjured human skin. These results suggest that incubation of CSS in vitamin C promotes formation of epidermal barrier for more rapid wound closure. The benefits of vitamin C on wound contraction are shown in Fig 6(B). Wounds treated with CSS incubated in 0.1 mM vitamin C remained statistically larger between 2 and 4 wk after grafting, demonstrating a delay in wound contraction. Mean wound area was greater at all time points up to 8 wk after grafting of CSS incubated in vitamin C. These responses may be attributed to the greater viability, epidermal barrier, and basement membrane formation in CSS incubated in vitamin C. It was also found that greater numbers of healed wounds stained positively for HLA-ABC after grafting with CSS incubated in 0.1 mM vitamin C (seven out of nine, 78%) than in 0.0 mM vitamin C (four of eight, 50%).

## DISCUSSION

Data reported here demonstrate that addition of vitamin C to incubation media improves the anatomy and physiology of CSS before grafting, and improves wound healing after grafting. Multiple benefits were found, including improvement of epidermal barrier, increased metabolic viability, greater DNA synthesis, and decreased wound contraction during the early phase of healing. The data support the intuitive postulate that improvements in wound healing should result from skin grafts with greater homology to native skin. Perhaps the most important of the physiologic advantages resulting from addition of vitamin C to incubation media is the sustained proliferation of keratinocytes in CSS for periods up to 5 wk. Without keratinocyte proliferation, there would be no expectation of stable wound closure. Results of this study also show early development of basement membrane antigens including collagen IV, collagen VII, and laminin 5. The regulatory actions of these basement membrane proteins on keratinocyte proliferation are well understood (Streuli *et al*, 1995; Lelievre *et al*, 1996), and account in part for the sustained DNA synthesis of keratinocytes by vitamin C that was observed in this study.

Vitamin C supported retention of cellular viability (BrdU, MTT) for up to 5 wk of incubation (Figs 2, 3). DNA synthesis in keratinocytes decreased between days 14 and 35 in all conditions tested, but was significantly greater in 0.1 mM vitamin C than 0.0 mM vitamin C at day 35. This demonstrates that the general rate of keratinocyte proliferation decreases over time, but is maintained at a higher level by vitamin C. The retention of keratinocyte proliferation is believed to relate directly to the efficacy of CSS to engraft and persist after transplantation. Therefore, although the absolute differences between 0.0 and 0.1 mM vitamin C seem small, the retention of a proliferative



**Figure 6. Vitamin C promotes barrier formation and reduces wound contraction during wound healing.** (A) More rapid formation of epidermal barrier and wound closure is demonstrated by statistically lower SEC values for CSS incubated in 0.1 mM than in 0.0 mM vitamin C at 2 and 3 wk after grafting of CSS to athymic mice. (B) Statistically less wound contraction was found at postoperative week 2, 3, and 4 for CSS incubated in 0.1 mM vitamin C than in 0.0 mM vitamin C. Mean values for CSS incubated in 0.1 mM vitamin C remained higher through 8 wk after grafting to athymic mice. Greater numbers of healed wounds stained positively for HLA-ABC after grafting with CSS incubated in 0.1 mM vitamin C (seven out of nine, 78%) than in 0.0 mM vitamin C (four out of eight, 50%).

population of keratinocytes promotes permanent closure of treated wounds. Absolute differences in MTT values were smaller as a function of time for two possible reasons. First, the measurement of MTT included both fibroblasts and keratinocytes, and the fibroblast component may maintain a higher metabolic rate than the epidermal component. Second, MTT measures mitochondrial metabolism, which is an index of cellular viability rather than proliferation. Therefore, the differences in absolute values between BrdU and MTT illustrate the simultaneous decrease in proliferation

rate and retention of metabolic rate in the presence of vitamin C. Both measures, however, recorded statistically greater cellular viability as a function of vitamin C in the medium.

Greater efficacy of wound healing by CSS incubated in vitamin C was measured by more stable formation of epidermal barrier (Fig 5A), and more rapid wound closure and less wound contraction after grafting (Fig 6). These results emphasize the importance of cellular physiology at the time of grafting, and verify the retention of potency of CSS for up to 5 wk as a function of vitamin C. This extension of viability by nutritional regulation translates into greater availability and longer storage of cultured skin grafts for clinical applications in surgery and dermatology.

Nutritional deficiency of vitamin C results in scurvy, which is characterized by cutaneous lesions resulting from dermal and epidermal atrophy (Hirschmann and Raugi, 1999). CSS without vitamin C exhibit metabolic and physiologic symptoms of scurvy including deficient epithelial attachment, reduced development of the connective tissue component, and defective epidermal barrier. In the presence of vitamin C, it was also noted qualitatively after 5 wk incubation that fibroblasts in CSS were much more numerous than without vitamin C. This observation is consistent with other studies that have reported the stimulation of fibroblast proliferation and collagen synthesis by vitamin C (Barnes, 1975; Prockop *et al*, 1979). Although not measured in this study, it may be inferred that synthesis of collagen, carbohydrate-based extracellular matrix (e.g., hyaluronic acid, GAG), and peptide cytokines (e.g., basic fibroblast growth factor, insulin-like growth factors) are also increased by the availability of vitamin C to cells in CSS. The multiple and broad responses of CSS to vitamin C *in vitro* also suggest that it serves as a cofactor in a general biochemical activity (i.e., hydroxylation) for virtually all metabolic pathways in which that activity occurs. The result is much greater vitality and stability of the CSS, and correction of the nutritional deficiency that leads to the symptoms of scurvy. Although scurvy has historically been viewed as a connective tissue disease, it may also be considered that deficiency of vitamin C causes a direct block in the biochemical pathway to the formation of epidermal barrier by keratinocytes.

Earlier studies from this laboratory and others had determined that epidermal barrier in CSS was deficient, but barrier function was restored after grafting to experimental wounds (Boyce *et al*, 1991; Higounenc *et al*, 1994; Vicanova *et al*, 1998) or clinical burns (Gallico *et al*, 1984; Boyce *et al*, 1993b). This observation is also explained, in part, by the availability of vitamin C in wounds of well-nourished recipients. It was also noted, however, that the time required to form functional epidermal barrier and accomplish wound closure was 2 wk or more. Conversely, if functional epidermal barrier forms *in vitro*, then biologic wound closure occurs at the time of grafting and becomes stable after vascularization of the graft. Clinical studies with CSS incubated in vitamin C have shown anecdotally that formation *in vitro* of epidermal barrier and stronger epithelial-mesenchymal attachments in CSS result in formation of functional epidermal barrier within 1 wk after grafting (Boyce *et al*, 1999). Additional studies are planned to verify and quantify those observations.

Continued progress in development of advanced therapies for treatment of full-thickness skin wounds can be expected to result from availability of a laboratory-generated skin graft with homology to healthy human skin. Results of this study suggest that medium conditions in which cellular metabolism approaches normal both qualitatively and quantitatively will contribute to CSS with greater clinical efficacy. Greater availability, stability, and efficacy of CSS may be expected to contribute to reduction of morbidity and mortality from cutaneous wounds.

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