Topical Nutrients Promote Engraftment and Inhibit Wound Contraction of Cultured Skin Substitutes in Athymic Mice

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Routine treatment of burns with cultured skin substitutes (CSS) has been limited by poor engraftment and by scarring. Hypothetically, topical application of essential nutrients and/or growth factors may support epithelial survival temporarily during graft vascularization. CSS, composed of human epidermal keratinocytes and dermal fibroblasts attached to collagen-glycosaminoglycan substrates, were incubated for 19 d in media optimized for keratinocytes. CSS, human xenografts, murine autografts, or no grafts were applied orthotopically to full-thickness skin wounds (2 × 2 cm) in athymic mice. Wounds were irrigated for 14 d with 1 ml/d modified cell culture medium or with saline containing epidermal growth factor, or were treated with dry dressings. After 6 weeks, treated sites were scored for percentage original wound area (mean ± SEM) and percentage HLA-ABC–positive healed wounds [(number positive/n) × 100], and tested for significance (analysis of variance, $p < 0.0001$; Tukey test, $p < 0.05$). The data showed that CSS irrigated with nutrient medium were not statistically different in wound area (67.8 ± 5.1%) from murine autografts (63.3 ± 2.9%) but were statistically larger than human xenograft, no graft, or CSS treated with saline irrigation or dry dressings. HLA-ABC expression was 100% in CSS with nutrient irrigation, 86% in CSS with saline irrigation, 83% in CSS without irrigation, and 75% in xenografts with nutrient irrigation. These findings suggest that availability of essential nutrients supports keratinocyte viability during graft vascularization of CSS. ***J Invest Dermatol 104:345–349, 1995***

Models of cultured skin [1–3] have been developed and proposed for treatment of full-thickness skin loss conditions including burns, congenital giant nevus, reconstructive surgery, and chronic wounds [4–9]. By definition, autologous epithelium must be restored on wounds to accomplish permanent skin healing. This requirement is met by cultured epithelial autografts [10,11]. To restore the dermal component of skin, dermal substitutes have been combined with cultured keratinocytes [12]. However, none of these models has displaced split-thickness skin autograft as the preferred treatment for full-thickness wounds [13,14], because none compares favorably with skin autograft in functional or cosmetic outcome.* Common deficiencies of these materials include, but are not limited to, absence of a vascular plexus, highly variable rates of engraftment [15], debilitating wound contracture [16], chronic ulceration of epithelialized areas [17], and hypopigmentation [18].

Previous reports have described a composite skin substitute consisting of cultured human epidermal keratinocytes attached to an implantable collagen-glycosaminoglycan substrate populated with dermal fibroblasts [5,18,19]. Preclinical studies have demonstrated that 1) the composition of collagen-glycosaminoglycan substrates may be controlled within narrow limits by biophysical processes [20]; 2) cellular proliferation and differentiation are regulated by incubation conditions [3,19–21]†; and 3) engraftment of cultured skin with melanocytes in athymic mice regenerates human skin with full pigmentation and inhibits wound contraction [22]. Preclinical and clinical reports have provided direct qualitative evidence of complete healing of excised burns with regeneration of epidermal and connective tissues [6,18]. This report presents quantitative data demonstrating that application of topical nutrients promotes complete engraftment of skin substitutes on full-thickness wounds in athymic mice. These findings suggest that irrigation of cultured skin grafts with topical nutrients, in combination with appropriate antimicrobial agents, may promote engraftment of cultured skin and regeneration of epithelial and connective tissue for closure of full-thickness skin wounds.

MATERIALS AND METHODS

Experimental Design To test whether topical nutrients plus growth factors enhance engraftment of cultured skin, cell-biopolymer composites were prepared under constant conditions and grafted to full-thickness wounds in athymic mice. After grafting, wounds were irrigated for 14 d with keratinocyte growth medium (see below) supplemented with 5 μg/ml bovine insulin plus 10 ng/ml epidermal growth factor (EGF). Experimental grafts were compared directly with murine autografts (n = 7), human xenografts (n = 8), or no grafts (n = 7), and scored for percentage original wound area and percentage human leukocyte antigens (HLA)-ABC–posi-

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Abbreviation: CSS, cultured skin substitute(s).

* Warden GD: Presidential Address at the 25th Annual Meeting of the American Burn Association, 1993, Cincinnati, Ohio.

† Boyce ST, Hoath SB, Wickett RR, Harriger MD, Williams ML: Loss of requirement for exogenous epidermal growth factor by cultured analogues of normal human skin. (abstr) *J Invest Dermatol* 9:579, 1993.

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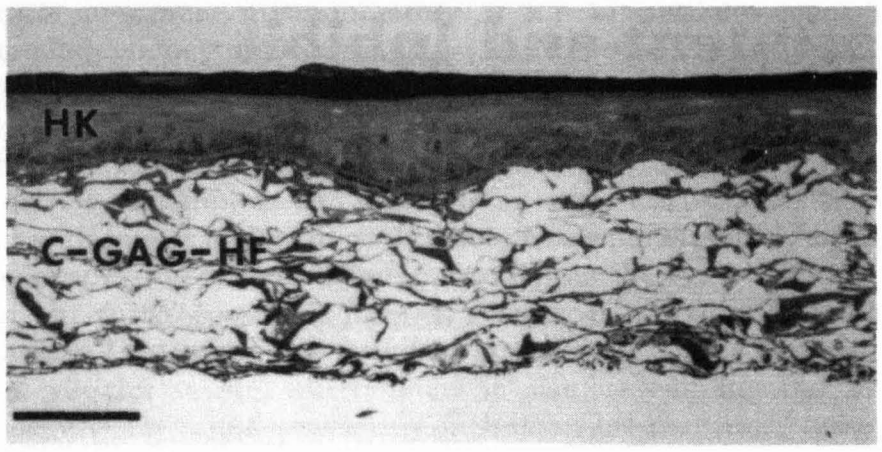

Figure 1. Histology of CSS. Cultured human keratinocytes (HK) form a uniformly stratified epithelium that includes cornified layers analogous to stratum corneum. Keratinocytes are attached to a collagen-glycosaminoglycan substrate that is populated with cultured human fibroblasts (C-GAG-HF). *Bar*, 0.1 mm.

tive wounds (cultured grafts and xenografts). Experimental irrigations with nutrients and antimicrobial agents were compared directly to saline with 10 ng/ml EGF and antimicrobial agents, and to dry dressings consisting of N-Terface (Winfield Laboratories, Richardson, TX) covered with cotton gauze and OpSite as controls.

Cultured Skin Substitutes (CSS) CSS were prepared as described in previous studies [6,19,20,22] from collagen-glycosaminoglycan substrates populated sequentially with human dermal fibroblasts and epidermal keratinocytes. Beginning with inoculation of cultured keratinocytes at day 0 of incubation, cultured cell-biopolymer composites were incubated for 2 d submerged in MCDB 153 medium [21] containing 0.2 mM calcium. On culture day 3, the medium was changed to replace bovine pituitary extract with a lipid supplement plus carnitine, increased serine [23], and calcium at 0.5 mM. On culture day 4, constructs were lifted to the air-liquid interface on stainless-steel supports, EGF was decreased to 1 ng/ml, and calcium was increased to 1.0 mM. On culture d 7, calcium was increased to 1.5 mM, and grafts were incubated in these conditions with daily medium changes until 19 d, at which time grafting to athymic mice was performed.

Grafting to Athymic Mice and Irrigation All animal studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Grafting to athymic mice was performed as described in previous studies [19,22]. The entire wound area was covered with a semipermeable adhesive film (OpSite; Smith & Nephew United, Largo, FL) to produce a liquid-tight compartment over the wound. Immediately after surgery, 1.5 ml of sterile keratinocyte growth medium containing antimicrobials (Norfloxacin, 20 μg/ml, and Nystatin, 100 U/ml) was injected through the Opsite into the cotton gauze. Constant conditions for irrigation media included 0.5 mM calcium, 10 ng/ml EGF, 5.0 μg/ml bovine insulin, and 0.5 μg/ml hydrocortisone. CSS, autografts, xenografts, and no-graft conditions were irrigated. Dressed grafts were then covered with a self-adherent bandage (Coban; 3M Medical Division, St. Paul, MN) to protect the treated sites from mechanical disturbance. Dressings covering the treated sites were injected with 1.0 ml/d of irrigants as described above for 13 d post-grafting, except for dry dressings, which received no irrigation. At 14 d after grafting, dressings and stent sutures were removed from all animals. Mice were rebandaged on day 14 and day 28 after grafting. On day 42, all dressings were removed, the animals were photographed, and the wounds were traced. The mice were then sacrificed and tissue samples were collected for microscopy.

Data Collection and Analysis Data for wound contraction are expressed as percentage of original wound area (mean $\pm$ SEM) [19,22]. Data from multiple test groups in wound-contraction studies were subjected to analysis of variance ($p < 0.0001$) and Tukey studentized range test, with significance accepted at the 95% confidence level ($p < 0.05$). Graft acceptance was determined by direct immunofluorescence staining of healed epidermis with fluorescein-labeled monoclonal antibody against a common hapten of the HLA-ABC histocompatibility antigens [24]. Data for graft

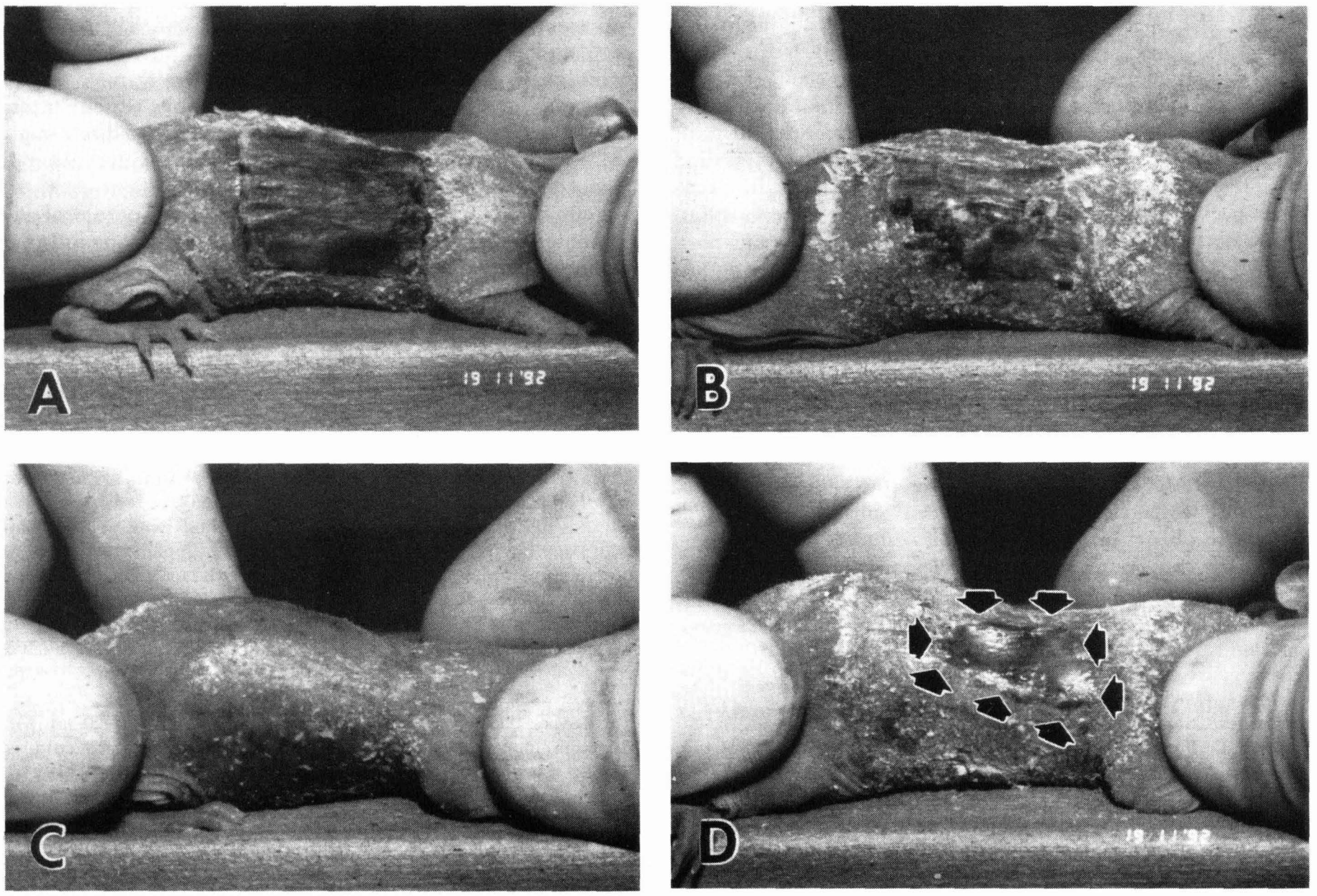

Figure 2. External appearance of athymic mice after treatment. *A*) Composite cultured skin; *B*) human xenograft; *C*) murine autograft; *D*) no graft. All grafts were irrigated for 14 d with nutrient medium containing 10 ng/ml EGF. Wounds treated with no graft (*D*) remained open at 6 weeks (*arrows*).

acceptance are expressed as percentage of HLA-ABC–positive wounds in each test group. Microscopic analyses were performed using standard methods.

RESULTS

CSS Histology The histologic appearance of composite cultured skin is shown in **Fig 1**. Incubation of CSS in lipid-supplemented medium with air exposure for 15 d promoted development of a well-stratified epithelium with distinct external layers analogous to stratum corneum. Fibroblasts sparsely populated the collagen-glycosaminoglycan substrate, and the total graft thickness was approximately 0.3 mm. Although the biopolymer substrate was thin, it was avascular and separated the epithelium from the wound after grafting.

Wound Closure in Athymic Mice **Figure 2** shows the external appearance of representative mice from control and experimental treatment conditions at 6 weeks after grafting. CSS irrigated with medium containing EGF **(Fig 2*A*)** were epithelialized completely and were hyperkeratotic, which is characteristic of human skin on athymic mice. Similarly, human xenograft **(Fig 2*B*)** was hyperkeratotic and remained incompletely healed at 6 weeks. Incomplete healing of human xenograft was associated with acantholysis of epidermis during the irrigation period, which may be attributed to an insufficient concentration (0.5 mM) of calcium in the irrigation medium. Murine autograft **(Fig 2*C*)** was fully healed and fused to the wound margin. Treatments with no graft and irrigation medium for 14 d showed greater wound contraction than grafted animals. Some wounds in this group were not closed at 6 weeks after grafting **(Fig 2*D*)**.

HLA-ABC Expression and Biopolymer Degradation in Healed CSS Closure of wounds with cultured human epithelium was confirmed by direct immunofluorescence staining of cryostat sections with fluorescein-labeled monoclonal antibody against a common hapten of the HLA-ABC antigens. Persistence of cultured epithelium **(Fig 3*A*)** was confirmed by net-like staining of the plasma membranes of nucleated keratinocytes. Also noteworthy in this experiment was the persistence of implanted collagen at 6 weeks, which stained nonspecifically and showed a reticulated pattern below the regenerated epidermis. Light micrographs of skin regenerated from cultured cell-biopolymer grafts at 6 weeks also showed remnants of implanted collagen-glycosaminoglycan. **Figure 3*B*** shows fully keratinized epidermis attached to regenerating connective tissue that contains implanted collagen, fibroblasts, a neovascular plexus, and a mild inflammatory infiltrate. At higher magnification in **Fig 3*C***, reticulations of the implanted biopolymer are clearly visible, and are associated with phagocytes consisting predominantly of macrophages.

Inhibition of Wound Contraction by Topical Nutrients Measurements of wound area at 6 weeks after grafting are presented in **Figs 4 and 5**. **Figure 4** shows that graft areas of cultured skin (67.8 ± 5.1%) were not different statistically from murine autografts (63.3 ± 2.9%) but were statistically different from areas of human xenografts (41.8 ± 3.9%) and treatment with no graft (21.8 ± 2.6%). Areas **(Fig 5)** of CSS irrigated with nutrients (45.8 ± 4.3%) were statistically larger than those with saline irrigation (23.1 ± 2.5%) or dry dressings (28.0 ± 3.3%).

Topical Nutrients Promote Engraftment of CSS Analysis of HLA-ABC expression at 6 weeks in cultured grafts and human xenografts is shown in **Fig 6**. Engraftment of CSS occurred in 100% of treated wounds that were irrigated with nutrients, as demonstrated by positive expression of HLA-ABC. Engraftment of CSS was not supported in all animals treated with saline irrigation or dry dressings: 86% (six of seven) and 83% (five of six), respectively. Positive staining for HLA-ABC was detected in 75% (six of eight) of the wounds treated with human xenograft. This relatively low value for engraftment of human skin correlates with significantly reduced wound area and was associated with epidermal acantholysis at the dressing change at 14 d. Acantholysis was attributed to a low calcium concentration in the irrigation medium.

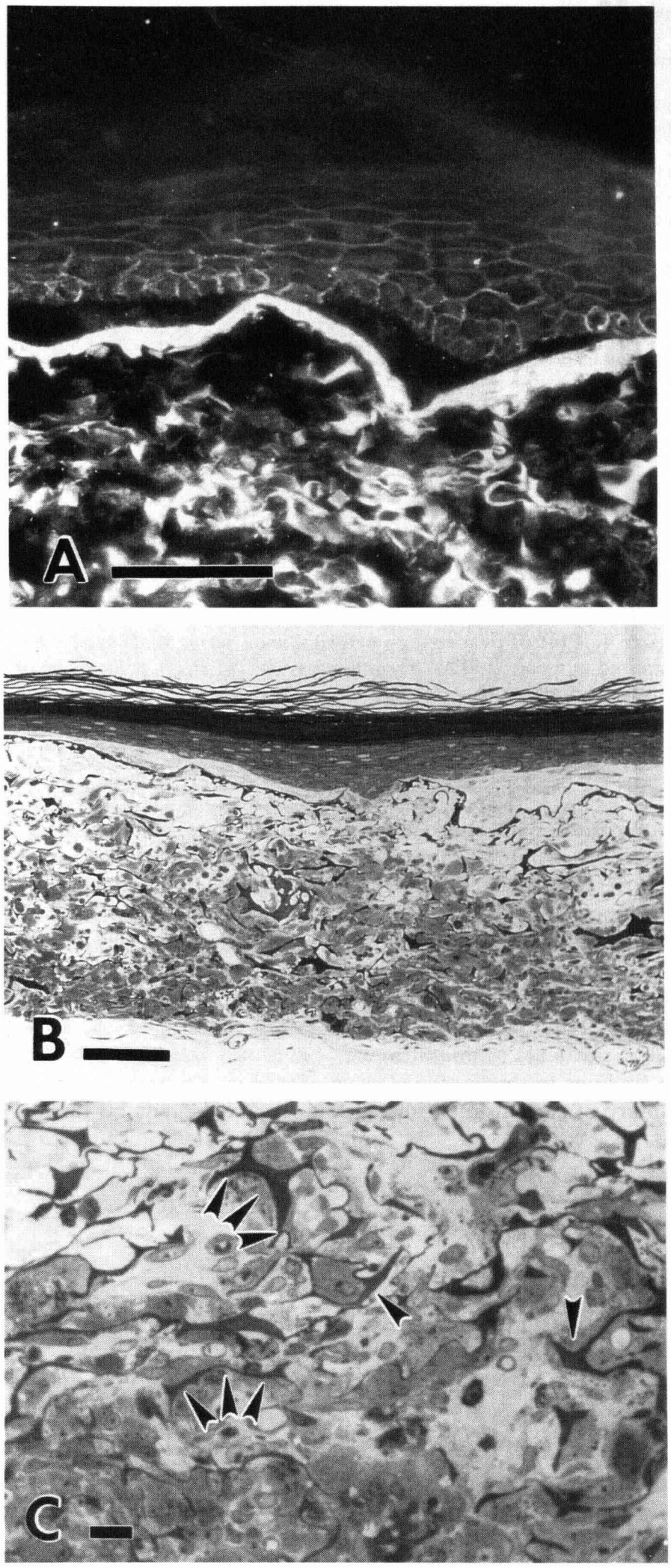

Figure 3. Histologic examination of healed skin after treatment with composite cultured skin. *A*) Staining of HLA-ABC antigens on plasma membranes of nucleated keratinocytes; *B*) light micrograph of excised skin showing keratinized epidermis and remnants of implanted collagen; and *C*) remnants of implanted collagen (*arrowheads*) in regenerating connective tissue. *Bars*: *A*, B, 0.1 mm; *C*, 10 μm.

DISCUSSION

The data presented here demonstrate that treatment of full-thickness wounds with grafts of cultured skin cells and biopolymers, in

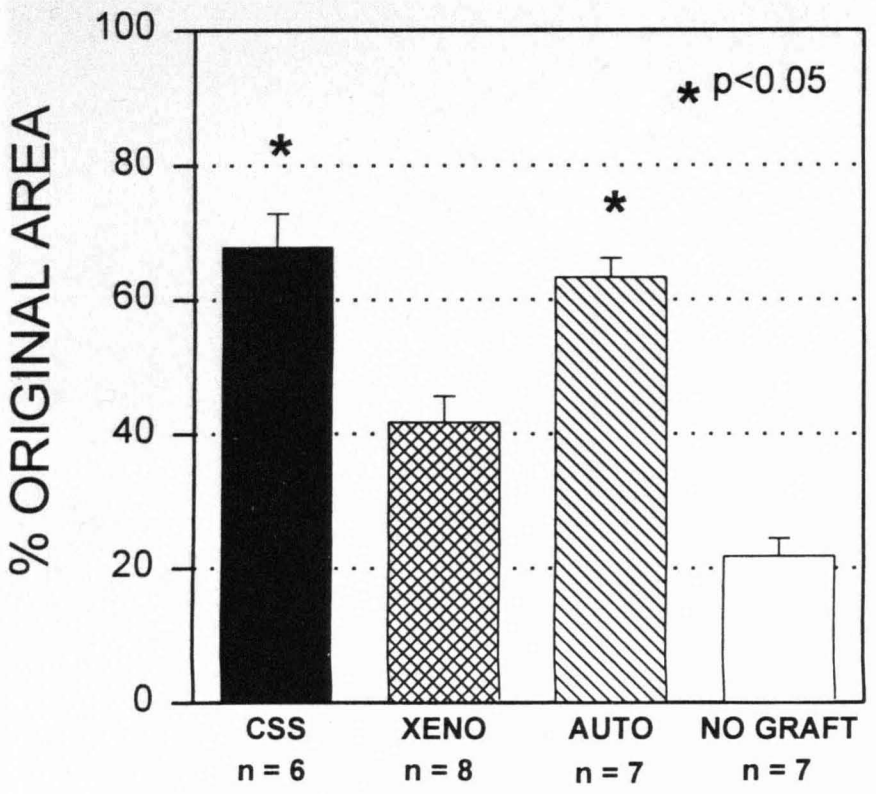

Figure 4. Plot of percentage original area *versus* graft type. Data are expressed as mean ± SEM. Treatments with CSS (67.8 ± 5.1%) were not statistically different from murine autografts (auto; 63.3 ± 2.9%) in wound area at 6 weeks after grafting. Human xenografts (xeno; 41.8 ± 3.9%) and no grafts (21.8 ± 2.6%) were significantly smaller in area.

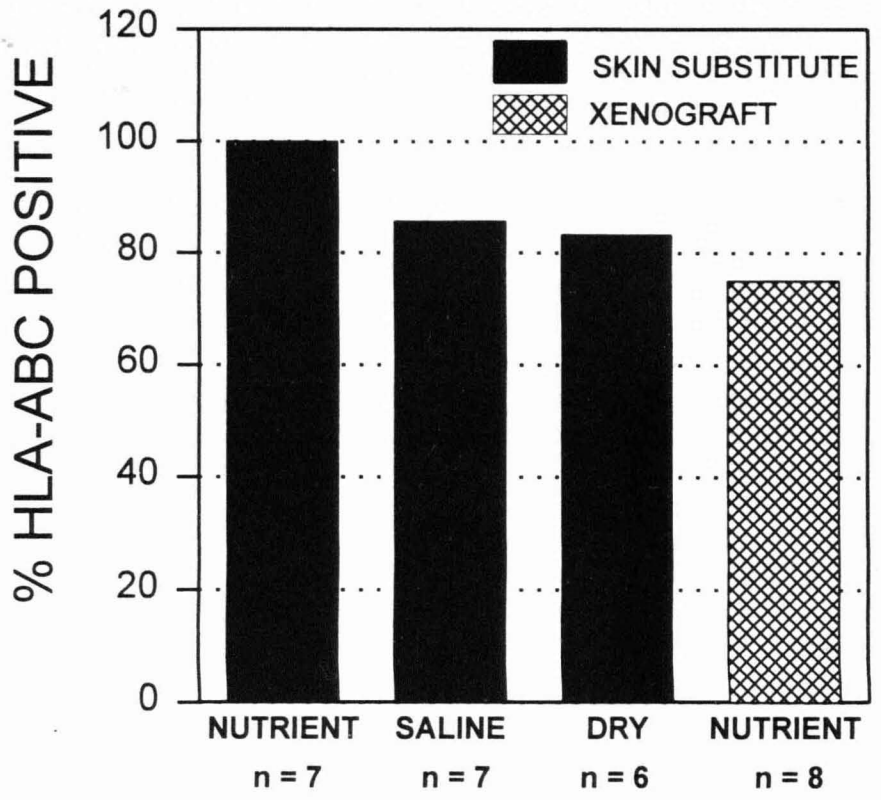

Figure 6. Plot of percentage HLA-ABC–positive wounds *versus* treatment condition. All skin substitutes treated with nutrient irrigation (n = 7) were positive for HLA-ABC expression in healed epidermis. Treatment with saline containing EGF (10 ng/ml) and antimicrobials promoted HLA expression in 86% of wounds (six of seven), and dry dressings covered with Op-Site supported development of human epithelium in 83% of subjects (five of six). Nutrient irrigation of human xenograft showed HLA-ABC expression in 75% (six of eight) of treated wounds.

combination with short-term irrigations of topical nutrients, accomplishes wound closure. Topical nutrients may serve two main actions, one direct and one indirect. Direct action promotes keratinocyte survival and viability by nutritional support. Indirect action may result from the release of angiogenic compounds by viable keratinocytes, which stimulate and accelerate fibrovascular ingrowth to provide an indefinite supply of essential nutrients and other native metabolites. These interpretations of the roles of topical nutrients in engraftment of cultured skin are supported by the findings in this study. However, it must also be noted that the viability of composite cultured skin depends heavily on incubation

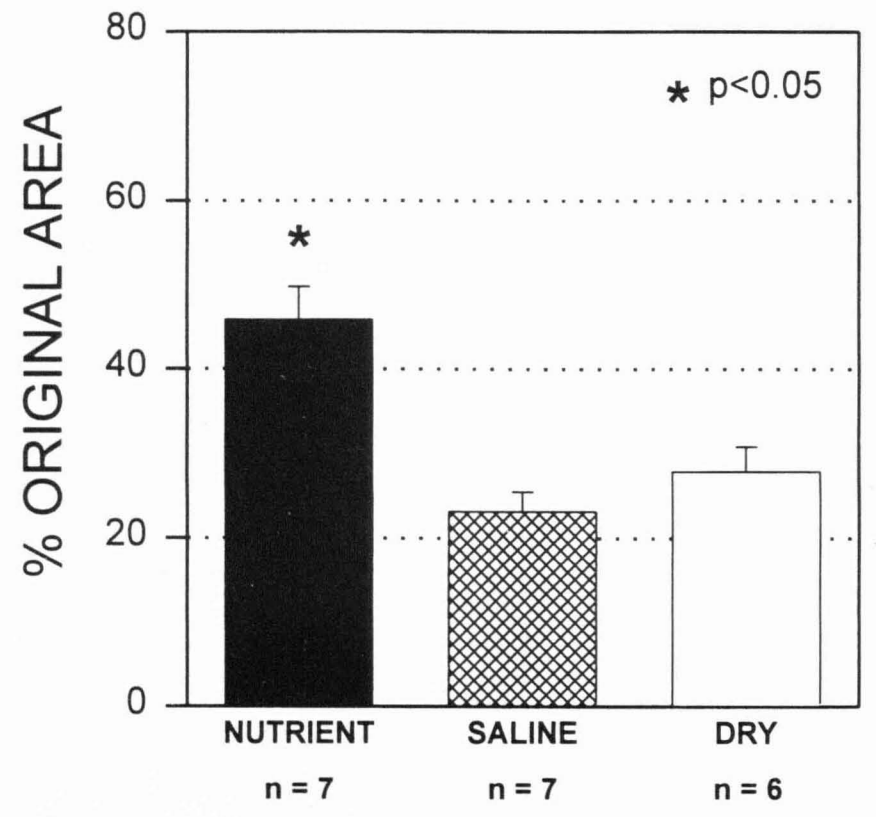

Figure 5. Plot of percentage original area *versus* dressing condition. Data are expressed as mean ± SEM. Wounds treated with CSS and nutrient irrigation (nutrient; 45.8 ± 4.3%) were significantly larger in area than CSS irrigated with saline containing EGF (10 ng/ml) and antimicrobials (saline; 23.1 ± 2.5%) or CSS that received dry dressings covered with Op-Site (dry; 28.0 ± 3.3%).

media to promote and maintain a balance of proliferation and differentiation in the graft at the time of surgery [3,19,21,22].

Production of a wide variety of cytokines, attachment factors, and cellular antigens by epidermal keratinocytes is well documented [25,26], as is specific expression of individual factors in response to specific molecular mediators. Examples include expression of HLA-DR in response to interferon-γ; release of interleukins-1, -3, -6, -7, and -8 in response to injury; and expression of keratins in response to transforming growth factor-β.‡ In addition, cultured keratinocytes are known to express many cytokines constitutively during active replication [27]. Therefore, it is not surprising that simple topical application of factors has failed to duplicate completely the complex and highly regulated metabolism of keratinocytes during wound healing. Conversely, persistence of grafted keratinocytes may provide regulated delivery of many factors needed for wound healing, but requires survival of epithelial cells during the development of a neovascular plexus. Engraftment of CSS in this study suggests that application of topical nutrients satisfies this requirement fully.

Grafting of CSS onto subcutaneous fat has produced very poor results [4,6,15,18] because of the limited vascular supply in fat. Poor vascular supply can result in nutrient deprivation of cultured grafts for 5 d or more, allowing transplanted cells to die and/or be destroyed by microbial contamination. Based on the results of this study, we predict that temporary use of topical nutrients can provide dramatic improvement in engraftment of both composite cultured skin and keratinocyte sheets on poorly vascularized wounds. In combination with effective antimicrobial formulations that are nontoxic to cultured keratinocytes, topical nutrients reduce the stringency for survival of grafted keratinocytes on acute traumatic wounds such as burns. However, wounds with an etiology of associated vascular insufficiency (i.e., venous stasis ulcers) may not benefit from increased short-term survival of cultured skin grafts. A reduced stringency for engraftment of cultured skin on responsive

‡ Jiang C-K, Freedberg IM, Blumenberg M: Regulation of keratin gene expression by TGF-β (abstr). *J Invest Dermatol* 100:502, 1993.

or "normal" wounds [28] may help achieve the goal of a dermal-epidermal skin substitute that may be grafted to debrided wounds in a single procedure. Attainment of this goal would represent an important advance toward reducing morbidity from acute or chronic full-thickness skin loss conditions.

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ANNOUNCEMENT

An International Sumposium "Mast Cells and Cellular Interactions" will be held in Berlin, Germany, September 15–16, 1995, organised by the Department of Dermatology, University Hospital Rudolf Virchow, Freie Universität, Berlin. The organising committee is B. M. Czarnetzki, J. Grabbe, G. Kolde, S. Krüger-Krasagakes, R. Paus, and T. Zuberbier.

The symposium will consist of a Preliminary Program, followed by overview lectures in Mast Cells and Nerves (J. Foreman, London; C. T. Theoharides, Philadelphia; J. Bienenstock, Hamilton), Mast Cells and Fibroblasts (S. Galli, Boston; L. B. Schwartz, Richmond; L. Ashmann, Adelaide), Mast Cells, Endothelial and Inflammatory Cells (G. Murphy, Philadelphia; P. Askenase, New Haven; G. Marone, Napoli), and Mast Cells and Wound Healing/Hair Growth (H. Claman, Denver; R. Paus, Berlin; K. Norrby, Göteborg), and a Round Table Discussion, Novel Perspectives—Mast Cells and Tissue Homeostasis (M. Church, Southampton; B. M. Czarnetzki, Berlin, S. Galli, Boston; T. C. Theoharides, Philadelphia).

The abstract deadline is 1 May 1995. For further details and abstract forms, please contact Frau Fuchs, Department of Dermatology, UKRV, FU Berlin, D-13353 Berlin. Telephone, +(0)30-450-65042; fax, +(0)30-450-65900.