Pigmentation and Inhibition of Wound Contraction by Cultured Skin Substitutes with Adult Melanocytes After Transplantation to Athymic Mice

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Wound closure with cultured skin substitutes results in epithelium that is consistently hypopigmented. Hypothetically, addition of human melanocytes to cultured skin grafts may result in normal pigmentation of healed skin. Skin substitutes were composed of human epidermal keratinocytes and melanocytes, dermal fibroblasts, and collagen-glycosaminoglycan substrates, and were incubated for 12 d in media for keratinocyte growth (KG, n = 4), for keratinocyte differentiation containing four fatty acids and vitamin E with basic fibroblast growth factor (KDF, n = 6) or epidermal growth factor (KDE, n = 6), or for melanocyte growth (MG, n = 6) with phorbol ester and 5% fetal bovine serum. Skin substitutes were grafted orthotopically to full-thickness skin wounds (2 × 2 cm) on athymic mice, and scored for percent original wound size (± SEM), visible pigmentation (number pigmented/n), and positive staining for human leukocyte antigens (HLA)-ABC after 6 weeks on the mice.

The data show that cultured skin grafts containing human melanocytes that are incubated in KDE or MG media have statistically significant reduction in wound contraction, 1:1 correlation of expression of pigment and HLA-ABC, and increased frequency of pigmentation after healing compared to incubation in KG or KDF media. Transmission electron microscopy confirmed the presence of melanocytes, melanosomes, and pigment transfer to keratinocytes in pigmented skin. These results suggest that survival and differentiated function of cultured epithelium can support melanization of skin, and that skin analogues exposed to phorbol ester *in vitro* can support skin pigmentation after wound healing. *J Invest Dermatol 100:360–365, 1993*

Cultured analogues of human skin have contributed greatly to the understanding of skin biology, pathology, and toxicology, and to treatment of skin wounds including ulcers and burns [1–3]. It has been noted that selective cultures of epidermal keratinocytes often contain small populations of melanocytes that retain the capacity to regenerate pigment after transplantation [4,5]. However, in skin wound treatment, application of cultured epithelium as keratinocyte sheets [6] or composite grafts [7] results in regeneration of markedly hypopigmented skin with associated deficiencies of cosmesis and solar protection. Hypothetically, repopulation of melanocytes into cultured skin grafts would correct these deficiencies. Certain depigmentation disorders, such as piebaldism, have been treated by transplantation of cultured human melanocytes [8].

This study tested whether the addition *in vitro* of cultured human adult melanocytes to cultured skin grafts could form pigmented human skin after transplantation to full-thickness wounds on athymic mice. Cultured human melanocytes were added to composite grafts of cultured human keratinocytes and fibroblasts attached biologically to an implantable collagen-glycosaminoglycan substrate [3,9] by simultaneous inoculation with kertinocytes. Culture of skin analogues containing three cell types raised the question in which medium formulation the composites should be incubated. Four medium formulations were tested that promote 1) keratinocyte growth (KG), 2) keratinocyte differentiation supplemented with basic fibroblast growth factor (KDF), 3) keratinocyte differentiation supplemented with epidermal growth factor (KDE), or 4) melanocyte growth (MG). These formulations were chosen for testing based on studies *in vitro* that have shown either selective promotion of cell division or differentiated function of human epidermal keratinocytes or melanocytes. KG medium contains bovine pituitary extract and EGF that promotes rapid cell division in keratinocytes [10,11]. KDE and KDF media are supplemented with four fatty acids (arachidonic, linoleic, oleic, palmitic) and vitamin E, which promote kerat-

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Abbreviations:
- bFGF: basic fibroblast growth factor
- C-GAG: collagen-glycosaminoglycan
- CSS: cultured skin substitute
- DEJ: dermal-epidermal junction
- EGF: epidermal growth factor
- HF: human fibroblasts
- HK: human keratinocytes
- HLA-ABC: human leukocyte antigens of A, B, and C types
- HM: human melanocytes
- KDE: keratinocyte differentiation (medium) with EGF
- KDF: keratinocyte differentiation (medium) with bFGF
- KG: keratinocyte growth (medium)
- MG: melanocyte growth (medium)
- TPA: tetradecanoyl phorbol acetate

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Table I. Incubation Media[a]

Supplements[b]	Medium Composition KG	KDF	KDE	MG
Calcium (mM)	0.2	0.5	0.5	0.2
FBS (% v/v)	0	0	0	5.0
BPE (% v/v)	0.5	0	0	0.2
BSA (mg/ml)	0	1.5	1.5	0
EGF (ng/ml)	1.0	0	10	0
bFGF (ng/ml)	0	10	0	0.3
TPA (ng/ml)	0	0	0	10
Insulin (μg/ml)	5.0	5.0	5.0	5.0
Hydrocortisone (μg/ml)	0.5	0.5	0.5	0
Transferrin (μg/ml)	0	0	0	5.0
Vitamin E (μg/ml)	0	10	10	0
Fatty acids (μg/ml)	0	20	20	0
Carnitine (μg/ml)	0	1.6	1.6	0

[a] Basal medium: MCDB 153.
[b] FBS, fetal bovine serum; BPE, bovine pituitary extract; BSA, bovine serum albumin.

inocyte differentiation.* MG medium contains tetradecanoyl phorbol acetate, which is toxic to keratinocytes and stimulates melanocyte proliferation [12].

Skin analogues incubated in each medium were grafted to full-thickness, excised skin wounds on athymic mice [5]. After 6 weeks, wounds treated with grafts from KDE or MG media showed complete pigmentation and significant inhibition of wound contraction ($p = 0.03$) compared to grafts cultured in KG or KDF media. These results demonstrate restoration of uniform pigmentation of wounds healed with composite grafts of cultured skin cells and biopolymers, and importance of incubation conditions on efficacy of transplanted cells for wound closure.

MATERIALS AND METHODS

Experimental Procedures All animal studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Cultured skin substitutes were grafted to full-thickness skin wounds on athymic mice (BALB/c, nu/nu). Mice were anesthetized with 0.3 ml Avertin (tribromoethanol in tertiary amyl-alcohol) and 2 × 2 cm full-thickness skin wounds were prepared leaving the panniculus carnosus intact [13,14]. Cultured skin substitutes were administered; non-adherent wound dressing (N-Terface, Winfield Laboratories, Richardson, TX) was placed over the grafts, and eight stent-type sutures secured the grafts and N-Terface to the wound margins and wound bed. Grafts were dressed with cotton gauze, and sutures were tied over the gauze to hold the dressings in place [5]. Wound perimeters were coated with sterile benzoin tincture (3M Health Care; St. Paul, MN), which was allowed to dry (30 seconds) before covering the entire wound area with a semi-permeable adhesive film (OpSite, Smith & Nephew United, Largo, FL). This produced a liquid-tight compartment over the wound into which 1.5 ml of sterile keratinocyte growth medium (KG in Table I) containing antimicrobials [Norfloxacin (20 μg/ml) + Nystatin (100 U/ml)] was injected through the Opsite into the cotton gauze immediately following surgery. Dressed grafts were then covered with a self-adherent bandage (Coban, 3M Medical Division, St. Paul, MN) to protect treated sites from mechanical disturbance. Treated sites received 1.0 ml KG medium with antimicrobials daily for 13 d post-grafting. On day 14 post-grafting, dressings and stent sutures were removed from all animals and wound-size data were collected. Mice were re-bandaged from days 14–27 with a dressing consisting of N-Terface, Xeroform (Sherwood Laboratories, St. Louis, MO), cotton gauze, and Coban. Dressings from days 28–41 post-grafting consisted of N-Terface, cotton gauze, and Coban. On day 42, all animals were removed from their dressings and photographed, and wounds were traced; most animals were sacrificed and tissue samples were collected for microscopy. Wound tracings and photographs were performed at 2, 3, 4, 5, and 6 weeks after surgery. Selected animals were not sacrificed, and were photographed at later time points (i.e., 10 weeks and 6 months).

Experimental Conditions and Preparation of Skin Substitutes Cultured skin substitutes (CSS) were prepared from separate cultures of HK, HM, and HF combined on 10 × 10 cm acellular collagen-glycosaminoglycan (C-GAG) substrates as previously described [5,15] with minor modifications. HF were inoculated onto the porous side of two substrates at 5×10^5 cells/cm^2 in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Human adult melanocytes were pooled from multiple donors, cultured as described by Medrano and Nordlund [12], and prepared as suspensions by standard trypsinization. Suspensions of melanocytes and keratinocytes [10] were mixed at a ratio of 1:30, and inoculated at 1×10^6 cells/cm^2 onto the non-porous side of C-GAG substrates. Composite grafts were incubated 2 d submerged in keratinocyte growth medium, cut into quarters 4.2 × 4.2 cm each, randomized into the four test media, and incubated 2 d submerged in the four experimental media described in Table I. On day 4, all grafts were lifted to the air-liquid interface and incubated in the four media for 15 d with daily medium changes. Media consisted of a) serum-free MCDB 153 containing 0.5% (v/v) bovine pituitary extract (KG) [11,16]; b) lipid-supplemented medium* containing 10 ng/ml recombinant human basic fibroblast growth factor (KDF); c) lipid-supplemented medium containing 10 ng/ml recombinant human epidermal growth factor (KDE); and d) melanocyte growth medium (MG). All four media conditions were supplemented with 10 ng/ml bFGF on days 11–15 of lifted culture.

Data Collection and Analysis Direct tracings of wound perimeters onto frosted mylar and photography were performed at weekly intervals, from weeks 2–6 after grafting, for measurement of wound contraction, and examination of pigmentation. Wound contraction was measured by direct image analysis (Image-1, Universal Imaging Corporation, Media, PA) of the tracings of wound perimeters. Data for wound contraction are expressed as percentage of original wound size (mean ± SEM) [5]. Data from multiple test groups in wound contraction studies were subjected to Tukey's studentized range test with significance accepted at the 95% confidence level ($p < 0.05$) and analysis of variance. Data for pigmentation are expressed as a percentage of the total number of animals in each test group that expressed visible pigment in the grafted region by 6 weeks after surgery. 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 (Accurate Chemical and Scientific Corporation, Westbury, NY) [17]. Tissue samples used for immunohistochemical staining were prepared from cryostat sections of unfixed, fresh-frozen excised skin from wound sites. Data for graft acceptance are expressed as percentage of HLA-ABC positive wounds in each test group. Correlation between the percent pigmented and the percent HLA-ABC positive for each test group was determined using the Spearman's rank order correlation coefficient and Kendall's tau. Microscopic (light and electron) analyses were performed by standard methods.

RESULTS

Composite grafts cultured in KDE media (Fig 1*A,B*) demonstrate pigmented melanocytes (M, *arrows*) in the epithelial compartment of the skin analogue at 19 d after inoculation of the melanocyte-keratinocyte mixture. Fibroblasts are distributed throughout the reticulated part of the collagen-GAG substrate. Transmission electron microscopy (Fig 1*C*) confirms presence of human melanocytes (HM) that contain sparse distributions of melanosomes (ms) located at the surface of the collagen-GAG substrate. Melanosomes were found in keratinocytes of CSS samples only very infrequently *in vitro*. Figure 2*A,B* are photographs of athymic mice from separate experiments with uniformly pigmented human skin of medium to

* Boyce ST, Williams ML: Free fatty acids and vitamin E induce lamellar bodies and precursors of barrier lipids in lifted dermal-epidermal skin substitutes (abstr). J Invest Dermatol 96(4):620A, 1991.

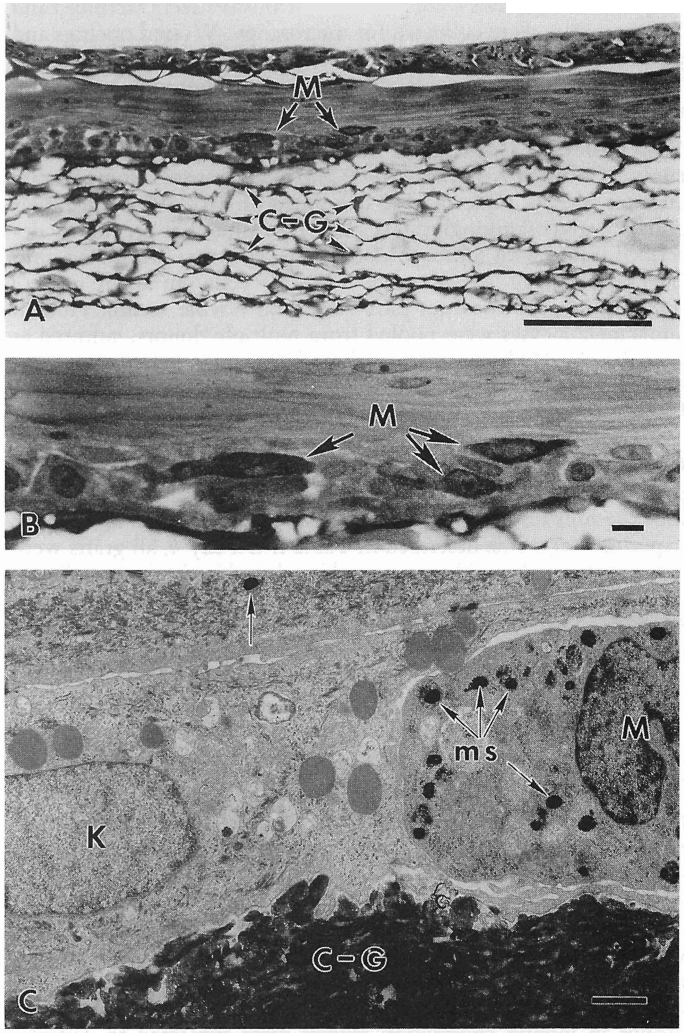

Figure 1. Light and transmission electron micrographs CSS *in vitro* containing cultured adult human melanocytes. *A*) Light micrograph of CSS showing the reticulated structure of the collagen-GAG substrate populated with human fibroblasts, partially stratified kertinocytes, and melanocytes (M) expressing pigment; *scale bar,* 0.1 mm. *B*) Higher magnification of *A* showing pigmented melanocytes adjacent to the keratinocyte-substrate interface; *scale bar,* 10 μm. *C*) Transmission electron micrographs of interface of keratinocytes (K) and melanocyte (M) with the C-GAG substrate. Sparse distributions of incompletely-formed melanosomes (ms) are seen in melanocytes, and occasionally in keratinocytes *(single vertical arrow); scale bar,* 1 μm.

dark color at 6 weeks after grafting. The original square shape of the wound is retained, and sides of the square are 1.25–1.5 cm in length. External texture of the healed skin is more rough than surrounding murine skin. Rough texture results from hyperkeratosis of human epidermis on athymic mice that is characteristic of the human-murine model. Adjacent borders of murine and human skin are fully fused with continuous epithelium across wound margins. Melanocytes did not migrate out of the grafted area as confirmed by histologic examination of the wound margin. Pigment is retained at 10 weeks (Fig 2*C*), and 6 months after grafting (Fig 2*D*). Although the original square shape of the wound is retained at 6 months, the pigmented area has receded to approximately 50% of the treated site. The mechanism for pigment recession is not currently understood, but is believed to be an artifact of the animal model. Redevelopment of pigment in clinical cases [15,18] has not shown subsequent recession. All animals that received grafts incubated in KDE or MG media showed complete pigmentation within the grafted site. Wounds treated with grafts incubated in KDF or KG media expressed partial or no pigmented areas within the grafted site.

Inhibition of wound contraction as a function of culture condition is shown in Fig 3. Mean wound sizes were 28.1 ± 3.7% for grafts incubated in KG medium, 28.2 ± 2.4% for KDF medium, 42.6 ± 2.9% for KDE medium, and 51.0 ± 2.9 for MG medium. Tukey's Studentized range test detects significantly larger ($p < 0.05$) healed wounds treated with grafts cultured in KDE and MG media. Analysis of variance showed highly significant differences ($p < 0.0001$) among quantitative wound sizes of the four test conditions.

Positive staining of biopsy tissue for HLA-ABC and gross examination of pigmentation showed complete correlation ($r_s = 1.0$) between these two parameters by Spearman's rank order nonparametric correlation analysis of all test wounds. These results are presented in Fig 4. HLA staining and pigmentation were detected in 50% (two of four) of wounds that received grafts from KG medium, 67% (four of six) of wounds with grafts from KDF medium, and 100% of wounds treated with grafts from both KDE and MG media. Although both KDE and MG media showed 100% of wounds positive for these two markers, it is important to note that not all wounds treated with grafts from KDE medium were completely pigmented, but that all from MG medium were completely pigmented. These results suggest that all pigmented areas of grafts are covered with human epithelium, and that melanocytes may act as reporter cells in cultured grafts.

Histology of biopsy tissue and immunostaining for HLA-ABC of regenerated skin tissue (not shown) shows hyperkeratotic epidermis compared to surrounding murine skin, which accounts for the rough, scaly external texture seen in Fig 2. Melanocytes are distributed predominantly on the epidermal side of the dermal-epidermal junction (DEJ), but are also found in subjacent connective tissue. Positive staining for HLA-ABC antigens of plasma membranes of keratinocytes in healed epidermis (not shown) confirms its human identity, and analogy to grafting native human skin.

Figure 5 shows transmission electron micrographs of biopsies of healed, pigmented skin. After engraftment and histogenesis of skin, melanocytes (HM) express very high frequencies of melanosomes (ms) that are transferred in high numbers to surrounding keratinocytes (HK). Small numbers of melanocytes are located in connective tissue (Fig 5*A*). However, the majority of melanocytes are distributed along the epidermal side of the basement membrane (Fig 5*B*) with dendritic processes extending between and among surrounding keratinocytes (Fig 5*C*). Pigment synthesis and transfer to keratinocytes accounts for uniform pigmentation of healed skin within the borders of the original wound site. Melanosomes are retained by keratinocytes throughout differentiation, and are found within mature corneocytes (Fig 5*D*) that are shed by desquamation.

DISCUSSION

Data presented here extend earlier reports of selective culture of skin cell types (keratinocytes, melanocytes, fibroblasts) [2,10,12,18–21], co-culture of keratinocytes and melanocytes [22], combination of cultured cells with implantable substrates to form tissue analogues [1,3,23], grafting to athymic mice of composite human cell-biopolymer constructs of skin [5,13,14,24], and clinical histogenesis of cultured skin cells into functional skin on full-thickness wounds [6,15,18]. Deliberate addition of melanocytes to cultured skin demonstrates restoration of a physiologic function and cosmetic factor that is not presently provided by skin formed from cultured cell grafts. Although not tested in this study, pigmented skin is expected to help protect basal keratinocytes and dermal cells from injury from ultraviolet radiation, and to allow color matching of grafted site with surrounding uninjured skin.

Pooled strains of non-transformed adult human melanocytes were used in this study, and each pool contained one or more strains of melanocytes from black donors. Preliminary studies† containing five pooled strains showed development of virtually black skin on

† Boyce ST, Medrano EE, Abdel-Malek ZA, Nordlund JJ: Pigmentation of cultured epithelium by human melanocytes in a composite skin substitute model (abstr). J Cell Biol 115(3):357a, 1991.

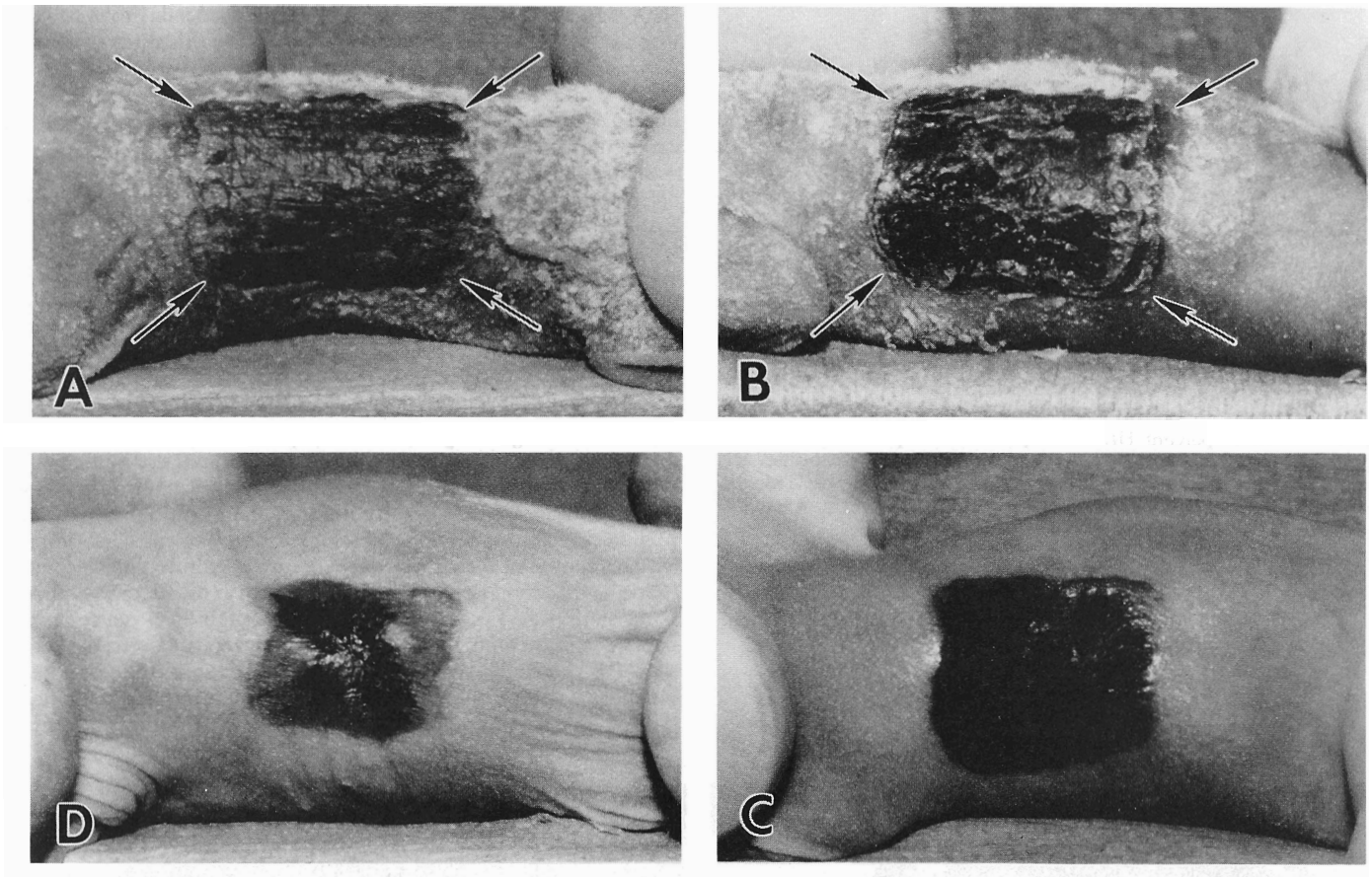

Figure 2. Photographs of athymic mice with pigmented human skin from cultured skin substitutes with human melanocytes. *A,B*) Completely and uniformly pigmented human skin develops from cultured skin substitutes containing cultured human melanocytes from random multiple strains. Two experiments show lighter *(A)* or darker *(B)* pigmentation after 6 weeks of healing, coincident with random melanocyte strains in CSS. *C,D*) Animal shown in *B* above, at 10 weeks *(C)*, and 6 months *(D)* after grafting.

the animals as shown in Fig 1. Animals grafted in subsequent experiments from different pooled strains also developed pigment, but the color at an equivalent timepoint was closer to brown than black. In all cases, the same number of melanocytes, and ratio of melanocytes to keratinocytes, was used. These results suggest that individual genotypes of grafted melanocytes are responsible for phenotypic expression of pigment, and that all melanocytes are affected uniformly by host. Native human skin grafted to athymic mice is known to hyperpigment spontaneously as a result of melanocyte proliferation and increases of melanosomes per cell with time.‡ Similarly, this study shows that cultured skin becomes uniformly hyperpigmented after transplantation as confirmed by high frequencies of melanosomes in melanocytes and keratinocytes in healed skin (Fig 5). Conversely, only very low frequencies of melanosomes are seen *in vitro* (Fig 1). Therefore, standardization of pigment expression in this model must consider the genotype of the melanocyte strain(s), incubation conditions *in vitro*, melanocyte responses to cellular or humoral effects from the host, and time subsequent to grafting.

Earlier studies from this laboratory showed that wound size after treatment with cultured skin was not statistically different from treatment with native human skin [5]. That study compared cultured skin grafts to human xenograft, murine autograft, and sham treatment with no graft. Although that study used a dry dressing technique rather than irrigation of dressings with culture medium, values for wound contraction at 6 weeks after treatment with no graft were 20% of original size, and after treatment with human xenograft were 50% of original size. This latter value agrees well with the wound size achieved with cultured skin substitutes in this study. It is important to recognize that reduced contraction of cultured skin grafts depends on keratinocyte survival, not melanocyte survival. In no instance has pigmented skin contained HLA-ABC– negative keratinocytes (murine). These findings may be interpreted that survival of transplanted melanocytes depends on transplanted keratinocytes, which depends, in large part, on incubation media. Earlier studies also have used immunostaining of healed skin for

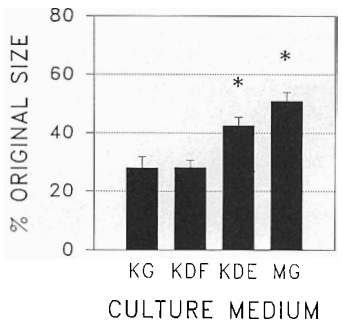

Figure 3. Plot of original wound size (mean ± standard error of the mean) versus culture medium of cultured skin substitutes. Skin substitutes incubated in KDE or MG media show statistically significant ($p < 0.05$) inhibition of wound contraction compared to incubations of CSS in other media. Cultured skin restores function and inhibits wound contraction.

‡ Robb E, Farooqui J, Boissy R, Warden GD, Nordlund JJ: The role of melanocytes in hyperpigmented split-thickness human skin (abstr). Proc Am Burn Assn 24:186, 1992

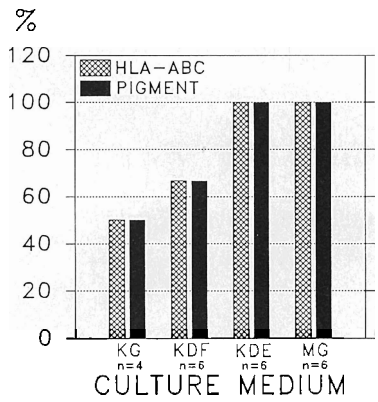

Figure 4. Plot of percent HLA-ABC positive and percent pigmented animals per group versus culture medium of cultured skin substitutes. All animals treated with cultured skin grafts incubated in KDE or MG media (see Table I for description) had complete healing of wounds. Absolute correlation is demonstrated between expression of HLA-ABC and pigment in healed wounds.

HLA-ABC to confirm the persistence of human cells in the healed wounds. However, the complete correlation in this study of HLA-ABC staining and visible pigmentation suggests that grafted melanocytes may be acceptable as reporter cells for engraftment of human epithelium. This correlation may be further tested for validity with greater numbers of pigmented skin grafts.

Standardization of this model opens important prospects for understanding of the cellular or humoral mechanisms of skin pigmentation in normal and pathologic conditions. Grafting of wounds with native or cultured skin grafts results in hyper- or hypopigmentation, respectively. Identification of mechanisms responsible for hyperpigmentation associated with burn scar may provide insights for corrective intervention. Addition of autologous or allogeneic melanocytes could restore pigment permanently if grafted cells are tolerated immunologically by the recipient. Correction of these defects in wound pigmentation may reduce morbidity during recovery from skin grafting. Other very powerful prospects for this model include establishment of uniform populations of human skin *in vivo* that contain non-transformed or dysplastic melanocytes for study of mechanisms and probabilities of malignant transformation [25], or for study of skin photobiology. Critical to consideration of transplantation of melanocytes is exposure of cultured melanocytes to media that contain tumor promoters, such as tetradecanoyl phorbol acetate (TPA) used in this study. Although TPA is a selective factor in culture for melanocytes and against keratinocytes, the keratinocytes in composite grafts retain sufficient viability to heal wounds and form stable epidermis. This result implies detoxification of TPA by high cell numbers in cultured grafts, and serum

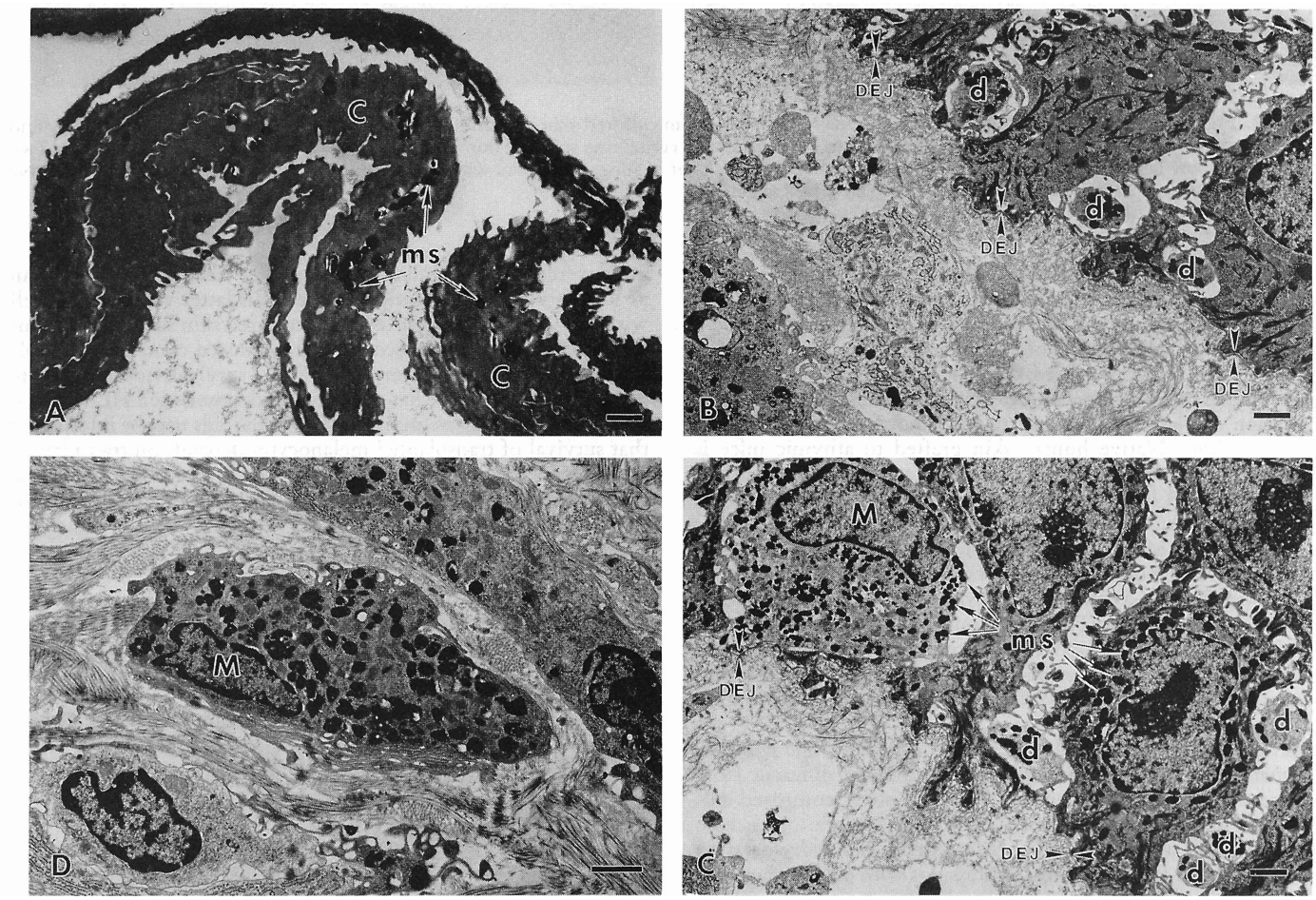

Figure 5. Transmission electron micrographs of melanocytes in healed skin biopsies from athymic mice. *A*) Melanosomes (ms) remain mostly within keratinocytes until desquamation of mature corneocytes (C). *B,C*) Melanocytes (M) are distributed predominantly on the epidermal side of the dermal-epidermal junction (DEJ), and extend dendritic processes (d) along the DEJ between keratinocytes. Skin pigmentation results from transfer of high frequencies of melanosomes (ms) to surrounding keratinocytes. *D*) Small numbers of melanocytes (M) are located in connective tissue immediately subjacent to the DEJ. *Scale bars*, 1 μm.

protein in MG medium. Risks of melanocyte transformation by culture agents must be evaluated carefully to determine whether transplantation of cells after exposure to phorbol esters is safe. Culture of keratinocytes in serum-free medium without phorbol ester has been determined not to increase risk of epithelial transformation [26], but in this study all cells in cultured grafts incubated in MG medium were exposed to TPA. Therefore, protocol design for transplantation of cultured skin must consider seriously the culture conditions and media formulations as risk factors that must be evaluated rigorously before initiation of clinical studies.

This report demonstrates reproducible and statistically significant improvement of wound healing and pigmentation by composite grafts of cultured skin cells and implantable biopolymers. Pigmented human skin *in vivo* provides a powerful model for mechanistic studies of wound healing, carcinogenesis of melanoma, and depigmentation disorders. Restoration of skin pigmentation by transplanted melanocytes constitutes an advancement in physiologic function of healed skin that offers reduced morbidity in recovery from conditions that require skin grafting.

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