Regulation of Pigmentation in Cultured Skin Substitutes by Cytometric Sorting of Melanocytes and Keratinocytes

Viki B. Swope,† Andrew P. Supp,‡ James R. Cornelius,‡ George F. Babcock,†‡ and Steven T. Boyce†‡
†Department of Surgery, University of Cincinnati, and ‡Shriners Burns Institute, Cincinnati, Ohio, U.S.A.

Unpredictable pigmentation in cultured skin substitutes (CSS) is an anatomic deficiency after wound treatment and can require years to normalize. Variable numbers of human melanocytes (HM) survive in cultures of human keratinocytes (HK) as demonstrated by focal areas of pigmentation in CSS after healing. The purposes of this study were to deplete HM from HK cultures and to regulate the numbers of HM contained in CSS. A highly pigmented HM cell strain was chosen for these studies to emphasize the differences in light scattering between HK and HM by flow cytometry. Cytometric gates were set with selective cultures of HM and HK and were used to sort a mixed population of HK + 4% HM. After sorting, CSS were prepared from human fibroblasts attached to collagen-glycosaminoglycan sponges combined with cells from the HK + 4% HM (pre-treatment control), the sorted HK (experimental), or sorted HK + 3% HM (post-treatment positive control) subpopulations and grafted to athymic mice. Grafted wounds were assessed for 6 wk by planimetry for area of pigment and by a Minolta Chromameter for color density and hue *in situ*. Histology and staining of HLA-ABC were performed at 6 wk. Data from percent pigmented area and chromameter measurements identified quantitative and statistically significant decreases in color of healed skin after flow cytometric separation of HK and HM. Therefore, a purified HK subpopulation depleted of HM was isolated by flow cytometry that generated healed skin with reduced pigmentation. These results suggest that HM can be selectively depleted from HK cultures and then added to cultured skin substitutes at specific densities to generate predictable pigmentation for improved function and cosmesis in healed wounds.
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Epidermal melanocytes serve both physiologic and psycho-social functions. Clinical morbidity caused by pigment abnormalities from congenital, pathologic, or traumatic etiologies can compromise an individual's physical and psychologic health. Protection from ultraviolet radiation, and paracrine interactions with other cutaneous cells are recognized functions of epidermal melanocytes (Abdel-Malek, 1988; Nordlund *et al,* 1989; Halaban *et al,* 1991; Swope *et al,* 1991). Several cutaneous diseases cause dysfunction or failure of the pigmentation system resulting in either hypo-pigmentation or hyper-pigmentation. Post-inflammatory hyper-pigmentation is a highly variable sequela of skin grafting, wound healing, and dermatologic diseases, including dermatitis and acne (Nordlund, 1988; Pelc and Nordlund, 1993). If the melanin deposition is epidermal in origin, the skin dyschromia can take months to years to normalize as inflammation resolves and epidermal turnover proceeds. By comparison, dermal melanosis caused by basement membrane damage, macrophage phagocytosis of melanin, and the resistance of melanin to enzymatic degradation may never resolve, leading to permanent cosmetic morbidity (Pelc and Nordlund, 1993). The molecular mechanisms of post-inflammatory hyper-pigmentation are not yet understood, but an overexpression of melanogenic factors by the hyper-trophic epidermis is an intuitive possibility. Increased numbers of active human melanocytes (HM) have been observed as early as 7–14 d after surgical wounding as determined by elevated numbers of pre-melanosomes and mature melanosomes compared to uninjured controls (Tsukada, 1977). Twelve months after skin engraftment, the cellular density of pre-melanosomes decreased, suggesting reduced rates of pigment synthesis. Human xenografts on athymic mice result in an increase in HM density in the grafted epidermis compared to the HM number in the ungrafted control skin (Farooqui *et al,* 1993). In another pre-clinical study of experimental burns, repigmentation was shown to occur rapidly in the healing epidermis with progressive hyper-pigmentation beginning at the periphery and migrating to the center of the wound (Sowemimo *et al,* 1981).

Cultured skin substitutes (CSS) have become adjunctive treatments for severely burned patients to reduce the requirements for donor skin and numbers of surgical procedures. CSS consisting of human fibroblasts and keratinocytes (HK) attached to collagen-glycosaminoglycan substrates have been used for clinical treatment of burns (Boyce and Hansbrough, 1988; Hansbrough *et al,* 1989; Boyce *et al,* 1993, 1995; Harriger *et al,* 1995), and chronic wounds (Boyce *et al,* 1995). Passenger melanocytes have been reported to

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Reprint requests to: Dr. Steven T. Boyce, Shriners Burns Institute, Research Department, 3229 Burnet Avenue, Cincinnati, OH 45229-3095.
Abbreviations: CSS, cultured skin substitutes; HM, human melanocytes; HK, human keratinocytes; CIELAB, Commission International d'Eclairage 1976 L*a*b*.

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Table I. Experimental Design and Procedural Steps

Step 1	Step 2	Step 3	Step 4	Step 5
Pre-Cytometry Cell Populations	Flow Cytometer Calibration	Cytometric Sorting	CSS Epithelium (1 wk culture)	Data Collection
(A) HM (selective)	(A) Set HM gate	(C) HK + 4% HM	(C) HK + 4% HM	(1) Photography and microscopy
(B) HK (selective)	(B) Set HK gate	(C_1) Sorted HK	(C_1) Sorted HK	(2) % area pigmented
(C) HK + 4% HM		(C_2) Sorted HM	(D) Sorted HK + 3% HM[a]	(3) Chromameter values

[a] HM added to sorted HK were from selective cultures such as population A and were not from sorted HM (population C_2).

survive in HK cultures (DeLuca *et al,* 1988) and are responsible for focal hyper-pigmentation. Previous studies from this laboratory have reported focal pigmentation of CSS after transplantation to athymic mice (Boyce *et al,* 1991), HM pigment synthesis and transfer to HK in CSS *in vitro*; hyper-pigmentation after grafting to athymic mice (Boyce *et al,* 1993); and focal pigmentation of autologous CSS grafted to burn patients (Harriger *et al,* 1995). The purposes of this study were to develop a method using flow cytometry to deplete passenger HM from HK populations and regulate by titration the distribution of HM in CSS to restore uniform pigmentation in healed skin. Boissy *et al* (1989) showed that HM with different pigment densities could be segregated based on the light scatter patterns and level of endogenous peroxides. An advantage of using definitive anatomic differences between HK and HM is the reduction of cell manipulation and preservation of cell viability. The sorted HK subpopulation was used to prepare CSS for engraftment to athymic mice and was compared to CSS that contained HM for development of pigmented human skin after wound healing.

MATERIALS AND METHODS

Experimental Design A diagram of the experimental design is presented in **Table I**. Three cell populations were subjected to flow cytometry: (i) HM after selective cell culture **(Fig 1)**, (ii) HK after selective cell culture, and (iii) HK + 4% HM. The selective cultures of HM and HK were used to calibrate cytometric gates for minimum overlap of HM and HK populations **(Fig 2)**. After gates were set, the HK + 4% HM population was sorted to separate HK and HM subpopulations. After cytometric sorting and 1 wk in expansion culture, the three cell populations were used to prepare the epithelial component of cultured skin substitutes: (i) HK + 4% HM (pre-treatment positive control), (ii) sorted HK subpopulation, and (iii) sorted HK subpopulation + 3% HM (post-treatment positive control). At the time of CSS inoculation, HM from selective culture were added to a portion of the sorted HK subpopulation to approximate the HM proportion in human skin (Fitzpatrick *et al,* 1967). The percentage of HM (4%) in the pre-treatment control was predicted to be diluted by the faster rate of HK proliferation during the expansion process in cell culture. This expected HM dilution contributed to the decision to reduce the post-treatment HM to 3%. These three preparations of CSS were grafted to full-thickness wounds on athymic mice. Data collection *in vitro* included (i) photomicrography, (ii) immunocytochemistry, and (iii) chromameter readings *in situ*. Data collection *in vivo* included (i) photography, (ii) histology, (iii) planimetry of pigmented area, and (iv) chromameter readings.

Cell Culture Human epidermal keratinocytes and fibroblasts were co-isolated from surgical-discard tissue by using selective growth media and were cryopreserved at an early passage to provide a stock of cells for experimentation (Boyce and Ham, 1983, 1985). Human epidermal melanocytes were derived from the same epidermal suspension used to establish the HK by inoculating a portion of the epidermal cell suspension into flasks with melanocyte growth medium. The melanocyte growth medium did not contain either phorbol esters or cholera toxin, and the medium selected for

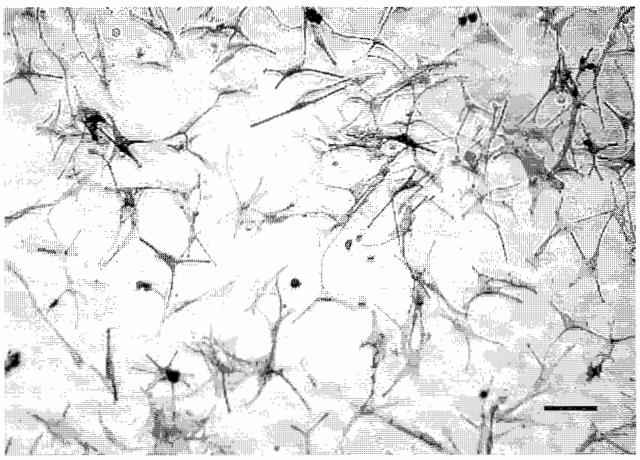

Figure 1. Pigmented phenotype of cultured HM *in vitro*. Phase-contrast photomicrograph of the HM used for the flow cytometric sort. Melanin content was 477 μg per 10^6 cells. *Scale bar,* 100 μm.

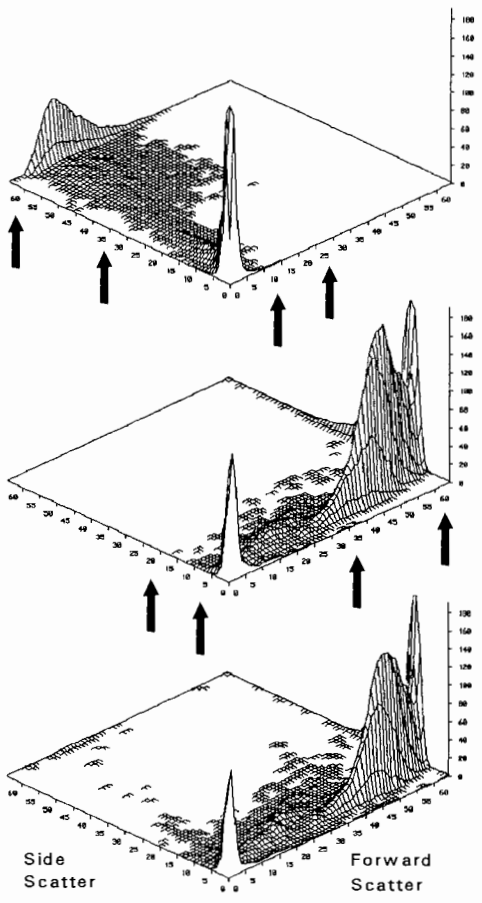

Figure 2. Selection of cytometric gates for sorting of melanocytes and keratinocytes. → indicate locations of sorting gates on forward and side scatter axes. Flow cytometric histograms: *top,* HM after selective culture; *center,* HK after selective culture; *bottom,* HK + 4% HM. Top and center histograms were used to set the sort gates for maximum exclusion of HM from the HK population.

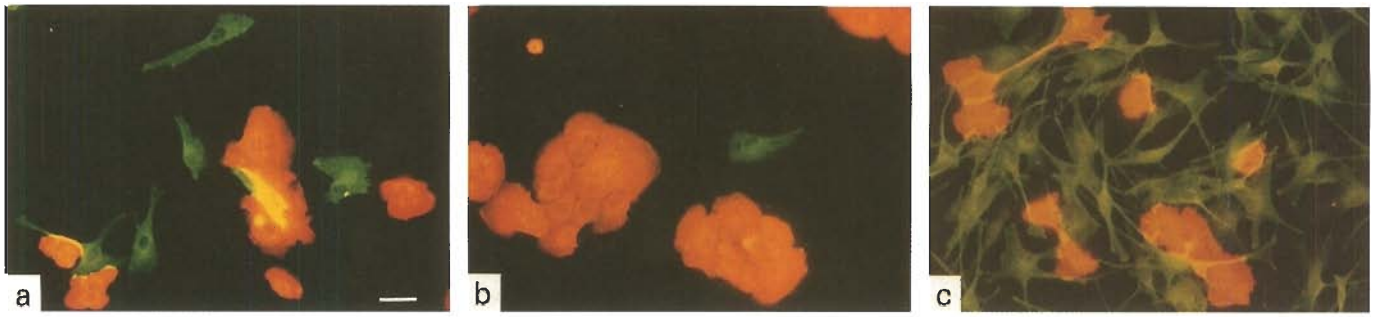

Figure 3. Enrichment of the HK after cytometric sorting. Fluorescence photomicrographs represent double labeling of HM (Mel-5 antibody + fluorescein isothiocyanate-conjugated secondary) and HK (anti-cytokeratin antibody + Texas red-conjugated secondary) 48 h after the sort. *(a)* HK + 4% HM. *(b)* Sorted HK. *(c)* Sorted HM cell subpopulations. *Scale bar,* 50 μm.

the proliferation of HM and not for HK (Swope *et al,* 1995). Two HM cell strains were tested by using the flow cytometer and the HM with the greatest degree of pigmentation resulted in the best HM-HK separation. This pigmented HM strain was chosen for this experiment. The human fibroblasts, HK, and HM used in this study were obtained from a single African-American donor. The HM were heavily pigmented with a melanin content of 477 μg per 10^6 cells (Lee *et al,* 1972).

Flow Cytometry/Cell Sorting A Coulter EPICS 753 flow cytometer (Coulter, Miami, FL) and the Cytomation data collection and software system (Cytomation, Fort Collins, CO) were used to analyze three experimental cell populations: (i) HM, (ii) HK, and (iii) the HK + 4% HM **(Table I, Fig 2)**. The cells were passed through a multiline ultraviolet beam of a Coherent I90-5 laser (351.1–363.8 nm), and the forward and side angle light scatter parameters were collected for analysis (Loken and Stall, 1982). Populations of HM and HK above were used to position the sort gates for maximum exclusion of HM from the HK region. Next, the HK + 4% HM cell population was sorted, and the sorted cells were maintained on ice throughout the procedure. Simultaneously, cells were collected from the gated HM region to isolate the reciprocal subpopulation of sorted HM. The HK + 4% HM population and sorted HK subpopulation were inoculated into T75 flasks at a density of 2.7×10^3 cells per cm^2 and expanded *in vitro* for 1 wk before preparation of cultured skin substitutes. The HM-HK sort procedure was carried out three times on two HM cell strains prior to this experiment. The quality of the separation was verified with the immunocytochemistry protocol described below and was found to be highly reproducible for the pigmented HM cell strain.

Immunocytochemistry HK + 4% HM, sorted HK, and sorted HM cells were inoculated into eight-well chamber slides and incubated for 48 h after the sorting procedure **(Fig 3)**. The cells were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde and then were blocked with 2% bovine serum albumin and 2% normal goat serum. A double-labeling procedure consisted of sequential incubation of cells in MEL-5 murine monoclonal antibody (Signet Labs, Dedham, MA) directed against a pigment-associated glycoprotein followed by a goat anti-mouse IgG-fluorescein isothiocyanate secondary antiserum (Sigma, St. Louis, MO). The keratinocyte-specific antiserum was a polyclonal rabbit anti-cytokeratin (Zymed Labs, South San Francisco, CA) that was reacted with a goat anti-rabbit IgG/IgM-Texas Red antibody (Southern Biotechnology Associates, Birmingham, AL). Negative controls included (i) no MEL-5 antibody, (ii) no anti-cytokeratin antibody, (iii) manufacturer's negative control for MEL-5, and (iv) both secondary antibodies with no primary antibodies. No cross-reaction was observed between the staining for the HK and HM.

Cultured Skin Substitutes Cultured human fibroblasts were harvested and inoculated onto the porous side of collagen-glycosaminoglycan biopolymer substrates (Hansbrough *et al,* 1989). Twenty-four hours later, cells from the HK + 4% HM cultures, sorted HK subpopulation, or sorted HK + 3% HM cell preparation were inoculated onto the opposite laminated surface and designated day 0 of *in vitro* culture. On culture day 3, CSS were lifted onto a cotton pad and maintained at the air-liquid interface in saturated relative humidity at 37°C and 5% CO_2 (Boyce *et al,* 1993). Daily changes of a lipid-enriched nutrient medium (Boyce and Williams, 1993) were performed, and the CSS were surgically applied to athymic mice on incubation days 13 and 14.

Surgical Grafting Procedure All animal studies were previously approved by the University of Cincinnati Institutional Animal Care and Use Committee. On days 13 and 14 of *in vitro* incubation, CSS from each epidermal preparation (**Table I**, *Step 4*) were cut into grafts of 2 × 2 cm and applied orthotopically onto full-thickness wounds prepared in athymic mice (n = 6, 5, or 6 per group, respectively; Boyce *et al,* 1991). Grafted wounds were dressed with cotton gauze, covered with Op-Site (Smith & Nephew Medical, Hull, England), and bandaged with Coban (3M, Minneapolis, MN). One milliliter of irrigation fluid consisting of nutrient medium and anti-microbials was administered into each dressing for 10 d (Boyce *et al,* 1993). On day 10 after surgery, stenting sutures were removed and the grafted site was covered with a semi-dry dressing consisting of N-Terface (Winfield Laboratories, Richardson, TX), Xeroform gauze (Sherwood Laboratories, St. Louis, MO.), cotton gauze, and Coban. On day 21, the dressings were replaced by dry dressings without the Xeroform, and on day 28 the dressings were removed completely. Photographs, chromameter measurements, and tracings of pigmented area and wound area were collected on days 10, 14, 21, 28, 35, and 42 after grafting **(Figs 4-6)**. The percent pigmented area was quantified by using computerized planimetry and defined as the pigmented area divided by the total wound area × 100 **(Fig 5)**. The animals were sacrificed on day 42 and frozen sections of graft biopsies were prepared for direct immunofluorescence detection of HLA-ABC (Briggaman, 1985).

Chromameter Measurement Reflectance spectroscopy *in situ* was used to measure the visible spectrum of light reflected from the skin. Color has been assessed and described by the principles of the Commission International d'Eclairage 1976 $L^*a^*b^*$ (CIELAB; Muizzuddin *et al,* 1990; Weatherall and Coombs, 1992) because color vision is trichromatic. The three CIELAB color space values (L^*, a^*, and b^*) provide a means to measure color objectively and record three color dimensions. The L^* value describes the psychometric correlate of perceived lightness (Weatherall and Coombs, 1992) and ranges from absolute black (0) to absolute white (+100). Chromaticity is represented by the a^* value ranging from green (−60) to red (+60) and the b^* value ranging from blue (−60) to yellow (+60). The CIELAB color space values can be used to measure the difference between any two colors (Weatherall and Coombs, 1992). By using coordinate geometry, the color difference (ΔE) is calculated as the length of the line connecting their coordinate locations:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}.$$

The chromameter was used to evaluate CSS color by reflectance spectroscopy *in situ* and to assign numerical values to the relative color components of lightness/darkness and chromaticity. The Chroma Meter CR-300 (Minolta, Osaka, Japan) has an 8-mm-diameter measurement aperture that uses diffuse illumination of a pulsed xenon arc lamp, and a 0° viewing angle. After calibration, chromameter measurements were collected from the *in vitro* CSS on day 15 after HK inoculation and from the center of each *in vivo* graft on days 10, 14, 21, 28, 35, and 42 after surgery **(Fig 6)**. The color difference ΔE was calculated in reference to the sorted HK + 3% HM CSS because this group showed the CIELAB color space values of greatest magnitude.

Statistical Analysis The percent pigmented area was analyzed statistically as a one-between (group) one-within (time) repeated measures analysis of variance. Univariate analysis of variance was preformed at each time point among the three groups, and comparisons of groups were made by Tukey's test. Analysis of variance and the Student-Newman-Keuls method for multiple pairwise comparisons were used to analyze the chromameter values L^*, a^*, and b^*. A repeated measures analysis of variance was used to analyze the *in vivo* ΔE time points and Student's t test was used to compare the *in vitro* ΔE.

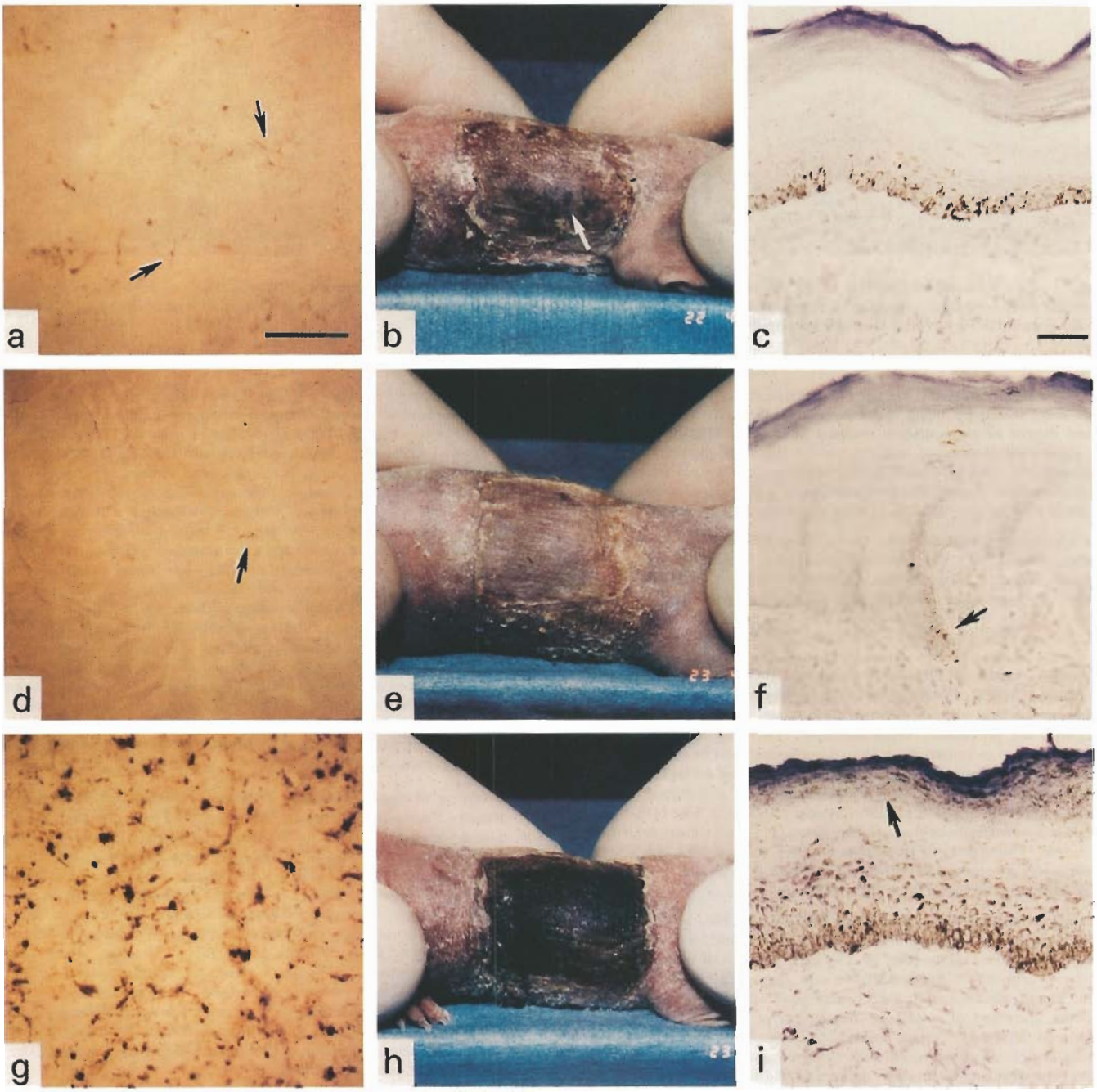

Figure 4. Pigmentation of cultured skin substitutes *in vitro* and *in vivo*. *(a–c)* HK +4% HM group. *(d–f)* Sorted HK group. *(g–i)* Sorted HK + 3% HM group. *(a,d,g) En face* photomicrographs demonstrate the frequencies of HM and melanin in CSS *in vitro*. → indicate melanocytes. *Scale bar,* 500 μm. Grafted athymic mice from corresponding conditions show focal pigmentation in the HK + 4% HM CSS (*b*, ⇨), the hypo-pigmentation in the sorted HK CSS *(e)*, and hyper-pigmentation in the sorted HK +3% HM CSS *(h)* at 21 d after grafting. Histologic micrographs of healed skin at 42 d after grafting show moderate distributions of HM in wounds treated with HK + 4% HM CSS *(c)*, rare frequencies of HM in wounds treated with sorted HK CSS *(f)*, and very high amounts of pigment in wounds treated with sorted HK + 3% HM CSS *(i)*. → indicate HM melanin. *Scale bar,* 50 μm.

RESULTS

Preliminary experiments demonstrated that the greatest difference between the light-scatter patterns of HK and HM as detected by flow cytometry resulted from use of a pigmented HM cell strain **(Fig 1)**. In previous CSS experiments using this keratinocyte strain, the presence of passenger HM was confirmed by pigmented grafts on athymic mice (data not shown). The HK-HM separation was based on the unique light scattering characteristics of each cell type detected by the flow cytometer with an ultraviolet laser to illuminate the cells. Because no antibody labeling was used for these sorting procedures, the cell handling and processing time was reduced. The histogram plots of forward *versus* side angle light scatter of the HM and HK after selective culture and the HK + 4% HM cell population are shown in **Fig 2**, top, center, and bottom, respectively. **Figure 2,** top and center, was used to set the gates for maximum exclusion of the HM from the HK population. The optimized sort region contained 0.2% of all HM and 40.3% of all HK. The relative abundance of HM in the HK + 4% HM cell inoculum is shown in **Fig 3*a***, and the depletion of HM is demonstrated in the sorted HK subpopulation in **Fig 3*b***. The sorted

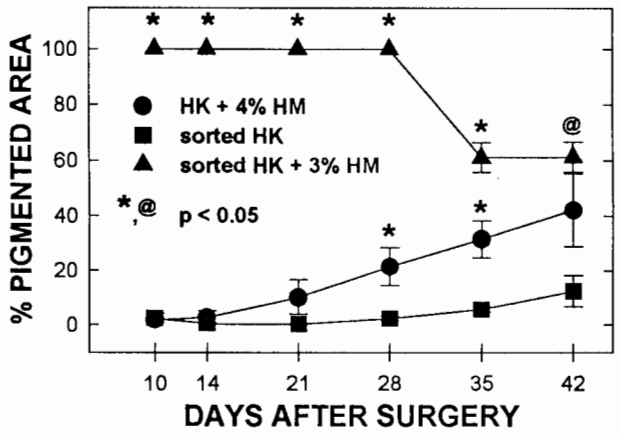

Figure 5. Kinetic development of skin pigment in grafted CSS. The pigmented areas on each animal were traced and divided by the total wound area of healed wound at each time point. *Error bars,* SEM (n = 5 or 6). *, Significant difference as compared to the other two groups; @, significant difference between the sorted HK group and the sorted HK + 3% HM group at individual time points ($p < 0.05$).

HM subpopulation was verified to contain predominantly melanocytes **(Fig 3*c*)**.

In **Fig 4**, the *en face* photomicrographs of the CSS *in vitro*, photographs of healed skin on athymic mice, and histologic micrographs show obvious differences in melanin deposition in the following order of increasing magnitude: sorted HK, HK + 4% HM, and sorted HK +3% HM. Individual melanocytes and their dendrites can be readily identified in the photomicrograph of the HK + 4% HM CSS **(Fig 4*a*)**. The frequency of HM is greatly reduced by cytometric sorting **(Fig 4*d*)**. The melanin deposited in CSS prepared from the sorted HK subpopulation + 3% HM is so extensive that individual HM are indiscernable **(Fig 4*g*)**. Twenty-one days after grafting, focal areas of pigmentation were noted in the HK +4% HM CSS grafted animals **(Fig 4*b*)**, and the sorted HK CSS remained hypo-pigmented **(Fig 4*e*)** throughout the study. CSS prepared from the sorted HK subpopulation + 3% HM exhibited the greatest degree of pigmentation throughout the experiment as shown in **Fig 4*h***. Similarly, histologic micrographs of healed skin 42 d after grafting to athymic mice shows moderate distribution of HM melanin in pigmented spots in HK + 4% HM CSS **(Fig 4*c*)**, rare frequencies of HM in healed skin from sorted HK CSS **(Fig 4*f*)**, and very heavy pigmentation in grafts from the sorted HK+ 3% HM condition **(Fig 4*i*)**. In all cases, HM were distributed in the basal layer of the epidermis and transferred pigment to keratinocytes after healing. Staining for human leukocyte antigens-ABC verified the presence of human cells in all of the healed wounds at the conclusion of the experiment (data not shown).

Figure 5 shows results from the tracings of the pigmented and the total areas of healed wounds on the athymic mice at each time point. The HK + 4% HM CSS slowly developed focal pigmented areas that continued to increase in diameter and coalesce to a maximum of 42% of the healed area by 42 d after grafting. The sorted HK CSS pigmented area was not detectable visually until the end of the experiment at which time several small foci of pigment developed constituting about 12% of the healed area at the conclusion of the experiment. Pigmentation in the sorted HK + 3% HM CSS was 100% until 5 wk after grafting at which time selected animals in this group had migration of normal nonpigmented murine epidermis into the sites treated with CSS. This migration is considered an artifact of the human-murine model and resulted in a decrease of the mean percent pigmented area to 61%. As demonstrated in **Fig 5**, the percent pigmented area of the sorted HK + 3% HM group was significantly greater than the sorted HK group at all time points and all three groups were significantly different from each other at days 28 and 35. The percent pigmented area of the HK + 4% HM and sorted HK + 3% HM groups were not different from each other at day 42 due to the increased pigmentation of the HK + 4% HM CSS and the ingrowth of

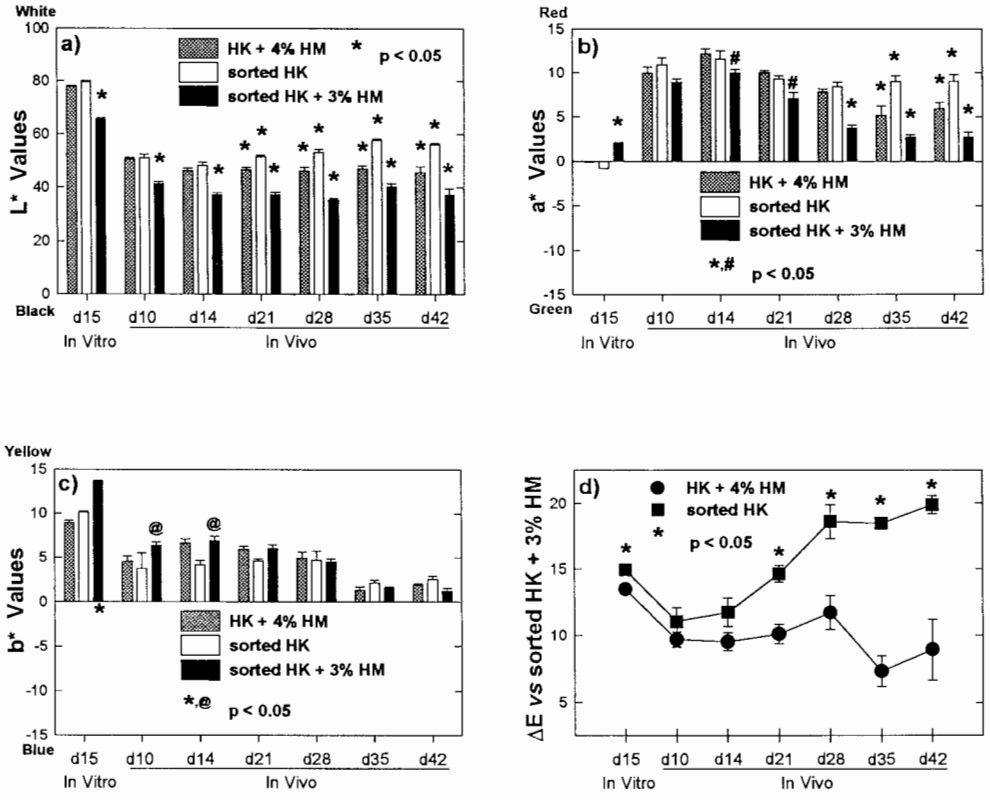

Figure 6. Quantification of color in cultured skin substitutes before and after grafting by chromameter measurements. *(a)* The L* value represents the scale of absolute black (0) to absolute white (+100). *(b)* The a* value represents the scale of green (−60) to red (+60). *(c)* The b* value represents the scale of blue (−60) to yellow (+60) in CSS *in vitro* and *in vivo*. *(d)* ΔE represents the difference between the combined tricolor stimulus values in reference to the sorted HK+ 3% HM CSS. *Error bars,* SEM (n = 5 or 6). *(a–c)* *, significant difference compared to the other two groups; #, significant difference between the sorted HK + 3% HM CSS and the HK+ 4% HM CSS only; @, significant difference between the sorted HK + 3% HM CSS and the sorted HK CSS at individual time points ($p < 0.05$). *(d)* *, significant difference between the HK + 4% HM CSS and sorted HK CSS ΔE in reference to the sorted HK + 3% HM CSS at individual time points ($p < 0.05$).

normal nonpigmented murine epidermis in the sorted HK + 3% HM CSS.

The chromameter quantified the CIELAB color space values of the CSS *in vitro* and *in vivo* as shown in **Fig 6**. L^* values, which measure the scale of black to white, showed significant differences between the sorted HK + 3% HM CSS and the other experimental groups at all time points **(Fig 6*a*)**. At the early time points after grafting, the underlying wound bed influenced the color values because the CSS were thinner and more translucent. This accounts for the substantial change from the *in vitro* day 15 to *in vivo* day 10 measurements of L^*, a^*, and b^*. As the grafts healed and the contribution of the wound bed decreased, L^* values of the sorted HK CSS increased representing greater whiteness, and L^* values stabilized for CSS prepared with HK + 4% HM and sorted HK + 3% HM populations. The a^* value, which measures the green to red scale of hues, increased in all conditions after surgery due to the contribution of red hue from the wound bed. As graft healing and pigmentation proceeded, the a^* value decreased in the HK + 4% HM CSS and sorted HK+ 3% HM CSS **(Fig 6*b*)**. The b^* value, representing blue to yellow scale of hues, showed the fewest significant differences among CSS groups at each time point although all groups tended to decrease, indicating a reduction in the yellow hue after engraftment **(Fig 6*c*)**. The ΔE value is a composite value that accounts for all three color parameters and assigns a set of spatial geometric coordinates to compare total color of individual samples **(Fig 6*d*)**. Because ΔE represents a difference between two composite color values, the sorted HK +3% HM experimental group was selected as the reference condition at each time point. There was a significant difference between the ΔE values for *in vitro* HK + 4% HM CSS and sorted HK CSS because the standard errors within each group were very small. As the HK + 4% HM CSS developed pigment *in vivo*, the absolute difference of ΔE between the HK + 4% HM CSS and sorted HK+ 3% HM CSS decreased. The sorted HK CSS ΔE value increased steadily with time because the sorted HK CSS L^* value increased and the sorted HK+ 3% HM CSS a^* value decreased after engraftment. Statistical significance for ΔE was found at *in vitro* day 15 and *in vivo* days 21, 28, 35, and 42.

DISCUSSION

The irregular patterns of pigmentation in CSS must be regulated to achieve an acceptable cosmetic result in burn patients. CSS pigmentation may be reduced by modulating the epidermal melanogenic factors or adjusting the melanocyte density. Because clinical use of CSS for treatment of burns generates variable pigmentation (Harriger *et al,* 1995), regulation of melanocyte numbers in CSS was selected as an initial approach to predictable pigmentation in healed skin. Complete predictability of skin pigmentation with transplanted skin cells, however, will also require regulation of rates of melanin synthesis and degradation that are not yet fully understood.

The CSS prepared from the HK + 4% HM and sorted HK cell cultures after 1 wk of incubation were not visibly pigmented *in vitro*. In contrast, the post-treatment positive control CSS with 3% HM added to sorted HK cells at the time of CSS preparation were pigmented in culture. This control was included to approximate the epidermal-melanin unit of 1:36 reported for adult human skin (Fitzpatrick *et al,* 1967). The *en face* photomicrographs of CSS *in vitro* and the corresponding animal photographs and histologies agreed well with the percent pigmented area **(Figs 4, 5)**. The HK + 4% HM CSS were not obviously pigmented *in vitro* due to the HM dilution resulting from the faster rate of HK proliferation during the cellular expansion. *In vivo* the HK + 4% HM CSS developed pigmented foci and the sorted HK CSS remained relatively nonpigmented except for infrequent small pigmented foci that developed *in vivo*. The sorted HK +3% HM CSS were heavily pigmented *in vitro* and *in vivo* **(Figs 4–6)**. This HK strain has been used for other CSS grafting experiments. The passenger HM surviving within the HK frozen stock resulted in notable pigmented foci by 3 wk after grafting, which continued to progress with time. The lack of significant pigmentation in the sorted HK CSS by 6 wk verifies the HM-HK separation by sorting. It is reasonable to expect that multiple HK subcultures would result in an effective HM reduction by limiting dilution. But postnatal HK cannot be repeatedly subcultured without greatly reducing their proliferative rate and colony-forming efficiency. Clinical HK are used to prepare CSS at passage 1 or 2. Cytometry as used in this study depends on pigment for sorting of melanocyte and keratinocyte populations. Application of sorting to melanocyte populations with little or no pigment will require modification of this general technique to label surface markers specific to one of the two cell types. Preliminary experiments have been performed based on separation of keratinocytes after labeling of desmosomal proteins on the cell surface with a fluorescent antibody. Although the cytometric method in this approach is based on detection of a fluorescent label rather than direct light scattering, the result of separation of melanocyte and keratinocyte populations is the same.

Noninvasive biophysical assessment of skin pigmentation can be performed with the chromameter. This instrument is used to characterize the tristimulus color values for accurate color comparisons. The color space values described by the principles of the Commission International d'Eclairage 1976 $L^*a^*b^*$ (CIELAB) objectively record 3-dimensional color (Muizzuddin *et al,* 1990; Weatherall and Coombs, 1992). The CIELAB color space value L^* describes the perceived lightness/darkness and ranges from absolute black to absolute white. Chromaticity is represented by the a^* color hue value on the green to red scale in combination with the b^* color hue value on the blue to yellow scale. As shown in **Fig 6**, the wound bed had the greatest influence on the L^* and a^* values at the early *in vivo* time points because the CSS were more translucent and less pigmented. The HK + 4% HM CSS and sorted HK + 3% HM CSS L^* values stabilized and the sorted HK CSS L^* value increased slightly as the result of healing and keratinization. As engraftment and pigmentation proceeded in the HK + 4% HM CSS and sorted HK + 3% HM CSS, the a^* value decreased, but the relatively high a^* value for the nonpigmented sorted HK CSS may have resulted from vascularization contributing to the red hue. The composite tricolor CIELAB values are described by ΔE and the sorted HK + 3% HM CSS was chosen as the point of reference for color comparison at each time point. The increasing sorted HK CSS ΔE is an indicator of the successful separation of the HK and HM. The overall HK + 4% HM CSS ΔE decreased as the pigmentation of the HK + 4% HM CSS increased and the color difference decreased between the HK + 4% HM CSS and the reference sorted HK +3% HM CSS. The chromameter provided a convenient and efficient technique to quantify serially the color changes in the CSS without compromise of sample integrity.

It has been reported that the relationship between the keratinocyte and the melanocyte is strictly regulated. The *in vitro* HK/HM ratio was shown by De Luca *et al* (1988) to be maintained in a defined range and the HM became basally situated in skin substitutes *in vitro* even in the absence of a dermal component. As pointed out by Scott and Haake (1991), the basal HK/HM ratio is regulated by the HK and the developmental stage of the HK also influences the relationship. Skin constructs prepared from fetal-derived HK and HM of either fetal or neonatal source had a significantly lower basal HK/HM ratio than constructs from neonatal HK irrespective of the HM source. These ratios were consistent with that found in the *in vivo* situation for the fetal and postnatal epidermis (Fitzpatrick *et al,* 1967; Holbrook *et al,* 1989). The source of the HM did not appear to affect the outcome of the basal HK/HM ratio but the keratinocyte source definitely altered the basal HK/HM ratio. Further investigations will contribute to understanding of the factors that regulate and maintain this relationship.

To experimentally regulate pigmentation, flow cytometry was employed to deplete HM from the HK/HM mixed population. CSS prepared from HK populations depleted of HM by cytometric sorting developed minimal pigmentation after wound closure. Improvement of methods for complete elimination of highly or poorly pigmented HM from HK cultures should allow preparation

of nonpigmented CSS to which HM can be added in predictable distributions. Regulation of melanocyte distribution is an initial step to predictable skin color after wound treatment with CSS.

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