Regulation of cutaneous pigmentation by titration of human melanocytes in cultured skin substitutes grafted to athymic mice

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Pigmentation of healed cultured skin substitutes in burn patients is frequently irregular and unpredictable which compromises solar protection and the patient's self-image. To address these morbidities, human fibroblasts were inoculated on a collagen-glycosaminoglycan substrate followed 1 day later by the addition of keratinocytes at 1.1×10^6/cm^2 combined with either 0, 1.1×10^2, 1.1×10^3, or 1.1×10^4 melanocytes/cm^2. The skin substitutes were incubated in vitro for 3 weeks and grafted to athymic mice. In vitro, the number of L-Dopa–positive melanocytes in the skin substitutes increased proportionately to the number of melanocytes inoculated. The melanocytes localized to the basal epidermis when labeled for MEL-5. The skin substitutes with 1.1×10^4 melanocytes/cm^2 were significantly darker than other groups in vitro by chromameter evaluation. By 12 weeks after grafting, the cultured skin ranged from no pigment in the control group, to 75% pigmented area in the 1.1×10^3 melanocytes/cm^2 group, to complete pigmentation in the 1.1×10^4 melanocytes/cm^2 group. In vivo, the mean chromameter values were significantly darker for the grafts with 1.1×10^3 and 1.1×10^4 melanocytes/cm^2. These results suggest that complete restoration of cutaneous pigmentation can be accomplished by addition of between 0.1 and 1.0×10^4 melanocytes/cm^2 to skin substitutes.**(WOUND REP REG 2002;10:378–386)**

Rapid and permanent closure of cutaneous burn wounds is a major goal in the acute phase of recovery from extensive burn injuries. Although split-thickness skin autograft is the method of choice to cover excised burn wounds, massive burns have necessitated the development of autologous cultured skin substitutes (CSS) to supplement the autografts. Clinical studies in this laboratory have shown that CSS can result in rapid wound closure with high rates of engraftment to full-thickness burn wounds.[1] The use of CSS has reduced the area of donor sites needed to heal burn wounds with consequent reductions in morbidity, pain, scar formation, and numbers of surgical procedures.[1,2]

CSS	Cultured skin substitutes
L-Dopa	L-3,4-dihydroxyphenylalanine
HF	Human fibroblast
HK	Human keratinocyte
HM	Human melanocyte
PBS	Phosphate buffered saline solution

Despite the success with CSS in treating burn wounds, CSS are deficient in many of the structures and functions of uninjured skin, such as sebaceous glands, melanocytes, hair follicles, endothelial cells, nerve cells and cell-mediated immunity.[3] In postburn healing wounds, abnormal pigmentation patterns include both hyperpigmentation and hypopigmentation.[4–6] The skin dyschromia can take months to years to normalize. Incomplete pigmentation is an anatomic and physiologic deficiency of CSS that can result in an undesirable cosmetic outcome and increased sensitivity to solar exposure for the patient.[7] Thus, deficiencies of pigmentation

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may adversely impact the ability of the CSS to avoid ultraviolet damage, and irregular pigmentation may negatively affect the psycho-social recovery of the patient. Previous studies by other investigators have shown restoration of pigmentation by clinical transplantation of cultured melanocytes to treat vitiligo[8] or hypopigmented burn scar.[9]

Historically, the epidermal-melanin unit has been described as the distribution of melanosomes from a single human melanocyte (HM) to 30–40 keratinocytes (HKs).[10,11] DeLuca et al.[12,13] showed that the HK to HM ratio in vitro was relatively constant and that HMs organize basally in cultured epithelium and could be transplanted for treatment of vitiligo.[14] In an insert model, HMs donated melanosomes to HKs and were situated in a basal location where melanosome containing dendrites extended among the HKs.[15,16] Epidermal substitutes have also demonstrated functional melanin units.[17–19] Co-cultures of HKs and passenger HMs inoculated on an acellular dermis form pigmented foci, which progressed to pigment the entire graft by 8 weeks on athymic mice.[20] Skin composites have been used to study the interaction of fibroblasts, HKs, and HMs in a three-dimensional structure.[21,22] HKs and HMs establish a defined ratio when cultured on a contracted collagen gel.[23,24] The ratio of basal HK to HM was regulated based on the fetal or neonatal origin of the HKs, but was not influenced by the HMs' origin. Other investigators have studied the effect of ultraviolet light on skin substitutes populated with HMs. Ultraviolet irradiation caused the HMs in skin composites to become more dendritic, stimulated melanin production, and increased the number of HMs.[25–28]

Hypopigmentation with multiple, discrete foci of pigmentation is frequently observed clinically with CSS, although the degree of pigmentation varies greatly among patients.[2,6,29] The pigmented foci result from passenger HMs that are cultured along with the HKs. Earlier studies in this laboratory showed that selectively cultured HMs mixed with HKs at a ratio of 1 : 30 and seeded onto fibroblast-inoculated collagen-glycosaminoglycan substrates become pigmented on athymic mice.[30] Flow cytometry was used to remove passenger HMs from the HK population and 3% HMs were added to HKs in CSS to produce uniform pigmentation in healed grafts.[31] The current study focuses on the selective culture and addition of HMs to CSS at increasing densities to repigment the CSS in vivo. The objective of the study was to determine a minimum density of melanocytes that was required to restore pigmentation completely. These studies were performed as an initial step toward regulation of pigmentation to improve the overall functional and cosmetic outcome of CSS.

MATERIALS AND METHODS

Epidermal HKs and dermal human fibroblasts (HFs) were coisolated from surgical discard tissue using selective growth media and cryopreserved at an early passage to provide a stock of cells for experimentation.[32,33] Epidermal HMs were derived from a neonatal foreskin and established in melanocyte selective growth medium as previously described.[34] The HMs were not exposed to phorbol esters or cholera toxin, and they were pigmented with a melanin content of 90.8 ± 6.99 μg/10^6 cells.[35] The HK strain used for the in vivo studies was chosen after a preliminary experiment showed that CSS grafted to athymic mice were virtually absent of pigment.

CSS preparation

The CSS were prepared by sequential inoculation with HFs (5.5×10^5/cm^2) followed 1 day later with HKs (1.1×10^6/cm^2; 8×10^7 HKs were inoculated onto 72 cm^2) mixed with HMs (0, 1.1×10^2, 1.1×10^3, or 1.1×10^4/cm^2; equivalent to 0.00, 0.01, 0.10, and 1.00% of HKs, respectively) onto acellular collagen-glycosaminoglycan biopolymer substrates as previously described.[36] The control group had no HMs added to the HK cell suspension. The CSS growth medium consisted of Dulbecco's modified Eagle's medium supplemented as reported by Chen with modifications.[37] Day 0 was designated as the day of HK inoculation. On day 3 after HK inoculation, the CSS were lifted and maintained at the air liquid interface in saturated relative humidity at 37°C and 5% CO_2. The growth medium was replaced daily. In vitro biopsies were collected weekly for light microscopy and L-3,4-dihydroxyphenylalanine (L-Dopa) staining and at week 3 for immunohistochemistry. Chromameter evaluation was performed weekly in vitro to record changes in CSS color. For light microscopy, CSS biopsies were fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer, embedded in glycol-methacrylate resin, sectioned, and stained with Toluidine Blue.

Immunohistochemistry

Frozen sections of CSS at week 3 in vitro were fixed in methanol and dried with acetone at −20°C. All steps were carried out at room temperature and three phosphate buffered saline solution (PBS) washes were performed between each step. The CSS sections were blocked with 2% bovine serum albumin and 2% normal goat serum. The double labeling procedure involved incubating the MEL-5 monoclonal antibody (1 : 40; Signet Labs., Dedham, MA) directed against a pigment-associated glycoprotein followed by a goat antimouse IgG-FITC secondary (1 : 80; Sigma Chemical Co., St. Louis, MO). The primary antibody directed against epidermal keratins was a polyclonal rabbit anticytokeratin (1 : 60; Zymed Laboratories., Inc., South

San Francisco, CA) which was subsequently reacted with a goat anti-rabbit IgG/M-Texas Red secondary antibody (1 : 100; Southern Biotechnology Associates, Inc., Birmingham, AL). The negative control lacked the MEL-5 antibody and no cross reaction was observed between the staining for the HKs and HMs.

L-Dopa staining

L-Dopa (Sigma Chemical Co.) is the substrate for the melanin synthetic pathway. Weekly, 1 cm^2 biopsies from the 0, 1.1×10^3, and 1.1×10^4 HM/cm^2 CSS ($n = 3$/group) were floated in a 16.8-mM ethylenediamine tetraacetic acid solution for 2 hours at 37°C.[38] The epidermis was gently separated from the dermal portion of the CSS in PBS. Each isolated epidermal layer was floated in L-Dopa for 3–4 hours at 37°C. Following dehydration, the epidermal isolates were mounted onto microscope slides. L-Dopa–positive cells were counted in four fields (area $= 3.8 \times 10^{-3}$ cm^2) for each of three CSS per group. These counts were normalized to a 1-cm^2 area (mean ± SEM). L-Dopa staining was not performed with the 1.1×10^2 HM/cm^2 group.

Chromameter measurement

Reflectance spectroscopy in situ was used to measure the visible spectrum of light reflected from the cultured skin.[31] A uniform, three-dimensional color space has been described by the principles of the Commission of d′ Eclairage 1976 L*a*b* because color vision is trichromatic.[39,40] The L* value, which correlates to perceived lightness and can range from absolute black (0) to absolute white (+100), has been the most sensitive of the trichromatic values to CSS color change following HM inoculation. For this reason, only the L* value (mean ± SEM) is presented here. The Chroma Meter CR-300 (Minolta, Osaka, Japan) with an aperture of 0.5 cm^2 was used to evaluate CSS melanization both in vitro ($n = 4$ CSS/group) and in vivo ($n = 3$–6 animals/group).

Surgical grafting procedure and graft assessment

All animal studies were previously approved by the University of Cincinnati Institutional Animal Care and Use Committee. At day 21 of in vitro incubation, CSS were cut into 4 cm^2 grafts and grafted orthotopically onto full-thickness wounds surgically created in athymic mice (nu/nu, Jackson Labs, Bar Harbor, ME; $n = 3$–6 per group). An occlusive dressing with antibiotic ointment was applied to the grafted area as previously described.[41] The dressings and stent sutures were removed at week 3 and the animals were maintained without dressings for the duration of the in vivo evaluation period. Photographs, tracings for the percent pigmented area, and chromameter measurements were collected from weeks 3–12 after grafting. The percent pigmented area was quantitated using computer planimetry and defined as the pigmented area divided by the total wound area × 100. The animals were euthanized and the grafts biopsied at week 12. Frozen sections from the graft biopsies were utilized for direct immunofluorescence to detect HLA-ABC. This confirmed the persistence of human cells in the healed grafts.[42]

Statistical analysis

The L-Dopa–positive cells, chromameter measurements, and percent pigmented area data were analyzed by one-between (group), one-within (time) repeated measures analysis of variance. Following verification of overall significance, univariate ANOVAs were performed at each time point and the multiple comparisons of groups were made by Tukey's test to determine significant differences ($p < 0.05$).

RESULTS

An 11-year-old black burn victim was grafted with both meshed autograft and autologous CSS (Figure 1). Hypopigmentation is evident in the CSS on the abdomen and upper left thigh with foci of pigment when compared to the more evenly pigmented autograft on the arms and upper right thigh. These pigmented foci are the result of passenger HMs that survive in the HK cultures. This patient is an example of incomplete CSS pigmentation and a poor cosmetic consequence.

Initially, an in vitro study was performed to examine the nature of pigmentation in CSS. Collagen-glycosaminoglycan substrates were inoculated sequentially with HFs (5×10^5/cm^2) and a mixture of HKs (1.1×10^6/cm^2) and HMs (0, 1.1×10^2, 1.1×10^3, or 1.1×10^4/cm^2). Figure 2A shows a histological section of CSS in vitro stained with Toluidine Blue at day 21. A mature epithelium with multiple layers of cornification was present attached to a dermal substitute containing HFs. This histology from the 1.1×10^4 HM/cm^2 density was representative of all experimental groups. A double labeling immunohistochemical method identified HMs located in the basal epidermis (Figure 2B). The 1.1×10^4 HM/cm^2 density shown in Figure 2 shows the presence of HMs at the dermal-epidermal junction at day 21. The lower HM densities had fewer HMs in the epidermis stained by this technique (data not shown).

L-Dopa, the substrate for the melanin synthetic pathway, was incubated with the isolated epidermal layer of the CSS. Figure 3A shows a representative histological section of CSS for all study groups at day 7 in vitro. Figures 3B, C, and D are the L-Dopa–positive cells in epidermal isolates at day 7 from the 0, 1.1×10^3, or 1.1×10^4 HM/cm^2 groups, respectively. The L-Dopa–positive cells in the control group without added HMs

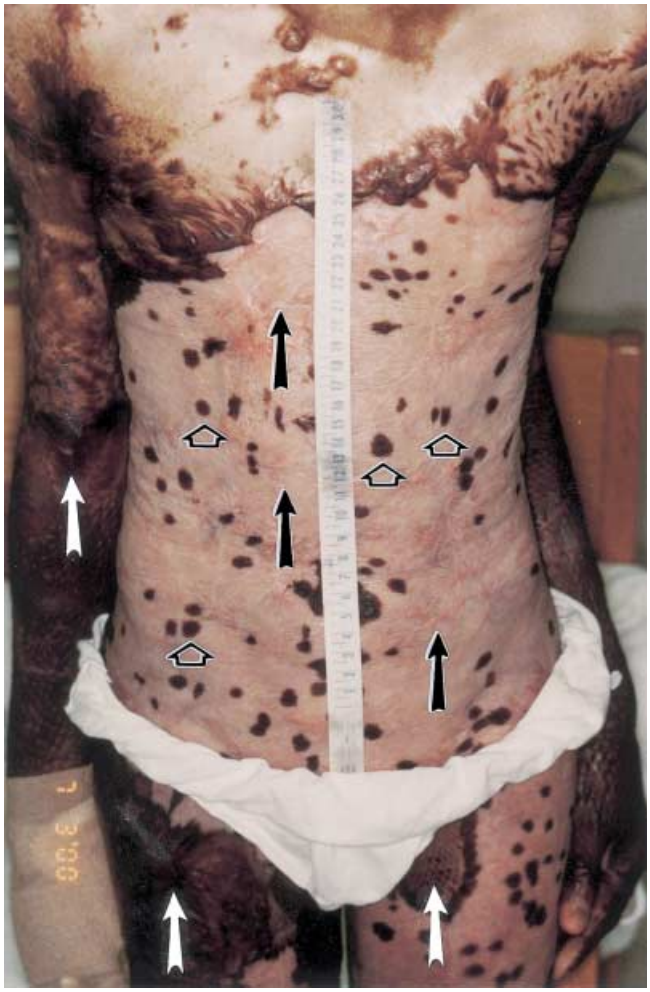

FIGURE 1. Healed cultured skin substitutes from a burn patient. CSS from the abdomen and anterior thighs of an 11-year-old black patient were photographed approximately 9 months after grafting. Hypopigmentation is evident in the CSS (black arrows) as compared to the pigmented meshed autograft (white arrows). Foci of pigment represent passenger HMs that survived in the HK cultures and were inoculated with the HK at the time of CSS preparation (open arrowheads).

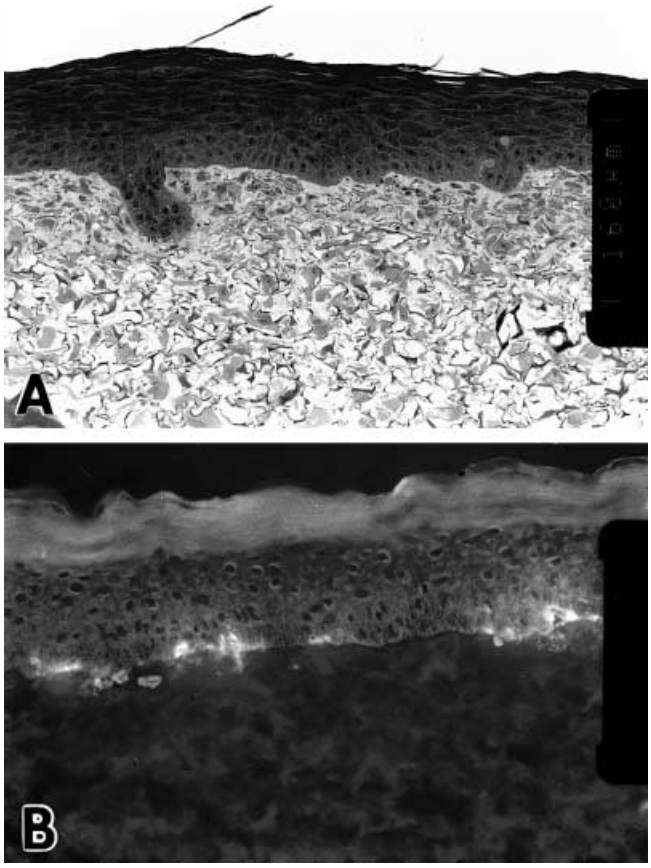

FIGURE 2. Photomicrograph of melanocytes in cultured skin substitutes. (A) CSS histologic sections were stained at day 21 with Toluidine Blue and (B) the monoclonal antibody, Mel-5 + a FITC-labeled secondary antibody to identify HMs. The HMs were located in the basal epidermis. Bar = 160 μm.

(Figure 3B) represent passenger HMs that were cultured with the HKs. The addition of 1.1×10^3 HM/cm^2 to the CSS resulted in more L-Dopa–positive cells than the control group (Figure 3C). In Figure 3D, the highest HM density, 1.1×10^4 HM/cm^2, has numerous L-Dopa–positive HMs in the epidermal isolates. The quantitative assessment of L-Dopa–positive cells is shown in Figure 3E. There was a statistically significant increase in the number of L-Dopa–positive cells in the 1.1×10^4 HM/cm^2 group as compared to the other two groups at all time points.

The color scoring or L* value ranges from absolute black (0) to absolute white (+ 100) was measured weekly on the CSS in vitro. The mean L* values from 0, 1.1×10^2, 1.1×10^3, and 1.1×10^4 HM/cm^2 groups (Figure 4) indicated that the highest HM density, 1.1×10^4 HM/cm^2, was significantly darker than the other treatment groups at each weekly time point. Also, the 1.1×10^4 HM/cm^2 density became darker in a time-dependent manner over the 3-week time course. No time-dependent statistical differences in chromameter measurements were observed within the other three study groups.

Athymic mice were grafted with CSS prepared with 0, 1.1×10^2, 1.1×10^3, and 1.1×10^4 HM/cm^2 and were evaluated for changes in pigmentation from weeks 3–12 (Figure 5 and Figure 6A). The HK strain used for these animal studies was carefully chosen in an initial in vivo study to be virtually devoid of passenger HMs. The control group without added HM had little to no pigment (Figures 5A and B), and the percent pigmented area was near 0 (Figure 6A) throughout the study. The 1.1×10^2 HM/cm^2 had small 1–2 mm foci of pigment that increased in size to about 35% by 12 weeks (Figures 5C and D), and the percent pigmented area was significantly greater than the control. The 1.1×10^3 HM/cm^2 group had pigmented foci that coalesced to pigment about 75% of the CSS graft (Figures 5E and F). The percent pigmented area for the 1.1×10^3

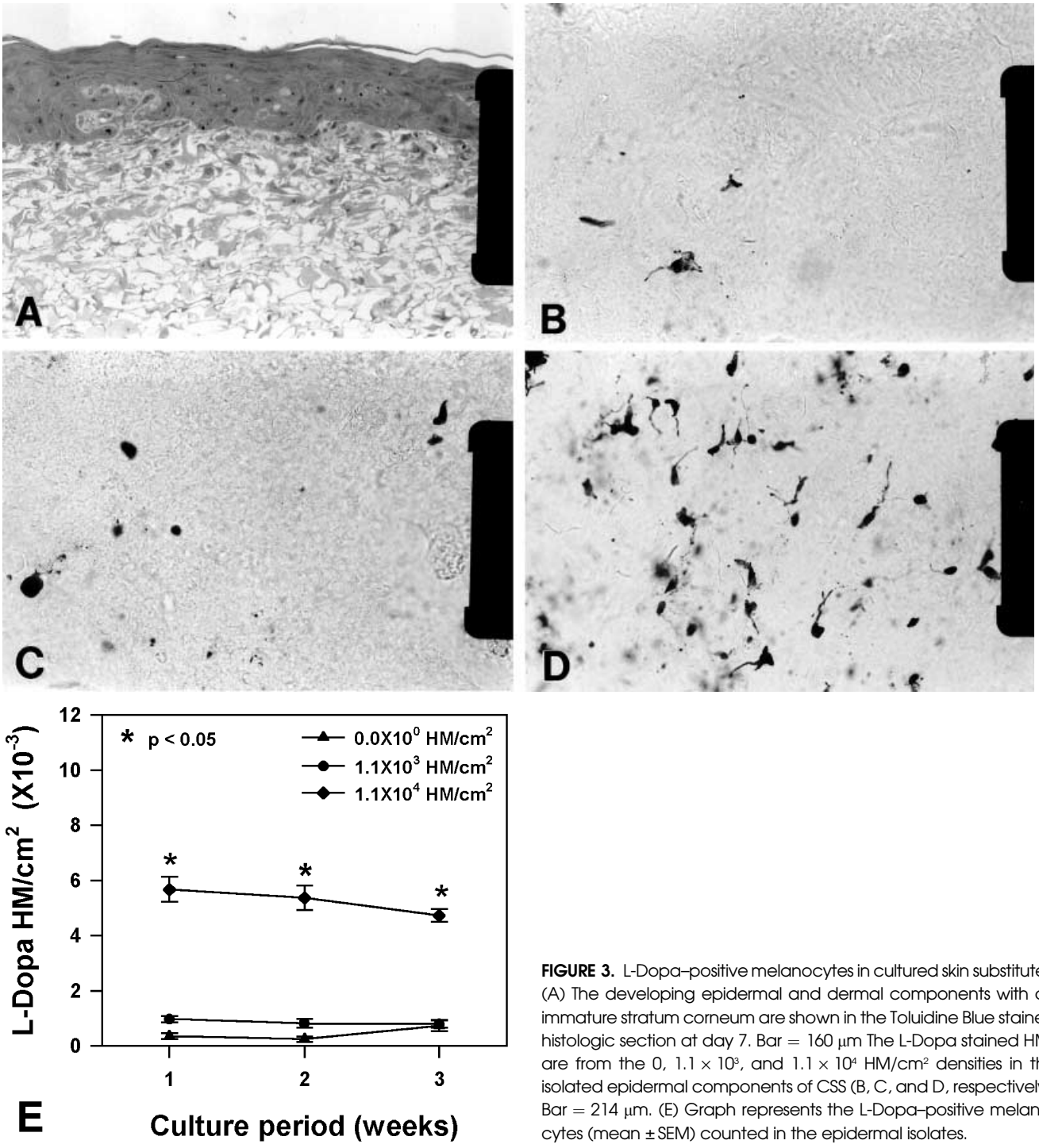

FIGURE 3. L-Dopa–positive melanocytes in cultured skin substitutes. (A) The developing epidermal and dermal components with an immature stratum corneum are shown in the Toluidine Blue stained histologic section at day 7. Bar = 160 μm The L-Dopa stained HMs are from the 0, 1.1×10^3, and 1.1×10^4 HM/cm^2 densities in the isolated epidermal components of CSS (B, C, and D, respectively). Bar = 214 μm. (E) Graph represents the L-Dopa–positive melanocytes (mean ± SEM) counted in the epidermal isolates.

HM/cm^2 group was significantly greater than the control and 1.1×10^2 HM/cm^2 groups during weeks 4–12 (Figure 6A). The 1.1×10^3 HM/cm^2 density showed a steady rate of increasing melanization that continued through the end of the experiment. The highest density, 1.1×10^4 HM/cm^2 grafts were pigmented almost completely by week 5 (Figures 5G and H). The 1.1×10^4 HM/cm^2 pigmented area was significantly greater than all other groups during the entire 12-week test period (Figure 6A).

The chromameter was used to measure the color progression of the healing grafts from weeks 3–12 after surgery (Figure 6B). The L* values for the control group without added HMs and the 1.1×10^2 HM/cm^2 CSS were not significantly different from each other at any time point. The 1.1×10^3 HM/cm^2 group was significantly darker than the control group at weeks 6 and 12, and vs. the control and 1.1×10^2 HM/cm^2 groups at weeks 8 and 10. The grafts with the highest HM density, 1.1×10^4 HM/cm^2

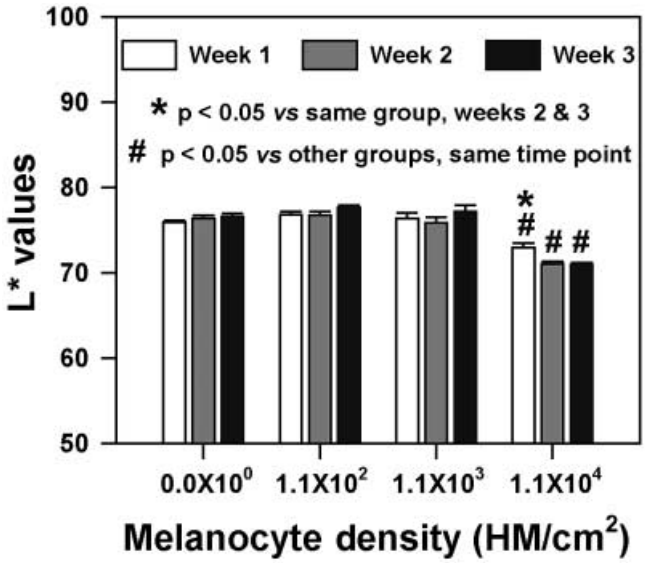

FIGURE 4. Chromameter measurements from cultured skin substitutes in vitro. The bar graph represents the chromameter L* values (mean ±SEM), which can range from absolute black (0) to absolute white (+ 100) from the CSS at weeks 1, 2, and 3. The 1.1×10^4 HM/cm^2 group was significantly darker than the other groups at all time points.

were significantly darker than all other groups at weeks 3–6 after surgery and versus the control and 1.1×10^2 HM/cm^2 groups at weeks 8, 10, and 12. The two highest HM densities were different from each other in the early postgrafting phase, but the pigment in the 1.1×10^3 HM/cm^2 group increased with time so that the chromameter readings for these groups were not statistically different 8 weeks after grafting.

DISCUSSION

The clinical success of CSS is compromised by abnormalities in the distribution of color in healed skin. Pigmentation is influenced by the homogeneity of the melanocyte distribution and the magnitude of pigment expression. In Figure 1, the melanization of the clinical CSS is heterogeneous with multiple pigmented foci. Passenger HMs persist in the HK culture and lead to a range of pigment patterns from diffusely hyperpigmented to irregularly hypopigmented.[4–6] Hypopigmentation is most commonly seen in CSS grafts prepared from cryopreserved HKs because the passenger HMs do not survive the cryopreservation process well. Initial sets of CSS are prepared from unfrozen HKs and have a higher percentage of passenger HMs than CSS generated from cryopreserved HKs.[6] Therefore, the CSS inoculated with unfrozen HKs are more pigmented than CSS made from cryopreserved cells. The final set of CSS for the back of the patient in Figure 1 was prepared from cryopreserved HKs and was almost totally devoid of pigment (photograph not shown). Because HMs protect epidermal cells by transferring melanosomes to surrounding HKs, the absence of HMs compromises this protective function.[7] Selective melanocyte culture and inoculation into the CSS would prospectively restore the normal structure and function of HMs to the skin.

These studies have examined the number of HMs needed to fully restore the pigment in CSS in vivo. The HFs, HKs, and HMs were selectively cultured individually and added to the collagen-glycosaminoglycan substrate in a controlled manner. Importantly, HMs were cultured in media that did not contain phorbol esters, thereby reducing the risk of neoplastic transformation after grafting. The HKs and HMs were mixed and inoculated onto the substrate 1 day after the HFs. The HMs were situated in the basal epidermis (Figure 2) at 3 weeks in vitro and the number of L-Dopa–positive HMs in the epidermal isolates correlated directly with the HMs' inoculation density (Figure 3). The CSS color was assessed with a Minolta Chroma Meter, a noninvasive biophysical instrument. The in vitro chromameter readings indicated that the 1.1×10^4 HM/cm^2 density was significantly darker than the other groups during the 3-week culture period (Figure 4). This highest HM density progressively darkened with time. When CSS prepared with HMs were grafted to athymic mice, the control CSS with no additional HMs remained virtually unpigmented throughout the 12-week experiment (Figures 5 and 6). CSS with added melanocytes showed density-dependent and time-dependent increases in pigmentation. These data suggest that CSS with 1.1×10^3 HM/cm^2 would have repigmented completely because the percent pigmented area was still increasing at the end of the experiment. Because pigmentation in wounds grafted with CSS continues to develop over several years, the 1.1×10^3 HM/cm^2 density may be sufficient to repigment the CSS completely over time. To gain an additional degree of control of CSS pigmentation, an estimate of the passenger HM density will be necessary for each HK culture. At the time of HK expansion for CSS, a cell suspension could be labeled with the monoclonal antibody, Mel-5 and the percent HMs determined by either immunocytochemistry or flow cytometry.[31]

The efficiency at which the HMs survive the CSS preparation and culture procedure must be studied because a significant HM loss takes place between inoculation and healing of CSS grafts. The highest melanocyte density inoculated in this experiment was approximately 1.1×10^4 HM/cm^2, but the extrapolated L-Dopa data in Figure 3 indicates that there were about 5.0×10^3 HM/cm^2 at 3 weeks in culture. This represents a 50% decrease in HMs in the CSS. At the 10-fold lower

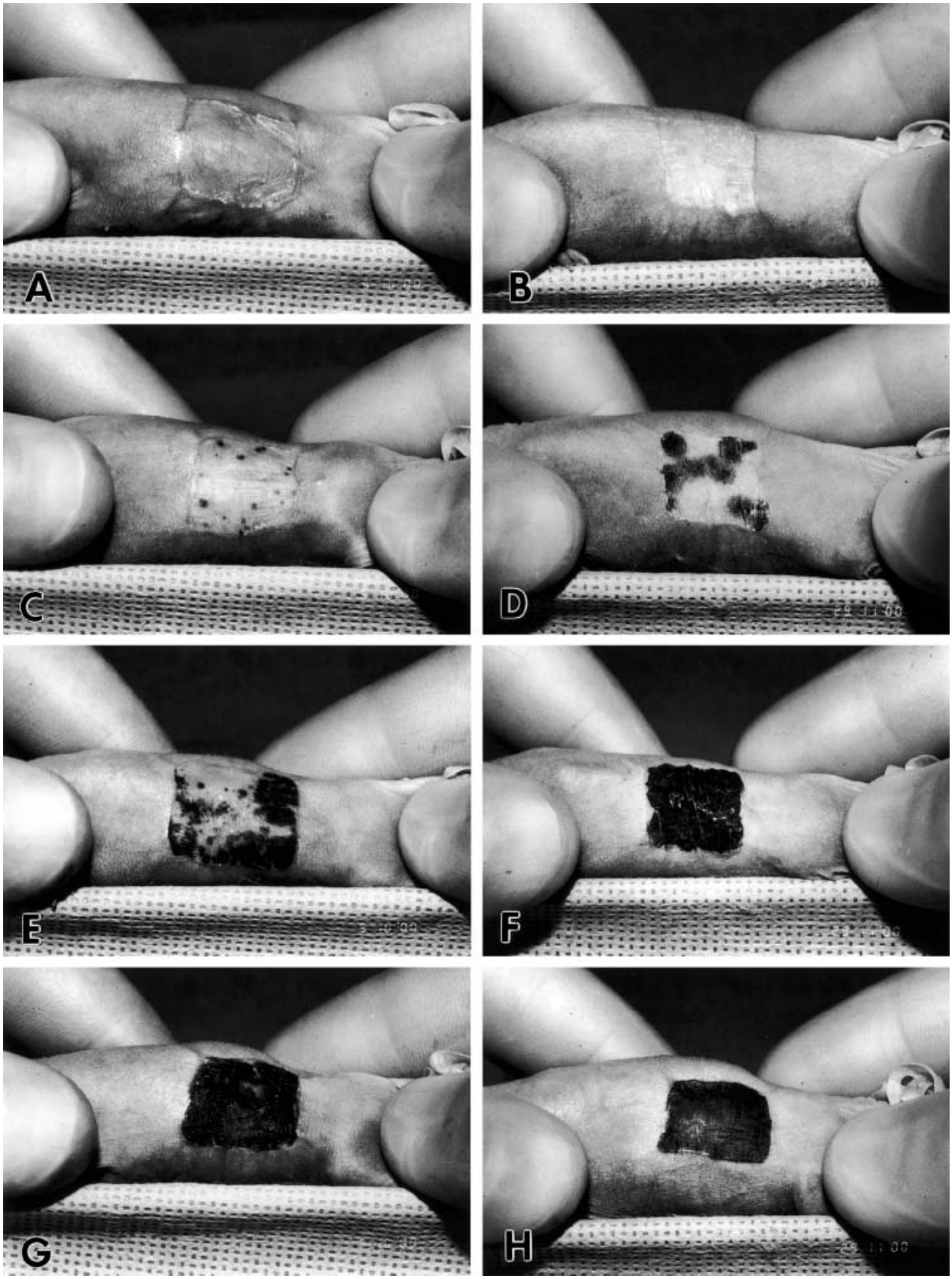

FIGURE 5. Pigment development of cultured skin substitutes on athymic mice. The progression of pigment is shown at weeks 5 (A, C, E, G) and 12 (B, D, F, H) after grafting with CSS inoculated with 0, 1.1×10^2, 1.1×10^3, and 1.1×10^4 HM/cm^2. The control CSS with no additional HMs remained unpigmented (A, B). The 1.1×10^2 HM/cm^2 group had pinpoint foci of pigment at 5 weeks that increased to partially repigment the CSS by 12 weeks (C, D). The foci of pigment enlarged and coalesced in the CSS with 1.1×10^3 HM/cm^2 by 12 weeks (E, F) and the 1.1×10^4 HM/cm^2 group was completely pigmented (G, H).

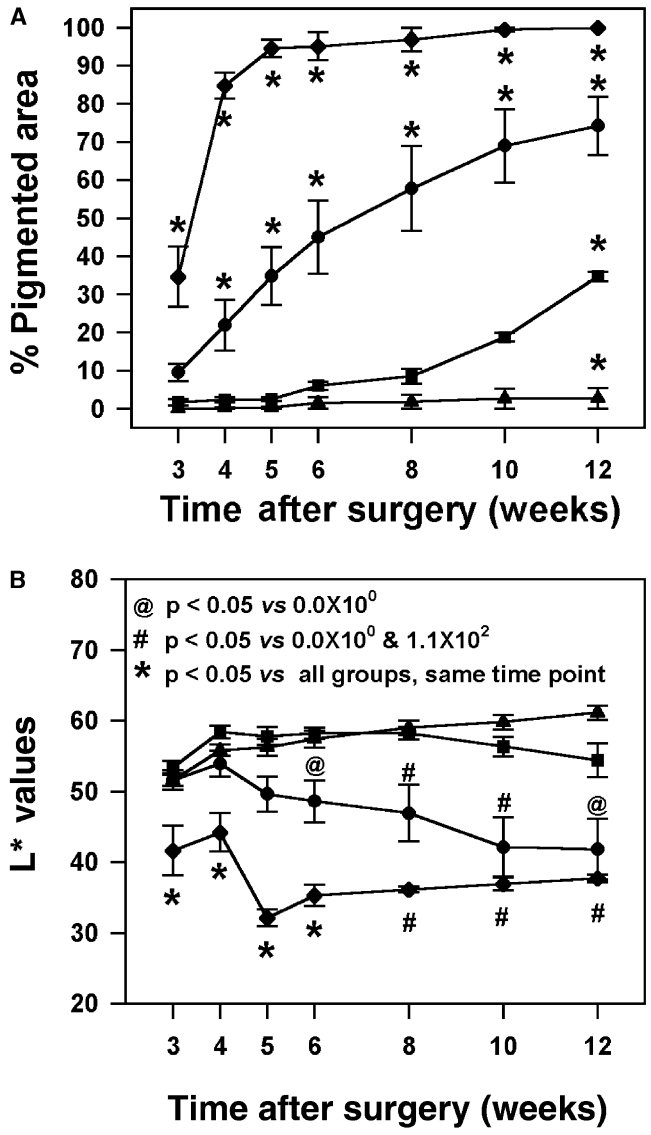

FIGURE 6. In vivo assessment of pigment in cultured skin substitutes. The graphs of percent pigmented area (A) and chromameter L* values (B) show the change in pigmentation of CSS inoculated with 0 (▲), 1.1×10^2 (■), 1.1×10^3 (●), and 1.1×10^4 (◆) HM/cm^2 from 3 to 12 weeks after grafting to athymic mice. The 1.1×10^3 and 1.1×10^4 HM/cm^2 groups had the largest percent pigmented areas (A; *, $p < 0.05$ vs. all groups at the same time point). The CSS with 1.1×10^3 and 1.1×10^4 HM/cm^2 were darkest (lower L* values) by chromameter evaluation (B).

density, 1.1×10^3 HM/cm^2, the HM loss equaled about 30%, decreasing to 8×10^2 HM/cm^2. This inefficiency of HM survival in CSS requires the propagation of 30–50% more HMs to adequately repopulate the CSS. Because HMs tend to grow more slowly in vitro than HKs, the HM culture time may become a limiting factor. About 2000 cm^2 of collagen substrate must be inoculated to provide 1200 cm^2 to a patient because of substrate shrinkage during CSS maturation. This would require 2.2×10^6 or 2.2×10^7 HMs for either the 1.1×10^3 or 1.1×10^4 HM/cm^2 densities, respectively. Several weeks are required to establish actively growing HMs depending on the size of the initial biopsy that can be dedicated to selective HM culture and the inherent growth rate of each strain of HM. The priority for biopsy utilization from a burn patient must be for culturing HFs and HKs, so the portion that can be used for HMs may be very small. The first set of CSS is inoculated at about 2.5 weeks after beginning the primary culture, making the ability to generate HMs for clinical CSS a challenge.

Data presented in this report show that normal human melanocytes may be cultured selectively in media without phorbol esters and added to the epidermal component of cultured skin substitutes for treatment of full-thickness skin wounds. Cutaneous pigment develops as a function of melanocyte density, and time after grafting and complete restoration of pigment can be obtained approximately 1 month after grafting of CSS. Improvements in efficiency of melanocyte transplantation are expected to reduce either the numbers of melanocytes or the time required to restore skin color. These results indicate that clinical morbidity from hypopigmentation may be reduced or eliminated in the future by regulation of melanocyte densities in CSS for closure of full-thickness skin wounds.

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