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doi: 10.1111/j.1600-0749.2006.00325.x

Increased expression of integrins and decreased apoptosis correlate with increased melanocyte retention in cultured skin substitutes

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Summary

Losses of human melanocytes (HM) in transplantation of cultured skin substitutes (CSS) may result from poor cellular attachments. To test this hypothesis, HM integrin expression was measured in four culture media: (a) melanocyte growth medium (MGM), an HM proliferation medium; (b) UCMC 160, a CSS maturation medium; (c) mMGM, modified MGM with 1.8 mM calcium; and (d) modified UCMC 160 with HM supplements (mUCMC 160). HM grew well in all media except UCMC 160. Increased expression of β1, β4, $\alpha 3\beta 1$ and α5 integrins on HM cultured in MGM and mMGM versus UCMC 160 was found by flow cytometry. Annexin V-allophycocyanin (APC) labeled HM in apoptosis and increased significantly in UCMC 160 (31.1%) compared with MGM (11.9%) or mMGM (13.9%). CSS were incubated in UCMC 160, mMGM or mUCMC 160 media, and grafted to athymic mice. In the mMGM group, grafts were darker as measured with a chromameter through 6 weeks and the average number of basal HM per field was greater at 12 weeks postgrafting. Increased graft loss was observed in the mMGM group which corresponded with the poor epidermal morphology in vitro. Although HM retention improved in vivo using mMGM to culture the CSS, the stability of the epidermis decreased. These results indicate that expression of integrins on HM in vitro correlates with HM retention in CSS and short-term survival after transplantation, but that long-term survival depends also on stable epithelium.

Key words: Pigmentation/melanocytes/integrins/apoptosis/engineered skin/wound healing

Received 6 December 2005, revised and accepted for publication 12 May 2006

Introduction

Transplantation of human melanocytes (HM) has been performed for treatment of conditions of lost pigmentation including vitiligo and burn scars (Beck et al., 2003; Guerra et al., 2000; Olsson and Juhlin, 1993; Pianigiani et al., 2005; Stoner and Wood, 2000). Models of reconstructed skin containing epidermal human keratinocytes (HK) and human fibroblasts (HF) prepared with high HM inoculation densities (5–10% of HK inoculum) have resulted in a low efficiency of melanocyte survival at the dermal-epidermal junction (Duval et al., 2002; Meier et al., 2000). Cultured skin substitutes (CSS) have been used for clinical treatment of burns, scars, chronic wounds, and congenital skin defects (Boyce and Warden, 2002; Passaretti et al., 2004), but generate skin that is consistently hypopigmented (Figure 1). Treatment of full-thickness wounds, such as acute burns, with CSS requires the rapid expansion of epidermal HK to construct the first CSS by 3 weeks after collection of the clinical biopsy. Reduced pigmentation in CSS results from the dilution of the slower growing passenger HM in the HK cultures and decreased HM survival after cryopreservation. Selective HM culture is necessary to generate the quantity of HM needed to repopulate repeated CSS preparations (approximately 2000 cm^2 per CSS set) beginning by about 3 weeks in culture, and continuing until wound closure is completed. This demanding requirement for HM proliferation necessitates improving the efficiency and predictable transplantation of HM to achieve uniform pigmentation of CSS. Hypothetically, the HM losses may result from inadequate expression of adhesion molecules or insufficient synthesis of basement membrane proteins in the immature CSS.

Human melanocytes have been shown to express α3, α6, αv, and β1 in normal human skin (Zambruno et al., 1993). In culture, HM express α2, α3, α5, α6, αv, and β1 subunits and the following heterodimers were detected on HM in vitro: $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, and $\alpha v\beta 3$ (Danen et al., 1996; Hara et al., 1994; Mengeaud et al., 1996; Morelli et al., 1993; Zambruno et al., 1993). The α2 subunit was

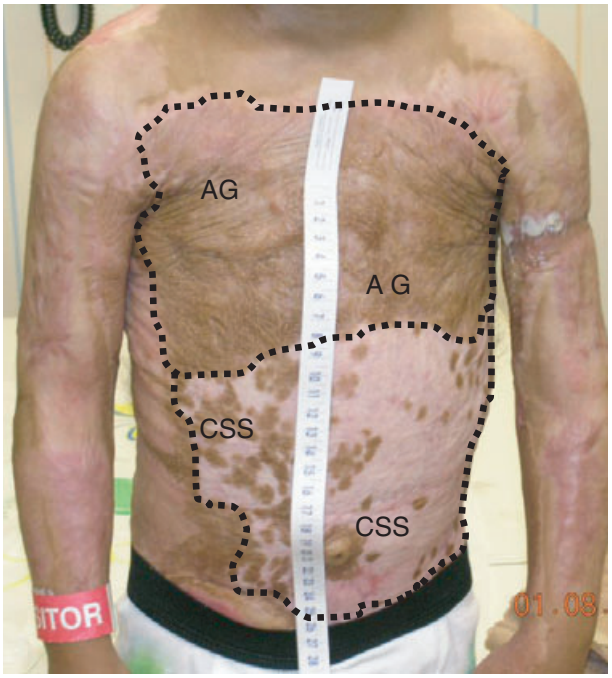

Figure 1. Clinical results at 1 yr after stable engraftment and healing of split-thickness skin autograft (AG), and autologous cultured skin substitutes (CSS). AG develops uniform pigmentation, but CSS remains hypopigmented indefinitely. Scale in cm.

localized along the length of HM dendrites, whereas the $\alpha v\beta 3$ and $\alpha 5$ integrins were found at the midpoints and tips of dendrites in focal contacts. Conversely, the $\alpha 6$ and $\beta 1$ subunits were identified on the entire HM cell surface (Hara et al., 1994; Zambruno et al., 1993). HM attachment to laminin-5 in the basement membrane is directed via $\alpha 3\beta 1$ integrin and laminin-1 interacts with $\alpha 6\beta 1$ integrin (Danen et al., 1996; Hara et al., 1994; Kreidberg, 2000; Krengel et al., 2005; Mengeaud et al., 1996; Scott et al., 1999). HM adhesion and migration on collagen IV is regulated by $\alpha 2\beta 1$ and $\alpha 3\beta 1$, while fibronectin attachment is mediated by the $\alpha 3\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 3$ integrins (Danen et al., 1996; Morelli et al., 1993; Scott et al., 1992, 1999; Zambruno et al., 1993).

Environmental factors can also regulate HM integrin expression. Media containing calcium, magnesium and/or manganese affected HM adhesion to various substrata in a concentration dependent manner (Hara et al., 1994). These investigators reported that HM adhesion to laminin was mediated by $\alpha 6\beta 1$ in the presence of magnesium and/or manganese and dendricity on laminin or collagen IV was a function of $\alpha v\beta 3$, $\alpha 2$, and $\alpha 5$ expression and extracellular calcium. Phorbol esters increased the $\alpha 3\beta 1$ and $\alpha 5\beta 1$ expression and decreased $\alpha 2\beta 1$, $\alpha 4\beta 1$, and $\alpha 6\beta 1$ expression (Danen et al., 1996; Zambruno et al., 1993). Ultraviolet light down-regulated $\alpha 6$ expression on HM and laminin-1 protected HM against this UV-induced effect (Krengel et al., 2005). An analog of α-melanocyte stimulating hormone (α-MSH) increased HM adhesion to fibronectin and promoted actin cytoskeletal reorganization, whereas endothelin-1 (ET-1) resulted in a distinct pattern of actin stress fiber reorganization and increased HM migration on fibronectin (Scott et al., 1997).

This study examined regulation of integrin expression by alternative culture media, and how culture conditions may contribute to HM loss of adhesion to the developing basement membrane in CSS. HM cultured in a serum-free, phorbol ester-free control medium [melanocyte growth medium (MGM)] were compared with HM grown in a medium for epithelial maturation, cultured skin substitutes maturation medium (UCMC 160), and modifications of these two media for cellular growth, expression of selected integrins and annexin V, an apoptosis marker. CSS prepared in alternative media were evaluated in vitro for morphological properties by histology, melanocyte retention by immunofluorescence microscopy, and epidermal barrier formation by surface hydration. CSS from modified media containing 1.8 mM Ca^{2+} were evaluated after grafting to athymic mice for pigmented area, HM density and average pigment intensity. The results suggest that HM survival before and after transplantation, and expression of pigment correlate with expression of integrins by HM in vitro which is regulated by the combined formulation of the basal medium, and the medium supplements.

Results

A representative clinical outcome of hypopigmentation of CSS is shown in Figure 1. Healed CSS is smooth, soft, and strong, but develops pigmented foci that increase in area over time, but usually are not sufficient to restore uniform distribution of skin color. Each pigmented area is interpreted as resulting from an individual melanocyte retained during preparation of CSS. Expansion of keratinocytes in selective culture effectively dilutes melanocytes in CSS and generates epithelium without pigmentation, analogous to piebald skin.

Four media were used to cultivate HM in vitro. The control MGM growth medium does not contain serum or phorbol esters and was modified melanocyte growth medium (mMGM) with the addition of 1.8 mM calcium, linoleic acid, and ascorbic acid to aid CSS maturation. The UCMC 160 is a CSS medium described in the methods that was modified cultured skin substitutes medium (mUCMC 160) with the addition of the HM supplements, bovine pituitary extract (BPE), ET-1, α-MSH and basic fibroblast growth factor (bFGF). MGM, mMGM, and mUCMC 160 media supported vigorous HM growth. The UCMC 160 medium resulted in HM detachment from the culture vessel by 48–72 h. The HM morphology became bipolar in the UCMC 160 compared with the multi-dendritic HM in the other media (Figure 2A–D). These differences are reflected by significantly lower HM cell numbers when cultured in UCMC

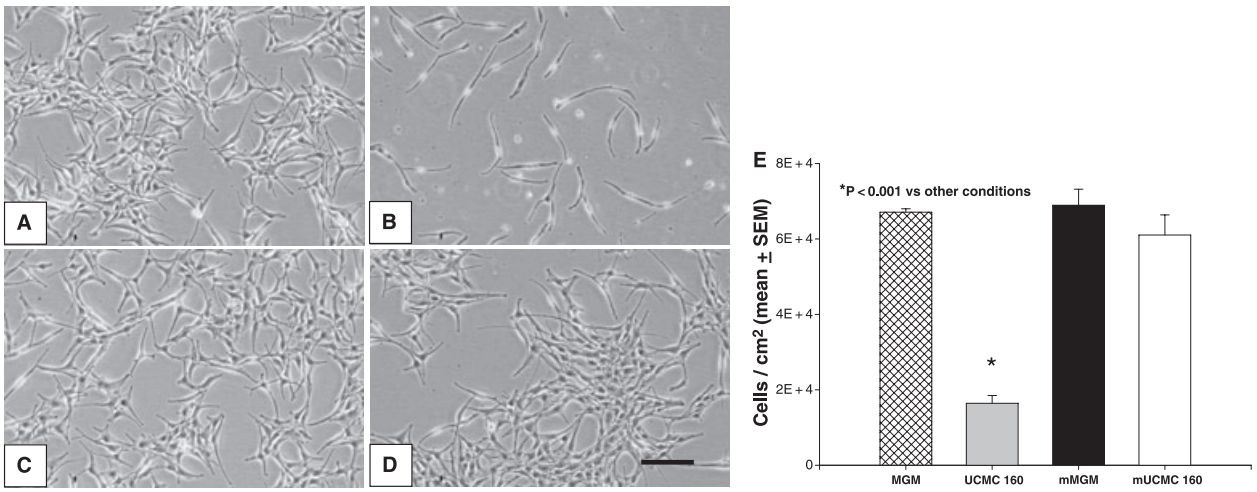

Figure 2. Photomicrographs of melanocytes cultured in alternative media: (A) MGM, (B) UCMC 160, (C) mMGM, and (D) mUCMC 160. HM grew equally well in the control MGM, mMGM, and mUCMC 160 media. Significant reduction in proliferation and increased detachment from the culture vessel were observed when HM were cultured in the CSS medium, UCMC 160 (B). Scale bar = 0.2 mm. (E) Cell counts of HM in the alternative media conditions showing a significant reduction in HM proliferation in UCMC 160 medium.

160 medium (Figure 2E). Flow cytometric analysis revealed that the percent HM positive for $\alpha 2$ and $\alpha 6$ integrins was very low (<10% positive), and conversely, the $\alpha 1$, αv, and $\beta 3$ expression was very high (80–99% positive) in all four media conditions (data not shown). The CSS medium, UCMC 160, significantly decreased the $\beta 1$, $\beta 4$, $\alpha 3\beta 1$, and $\alpha 5$ integrin expression versus the MGM control and the mMGM media (Figure 3). Although the percent of HM expressing these integrins increased in mUCMC 160 medium containing selected HM growth factors, there was no statistical difference from the HM grown in UCMC 160 medium. Calcium was elevated to 1.8 mM in the mMGM compared with 0.2 mM in MGM and no significant differences in HM growth or integrin expression were observed between these two media. Thus, high calcium alone was not responsible for integrin expression reductions seen with UCMC 160 medium.

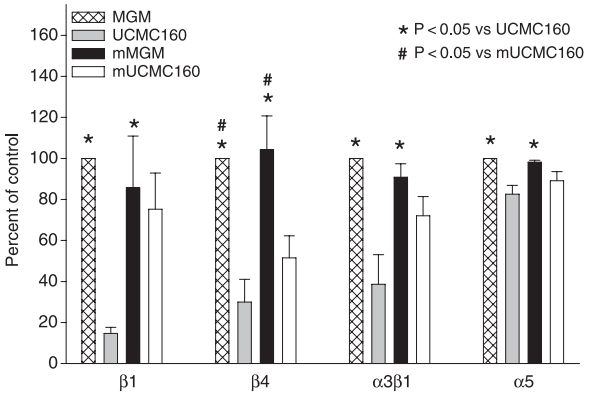

Figure 3. Flow cytometric analysis of HM integrin expression. Data are presented as a percentage of the control HM (cultured in MGM) that stained positively for specific integrin expression. Significant integrin reductions were measured after HM were cultured in UCMC 160 medium for 3 d.

Annexin V-allophycocyanin (APC) and flow cytometry was used to evaluate the role of apoptosis in HM survival when cultured for 4 d in the test media (Figure 4). The annexin V-APC-positive/PI-negative HM represent the early apoptotic subpopulation, the annexin V-APC-positive/PI-positive HM are the late apoptotic fraction and the annexin V-APC-negative/PI-positive HM represent the necrotic cells (Figure 4A,B). A significant proportion of apoptotic HM (annexin V-APC-positive cells) were observed in the upper-right quadrant after incubation in UCMC 160 medium (Figure 4B). The HM cultured in MGM had the lowest percentage of annexin V-positive cells (11.9%), whereas the annexin V-positive fraction increased significantly to 31.1% when cultured in UCMC 160 (Figure 4C). The percent annexin V-positive HM cultured in mMGM medium (13.9%) was not statistically different from the control MGM cells. About 10% fewer annexin V-positive HM were observed in cultures with mUCMC 160 medium (19.7%) compared with the UCMC 160 medium.

Cultured skin substitutes were prepared with mMGM, UCMC 160, and mUCMC 160 media; all of which contained 1.8 mM calcium necessary for epithelial differentiation. The goal was to improve HM survival in the CSS because the mMGM medium did not downregulate HM integrin expression and did not induce HM apoptosis in contrast to the CSS medium, UCMC 160. At 2 weeks of in vitro culture at the air liquid interface (Figure 5), CSS histologies show well-developed dermal and epidermal components in the UCMC 160 (Figure 5A) and mUCMC 160 (Figure 5E) media. The epidermis was poorly organized in the mMGM (Figure 5C). Immuno-

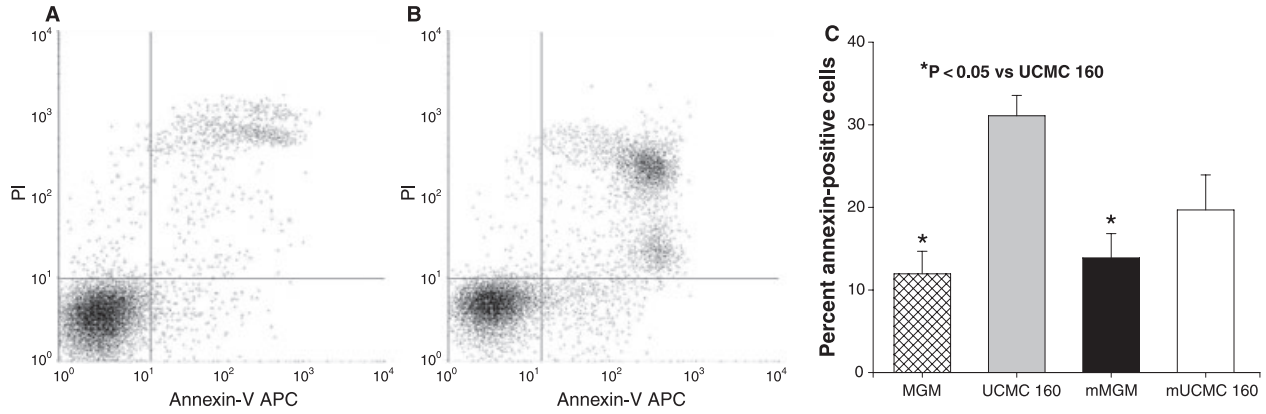

Figure 4. Flow cytometric scattergrams and graphic representation of melanocytes stained for annexin V-APC and propidium iodide after 4 d in test media. (A) and (B) are plots of annexin V-APC and propidium iodide labeled HM cultured in (A) control MGM medium, or (B) UCMC 160 medium. The right hand quadrants represent the annexin V-positive HM consistent with cells in apoptosis. (C) Bar graph of the percent annexin V-positive HM cultured in the four media conditions. The percent annexin V-positive HM cultured in UCMC 160 were significantly higher when compared with either MGM or mMGM.

histochemisty using the Mel-5 antibody to tyrosinase related protein-1 demonstrated clusters of HM (arrows) in CSS cultured in these three media conditions (Figure 5B, D, F). Counts of HM labeled with Mel-5 showed a statistically significant increase of HM in CSS incubated in mMGM medium (Figure 5G). Surface electrical capacitance (SEC) measures surface hydration, an indicator of barrier formation and epidermal maturation. The SEC values (Table 1) from mMGM cultured CSS were elevated indicating deficient barrier development which was consistent with the histologic observations of a poorly stratified epithelium (Figure 5).

Figure 6 shows representative CSS in vivo at 4 weeks (Figure 6A, D, G) and 12 weeks (Figure 6B, E, H) post-grafting from the three media conditions and the corresponding immunohistochemical staining for Mel-5 (Figure 6C, F, I). The pigmentation in vivo increased by 12 weeks post-grafting in the UCMC 160 and mUCMC 160 groups, whereas the mMGM group had unstable epidermis with consequent loss of pigment over time. Counts of basal Mel-5-positive cells at 12 weeks post-grafting showed greater frequencies of HM in CSS cultured in mMGM (Figure 6J). The density of basal HM in vivo increased in CSS in all three media conditions compared with the HM density before engraftment (Figure 5G). Pigmentation of CSS grafted to mice was evaluated using a chromameter. The chromameter L^* value measures the range from black (0) to white (100). The mMGM grafts were statistically darker (lower L^* value) from 3 to 6 weeks post-grafting, but were not statistically different by 8–12 weeks (Figure 7A). The percent pigmented area was highest for the mMGM grafts at week 2, but decreased with time due to epidermal instability (Figure 7B). These data indicate that the mMGM supported improved pigmentation in CSS, but because the medium did not sustain a healthy epithelium, the grafts failed with time.

Discussion

The recovery of patients with extensive burn injuries is dependent on rapid wound coverage. Preparation of CSS may be the only alternative to heal a large burn depending on donor site availability. Due to the slower rate of HM proliferation relative to HK, the addition of adequate HM to the CSS to achieve uniform pigmentation is challenging for acute burn patients. This laboratory prepares approximately 2000 cm^2 of CSS each week for each patient until the wounds are closed. At the current HM inoculation density of $1 \times 10^4/cm^2$ (1% of HK inoculum) 2.0×10^7 HM would be required for each CSS preparation beginning 3 weeks after biopsy collection. The size of the clinical biopsy is determined by area burned and available donor sites with the primary requirement for HK expansion. Predictable pigmentation at lower HM inoculation densities ($<1 \times 10^4/cm^2$) continues to be inconsistent (Figure 7B) and significant losses of HM occur in the CSS (Swope et al., 2002). These considerations require a better understanding of HM adhesion and survival in the CSS so that the HM population can be conserved to create a more physiologically correct CSS.

The CSS have an excellent dermal and epidermal morphology when cultured in the UCMC160 medium (Figure 5A). Epidermal morphology in vitro has been shown to correlate with efficacy of wound closure, and repigmentation after grafting (Boyce et al., 2002). However, pigmentation of healed CSS has been deficient due to the unpredictable retention of HM in vitro at the dermal-epidermal junction in the UCMC 160 medium. A high percentage of HM tend to move through the epidermal compartment into the stratum corneum whether HM are inoculated with HK, or 24 h before HK addition (unpublished observation). The UCMC 160 medium with 1.8 mM calcium significantly reduced the HM growth

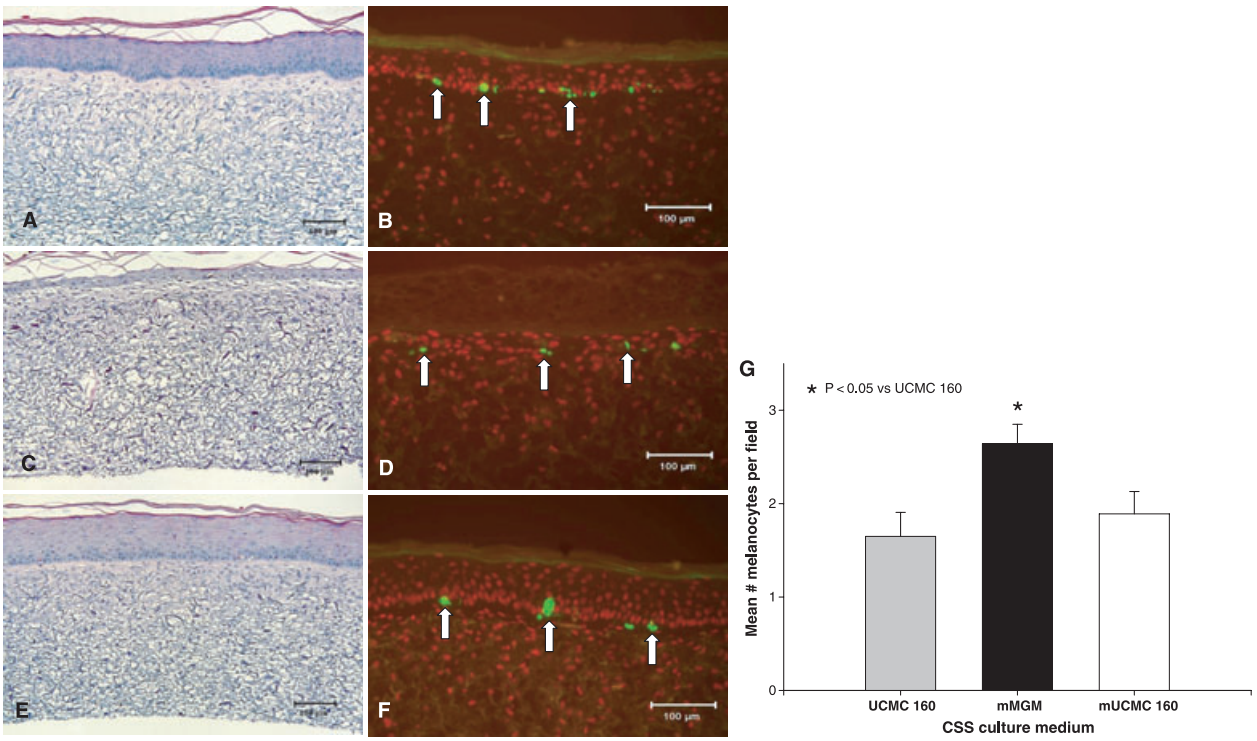

Figure 5. Histologies of CSS cultured in vitro for 14 d. (A), (C), and (E) are paraffin sections stained with hematoxylin and eosin. (B), (D), and (F) are frozen sections stained with Mel-5/goat anti-mouse IgG Alexa fluor 488 antibodies to identify HM (arrows), and with propidium iodide to label all nuclei. CSS were cultured in the following media: (A, B) UCMC 160; (C, D) mMGM and (E, F) mUCMC 160. The CSS morphology after culture with UCMC 160 or mUCMC 160 was very good, in contrast to CSS cultured in mMGM where the epidermis was very thin. Scale bars = 0.1 mm. (G) Counts of basal Mel-5-positive melanocytes showed greater cell numbers/field (550 μm length) in CSS cultured in mMGM than in other conditions.

Table 1. Surface electrical capacitance in vitro

CSS culture media	Day 8 (DPM units)	Day 12 (DPM units)
UCMC 160	666.8 ± 32.2	183.2 ± 11.5
mMGM	801.2 ± 13.0*	373.5 ± 57.5*
mUCMC 160	616.2 ± 15.6	229.2 ± 16.2

*P < 0.05 versus UCMC160 and mUCMC160.

and expression of β1, β4, $\alpha 3\beta 1$, and α5 integrins by flow cytometric analysis compared with the MGM control medium containing 0.2 mM calcium, or mMGM with elevated calcium (Figure 3). In fact, β1, $\alpha 3\beta 1$, and α5 expression recovered in the mUCMC 160 with the addition of HM supplements versus the UCMC 160 medium. These data suggest that factors other than calcium are responsible for the altered integrin expression reported here. The annexin V-positive HM increased about 2.5 times when cultured in UCMC 160 compared with either the MGM or mMGM (Figure 4). These results indicate that programmed HM death was induced by the CSS medium, UCMC 160, in monolayer culture. This result was partially reversed in mUCMC 160 by the HM mitogenic supplements (α-MSH, bFGF, ET-1, and BPE).

Bovine collagen I sponges were inoculated with HF and allowed to mature for 5 d in the UCMC 160. The extracellular matrix molecules detected by immunohistochemistry after HF were inoculated on the collagen I sponge included laminin (except laminin-5), collagen IV and fibronectin (data not shown; Fleischmajer et al., 1998). The HM were inoculated onto the HF-collagen I substrate in the test media before HK inoculation. Because the calcium concentration of mMGM did not adversely affect HM growth or integrin expression, pigmentation was hypothesized to improve in the CSS incubated in mMGM noting that the elevated calcium was needed for epithelial maturation. The HM density in the basal epidermis in the mMGM condition was greater in vitro and in vivo versus the UCMC 160 control CSS (Figures 5G and 6J). During 3–6 weeks after grafting, the pigmentation increased in the CSS incubated in mMGM as anticipated (Figures 6 and 7). However, due to the poor epithelial development in mMGM, these CSS began to be displaced by the surrounding mouse epithelium and pigmented grafts were progressively lost (Figures 5–7). The basal medium is the same for selective cultures of HM and HK, but differs from the UCMC 160 for CSS. Surprisingly, the mMGM did not support the CSS epithelium even though monolayer HK grew

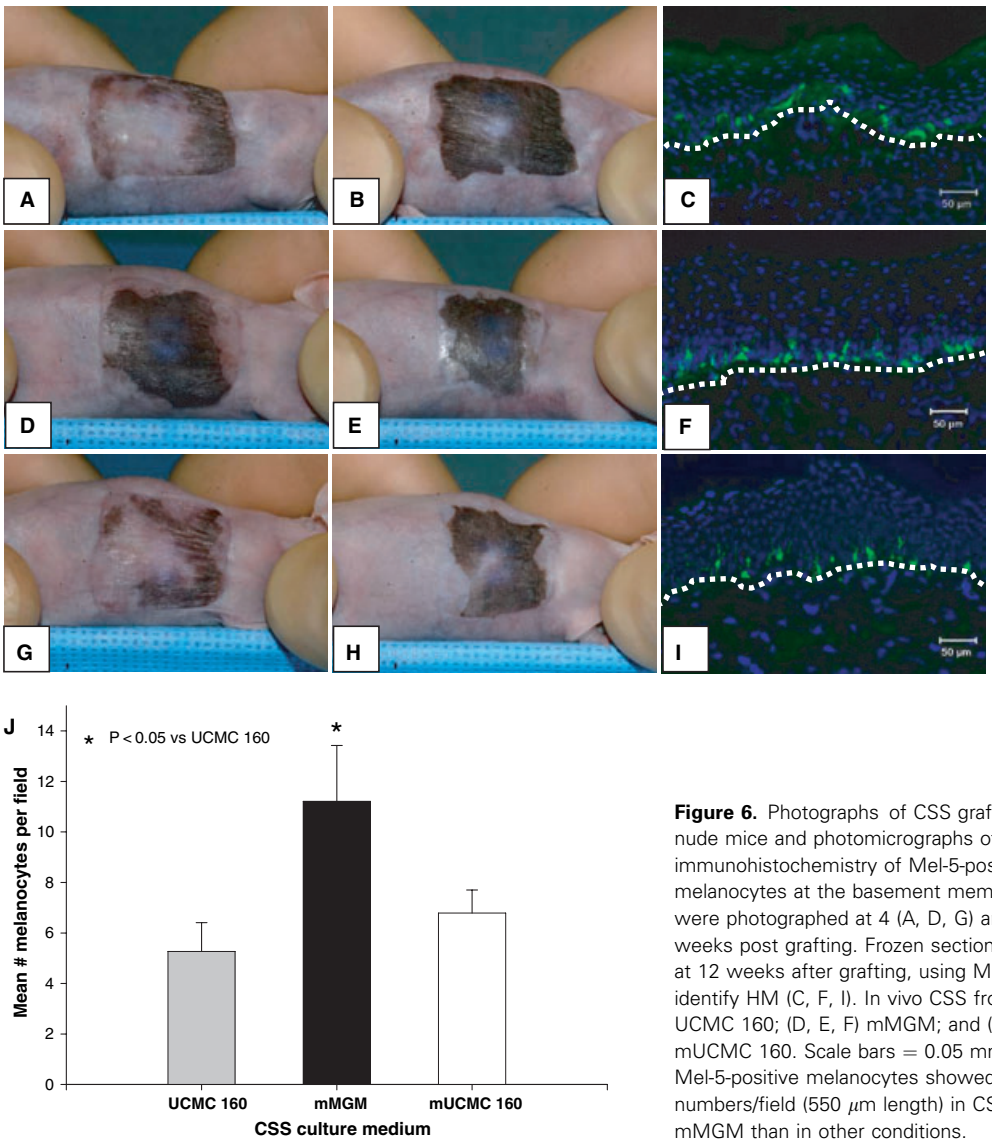

Figure 6. Photographs of CSS grafted to athymic nude mice and photomicrographs of corresponding immunohistochemistry of Mel-5-positive melanocytes at the basement membrane. Mice were photographed at 4 (A, D, G) and 12 (B, E, H) weeks post grafting. Frozen sections were stained at 12 weeks after grafting, using Mel-5 antibody to identify HM (C, F, I). In vivo CSS from: (A, B, C) UCMC 160; (D, E, F) mMGM; and (G, H, I) mUCMC 160. Scale bars = 0.05 mm. (J) Counts of Mel-5-positive melanocytes showed greater cell numbers/field (550 μm length) in CSS cultured in mMGM than in other conditions.

well in the mMGM (data not shown). Further studies will investigate factors necessary for a functional epithelium that will also support the HM at the basement membrane.

Loss of the appropriate cell–matrix interaction results in apoptosis through a process called anoikis (Frisch and Screaton, 2001). Normal tissue homeostasis regulates the progression of cells from proliferation to differentiation and finally to apoptosis. The requirement is not only for attachment of cells to a matrix, but for adhesion to a physiologically functional extracellular matrix which supports growth (Aplin et al., 1999). After 2–3 d of monolayer culture in UCMC 160 CSS medium, HM integrin expression decreased and the HM began to release from the culture surface possibly due to an acquired integrin–matrix deficiency. At the time of HM inoculation onto the collagen I sponge, the HF have synthesized the extracellular matrix proteins, laminin-1, fibronectin, and collagen IV, but an organized basement membrane is lacking in the immature CSS. Laminin-5 is absent until HK are inoculated onto the CSS subsequent to HM addition (data not shown; Fleischmajer et al., 1998). Laminin in the presence of HM growth factors increased melanocyte proliferation and the growth stimulation was abrogated by the addition of antibodies to β1 and α6 integrins (Mortarini et al., 1995). The HM attachment to laminin-1 is mediated by $\alpha 6\beta 1$ via a magnesium and/or manganese dependent process, to collagen IV via the α2, α5, and $\alpha v\beta 3$ integrins, and to fibronectin by way of the α3, α5, αv, and β1 integrins (Hara et al., 1994). Neonatal HM had the greatest attachment to laminin-5 and fibronectin, then laminin-1 and collagen IV (Scott et al., 1999; Mengeaud et al., 1996). Laminin-5 is an important ligand for HM adhesion via the α3-integrin both in vitro

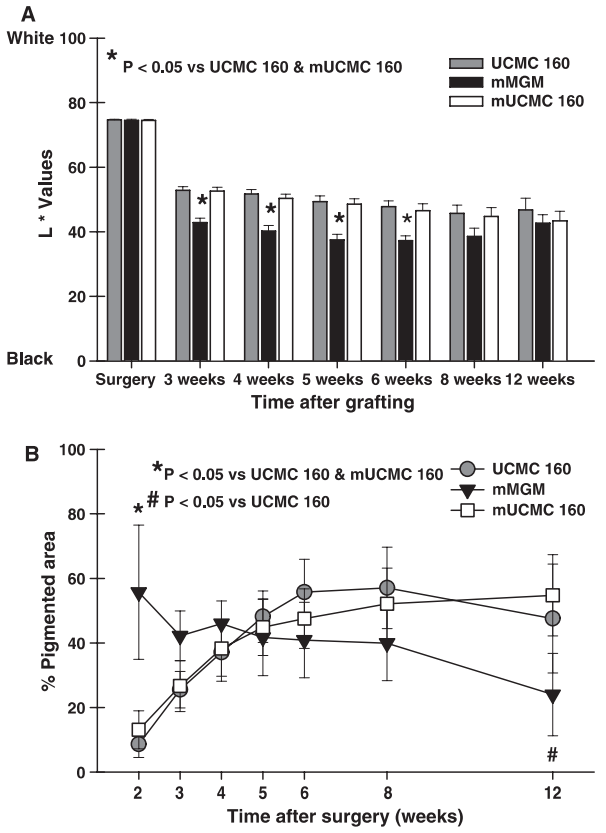

Figure 7. Chromameter measurements and percent pigmented area of CSS in vivo. (A) The *L** value of the chromameter measurement gives a numerical value from black (0) to white (100). The CSS cultured in mMGM in vitro were significantly darker from weeks 3 to 6 after grafting to mice. (B) The pigmented region in each graft in vivo was traced and the percent pigmented area was determined using image analysis. The mMGM grafts had a greater pigmented area at week 2, but progressively declined with time when compared with the increasing pigmented area for the other two conditions. These data indicate that mMGM grafts had the greatest pigmentation early, but it was lost subsequently due to the instability of the epidermis.

and in salt split skin (Mengeaud et al., 1996; Scott et al., 1999). Fibronectin, laminin-1 and laminin-5 are synthesized and secreted by neonatal HM (Scott et al., 1999) suggesting a role for these extracellular matrix molecules in HM attachment. Our studies with neonatal HM cultured in CSS medium resulted in decreased integrin expression of β1, β4, $\alpha3\beta1$, α5 and the low expression of α2 and α6 in all media tested. Downregulation of HM integrins by the CSS medium, the absence of extracellular matrix molecules in the immature CSS at the time of HM inoculation, such as laminin-5 and an unorganized basement membrane predicts an adverse effect on the adhesion of HM to the CSS.

Apoptosis has been reported in melanocytes as a function of attachment substrate and growth factors. HM adherence to fibronectin suppresses apoptosis, and apoptosis was induced when HM attachment to matrix was inhibited (Scott et al., 1997). In addition, β1 blocking antibodies increased apoptosis in HM attached to fibronectin. Growth factors, 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA), insulin, α-MSH and ET-1 act as survival factors for HM (Eberle et al., 2002; Kadekaro et al., 2005; Oka et al., 2004; Scott et al., 1997). In this study, annexin V staining, an indicator of apoptosis, increased after culture of HM in UCMC 160 medium versus the MGM control medium. The mUCMC 160 medium reduced the percent annexin positive HM by about 10% when compared with UCMC 160. Furthermore, the mUCMC 160 medium did not result in the release of HM in monolayer culture, and HM proliferation in mUCMC 160 medium was equivalent to the control MGM. The HM integrin expression was partially restored in the mUCMC 160 medium versus the UCMC 160 medium. More studies are necessary to determine if the α-MSH and ET-1 added to the mUCMC 160 medium helps to protect HM from premature apoptosis.

This report demonstrates that HM integrin expression is regulated by the culture conditions in vitro, and correlates with retention of HM. Regulation of HM integrins via the culture conditions will be studied to stimulate expression of integrins important for HM attachment to matrix. The removal of proliferation factors, such as TPA switches the HM to a differentiation phenotype and increases the α6 expression (Krengel et al., 2005; Zambruno et al., 1993). A critical consideration for HM retention in CSS is stability of the composite epithelium which is necessary for engraftment and survival of pigmented CSS. The extracellular matrix will be evaluated to see if the addition of a low number of HK to the HF-collagen substrate before HM inoculation would promote laminin-5 deposition. Concurrently, by increasing the α3 and α6 integrin expression on HM, improved HM survival may occur at the developing basement membrane in the CSS. These kinds of regulatory factors for adherence of HM in CSS for transplantation in wound treatment may be expected to lead to reduced morbidity from hypopigmentation in CSS, and provide predictable restoration of skin color.

Materials and methods

Cell culture

Human melanocytes were isolated from neonatal foreskins and cultured in the following serum-free and phorbol-ester-free melanoctye growth medium (MGM): modified MCDB 153 (Boyce, 1999), 0.2 mM calcium, 0.4% BPE, 20 pM triiodothyronine, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 1 ng/ml bFGF, 10^{-9} M ET-1, 10^{-8} M α-MSH and 100 units/ml penicillin G, 100 μg/ml streptomycin and 250 ng/ml Fungizone® (PSF) (Swope et al., 1995). HK and HF were isolated simultaneously from surgical discard tissue. These cells were grown in selective growth media and cryopreserved at the end of the primary culture (passage 0), providing a stock of cells for preparation of CSS (Boyce and Ham, 1983). Modified MGM (mMGM) consisted of MGM with the following modifications:

1.8 mM calcium, 2 μg/ml linoleic acid/BSA and 10^{-4} M ascorbic acid-2-phosphate. The CSS medium designated UCMC 160, consisted of DMEM with 1.8 mM calcium, supplemented with 20 pM triiodothyronine, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 2 μg/ml linoleic acid/BSA, 10^{-4} M ascorbic acid-2-phosphate, 1 mM strontium chloride, 10 ng/ml epidermal growth factor, 756 pM progesterone and antimicrobials (Swope et al., 2001). Modified UCMC 160 (mUCMC 160) had 0.4% BPE, 1 ng/ml bFGF, 10^{-9} M ET-1, 10^{-8} M α-MSH added to the UCMC 160 CSS medium. Cell counts were performed in triplicate cultures and repeated (Figure 2).

Cultured skin substitutes preparation

Human fibroblasts were harvested, inoculated (0.5×10^6/cm^2) onto collagen substrates, and incubated at 37°C and 5% CO_2. Beginning on the following day, the HF-collagen substrates were floated in UCMC 160 medium for 5 d. The HF-collagen substrates were subsequently inoculated with 1×10^4/cm^2 HM in the CSS test media: mMGM or mUCMC 160, and control medium UCMC 160. After 24 h, the HF/HM-collagen substrates were floated in the respective media for two more days. HK (1×10^6/cm^2) were harvested and inoculated on to the HF/HM-collagen substrates in the test or control media, and were returned to the incubator ($n = 4$ CSS per condition). On the following day, the CSS were transferred to steel lifting platforms with cotton pads in dishes with the appropriate media. The CSS media were replaced daily, and the HK inoculation onto the CSS was designated as incubation day 0. On day 4, after HK inoculation, progesterone and epidermal growth factor were removed from UCMC 160 and mUCMC 160 media. Paraffin and frozen histologies were collected on day 14 for light microscopy (Figure 5).

Flow cytometric analysis of melanocyte integrin expression

Human melanocytes were inoculated in MGM at 6×10^3/cm^2 onto serum precoated 225 cm^2 flasks for 48 h, and the culture medium was changed to either MGM, mMGM, UCMC 160, or mUCMC 160 for 72 h. The HM were released with 5 mM EDTA for 5 min at 37°C, and pooled with any floating HM from the culture media. The cells were rinsed with cold PBS and fixed for 1 h at room temperature in 4% paraformaldehyde and 0.05% glutaraldehyde. The HM were stored in PBS at 4°C. The following staining protocol was performed at room temperature and the HM were washed twice between each incubation with PBS. The HM were permeabilized with 0.2% Triton-X 100 for 10 min and blocked with PBS + 2% bovine serum albumin + 2% normal goat serum for 45 min. Two million HM were incubated with 2 μg primary anti-integrin antibody for 1 h and washed with two rinses of 0.1% Triton-X 100. The HM were incubated with either goat anti-mouse IgG Alexa Fluor 488 or chicken anti-rat IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). The cells were analyzed using a BD LSR (Becton-Dickinson, San Jose, CA, USA). The instrument was aligned with AlignFlow 2.5 μm beads (Molecular Probes) for the 488 nm lasers. Data were collected and analyzed with CellQuest Pro software (Becton-Dickinson). Cells were excited with the 488 nm line of the argon-ion laser using the Alexa 488 fluorochrome. Dead cells and debris were excluded from analysis by setting the appropriate threshold trigger on forward-angle light scatter. Log fluorescence was collected for Alexa Fluor 488 using a 530/30 band pass filter. In all experiments, 1×10^4 gated events were collected. The isotype control antibody concentration was matched to that of the primary antibodies. The two different α6 antibodies were tested on fixed and unfixed HM with similar results. The following primary antibodies were obtained from BD Biosciences Pharmingen (San Diego, CA, USA): α2 (clone AK-7), α6 (clone GoH3), β1 (clone MAR4), and β4 (clone 439–9b). The α1 (clone FB12), α6 (clone 4F10) and $\alpha 3\beta 1$ (clone M-K1D2) antibodies were purchased from Chemicon (Temecula, CA, USA). Anti-integrin α5 (clone P1D6) and αv (clone 313.6F8) were acquired from Calbiochem (San Diego, CA, USA) and β3 (clone PM6/13) was obtained from Cymbus Biotechnology (Hants, UK). The mouse IgG_1 isotype control was purchased from R&D Systems (Minneapolis, MN, USA) and the rat IgG isotype control was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Two HM cell strains were tested, and four experiments were combined for statistical analysis (Figure 3).

Annexin staining for apoptosis

Human melanocytes were inoculated at 6×10^3/cm^2 and incubated for 48 h in MGM then the culture medium was changed to four test media: MGM, mMGM, UCMC 160, and mUCMC 160 for 96 h. Two HM strains were tested and the experiment performed twice with each strain. For each condition, the floating HM were pooled with the trypsinized HM, centrifuged and rinsed with cold PBS. The cells were resuspended in 1X binding buffer and 1×10^5 HM were incubated with 5 μl annexin V-APC (APC, allophycocyanin; BD Biosciences Pharmingen) and 0.9 μg propidium iodide (PI) for 15 min at room temperature in the dark. Following the addition of 400 μl 1X binding buffer, the cells were analyzed as described above using the 488 and 633 nm lasers. The cells were excited with the 488 nm line of the argon-ion laser and a 633 nm helium neon laser using PI and APC fluorochromes. Log fluorescence was collected for PI using a 575/26 band pass filter and for APC using a 660/13 band pass filter. In all experiments, 1×10^4 gated events were collected (Figure 4). Figure 4C represents data combined from three experiments.

Immunohistochemistry

Frozen sections of CSS at 2 weeks in vitro were fixed in absolute methanol and dried with acetone at –20°C. All steps were carried out at room temperature and three PBS washes were performed between each step. The CSS sections were permeabilized with 0.2% Triton X-100/PBS and then blocked with PBS + 2% BSA + 2% normal goat serum. The sections were incubated with Mel-5, a monoclonal antibody directed to TRP-1 in the melanin synthetic pathway (Signet Labs, Dedham, MA, USA) or the purified mouse IgG2a isotype control (BD Biosciences Pharmingen). The primary antibodies were followed by goat anti-mouse IgG Alexa Fluor 488 (Molecular Probes). Propidium iodide 0.5 μg/ml (Sigma Chemical Co., St Louis, MO, USA) stained the nuclei of all cells. The slides were coverslipped with Fluoromount G (Southern Biotechnology Associates). Basal HM were counted per microscope field (550 μm field length) from four unique sections per CSS and three to four CSS per condition (12–19 fields per condition; Figure 5).

Cultured skin substitutes grafted to athymic nude mice were prepared for frozen sectioning at 12 weeks post-grafting. The sections were fixed as above. A Mouse-On-Mouse (MOM) fluorescein immunodetection kit (Vector Labs, Burlingame, CA, USA) designed to localize mouse primary antibodies on mouse tissues was used to label HM in the grafted CSS using the Mel-5 antibody. Sections from HLA-ABC positive CSS were stained and the basal HM quantitated per microscope field (550 μm field length). HM from three unique CSS sections per animal were counted ($n = 3$ mice for mMGM, $n = 6$ mice for UCMC 160 and mUCMC 160; 140–190 fields per condition; Figure 6).

Surface electrical capacitance

Skin surface hydration is measured by SEC and is inversely proportional to the electrical impedance (Boyce et al., 1996; Supp

et al., 1999). SEC was collected using the NOVA Dermal Phase Meter (DPM 9003; NOVA Technology, Portsmouth, NH, USA) from the CSS grafts in vitro. By applying an alternating current between two electrodes at the skin surface, an electrical phase shift can be measured by the NOVA meter. Lower SEC readings correspond to less water movement through the stratum corneum, an indicator of a functional CSS barrier. On culture days 8 and 12, SEC data were collected from four sites on each CSS in vitro (quadruplicate readings from 4 CSS per condition), SEC data (mean ± SEM) are expressed in DPM units (arbitrary value measured by the DPM 9003) and represent the value obtained after 10 s of direct contact of the DPM 9003 probe with the CSS surface (Table 1).

Surgical grafting of CSS and assessments

All animal studies were previously approved by the University of Cincinnati Institutional Animal Care and Use Committee. At culture day 14, 4 cm^2 CSS grafts were grafted orthotopically onto full thickness wounds prepared surgically in athymic mice (nu/nu; Jackson Labs, Bar Harbor, ME, USA; n = 7–8 per CSS condition). A semi-occlusive dressing with antibiotic ointment was used to cover the grafted area (Swope et al., 2002). The dressings and stent sutures were removed at week 2 and the animals were maintained without dressings for the remainder of the in vivo assessment period. Photographs, tracings of the pigmented area and chromameter measurements were taken of the CSS in vivo. The percent pigmented area (n = 7–8 animals/group; Figure 7B) was quantified using computer planimetry and defined as the percent pigmented area divided by the total wound area X 100. The animals were euthanized and biopsies collected at week 12 for frozen and paraffin sections. Frozen sections of the grafts were evaluated for HLA–ABC to confirm the persistence of human cells in the healed grafts (Boyce, 1999).

Chromameter measurements

Reflectance spectroscopy in situ was used to measure the visible spectrum of light reflected from the cultured skin (Swope et al., 1997). 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 (Muizzuddin et al., 1990; Weatherall and Coombs, 1992). The L^* value correlates to perceived lightness and ranges from absolute black (0) to absolute white (100) and 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 (Figure 7A). 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 = 5–6 animals/group).

Statistical analysis

The flow cytometry data, annexin V-APC results and SEC data were analyzed by a one-way analysis of variance (ANOVA; Sigma Stat). Significance was determined on all pairwise multiple comparisons by Tukey's Test ($P < 0.05$). Significant differences in pigmentation analyses (L^* values and percent pigmented area) were identified by RM-ANOVA using the MIXED procedure method in SAS, followed by pairwise comparisons of the least-square-means for all significant ANOVAs.

Acknowledgements

The authors thank Jodi Miller and Deanna Snider for their technical assistance. These studies were supported by grants from the National Institutes of Health (GM50509), and Shriners Hospitals for Children (8450 and 8670).

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