

PIGMENTATION AND MICROANATOMY OF SKIN REGENERATED FROM COMPOSITE GRAFTS OF CULTURED CELLS AND BIOPOLYMERS APPLIED TO FULL-THICKNESS BURN WOUNDS^{1,2}

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Rapid coverage and epithelial closure of extensive burns remains a major requirement for patient recovery. Although many skin substitutes have been described, permanent regeneration of both epithelial and connective tissues after a single surgical application of a skin substitute has not become routine. To replace both dermal and epidermal skin, cultured skin substitutes (CSS) were prepared from autologous keratinocytes and fibroblasts seeded onto collagen-glycosaminoglycan (C-GAG) substrates. CSS were applied to excised, full-thickness burns on 5 patients. Histologic analysis showed a fully stratified, hyperkeratotic epidermis within 12 days of grafting with little to no evidence of an inflammatory reaction. Epidermal and connective tissues are interdigitated in analogy to rete pegs and dermal papillae, and the neovascular plexus approximates the dermal-epidermal junction. Transmission electron microscopy identified a continuous basement membrane with hemidesmosomes and anchoring fibrils that connected the epidermis with the underlying connective tissue. Within 14-28 days, the C-GAG had been degraded and replaced by newly synthesized collagen in regenerated connective tissue. Spontaneous repigmentation of healing CSS from passenger melanocytes in keratinocytes culture was observed within 2 months after grafting. Electron microscopy revealed the presence of numerous melanosomes within the keratinocytes, illustrating pigment transfer between melanocytes and keratinocytes after wound closure. These results demonstrate that the CSS develop into functional permanent skin tissue capable of spontaneous repigmentation after grafting onto burn wounds.

Rapid healing of large skin defects is a major objective for treatment of patients with extensive burns. However, a serious problem is the lack of available donor sites on the patient from which autografts can be harvested, and poor healing of donor sites on patients with burn wounds. These clinical limitations have led to the development of alternative materials for the permanent coverage of skin wounds (1-8). A limitation with cultured epidermal autografts is the slow

formation of the dermal-epidermal junction (DEJ),* which is associated with enzymatic dissociation of the keratinocyte sheets leading to blistering and ulceration (4, 8-10). Composite skin substitutes with greater homology to native skin comprising dermal and epidermal components that are applied in one procedure have been reported not to blister after grafting (6, 7).

Permanent closure of full-thickness burns has been reported previously using cultured skin substitutes (CSS) consisting of cultured autologous keratinocytes and fibroblasts attached to collagen-glycosaminoglycan (C-GAG) substrates (6, 11-14). These grafts become fully integrated with fibrovascular tissue and develop functional properties approximating that of the adjacent skin (6, 15, 16). Within 9 days after grafting, the development of a continuous basement membrane with frequent hemidesmosomes and normal markers for epidermal differentiation are expressed (16).

Pigmentation of healing skin is a functional as well as an aesthetic feature. Skin autograft will eventually repigment over time after grafting, but CSS often result in hypopigmentation of the healed wound (2). Hypopigmentation can leave the healed skin susceptible to ultraviolet damage and cause psychosocial disability to the patient. Transplantation of melanocytes with keratinocytes in CSS regenerates uniform pigment in healed skin after grafting to mice (17). Also, cultures of keratinocytes often contain viable populations of passenger melanocytes that are able to regenerate focal epidermal pigmentation after grafting (18, 19).

Microscopic analyses of patient biopsies as early as 10 days after grafting substantiate previous conclusions of CSS approximating the anatomy and ultrastructure of normal human skin (15, 16). This preliminary study confirms that early formation of the DEJ prevents skin blistering and ulceration in this cultured skin graft. Pigmented areas, resulting from transplantation of passenger melanocytes, develop as individual foci in the healing epidermis. As early as 3 weeks after grafting, passenger melanocytes express melanosomes that are transferred to surrounding keratinocytes. Melanosomes remain in the keratinocytes throughout differentiation and are found within mature corneocytes that are shed by desquamation. Stable pigmentation is observed up to 2 years after grafting, with pigmented areas increasing in diameter and fusing together. These findings demonstrate that a cultured cell-biopolymer skin substitute can not only regenerate a stable, functional tissue for permanent wound repair, but

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* Abbreviations: C-GAG, collagen-glycosaminoglycan; CSS, cultured skin substitute; DEJ, dermal epidermal junction; TEM, transmission electron microscopy.

also may develop into a cosmetically acceptable epidermal surface.

MATERIALS AND METHODS

Patient information. Four patients admitted to the Shriners Burns Institute, Cincinnati Unit and one at the University of Cincinnati Hospital Burn Special Care Unit were enrolled by informed consent into a clinical study that was approved by the University of Cincinnati Institutional Review Board. Patient demographics are summarized in Table 1. The patients ranged in age from 4 to 38 years (mean age 15.6 years). There were 4 males and 1 female with a 77.8% average total body surface area burn (range 58–87%).

Preparation of grafts. Biopsies ranging in size from 10 to 20 cm² were collected at the first scheduled skin graft procedure after acquisition of informed consent. Proliferative epidermal keratinocytes and dermal fibroblasts were isolated by enzymatic digestion and expanded in number in selective nutrient media for each cell type, as described previously (6, 11–14, 19). After cells were propagated to large numbers, dermal fibroblasts were inoculated into the porous surface of the C-GAG membrane (6, 13, 14). Following 1 day of culture, the membranes were inverted and keratinocytes inoculated onto the nonporous, laminated surface of the C-GAG membrane, and incubated 4 days submerged and up to 10 days at the air-liquid interface (20, 21).

Surgical application and graft maintenance. Wounds were excised to viable tissue and irrigated with saline. Cultured grafts were applied to the wounds, covered with N-terface, and stapled in place, as described previously (16). Wet dressings were applied, irrigated with antimicrobial solutions, and changed at 2- to 3-day intervals for 2 weeks. At 2 weeks and thereafter, dressings consisted of Adaptic with antimicrobial ointments until healing. After complete reepithelialization, pressure garments were applied to the grafted sites.

Clinical observations. Clinical follow-up began at the first dressing change and was performed weekly during the first 4 weeks and then at 2, 4, and 6 months. A skin biopsy was collected from the center of treated sites under local anesthesia using a 3-mm punch at time points as indicated in Table 1. Patients CSS018, CSS022, and SBI20 had 1 biopsy taken for histological examination at the designated time point, while patients CSS017 and CSS027 had a biopsy taken at 2 designated time points.

Preparation of samples for microscopy. Biopsies were rinsed in saline and fixed with 2% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4). Samples for light microscopy and transmission electron microscopy (TEM) were washed in 0.1 M sodium cacodylate (pH 7.4) and post-fixed in 1% osmium tetroxide in the same buffer. The tissue was washed, dehydrated in acetone, and embedded in an epon-araldite mixture.

Polymerized blocks were sectioned with a Reichert-Jung Ultracut E ultramicrotome. For light microscopy, semithin sections (~1 μ m) were cut and mounted on chrom-alum gelatin-coated slides. Sections were stained with toluidine blue and observed using a Nikon Microphot-FXA microscope. Thin sections for TEM were mounted onto 300-mesh copper grids, counterstained with uranyl acetate and lead citrate, and observed with a JEOL JEM-100 CXII transmission electron microscope.

RESULTS

Single biopsies were removed from the centers of CSS-treated areas, as described in Table 1. The patients in this study responded similarly after grafting in that: (1) reepithelialization occurred within 2 weeks, (2) epithelial attachment and reorganization of a dermal-epidermal junction is evident by 1 month, (3) degradation and replacement of the C-GAG implant with regenerated connective tissue is observed within 2–3 weeks, and (4) spontaneous repigmenta-

TABLE 1. Patient demographics and evaluation period

Patient	Age	Race	Sex	% Burn	CSS biopsy days after grafting
CSS017	16	W	M	58	14 136
CSS018	38	W	M	87	24
CSS022	12	W	M	80	10
SBI20	8	B	F	87	797
CSS027	4	W	M	77	12 28
Mean	15.6			77.8	

tion, when observed, occurs by radial outgrowth and fusion of pigmented areas.

Before grafting, the cultured epithelium of the CSS becomes stratified and partially keratinized by air exposure in vitro for 10 to 14 days (Fig. 1). Enucleated squamous cells detach from the surface of the epidermis while the basal cells are cuboidal and remain attached to the C-GAG matrix. The C-GAG substrate serves as a culture surface and delivery vehicle of the epidermis to the wounds without disruption of cell-substrate attachments and permits application in a single surgical procedure.

Histologic examination of the healing skin 12 days after grafting (Fig. 2A) demonstrates a well-keratinized epidermis with an interdigitated DEJ which persists at 28 days (Fig. 2B). The connective tissue is fully vascularized at 12 days and fragments of degraded C-GAG implant present at 12 days are cleared by 28 days after grafting.

By day 12, the DEJ contains keratin tonofilaments attached to hemidesmosomes of the basement membrane (Fig. 3A). Anchoring fibrils develop and extend from the hemidesmosomes into the connective tissue of the healing skin. By day 136, the DEJ is continuous, accounting for the clinical observation that epidermal blistering does not occur with this cultured skin graft (Fig. 3B).

Degradation and clearance of the C-GAG implant at 12 days is associated closely with the presence of infiltrated

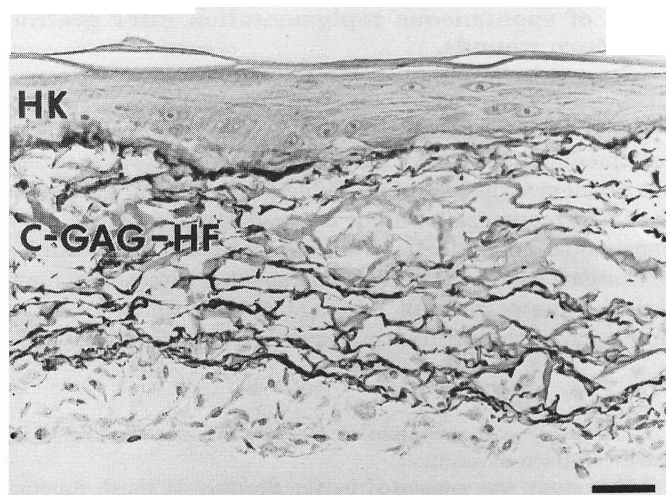


FIGURE 1. Histology of composite CSS. The C-GAG substrate seeded with human fibroblasts (C-GAG-HF) serves as a simple dermis in the human skin substitute. Human keratinocytes (HK) differentiate, forming the epidermis (bar = 50 μ m).

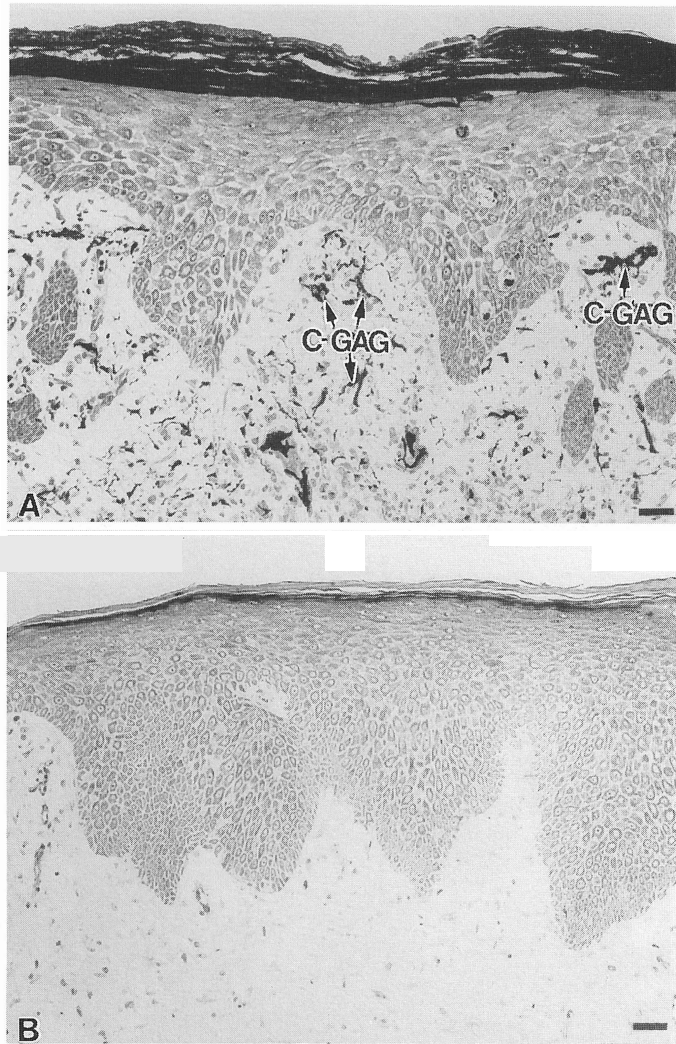


FIGURE 2. Histology of healed skin 12 and 28 days after grafting. Biopsies taken 12 days after application of cultured skin (A) shows a hypertrophic, hyperkeratotic epidermis interdigitated with a well-vascularized connective tissue analogous to rete pegs and dermal papillae. Fragments of degraded C-GAG implant persist in the connective tissue. By 28 days (B), the epidermis has become less hyperkeratotic and no implanted collagen is evident in the connective tissue. Bars = 50 μ m.

macrophages and granulocytes. The intensity of leukocyte infiltrate is mild to moderate. Fibroblasts in the wound site may enter either by transplantation or migration from the wound bed. Native collagen is distinguished by fibril diameter and distribution from implanted C-GAG. Presence of mature collagen is concurrent with degradative of the C-GAG implant. A macrophage adheres to the C-GAG matrix 12 days after grafting (Fig. 4A), suggesting degradation activity. Two infiltrated granulocytes adhere to C-GAG, while an adjacent fibroblast simultaneously contacts native collagen and C-GAG (Fig. 4B).

Regeneration of skin pigment by transplanted passenger melanocytes is evident by 2 months after grafting. Focal areas of CSS develop pigmentation (Fig. 5A), are uniform in color, increase in area, and fuse together with time (Fig. 5B). In this black patient, approximately 16.3% of the grafted area has regained normal pigmentation by 2 years after

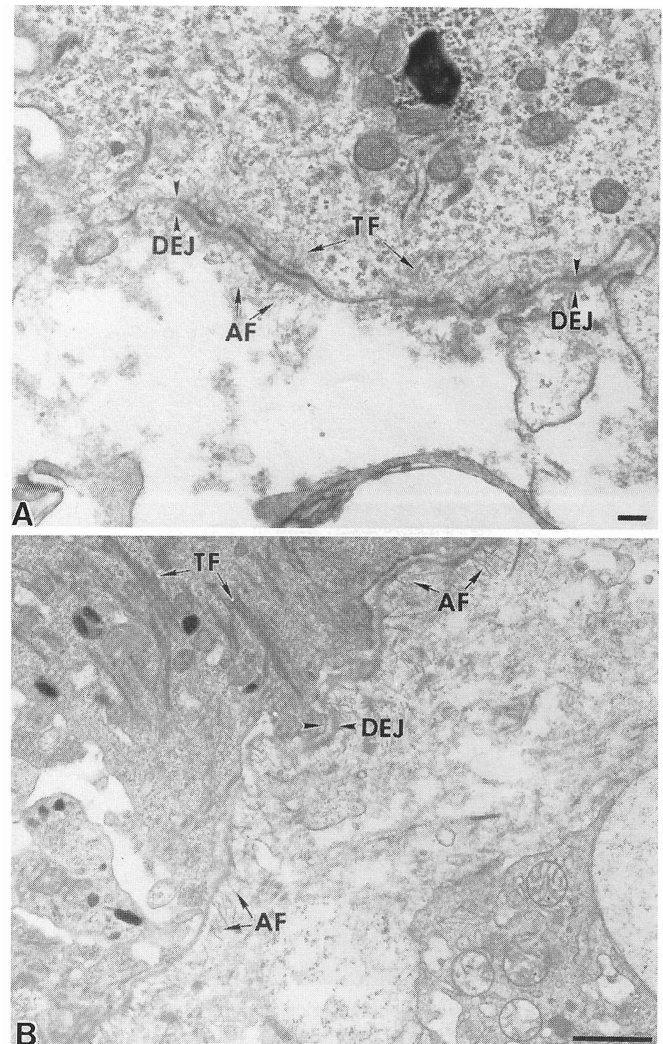


FIGURE 3. Transmission electron micrographs of cultured skin biopsies showing DEJ and anchoring fibrils (AF) extending from hemidesmosomes. At day 12 (A), the DEJ is not continuous but is developing normally, with tonofilaments (TF) extending from the basal cells into the hemidesmosomal densities. By day 136 (B), the DEJ is continuous, accounting for the absence of epithelial blistering. Bars = 1 μ m.

surgery. TEM of a biopsy from a repigmented area shown in Figure 5B reveals transfer of high frequencies of melanosomes to keratinocytes (Fig. 6A). Indeterminant cells, hypothesized to be precursors of melanocytes (22), are distributed along the basal layer of the epidermis. Melanosomes remain in the keratinocytes throughout differentiation and are found within mature corneocytes that are shed by desquamation (Fig. 6B).

DISCUSSION

These data support earlier reports from this laboratory that permanent closure of skin wounds can be achieved with a composite skin substitute (15, 16). Autologous human keratinocytes and fibroblasts attached to an implantable C-GAG substrate allow for rapid attachment to the wound bed while permitting ingrowth of fibrovascular tissue. Histologic and ultrastructural analysis of the healed skin after grafting

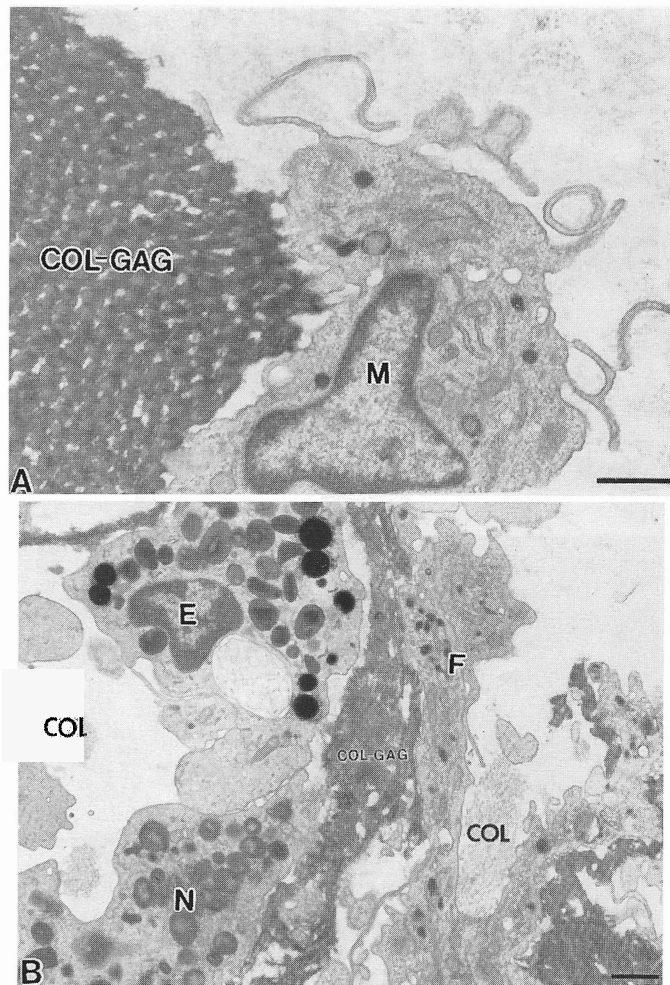


FIGURE 4. Transmission electron micrographs of cultured skin biopsies showing degradation of implanted C-GAG at 12 days after application. (A) C-GAG (COL-GAG) is actively phagocytized by an infiltrated macrophage (M). (B) An eosinophil (E) and neutrophil (N) are shown degrading COL-GAG, while a fibroblast (F) deposits collagen (COL) in regenerating dermis. Bars = 1 μ m.

to burn patients revealed attributes of fully healed and functional skin. Spontaneous focal pigmentation from passenger melanocytes in the skin graft may add protection from solar radiation and allow for cosmetic restoration in dark-skinned patients.

Conventional treatment of massive full-thickness skin injuries includes coverage with meshed, split-thickness autologous grafts (23). Current alternatives to skin autograft for permanent closure of skin wounds include cultured epidermal keratinocyte sheets (1, 2), epidermal cells added to de-epidermized dermis (3), and composite skin substitutes (7, 13, 24, 25). Although these have been studied, none has replaced the split-thickness autograft for treatment of full-thickness burns.

The culture of human epidermal keratinocytes on a substrate prior to grafting provides a human skin analog that meets requirements for skin substitutes as a suitable surgical replacement (26). Previous reports from this laboratory have shown that this skin substitute functions as a barrier against microbial contamination and fluid loss, is stable, durable, flexible, and easily manipulated by the surgeons. To

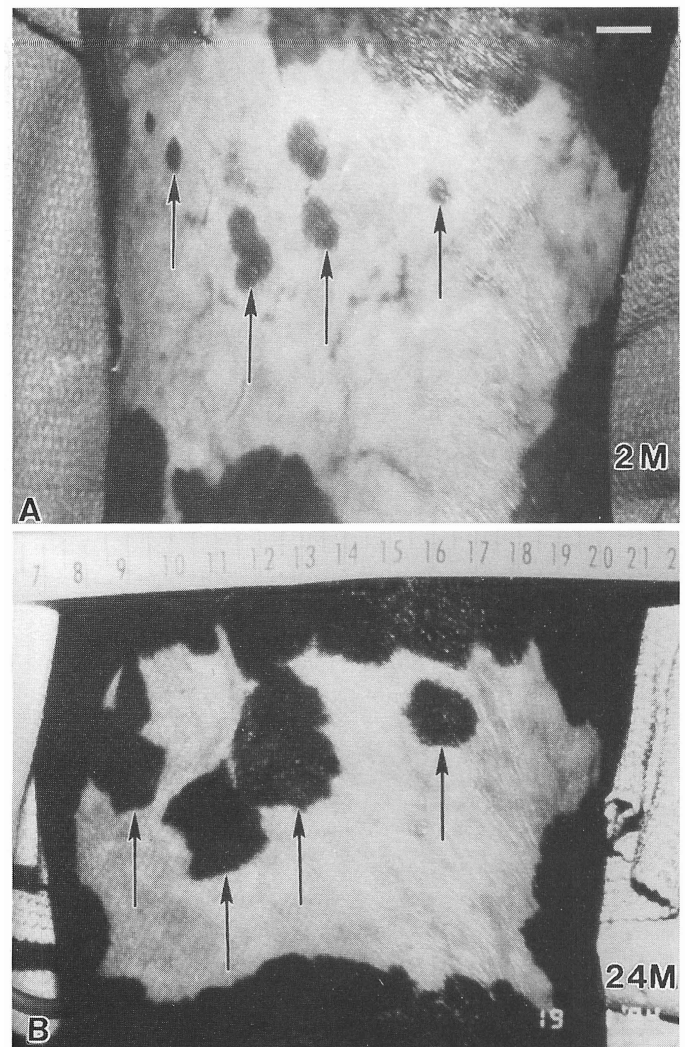


FIGURE 5. Clinical result in a black patient showing repigmentation of healing skin by passenger melanocytes. (A) Two months after grafting, focal areas of regenerated skin pigment are observed in the healed site (arrows) (bar = 1 cm). (B) By 24 months, these focal areas have increased in area and fused (arrows), forming uniformly pigmented areas.

be considered as a permanent skin substitute, the cultured material should permit rapid attachment to the wound bed and simultaneous ingrowth of fibrovascular tissue. Enzymatic release of cultured epithelial sheets immediately before grafting is associated with epithelial blistering necessitating the need for regrafting (4, 8, 10). The use of a CSS eliminates the need for enzymatic treatment and allows for the progressive formation of the DEJ *in vitro* prior to grafting. The persistence of this cell substrate attachment may contribute to improved epidermal strength, and account for the absence of epidermal blistering supported by this study.

The early degradation of the C-GAG implant by infiltrated leukocytes suggests compatibility between the host and implant material. The results of this study support earlier reports that the C-GAG implant is degraded completely within 1 month after grafting (6, 16, 19). This early degradation is simultaneous with regeneration of new connective tissue and vascularization. Clinical evaluations (data not shown) sug-

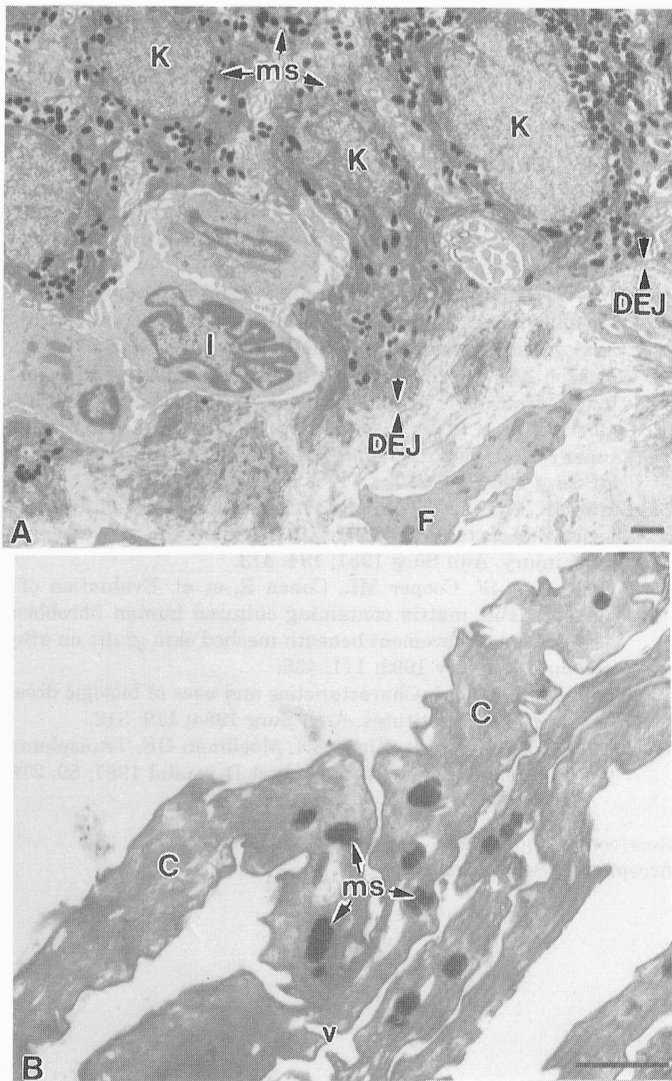


FIGURE 6. Transmission electron micrographs of biopsies from pigmented areas shown in Figure 5B. (A) Skin pigmentation results from transfer and retention of high frequencies of melanosomes (ms) to keratinocytes (K). Indeterminant cells (I) are evident along the DEJ. (B) Melanosomes (ms) remain within corneocytes until desquamated as mature corneocytes (C). Bars = 1 μ m.

gest no greater persistence of erythema in healed skin treated with cultured or native skin grafts.

Skin autograft is known to repigment over time after grafting. However, cultured grafts tend to be hypopigmented (2), leading to an increased risk of ultraviolet damage as well as psychosocial adjustment for dark-skinned patients. This report supports previous findings that passenger melanocytes in keratinocyte cultures retain the capacity to regenerate pigment after transplantation (18, 19). Focal pigmentation in healed skin is observed at 2 months after grafting in a patient in this study. After 2 years, the focal pigmentation increased in area and fused together, forming areas of uniform pigmentation. Incidence of repigmentation is observed in both dark-skinned and light-skinned patients, with greater intensity of color in dark-skinned patients. At present, frequency of pigmented foci is random and most probably dependent upon survival of passenger melanocytes

during propagation of keratinocyte cultures. Ultrastructural analysis of the pigmented areas verified that the enlargement of pigmented sites corresponded to transfer of melanosomes to adjacent keratinocytes. The limitation to pigmentation in this condition is the ability to transfer melanosomes from an isolated population of melanocytes to the keratinocytes in the graft field. As observed in this study, this is a slow process, with complete pigmentation not readily attainable. Repopulation of melanocytes into cultured skin grafts may correct this deficiency. Depigmentation disorders, such as piebaldism, have been treated by transplantation of human melanocytes (27). Complete pigmentation may hypothetically protect the newly grafted skin from ultraviolet radiation as well as restore proper pigmentation to the individual. This laboratory has recently demonstrated complete restoration of pigmentation after the addition of melanocytes to cultured skin grafted to athymic mice (17). The application of this system to humans may lead to permanent pigmentation. Grafts may be prepared with autologous or allogeneic melanocytes if grafted cells are tolerated immunologically. One factor for consideration in the current culture condition for growth of melanocytes is the inclusion of tumor promoters such as tetradecanoyl phorbol acetate in the growth media. Risks of transformation by culture conditions must be evaluated critically for safety of transplantation to humans. Therefore, current culture conditions for melanocytes will require revision before the advent of clinical studies.

The findings of this report provide additional evidence to suggest that composite cultured skin substitutes form a functional full-thickness skin after grafting to excised burns. The spontaneous pigmentation with passenger melanocytes provides strong evidence that complete restoration of skin pigmentation by melanocytes cocultured with keratinocytes in humans is possible. This human model may lend itself to comprehensive studies of carcinogenesis and pigmentation disorders.

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