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Ultrastructural features of composite skin cultures grafted onto athymic mice

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ABSTRACT

Skin substitutes composed of cultured keratinocytes with or without a dermal substrate are now being used in the treatment of burns and other cutaneous wounds. Composite skin cultures (Graftskin, LSE), consisting of epidermal keratinocytes seeded on a fibroblast-containing collagen matrix and maintained at the air–liquid interface, develop a well differentiated epidermis in vitro with many of the morphological and biochemical features of intact skin. Basement membrane-associated antigens, developing hemidesmosomes and short segments of lamina densa are present at the dermal–epidermal junction in vitro, although the LSE lacks a continuous basement membrane. As epidermal differentiation proceeds, the culture develops a stratum corneum composed of electron-dense corneocytes surrounded by extracellular lipid. However, the intercorneocyte lipid lamellae do not exhibit the repeating pattern of broad and narrow electron lucent bands observed in electron micrographs of the stratum corneum of intact skin. In this study, LSE were grafted onto full thickness wounds in athymic mice. Animals were killed 6, 15, 30 and 60 d after surgery for examination by light and electron microscopy to identify any ultrastructural changes which occurred in the culture in response to the host environment. The grafted LSE integrated well into the host tissue and remained intact throughout the 60 d study period. At the dermal–epidermal junction, a continuous basement membrane with a well defined lamina densa was established by 15 d after surgery. An extensive network of anchoring fibrils was present by 30 d after surgery. Collagen fibrils within the dermal matrix condensed by 6 d after surgery and began organising into loosely packed bundles by 15 d after surgery. Tightly packed bundles of collagen fibrils with a circular cross-section were observed at 30 d after surgery. Landmann unit repeats were identified in the intercorneocyte lipid lamellae at 30 d after surgery. The ultrastructural analysis of the grafted LSE demonstrates that the culture, highly differentiated in vitro, not only persisted after grafting, but responded to the biochemical features of the in vivo environment, rapidly developing additional morphological features of intact skin which may be critical to the establishment of a stable and durable skin replacement.

Key words: Skin substitutes; basement membrane; collagen.

INTRODUCTION

The application of cell culture techniques to the treatment of burns and other cutaneous wounds has generated a number of cultured skin substitutes designed to provide permanent wound closure. Epithelial sheets consisting of a basal keratinocyte layer and a variable number of noncornified suprabasal layers (Banks-Schlegel & Green 1980; Fauré et al. 1987; Kanitakis et al. 1987; Compton et al. 1989) have been successfully grafted onto humans (O'Connor et al. 1981; Gallico et al. 1984) and mice (Billingham & Reynolds 1952; Banks-Schlegel & Green 1980). A stratified, cornified epidermis with a morphological appearance similar to that of intact skin develops at the graft site within 1 wk after surgery (Fauré et al. 1987; Kanitakis et al. 1987; Compton et al. 1989). While there is a continuous basement membrane by 3–4 wk, anchoring fibril size and density are not comparable to normal skin until

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1–2 y after surgery (Compton et al. 1989), making the grafts fragile and prone to blistering (Woodley et al. 1988; Hansbrough et al. 1989; Carver & Leigh 1991). When grafted directly onto the wound bed, the epidermal sheet appears to stimulate the gradual formation of a collagen-containing neodermis which replaces the underlying granulation tissue (Compton et al. 1989; Woodley et al. 1990).

Composite grafts take advantage of the physical support provided by a dermal layer and chemical interactions between the epidermal and dermal components, establishing a functional tissue at the graft site more quickly and effectively than epithelial sheets alone (Bell et al. 1983; Bosca et al. 1988; Boyce & Hansbrough 1988; Langdon et al. 1988; Hansbrough et al. 1989; Cooper & Hansbrough 1991). When an epithelial sheet is supported by a collagen-glycosaminoglycan (GAG) membrane inoculated with fibroblasts (Cooper & Hansbrough, 1991), a histologically differentiated epidermis and complete basement membrane are observed by 9–10 d after grafting (Hansbrough et al. 1989; Cooper & Hansbrough 1991). Bundles of collagen fibrils assemble in the developing neodermis as the dermal membrane is resorbed (Cooper & Hansbrough 1991).

A continuous basement membrane is observed 7 d after surgery (Bell et al. 1983; Madden et al. 1986) when constructs of keratinocytes seeded on a collagen lattice are grafted onto animals (Bell et al. 1983; Bosca et al. 1988; Nanchahal et al. 1989; Hansbrough et al. 1994) and humans (Madden et al. 1986; Nanchahal et al. 1989). In these studies, however, the degree of epidermal development at the time of grafting varies widely. We have developed a skin equivalent (LSE) composed of keratinocytes seeded on fibroblast-containing collagen lattices and maintained at the air–liquid interface (Bell et al. 1983; Parenteau et al. 1992) which develops many of the morphological and biochemical characteristics of intact skin in vitro (Parenteau et al. 1992). Basement membrane components have been identified at the dermal–epidermal junction and short regions of lamina densa can be found beneath developing hemidesmosomes (Parenteau et al. 1992). The dermal matrix contains short, banded collagen fibrils and extracellular matrix proteins, such as GAGs, produced by the dermal fibroblasts (Bell et al. 1991).

Achieving a high level of differentiation in skin cultures has been an important goal in vitro. However, a differentiated epidermis has not been considered important, or even desirable, for grafting purposes. There is concern that the protocols and culture periods required to achieve differentiation in vitro may compromise the biological effectiveness of the culture, impairing its ability to persist in vivo. In this study, LSE were grafted onto athymic mice and examined at selected timepoints after grafting. In addition to following the morphological changes which occur at the dermal–epidermal junction, we examined the ultrastructural features of the stratum corneum to determine if the lipid metabolism of the cultures, and thus the organisation of the intercorneocyte lipid lamellae, was altered by the host environment. The data indicate that the differentiated LSE integrates well with the host tissue, persists long term and acquires additional morphological characteristics of intact skin without compromising its ability to stimulate remodelling of the underlying dermal matrix.

MATERIALS AND METHODS

LSE culture method

LSE were constructed as described by Parenteau et al (1991). Briefly, passaged human dermal fibroblasts isolated from foreskin were combined with bovine collagen in a modified Transwell culture insert (Costar Instruments, Cambridge, MA, USA). The cells contract the collagen to form a cellular dermal matrix in 4–6 days. Human foreskin keratinocytes were seeded onto the surface of the lattice and the construct was cultured submerged for 4 d in a defined minimally supplemented basal medium (Johnson et al. 1992) to allow the keratinocytes to cover the lattice and stratify. The construct was then cultured at the air–liquid interface to promote differentiation and cornification of the epidermis. The LSE were grafted after 11 d of culture at the air–liquid interface.

Surgery and necropsy

The LSE were applied to 2×2 cm full-thickness wound in 24 athymic mice and sutured in place, as described by Hansbrough et al (1994). Briefly, LSE grafts of 2×2 cm were sutured onto the woundbeds and dressed. Six mice each were killed at 6, 15, 30 and 60 d after surgery. At the time of killing, the grafts were examined macroscopically. The entire graft and adjacent mouse skin were excised and divided for light and electron microscopic fixation, making sure the host–graft junction was present in all samples.

Light microscopy

Samples were fixed in 10% formalin, dehydrated in a graded ethanol series, infiltrated with Tissue Prep 2

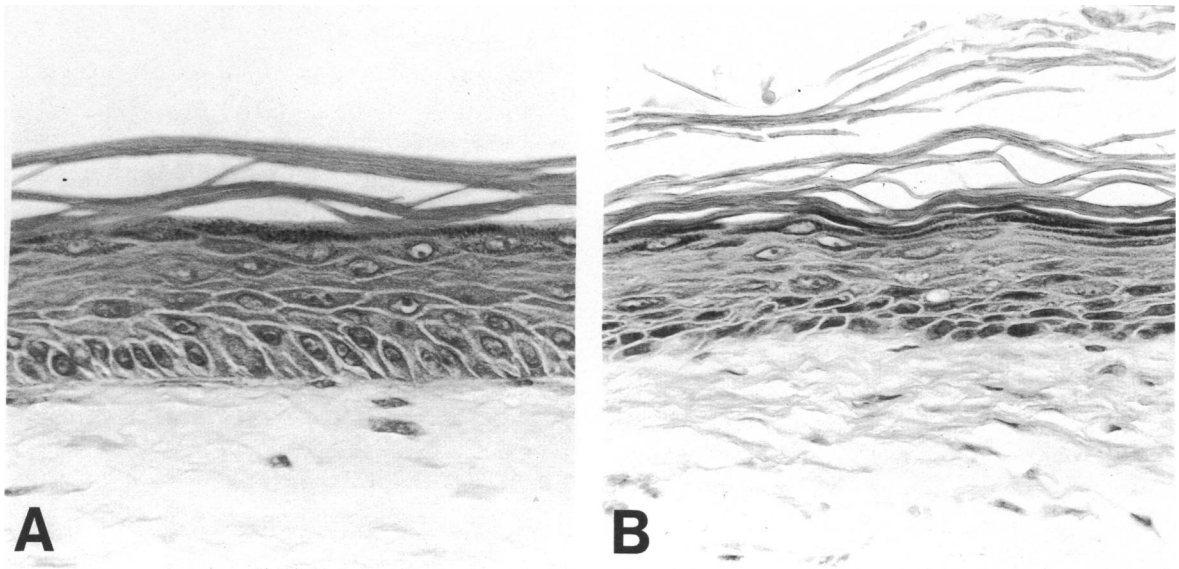

Fig. 1. Appearance of the LSE in vitro and 60 d after surgery. (*A*) At 11 d of culture at the air–liquid interface in vitro, the LSE had a differentiated epidermis which was firmly attached to the underlying fibroblast-containing dermal matrix. ×660. (*B*) At 60 d after surgery, the epidermis retained the differentiated phenotype of the in vitro culture. The dermal-epidermal junction remained linear and the matrix lacked dermal appendages. ×660.

paraffin (Fisher Scientific, Fair Lawn, NJ, USA) and embedded in Paraplast Xtra (Sherwood Medical, St Louis, MO, USA). 5 μm sections were stained with haematoxylin and eosin.

Transmission electron microscopy

For transmission electron microscopy, samples were fixed for 4 h in a solution of 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4. Samples were postfixed in 1% OsO_4 (in 0.1 M sodium cacodylate) and stained en bloc with 2% uranyl acetate (aqueous). Ultrathin sections were stained with uranyl acetate and lead citrate. For examining the ultrastructural features of the corneum, certain samples were postfixed for 1 h in darkness at room temperature in a solution of 0.25% ruthenium tetroxide (Polysciences, Warrington, PA, USA) with 0.5% potassium ferricyanide in 0.1 M sodium cacodylate, pH 7.4 (Hou et al. 1991). After secondary fixation all specimens were dehydrated in a graded ethanol series and propylene oxide and embedded in Epox 812 (Ernest F. Fullam, Rochester, NY, USA). Thin sections (~ 700 nm) were stained with lead citrate and examined on a JEOL JEM100S at 80 kV.

RESULTS

By the time of grafting, at 11 d of culture at the air–liquid interface, the epidermis of the LSE in vitro contained distinct basal, spinous and granular layers (Fig. 1*A*). The apical surface of the culture was covered by a multilayered corneum. Fibroblasts were extended in the contracted collagen matrix.

The epidermis of the grafted LSE maintained its differentiated state throughout the 60 d study period (Fig. 1*B*). The graft was easily distinguishable from the surrounding mouse tissue at all timepoints after grafting by the lack of epidermal appendages and the linear dermal–epidermal junction.

In electron micrographs of the LSE in vitro and throughout the 60 d study period, the suprabasal keratinocytes were interconnected by complete desmosomes and contained abundant tonofilaments and lamellar granules (Fig. 2). Granular layer keratinocytes contained a heterogeneous population of keratohyalin granules of irregular shapes and variable sizes. The number and size of the keratohyalin granules appeared to increase with time after grafting. Extruded lamellar granules were observed at the junction between the granular layer and the corneum and in the intercorneocyte space.

The intercorneocyte space of the construct's stratum corneum contained stacks of lipid lamellae in vitro. At 6 and 15 d after surgery, all the lamellar lipid sheets contained electron-lucent bands of equal thickness (Fig. 3*A*) rather than the Landmann unit repeat pattern of alternating broad and narrow bands present in intact skin (Swartzendruber et al. 1989).

By 30 d after surgery, the amount of extruded lamellar granules at the junction between the granular layer and the corneum had significantly increased.

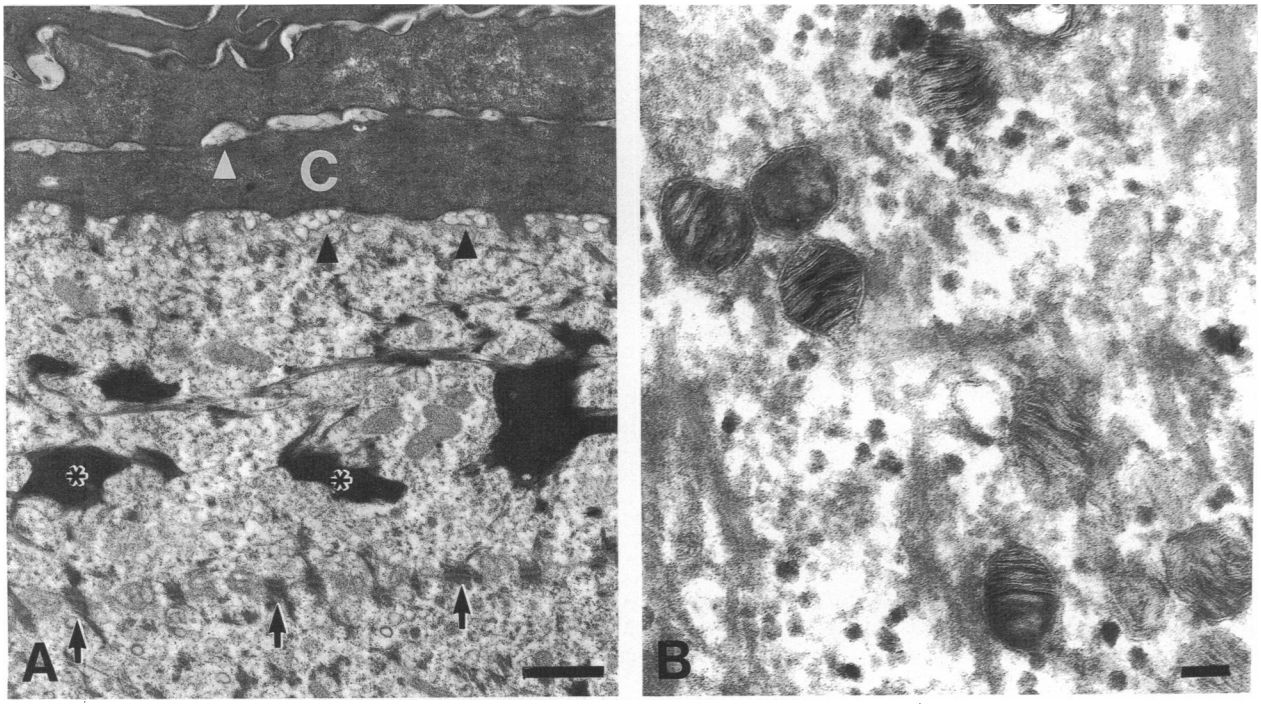

Fig. 2. Suprabasal cell layers of the LSE 15 d after surgery. (*A*) Throughout the epidermis, keratinocytes were interconnected by complete desmosomes (arrows). Keratohyalin granules (asterisks) were present throughout the granular layer. The corneum (C) was composed of flattened, electron-dense corneocytes. Evidence of the extrusion of lamellar granule contents (arrowheads) was observed at the granular layer-corneum junction and between corneocytes. Bar, 1 μm. (*B*) Lamellar granules containing stacks of lipid lamellae were present in the keratinocyte cytoplasm. Bar, 0.1 μm.

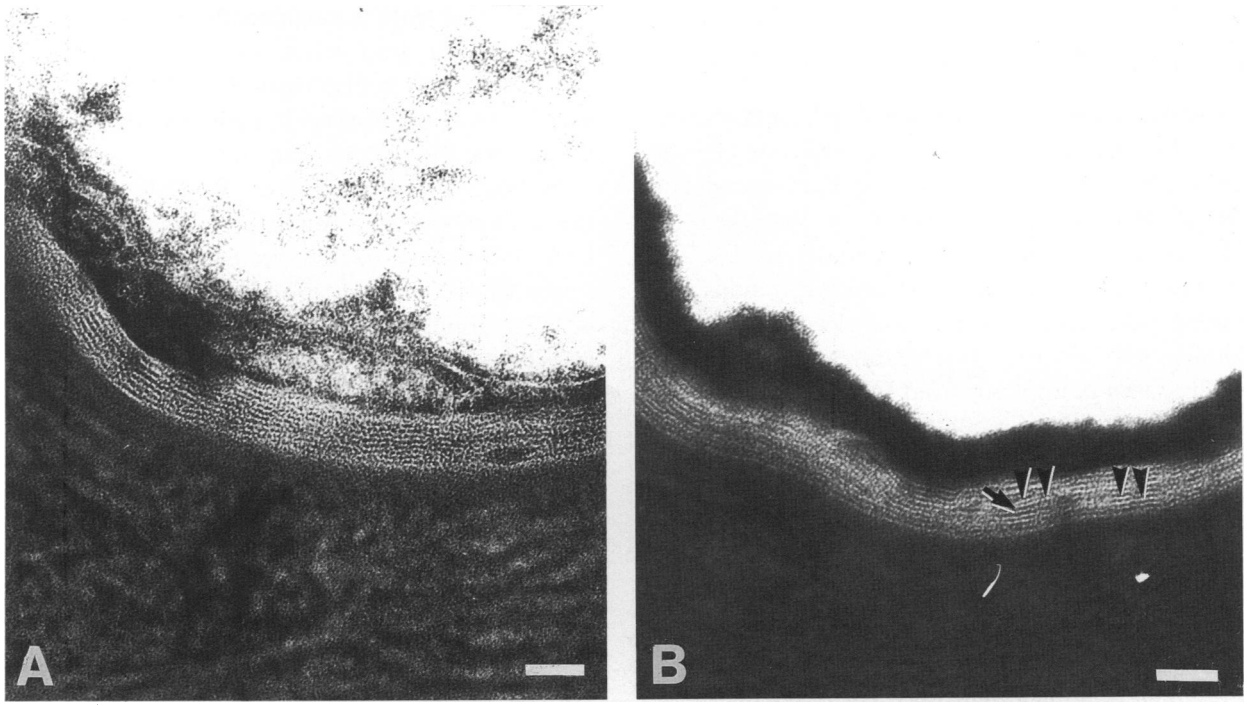

Fig. 3. Lamellar lipid structures of the LSE in vitro and after grafting. (*A*) Intercorneocyte lipid is organised into stacks of lamellae of varying thickness which appear in electron micrographs as electron-lucent bands representing the hydrocarbon tails of the lipid lamellae. In the corneum of the construct in vitro and up to 15 d after surgery, all intercorneocyte lamellar stacks had electron-lucent bands of equal thickness. (*B*) By 30 d after surgery, lamellar stacks with the broad-narrow-broad configuration representing lipid bilayers (arrowheads) and intervening monolayers (arrow) were observed in the intercorneocyte space. Bar, 0.05 μm.

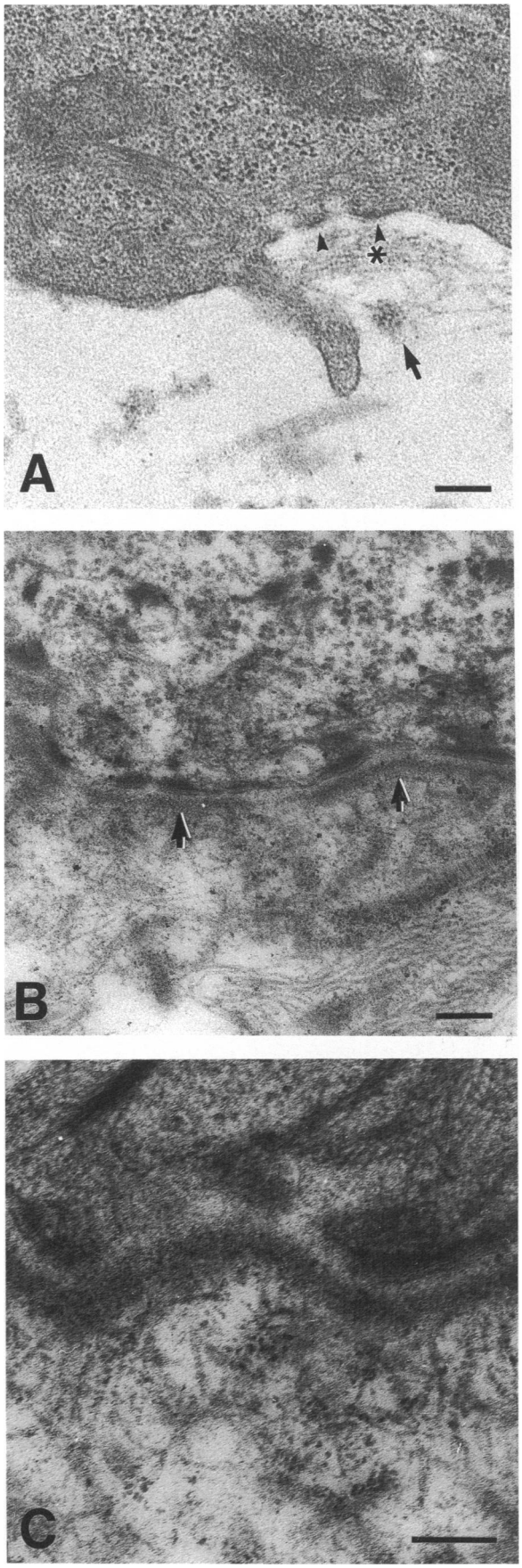

Fig. 4. Dermal–epidermal junction of the LSE in vitro and after grafting. (*A*) The dermal–epidermal junction of the LSE in vitro contained thin fibrils (asterisk) in the dermal matrix beneath developing hemidesmosomes (arrowheads). Arrow indicates an anchoring plaque, with connecting anchoring fibrils. Bar, 0.2 μm. (*B*) At 15 d after surgery, a continuous basement membrane was observed with a distinct lamina densa (arrows). Bar, 0.2 μm. (*C*) At 30 d after surgery, the dermal–epidermal junction contained well-developed hemidesmosomes and a compact lamina densa of homogeneous thickness. Numerous anchoring fibrils extended into the dermal matrix. Bar, 0.1 μm.

The interncorneocyte space contained regions with stacks of lipid lamellae and others where the lipid envelopes were directly apposed. The lamellar stacks were more homogeneous in thickness than those of the LSE in vitro or 6–15 d after surgery. By 30 d after surgery, the Landmann unit repeat pattern was observed in some regions of the intercorneocyte lipid lamellae (Fig. 3*B*).

At 11 d of culture at the air–liquid interface in vitro, the LSE lacked a continuous basement membrane. Thin fibrils and amorphous material were observed in invaginations of the basal membrane of basal keratinocytes. Developing hemidesmosomes were found in association with these structures (Fig. 4*A*). These structures are similar to those reported at the dermal epidermal junction of other skin cultures (Cooper et al. 1993).

At 6 d after surgery, the basement membrane was still discontinuous and remained primarily localised to invaginations in the basal keratinocyte plasma membrane. By 15 d after surgery the basement membrane was continuous, with a distinct lamina lucida and lamina densa (Fig. 4*B*). The hemidesmosomes were small and many had not developed tonofilament attachments in the keratinocyte cytoplasm. The presence of anchoring fibrils was variable. When present, they were sparse when compared with the surrounding mouse skin.

By 30 d after surgery the hemidesmosomes were broader and had extensive tonofilament attachments while the lamina densa was more compact. A dense array of anchoring fibrils extended from the lamina densa into the dermal matrix, with numerous attachments to anchoring plaques (Fig. 4*C*).

Significant morphological changes occurred in the ultrastructure of the dermal matrix collagen after grafting, though the dermal matrix of the graft remained readily distinguishable in light micrographs throughout the study period. The dermal matrix of the cultures in vitro consisted of thin, banded fibrils (Fig. 5*A*) which were not organised into bundles and were not circular in cross-section. By 6 d after surgery these collagen fibrils had begun condensing into loosely packed bundles (Fig. 5*B*). By 30 d after

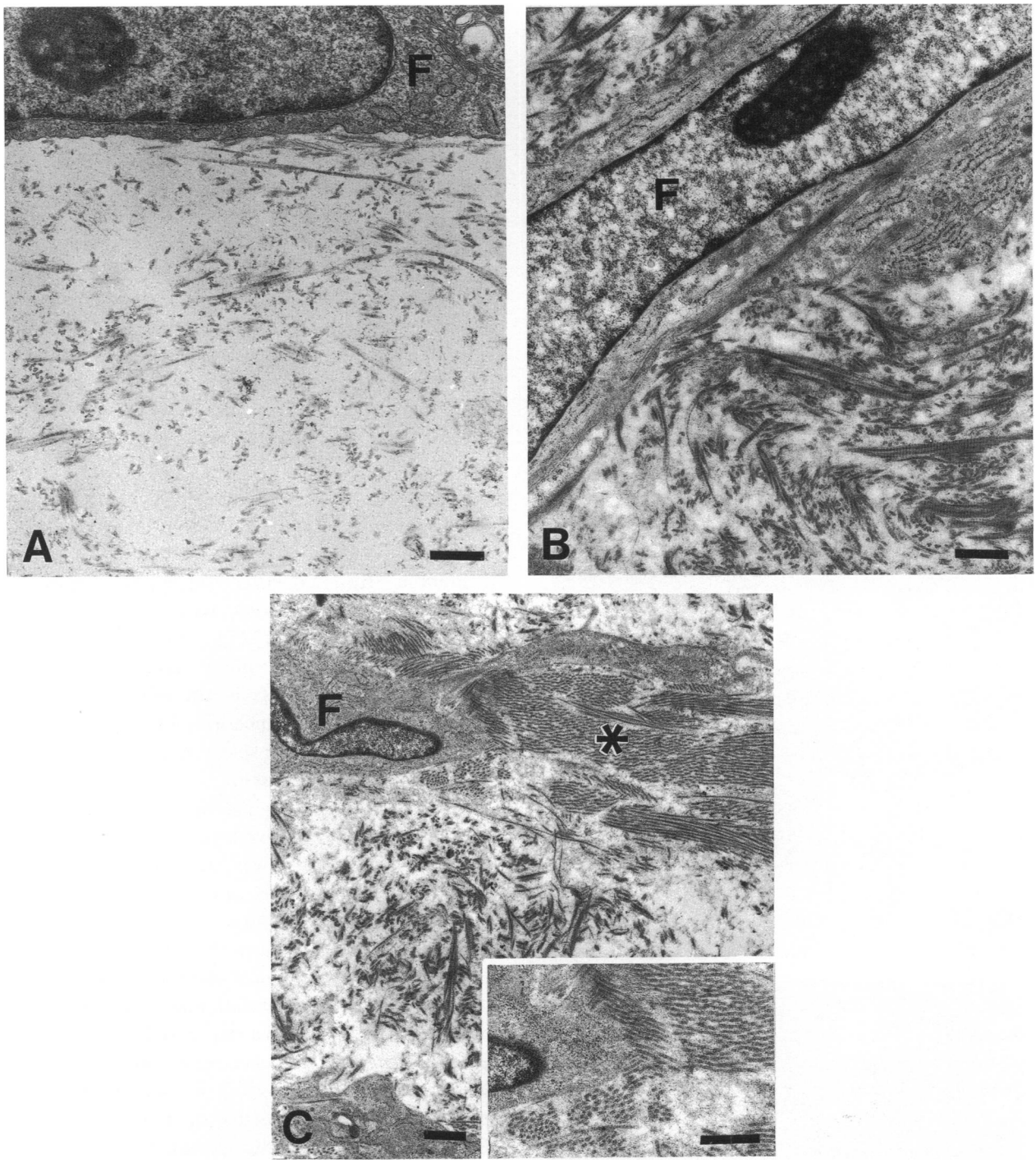

Fig. 5. The dermal matrix of the LSE in vitro and after grafting. (*A*) The dermal matrix of the construct in vitro consisted of a loose network of banded collagen fibrils. (*B*) At 6 d after surgery, the density of the collagen fibrils in the matrix increased. Individual fibrils appeared to have thickened and were organized into small bundles. Bar, 1 μm. (*C*) By 30 d after surgery, large bundles of collagen fibrils (asterisks) were observed intermixed with disorganized clusters of fibrils. Bar, 1 μm. Inset, the collagen fibrils contained in these bundles were of uniform thickness and circular in cross-section. F, fibroblast. Bars 1 μm.

surgery, thick tightly packed bundles of collagen fibrils were observed, concentrated around fibroblasts throughout the dermal matrix (Fig. 5 *C*). The fibrils in many of these bundles appeared circular in cross-section. Developing elastic fibrils were not detected in the dermal matrix at any time during the study period.

DISCUSSION

The grafted LSE epidermis stains positive for human involucrin at 60 d after surgery (Hansbrough et al. 1994) confirming that, like intact mammalian skin (Rygaard, 1974) and split-thickness human cadaver skin (Hansbrough et al. 1994), the grafted LSE persists

on the athymic mouse. Therefore, the ultrastructural changes we observed in the epidermis at the graft site occurred in the epidermal cells of the LSE, rather than in invading mouse epidermis. Demarchez et al. (1992) have shown that when viable human fibroblast-containing collagen lattices are grafted onto athymic mice, the human fibroblasts persist and the bovine type I collagen is replaced by human type I collagen. The dermal matrix of the LSE contains viable, metabolically active fibroblasts at the time of grafting. Studies are underway to determine whether the fibroblasts in the dermal matrix of the grafted culture are of human or murine origin.

The dermal matrix of the LSE supported the rapid assembly of a continuous basement membrane by 15 d after grafting. In contrast, a complete basement membrane is not established in epithelial sheet grafts until 3–4 wk after surgery (Compton et al. 1989). In preparation for grafting epithelial sheets, hemidesmosomes are disrupted by the enzymatic removal of the cells from the culture dish (Compton et al. 1989) and must be reestablished after grafting. The dermal matrix preserves the basement membrane components and assemblies established in vitro, reducing the amount of time necessary after grafting for the formation of a continuous basement membrane (Hansbrough et al. 1989; Cooper & Hansbrough 1991).

The strength of the epidermal attachment is critical to the long-term maintenance of graft integrity. The blistering and epithelial erosion observed in epithelial sheet grafts has been at least partially attributed to incomplete anchoring fibril formation at the junction between the epidermis and the neodermis (Woodley et al. 1988). The authors attributed these anchoring fibril abnormalities to compositional deficiencies in the neodermis which forms in the granulating wound bed beneath the epidermal sheet rather than the length of time after grafting when the observations were made. The appearance of significant numbers of anchoring fibrils by 30 d after surgery in the LSE indicates that the presence of a dermal component contributes to the formation of a sturdier basement membrane zone.

The ultrastructure and organisation of the dermal collagen of the grafted LSE was morphologically quite different from that of the collagen lattice formed in vitro. There was significant condensation of the constituent bovine collagen fibrils. In addition, bundles of collagen fibrils with a circular cross section surrounded the fibroblasts in the dermal matrix of the grafted LSE. These circular fibrils most likely represented collagen formed and deposited by the fibroblasts after grafting.

While there have been numerous studies on the ultrastructural changes which occur at the dermal–epidermal junction and in developing neodermis of cultured skin grafts, there have been few published reports on the ultrastructure of the stratum corneum or barrier function of these grafts (Higounenc et al. 1994). The LSE (Parenteau et al. 1992) and other skin constructs maintained at the air–liquid interface (Ponec et al. 1988; Madison et al. 1989; Mak et al. 1991; Régnier et al. 1992) develop a stratum corneum and barrier function in vitro, although the cultures are more permeable than intact skin (Cumpstone et al. 1989; Mak et al. 1991; Gay et al. 1992; Régnier et al. 1992). The stratum corneum lipids organise into stacks of lamellae in the intercorneocyte space (Ponec et al. 1988; Madison et al. 1989; Boddé et al. 1990; Parenteau et al. 1992) which lack the Landmann unit repeat pattern (Swartzendruber et al. 1989) of electron-lucent bands of varying thickness observed in intact skin (Madison et al. 1989; Nolte et al. 1993).

A normalisation of lipid composition and transepidermal water loss was reported after skin constructs composed of human keratinoytes seeded on de-epidermised dermis and maintained at the air–liquid interface were grafted onto athymic mice (Higounenc et al. 1994). While no lipid analysis or transepidermal permeability measurements were performed on the LSE after grafting, the appearance of Landmann unit repeats in the intercorneocyte lipid lamellae suggests that the epidermal lipid metabolism of the culture, and consequently the formation of the lamellae, was affected by the host. Since the presence of specific lipid species in the appropriate ultrastructural arrangement is crucial to the establishment of a barrier-competent stratum corneum (Elias, 1992; Fartasch et al. 1993; Mao-Quiang et al. 1993), it is possible that the permeability of the culture decreased after grafting, approaching that of intact skin.

The development of permanent skin replacements is critical to improving the survival of severe burn patients with limited donor site availability. The data presented in this study indicate that the LSE, which has both a highly differentiated epidermis and a viable dermal component in vitro, remains able to undergo physiological and structural changes after grafting which result in a morphology more like that of intact skin. Because the culture persists and is able to respond rapidly to the host environment, it may provide a means for permanent wound closure. The rapid attachment of the epidermal component through basement membrane formation and anchoring filament development should provide the necessary protection from mechanical stress and reduce

blistering in the clinical setting. The improved stratum corneum lipid organisation may inhibit desiccation of the fragile wound tissue. Studies are now underway to characterise further the response and behaviour of the grafted LSE for clinical use in the treatment of cutaneous burn wounds and venous stasis ulcers and in dermatological excision surgery.

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