Reconstitution of Structure and Cell Function in Human Skin Grafts Derived from Cryopreserved Allogeneic Dermis and Autologous Cultured Keratinocytes

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Grafts of allogeneic dermis plus autologous epidermal cell cultures were used to replace extensively burned skin. Cryopreserved split-thickness cadaveric skin was grafted onto debrided burn wounds, and autologous keratinocytes were cultured from uninjured donor sites. Several weeks later, allograft epidermis was abraded and replaced with the keratinocyte cultures. The final grafts were thus composites of autologous cultured epidermis and allogeneic dermis. In a case with 28 months follow-up, reconstitution of the dermal-epidermal (BMZ.1) and microvascular (BMZ.2) basement membrane zones was studied immunohistochemically and ultrastructurally. Immediately before grafting, thawed cryopreserved skin reacted with antibodies against laminin and type IV collagen in normal patterns. Twenty-nine days after grafting, BMZ.1 reacted weakly with both antibodies, and anticalcine reactivity was absent from BMZ.2. Antilaminin reactivity of BMZ.2, however, was moderately intense, consistent with recent neovascularization. On day 29, the allograft epidermis was replaced with autologous keratinocyte cultures. Twenty-five days later (54 d after allografting), staining of both BMZs was intense with both antibodies. Ultrastructurally, at day 76 (47 d after culture placement) BMZ.1 revealed only small hemidesmosomes, few incipient anchoring fibrils, and a discontinuous lamina densa. BMZ.2, however, was fully reconstituted. By 124 d, both BMZs appeared normal. Observations in the dermis at 76 d included the presence of lymphocytes, organellar debris, and hyperactive collagen fibrillogenesis, all indicative of dermal remodelling. The microvasculature was well differentiated, but no elastic fibers or nerves were found. In the epidermis, melanocytes and evidence of melanosome transfer were seen at 5, 47, and 95 d after grafting of keratinocyte cultures. We conclude that the composite procedure reconstitutes skin with excellent textural and histologic qualities. J Invest Dermatol 91:478-485, 1988

Skin grafts, consisting of allogeneic dermis and stratified keratinocyte cultures derived from autologous epidermis, have been used to provide skin replacement in six severely burned individuals, one of whom has been followed for 28 months [1,2]. Debrided burn wounds were first resurfaced with cryopreserved, meshed, split-thickness autologous skin. In each case, donor skin was obtained from several cadaveric sources and was not matched with the recipient for transplantation antigens. Allografts were consistently accepted. Because the allograft epidermis was considered to be the principal target of eventual immune rejection [3], it was removed at 3-4 weeks by abrasion. The dermal bed was resurfaced with stratified autologous keratinocyte cultures, which had been initiated from unburned skin of the patient at the time of burn debridement.

Except for occasional loss due to local bacterial infection, the grafted keratinocyte cultures formed a histologically differentiated epidermis of normal or increased thickness, and began to epithelialize intervening exposed dermis by radial outgrowth within 7d after application. In one case, a few scattered blisters, mostly less than 1 cm in diameter, developed during the first 6 weeks and resolved spontaneously. No clinical signs of inflammation suggestive of tissue rejection have been observed, and the healed grafts are minimally scarred. Melanocyte function was indicated by the clinical observation of patchy pigmentation in the grafted areas. In some of the grafted areas sparse hair regrowth was evident.

Here we report immunohistochemical and ultrastructural findings indicative of normal cell function in the composite skin graft, including complete reconstitution of the dermal-epidermal basement membrane zone (BMZ), epidermal melanin units, the dermal microvasculature, and the matrix remodelling activity of dermal fibroblasts.

MATERIALS AND METHODS

Patients Six severely burned individuals have been treated with the composite grafting technique. Of these, one has been followed for 28 months and two died early in the post-grafting period. The
other three have been successfully grafted with shorter follow-up and will be the subjects of subsequent reports. The grafts of the first two patients are the subjects of this study; the clinical details of these cases have been presented [2].

**Cryopreserved Cadaveric Skin** Cryopreserved allogeneic skin was obtained from the Yale Skin Bank. The skin was harvested by dermatome and is 0.3-0.5 mm thick. The details of its acquisition, storage, and thawing are described elsewhere [2]. Cellular viability of skin preserved in this manner and used as allografts has been documented by several independent methods. These include outgrowth of both keratinocytes and fibroblasts in explant culture, incorporation of tritiated thymidine as assessed by autoradiography [4], and synthesis of $^3$H-methionine labeled proteins by the keratinocytes that grew from the explants.

**Autologous Keratinocyte Cultures** Keratinocyte cultures were initiated from uninjured skin of the patients as reported [1,2]. The primary culture medium consisted of DMEM supplemented with 20% fetal bovine serum, hydrocortisone (1 μM), cholera toxin (0.1 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and amphotericin B (1.25 μg/ml). Cells were plated onto mitomycin C-treated 3T3 fibroblasts at a density of 4 × 10^6/60 mm dish or onto type I collagen-coated dishes at 8 × 10^5/60 mm dish (5). Medium was replaced every other day.

When primary cultures were approximately 90% confluent (10-12 d), the cells were released from the dishes by treatment with trypsin-EDTA and passage into serum-free MCDB-153 medium (Irvine Scientific). Additions included hydrocortisone (0.5 μM), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), insulin (5 μg/ml), epidermal growth factor (10 ng/ml), and bovine pituitary extract (60-100 μg protein/ml) as described [6]. The following ingredients were supplemented to final concentrations as follows: CaCl₂ (0.1 mM), histidine (0.24 mM), isoleucine (0.75 mM), methionine (0.09 mM), phenylalanine (0.09 mM), tryptophan (0.045 mM), and tyrosine (0.075 mM) [7]. For passages in the MCDB-153 medium, cells were seeded at 2.5 × 10^5/60 mm dish, onto untreated plastic. The splitting ratio for a passage under these conditions varied from 1:6 to 1:12. The cells proliferated rapidly (population doubling time 30-48 h) as a monolayer [8], and cultures were nearly confluent in 4-6 d.

In preparation for grafting, cultures in the second to fourth passages were returned to primary culture medium that had been modified by omitting cholera toxin. The medium was changed every other day. The cells continued to proliferate in this medium [8] and the cultures became stratified [9]. Within 4-6 d the cultures were 2-4 cell layers thick and confluent.

**Transfer of Keratinocyte Cultures** The medium was aspirated and the cultures were washed once in calcium and magnesium free phosphate buffered saline (CMF-PBS). The cultures were partially released from the dishes by incubation at 37°C in 2 ml of DMEM containing Dispase II (Boehringer-Mannheim), 6U/ml. A polyolefin transfer membrane (Delnet®), cut into discs matching the inner diameter of the dish, was submerged in the Dispase solution in direct opposition to the culture. The edge of the culture was loosened with fine forceps and grasped together with the edge of the transfer membrane. Next, the culture supported by the transfer membrane was peeled from the surface of the dish. Cultures adhered to the transfer membranes without shrinking and without need for mechanical fixation. Each culture-membrane duplex was then transferred to a fresh dish, culture side up, and moistened with DMEM. For grafting, the duplexes were placed culture side down on the recipient site, covered with Vaseline impregnated gauze, and left undisturbed for 1 week.

The transfer of autologous keratinocyte cultures to the abraded allograft completes the construction of a composite skin graft, and denotes day zero in the chronology of a composite graft.

**Light Microscopy** Sites selected for biopsy were anesthetized with 1% lidocaine containing epinephrine 1:100,000. Punch biopsy specimens were fixed in 10% formalin and embedded in paraffin. A keratinocyte culture, prepared for grafting and removed from the dish as described above, was fixed and embedded in the same way. Sections were stained with hematoxylin and eosin.

**Immunohistochemistry** Tissues were fixed and embedded in paraffin as for light microscopy. Immunoperoxidase labeling of 6-μm sections with antibodies against laminin and type IV collagen was performed as described [10-13].

**Electron Microscopy** Punch biopsy specimens, 3 mm in diameter, were obtained as described above. These were cut vertically into 1-mm slices and fixed overnight at 4°C in Karnovsky’s mixture of 2% formaldehyde and 2.5% glutaraldehyde [14], buffered to pH 7.2 with 0.1 M sodium cacodylate buffer. In order to enhance the cross striations of anchoring fibrils, the specimens were then fixed in buffered 1% osmium tetroxide containing 1.5% potassium ferrocyanide [15]. The specimens were dehydrated in ethanol and embedded in Spurr’s epoxy resin [16]. Ultrathin sections were cut by diamond knife. To further accent the cross striations, the sections were stained with lead citrate only [17]. Specimens examined for the presence of collagen fibers were stained with uranyl acetate and lead citrate.

**RESULTS**

**Histology** A biopsy of cryopreserved cadaveric skin 29 d after placement on the eschar-exfoliated burn site showed healthy epidermal and dermal components (Fig 1A). The grafted area was then abraded in order to remove all-epidermis. A biopsy confirmed that the epidermis had been completely removed (Fig 1B). A biopsy taken 25 d after transfer of autologous keratinocyte cultures onto the abraded allograft exhibited a well-developed epithelium without rete ridges (Fig 1C). The dermis at this time was hypercellular with an increased number of fibroblasts, a mild lymphohistiocytic infiltrate, and prominent, patent microvasculature. A biopsy taken 11 months after application of the keratinocyte cultures showed further maturation of the dermal-epidermal junction with increased undulation suggestive of rete ridge formation and resolution of the dermal hypercellularity (Fig 1D). Hair follicles and eccrine sweat glands were occasionally demonstrable (not shown).

**Immunohistochemistry** Exposure of thinned cryopreserved skin to antibodies against laminin and type IV collagen revealed staining of the dermal-epidermal and microvascular basement membranes with both antibodies (Fig 2A, B). Sections of allograft 29 d after placement (immediately before removal of the allogeneic epidermis) revealed significant reduction in staining of the dermal-epidermal BMZ. A marked angiogenic response was accompanied by

![Figure 1](image-url)
moderate basement membrane staining of microvascular walls for laminin (Fig 2C), but none for type IV collagen (Fig 2D), a pattern consistent with neo-vascularization [13]. Keratinocyte cultures after Dispase treatment stained weakly for both basement membrane antigens without evidence of polarity (Fig 2E,F). On days 25 and 38 after culture transfer, the composite graft revealed intense staining for both laminin and type IV collagen in the dermal-epidermal BMZ, indistinguishable from that of normal skin. Vascular basement membranes stained for both antigens as intensely as in normal skin (Fig 2G-J).

**Ultrastructure**

The fine structure of the dermal-epidermal BMZ in specimens obtained 47 and 95 d after application of keratinocyte cultures to abraded allograft dermis was compared with that of an uninjured control site a few centimeters distant. The undulating BMZ of the patient's normal flank skin is shown in Fig 3A. The individual components of the zone are delineated clearly and consist of the plasma membrane of the basal keratinocyte, modified by extensively confluent hemidesmosomes; a sharply delineated lamina lucida traversed by anchoring filaments at the sites of hemidesmosomes; and a continuous lamina densa, which is the site of attachment of the branched cross-striated anchoring fibrils. The anchoring fibrillar meshwork is densest in the recesses of the basal serratations.

In the 47-d composite graft, by comparison, the BMZ resembled that of a simple glandular epithelium rather than epidermis (Fig 3B). There were no rete ridges on light microscopy and by electron microscopy basal serratations were scarce. Hemidesmosomes were relatively few, discrete, and of small diameter. The lamina lucida was of normal width, traversed by anchoring filaments subjacent to hemidesmosomes, at which sites the lamina densa appeared to be of normal density. At other sites, the lamina densa was pale, thin, and occasionally discontinuous. Incipient anchoring fibrils occurred preferentially in the vicinity of hemidesmosomes. At blister sites the separation was immediately beneath the anchoring fibrils, which remained attached to the lamina densa at the blister roof (not illustrated). In the 95-day-old composite graft, the BMZ was essentially normal (Fig 3C).

A basal melanocyte, 5 d after application of autologous keratinocyte cultures, is shown in Fig 4A. All stages of melanosome synthesis are represented. In older grafts, melanocytes were observed in a parabasal position, i.e., separated from the BMZ by projections of basal keratinocytes (not illustrated). Portions of two basal keratinocytes are shown in Fig 4B to illustrate the abundant uptake of melanosomes from an adjacent melanocyte. Melanocytes and melano-phagosomes in keratinocytes were seen at 5, 47, and 95 d after application of the epidermal cultures.

Examination of the papillary dermal microvasculature in the 47-d composite graft revealed a morphologically normal, typical BMZ as illustrated in Fig 5A. Pericytic microvessels were also encased in morphologically normal basement membrane material (not illustrated). Their endothelium contained prominent Weibel-Palade granules. As already indicated by light microscopy, the density of collagen fibers appeared increased for this area. Fibroblasts had the characteristic appearance of active, protein-synthesizing cells. They had an extensive rough-surfaced endoplasmic reticulum and Golgi apparatus, and myriad small coated and uncoated vesicles (Fig 5B). The smooth membranes that surround nascent collagen fibrils (Fig 5B,C) have been shown to be deep recesses of fibroblast plasma membrane [18].

The papillary dermis contained numerous small lymphocytes and cellular debris, including free mitochondria and melanin granules. No elastic or nerve fibers were evident at 47 d.

**DISCUSSION**

The BMZ of the dermal-epidermal junction is a complex region composed of several structural components including the basal cell plasma membrane with its hemidesmosomes, the lamina lucida and anchoring filaments, and the lamina densa and subjacent anchoring fibrils. The BMZ can also be defined antigenically by the use of

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**Figure 2.** Immunoperoxidase staining of laminin and type IV collagen. Photomicrographs of the cadaveric allograft before (a,h) and 29 d after placement (c,d); an autologous keratinocyte culture (e,f); and the complete composite graft 25 d (g,h) and 38 d (i,j) after keratinocyte culture placement. Immunoperoxidase stains for lamin (a,c,e,g, and i). Immunoperoxidase stains for type IV collagen (b,d,f,h, and j). Scale bars: 10 μm.
Figure 3. Fine structure of dermal-epidermal junction. Uninjured control site less than 10 cm from graft (a); BMZ 47 d after application of autologous keratinocyte culture to freshly abraded dermal allograft (b); and BMZ 95 d after placement of keratinocyte culture (c). K: basal keratinocyte; D: dermis; vertical bracket: BMZ at control site; gK: graft keratinocyte; m: melanin granule; cD: dermis of cadaveric origin; horizontal brackets: incipient anchoring fibrils extending from hemidesmosomes. Note basal serrations of keratinocytes in control biopsy (a) and older epidermal graft (c). Stars denote regions of highest density of anchoring fibrils. The prominent electron dense dots in (c) are glycogen. Sections were stained with lead citrate. Scale bars: 0.5 um.

Antibodies to several specific molecular components, such as laminin and type IV collagen, the two antigens examined in this report. These components and the lamina densa are the products of keratinocytes [19]; anchoring fibrils have been thought to be produced by dermal fibroblasts [20]. Cultured keratinocytes synthesize laminin [21], bullous pemphigoid antigen [21], and Type IV collagen [22] in vitro. Keratinocytes also synthesize the collagenous component of anchoring fibrils, type VII collagen [23,24]. The cell responsible for the production of anchoring fibrils has not been clearly identified and may be either or both the keratinocyte and the fibroblast.

Our data demonstrate that in composite skin grafts autologous
Figure 4. Functional melanocytes in epidermis of composite graft. a: Melanocyte with melanosomes of all stages of maturation in basal layer of epidermis 5 d after grafting of autologous epidermal culture. N: nucleus of melanocyte; K: cytoplasm of basal keratinocyte. b: Evidence of melanin transfer to basal keratinocytes in 95-d composite graft. 1: melanocytic dendrite in longitudinal section; 2: in cross section; 3 and 4: large and partially digested melanophagosomes in keratinocytes; 5: small melanophagosome. N: nucleus of basal keratinocyte; arrows: basal plasma membrane. Sections were stained with lead citrate. Scale bars: 0.5 um.
Figure 5. Papillary dermal components of composite graft 47 d after placement of autologous keratinocyte culture (76 d after placement of cadaveric allograft). a: Capillary endothelial cell in contracted state with collapsed lumen; b,c: Fibroblasts active in the synthesis of collagen. Arrows in a point to collapsed capillary lumen outlined by endothelial plasma membrane that is studded with pinocytotic vesicles; arrowheads point to capillary BMZ. Arrows in b and c point to sites of collagen fibrillogenesis, sectioned longitudinally in b and transversely in c. Asterisks denote oblique sections of individual collagen fibrils. N: nucleus of endothelial cell; V: veil cells; G: Golgi apparatus. Sections were stained with lead citrate (a) or uranyl acetate followed by lead citrate (b,c). Scale bars: 0.5 um.
keratinocyte cultures form a morphologically unremarkable epidermis after engraftment onto allogeneic dermis. Adnexal structures, including hair follicles and eccrine sweat glands, are evident in some grafted areas, presumed to be sites at which the burn injury did not include the full thickness of skin and spared the deepest components of these appendages. These appendages were able to reestablish communication with the overlying epidermis by penetrating the layer of allogeneic dermis included in the composite graft.

We have also demonstrated the presence of suprabasilar dendritic cells that stain for S100 antigen in composite graft epidermis as early as 7 d after transfer of autologous keratinocyte cultures (data not presented) [30]. In the largest representative Langdon cell cultures of host origin that have migrated into the new epidermis, because similar S100 positive cells were not seen in cryopreserved skin before engraftment.

The grafted keratinocyte cultures become fully integrated with the underlying dermis through the formation of a normal BMZ, including anchoring fibrils, which are numerous and morphologically typical. In one of four evaluable patients, early subepidermal blisters appeared and resolved by 6 weeks. Although the immunohistochemical data from this patient's composite grafts showed intense staining for laminin and type IV collagen 25 d after the cultures were applied, hemidesmosomes and anchoring fibrils were significantly reduced, both in number and in size, even at 47 d, and the lamina densa was discontinuous. The eventual normalization of the BMZ and the return of greater numbers of anchoring fibrils by day 95 correlated with the resolution of clinical blistering. These findings are in contrast to those of Woodley et al [25] who examined the BMZ of autologous keratinocyte cultures grafted directly onto debrided burn wound beds in four patients. These investigators found greatly diminished or absent anchoring fibrils at the dermoepidermal BMZ as late as 4.5 months after application of keratinocyte cultures, although type VII collagen, the major component of anchoring fibrils, was present. In addition, three of their patients experienced spontaneous blistering and all four exhibited mechanical fragility in the grafted areas up to 7 months after grafting. Other investigators have been able to detect anchoring fibrils beneath autologous keratinocyte cultures grafted onto debrided burn wounds [26,27]. Our composite grafts differ from those in that keratinocyte cultures were applied to a dermal surface rather than excised or granulating burn wounds. This dermis, with its plentiful microvasculature and fibroblasts, may be responsible for the attendant functional normalization of the cellular components and the structural durability of the composite graft [28].

The normal undulating pattern of rete ridges is absent in histologic sections up to 11 months. It has been shown, at least for deeply ridged palm skin, that the epidermal basal cell population is heterogeneous with respect to proliferative activity. Highly proliferative cells have relatively smooth, non-serrated basal contours and reside at the base of deep rete ridges, whereas basal cells with serrated contours are slowly cycling and are thought to contribute to the adhesion of the epidermis to the papillary dermis [29]. We do not know whether proliferative heterogeneity exists among the basal cells of the composite skin graft. It appears from our study that, similar to the plasmalemmal pockets of fibroblasts that promote assembly of collagen fibrils, the crevices formed by the base serrations of keratinocytes constitute a microenvironment conducive to anchoring fibrillogenesis.

Melanocytes are present in the epidermis of the composite graft from the outset and function normally in pigment transfer to keratinocytes. The slightly elevated location, above the BMZ, appears to be characteristic of sites in which there has been melanocyte proliferation, such as embryonic skin, newborn epidermis, solar lentigo, repigmenting vitiligo, and epidermal melanosis in metastatic melanoma [30]. The melanocytes in the composite grafts are most likely autologous because melanocytes frequently persist through several passages with keratinocytes in culture. This finding suggests possibilities for the surgical repigmentation of scars and vitiliginous or piebald skin through cocultivation of melanocytes and keratinocytes before transplantation.

Blood vessels within engrafted split thickness skin are derived from the host [31]. In a corneal model of neovascularization, laminin and type IV collagen appeared sequentially, i.e., laminin appeared before type IV collagen [13]. Similarly, we found moderately intense staining for laminin at the microvascular BMZ of the allograft skin 29 d after placement, but no staining for type IV collagen. By 55 and 68 d after alloengraftment (days 25 and 38 of the complete composite graft), both antigens were detected with equal staining intensity. The sequential appearance of these components is also supported by the electron microscopic appearance of the dermoepidermal BMZ, because the lamina lucida (which contains laminin) is established before the lamina densa (which contains type IV collagen) has attained its full thickness.

The composite skin graft appears to permit structural and functional integration of a layer of allogeneic dermis between an epidermis derived from autologous keratinocyte cultures and underlying host tissues. Of the original cellular components of an allograft dermis, it is likely that fibroblasts are capable of long-term survival [32]. Neovascularization of the allograft is probably via ingrowth of host blood vessels [31]. Allogeneic endothelial cells most likely degenerate, although it is possible that they anastomose with the ingrowing host vessels and persist. Epithelial adnexae of the allograft are not expected to persist or regenerate because the proliferative cells of such structures are not included in grafts of the thickness used (0.3-0.5 mm). The survival of allogeneic cells in the composite graft has not been established. Nevertheless, the allogeneic dermal component of the graft is clearly capable of supporting rapid and permanent engraftment of host-derived keratinocyte cultures. Ex vivo biopsies of such grafted epidermis synthesize proteins characteristic of normal epidermis in vivo, including the 67-kD keratin molecule [8].

During the first 2 months after establishment of the composite graft, fibroblasts in the superficial dermis were abundant and produced dense accumulations of collagen. By 1 year the dermis was no longer hypercellular. The ultrastructural demonstration at day 47 after placement of the keratinocyte cultures of extracellular organelles and increased numbers of small lymphocytes suggests that there has been destruction of allogeneic cellular elements such as nerve fibers, Schwann cells and blood vessels, as well as adnexal cells. The healthy fibroblasts observed at day 47 may be of host or allograft origin.

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