Rapid Normalization of Epidermal Integrin Expression After Allografting of Human Keratinocytes

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Allogeneic keratinocyte grafts have beneficial effects on skin wounds, but the underlying interactions between graft and woundbed remain to be explored in detail. The epidermal integrins play a pivotal role in mediating cell-to-cell and cell-to-matrix interactions. In unwounded epidermis, $\alpha_2\beta_1$-, $\alpha_3\beta_1$-, $\alpha_6\beta_4$-, $\alpha_5\beta_1$-, and $\alpha_v\beta_5$-integrins are confined to basal cells. During healing of incisional wounds, these integrins are also expressed in suprabasal cells, where they remain detectable even after epidermal integrity is fully reestablished. We examined the integrin subunits α_2, α_3, α_6, α_5, and α_v in partial thickness burn wounds grafted with allogeneic keratinocytes and asked whether the effect of allogeneic keratinocyte grafts, i.e., fast reepithelialization, is reflected by an accelerated reversion to a normal integrin pattern. Biopsies were taken after wound debridement before grafting and 10 d after transplantation. After 10 d, a stratified epidermis had developed in all cases and integrins were mainly restricted to the basal cell layer of the neo-epidermis. α_2-, α_3-, α_6-, and α_v-subunits were present at basal and/or lateral cell borders, duplicating the integrin pattern in normal epidermis. The findings indicate that grafting accelerates the shift of the epidermis from an inflammatory to a regenerative state, as reflected by the reversion of the integrin pattern from a "spread-and-migrate" to the "steady-state" phenotype. ***Key words: allogeneic/grafts/wound repair. J Invest Dermatol 107:423–427, 1996***

Allogeneic human keratinocyte sheets have been successfully used (Hefton *et al*, 1983; Madden *et al*, 1986; Phillips *et al*, 1990; Oliver *et al*, 1991; Maier, 1993; Schönfeld *et al*, 1993) to reepithelialize human split thickness burn wounds. It is assumed that the vital cells of the graft provide a cover with a strong stimulus for wound healing, but the molecular interactions of the keratinocyte allografts with the underlying wound tissue remain to be investigated (Woodley *et al*, 1993). Integrins play a pivotal role in mediating the intercellular and cell-to-matrix binding of keratinocytes (Albelda and Buck, 1990; Klein *et al*, 1990; Rouslathi, 1991; Sonnenberg *et al*, 1991; Cheresh, 1992; Hynes, 1992; Sastry and Horwitz, 1993; Sonnenberg, 1993). The function and distribution of integrins in the normal epidermis (De Luca *et al*, 1990; Klein *et al*, 1990; Larjava *et al*, 1990; Hertle *et al*, 1992) and during the healing of incisional wounds have been analyzed (Cavani *et al*, 1993; Juhasz *et al*, 1993). In unwounded human skin (Hertle *et al*, 1992; Cavani *et al*, 1993; Juhasz *et al*, 1993), and in keratinocyte cultures (Marchisio *et al*, 1990, 1991, 1993), the integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_4$, and $\alpha_v\beta_5$ are confined mainly to basal epidermal keratinocytes, in contact with the basement membrane or, *in vitro*, with matrix molecules of the culture substrate. After wounding (Cavani *et al*, 1993; Juhasz *et al*, 1993) or in pathologic conditions such as psoriasis (Hertle *et al*, 1992) or dystrophic epidermolysis bullosa (Hertle *et al*, 1991), these integrins are also detectable on suprabasal cells, probably reflecting the activated state of these cells. Understanding the integrin pattern might therefore help to clarify whether keratinocytes are still actively spreading and migrating or whether they have returned to a steady-state phenotype as in normal epidermal homeostasis. The aim of the current study was to investigate whether the beneficial effect of allogeneic keratinocyte transplantation is reflected in the distribution of the integrin subunits α_2, α_3, α_5, α_6, and α_v in the regenerated epidermis of human split thickness burn wounds. Within 10 d after transplantation, the integrin pattern in the transplanted area resembled that of a normal epidermis, indicating that allogeneic keratinocyte grafts favorably influence reepithelialization and accelerate the reversion to a "steady-state" epidermal integrin pattern.

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MATERIALS AND METHODS

Cell Culture Human keratinocytes were isolated from skin biopsies of young hepatitis B virus-, hepatitis C virus-, cytomegalovirus-, and human immunodeficiency virus-negative donors. Cells were cultivated using the feeder-layer technique under differentiating conditions (Rheinwald and Green, 1975) in Dulbecco's modified Eagle's medium/Ham's F12 (Gibco, Eggenstein, Germany) containing 10% fetal bovine serum (Sigma, Deisenhofen, Germany) and supplements. The confluent cultured grafts were detached with 2.4 mg Dispase per ml (Boehringer, Mannheim, Germany) in Dulbecco's modified Eagle's medium, washed three times in Dulbecco's modified Eagle's medium, and attached with Ligaclips (Ethicon GmbH, Norderstedt, Germany) to Cuticerin (Beiersdorf, Hamburg, Germany), a nonsticking vaseline gauze. The second passage keratinocyte sheets were used as fresh allografts.

Patients, Wound Treatment, and Grafting Procedure Five patients of ages 2 through 7 y with partial thickness scalds or burns on the thorax or

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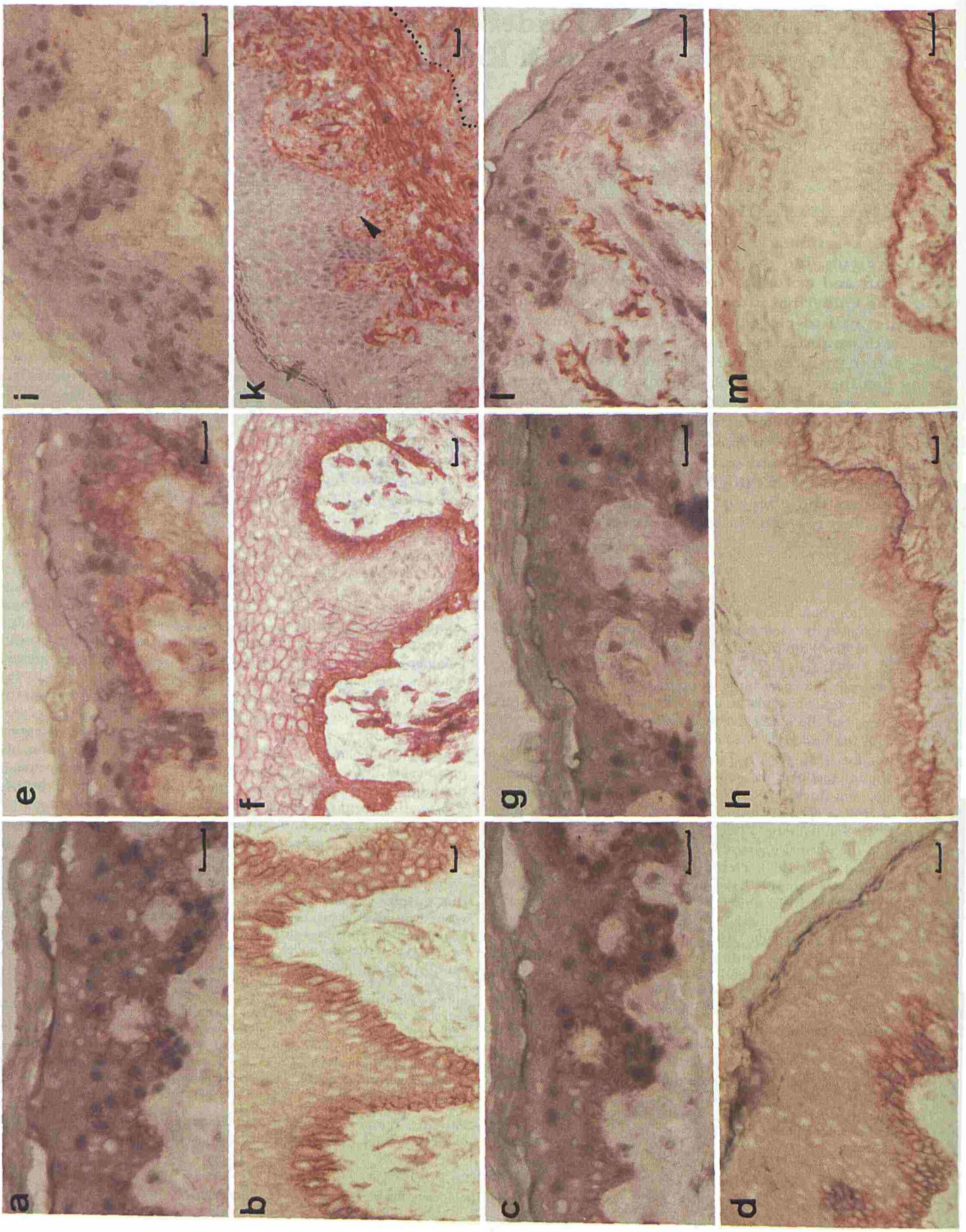
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Table I. Integrin Distribution in the Neo-Epidermis 10 d After Allografting[a]

	Integrin Chain														
	α_v			α_5			α_2			α_3			α_6		
	b[b]	lat	sb	b	lat	sb	b	lat	sb	b	lat	sb	b	lat	sb
P1[c]	++[d]	+	−	n.t.	n.t.	n.t.	−	++	−	+/−	++	−	++	+	−
P2	++	+	+/−	n.t.	n.t.	n.t.	+/−	++	−	+/−	++	−	++	+	+/−
P3	++	+	−	n.t.	n.t.	n.t.	−	++	−	+/−	++	−	++	+	+
P4	++	+	−	+/−	−	−	−	++	−	+/−	++	−	++	+	+/−
P5	++	+	−	+/−	−	−	−	++	−	+/−	++	−	++	+	+/−

[a] Ten days after grafting of allogeneic keratinocytes, frozen sections of the neo-epidermis were stained (alkaline phosphatase anti-alkaline phosphatase technique) by using moAbs against integrin chains. Stainings were evaluated semiquantitatively.
[b] b, basal; lat, lateral; sb, suprabasal.
[c] P1–P5, patient number.
[d] Semiquantitative evaluation scale, ++, strong staining; +, moderate staining; +/−, faint staining; −, no staining; n.t., not tested.

limbs were studied. The protocol of the study was approved by the Ethics Committee of the Klinikum Mannheim, Germany. All patients received cultured allografts 1 to 2 d after burn injury. Prior to debridement the wounds were cleaned with antiseptics (Braunol; Braun-Melsungen AG, Melsungen, Germany). Necrotic tissue was debrided and wounds were brushed with antiseptics (Braunol). Finally, the wound bed was rinsed with physiologic saline solution, and wounds were covered with the keratinocyte sheets attached to the vaseline-gauze and dressed with multiple layers of dry gauze.

Tissue Specimen Biopsies were taken from the center of the wounds immediately after wound debridement before grafting and on day 8 or 10 after grafting. Control biopsies of normal epidermis were taken from healthy volunteers. Biopsies were immediately embedded in Tissue Tek cryogel (Miles Inc., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −80°C. Frozen sections (5 μm) were cut with a cryotome (Jung-Frigocut 2700, Leica, Bensheim, Germany) at −20°C, fixed in acetone, air-dried, and immunolabeled at room temperature. Hematoxylin-eosin staining was performed by standard methods.

Immunohistochemistry was performed with a commercially available staining kit by an alkaline phosphatase anti-alkaline phosphatase technique (Dakopatts, Hamburg, Germany) with fast red as chromogenic substrate. For counterstaining, Mayer's hemalaun (hematoxylin, Merck, Darmstadt, Germany) was used. After mounting of the sections with Kaiser's glycerin-gelatine (Merck), photomicrographs were taken on Ektachrome 64T (Kodak, Rochester, NY) using an integrated photomicroscope (Zeiss, Oberkochen, Germany).

Antibody Preparations The following monoclonal antibodies (moAbs) against human integrin subunits were used: mouse-anti-α_2-chain, diluted 1:100 (A042, Biomol, Hamburg, Germany); mouse-anti-α_3-chain, diluted 1:100 (A043, Biomol); mouse-anti-α_5-chain, diluted 1:100 (A045, Biomol); rat-anti-α_6-chain, diluted 1:100 (No. 0769, Immunotech, Hamburg, Germany); mouse-anti-αv-chain, diluted 1:100 (A049, Biomol). Vitronectin or fibronectin were stained by using a mouse-anti-vitronectin moAb, diluted to 10 μg/ml (clone VN 7; a gift of Dr. Preissner, Bad Nauheim, Germany), and a mouse-anti-fibronectin moAb diluted to 10 μg/ml (Clone FN5[c]). The moAb FN5[c] was raised against fibronectin (kindly provided by Prof. N. Heimburger, Behringwerke AG, Marburg, Germany). The purified IgG was tested by enzyme-linked immunosorbent assay and immunoblot (data not shown). Furthermore the staining pattern of FN5[c] was compared to the commercially available mouse-anti-fibronectin-moAb (TaKaRa M001, supplied by Dianova, Hamburg, Germany). Staining was performed on normal skin and the biopsies of patients included in the study (data not shown). Phosphate-buffered saline, mouse-IgG_1 isotype-control (No. 571, Dianova) diluted 1:10, mouse-IgG_2 isotype control (No. 572, Dianova) diluted 1:20, normal mouse IgG (No. 015–000-002, Dianova), as well as normal rat IgG (No. 012–000-002, Dianova) were used to detect nonspecific binding. Secondary antibody for alkaline phosphatase anti-alkaline phosphatase detection was rabbit-anti-mouse IgG (Dianova) diluted 1:50. In the case of the rat antibody (anti-α_6-chain), further incubation with mouse-anti-rat IgG (Dianova) diluted 1:50 was performed prior to adding the secondary rabbit-anti-mouse IgG. All reagents were diluted in phosphate-buffered saline.

RESULTS

Histology of Wound Biopsies Hematoxylin and eosin staining of biopsies obtained before grafting, i.e., directly after wound debridement, revealed dermal connective tissue and adnexal structures. There was no stratified epidermis detectable at the wound surface. Ten days after allografting, however, a newly formed, stratified epidermis was present in all biopsies examined.

Integrin Pattern of Normal Epidermis The biopsies taken from healthy human volunteers were used as controls for integrin expression in normal epidermis. α_2-Chain–specific staining was concentrated primarily at the lateral aspects of basal cells, but isolated single cells of the spinous layer were also stained. No α_2-chain–specific staining was present on other suprabasal cells **(Fig 1*a*)**. Staining for the α_3-chain was also confined to the basal cell layer and was most pronounced at the lateral and apical cell borders, and α_3-staining, like α_2-staining, was not present in suprabasal cells **(Fig 1*c*)**. Discontinuous staining of α_6-integrin subunits was present on basal cells **(Fig 1*e*)** and was also detectable on some spinous cells. Staining of the α_5-integrin subunit was not found with the antibody used (data not shown). The corresponding α_5-integrin ligand, fibronectin, was also found sparsely in the dermal connective tissue of normal skin **(Fig 1*i*)**. The basal keratinocytes of the normal epidermis displayed a faint and discontinuous staining for the α_v-chain **(Fig 1*g*)**; staining was completely absent in suprabasal cells. Antibody to the α_v-integrin-ligand, vitronectin, was stained as fiber-like structures in the dermis **(Fig 1*l*)**.

Integrin Pattern After Reepithelialization Following Allografting

α_2-Chain After grafting of allogeneic keratinocytes, the basal cell layer of the epidermis reacted strongly with the anti-α_2-chain antibody in all biopsies tested. Staining was focused at the lateral and in some cells the apical cell borders of the basal keratinocytes **(Table I)**. In most basal cells, staining for the α_2-chain was absent at the basal cell pole, i.e., in the vicinity of the dermal-epidermal junction **(Fig 1*b*)**. In suprabasal cells, the α_2-chain could not be

Figure 1. Integrins in the neo-epidermis of transplanted wound sites resemble that in normal epidermis. Staining (alkaline phosphatase anti-alkaline phosphatase technique) of integrin chains, fibronectin, and vitronectin was performed on frozen sections of partial thickness burns grafted with allogeneic keratinocytes and in frozen sections of normal epidermis as described in *Materials and Methods*. Staining of α_2- (*a*), α_3- (*c*), and α_v- (*g*) chain in normal epidermis; staining of α_2- (*b*), α_3- (*d*), and α_v- (*h*) chain in the neo-epidermis 10 d after grafting. Staining of the α_6-chain in normal epidermis (*e*) or in the neo-epidermis 10 d after grafting (*f*). Staining of fibronectin in normal connective tissue (*i*) and in connective tissue of wounds 10 days after grafting (*k*). ➧ in (*k*): discontinuous fibronectin staining at the dermo-epidermal junction; the *dotted line* in (*k*) delineates the border between upper and deeper dermis. Staining of vitronectin in normal skin (*l*) and in the dermo-epidermal junction of the neo-epidermis 10 d after grafting (*m*). *Scale bars*, 10 μm.

Table II. Integrin Pattern in the Neo-Epidermis 10 d After Allografting Resembles That in Normal Epidermis[a]

	Normal Epidermis			Neo-Epidermis 10 Days After Allografting		
	Basal Cells			Basal Cells		
Integrin Chain	Basal Poles	Lateral Cell Borders	Suprabasal Cells	Basal Poles	Lateral Cell Borders	Suprabasal Cells
α_v	+[b]	+	−	++	+	+/−
α_5	−	−	−	−	−	−
α_2	−	+	−	−	+	−
α_3	−	+	−	+/−	+	−
α_6	+	+	−	++	+	+ or −

[a] Frozen sections of normal epidermis and of neo-epidermis of burn patients 10 d after grafting of allogeneic keratinocytes were stained (alkaline phosphatase anti-alkaline phosphatase technique) by using monoclonal antibodies against integrin chains. Localizations of integrin chains were evaluated semiquantitatively.

[b] Semiquantitative evaluation scale: ++, strong staining; +, staining; +/−, faint or granular staining; −, no staining.

detected. The staining pattern was similar to that found in normal epidermis **(Table II)**.

α_3-Chain After transplantation, the α_3-chain was detected in the basal cells of the neo-epidermis **(Fig 1*d*)** and was focused at the lateral and the apical cell borders **(Table I)**. Toward the dermal-epidermal junction, basal keratinocytes rarely stained **(Fig 1*d*)**.

α_5-Chain With the moAb specific for the integrin subunit α_5, the basal cells displayed very faint uniform label that was also present in suprabasal keratinocytes of the neo-epidermis **(Table I)**. The staining appeared granular and without intensification at the cell borders (data not shown), similar to the staining in the epidermis of unwounded skin **(Table II)**. The α_5-integrin ligand, fibronectin, was stained in the subepidermal connective tissue **(Fig 1*k*)**, where it was even more pronounced than in the deeper dermis (below the *dotted line* in **Fig 1*k***). Fibronectin-specific staining in the dermal-epidermal junction, however, was fainter and discontinuous (◗ in **Fig 1*k***).

α_6-Chain After transplantation, strong staining for the α_6-chain was present at the lateral and basal cell borders of basal keratinocytes of the neo-epidermis **(Fig 1*f*)**. The staining of suprabasal cells varied between the biopsies: in some, only weak membrane-associated and faint granular intracellular staining was observed, whereas in others, suprabasal cells were clearly stained. In a third case no α_6-specific staining was observed **(Table I)**.

α_v-Chain α_v-Specific staining was present in the basal cell layer of the newly formed epidermis in all biopsies **(Table I)**. In contrast to the normal epidermis **(Table II)**, staining was mainly restricted to the basal cell borders, i.e., near the dermal-epidermal junction **(Fig 1*h*)**. The lateral aspects of the basal keratinocytes and some spinous suprabasal cells revealed faint, membrane-associated staining. Ten days after allografting the ligand for the α_v-integrins, vitronectin, was concentrated at the dermal-epidermal junction **(Fig 1*m*)**, in close association with α_v-specific staining **(Fig 1*h*)**.

DISCUSSION

Although there were no epidermal keratinocytes detectable on the wound surface after initial debridement, we observed rapid and complete reepithelialization of the partial thickness burns within 10 days after allografting, with no signs of rejection. The most striking observation was that by 10 days after grafting integrins were already absent (α_5-chain) or polarized to the basal (α_3, α_6, α_v) and/or lateral (α_2, α_3, α_6, α_v) poles of the basal keratinocytes of the neo-epidermis. This is in contrast to healing incisional wounds, in which the integrins are still present on suprabasal cells even after the epidermis is fully healed (after 18 days) (Cavani *et al*, 1993).

In healing incisional wounds (Cavani *et al*, 1993), expression of α_2-integrin-chains was faint, even after the wounds were fully reepithelialized. In contrast, we found strong staining for the α_2-chain on basal keratinocytes, predominantly at the lateral poles of basal keratinocytes, suggesting rapid reversion of the expression of α_2-subunits to normal, probably due to acceleration of reepithelialization by the transplantated allografts. Moreover, its concentration at the lateral cell aspects indicated that the α_2-chain was not associated with type I collagen at the basal poles of basal keratinocytes, as *in vitro* (Scharffetter-Kochanek *et al*, 1992). Thus, the α_2-containing integrins are likely to be involved in cell-to-cell interactions (Marchisio *et al*, 1991; Scharffetter-Kochanek *et al*, 1992) rather than in mediating adhesion to type I collagen of the extracellular matrix. The localization of the α_3-chain also indicates a role for this integrin subunit in intercellular contacts (Larjava *et al*, 1990; Marchisio *et al*, 1991; Cavani *et al*, 1993) rather than in mediating interaction with the extracellular matrix (Sonnenberg, 1993).

Our finding that the α_5-subunit, which is part of the fibronectin receptor $\alpha_5\beta_1$, was sparse in the neo-epidermis is also compatible with an early reversion to normal. This observation is consistent with the hypothesis that the fibronectin receptor is predominantly involved in keratinocyte migration over a provisional fibronectin-rich matrix (Grinell, 1992; Cavani *et al*, 1993) and is decreased after wound closure and induction of terminal differentiation (Nicholson and Watt, 1991; Hotchin *et al*, 1993). Fibronectin itself was present in the dermis but was clearly separated from the dermal-epidermal junction, suggesting the presence of a basal lamina, which was also more directly indicated by a continuous layer of laminin (data not shown). Since the basal lamina is not permeable to high molecular proteins such as fibronectin (250 kDa), basal lamina synthesis is possibly involved in this visible separation of fibronectin from basal keratinocytes (Yurchenco and Schittny, 1990; Brown *et al*, 1991). We speculate that the decrease of α_5-specific staining reflects the downregulation of α_5-containing integrins, possibly as a consequence of the disappearance of fibronectin (Hotchin *et al*, 1993).

In contrast to the α_5-chain, the α_v-chain, which is part of vitronectin-specific integrins, was still found at the basal, i.e., basement membrane-associated, aspect of the basal keratinocytes, suggesting an interaction with the continuous vitronectin layer adjacent to the dermal-epidermal junction. Vitronectin (75 kDa) can penetrate the basal lamina (Yurchenco and Schittny, 1990; Brown *et al*, 1991), so that an interaction between vitronectin and the vitronectin-specific α_v-integrins can occur even after the synthesis of a basal lamina (Charo *et al*, 1990). *In vitro* data show that vitronectin is involved in counteracting fibronectin-induced keratinocyte motility (Brown *et al*, 1991). Thus, we suggest that allografted keratinocytes attach to the provisional fibronectin- and vitronectin-rich matrix by means of α_v-containing integrins. Vitronectin would displace fibronectin from polyvalent α_v-integrins due to its higher affinity and could thus contribute to the terminal differentiation of the keratinocytes (Adams and Watt, 1990; Charo *et al*, 1990; Nicholson and Watt, 1991; Hotchin *et al*, 1993).

The predominance of the α_6-chain at the basal and lateral aspects of the basal cell layer is reminiscent of normal epidermis. The expression at the basal pole of the basal keratinocytes might indicate the interaction of hemidesmosome-associated $\alpha_6\beta_4$ with the basement membrane and its function as a laminin receptor. $\alpha_6\beta_4$ is presumably involved in the control of basement membrane formation (Sonnenberg *et al*, 1991; Cavani *et al*, 1993; Marchisio *et al*, 1993), which would explain the intense α_6-specific staining along the dermal-epidermal junction.

Our data document a rapid and complete reepithelialization of partial thickness burn wounds by a stratified epidermis within 10 d after allografting with cultured keratinocytes. Immunohistochemical data revealed that this beneficial effect is reflected by the expression pattern of integrins, which unexpectedly resembled that in normal epidermis or in epidermis of incisional wounds after more than 18 d of healing (Cavani *et al*, 1993; Juhasz *et al*, 1993). The localization of the α_2-, α_3-, α_6-, α_v-, and α_5-integrin chains as well

as of the extracellular matrix proteins, vitronectin and fibronectin, are consistent with the assumption that transplantation of allogeneic keratinocytes accelerates the reformation of an apparently normal epidermis and the regeneration of a basement membrane. Elucidation of the underlying mechanisms, however, awaits further investigation.

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