

Pretreatment of Human Keratinocyte Sheets with Laminin 5 Improves their Grafting Efficiency

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Laminin 5 is essential in epithelial attachment to stromal tissues, suggesting that it might improve keratinocyte attachment in a variety of clinical situations. In this study, we examined the effect of exogenous laminin 5 upon the efficiency of transplantation of keratinocyte sheets in animal models. Keratinocyte sheets were prepared according to the method of Rheinwald and Green (1975). Purified laminin 5 was added to the sheets of group 1 (1.0 μg per cm^2), Dulbecco's modified Eagle's medium alone was added to group 2. The sheets were grafted to the panniculus carnosus of nude mice (BALB/C nu/nu) ($n = 12$) and nude rats (Fisher 344) ($n = 15$). The take rate was assessed by measurement of the area of surviving epithelium at 7 d postgrafting. Laminin 5 bound the keratinocyte sheets of group 1. At 7 d postgrafting,

the area of epithelialization of group 1 was significantly larger than that of group 2. Immunohistochemistry staining showed that collagen IV, laminin 5, and collagen VII stained more strongly at the dermal-epidermal junction in group 1 than in group 2. Integrin chains $\alpha 6$ and $\beta 4$ were similar in both groups. Electron microscopy at day 3 after grafting, showed the lamina densa of group 1 to be more continuous than in group 2. Pretreatment of cultured human keratinocyte sheets with laminin 5 improved the extent of epithelial coverage and increased the rate of neobasement membrane formation. The results suggest that laminin 5 promotes epithelial attachment by increasing the rate of basement membrane assembly. **Key words:** basement membrane/keratinocyte/laminin 5/transplantation. *J Invest Dermatol* 113:38-42, 1999

Laminin 5, localized to the epidermal basement membrane zone, has recently been characterized as a component of anchoring filaments. The molecule consists of three disulfide-linked subunits: $\alpha 3$ chain (165 kDa), $\beta 3$ chain (140 kDa), $\gamma 2$ chain (105 kDa) (Burgeson *et al*, 1994). It has been reported that the absence of laminin 5 in humans results in an increased susceptibility to blister formation within the epidermal basement membrane zone at the level of lamina lucida, resulting in Herlitz junctional epidermolysis bullosa (Meneguzzi *et al*, 1992), and in previous *in vitro* studies in cell culture, laminin 5 was deposited upon the culture substrate by growing and migrating human keratinocytes. Addition of monoclonal antibody BM-165 (anti-laminin $\alpha 3$) to the culture of keratinocytes causes the cells to round and detach, and skin fragments incubated with antibody extensively de-epithelialize (Rousselle *et al*, 1991). These results strongly suggest that this molecule plays an important part in the adherence of epidermis to dermis in normal skin.

Autologous transplantation of cultured human keratinocyte sheets has been used clinically for the treatment of extensive burns, giant nevus, and other cutaneous wounds. It is known that the take of cultured keratinocyte sheets and the reconstitution of skin in the grafted site are influenced by the condition of the recipient beds and that the graft take well on the dermal wounds. The take on

dermabraded wounds was almost completely (Kumagai *et al*, 1994, 1995). In practice, the take rate of the sheets transplanted directly to granulation tissue without dermal components has been poor. The two-stage grafting procedure combining dermal and epidermal equivalents *in situ* (Cuono *et al*, 1987) have been used in burn patients to solve the problem of graft fragility and absence of the dermis. In this study, we have tested the effect of the addition of exogenous laminin 5 during keratinocyte sheet transplantation to the panniculus carnosus of nude mice and nude rats, as a model for severely burned skin in absence of the dermis.

MATERIALS AND METHODS

Preparation of keratinocyte sheets

Culture of keratinocytes The harvested skin was immersed in Dulbecco's modified Eagle's medium (DMEM) containing dispase (1000 U per ml) for 20 h at 4°C. After this enzymatic treatment the epidermis was mechanically separated from the dermis. Strips of the separated epidermis were rinsed with Hanks' solution and incubated with 0.25% trypsin in Hanks' solution for 15 min at 37°C, after which they were placed in DMEM supplemented with 10% fetal bovine serum and stirred for 5 min at room temperature to obtain cell suspensions. The suspended cells were harvested by centrifugation for 5 min at $490 \times g$, and the resulting cell pellet was then suspended and cultured on a 3T3 feeder layer in keratinocytic growth medium prepared according to the method reported by Rheinwald and Green (1975).

Purification of laminin 5 from human keratinocyte-conditioned medium Human keratinocytes were grown in keratinocyte growth medium (Gibco BRL, Grand Island, NY) until they became confluent in Cell Factories (Nalge Nunc International, Naperville, IL) and then the culture medium was

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changed to DMEM containing 10% fetal bovine serum. The spent medium was collected every other day and centrifuged to remove cells, and then ethylenediamine tetraacetic acid, phenylmethylsulfonyl fluoride, and *N*-ethylmaleimide were added in final concentrations of 5 mM, 50 μ M, and 50 μ M, respectively, to minimize endogenous protease activities. In order to remove nonspecific binding by fibronectin, the medium was first passed through gelatin-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) and then through an anti-laminin β 3 affinity column (6F12-conjugated Sepharose). After washing, laminin 5 was eluted with 1 M acetic acid, followed by dialysis against phosphate-buffered saline. The purified laminin 5 was composed of 165 kDa (α 3 chain), 140 kDa (β 3 chain), and 105 kDa (γ 2 chain) (data not shown).

Purified laminin 5 coating Keratinocytes were detached from the culture dish using dispase (30 protease units per cm^2 , Godoshuseki, Tokyo, Japan) by incubation at 37°C for 2.5 h. Sheets were washed with Hanks' solution twice, then cut into 1.5 \times 1.5 cm^2 fragments. MEIPAC^R (aterocollagen sheet, Meijiseika, Tokyo, Japan) was used as a carrier for the keratinocyte sheets. MEIPAC^R was cut into 1.0 \times 1.0 cm^2 fragments and placed on to a piece of keratinocyte sheet. The edges of the keratinocyte sheet were folded on to MEIPAC^R, followed by clipping at the four corners using silver surgical clips. The sheets were turned over (the surface of the basal keratinocyte sheet facing up), and placed in a new dish. For group 1, purified laminin 5 was added to the sheets (diluted with DMEM to provide a final dose of 1 μ g per cm^2). As a control (group 2), DMEM alone was added to the sheets. The sheets of groups 1 and 2 were incubated at 37°C for 15 min. After incubation, the sheets were washed twice with Hanks solution and then grafted.

Grafting procedure We used nude mice (BALB/C nu/nu) (5–7 wk of age, weighing 20–25 g) from Japan SLC (Hamamatsu, Japan) and pathogen-free male nude rats (Tokyo, Japan) (Fisher 344) (5–7 wk of age, weighing 150–200 g) from CLEA JAPAN. On the day of the experiment the mice or rats were anesthetized with sodium pentobarbital (40 mg per kg i.p.; Abbott Laboratories, North Chicago, IL). Skin flaps 1.5 cm square were elevated and full thickness skin defects were made in their backs. The cultured human keratinocyte sheets were grafted on to the panniculus carnosus. The sheets of group 1 (mice, $n = 12$, rats, $n = 15$) and group 2 (mice, $n = 12$, rats, $n = 15$) were grafted. Silicone gauze (Release^R, Johnson & Johnson, Arlington, TX) was applied over the grafted sheets and the skin flap replaced. The margin was then sutured with nylon (Medical Division of Kyowa Precision Instruments, Chiba, Japan).

Assessment of take rate by statistical analysis The take rate was assessed by measurement of the area of existing epithelium 7 d after grafting. The area of resurfaced epithelium was confirmed by naked eye. We photographed the wounds, and then quantitated the areas of epithelialization. The rate of graft take was shown in percentages compared with original size of grafting cultured human keratinocyte sheets.

Mean values of the areas of epithelialization were obtained using NIH imaging software. All data were expressed as the mean and standard error of the mean (SEM). Comparisons between means in each group were performed by *t* test (Statview 4.01). Differences having $p < 0.05$ were considered significant.

Histologic examination Excised grafts were fixed by the AMeX method (Sato *et al.*, 1986). The pieces were incubated overnight in acetone at -20°C then placed in acetone and incubated for 15 min at 4°C. The pieces were then incubated twice with methyl benzoate for 15 min at room temperature and twice with xylene for 15 min at room temperature. After incubation with solvent, the grafts were incubated with paraffin for 3 h at 60°C. These embedded tissues were used for hematoxylin and eosin stains and immunohistochemical examination.

Immunohistochemistry Paraffin sections were deparaffinized with xylene, immediately immersed twice in acetone, washed in phosphate-buffered saline, and immunostained using streptavidin-biotinylated-peroxidase complex with the monoclonal antibodies: laminin 5 (BM-165, 2 μ g per ml), collagen type IV [JK-199, culture medium (1:5)], collagen type VII (NP-185, 6 μ g per ml), integrins α 6 and β 4 (Chemicon International, Temecula, CA, 1:100). The sections were incubated with 2% normal swine serum for 20 min, then primary antibodies were added and incubated overnight at 4°C. Sections were then rinsed in phosphate-buffered saline, incubated with biotinylated anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA) for 1 h at room temperature, then incubated with streptavidin-biotinylated-peroxidase complex (1:100, Amersham, Cleveland, OH) for 1 h at room temperature. After rinsing in phosphate-buffered saline, the sections were incubated in a solution of diaminobenzid-

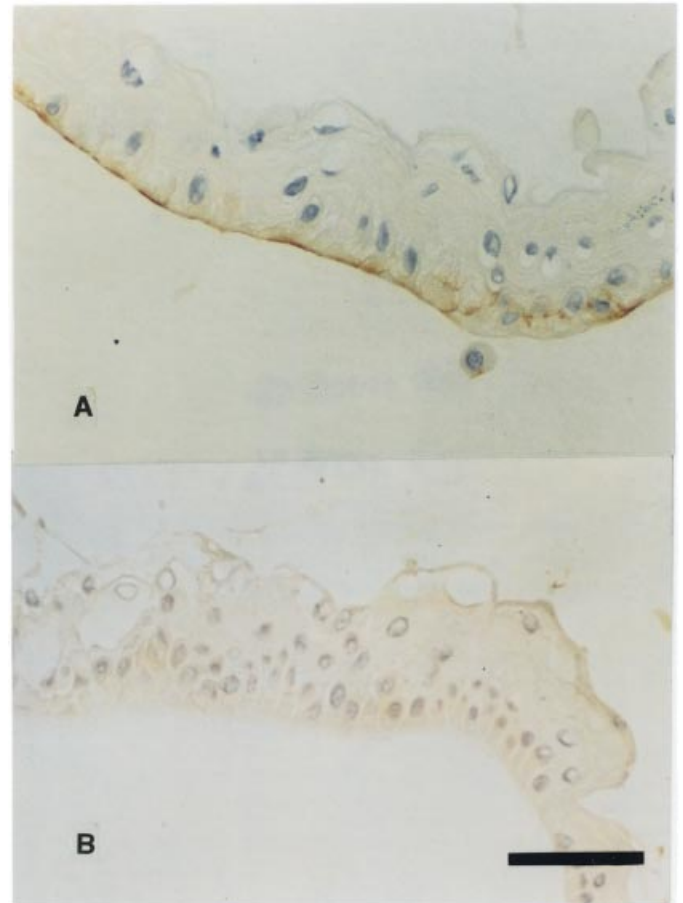


Figure 1. Vertical section of a keratinocyte sheet is stained positively with BM165 after exogenous application of laminin 5. This indicates that laminin 5 bound to keratinocyte sheets detached from the culture substrate by dispase (A), which was not seen in DMEM-treated sheets (B). Scale bar: 50 μ m.

ine for 5 min. Nuclear counterstaining was performed with Carazzi hematoxylin solution.

Electron microscopy Grafted skins were excised with the surrounding tissue and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, then postfixed with 1% OsO₄ in 0.1 M cacodylate buffer, dehydrated through an ethanol series and embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Ultra thin sections were prepared with an ultramicrotome (Portor-Blum MT-IIB or LKB NOVA), stained with uranyl acetate and lead citrate, and examined under a JEM-100SX or H-600 electron microscope.

RESULTS

The monoclonal antibodies used in this study are human specific.

Cultured epithelial sheets were removed from the dishes using the dispase. After treatment with the dispase, immunohistochemistry did not detect laminin 5 at the base of the keratinocyte sheets. Laminin 5 stained positively at the base of the sheets in group 1 after treatment with laminin 5 (1 μ g per cm^2), indicating that laminin 5 bound the keratinocyte sheets (Fig 1a). It was not seen in DMEM-treated sheets (Fig 1b).

At 7 d postgrafting, attached and surviving keratinocyte sheets were observed on the transplanted area (Fig 2). Keratinocyte sheets were implanted into the center of the exposed bed of the panniculus carnosus beneath silicone gauze. The silicone gauze served as a barrier protecting ingrowth of the recipient keratinocytes. The human keratinocyte sheets were physically separated from the host mouse skin in our model. The keratinocyte sheet-covered wound, supported by the silicone gauze covering the apical surface of the sheet was then covered with the skin flap. The silicone gauze

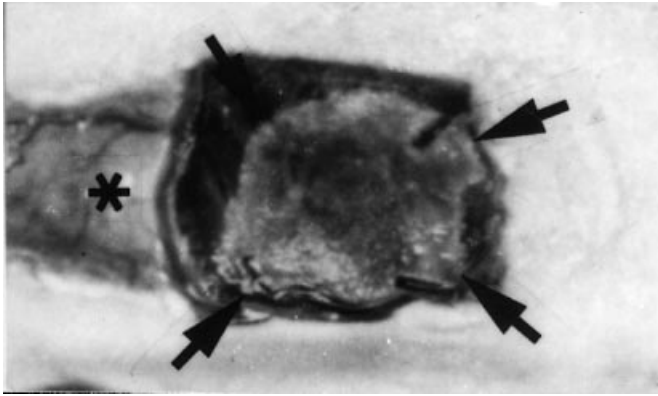


Figure 2. Keratinocyte sheets attached and survived on the transplanted area at 7 d postgrafting. The skin flap (*) was elevated and the silicone gauze was removed. Arrows indicate the margin of the surviving keratinocyte sheet.

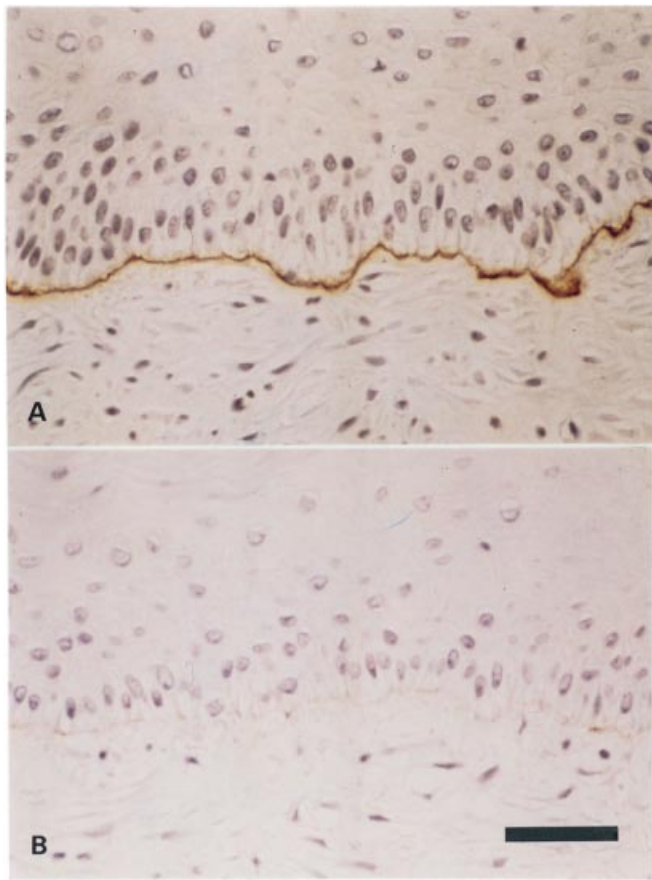


Figure 3. Immunodetection of type IV collagen at the dermal-epidermal junction of grafts at 7 d postgrafting. Type IV collagen stained more strongly at the dermal-epidermal junction in group 1 (A) than in group 2 (B). Scale bars: 50 μ m.

mechanically stabilized the grafts until they were biopsied. This model was used to test the grafting efficiency of keratinocyte sheets.

The graft take was measured as the area of epithelialization in both nude mice and nude rats, the area of epithelialization being larger in group 1 than in group 2 (nude mice, $58.5 \pm 21.9\%$ in group 1, $38.3 \pm 30.5\%$ in group 2; nude rats, $53.1 \pm 21.9\%$ in group 1, $35.3 \pm 22.5\%$ in group 2). By statistical analysis, the difference was significant in both groups.

At 7 d postgrafting, type IV collagen stained more strongly at the dermal-epidermal junction in group 1 than in group 2 (Fig 3a, b) by immunohistostaining.

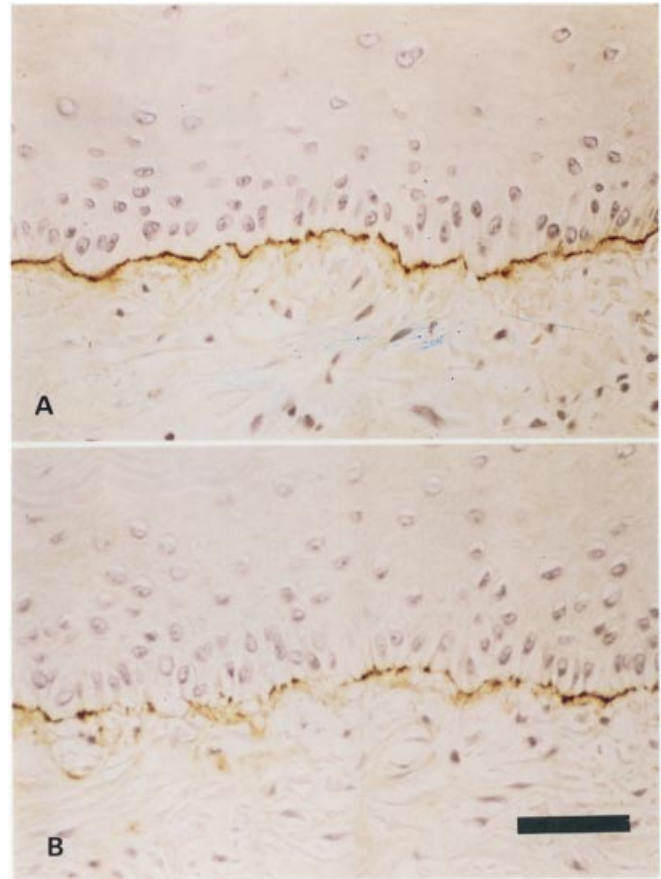


Figure 4. Immunodetection of laminin 5 at the dermal-epidermal junction in grafts at 7 d postgrafting. Laminin 5 stains more strongly at the dermal-epidermal junction in group 1 (A) than in group 2 (B). Scale bars: 50 μ m.

Laminin 5 also stained more strongly at the dermal-epidermal junction in group 1 than in group 2 (Fig 4a, b), as did type VII collagen (Fig 5a, b).

The effect of laminin 5 upon the ultrastructural organization of the basement membrane was examined by electron microscopy as shown in Figs 6 and 7. At day 3 after grafting, the lamina densa of group 1 was more continuous compared with group 2. This shows the early morphologic organization of basement membrane components caused by pretreatment with laminin 5 (Fig 6a, b).

At day 7 after grafting, electron-dense plates characteristic of hemidesmosomes were observed in both groups and no significant differences in the continuity of the basement membrane were observed (Fig 7a, b).

DISCUSSION

Autologous transplantation of cultured human keratinocyte sheets has been used clinically for the treatment of extensive burns (O'Connor *et al*, 1981), giant nevus (Gallico *et al*, 1989), and other cutaneous wounds. These methods, however, suffer problems with take rate and stability of the epithelium after grafting. In practice, the take rate of the sheets transplanted directly to granulation tissue without a dermal component has been poor. Spontaneous blistering has also been described in patients with burns grafted with keratinocytes (Woodley *et al*, 1988), particularly prominent in areas with extensive dermal loss (Kumagai *et al*, 1988; Desai, 1991), indicating instability of the dermal-epidermal junction. As greater understanding of the complex interaction of cells and matrix evolves, so have new techniques in the field of cultured keratinocytes for grafting. The two-stage grafting procedure combining dermal and epidermal equivalents *in situ* (Cuono *et al*, 1987) have been used successfully. In order to solve eventually the problems associated with the initial

fragility of skin regenerating from cultured human keratinocyte sheets, we need to understand the mechanisms underlying the regeneration of newly formed epidermis and dermis, in particular, the formation of dermal–epidermal junction *in vivo*. A possible solution to this problem might be transplantation of a construct consisting of epidermal and dermal equivalents, bound together by an already formed dermal–epidermal junction. Even though several groups are active in this field, the complete connection of epidermis and dermis with functional dermal–epidermal junction has not yet become a reality, and high concentration of seeding keratinocyte have a limitation of clinical use. On the other hand, promoting the formation of the basement membrane is important to grafting

of any kind of skin equivalent. This study focused on the role and influence of laminin 5 in the take of grafted cultured human keratinocyte sheets and the formation of the basement membrane. Laminin 5 plays an important part in the adherence of epidermis to dermis in normal skin and the present study suggests that exogenous application of laminin 5 improves transplanted keratinocyte sheet attachment to the substrate as a biologic “glue”.

Integrin chains $\alpha 6$ and $\beta 4$ were positively stained at the base of keratinocyte sheets after separation from culture dishes by dispase (not shown). Integrin $\alpha 6\beta 4$ is known to be a receptor for laminin 5 and to provide attachment of keratinocytes to basement membrane. After treatment of the dispase-released keratinocyte sheets with laminin 5, the treated sheets showed localization of laminin 5 to the basolateral surface of the sheets (Fig 1a), which was not seen in DMEM-treated sheets (Fig 1b). These observations indicate that laminin 5 binds specifically to the keratinocyte surface previously attached to the growth substrate, possibly through interactions with the integrins $\alpha 6\beta 4$, $\alpha 6\beta 1$, and/or $\alpha 3\beta 1$. The apparent concentration of laminin 5 to this cell surface may account for the effectiveness of the low concentrations of laminin 5 applied to the transplanted group 1 sheets.

The percentage of take for cultured keratinocyte sheets during the graft process has been reported to vary from 0% to 100% (Donati *et al*, 1992). At present, it appears that the variance between those values were due to multiple factors, including technical problems such as insufficient graft immobilization, lack of graft adherence caused by blood or serum collection, the presence of numerous virulent bacteria on the wound bed, and differing conditions of the recipient wound beds. These results, therefore, could not be compared directly, and an optimal control study was necessary. In our evaluation of the area of surviving epithelium after grafting, it was significantly larger in the laminin 5 pretreated group. These results suggest that exogenous application of laminin 5 provides additional graft stability and increases the take rate.

The use of soluble laminin 5 to promote cell attachment and basement membrane assembly has been reported in two related systems. The addition of laminin 5 to explanted human corneal rims induces the assembly of hemidesmosomes beneath the corneal epithelium, thus increasing the stability of epithelial attachment (Baker *et al*, 1996). Soluble laminin 5 has also been applied to a titanium alloy to provide a biologic coating for the attachment of gingival epithelial cells (Tamura *et al*, 1997). These studies suggest that laminin 5 may have broad uses in clinical settings where increased epithelial attachment and hemidesmosome assembly are required. Our data indicate that laminin 5 may improve the transplantation take rate of keratinocyte sheets transplanted to animal wound beds. In this case, the data suggest that the increase in successful keratinocyte sheet transplantation is due to both increased cell attachment and increased basement membrane assembly. Therefore, soluble laminin 5 may be clinically useful for the treatment of severe burns either by partially or wholly overcoming the problems of keratinocyte graft stability, or in combination with dermal grafting methods. It has been shown that prolonged graft stability and maintenance of a proliferating basal layer are

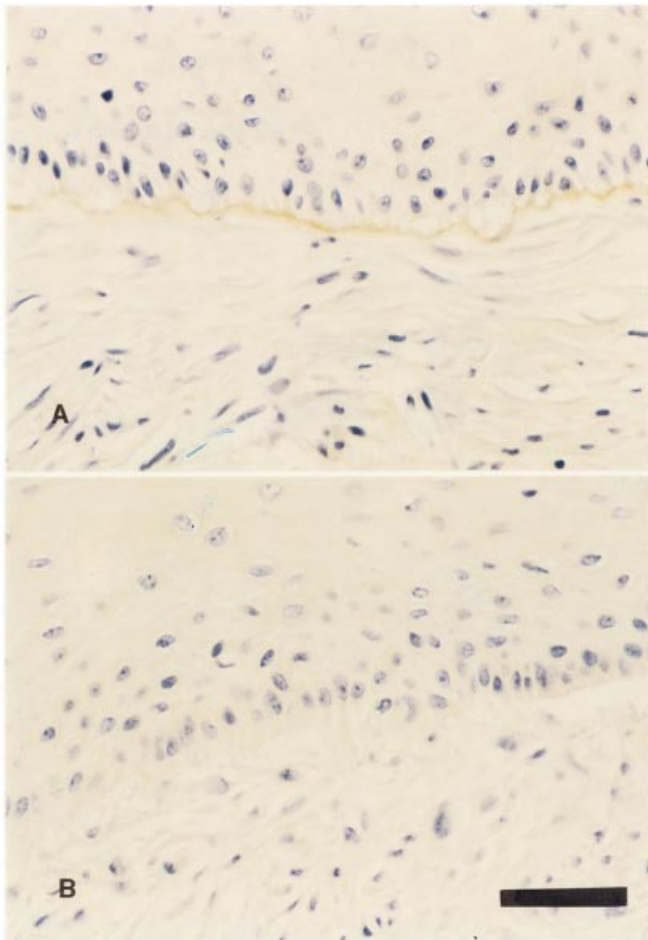
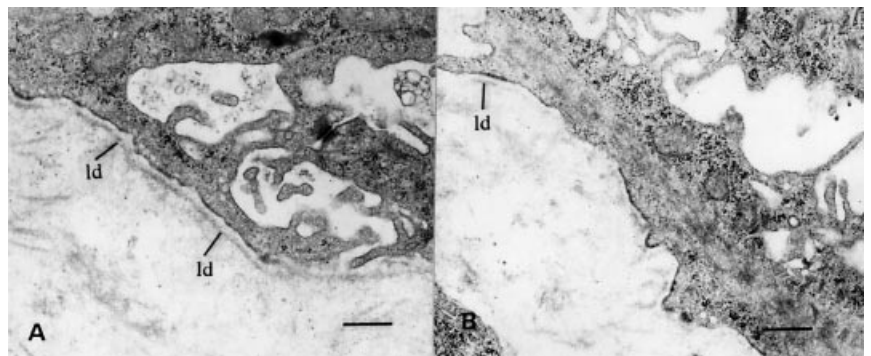


Figure 5. Immunodetection of type VII collagen expression at the dermal–epidermal junction in immunostaining at 7 d postgrafting. Type VII collagen stains more strongly at the dermal–epidermal junction in group 1 (A) than in group 2 (B). Scale bar: 50 μ m.

Figure 6. The effect of exogenous laminin 5 on ultrastructural organization of the basement membrane zone of keratinocyte grafts at day 3 after grafting. The lamina densa of group 1 (A) is more continuous when compared with that of group 2 (B). Scale bars: 0.5 μ m. This shows the early morphologic organization of basement membrane components caused by pretreatment of laminin 5. Id, lamina densa. In the immunodetection, type IV collagen (main component of lamina densa) was stained more strongly at the dermal–epidermal junction of grafts at 3 d postgrafting in group 1 (data not shown). The results of transmission electron microscopy correlate them.



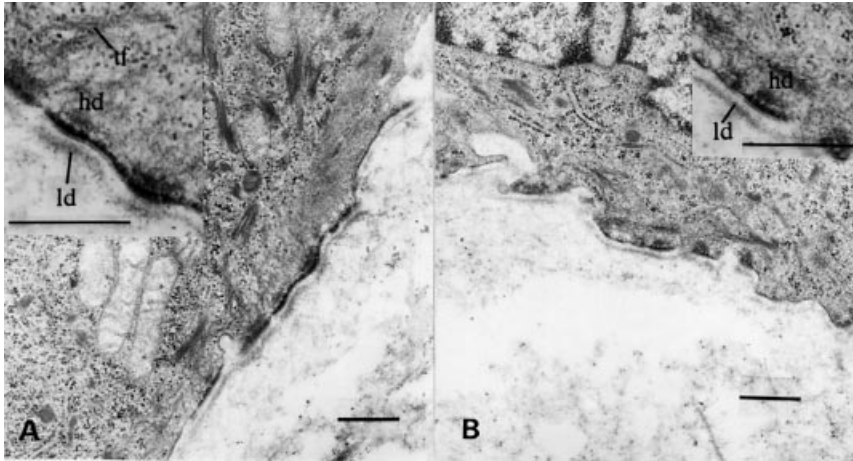


Figure 7. The effect of exogenous laminin 5 on ultrastructural organization of basement membrane zone at day 7 after grafting. Electron-dense plates characteristic of hemidesmosomes are observed in both groups. The regions of hemidesmosomes were a well-organized basement membrane structure consisting of attachment plaques, sub-basal dense plate, tonofilaments. The number of hemidesmosomes of group 1 were larger than that of group 2 in morphometrical observation. hd, hemidesmosome; ld, lamina densa; tf, tonofilament. Scale bars: 0.5 μ m.

dependent upon a completely formed basement membrane. Disperse release of keratinocyte sheets from substrate causes damage to the basal cell layer and loss of laminin 5 deposited during culture. By immunohistochemical evaluation after grafting, collagen IV, laminin 5 and collagen VII stained more strongly at the dermal-epidermal junction in group 1 than in group 2, suggesting higher concentrations of basement membrane components in the laminin 5 pretreated group.

By electron microscopy, we observed that the lamina densa was detected as early as 3 d after grafting in the treated group, whereas the lamina densa of the control group remained discontinuous, and in both groups, anchoring fibrils were not detected. It has been previously reported that lamina densa could be detected by electron microscopy between 31 d (Mommaas *et al*, 1992) and 6 wk (Aihara, 1989), in human cases. Despite the proteolysis resulting from the use of dispase to release the keratinocyte sheets from the growth substrate, we observed well-formed lamina densa earlier in the treated cases than in the controls. Exogenous application of laminin 5 stimulates the assembly of the basement membrane soon after transplantation. Compton *et al* (1989) found that full maturation of anchoring fibrils required more than a year. Our findings are in broad agreement with this report. In some reports, cultured keratinocyte sheets did not attach and vascularize sufficiently well to close the wound (Kumagai *et al*, 1988). Keratinocyte plasma membrane damage secondary to sheet separation of the keratinocyte sheets from the culture surface (Merrick *et al*, 1990), and abnormal anchoring fibril formation (Woodley *et al*, 1988) have been suggested as causes. In another study, however, a lamina densa was first identified at 6 wk and became continuous by 5 mo (Aihara, 1989), and by 1 y the basement membrane zone was complete and included anchoring fibrils (Compton *et al*, 1989).

When keratinocytes were grafted on to wound beds which contained a living dermis, however, the lamina densa and anchoring fibrils were identified within 5 d (Faure *et al*, 1987). These different results suggest that in the graft bed, the presence of living dermal components is essential for the formation of anchoring fibrils, further suggesting that the dermal mesenchymal cells may be the major source of type VII collagen under these conditions. In our study, anchoring fibrils were not observed after 7 d. The wound beds in our model were flesh excised fascia without dermal components.

Laminin 5 increased the percentage of graft take, apparently due to an increased rate of basement membrane assembly. We believe that these observations support the need for further studies of the efficacy of exogenous laminin 5 to increase keratinocyte graft take to human burn patients.

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