American Journal of Pathology, Vol. 143, No. 5, November 1993

Regulation of Extracellular Matrix Proteins and Integrin Cell Substratum Adhesion Receptors on Epithelium during Cutaneous Human Wound Healing *in Vivo*

Istvan Juhasz,* George F. Murphy,† Horng-Chin Yan,‡ Meenhard Herlyn,* and Steven M. Albelda*‡

From The Wistar Institute of Anatomy and Biology, Philadelphia, and the Departments of Dermatology† and Medicine,‡ University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania*

Although changes in extracellular matrix proteins during wound healing have been well documented, little is known about the regulation of corresponding extracellular matrix adhesion receptors (integrins). To study this process in a human* in vivo *model, full thickness human skin grafts were transplanted onto severe combined immunodeficient mice and deep excisional wounds involving both the epidermal and dermal layers were then made. The changes in the expression of cell matrix proteins and epithelial integrins over time were analyzed with specific antibodies using immunohistochemistry. Wounding was associated with alterations in extracellular matrix proteins, namely, loss of laminin and type IV collagen in the region of the wound and expression of tenascin and fibronectin. Changes were also noted in the integrins on the migrating keratinocytes. There was marked up-regulation of the α_v subunit and* de novo *expression of the fibronectin receptor ($\alpha_5\beta_1$) during the stage of active migration (days 1 to 3 after wounding). In the later stages of wound healing, after epithelial integrity had been established, redistribution of the α_2, α_3, α_6, and β_4 collagen/laminin-binding integrin subunits to suprabasal epidermal layers was noted. Thus, during cutaneous wound healing, keratinocytes up-regulate fibronectin/fibrinogen-binding integrins and redistribute collagen/laminin-binding integrins. This study demonstrates that the human skin/severe combined immunodeficient chimera provides a useful model to study events during human wound repair. (Am J Pathol 1993, 143:1458–1469)

The process of cutaneous wound healing is a complex and carefully orchestrated cascade of events that involves sequential alterations in extracellular matrix (ECM) proteins, release of growth factors, and secretion of migration-stimulating cytokines.[1–3] One of the most important components of this process is the migration of keratinocytes over the injured dermis to form a new epidermal layer. Under normal conditions, the basal keratinocytes rest on a basement membrane composed of proteins such as laminin, type IV collagen, and heparan sulfate proteoglycan.[2,4,5] During the wound healing process, however, the migrating epidermal keratinocytes are exposed to a provisional wound bed containing ECM material that includes fibrin, fibrinogen, fibronectin, tenascin, and vitronectin.[1,5,6–9] It is thought that the presence of fibronectin and fibrinogen has an important effect on the ability of the epidermal keratinocytes to migrate.[10–11]

Although the alterations in the composition of the extracellular wound matrix to which the keratinocytes are exposed during wound healing have been relatively well studied in animal models, much less is known about another important component of the

Supported by NIH grants HL-46311 (SMA), AR39674 (GFM), CA40358 (GFM), CA25874 (MH), a grant from the Polly Annenberg Levee Charitable Trust, and a research grant from the American Cancer Society (SMA). Dr. Albelda is an Established Investigator of the American Heart Association.

Dr. Juhasz is a Visiting Fellow from the Department of Dermatology, University Medical School, Debrecen, Hungary.

Accepted for publication July 14, 1993.

Address reprint requests to Dr. Steven M. Albelda, Pulmonary and Critical Care Division, 809 Maloney Building/HUP, 3600 Spruce Street, Philadelphia, PA 19104.

process: the changes in the specific cell-substratum adhesion receptors ultimately responsible for cellular migration. These receptors are primarily members of the integrin superfamily of proteins, a group of heterodimeric, calcium-dependent, transmembrane molecules found on virtually all cells of the body (reviewed in ref. 12).

Resting keratinocytes *in situ* express a number of integrins capable of binding to proteins normally found in basement membranes (ie, laminin and collagen), namely, the $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$ integrins.[13–19] They normally do not express the $\alpha_4\beta_1$ or $\alpha_v\beta_3$ integrins that bind to matrix proteins of inflammation such as fibronectin, fibrinogen, vitronectin, or thrombospondin and express little[18] or none[13,14] of the $\alpha_5\beta_1$ fibronectin receptor. With the exception of the $\alpha_3\beta_1$ integrin, which has the capability of binding to collagen, laminin, and fibronectin, the only receptor normally found on keratinocytes with the ability to adhere to matrix proteins of inflammation is an integrin containing the αv subunit, perhaps in combination with the β_5,[20] β_6, or β_8 subunits.

In vitro data,[21–24] and some limited experiments in animal[25] and human[18] models of wounding, suggest that the adhesion receptor profile of keratinocytes is altered during wound-induced migration. The purpose of this study was to test this hypothesis by carefully analyzing changes in ECM proteins during *in vivo* wound healing and correlating these changes to alterations in the corresponding expression and distribution of integrins on keratinocytes. There were a number of reasons to study this process in human skin. First, such studies have been difficult to perform previously because a complete panel of anti-integrin monoclonal antibodies with reactivity to nonhuman species has not been available. Second, there are clear-cut differences in the wound healing process between human and nonhuman species.[26,27] Because the ethical and logistical problems of human experimentation make these kinds of studies difficult, we have developed an alternative model: full thickness human skin grafts have been transplanted onto severe combined immunodeficient (SCID) mice.[28,29] In this model, the skin grafts closely resemble normal human skin histologically and maintain their human phenotype for at least 3 months. Four weeks after transplantation, the human grafts were subjected to dermal-epidermal excisional skin wounds. Changes in ECM composition and cell adhesion molecule expression during the wound healing process were analyzed by immunohistochemistry using a panel of antibodies to specific matrix molecules and integrin subunit-specific monoclonal antibodies.

Using the human/SCID chimeras, we observed the expected changes in the ECM composition in the wound bed, including loss of type IV collagen and laminin and increased expression of tenascin and fibronectin. Accompanying these changes were clear-cut alterations in the profile of cell adhesion receptors on keratinocytes. In the early phases of wound healing, *de novo* expression of the α_5 subunit and up-regulation of the α_v subunit on migrating keratinocytes was observed. In the later phases, when epidermal integrity had been reestablished, expression of the α_2, α_3, α_6, and β_4 subunits in multiple keratinocyte layers, in addition to expression in their normal basilar location, was documented.

Materials and Methods

Animals

Inbred mice with severe combined immunodeficiency (C.B.-17 SCID mice)[30] were obtained from a colony maintained at the Wistar Institute Animal Facility. At 6 weeks of age, all mice were tested for endogenous immunoglobulin M production (leakiness), and only fully immunodeficient mice were used. The mice were housed under pathogenfree conditions in micro-isolator cages. All experimental protocols were authorized and approved by the Animal Use Review Board of the Wistar Institute and the University of Pennsylvania.

Skin Grafting

At 6 to 8 weeks of age, SCID mice were transplanted with full thickness human skin. Before skin grafting, a 3-cm^2 area of the lateral abdominal region was shaved using an electric shaver. A circular graft bed, approximately 1.5 cm in diameter, was prepared on the lateral abdomen of mice under anesthesia by removing the skin to the fascia with scissors. Donor skin was human neonatal foreskin. Use of human tissues was approved by the Human Subjects Approval Committee of the Wistar Institute and the University of Pennsylvania. The human donor skin was grafted onto the wound bed and secured with 5-0 monofilament suture (Dermalon, Davis+Geck Inc., Danbury, CT). The transplantation site was then covered with Adaptic nonadhering wound dressing (Johnson and Johnson, New Brunswick, NJ) and an adhesive bandage that was clipped to the dorsal and ventral skin of the animal

with a surgical staple (Autoclip, Clay Adams, Parsippany, NY). An additional layer of Micropore surgical tape was applied (3M, St. Paul, MN). After 4 weeks, the grafts were completely healed.

Wounding Procedure

Four to 6 weeks after engraftment, a longitudinal, dermo-epidermal excisional wound, approximately 8 mm long and 2 mm wide, was made with a double blade scalpel handle (Robbins Instruments Inc., Chatham, NJ). The depth of the wound was adjusted so that the incisions did not extend past the human dermis into the underlying mouse tissue.

Biopsy Procedures

At the designated time points after wounding, the mice were euthanized, and the wounded skin graft, with the surrounding murine skin, was removed in its entirety for histology. The graft was then bisected perpendicularly to the direction of the wound. Each half of the biopsy was then oriented so that sections would show the wound in cross-section. The specimens were then snap-frozen in OCT and stored at −70 C.

Immunohistochemistry

Thin sections (5 μ) were made from frozen tissue blocks embedded in OCT, fixed in acetone for 5 minutes and stored at −70 C. Before staining, the sections were blocked with phosphate-buffered saline (PBS) containing 5% serum appropriate for the secondary antibody. After subsequent washing in PBS (pH 7.3) for 10 minutes, the sections were incubated with primary antibodies diluted in PBS/4% bovine serum albumin for 60 minutes. After 10 minutes washing in PBS/bovine serum albumin, the slides were incubated for 30 minutes with biotinylated anti-mouse or anti-rabbit immunoglobulin G as secondary antibody. The streptavidin-biotin ABC peroxidase detection system (Vector Laboratories, Burlingame, CA) was applied, followed by 2 minutes incubation with 3-amino-9 ethylcarbazole substrate (Zymed, San Francisco, CA). Finally, the slides were covered with PBS-glycerol aqueous mounting medium, coverslipped, and evaluated microscopically.

Antibodies

A variety of monoclonal and polyclonal antibodies were used (see Tables 1 and 2). All were titered for optimal reactivity. Monoclonal antibodies directed against human α_2, α_3, and α_5 integrin subunits were purchased from Telios (La Jolla, CA). Dr. Martin Hemmler generously provided the monoclonal antibodies (MAbs) TS2/7 and B-5H10 that recognize the VLA-1 (α_1) and the VLA-4 (α_4) antigens respectively.[31] Dr. Arnoud Sonnenberg provided us with the MAb directed against α_6.[32] The anti-β_4 MAb was given by Dr. Steven Kennel.[33] The MAb LM142 directed against the vitronectin receptor α subunit (α_v) was kindly given to us by Dr. David Cheresh.[34] Drs. Joel Bennett and James Hoxie provided SSA6, the monoclonal antibody directed against the platelet glycoprotein IIIa, the β_3 subunit.[35] The MAb 302-9 to tenascin was previously described.[36] Dr. John McDonald provided the N294 MAb directed against human fibronectin.[37] An irrelevant MAb against the chicken β_1 receptor (CSAT) was used as a negative control.[38]

Polyclonal antibodies to type IV collagen and laminin were gifts from Dr. Joseph Madri.[39] These antibodies reacted to both human and murine ECM proteins. The human-specific polyclonal PECAM-1 antiserum was prepared as described previously.[40] Preimmune rabbit serum from the PECAM-1-injected rabbit was used as an negative control.

Results

Characteristics of the Wounds

Excisional wounds, 2 mm in width, were made in 12 transplanted human skin grafts, without suturing of the wound. After a brief period of bleeding, hemostasis was obtained and the skin bandaged. The

Table 1. *Expression of ECM Proteins in Skin before and after Wounding*

ECM protein	Antibody	Source	Expression pattern		
			Normal skin	Early wound bed	Late wound bed
Type IV collagen	Polyclonal	(39)	●/BM	NA	★/BM
Laminin	Polyclonal	(39)	●/BM	NA	★/BM
Human fibronectin	N294	(37)	★/BM	●/Diffuse	★/BM
Tenascin	302–9	(36)	●/BM	●/Diffuse	★/Diffuse and BM

● = strong expression; ★ = weak expression; BM = basement membrane expression; NA = not applicable, no basement membrane.

Table 2. *Expression of Integrins on Epidermal Keratinocytes before and after wounding*

Integrin	Antibody	Source	Intact epidermis	Early wound	Late wound
α_1	TS2/7	(31)	○	○	○
α_2	PIE6	Telios	●/B	●/B	●/ML
α_3	PIB5	Telios	●/B	●/B	●/ML
α_6	GoH-6	(32)	●/B	●/B	●/ML
β_4	—	(33)	●/B	●/B	●/ML
α_4	B-5H10	(31)	○	○	○
α_5	PID6	Telios	○	●/B	○
α_v	LM142	(34)	★/B	●/B	●/ML
β_3	SSA6	(35)	○	○	○

● = strong expression; ★ = weak expression; ○ = no detectable expression; B = basilar distribution; ML = multiple layers.

wounds remained uninfected over the course of the study. Although some variation in the healing rate of the wounds was noted, in general, 1 day after wounding, a clear-cut defect in the epidermis was evident with little evidence of wound closure (Figure 1C). By day 2, the wounded area uncovered by epidermis was clearly smaller, with evidence of keratinocyte migration (Figure 1D). In most cases, epidermal integrity was restored 3 to 4 days after wounding (Figure 1E), however, in some grafts, wound closure had occurred by day 2 (Figure 1G). By 7 days after wounding, the wounded area had healed by visual inspection. Skin grafts were removed *in toto* on days 1, 2, 4, 7, 10, 14, and 21 after wounding, and the specimens analyzed for the expression of ECM proteins and cell adhesion molecules using immunohistochemical techniques.

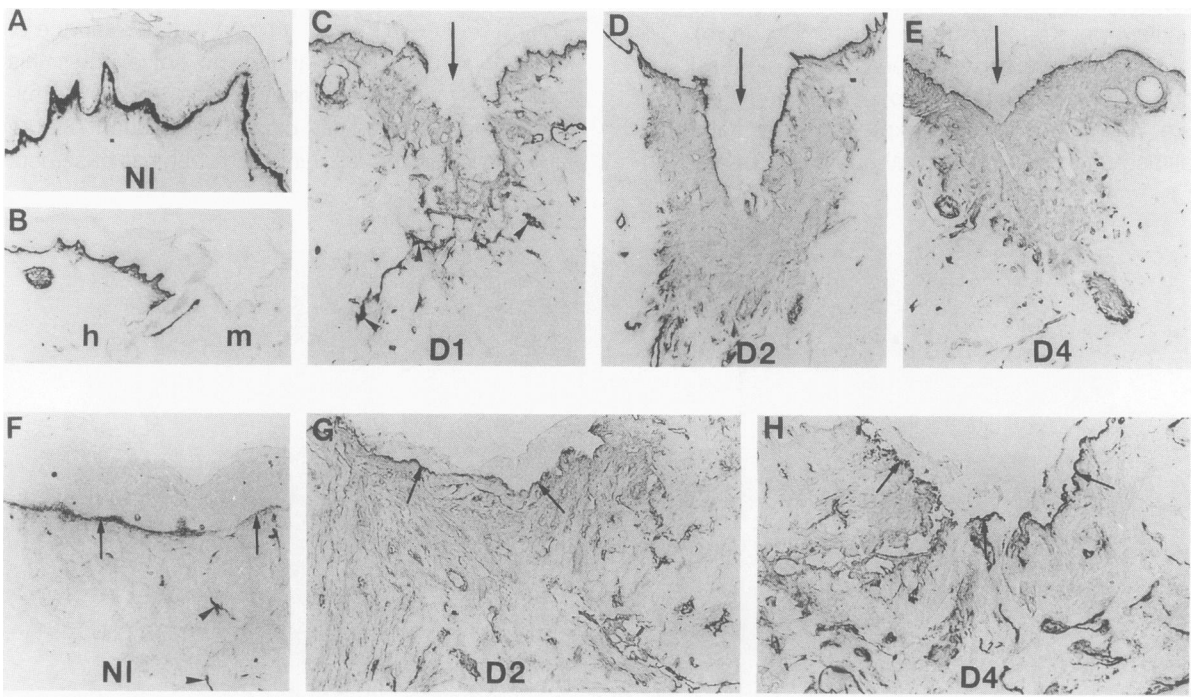

Figure 1. *Expression of tenascin and fibronectin in normal skin and during wound healing. Transplanted human skin grafts were removed, flash-frozen, sectioned, fixed in acetone, and stained, using an immunoperoxidase technique with an anti-human tenascin monoclonal antibody (upper panels) or an anti-human fibronectin antibody (lower panels). Skin was harvested before wounding (A, B, and F), or 1 day (C), 2 days (D and G) or 4 days (E and H) after wounding. Under normal conditions, tenascin staining was limited to the dermal-epidermal junction (A). The antibody was human specific as evidenced by staining at the junction of human (h)/mouse (m) skin (B). After wounding, tenascin expression increased throughout the dermis (C, D, and E) and on the basement membranes of wound capillaries (C, arrowheads). The large vertical arrows mark the sites of wounding. Under baseline conditions (F), fibronectin was expressed in a somewhat diffuse pattern at the dermal-epidermal junction (arrows) and in the basement membrane of some blood vessels (arrowheads). By day 2 (G), fibronectin was easily detected throughout the wounded area. In this wound, the keratinocytes had already reformed an intact epidermis by day 2. Large amounts of fibronectin were seen in the dermis, as well as underneath the newly formed epidermis (arrows). By 4 days postwounding, the expression of interstitial fibronectin markedly decreased (H), but strong staining of the dermal-epidermal junction persisted (arrows). Magnification 100×.*

ECM Protein Expression during Wound Repair

The wounding and healing process in the human skin grafts was associated with sequential changes in ECM protein expression (summarized in Table 1), similar to those reported in animal and human studies.[5–9] One of the earliest, and most striking changes, occurred in the expression of tenascin (Figure 1) detected using an antibody that was specific for human tenascin (Figure 1B). Consistent with a previous report in a rat wound model[8] and in human skin,[41] human tenascin was present exclusively at the dermal-epidermal border (Figure 1A) and in the basement membrane of some microvessels in unwounded skin. This distribution changed dramatically after wounding (Figure 1, C, D, and E). As early as 1 day after wounding (Figure 1C), tenascin was detected in the underlying wound matrix. Increased expression was also seen in the basement membrane of the wound capillaries (arrowheads). This striking expression of tenascin seemed to peak at day 2 postwounding (Figure 1D), persisted to day 4 (Figure 1E), a time after epidermal closure had occurred, but was, in fact, detectable until 14 days after wounding (data not shown).

Similar changes were seen in the expression of human fibronectin. Under baseline conditions (Figure 1F), fibronectin was expressed in a somewhat diffuse pattern at the dermal-epidermal junction (arrows) and in the basement membrane of some blood vessels (arrowheads). After wounding, fibronectin was easily detected throughout the wounded area in a similar distribution as tenascin. Figure 1G illustrates the distribution of human fibronectin 2 days after wounding. In this wound, the keratinocytes had already reformed an intact epidermis by day 2. Large amounts of fibronectin were seen in the dermis, as well as underneath the newly formed epidermis (arrows). By 4 days postwounding, the expression of interstitial fibronectin markedly decreased (Figure 1H), but strong staining of the dermal-epidermal junction persisted (arrows). By day 7 after wounding, fibronectin staining returned to baseline patterns (data not shown).

Alterations in laminin and type IV collagen, the normal ECM components of the basement membrane, were also observed during the wound healing process. Laminin was normally strongly expressed at the epidermal basement membrane (Figure 2A, small arrows) and in the basement membranes of the microvessels (Figure 2A, arrowheads). Immediately after wounding, laminin persisted under the epithelium at the edge of the wound (Figure 2B, small arrows), but the surface of the wound bed (large arrow) did not express this protein. By day 2, keratinocytes had begun to migrate and had partially closed the wound (Figure 2C). Although the laminin in the original basement

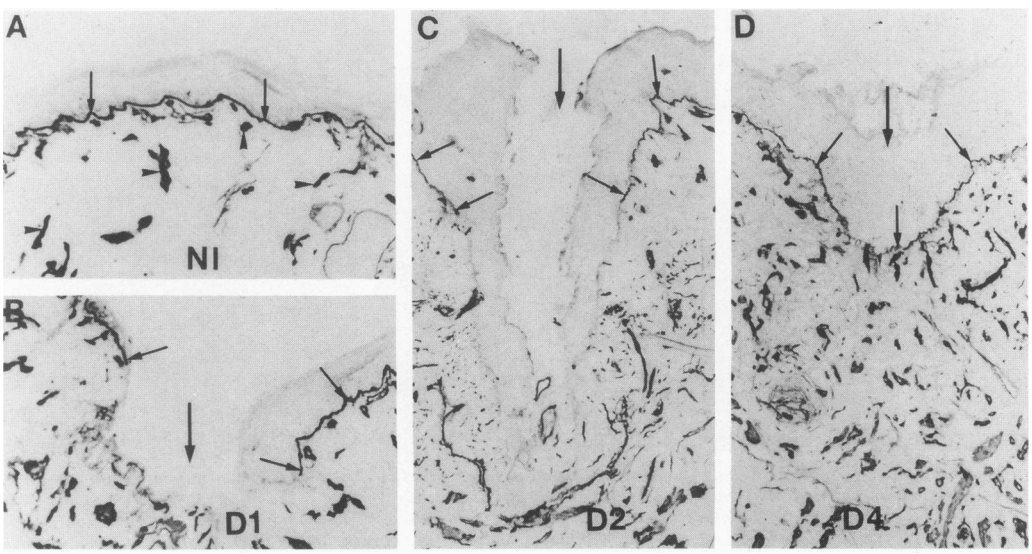

Figure 2. *Expression of laminin in normal skin and during wound healing. Skin was harvested before wounding* (A) *or 1 day* (B), *2 days* (C), *or 4 days* (D) *after wounding and stained with an anti-laminin polyclonal antibody. Under normal conditions, laminin was strongly expressed at the basement membrane of the epithelium* (A, *small arrows*) *and the vessels. On day 1* (B) *and 2* (C) *after wounding, the wound bed* (*large arrows*) *did not express laminin. Note the lack of laminin below the migrating keratinocytes in* C. *By day 4* (D), *a new basement membrane containing laminin had formed* (*small arrows*). *Magnification 100×.*

membrane could easily be identified (small arrows), there was *no* laminin present under the newly migrating keratinocytes. By day 4 (Figure 2D), a time point when the epithelium had regenerated, a new basement membrane containing laminin had been formed (small arrows). A similar sequence of events was observed for type IV collagen (data not shown). Thus, after wounding, the normal epidermal basement membrane components, laminin and type IV collagen, were lost beneath the epithelium that initially covered the wound bed. These ECM proteins were not re-expressed until after epidermal integrity had been restored.

Expression of Epithelial Integrins during Wound Repair

Initial studies comparing the expression of integrins in unwounded transplanted foreskins to those previously described in adult skin by us[14] and others[13,15–19] revealed no significant differences. Like normal adult skin, the transplanted foreskin showed strong expression of the α_2, α_3, α_6, and β_4 subunits, weak expression of the α_v subunit, and undetectable levels of the α_1, α_4, α_5, and β_3 subunits (described in detail below). The patterns of epithelial integrin expression in normal skin, as well as in the wounds, is summarized in Table 2.

Collagen/Laminin-Binding Integrins

The α_1 integrin subunit was not readily detectable on normal or wounded keratinocytes. In contrast, both the α_2 (data not shown) and α_3 integrin subunits were present in high amounts at both cell-cell and cell-matrix borders in the lower layers of the epidermis (Figure 3A, arrowheads), whereas the α_6 (Figure 4A) and β_4 subunits (Figure 4E) were strongly expressed by the basal keratinocytes in a strictly basilar distribution. Early in wound healing, the distribution of these integrins was relatively unchanged (Figure 4, B and F). However, as the keratinocytes began to migrate (Figures 3B and 4, C and G) integrin expression was not entirely restricted to a strictly basal distribution. This change in distribution was more obvious as the keratinocytes began to form an intact epidermal layer; the staining of α_2 (not shown), α_3 (Figure 3C), α_6 (Figure 4D), and β_4 (Figure 4H) became more diffuse and visible in multiple cell layers. Some degree of multilayered staining remained up to 10 days after wounding but had returned to normal by 14 days.

Fibronectin/Fibrinogen-Binding Integrins

The epidermis did not express the α_4 or β_3 integrin subunits under baseline or wounded conditions. However, there was patchy, weak expression of the α_v subunit, primarily in the basal keratinocytes, before wounding (Figure 5A). Presumably, this subunit paired with an alternative β subunit (ie, β_5, β_6, or β_8). Two days after wounding (Figure 5B), a marked up-regulation of the α_v subunit was observed on the cell membranes of migrating basal and suprabasal keratinocytes (arrows) but *not* on keratinocytes bordering the wound (arrowheads). This up-regulation of α_v persisted, accompanied by a more diffuse distribution (that is, staining in multiple layers of the

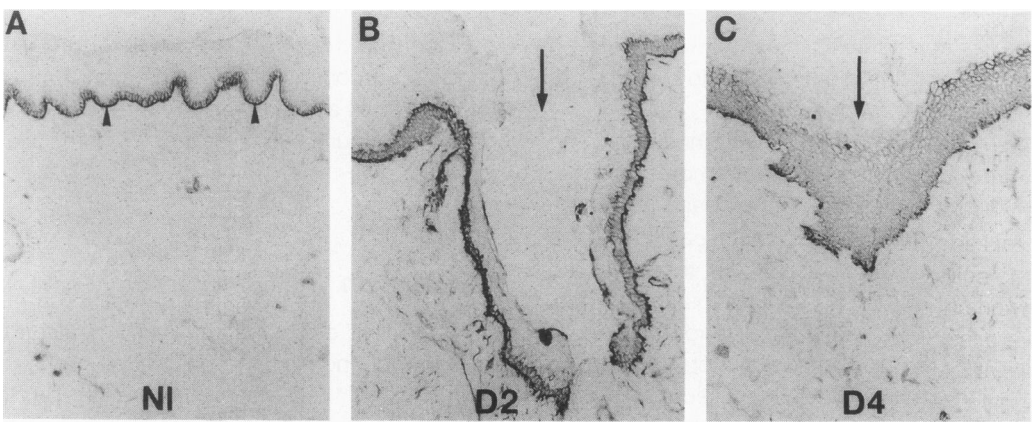

Figure 3. *Expression of the α_3 integrin subunit during wound healing. Grafts were stained with an anti-human α_3 monoclonal antibody. Skin was harvested before wounding* (**A**) *or 2 days* (**B**) *or 4 days* (**C**) *after wounding. The large arrows mark the site of the wound. In intact skin, α_3 was strongly expressed by the basal keratinocytes* (**A**). *On day 2 after wounding* (**B**), *α_3 expression remained relatively unchanged. By day 4* (**C**), *however, after the keratinocytes had formed an intact epidermal layer, expression of α_3 became more diffuse and visible in multiple cell layers. Magnification 100×.*

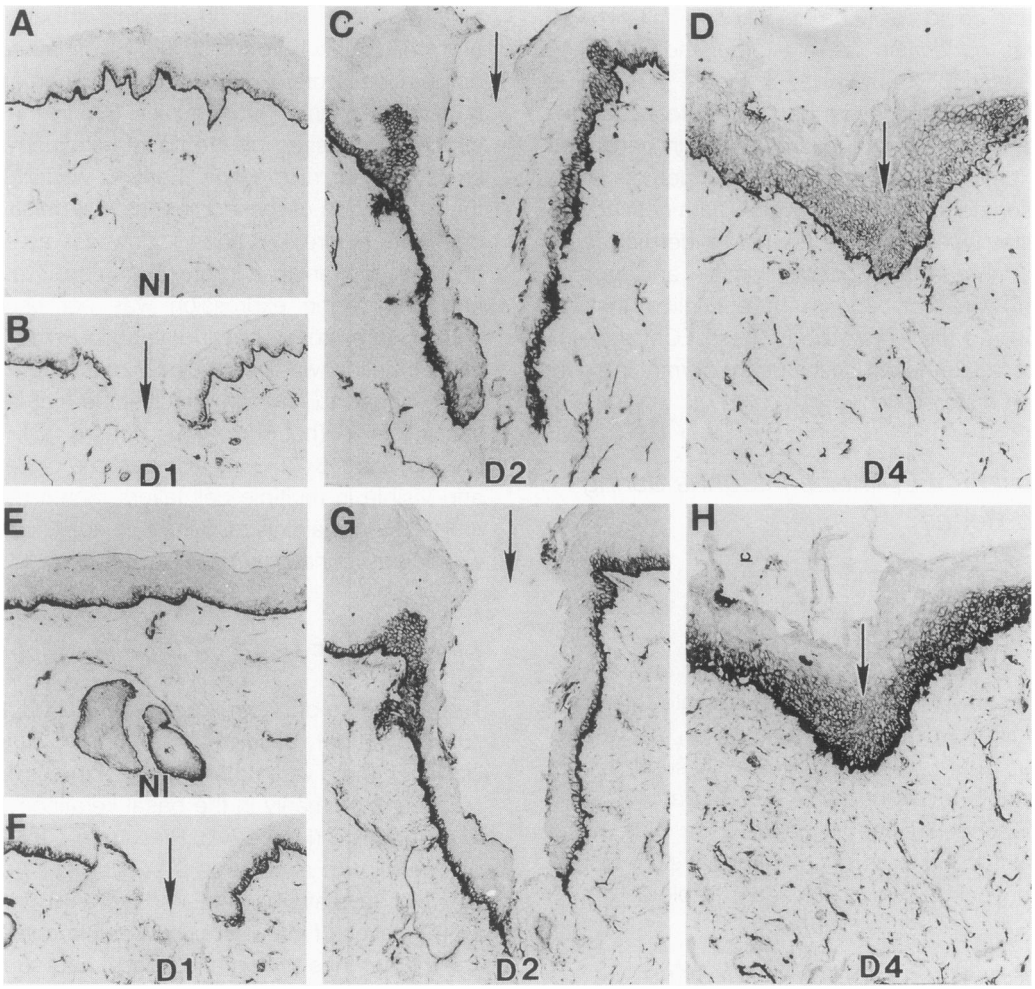

Figure 4. *Expression of the α_6 and β_4 integrin subunits during wound healing. Grafts were stained with an anti-human α_6 monoclonal antibody (upper panels) or an anti-human β_4 monoclonal antibody (lower panels). Skin was harvested before wounding (A and E) or 1 day (B and F), 2 days (C and G), or 4 days (D and H), after wounding. The large arrows mark the site of the wound. In intact skin, α_6 and β_4 were strongly expressed by the basal keratinocytes (A and E). On day 1 (B and F) expression remained unchanged. By day 2 (C and G) after wounding, subtle changes in the distribution of α_6 and β_4 began to appear. By day 4 (D and H), however, after the keratinocytes had formed an intact epidermal layer, expression of both integrin subunits became more diffuse and visible in multiple cell layers. Magnification 100×.*

epidermis) after epithelial integrity was achieved (day 4, Figure 5C) and did not return to baseline until 7 to 10 days after wounding.

The staining pattern of the α_5 fibronectin-binding subunit showed similar but even more dramatic changes during wounding. Under baseline conditions, staining of keratinocytes with α_5 antibody was identical to that of control antibody; there was no detectable expression of the α_5 subunit on basal epidermal cells (Figure 6A, arrowheads). However, as the keratinocytes became migratory (usually on day 2), they began to express the α_5 subunit to high levels (Figure 6, B, C, and D). The expression of α_5 was limited to keratinocytes that were adjacent to the wound bed (Figure 6B, arrowheads) and was not seen on the keratinocytes distant from the wound. Expression was primarily on the basal cells adjacent to the basement membrane (Figure 6, C and D). In contrast to the findings with the α_v subunit, as soon as the epidermis closed, expression of the α_5 subunit disappeared (Figure 6E, arrowheads).

Discussion

The process of cutaneous wound healing, induces a set of changes in the phenotype of the keratinocyte.[23] This keratinocyte activation program includes up-regulation of plasminogen activators, synthesis of a different set of keratins, and expression of growth factor and cytokine receptors.[5,42,43] Our results demonstrate that another important component of this activation program is the

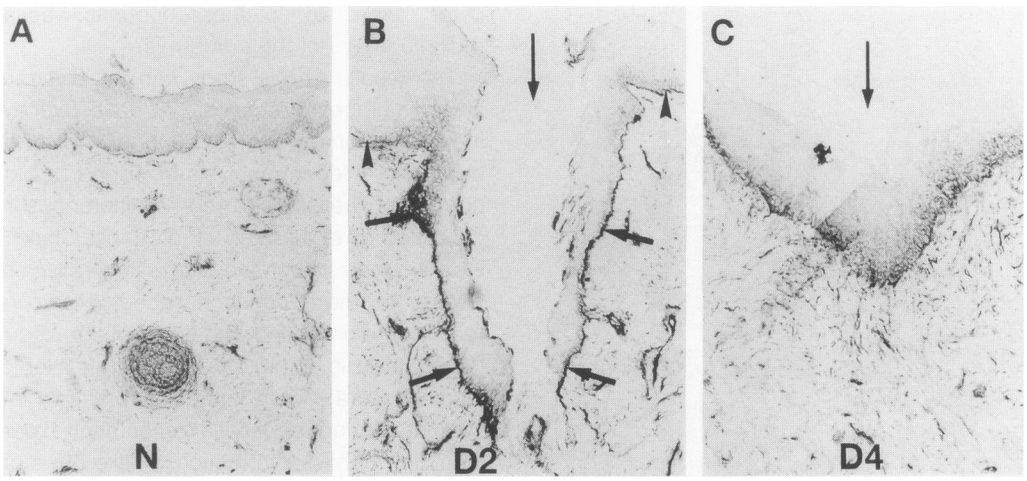

Figure 5. *Expression of the α_v integrin subunit during wound healing. Grafts were stained with an anti-human α_v monoclonal antibody. Skin was harvested before wounding* (**A**) *or 2 days* (**B**) *or 4 days* (**C**) *after full thickness wounding. The large arrows mark the site of the wound. In intact skin, there was patchy, weak expression of α_v by the basal keratinocytes* (**A**). *Stronger staining is observed in the follicular bulb seen in the lower third of the dermis. On day 2 after wounding* (**B**), *however, a marked up-regulation of the α_v subunit was observed on the migrating keratinocytes* (*large, short arrows*) *but not the keratinocytes further from the wound* (*arrowheads*). *This up-regulation persisted to day 4* (**C**), *accompanied by staining in multiple layers of the epidermis. The dark area near the wound site in* **C** *is an artifact and represents a fold in the tissue. Magnification 100×.*

changes in the specific cell-substratum adhesion receptors (integrins) ultimately responsible for cellular migration.

To analyze integrins during human wound healing, we have adapted the human skin/SCID mouse chimera model originally developed to study human endothelial cell adhesion molecules and tumor growth in an *in vivo* setting.[28,29] In this model, pieces of full thickness human skin are grafted onto SCID mice. We have found that these grafts are well accepted and maintain their human phenotype without evidence of inflammation, skin breakdown, or skin contraction. The histological appearance of the human skin remains normal; the distribution of integrins was virtually identical to that seen on normal human skin. It was thus possible to analyze sequentially the pattern of ECM proteins and the accompanying changes in integrin expression during the process of cutaneous wound healing using a well-characterized panel of antibodies.[14,44]

After cutaneous injury, the epidermal keratinocytes, which normally sit on a basement membrane composed primarily of laminin and type IV collagen, are exposed to an entirely different type of ECM. The defect in the skin is filled with a blood clot composed of platelets and a number of serum components including fibrin, fibrinogen, fibronectin, and vitronectin.[6] Newly recruited white blood cells, endothelial cells, and fibroblasts, as well as the resident cells, begin to produce other matrix proteins, such as cellular fibronectin and tenascin (Figure 1).[6–9,45] It is thought that the presence of fibronectin and fibrinogen (and perhaps tenascin) in the provisional wound matrix, coupled with the absence of laminin and type IV collagen,[46] is important in the process of epidermal keratinocyte migration. A number of studies have shown that the matrix proteins of inflammation markedly enhance keratinocyte migration rates both *in vitro*[10,47] and *in vivo*.[11] The role of tenascin, which has been shown to have some anti-adhesive properties, in the wound matrix has not yet been determined but could possibly increase migration of cells into the wound.[48]

During the early stages of wound healing, associated with the process of keratinocyte migration, the normal pattern of integrin expression was dramatically changed. The migrating keratinocytes showed marked up-regulation of integrin subunits involved in adhesion to matrix proteins of inflammation, including up-regulation of the α_v subunit (Figure 5) and strong expression of the α_5 subunit of fibronectin receptor (Figure 6), a subunit undetectable in nonmigrating keratinocytes. These changes, especially expression of the fibronectin receptor, were short-lived. As soon as epidermal integrity was restored (between days 2 and 4), expression of α_5 returned to baseline (ie, extremely low to undetectable levels) (Figure 6E). Up-regulation of the α_v subunit was more persistent, but returned to control levels by 7 days after wounding. Although it can be difficult to quantify levels of expression precisely using immunohistochemistry, we feel confident that true up-regulation of integrins occurred during wound healing, because clear differences in the

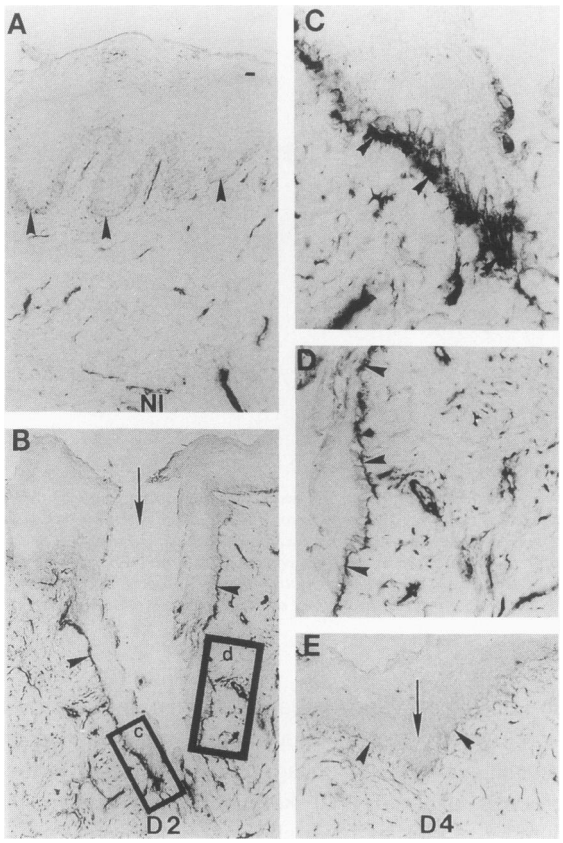

Figure 6. *Expression of the α_5 integrin subunit during wound healing. Grafts were stained using anti-human α_5 monoclonal antibody. Skin was harvested before wounding* (**A**) *or 2 days* (**B**) *or 4 days* (**C**) *after wounding. The large arrow marks the site of the wound. In intact skin, there was no detectable expression of α_5 by the basal keratinocytes* (**A**, *arrowheads*). *On day 2 after wounding* (**B**), *however, a marked up-regulation of the α_5 subunit was observed on the migrating keratinocytes* (*arrowheads*) *but not the keratinocytes further from the wound. At higher magnification* (**C** *and* **D**), *the α_5 subunit is seen to be strongly expressed by the migrating keratinocytes* (*arrowheads*). *By day 4* (**E**), *the expression of α_5 had returned to baseline* (*almost nondetectable*) *levels.* **A**, **B**, *and* **E**: *100×; C and* **D**: *400×.*

staining intensity were seen within the same microscope fields and cells not formerly expressing a given receptor showed strong staining at certain time points.

Alterations of a different sort were noted in the collagen/laminin integrin receptors during wound healing. There were relatively few changes in the amounts or distributions of the α_2, α_3, α_6, and β_4 subunits during the early stages of wound healing (Figures 3B and 4, C and G). In fact, it was interesting that these subunits remained strongly expressed despite the loss of immunodetectable laminin (Figure 2) and type IV collagen (data not shown) in the wound area under the migrating keratinocytes. After epidermal continuity was reestablished, however, the distribution of these integrins was markedly altered (Figures 3C and 4, D and H). Instead of being restricted to the most basal keratinocytes, expression of these subunits was noted in multiple layers of the epidermis.

The results of this study can be compared to a limited number of studies that have directly examined alterations in integrins during wounding. Stepp et al,[49] in a recent analysis of wound healing in rat corneal epithelium, showed no differences in the localization or expression of integrins. Clark[25] examined wounds in porcine skin using polyclonal antibodies to the fibronectin receptor and reported (although did not show) staining of the α_5 subunit in the basal cells of migrating epidermis. Hertle et al[18] studied alterations in integrin expression in human subjects during reepithelialization after experimental induction of blister formation. Unlike our study, they observed no changes in integrin expression during the initial stages of keratinocyte migration. However, similar to our results, they found that at the time of wound closure, the normal staining of the α_2, α_3, α_6, and β_1 subunits were no longer confined to the basilar layer of epidermis but were found in all the living suprabasal cell layers. One major difference between the Hertle study and ours was that their model of blister formation resulted in relatively superficial injury, whereas in our study, full thickness wounds were produced with resultant formation of granulation tissue and brisk angiogenesis (data not shown). It is interesting to note that in two recent studies examining the expression of integrins in psoriatic skin biopsies,[19,50] expression of the α_5 subunit was found on psoriatic keratinocytes, whereas in uninvolved skin, the fibronectin receptor was absent or poorly expressed, suggesting that up-regulation of the fibronectin receptor may be involved in other types of keratinocyte activation.

The physiological implications of the observed changes in integrin expression are currently unknown, although up-regulation of fibronectin/fibrinogen-binding integrins on the keratinocytes adjacent to the wounded area seem to correspond well with changes in the types of ECM proteins that appear during early wound healing and may enable these epidermal cells to migrate more efficiently over the provisional wound bed. An increase in expression of the fibronectin receptor during *in vivo* wound healing from very low levels at baseline is consistent with *in vitro* evidence showing that freshly explanted keratinocytes do not adhere or spread well on fibronectin, but acquire this ability after a number of days in culture.[21,23] This observation has recently been attributed to up-regulation of functional fibronectin integrin receptors ($\alpha_5\beta_1$) on the cultured cells.[22,51,52] This work, along with studies in skin explant models[24] has suggested that al-

terations of integrins on migrating keratinocytes, specifically the $\alpha_5\beta_1$ fibronectin receptor, are an important part of wound healing.

The biological significance of the redistribution of the collagen/laminin-binding integrins in the later stages of wounding is less obvious. Evidence exists to implicate $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins on keratinocytes in both cell-substratum and cell-cell interactions.[53] Suprabasal staining of integrins in psoriatic keratinocytes has also been observed.[18,19,49] Adams and Watt[54] have presented data to support the idea that loss of keratinocyte adhesiveness and integrin expression is associated with terminal differentiation. Together, these data suggest that increased and suprabasal expression of collagen/laminin-binding integrins is part of the hyperproliferative response to injury.

An interesting question raised by these studies is the mechanism(s) responsible for the observed alterations in integrin expression. Several possibilities exist. Certain growth factors and cytokines (transforming growth factor-β, for example) have been shown to alter the expression of integrins on some cells in culture.[55–60] A wide variety of bioactive mediators, including basic fibroblast growth factor, transforming growth factor-β, epidermal growth factor, platelet-derived growth factor, and tumor necrosis factor are present in the wound environment due to the leakage of serum or as a result of release from platelets, epidermal cells, macrophages, or mast cells.[1–3] Another signal for the up-regulation of integrins could be the alterations in ECM proteins in the wound bed (either loss of normal ECM components or presence of new matrix molecules). These changes in ECM proteins could act as direct signals to the cell, possibly transduced by integrins (see ref. 61). Alternatively, alterations in ECM proteins could function indirectly by stimulating the release of cytokines and/or growth factors from the keratinocytes, a pathway suggested by the recent demonstration that alterations in ECM proteins can affect the secretion of transforming growth factor-β by epithelial cells.[62] A third possibility is that mechanical signals (ie, loss of contact inhibition) occurring as a result of the tissue trauma could signal keratinocyte activation programs. Additional studies using cell and organ culture models will be necessary to further define these signaling mechanisms.

Finally, it is important to consider potential differences between the wound healing process in our chimeric model versus that which occurs in normal human skin *in vivo*. First, we have used neonatal skin rather than adult skin in these experiments. Although preliminary data indicated few differences in the ability of neonatal *versus* adult skin to heal, newborn skin was used because of greater accessibility and the fact that it was thinner, more vascular, and less fibrous than adult skin. Second, many of the proteins that make up the provisional matrix (ie, vitronectin, fibrin, and serum fibronectin) are derived from serum, as well as being produced by resident skin cells. In our model, these blood-derived factors are of murine rather than human origin. Third, some of the cells infiltrating the wound (ie, platelets and leukocytes) were also from the murine host. Although earlier studies using split thickness skin grafts transplanted onto nude mice indicated that almost all of the granulation tissue arose from the murine subdermis,[63] our study differs in that the granulation tissue examined was of human origin. This enhanced human component is probably the result of using full thickness skin grafts and may be due to differences between SCID and nude mice. Although it is impossible to predict the full implications of these various cross-species interactions, the rapid and normal-appearing repair reaction, our previous observations that murine leukocytes were able to participate normally in cytokine-induced inflammation in human skin transplants,[28] and the similarity of our results to those from studies using human skin biopsies[18,19] all suggest that the wound healing process in our model closely resembles the normal human repair process.

In summary, this study has demonstrated that during human cutaneous wound healing, alterations in the type and distribution of ECM components are accompanied by alterations in the expression of integrins on the migrating keratinocytes. By understanding more completely the patterns of expression of cell adhesion molecules and the mechanisms causing these changes, it is likely that novel methods to manipulate therapeutically the process of wound healing will be developed.

References

1. Clark RAF: Wound repair. Curr Opin Cell Biol 1989, 1:1000–1008
2. Clark RAF, Henson PM: The Molecular and Cellular Biology of Wound Repair. New York, Plenum Press, 1988
3. Woodley DT: Cutaneous would healing: a model for cell-matrix interactions. J Am Acad Dermatol 1985, 12: 420–433
4. Stanley JR, Alvarez OM, Bere EW Jr, Eaglstein WH, et al: Detection of basement membrane zone antigen during epidermal wound healing in pigs. J Invest Dermatol 1981, 77:240–243
5. Grinnell F: Wound repair, keratinocyte, activation and integrin modulation. J Cell Sci 1992, 101:1–5

6. Clark RAF, Lanigan JM, DellaPelle P, Manseau E, et al: Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. J Invest Dermatol 1982, 79:264–269
7. Grinnell F, Billingham RE, Burgess L: Distribution of fibronectin during wound healing in vivo. J Invest Dermatol 1981, 76:181–189
8. Mackie EJ, Halfter W, Liverani D: Induction of tenascin in healing wounds. J Cell Biol 1988, 107:2757–2767
9. Ffrench-Constant C, Van De Water L, Dvorak HF, Hynes RO: Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. J Cell Biol 1989, 109:903–914
10. Donaldson DJ, Mahan JT: Fibrinogen and fibronectin as substrates for epidermal cell migration during wound closure. J Cell Sci 1983, 62:117–127
11. Nishida T, Nakagawa S, Awata T, Ohashi Y, et al: Fibronectin promotes epithelial migration of cultured rabbit cornea in situ. J Cell Biol 1983, 97:1653–1657
12. Albelda SM, Buck CA: Integrins and other cell adhesion molecules. FASEB J 1990, 4:2868–2880
13. Peltonen J, Larjava H, Jaakkola S, Grainick H, et al: Localization of integrin receptors for fibronectin, collagen, and laminin in human skin. J Clin Invest 1989, 84:1916–1923
14. Albelda SM, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M: Integrin distribution in malignant melanoma: association of the β3 subunit with tumor progression. Cancer Res 1990, 50:6757–6764
15. Klein CE, Steinmayer T, Mattes JM, Kaufmann R, et al: Integrins of normal human epidermis: differential expression, synthesis and molecular structure. Br J Dermatol 1990, 123:171–178
16. Zambruno G, Manca V, Santantonio ML, Soligo D, et al: VLA protein expression on epidermal cells (keratinocytes, langerhans cells, melanocytes): a light and electron microscopic immunohistochemical study. Br J Dermatol 1991, 124:135–145
17. Hertle MD, Adams JC, Watt FM: Integrin expression during human epidermal development in vivo and in vitro. Development 1991, 112:193–206
18. Hertle MD, Kubler M-D, Leigh IM, Watt FM: Aberrant integrin expression during epidermal wound healing and in psoriatic epidermis. J Clin Invest 1992, 89: 1892–1901
19. Pellegrini G, De Luca M, Orecchia G, Balzac F, et al: Expression, topography, and function of integrin receptors are severely altered in keratinocytes from involved and uninvolved psoriatic skin. J Clin Invest 1992, 89:1783–1795
20. Adams JC, Watt FM: Expression of β1, β3, β4, and β5 integrins by human epidermal keratinocytes and non-differentiating keratinocytes. J Cell Biol 1991, 115: 829–841
21. Takashima A, Billingham RE, Grinnell F: Activation of rabbit keratinocyte fibronectin receptor function in vivo during wound healing. J Invest Dermatol 1986, 86: 585–590
22. Toda K, Tuan T, Brown P, Grinnell F: Fibronectin receptors of human keratinocytes and their expression during cell culture. J Cell Biol 1987, 105:3097–3104
23. Grinnell F: The activated keratinocyte: up regulation of cell adhesion and migration during wound healing. J Trauma 1990, 30:S144–S149
24. Guo M, Toda K-I, Grinnell F: Activation of human keratinocyte migration on type I collagen and fibronectin. J Cell Sci 1990, 96:197–205
25. Clark RAF: Fibronectin matrix deposition and fibronectin receptor expression in healing and normal skin. J Invest Dermatol 1990, 94:128s–134s
26. Cohen IK, Mast BA: Models of wound healing. J Trauma 1990, 30:S149–S155
27. Horne RSC, Hurley JV, Crowe DM, Ritz M, et al: Wound healing in foetal sheep: a histological and electron microscope study. Br J Plastic Surg 1992, 45: 333–344
28. Yan H-C, Juhasz I, Pilewski J, Murphy GF, Herlyn M, Albelda SM: Human/severe combined immunodeficient mouse chimeras: an experimental in vivo model system to study the regulation of human endothelial cell-leukocyte adhesion molecules. J Clin Invest, 1993, 91:986–996
29. Juhasz I, Albelda SM, Elder DE, Murphy GF, Adachi K, Herlyn D, Valyi-Nagy IT, Herlyn M: Growth and invasion of human melanomas in human skin grafted to immunodeficient mice. Am J Pathol, 1993, 143:528–537
30. Bosma GC, Custer RP, Bosma MJ: A severe combined immunodeficiency mutation in the mouse. Nature 1983, 301:527–530
31. Hemmler M, et al: VLA proteins in the integrin family: structures, functions and their role on leukocytes. Annu Rev Immunol 1990, 8:365–400
32. Sonnenberg A, Modderman PW, Hogervost R: Laminin receptor on platelets is the integrin VLA-6. Nature 1988, 336:487–489
33. Kennel SJ, Foote LJ, Falcioni R, Sonnenberg R, et al: Analysis of the tumor-associated antigen TSP-180. J Biol Chem 1993, 264:15515–15521
34. Cheresh D, Harper J: ARG-GLY-ASP recognition by cell adhesion receptor requires its 130 kDa α subunit. J Biol Chem 1987, 262:17703–17711
35. Brass LF, Shattil SJ, Kunicki TJ, Bennett JS: Effect of calcium on the stability of the platelet membrane glycoprotein IIb/IIIa complex. J Biol Chem 1985, 260: 7875–7881
36. Herlyn M, Graeven U, Speicher D, Sela B, et al: Characterization of tenascin secreted by human melanoma cells. Cancer Res 1991, 51:4852–4858
37. McDonald JA, Quade BJ, Broekelmann TJ, LaChance R, et al: Fibronectin's cell-adhesive domain and an amino-terminal matrix assembly domain participate in its assembly into fibroblast pericellular matrix. J Biol

Chem 1987, 262:2957–2967
38. Neff NT, Lowrey C, Decker C, Tovar A, et al: A monoclonal antibody detaches embryonic skeletal muscle from extracellular matrices. J Cell Biol 1982, 95:654–666
39. Madri JA, Roll FJ, Furthmayr H, Foidart JM: Ultrastructural localization of fibronectin and laminin in the basement membrane of murine kidney. J Cell Biol 1980, 86:682–687
40. Albelda SM, Muller WA, Buck CA, Newman PJ: Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. J Cell Biol 1991, 114:1059–1068
41. Schalkwijk J, Steijlen PM, van Wlijmen-Willems JJMI, Oosterling B, et al: Tenascin expression in human dermis is related to epidermal proliferation. Am J Pathol 1991, 139:1143–1150
42. Grondahl-Hansen J, Lund LR, Ralfkiaer E, Ottevanger V, et al: Urokinase- and tissue-type plasminogen activators in keratinocytes during wound reepithelialization in vivo. Invest Dermatol 1988, 90:790–795
43. Wenczak BA, Lynch JB, Nanney LB: Epidermal growth factor receptor distribution in burn wounds. J Clin Invest 1992, 90:2392–2401
44. Damjanovich L, Albelda SM, Mette SA, Buck CA: Distribution of integrin cell adhesion receptors in normal and malignant lung tissue. Am J Respir Cell Mol Biol 1992, 6:197–206
45. Winn HJ, Dvorak HF, Colvin RB, Clarke RAF, et al: Fibronectin beneath reepithelializing epidermis in vivo: sources and significance. J Invest Dermatol 1983, 80: 26s–30s
46. Woodley DT, Bachman PM, O'Keefe EJ: Laminin inhibits human keratinocyte locomotion. J Cell Biol 1988, 136:140–146
47. O'Keefe EJ, Payne RE, Russel N, Woodley DT: Spreading and enhanced motility of human keratinocytes on fibronectin. J Invest Dermatol 1985, 85:125–130
48. Koukoulis GK, Gould VE, Bhattacharyya A, Gould JE, et al: Tenascin in normal, reactive, hyperplastic, and neoplastic tissues: biologic and pathologic implications. Hum Pathol 1991, 22:636–643
49. Stepp MA, Spurr-Michaud S, Gipson IK: Integrins in the wounded and unwounded stratified squamous epithelium of the cornea. Invest Opthalmol Vis Sci 1993, 34:1829–1844
50. Kellner I, Knoter U, Sterry W: Overexpression of extracellular matrix receptors (VLA-3, 5 and 6) on psoriatic keratinocytes. Br J Dermatol 1991, 125:211–216
51. Guo M, Lawrence T, Akiyama SK, Gralnick HR, et al: Altered processing of integrin receptors during keratinocyte activation. Exp Cell Res 1991, 195:315–322
52. Kim LT, Ishihara S, Lee C-C, Akiyama SK, et al: Altered glycosylation and cell surface expression of $\beta 1$ integrin receptors during keratinocyte activation. J Cell Sci 1992, 103:743–754
53. Carter WG, Wayner EA, Bouchard TS, Kaur P: The role of integrin $\alpha 2\beta 1$ in cell-cell and cell-substrate adhesion of human epidermal cells. J Cell Biol 1990, 110: 1387–1404
54. Adams JC, Watt FM: Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding preceded $\alpha 5\beta 1$ integrin loss from the cell surface. Cell 1990, 63:425–435
55. Ignotz RA, Massagué J: Cell adhesion protein receptors as targets for transforming growth factor-β action. Cell 1987, 51:189–197
56. Roberts CJ, Birkenmeier TM, McQuillan JJ, Akiyama SK, et al: Transforming growth factor β stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. J Biol Chem 1988, 263:4586–4592
57. Heino J, Ignotz RA, Hemler ME, Crouse C, et al: Regulation of cell adhesion receptors by transforming growth factor-β. J Biol Chem 1989, 264:380–388
58. Heino J, Massagué J: Transforming growth factor-β switches the pattern of integrins expressed in MG-63 human osteosarcoma cells and causes a selective loss of cell adhesion to laminin. J Biol Chem 1989, 264:21806–21811
59. Santala P, Heino J: Regulation of integrin-type cell adhesion receptors by cytokines. J Biol Chem 1991, 266: 23505–23509
60. Bouvois B, Rouillard D, Sanceau J, Wietzerbin J: IFN-γ and transforming growth factor-$\beta 1$ differently regulate fibronectin and laminin receptors of human differentiating monocytic cells. J Immunol 1992, 148:3912–3919
61. Hynes RO: Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992, 69:11–25
62. Streuli CH, Schmidhauser C, Kobrin M, Bissell MJ, et al: Extracellular matrix regulates expression of the TGF-$\beta 1$ gene. J Cell Biol 1993, 120:253–260
63. Demarchez A, Desbas C, Prunieras M: Wound healing of human skin transplanted on to the nude mouse. Br J Dermatol 1985, 113:177–182