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The Spatial and Temporal Expression Patterns of Integrin α9β1 and One of Its Ligands, the EIIIA Segment of Fibronectin, in Cutaneous Wound Healing

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The fibronectins (FN) comprise a family of adhesive extracellular matrix proteins thought to mediate important functions in cutaneous wounds. Plasma fibronectin (pFN) extravasates for days from intact hyperpermeable vessels following injury whereas mRNAs encoding the cellular fibronectins (cFN) that include two segments, termed EIIIA (EDA) and EIIIB (EDB), are expressed by wound cells. Wounds in mice null for pFN appear to heal normally whereas those in EIIIA null mice exhibit defects, suggesting that cFN may play a role when pFN is missing. Integrin α9β1, a receptor for several extracellular matrix proteins as well as the EIIIA segment, is expressed normally in the basal layer of squamous epithelia. We report results from immunohistochemistry on healing wounds demonstrating that EIIIA-containing cFN are deposited abundantly but transiently from day 4 to 7 whereas EIIIB-containing cFN persist at least through day 14. Elevated expression of α9β1 is seen in basal and suprabasal epidermal keratinocytes in wounds. The spatial expression patterns of cFN and α9β1 are distinct, but overlap in the dermal–epidermal junction, and both are expressed contemporaneously. These observations suggest a role for α9β1–EIIIA interactions in wound keratinocyte function.

Key words: cutaneous wound healing/fibronectin/keratinocytes/integrin α9β1
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A central feature of the wound site is the assembly of a "provisional" extracellular matrix composed of plasma proteins, including clotted fibrin and plasma fibronectin (pFN). This provisional matrix arises initially as a result of hemorrhage but is maintained for some days by plasma extravasation mediated by endothelial cells in the post-capillary venules (Brown *et al*, 1992). During healing, this provisional matrix is gradually replaced by synthesis of extracellular matrix proteins, including variants of fibronectin (FN) (ffrench-Constant *et al*, 1989; Brown *et al*, 1993; Serini *et al*, 1998; Sakai *et al*, 2001).

The FN are a family of extracellular matrix proteins generated by alternative splicing in three segments termed EIIIA (or EDA), EIIIB (EDB), and V (IIICS) (Hynes, 1990). pFN lacks the EIIIA and EIIIB segments and the expression of FN that include these spliced segments tends to be quite restricted in adults. By contrast, the EIIIA and EIIIB segments are prominent in embryos, exhibiting characteristic patterns in specific developing tissues suggesting that each FN variant may serve important functions. Two integrins, α9β1 and α4β1, bind the EIIIA segment of FN (Liao *et al*, 2002), whereas no receptors have been identified for the EIIIB segment.

Although the fibrin–FN-rich provisional matrix is prominent at sites of injury, mice made conditionally null for pFN heal cutaneous wounds normally, suggesting that locally produced EIIIA- or EIIIB-containing FN may function in the absence of extravasated pFN (Sakai *et al*, 2001). Mice that are lacking the EIIIB segment are viable and show no obvious defects during rib fracture repair but exhibit subtle difference in fibronectin matrix assembly (Fukuda *et al*, 2002). Mice null for EIIIA segment are reported to show defects in reorganization of the dermal–epidermal junction following injury, raising the possibility that EIIIA+FN may influence keratinocyte function during wound healing (Muro *et al*, 2003).

Following injury, epidermal keratinocytes migrate from the edge of the wound over a matrix that likely includes remnants of dermal collagen as well as a newly synthesized provisional basement membrane rich in laminin-5 (LN-5) deposited by leading edge keratinocytes (Nguyen *et al*, 2000; Kubo *et al*, 2001). Integrin β1 family members are critical to keratinocyte adhesion, epithelial architecture, and migration during normal cutaneous wound healing (Raghavan *et al*, 2000; Grose *et al*, 2002). Normal skin keratinocytes

Abbreviations: cFN, cellular fibronectin; EIIIA (EDA), EIIIB (EDB), and V (IIICS), three segments of fibronectin generated by alternate splicing; FN, fibronectins; pFN, plasma fibronectin

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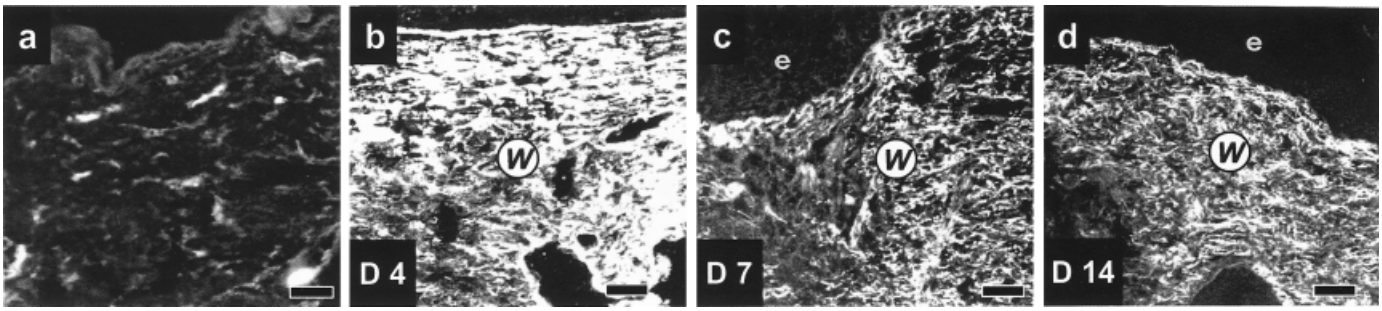

Figure 1
Analysis of fibronectin (FN) deposition in normal and wounded skin by immunohistochemistry. Cryostat sections of normal rat skin were reacted with antibodies to the V95 (*panel a*), or monoclonal antibody (mAb) BR5.3 (*panels b–d*). Excisional rat cutaneous wounds (4 mm diameter) were harvested at intervals of 4, 7, or 14 d (*panels b–d*) post-wounding. Sections are oriented with epidermis ("e") at the top. FN is present in the wound bed ("w") following injury and persists through day 14 (*d*). Magnification: *scale bar* = 50 μm.

express integrins including α2β1, α3β1, α5β1, α6β4, αvβ5, and αvβ6. Integrins α2β1 and α5β1 are upregulated following injury and are found in basal and suprabasal layers; however, α3β1 and α6β4 are also upregulated, but are confined to basal keratinocytes (Larjava *et al*, 1993; DiPersio *et al*, 1997; Nguyen *et al*, 2000). Integrin α9β1 is expressed by basal keratinocytes in normal skin (Palmer *et al*, 1993; Stepp *et al*, 2002). The expression pattern of α9β1 during cutaneous wound healing, however, remains unclear.

Integrin α9β1 is normally expressed in airway epithelial cells, the basal layer of squamous epithelia, smooth muscle, skeletal muscle, neutrophils, and hepatocytes (Palmer *et al*, 1993). Within 6–12 d of birth, null mice die with defects in the lymphatic system (Huang *et al*, 2000). Ligands for α9β1 include tenascin-C, osteopontin, vascular cell adhesion molecule-1 (VCAM-1), von Willebrand factor, tissue transglutaminase, ADAMs-12 and 15, and the EIIIA segment of FN (Liao *et al*, 2002; Stepp *et al*, 2002, and references contained therein). Increased expression of α9β1 in keratinocytes following injury has been reported in oral mucosal, corneal, and skin wounds (Stepp and Zhu, 1997; Hakkinen *et al*, 2000; Stepp *et al*, 2002; Pal-Ghosh *et al*, 2004). Although α9β1 is upregulated in corneal epidermal wounds during migration and re-stratification (Stepp and Zhu, 1997; Stepp *et al*, 2002), normal re-epithelialization and expression of α9β1 occurs in tenascin-C-deficient mice, suggesting that ligands other than, or in addition to, tenascin-C may bind to α9β1 during corneal healing (Iglesia *et al*, 2000).

A close examination of the expression of both EIIIA- and EIIIB-containing forms of FN, as well as α9β1 integrin, would provide clues about their functions during wound healing. We report here that keratinocytes express α9β1 during wound healing and that myofibroblasts do not. Our data support a model in which keratinocyte α9β1 serves multiple functions. These include EIIIA-independent functions, as for suprabasal keratinocytes that do not encounter EIIIA-containing FN, as well as EIIIA-dependent functions, and as for basal and migrating keratinocytes, which likely interact with EIIIA at the dermal–epidermal junction.

Results

FN in normal and wounded skin Prior work from our laboratories demonstrated that FN mRNAs were expressed at low levels in unwounded skin (ffrench-Constant *et al*, 1989). Using antibodies reactive with either the V95 (IP73) region or a constant region of rat FN (BR5.3), we observed that normal rat skin contained low levels of FN protein (Fig 1*a*). Small blood vessels within the papillary dermis were positively stained, however. We next delineated the spatial arrangement of total FN in healing wounds (Fig 1*b–d*). Following wounding, intense staining for total FN was observed in the wound bed at 4, 7, and 14 d ("w" in Fig 1*b–d*); little staining was observed in the adjacent unwounded dermis and none was evident in the overlying, regenerated epidermis ("e" in Fig 1*c*, *d*). Thus, we observe a striking increase in the total amount of FN deposited in healing wounds relative to normal skin.

When immunostaining was performed with antibodies specific to the EIIIA and EIIIB segments, we observed no significant staining in normal skin (Fig 2*a*, *e*). By 4 d following wounding, however, staining for both the EIIIA and EIIIB segments was observed in granulation tissue (Fig 2*b*, *f*). Staining for both segments increased in the granulation tissue at 7 d (Fig 2*c*, *g*) and exhibited a fibrillar pattern (inset, Fig 2*g*). Importantly, staining for EIIIB remained elevated through day 14 following wounding (Fig 2*h*) whereas staining for EIIIA was strikingly reduced (Fig 2*d*). Moreover, staining for smooth muscle cell (SMC) α-actin followed kinetics identical to those of the EIIIA segment with bright staining of myofibroblasts at day 7 and strikingly reduced staining at day 14 (not shown). Comparable staining was observed with different monoclonal antibodies (mAb) IST-9 (Fig 2*a–d*) and 3E2, as well as a goat polyclonal anti-EIIIA antibody (not shown). Arterioles, but not venules within the connective tissue subjacent to the panniculus carnosus, were also immunopositive for EIIIA + FN during the interval from 4 to 10 d (not shown). The epidermis was not stained at any time point with any antibody to FN, consistent with our analysis of FN mRNAs by *in situ* hybridization (ffrench-Constant *et al*, 1989). These results demonstrate that a temporal switch occurs in the composition of the granulation tissue between 7 and 14 d, with selective loss of the EIIIA segment, but retention of the EIIIB segment at 14 d.

Temporal pattern of integrin α9 expression in the epidermal keratinocytes migrating over the wound bed The transient but prominent expression of EIIIA + FN in cutaneous wounds (Fig 2*a–d*) prompted us to determine the

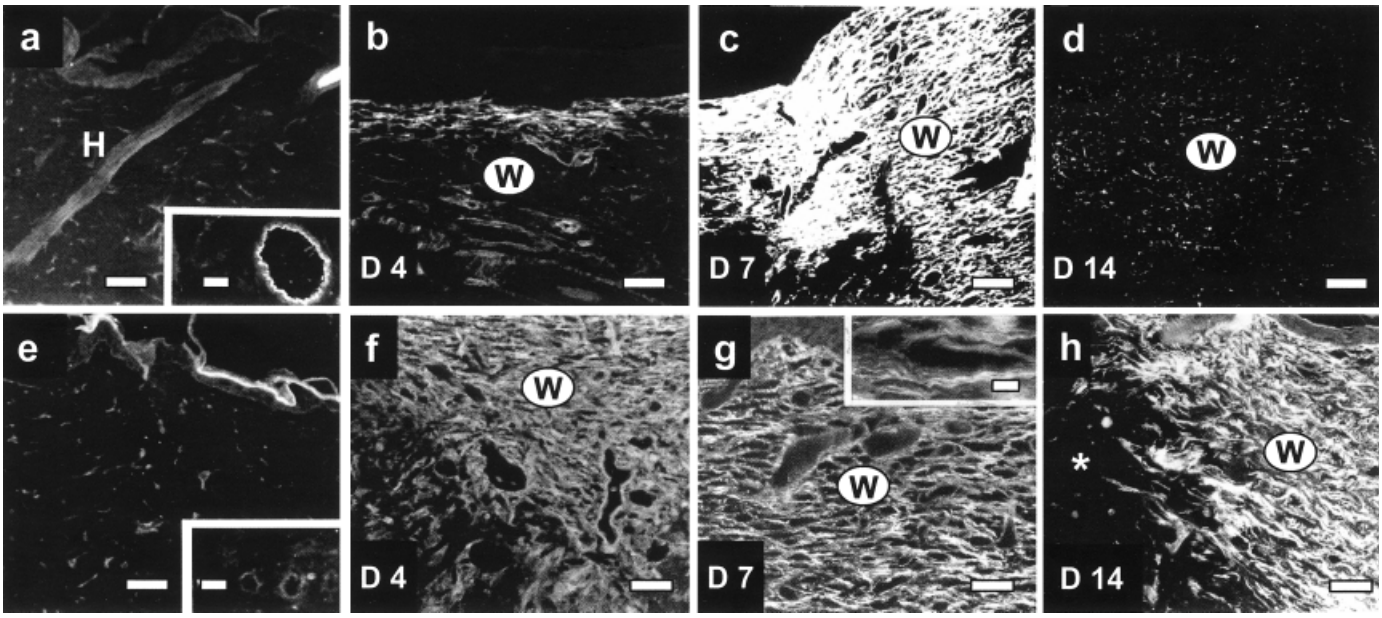

Figure 2
Temporal pattern of EIIIA and EIIIB + FN (fibronectin) in healing wounds. Cryostat sections were reacted with mouse monoclonal anti-EIIIA (*a–d*) or rabbit anti-EIIIB antibodies (*e–h*). Normal rat skin (*panels a*, *e*) or excisional cutaneous wounds (*panels b–d*, *f–h*) were harvested from rats at intervals of 4, 7, or 14 d post-wounding. In normal skin, EIIIA is present in the intima of some arterioles within the panniculus carnosus (*inset*, *a*); little to no staining for EIIIB was observed in vessels (*inset*, *panel e*). EIIIA and EIIIB staining is prominent between days 4 and 7. Staining has fibrillar appearance (*panel g*, *inset*). Little staining was observed in flanking, unwounded dermis (* in *panel h*). Staining for EIIIB persisted whereas EIIIA-decreased markedly between days 7 and 14 (cf. *panels d* with *h*). Wound bed denoted by "w". Magnification: *scale bar* = 50 μm, *inset bar* = 10 μm.

spatiotemporal expression pattern of one of its receptors, α9β1, in skin following injury. Earlier work established that α4β1 is expressed prominently by inflammatory cells, but also by fibroblasts and endothelial cells, and we did not study this further here (Massia and Hubbell, 1992; Gailit *et al*, 1993). We performed immunohistochemistry with a rabbit antibody that reacts with α9β1 on tissues harvested at intervals of 1–14 d (Fig 3). Images were obtained with equivalent exposure times. Increased levels of α9 expression were evident as early as day 1 post-injury in the basal and adjacent suprabasal layers of keratinocytes at the wound edge. The leading edge of keratinocytes migrating over the wound bed was brightly labeled with antibodies to α9 and staining was more intense at days 2 and 4 compared to day 1 post-wounding. By day 7, migrating keratinocytes had closed the wound and α9 staining peaked and was expressed in basal and suprabasal layers (Fig 3*d*). By days 10 and 14, we observed a marked decrease in α9 staining in the suprabasal keratinocytes. In contrast to the staining directly over the wound bed, staining in the epidermis at a distance from the wound remained confined to the basal layer of keratinocytes as it is in normal epidermis (not shown).

Expression of integrin α9 in arterioles and skeletal muscle cells We also noted α9β1 staining within the wound bed (Fig 3*d*). It was previously reported that blood vessels were positive for α9 in the granulation tissue of oral mucosa wounds (Hakkinen *et al*, 2000). To test whether or not blood vessels expressed α9 during cutaneous wound healing, we

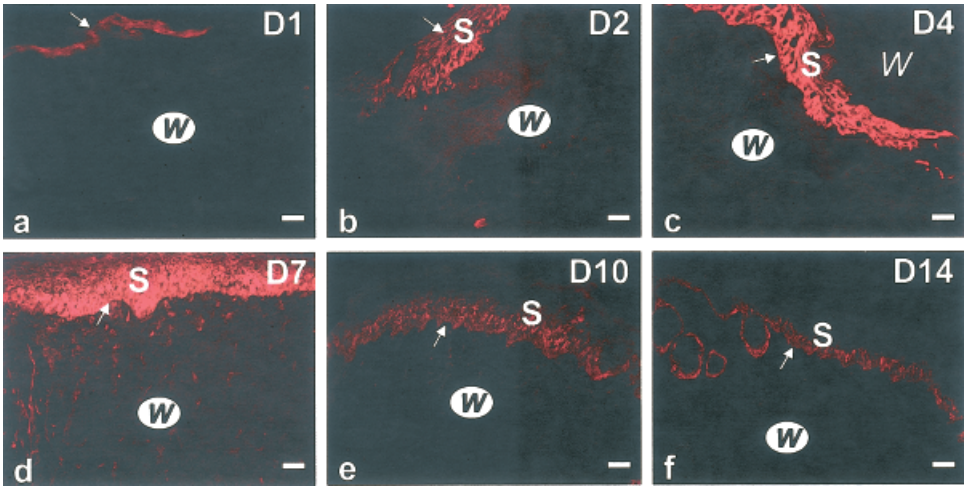

Figure 3
Spatiotemporal expression of α9 in the epidermal keratinocytes migrating over the wound bed. Excisional mouse cutaneous wounds were harvested at intervals of 1, 2, 4, 7, 10, and 14 d post-wounding. Harvested wounds were oriented with epidermis on top and panniculus carnosus below. Note progressive increase in α9β1 expression at day 1 through day 7 in basal (*arrow*) and suprabasal keratinocytes ("s"), over the wound bed ("w"). By contrast, a marked decrease in α9 expression is evident 10 and 14 d (*panels e*, *f*). Magnification: *scale bar* = 50 μm.

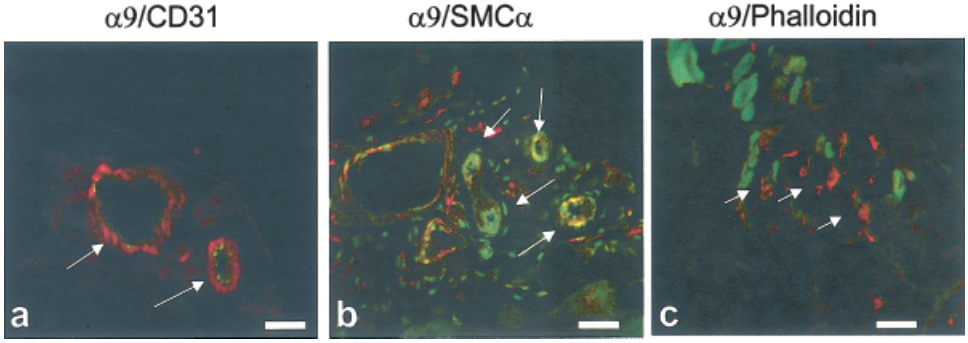

Figure 4
Expression pattern of α9 in blood vessels and skeletal muscle cells. Double label immunostaining was performed on cryostat sections of 7 day wounds using rabbit anti-α9 antibody (*a–c*), and either rat anti-CD31 (*a*), mouse anti-SMC α-actin (*b*), or phalloidin (*c*). Fields shown in panels *a* and *b* represent dermis adjacent to granulation tissue. Note that medial layer of arterioles labeled with α9 and SMC α-actin is observed in many but not all blood vessels (*b*). Integrin α9 did not co-localize significantly with CD31-positive endothelial cells (*a*). Selected skeletal muscle cells adjacent at the edge of injured panniculus carnosus stain for α9 (*c*). Arrow denotes blood vessel staining (*a*, *b*) and skeletal muscle cells (*c*). Magnification: *scale bar* = 50 μm.

performed immunohistochemistry on 7-d mouse wounds, a time marked by granulation tissue formation with prominent blood vessels. Tubular structures with the appearance of blood vessels were positive for α9 in granulation tissue and in the adjacent dermis. When we carried out double-label immunostaining with rabbit anti-α9 antibodies and either rat anti-CD31, or anti-SMCα, however, we observed that α9β1 did not co-localize appreciably with either marker in the tubular structure within granulation tissue. When we examined blood vessels in adjacent dermis, we observed that anti-CD31 stained the endothelial layer, whereas α9 localized primarily to the medial layer of blood vessels (Fig 4*a*, *b*). Selected skeletal muscle cells in the injured panniculus carnosus also stained positively for α9 at day 4 (not shown) and at day 7 (Fig 4*c*) post-injury. We observed that not all skeletal muscle cells express α9, but those at the end most proximal to injury were preferentially labeled.

Discussion

Alternative splicing of FN provides an efficient mechanism for regulating the expression of function-specific isoforms following tissue injury. Results reported here provide new data that reveal a distinct temporal pattern of FN splicing and protein deposition for the EIIIA and EIIIB segments in wounds. The EIIIA segment is deposited in the wound ECM between days 4 and 7 (Fig 2) and this pattern coincides temporally with the expression of α9β1 by keratinocytes (Fig 3). This integrin is expressed by basal keratinocytes in normal skin, and its expression is dramatically upregulated in both basal and suprabasal cells over and in close proximity to the wound (Fig 3). Although EIIIA-containing FN are deposited throughout the granulation tissue concomitant with the expression of SMC α-actin, they are also abundant in the dermal–epithelial zone through which keratinocytes migrate (Fig 2). Despite strong EIIIA–FN staining in the granulation tissue, α9β1 is not detectable in myofibroblasts. Our data suggest that keratinocyte–α9β1 interactions with the EIIIA segment of FN may be important to keratinocyte function during re-epithelialization.

In normal adult skin, low levels of FN are detected and these FN are largely devoid of the EIIIA or EIIIB segments (Figs 1 and 2*a*, *e*). Immediately after wounding, the extracellular matrix changes dramatically. Frank hemorrhage and prolonged blood plasma extravasation over several days result in the deposition of plasma proteins, such as pFN, fibrinogen, and vitronectin into the "provisional matrix" within the viable tissue of the wound bed (Grinnell *et al*, 1981; Clark *et al*, 1982). By 1–2 d after injury, wound macrophages contain increased levels of FN mRNAs that include the EIIIA and EIIIB segments (Brown *et al*, 1993) and by 4 d these cFN are deposited in the wound matrix (Fig 2). This temporal pattern of FN splicing occurs in other types of injury indicating that a general pattern exists in which pFN that lacks the EIIIA and EIIIB segments is deposited prior to "cellular" FN that includes these segments suggesting that the temporal pattern of FN variant expression has functional consequences for individual cell types in distinct repair settings.

Our observation of a concurrent appearance and cessation of EIIIA and SMC α-actin expression during granulation tissue formation is consistent with observations that EIIIA may be necessary for myofibroblast differentiation (Serini *et al*, 1998). Although we previously determined that α4β1 and α9β1 integrins function as receptors for the EIIIA segment (Liao *et al*, 2002), we show here that α9β1 is not detected in myofibroblasts. Indeed, we find that α9β1 is not expressed by cultured fibroblasts and myofibroblasts, whereas α4β1 is, when assayed by flow cytometry (Phillips and Van De Water, unpublished observations). By contrast, vascular smooth muscle cells do express α9β1 and this expression is markedly upregulated in some, but not all, arterioles in healing wounds as well as by injured skeletal muscle cells (Fig 4). Our observation that skeletal myofibrils within the panniculus carnosus express α9β1 at their injured ends (Fig 4) also suggests a potential role in cell fusion.

What is the potential importance of the concurrent expression of EIIIA and α9β1 during tissue repair? There are several ligands for α9β1 in addition to the EIIIA segment. Of these, osteopontin is present deep in the wound and not adjacent to keratinocytes (Liaw *et al*, 1998). VCAM-1 is expressed by endothelial cells (Taooka *et al*, 1999). Tenascin-C is prominently expressed in a pattern similar to the one for EIIIA-containing FN reported here (Mackie *et al*, 1988; Hakkinen *et al*, 2000). Interestingly, tenascin-C null mice heal normally suggesting that other ligands for α9β1 may function in the absence of tenascin-C (Iglesia *et al*, 2000). EIIIA–FN are expressed in a temporal pattern that is maximal between days 4 and 7 and in a spatial pattern that overlaps with keratinocyte α9β1 in the dermal–epidermal junction (Figs 2 and 3). Although moderate levels of keratinocyte

α9β1 are observed in the basal layer of normal epidermis, α9β1 expression is upregulated in keratinocytes at sites immediately over the injury. Because keratinocytes do not express α4β1 (Kubo *et al*, 2001), our data support a hypothesis in which EIIIA, along with tenascin-C, interactions with α9β1 regulate keratinocyte functions important to re-epithelialization such as migration, differentiation or proliferation.

Our data also support a model in which keratinocyte α9β1 serves multiple functions. These include EIIIA-dependent functions such as basal keratinocytes that likely interact with EIIIA at the dermal–epidermal junction and may modulate re-epithelialization. They also include EIIIA-independent functions, as for suprabasal keratinocytes that do not encounter EIIIA-containing FN. Because the α9β1 null mouse is perinatally lethal, these hypothesized roles have not been tested (Huang *et al*, 2000). Integrin α9β1 is expressed during murine embryogenesis at a time in which epithelial stratification occurs and eyelid fusion takes place, and α9β1 is found at sites and times where leading edges of epithelial sheets are merging during the closure of corneal wounds (Wang *et al*, 1995; Pal-Ghosh *et al*, 2004). Our data are consistent with these observations and show that elevated expression of α9 is seen in suprabasal and basal keratinocytes and persists until the migrating epidermis merges together from both ends by day 7 (Fig 3); beyond this day, α9 expression decreases markedly.

Materials and Methods

Materials A mouse mAb, BR5.3, reacts with all forms of FN (Peters and Hynes, 1996; Peters *et al*, 1996). Antibodies to EIIIA include mAb to rat and human EIIIA (clone IST-9, Accurate Scientific, Westbury, New York; clone 3E2, Sigma Chemical, St Louis, Missouri). IP73, IP153, and IP264 are polyclonal antibodies raised to the V95 segment, to an EIIIA-derived synthetic peptide, and to an EIIIB-GST fusion peptide, respectively (Peters and Hynes, 1996; Peters *et al*, 1996). Rabbit anti-α9 antibodies were raised against a synthetic peptide (Iglesia *et al*, 2000). Anti-SMC α-actin mAb, clone 1A4, was purchased from Sigma Chemical. Mouse anti-CD31 (MEC13.3, BD Pharmingen, San Diego, California) was used as an endothelial cell marker. All polyclonal antibodies were immunopurified on their antigens.

Methods

Wounds Punch biopsies (4 mm diameter) were made in the flanks of either female adult CD1 rats (~200 g, Charles River Laboratory, Wilmington, Massachusetts) or in the flanks of female adult BALB/C mice (~5–6 wk, Taconic labs, Tarrytown, New York) as described previously (Brown *et al*, 1993). Wounds were harvested at intervals, fixed in 3% paraformaldehyde (pHCHO) in phosphate-buffered saline (PBS, pH 7.4) for 1 h (4°C) or not fixed, quenched in 0.1 M glycine in PBS, pH 7.4 for 1 h (4°C), and sedimented (at unit gravity) in 0.6% sucrose in PBS, pH 7.4, for 4 h at 4°C. The tissues were embedded in OCT (Electron Microscopy Sciences, Washington, Pennsylvania) and frozen (−80°C). Some tissues were frozen immediately after biopsy in OCT, sectioned, and in some instances post-fixed in acetone (−20°C). All procedures for animal care and handling had the approval of the relevant Institutional Animal Care and Use Committees.

Immunofluorescence Cryostat sections from rat (4 μm) or mouse (10 μm) were placed on poly-lysine-coated slides and rinsed in PBS to remove OCT. Fixation protocols used with the respective antibodies were as follows: BR5.3, IP73, IP264 and 1A4 (pHCHO); IST-9 (pHCHO or acetone); 1A4, MEC13.3, and rabbit anti-α9 (unfixed). Tissue sections were treated with 5% nonfat milk, 10% heat-inactivated goat serum and 0.02% Tween 20 in PBS for 1 h followed successively by diluted primary antibodies, wash buffer (0.02% Tween 20 in PBS), appropriate fluorescent secondary IgG in a solution that included phalloidin 488 or 594 (Molecular Probes, Eugene, Oregon). For anti-EIIIB (IP264), sections were deglycosylated by incubation with PNGase (1:20, New England Biolabs, Beverly, Massachusetts) overnight at 37°C prior to immunostaining (Peters and Hynes, 1996; Peters *et al*, 1996). Controls included substituting either mouse or rabbit IgG for specific primary antibody. Rabbit anti-α9 was pre-incubated with peptide, CZ-RKENEDS-WDWVQKNQ, for 1 h. Photographs were taken under epifluorescence illumination on either a Zeiss Axiophot microscope using Kodak T3200 film or on a Nikon Eclipse TE2000 U using a SPOT camera (Diagnostic Instruments, Sterling Heights, Michigan).

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