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REPORTS

Fibronectin and Fibrin Provide a Provisional Matrix for Epidermal Cell Migration During Wound Reepithelialization

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Factors regulating the attachment and directional migration of a regenerating epidermis in wound healing are poorly understood. In studies of guinea pig 4-mm skin wounds, left uncovered for 1–28 days, biopsied and processed for 1-μm section and immunofluorescence, the epidermis migrated over an irregularly thickened provisional matrix containing fibrin and fibronectin. The provisional matrix lacked two major components of normal basement membrane, laminin and type IV collagen, which can mediate tenacious epithelial attachment to plastic *in vitro* and may limit epidermal cell migration *in vivo*. Upon completion of wound reepithelialization at 7–9 days after wounding, the basement membrane zone lost its thickened appearance, fibronectin and fibrinogen disappeared, and type IV collagen and laminin reappeared. Although these findings do not prove that epidermal cell migration during reepithelialization requires a fibrin and fibronectin matrix, they demonstrate that epidermal cells do move over such a substratum during *in vivo* wound repair.

Mechanisms responsible for adhesion of reepithelializing epidermis to the underlying dermis and granulation tissue during wound healing have not been identified. Striking ultrastructural changes occur in the basement membrane zone (BMZ) beneath the migrating epidermal cells, which include desiccation and dissolution of the basal lamina with subsequent migration of epithelial cells across a dense matrix that differs ultrastructurally from normal BMZ [1–3]. The two basement membrane proteins, type IV collagen and laminin, that can mediate epithelial cell adherence *in vitro* [4,5] are notably absent under migrating epidermal cells *in vitro* [6,7] as well as *in vivo* [8]. Bullous pemphigoid (BP) antigen, another BMZ protein that is more closely associated with the basal cell plasma membrane [9,10], is present beneath the migrating epidermis, but its role in epidermal adherance to underlying dermis is unknown [8]. The contents of the dense amorphous matrix over which the epidermal cells move, therefore, warrants investigation.

Fibrin and fibronectin are likely components of this matrix since they accumulate in the extravascular space during some forms of tissue injury and may provide a provisional matrix for macrophage, endothelial cell, and fibroblast localization to sites of injury [11–14]. *In vitro* data also support this possibility. Fibrinogen and fibronectin, when covalently cross-linked by factor XIII, promote the adherence of fibroblasts to tissue culture dishes [15], and fibronectin coating of plates enhances endothelial cell adherence [16,17]. Furthermore, fibronectin promotes the migration of fibroblasts and endothelial cells [18–20] and is involved in the full expression of macrophage immobilization by MIF (migration inhibitory factor) [21]. Although fibronectin does not enhance the adherence of epidermal cells to collagen *in vitro* [4], it has been shown to support the growth of keratinocytes when used to precoat tissue culture dishes [22]. This present study examines the BMZ under the migrating epithelial cells of reepithelializing wounds in guinea pigs for the presence of fibrinogen and fibronectin as well as type IV collagen, laminin, and BP antigen.

MATERIALS AND METHODS

Wound Healing Model

Standard wounds were made with a 4-mm skin biopsy punch down to the panniculus carnosus in chemically depilated flanks of 400–600 g male or female Hartley guinea pigs. At intervals thereafter, ranging from 1 to 28 days, the uncovered wound sites were harvested and bisected for immunofluorescence and 1-μm Epon sections (see below).

Antisera and Antibodies

Guinea pig fibronectin was purified as previously described [12] and guinea pig fibrinogen was isolated by the method of Blomback and Blomback [23]. Rabbits were immunized with 100 μg of protein in complete Freund's adjuvant (CFA) in the hind foot pads and boosted several times with 100 μg of protein in saline intravenously. Antisera were assayed by immunoelectrophoresis and indirect immunofluorescence to determine sensitivity and specificity of antifibronectin and antifibrinogen antibodies. Human embryonic lung fibroblasts (WI38), which synthesize fibronectin but not fibrinogen in tissue culture [24], were employed as a positive control for the antifibronectin antisera in an indirect immunofluorescence assay. Human or guinea pig fibrinogen, depleted of fibronectin by passage over a gelatin affinity column [25], clotted with 1 U human α-thrombin (Sigma Chemical Co., St. Louis, Missouri), and embedded after syneresis in Tissue-Tek II OCT compound (Miles Laboratories, Inc, Naperville, Illinois), was used as a positive control for the antifibrinogen antisera by indirect immunofluorescence. Since cross-reactivity between the two antisera was noted on occasion, antifibronectin antisera were routinely adsorbed with fibrinogen-Sepharose beads or insolubilized gelatin-absorbed (fibronectin-depleted) plasma and antifibrinogen antisera were routinely adsorbed with insolubilized guinea pig serum. The adsorbed antisera were applied to DEAE cellulose anion exchange columns equilibrated with 0.01 M PO_4 buffer, pH 8.0. The void volume peaks (IgG) were pooled and concentrated by precipitation with half-saturated $(NH_4)_2SO_4$. The purified IgG fractions were conjugated with fluorescein or rhodamine by the dialysis method [26]. The antifibrinogen antibodies we used react

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Abbreviations:
BMZ: basement membrane zone
BP: bullous pemphigoid
CFA: complete Freund's adjuvant
MIF: migration inhibitory factor

with fibrin, fibrinogen, and with certain of their degradation products. Fibrin can generally be recognized because of its characteristic fibrillar apppearance.

Anticollagenous basement membrane protein (Type IV collagen) antibodies were derived from two sources. For the first, the immunogen was isolated from neutral solutions of pepsin-digested human kidney slices which were subjected to salt precipitation and heat gelation [27] and injected into rabbits as described above to elicit antibodies. After adsorption with insolubulized normal human serum, this antibody reacted with the BMZ of epidermis, vessels, and renal tubules and glomeruli. No reaction with interstitial collagen was detected by immunofluorescence. The second source was antibodies against type IV collagen purified from the EHS mouse sarcoma [28] and kindly provided by Dr. George Martin. Antilaminin antibodies were kindly provided by Dr. Stephen Katz. This antigen was also purified from the EHS mouse sarcoma and antibodies prepared as previously described [29]. Anti-BP antiserum was obtained from a patient with high-titered levels (1:50,000) of antibodies to BP antigen.

Immunofluorescence Studies

One-half of each wound specimen including adjacent skin was embedded in Tissue Tek II O.C.T. (Lab Tek Products, Nashville, Illinois) and frozen at −70°C until used. Four-μm sections were cut in a cryostat and washed 3 times with 0.15 M NaCl, 0.01 M PO_4 buffer, pH 7.4 (PBS). For direct immunofluorescence studies, a 1:4 or 1:8 dilution of fluoresceinated rabbit IgG antibodies to guinea pig fibronectin or fibrinogen was applied to the tissue sections, and incubated in a moist chamber for 30 min. After further washing, sections were mounted in Elvanol-glycerin and viewed in a Zeiss darkfield fluorescence microscope. For indirect immunofluorescence a dilution of unlabeled first antibody or antiserum was applied to the tissue sections, incubated in a moist chamber for 30 min, washed, and followed with an apppropriate second fluoresceinated antiserum: goat antihuman IgG, goat antirabbit IgG, or rabbit antisheep IgG (Cappel Laboratories, Cochranville, Pennsylvania).

One-Micron Section Technique [30]

Portions of the other half of each biopsy were fixed in a solution of 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.025% $CaCl_2$ in 0.1 M cacodylate buffer pH 7.4 on a rotary shaker for 4 hr at 25°C, then transferred to cold 0.1 M cacodylate buffer. Tissue was postfixed in 1% or 2% osmium tetroxide, dehydrated, and embedded in Epon (Shell Chemical Co., Houston, Texas). Sections 1-μm thick were stained with Giemsa's reagent and examined by light microscopy.

RESULTS

One-Micron Section Studies of the Migrating Epidermis

A migrating epithelial tongue was first detected on the second day after wounding. The migrating epidermal cells always stained pale with Giemsa's stain compared to adjacent normal cells. This fact plus landmarks of the wound edge itself made the extent of the migrating epidermal tongue easy to identify. The migration rate was approximately 0.3 mm/day until the wound was completely reepithelialized. Most wounds completely reepithelialized by 7 or 9 days, after punch biopsy. Although the original defect was 4 mm in diameter, the span of newly formed epidermis never measured more than 2 mm in diameter. Wound contraction continued for another week after reepithelialization was complete, often reducing the new epithelial surface to 1 mm in diameter.

Our interest focused on the BMZ beneath the migrating epithelial cells and under the newly formed epidermis. The migrating epithelial tongue descended along the wound edge apparently separating necrotic dermis, inflammatory cells, and escar above from viable dermis below (Fig 1*A*). An accumulation of homogeneous ground glass material, which was at times fibrillar, appeared in the BMZ of the migrating epithelium and trailed off into the dermis or granulation tissue below. Material with these characteristics always stopped abruptly at the stationary epidermis where normal-appearing BMZ began (Fig 1*B*). However, along the wound edge which had not yet reepithelialized, there developed a discrete, 0.5–0.7 mm area comprised of homogenous fibrillar material interposed between collagen bundles. This fibrillar material became continuous with the homogenous material of the thickened BMZ region as the epidermal cells dissected through the area.

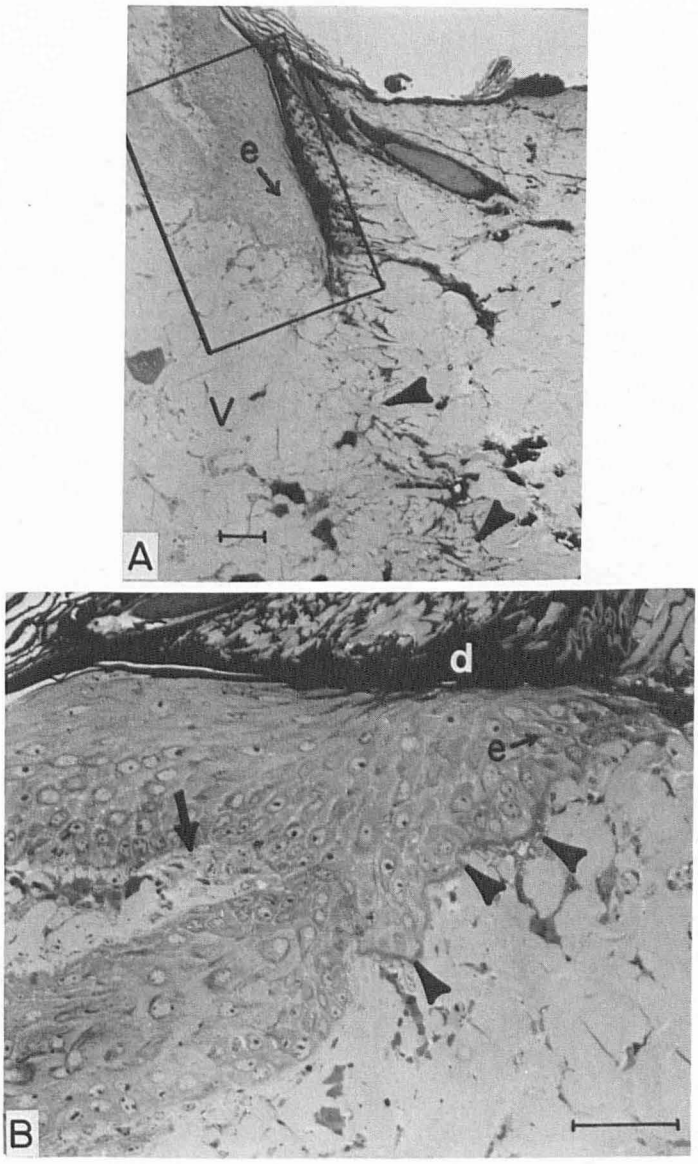

FIG 1. *A*, One-micron Epon section of the edge of a 3-day excisional wound in guinea pig skin. The migrating epithelial tongue (*e*) dissects between the necrotic tissue containing inflammatory cells (above) and the viable dermis (*V*) within which fibrin strands are interposed between collagen bundles (*arrowheads*). Bar = 50 μm. *B*, Higher magnification of the same section. Beneath the migrating epidermis (*e*) an irregular thickening of the basement membrane zone (BMZ) (*arrowheads*) is interconnected with fibrillar material deposited in the underlying dermis. This can be contrasted to histologically appearing normal BMZ beneath stationary epidermis (*arrow*). Dense fibrin eschar, post-syneresis (*d*), lies above the migrating epidermis. Bar = 50 μm.

Immunofluorescence Studies of the Migrating Epidermis

To identify the components of the thickened provisional BMZ we used antibodies to various known basement membrane proteins, BP antigen, type IV collagen, and laminin, and to fibrinogen and fibronectin, as fluorescent probes.

In normal skin, anti-BP antigen stained only the dermal-epidermal junction; whereas, anti-type IV collagen and antilaminin stained the dermal-epidermal junction as well as the BMZ of blood vessels, smooth muscles, fat cells, and nerves. The normal dermal-epidermal junction did not stain with antifibrinogen at any dilution and did not stain with antifibronectin at the dilution (1:8) used in this study.

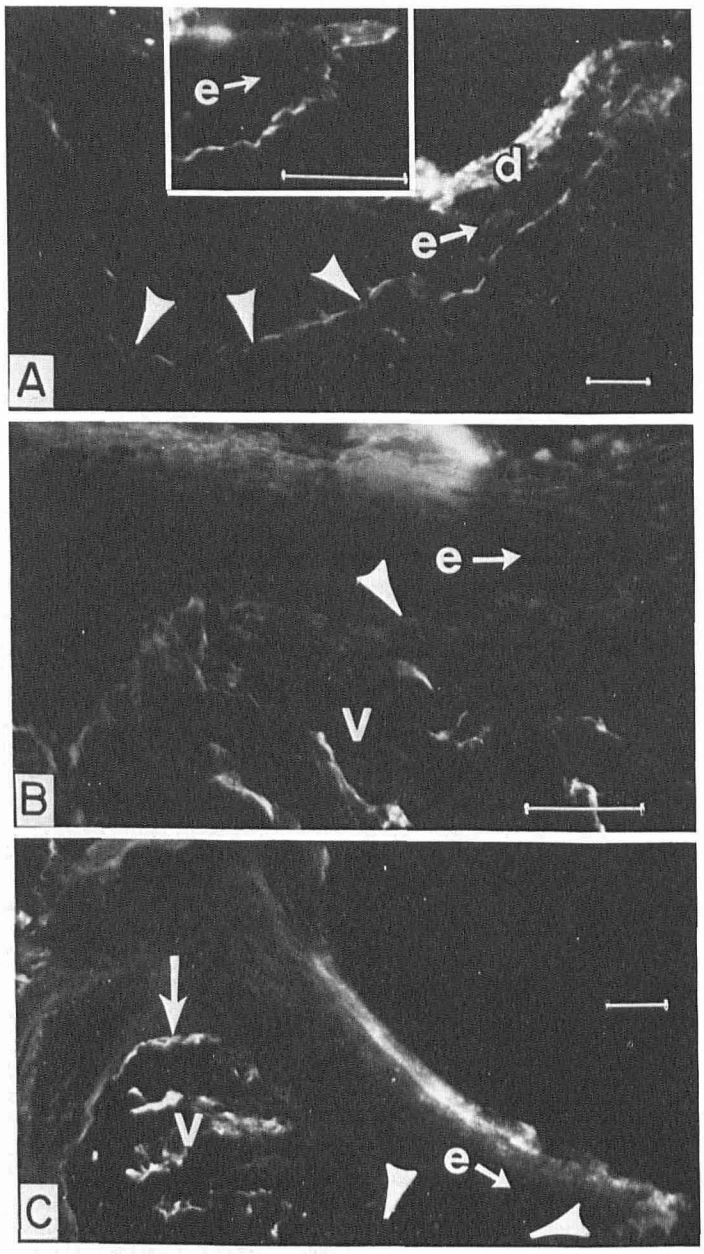

FIG 2. Immunofluorescence of the migrating epithelium in a guinea pig wound 3 days after excision. *A*, The BMZ (*arrowheads*) stains with anti-bullous pemphigoid (BP) antiserum to the tip of the wedge-shaped epidermis (*inset*). The eschar (*d*) on the upper surface of the epidermis (*e*) stained nonspecifically with all antisera used. *B*, With antihuman collagenous basement membrane (type IV collagen) antiserum, the normal BMZ of the dermal-epidermal junction, hair follicles, and blood vessels (*v*) stained intensely while the migrating epithelial tongue BMZ did not stain (*arrowhead*). *C*, Antilaminin antiserum gave a pattern similar to that of anti-type IV collagen although several breaks were seen in the staining of the BMZ of the stationary dermal-epidermal junction and hair follicule adjacent to the wound (*arrow*). No staining was present in the BMZ (*arrowheads*) beneath the migrating epidermis (*e*). *A–C*: Small *arrows* are pointing in the direction of epidermal migration. Bars = 50 μm.

In wounds, BP antigen was present in the dermal-epidermal junction out to the tip of the migrating epithelial tongue (Fig. 2*A*) as has been reported recently [8]. Type IV collagen (Fig. 2*B*) and laminin (Fig. 2*C*) persisted in the BMZ of the stationary epidermis at the wound edge while both were completely absent from the BMZ of epithelium in transit (Fig 2*B*,*C*). As the epithelial cells ceased to migrate, laminin and type IV collagen became detectable in the BMZ, beginning from the original wound edge and reappearing inward. Laminin staining reappeared after that of type IV collagen: analogous to the sequence in which these proteins appear *in vitro* [6].

The thickened BMZ of the migrating epidermis stained intensely with fluorescein-conjugated antifibrinogen antibodies (Fig 3*A*). In addition, strands of intensely staining fibrin extended from the BMZ into the dermis. The staining with antifibrinogen was limited to BMZ of the migrating and newly stationary epidermis of the wound surface and stopped abruptly at the BMZ of adjacent normal skin. Specificity of the antifibrinogen antibodies was demonstrated by the fact that all tissue fluorescence was abolished when the antibodies were absorbed with fibrinogen-Sepharose beads. Fluoresceinated antibodies to fibronectin also stained the BMZ of the migrating epidermis (Fig 3*B*) but not the BMZ of the newly stationary epidermis where type IV collagen reappeared nor the BMZ of the adjacent normal skin. Strands of fibronectin-staining material extended from the BMZ of the migrating epithelium into the dermis (Fig 3*B*) in a pattern identical to that of fibrin. Adsorption of fibronectin antibodies with fibronectin-Sepharose abolished the staining.

In the migrating tongue, BP antigen (Fig 2*A*) always appeared as a fine regular line indistinguishable from that in normal skin, while fibrinogen (Fig 3*A*) and fibronectin (Fig 3*B*) apppeared as thicker, more irregular bands involving the entire thickness of the BMZ with reticular strands extending vertically downward into the dermis at multiple points along the BMZ. The BMZ beneath the migrating epidermis did not stain with anti-guinea pig IgG or albumin.

One-Micron Section and Immunofluorescence Studies of a Newly Reepithelialized Wound

The epidermal cells of newly complete, reepithelialized wounds at 7 to 10 days after injury no longer had the pale

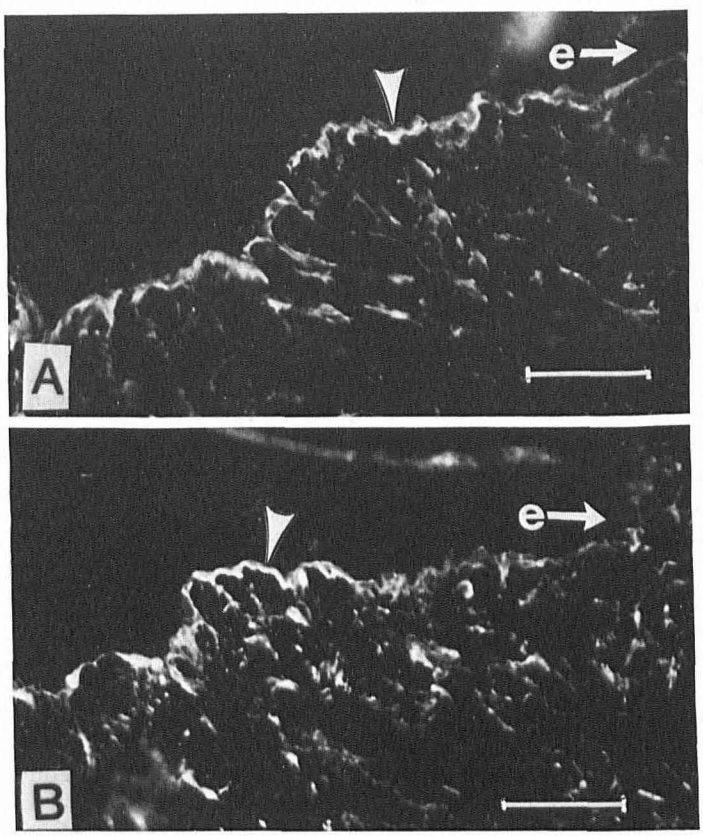

FIG 3. Specimens of 3-day excisional wounds in guinea pigs stained with antifibrinogen antibodies (*A*) and antifibronectin antibodies (*B*). *A*, Fibrin is disposed beneath the migrating epithelium (*e*) in an irregularly thick linear fashion along the BMZ and into the adjacent dermis (*arrowhead*). *B*, Fibronectin also appeared along the BMZ of the migrating epidermis (*e*) as a thick, irregular band with strands extending perpendicularly into the dermis (*arrowhead*). *Arrows* indicate tip of migrating epidermis. Bars = 50 μm.

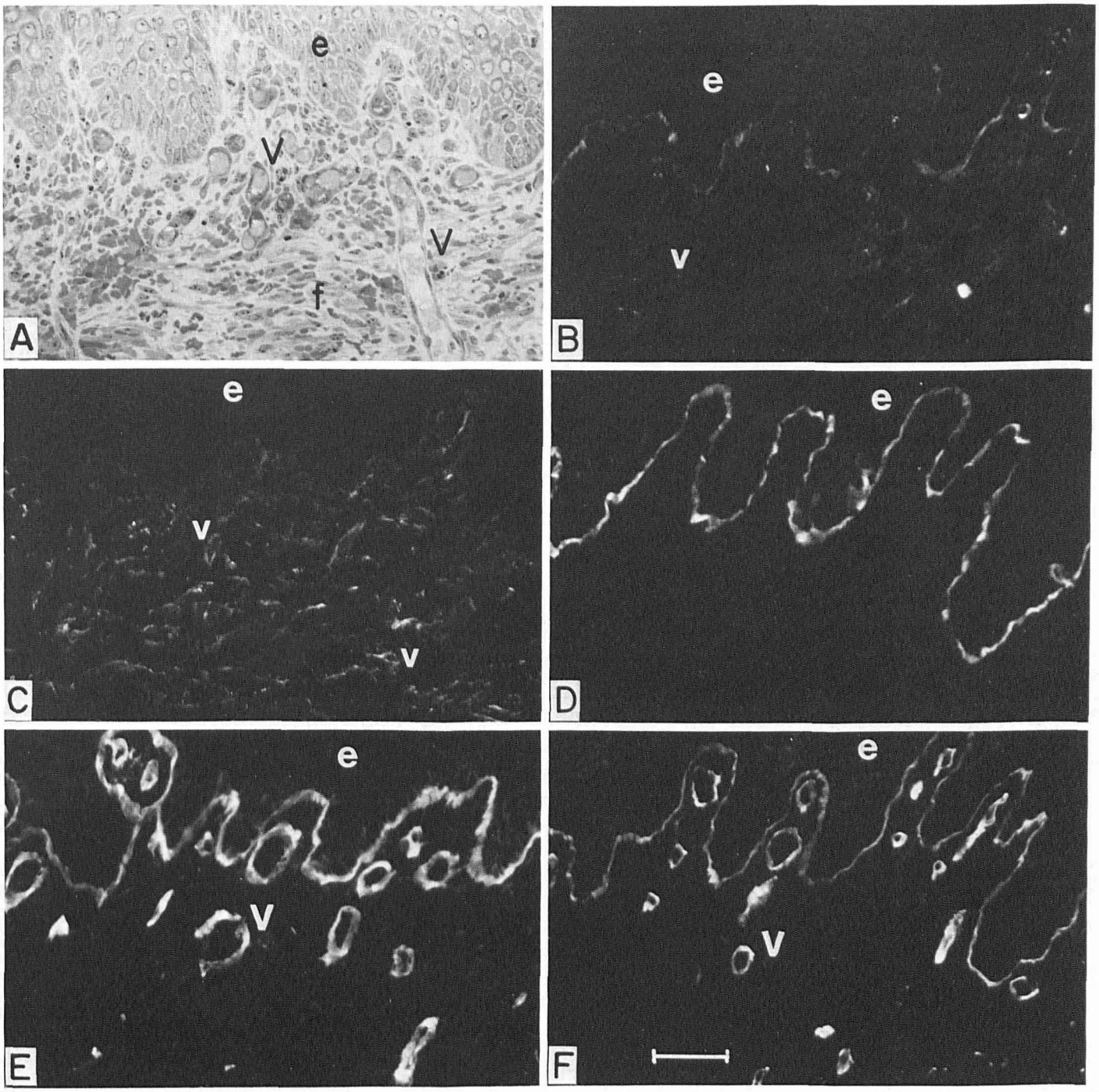

FIG 4. *A*, One-micron section of guinea pig skin 9 days after wounding. The wound has entirely reepithelialized (*e*) and the BMZ is no longer thickened. New blood vessels (*v*) course perpendicular to the epidermis while fibroblasts (*f*) are aligned parallel to the epidermis. *B–F*, Immunofluorescence of adjacent sections of a guinea pig wound 12 days after injury when reepithelialization has been completed. *B*, Antifibrinogen antibodies stain the BMZ of the newly formed epidermis although much less intensely than the BMZ beneath a migrating epidermis. *C*, Antifibronectin antibodies stain the BMZ weakly if at all, but do stain the granulation tissue in a reticular pattern. *D*, Dermal-epidermal junction is stained normally with BP antiserum. *E* and *F*, Anti-human type IV collagen and antilaminin, respectively, stain the BMZ of both the dermal-epidermal junction and the blood vessels. Bar = 50 μm.

appearance with Giemsa's stain that they had earlier when in transit, and the BMZ no longer appeared thickened (Fig 4*A*). Antifibrinogen antibodies gave weaker and more focal staining along the BMZ (Fig 4*B*) while antifibronectin no longer stained the BMZ, but did stain fine reticular fibrils in the underlying granulation tissue oriented parallel to the epidermis (Fig 4*C*). BP antigen appeared unchanged as a fine regular line along the dermal-epidermal junction (Fig 4*D*). Antibodies to type IV collagen (Fig 4*E*) and laminin (Fig 4*F*) gave similar patterns of bright linear fluorescence of the dermal-epidermal junction and the BMZ of the blood vessels coursing through the granulation tissue. By 28 days postexcision neither fibronectin nor fibrinogen was demonstrable in the BMZ beneath the reepithelialized wound (data not shown).

DISCUSSION

Anchoring of the normal epidermis to the underlying dermis is thought to be mediated to a large extent by two proteins of the BMZ, type IV collagen and laminin. This supposition is

derived from *in vitro* data of Terranova, Rohrback, and Martin [5] which show that these proteins mediate attachment of epidermal cells to tissue culture dishes. Two other BMZ proteins, BP antigen [8] and AB_2 collagen [7], are normally present in the dermal-epidermal junction and, in addition, have been observed, respectively, in the BMZ of migrating epidermis *in vivo* and during radial outgrowth from skin organ cultures. Neither BP antigen nor AB_2 collagen has thus far been implicated in epidermal attachment.

When a defect is created in the epidermis and underlying dermis, as in the present experiments, healing of the dermis occurs from below by proliferation of granulation tissue [31] while the epidermis is repaired from the edges of the wound by migration of a tongue of epidermal cells that dissects downward and centripetally between the eschar (above) and viable dermis (below). In this study, we noted that the advancing edge of migrating epidermis lacked a normal BMZ but rather rested on a homogeneous, dense fibrillar material that was thicker and more irregular than normal BMZ. This material differed from normal BMZ as judged by immunofluorescence in that both fibrin and fibronectin were present whereas laminin and type IV collagen were not. BP antigen was present at the dermal-epidermal junction, as in normal skin. After the epithelial defect was repaired and epidermal migration had ceased (i.e., after about day 7),, the BMZ was remodeled to a more normal histologic appearance and composition: laminin and type IV collagen returned as fibrin and fibronectin gradually disappeared. Epithelial migration over a fibrin-fibronectin provisional matrix also occurs in superficial rabbit corneal wounds [32].

Comparison of epithelial healing in the skin and cornea [32] reveals common features as well as significant differences. In a minimal corneal wound achieved by scraping of the epithelium of a rabbit cornea, the stroma and basement lamina remain. Fibrin and then fibronectin deposit on the surface within hours and the epithelium moves rapidly across this matrix (without cell division) to close a 6–8 mm defect in 2–3 days. Thus, the fibrin-fibronectin matrix occurs without disruption of vessels, formation of an eschar, or ingrowth of granulation tissue. In contrast to the skin, the BP antigen is notably absent until the corneal epithelium has completed its migration and begun cell division. While indicating BP antigen is not essential for epithelial adhesion to fibrin-fibronectin, the reasons for the difference are not known. Perhaps cell division or simply more time is required for BP antigen production; alternatively the method of migration may differ, e.g., sliding of the basal layer (cornea) vs. "hop-scotch" with the upper layer coming down in front of the leading edge and remaining fixed (skin).

These observations contribute to an understanding of the mechanism of reepithelialization of an epidermal defect. Normal epidermis is anchored firmly to the underlying BMZ which is itself a synthetic product of epidermis interacting with stroma. However, somewhat looser attachments may be necessary if epidermal cell migration is to occur. Consistent with this view, the homogeneous material or provisional BMZ over which epidermal cells migrate during reepithelialization in our experiments lacked the components of normal BMZ thought to be necessary for firm epidermal anchoring; i.e., laminin and type IV collagen. Indeed, these components appeared only when migration had ceased and the defect was bridged. Contrariwise, provisional BMZ contained fibrin and fibronectin, protein normally absent from mature BMZ.

Our studies do not establish whether fibrin or fibronectin is essential for normal epithelial repair. However, our *in vivo* observations do justify further analysis of this possibility. Fibronectin *in vitro* does not promote epidermal adhesion to collagen [4], but *in vivo* we find fibronectin associated with fibrin, not collagen, during wound reepithelialization. It is well known that fibronectin mediates the adhesion of fibroblasts and endothelial cells *in vitro* [15–17]; however, it has recently been demonstrated that fibronectin cross-bonded to fibrin(ogen) by factor XIII provides a much more adhesive surface for fibroblasts than does fibronectin alone [15]. For these reasons we suggest that fibronectin and fibrin, possibly cross-linked by factor XIII, may be important to epidermal cell migration and therefore to wound healing *in vivo*. Interestingly, the genetic absence of factor XIII, transglutaminase, which enzymatically mediates γ-glutamyl-ϵ-lysyl cross-links between fibrin monomers or between fibronectin-fibrin and fibronectin-collagen [33], is known to be associated with an incompletely characterized defect in wound healing [34].

Our studies did not establish the source of the provisional BMZ found underlying the advanced tongue of migrating epidermis. The fibrin, presumably, is derived exclusively from extravasated and subsequently clotted fibrinogen. Fibronectin may also have been derived, in part, from extravasated cold insoluble globulin (plasma fibronectin). However, underlying fibroblasts may also have contributed some of the fibronectin component of the provisional BMZ. Synthesis of mature BMZ containing laminin and type IV collagen occurred only after epithelial migration had ceased, at a time when firm anchoring of epidermis was required and when the staining properties of the epidermal cells had returned to normal. Taken together, these data suggest that migrating and normal epidermal cells may require substrata of different chemical composition.

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Blood Vessel Fibronectin Increases in Conjunction with Endothelial Cell Proliferation and Capillary Ingrowth During Wound Healing

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The regulation of angiogenesis and alterations in the structure of blood vessels taking part in wound healing are poorly understood. In studies of guinea pig 4-mm skin wounds, left uncovered for 1–28 days, biopsied and processed for 1-μm section and immunofluorescence, we found that fibronectin in blood vessel walls markedly increased in conjunction with endothelial cell proliferation and capillary ingrowth. Both the endothelial cell proliferation and the increased vessel wall fibronectin were restricted to a 0.5-mm area along the margin of the wound and occurred 3–7 days after injury. Fibronectin was easily demonstrated in capillaries of the peripheral granulation tissue but was difficult to demonstrate in central areas of the granulation tissue secondary to a brightly fluorescent reticular background staining probably attributable to fibroblast-related fibronectin. The fibronectin in blood vessel walls rapidly diminished as endothelial cell proliferation and capillary ingrowth ceased. These data suggest that fibronectin may provide a provisional substratum for endothelial cell mitosis and movement.

In the process of embryogenesis and wound healing, a specialized interaction must exist between mobile cell populations and their substratum to provide a sufficient degree of adherence to ensure attachment while permitting cell movement and mitosis. The ideal situation might be for the cells to control the degree to which they adhere to their substratum. Recent *in vitro* investigations have examined factors responsible for variations in cell adhesion to underlying substratum. Many cells bind to tissue culture substratum through extracellular glycoproteins [1–15]. One of these attachment proteins, fibronectin, is produced by fibroblasts [16–18], monocytes [19,20], and endothelial cells [21–23], as well as other cell types and is present at moderate concentrations in blood and serum [24]. In culture, fibronectin, derived from serum and/or produced by cells, can bind to the substratum and simultaneously bind to cell surfaces [6,13,14]. In doing so, fibronectin attaches cultured cells to the underlying surface. Culp and his coworkers [25,26] recently have postulated that fibroblasts may modulate their binding to fibronectin by locally modifying the concentration and type of glycosaminoglycan on their surface membrane. If this hypothesis is correct, not only for fibroblasts but also for endothelial cells and epidermal cells, it would provide a mechanistic explanation for the appearance of fibronectin in morphogenesis dur-

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Abbreviations:
CIG: cold-insoluble globulin