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Immunomorphological and Ultrastructural Aspects of Keratinocyte Migration in Epidermal Wound Healing

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Summary. Epidermal repair was studied after the induction of a suction blister on human abdominal skin. The investigation was concerned with keratinocyte migration from the epidermal wound margins and changes at the dermo-epidermal junction.

Specific antisera against actin and keratin proteins showed the distribution of fine and intermediate filaments within marginal epithelial cells. Pemphigus- and pemphigoid-autoantibodies allowed the evaluation of the decomposition of extracellular substances. Transmission and scanning electron microscopy were used to analyze the submicroscopical changes in intra- and extraepithelial structures.

It was found that suprabasal cells, defined by the presence of keratin polypeptides of 67000 daltons, moved out of the epidermis, thus covering the wound. These cells were strongly labelled by actin-antibodies at the wound margins. The expression of pemphigus antigen was not changed at the wound margins. Components of the basal membrane zone (bullous pemphigoid antigen) were not detected immunohistologically before keratinocytes were present at the surface of the wound. This finding supports the hypothesis that the substances at the junctional zone are partly of epithelial origin.

Transmission electron microscopy showed that the lamina densa was present across the wound hiatus. In addition, the development of desmosomes and hemidesmosomes during wound healing was demonstrated.

Scanning electron microscopy did not support the concept of cell movement as a coherent sheet, but rather that of an "ameboid" type of cell movement.

Key words: Epidermal repair – Migration – Fine and intermediate filaments – Keratinocyte differentiation

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Introduction

Epidermal wound healing is known to be effected by two properties of epidermal cells, i.e. migration and proliferation (review: Pollack 1979).

During the first 48 h, keratinocyte migration may play the principal role in epidermal repair (Krawczyk 1971; Marks et al. 1972). Migration of epidermal cells was demonstrated to occur from epidermal appendages and from the margins of the wound (Winter 1972; Eaglstein and Mertz 1978; Pang et al. 1978). The predominance of the one or the other source of migrating cells depends on the extension and depth of the wound and the region of skin affected. (Winter 1972; Pollack 1979).

The present study, performed by suction blistering of human abdominal skin, was concerned with the morphological phenomena at the epidermal wound margins during the first three days after induction of the wounds. The investigation was performed to elucidate:

1. the origin and type of marginal cellular migration,
2. the structural changes occurring in the migrating keratinocytes,
3. the alterations occurring in the basal membrane zone and the interepithelial spaces during epidermal repair.

Recently, a specific antiserum against the keratin polypeptide of molecular weight 67,000 daltons (67 K) was induced in guinea pigs (Viac et al. 1980; Viac et al. 1980). This immune serum allowed a distinction between the basal and the suprabasal cell compartment in the epidermis (Viac et al. 1980; Viac et al. 1980; Löning et al. 1980). The basal keratinocytes were generally not stained by 67K-antibodies, in contrast to the suprabasal epidermal cells. Hence, this antiserum appeared to be of value for the characterization of migrating keratinocytes. Using 67K-antibodies, we also tried to answer the question whether the marginal epidermis migrates as an intact coherent sheet (Weiss 1961) or whether the epidermal cells roll over each other in attempting to cover the wound hiatus (Krawczyk 1971; Martinez 1972).

In addition, antibodies against actin were used to investigate the distribution of microfilaments within migrating epidermal cells (Gabbiani and Ryan 1974).

Changes of intercellular substance during epidermal repair were studied using pemphigus-autoantibodies. Pemphigoid-autoantibodies served to analyze the formation of the basal membrane zone (Abell et al. 1973; Brickman et al. 1977; Hintner et al. 1979; Woodley et al. 1979).

This immunohistochemical study was completed by transmission and scanning electron microscopy. The ultrastructural part of our investigation was particularly concerned with the morphogenesis of epidermal and dermo-epidermal junctions (Colson et al. 1971; Krawczyk and Wilgram 1973; Beerens et al. 1975; Sciubba 1977).

Materials and Methods

Wounding

Suction blisters were induced on the abdominal skin of seven healthy volunteers following a technique first described by Kiistala and Mustakallio (1967). The suction chamber chosen (Dermovac Instrumentarium, Helsinki, Finland) contained a perforated disk with five apertures each of 6 mm diameter. The negative pressure was −220 mm Hg and the required suction time averaged 120 mn.

This procedure allowed the separation of epidermis and dermis. The basal lamina remained on the dermal site (Ortonne 1980). When dermal-epidermal dissociation was obtained, the roof of the blister was removed. The wounds were covered with a dressing taped to the skin about 2 cm from the edge. Punch biopsies were undertaken at 24, 48 and 72 h after induction of the wounds.

Antisera

67K-Antiserum. Adult (400 g) female Hartley guinea pigs were immunized with the keratin polypeptide of molecular weight of 67,000 daltons according to a procedure previously described (Viac et al.1978). The animals were bled by heart puncture 8 days after the last injection. The immune serum obtained was absorbed successively with human erythrocytes and liver powder (Olson et al. 1972) and used at a dilution of 1:200 in phosphate buffered saline (PBS) (Viac et al. 1980).

Actin-Antiserum. Actin-autoantibodies were kindly provided by Dr. G. Gabbiani and were used at a dilution of 1:20 in PBS (Gabbiani and Ryan 1974; Bussolati et al. 1980).

Autoantibodies against intercellular and basal membrane substances: Pemphigus vulgaris- and bullous pemphigoid-high titre sera were used diluted at 1:100 in PBS.

Immunofluorescence

The tissue samples were snap-frozen in liquid nitrogen. They were then sectioned in a cryostat at 4 microns. Incubation with the antisera described above was followed by fluorescein conjugated immune sera (goat anti-guinea pig IgG, 1:20 in PBS, NORDIC, for demonstration of keratin; rabbit anti-human IgG, 1:20 in PBS, Behring, for demonstration of actin, pemphigus and pemphigoid antigen). The slides were observed with a Leitz fluorescence microscope (epi-illumination orthoplan).

Immunoenzymatic Staining

The samples were fixed in Bouin's solution for 4–8 h and embedded in paraffin. On 6 μ sections, immunocytochemical staining was performed using the primary antisera described above and peroxidase-conjugated link antisera (goat anti-guinea pig IgG and rabbit anti-human IgG, 1:50 in PBS, NORDIC). 3′, 3′-diaminobenzidine (SIGMA) was used to reveal peroxidase activity (Graham and Karnovsky 1966). Parallel sections were stained with haematoxylin-eosin and the PAS-reaction. Control studies were performed in which the specific primary antibodies were replaced by 1. preimmune serum, 2. antibody absorbed with the antigen, 3. peroxidase-coupled link antisera only.

Transmission Electron Microscopy

Specimens for electron microscopy were fixed for 2 h in buffered 2% glutaraldehyde. The samples were rinsed in cacodylate buffer (pH 7.2), post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded in Epon. The tissue blocks were trimmed and sectioned with an ultramicrotome Reichert Om-U2. 2 μ sections were stained with toluidine blue and studied in a light microscope. Thin sections were stained with 3% uranyl acetate and lead citrate and examined with a Philips EM 300 at a voltage of 80 KV.

Scanning Electron Microscopy

The samples were fixed in 2% glutaraldehyde and then dehydrated in a graded ethanol series. The samples were left overnight in acetone under vacuum. Critical point drying was applied with liquid CO_2. The specimens were fixed on standard mounting blocks by means of double face tape with surface to be studied facing upwards. The tissue surface were uniformly coated in a vacuum evaporator with about 200 A of gold palladium to ensure good electrical conductivity in scanning electron microscopy. The samples were examined with an Cambridge Stereoscan 600.

Results

Light Microscopy

After 48 h, keratinocytes from the edges of the blister started to migrate (Fig. 1A). In conventionally stained sections (HE, PAS), elongated cells were

observed infiltrating the fibrin deposits of the wound. These cells were present not only across the surface of the wound, but also in the superficial parts of the corium.

Parallel immunoenzymatic studies revealed that the suprabasal keratinocytes of the unaltered skin reacted strongly with 67K-immune serum, whereas the basal cell layer remained negative (Fig. 1B). However, all keratinocytes located at the tongue of the newly formed epidermis were labelled by 67K-antibodies (Fig. 1C). The most advanced elongated epidermal cells in direct contact with the dermis were also 67K-positive (Fig. 1D) and some migrating keratinocytes in the superficial corium were stained with this antiserum (Fig. 1D). At this time (48 h), mitotic figures were not detected in these keratinocytes.

At the tip of the regenerating epidermis, cytoplasmic actin was demonstrated within the advancing epidermal cells (Fig. 2A, B). It was the suprabasal cell compartment which showed strong immunoreactivity at the wound margins (Fig. 2A). Mesenchymal cells resembling fibroblasts and reacting with actin-antibodies were also found in the granulation tissue of the wound (Fig. 2A).

By immunofluorescence and immunocytochemistry of frozen tissue sections, intercellular substance demonstrated by pemphigus autoantibodies was observed to be present even in the marginal epidermis adjacent to the wound hiatus. In the basal membrane zone, bullous pemphigoid autoantibodies reacted with the dermo-epidermal junction except for the centre of the wound, where no keratinocytes were present (Fig. 3A, B). Using the PAS-reagent, the basal membrane was continuously stained only beneath epidermal cells. In the centre of the wound, the reaction was present, but in a irregular manner.

Electron Microscopy

Transmission electron microscopy revealed structural changes of both the epidermal margins and the migrating keratinocytes at the surface of the wound. Distal to the advancing tip of the epidermis, the keratinocytes appeared to be rounded (Fig. 4A). Within the cytoplasm, loosely distributed filaments were observed. At the cellular junctions, both mature and incomplete, asymmetrical desmosomes were found (Fig. 4B). At the dermo-epidermal junction, hemidesmosomes appeared as condensations of the cellular membrane (Fig. 4C). Tonofilaments were seen to be fixed to these structures (Fig. 4B, C). In addition to the desmosome attached tonofibrils, parallelly arranged filaments were found along the inner leaflet of the plasma membrane (Fig. 4D). The lamina lucida and lamina densa of the basal membrane zone were both intact (Fig. 4C, D). In the centre of the wound, however, only the lamina densa was present (Fig. 3C, D), showing some focal disruptions (Fig. 3D). At the center the most advanced keratinocytes were flattened (Fig. 5A) and the cytoplasm of the migrating epidermal cells contained tonofilament bundles, which were frequently marginally condensed (Fig. 5A). Few desmosomal contactes were observed between cytoplasmic elongations (Fig. 5A, B). Instead of true desmosomes, interdigitations of the cell membranes were frequently formed. In the vicinity of the dermis, hemidesmosomes were found which appeared to be in a primitive stage of development (Fig. 5C). Few filaments inserted into these structures.

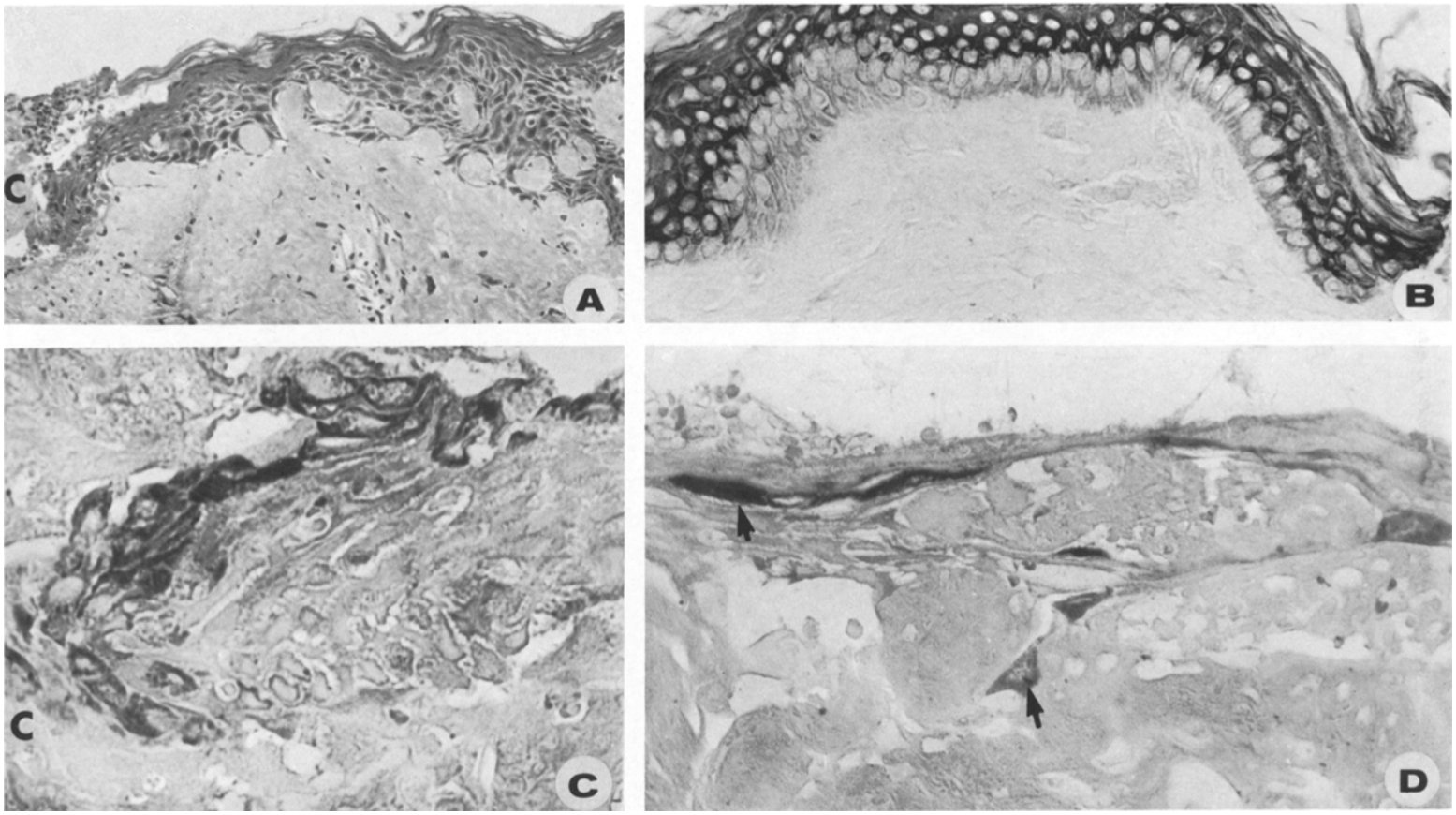

Fig. 1A–D. Epidermal wound margin after 48 h. **A** Topographical view of the advancing tip of the epidermis (*C*=centre of the wound). Haematoxylin-eosin. ×63. **B** Keratin staining of unaltered epidermis. Negative basal cell layer. Indirect immunoperoxidase method. ×160. **C** Demonstration of 67K-positive keratinocytes at the tip of the advancing epidermis (*C*=centre of the wound). Indirect immunoperoxidase method. ×250. **D** Migrating keratinocytes at the wound surface. Arrows point to 67K-positive epidermal cells. Indirect immunoperoxidase method. ×250

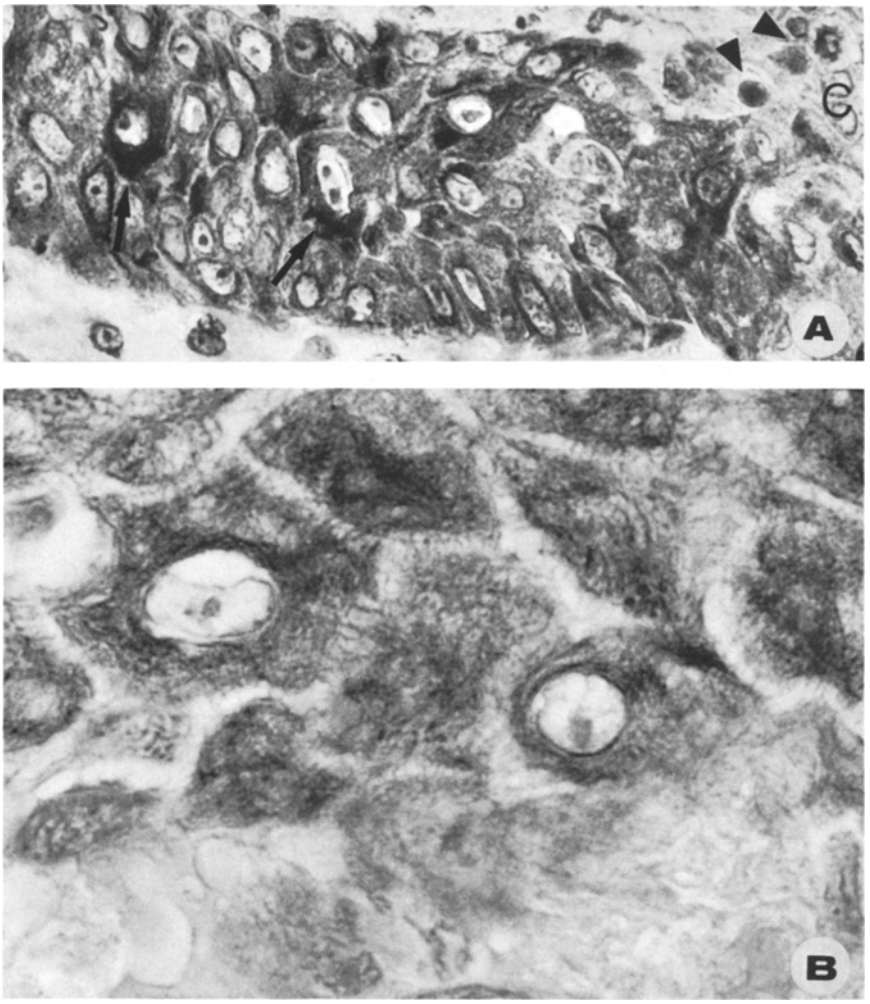

Fig. 2A, B. Demonstration of cytoplasmic actin at the wound margin. **A** Actin-positive suprabasally located keratinocytes (arrows). Some labelled mesenchymal cells (arrowheads) in the centre of the wound (C). Indirect immunoperoxidase method. ×250. **B** Higher magnification of stained epidermal cells at the leading edge of the newly formed epidermis. Indirect immunoperoxidase method. ×1,300

By scanning electron microscopy, flat wavy keratinocytes were observed at the wound margins (Fig. 6A, B). Their surfaces were generally smooth. Some of them showed a stellate shape and were apparently stretched on the surface of flat epithelial cells which had earlier covered the basal lamina. These keratinocytes were still connected to the neighbouring cells. However, focal widenings of intercellular spaces were observed (Fig. 6B). Most of the wound hiatus was covered by a well-established basal lamina (Fig. 6A).

Our findings are summarized in Table 1.

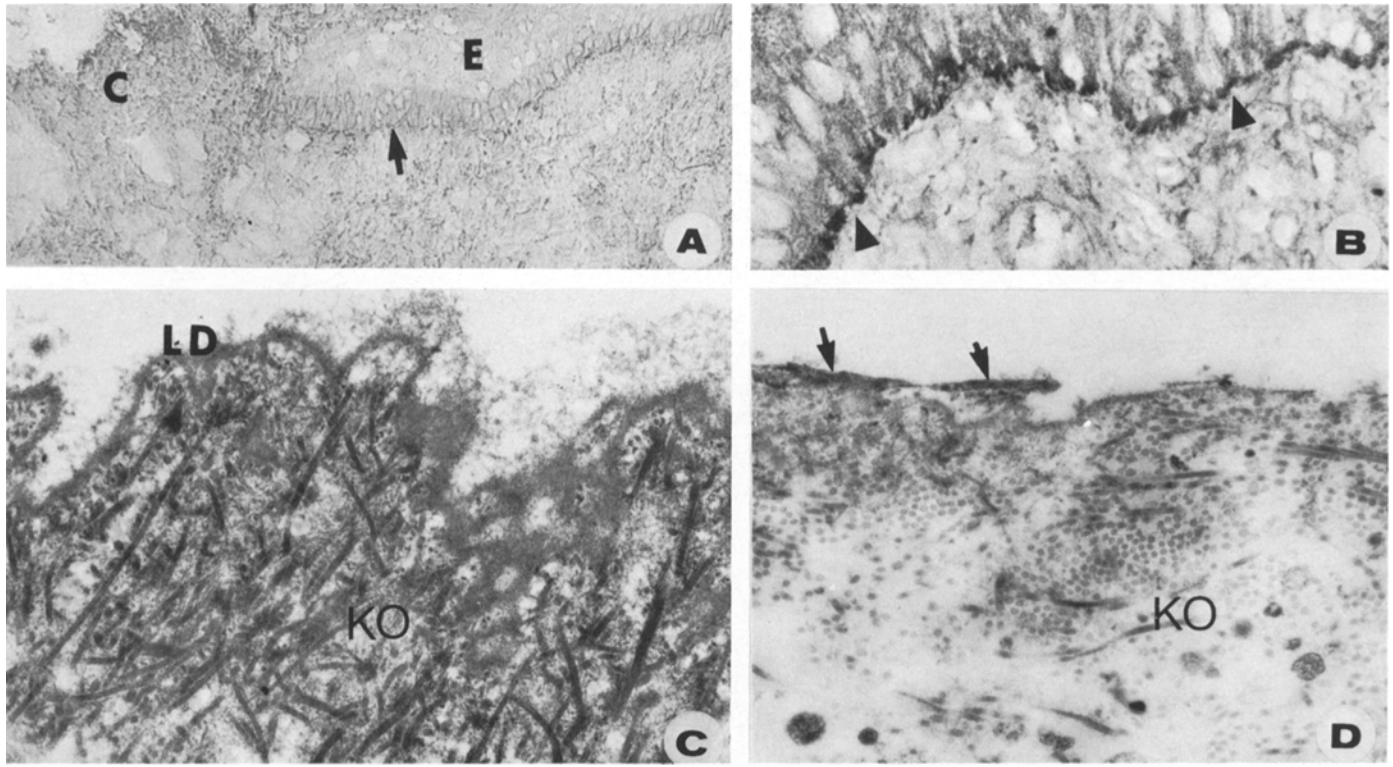

Fig. 3A–D. Demonstration of basal membrane zone by bullous pemphigoid autoantibodies and transmission electron microscopy. **A** Epidermal wound margin (*C*=centre of the wound). Presence of bullous pemphigoid antigenic sites beneath the leading epidermal tongue (arrow; *E*=epidermis). Indirect immunoperoxidase method. ×63. **B** Higher magnification of labelled basal membrane zone. Indirect immunoperoxidase method. ×500. **C** Ultrastructural view of the basal membrane zone in the centre of the wound. Lamina densa (*LD*) is present (*KO*= collagen). ×19,000 **D** Focal disruptions of lamina densa (arrows) in the centre of the wound. ×12,000

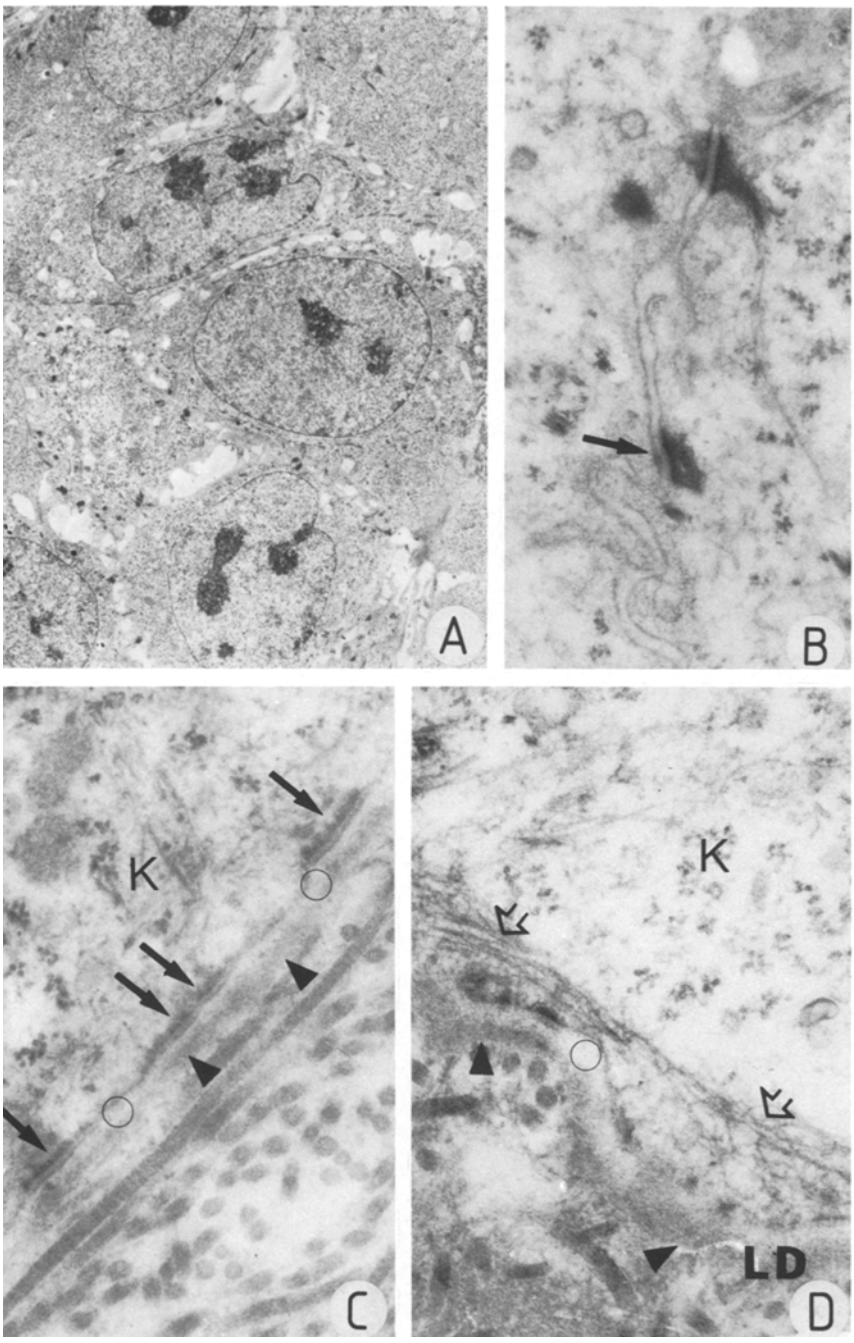

Fig. 4A–D. Ultrastructural view of the regenerating epidermis distal to the wound hiatus. **A** Aspect of rounded keratinocytes poor in tonofibrils. ×3,000. **B** Maturing asymmetrical desmosomes (arrow) with inserting tonofilaments. ×32,000. **C** Maturing hemidesmosomes (arrows) appearing as intracytoplasmic focal condensations (*K*=keratinocyte; open circles=lamina lucida; arrowheads=lamina densa). ×36,000. **D** Parallel orientation of filaments (open arrows) at the inner leaflet of the plasma membrane of keratinocytes (*K*). Collagen bundles are in close contact with the lamina densa (LD and arrowheads; open circle indicates lamina lucida). ×32,000

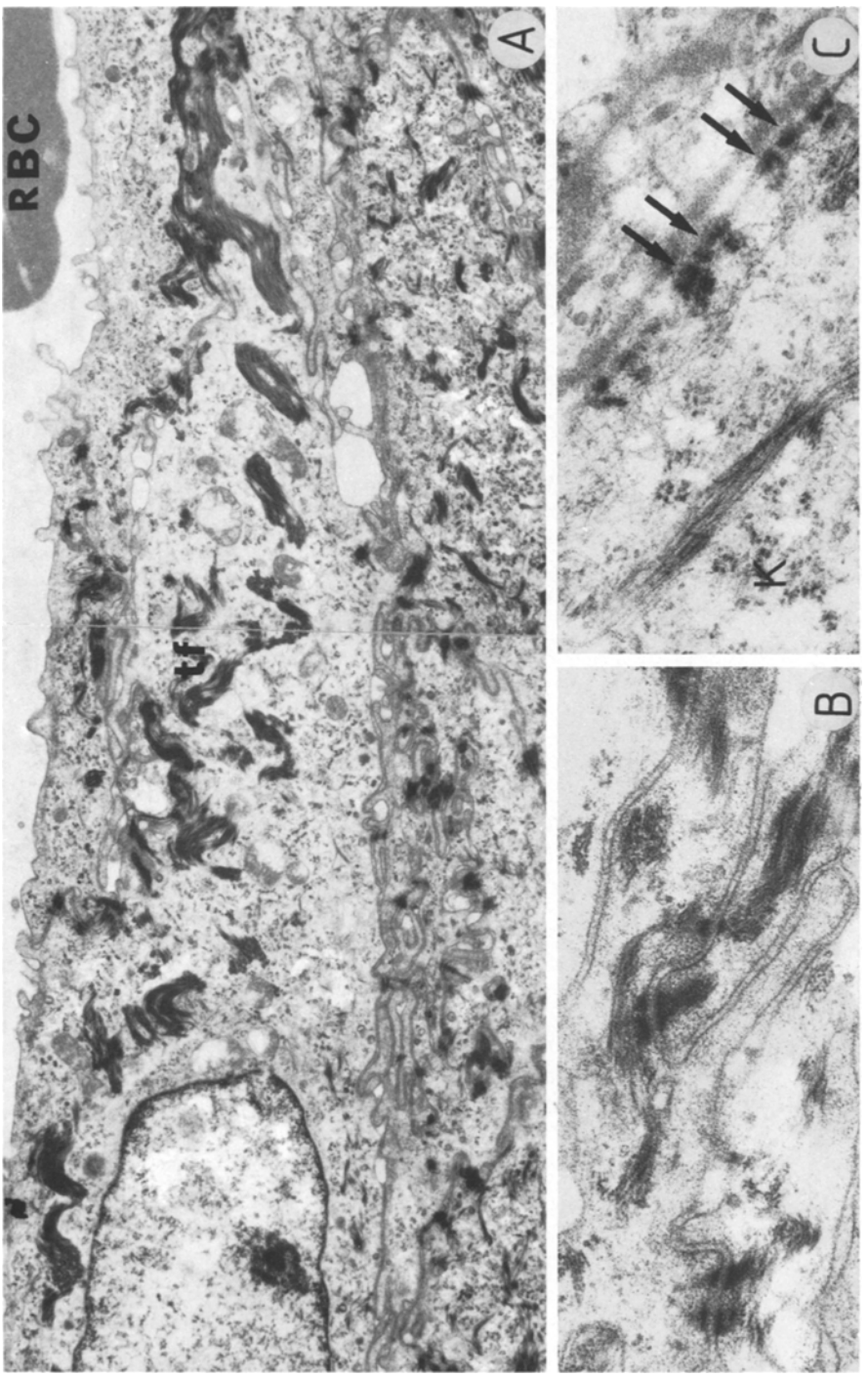

Fig. 5A–C Ultrastructural view of most advanced migrating keratinocytes. **A** Flat keratinocytes with dense bundles of tonofibrils at the cell poles (*RBC*=red blood cell). ×10,000. **B** Interdigitations of the cell membrane and maturing desmosomes with only a few number of inserting tonofilaments. ×42,000. **C** Maturing hemidesmosomes (arrows) beneath the leading keratinocytes (*K*) of the regenerating epidermis. ×51,000

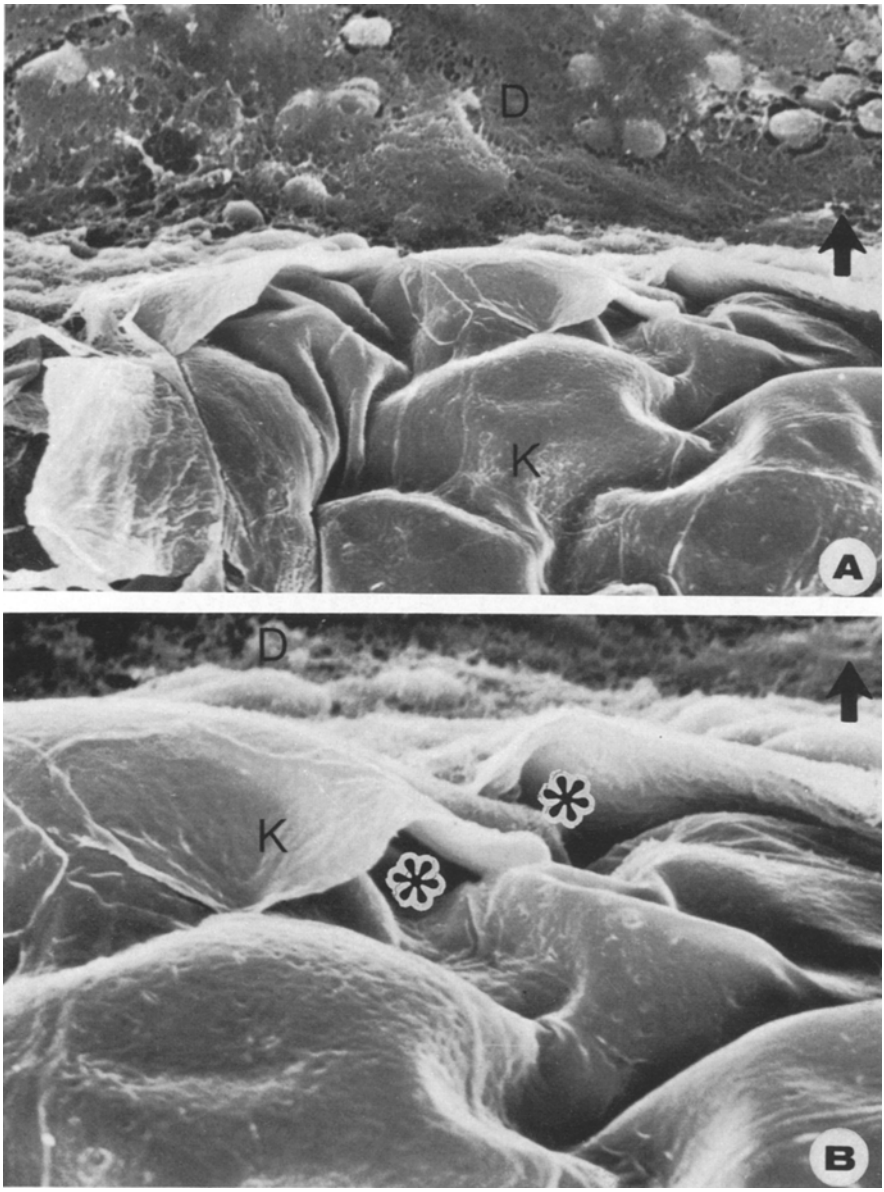

Fig. 6A, B. Scanning electron microscopy of the tip of the regenerating epidermis. **A** Flat keratinocytes (*K*) migrating from the epidermal margin. Most part of the dermis (*D*) of the wound is covered by the basal lamina. Arrow indicates the direction of keratinocyte migration. × 30,000. **B** High power view of the same area. The surface of the keratinocytes (*K*) is smooth. Focal widening of the intercellular spaces (asterisks) (*D* dermis; arrow shows direction of migration). × 50,000

Table 1. Histochemical, immunohistological and ultrastructural observations at the different wound areas

	Epidermal margin	Centre of the wound
Keratin (67K)		
suprabasal	++	+
basal	−	(single cells)
Actin		
suprabasal	++	+
basal	+	(single cells)
Pemphigus antigen	+	−
Bullous pemphigoid antigen	+	−
Lamina densa	+	+
PAS-reaction	+	+

Discussion

Epidermal repair has frequently been studied after superficial wounding in which, however, the papillary dermis is altered (Ordman and Gillman 1966; Croft and Tarin 1970). In those experiments, epidermal migration is known to be inhibited by the destruction of dermal structures, which finally leads to delayed wound healing (review: Pollack 1979).

For this reason, a model was used which allowed the evaluation of epidermal wound healing after purely epidermal wounding (Krawczyk 1971; Pang et al. 1978; Ortonne 1980). The detachment of the epidermis was obtained by suction according to a procedure first described by Kiistala and Mustakallio (1967).

Keratinocyte migration takes place from the intact epidermis of the wound edge and from the remaining adnexal structures within the dermis of the wounded area (Winter 1972; Pang et al. 1978). For this study, the abdominal skin, poor in adnexal organs was chosen, because our particular interest was analysis of keratinocyte migration from the margins of the wound, with special reference to the filament systems involved.

It is now the belief of most investigators that keratin filaments are a fundamental characteristic of epithelium-derived cells (Sun and Green 1978; Franke et al. 1979; Viac et al. 1980). Extracting keratins directly from sequential oblique sections of normal epidermis, Fuchs and Green (1980) showed that cells of the inner layers of the epidermis contain only small keratins (46,000 to 58,000 daltons), whereas the cells of the outer layers contain large keratins (63,000 to 67,000 daltons) in addition to small ones. In accordance to these biochemical findings, previous studies showed that specific antibodies against the keratin polypeptide subunit of 67,000 daltons permits an immunological distinction between the basal and the suprabasal cell compartment in the epidermis (Viac et al. 1980; Viac et al. 1980; Löning et al. 1980).

Using 67K-antiserum, the migrating epidermal cells were found to be labelled. In contrast to the suprabasal cell compartment, the marginal basal cells were 67K-negative.

In various experiments, Winter (1972) showed that keratinocytes of the basal and of the prickle cell layer are capable of migration across the wound. From our studies, however, it must be concluded that basal cells do not migrate laterally, in contrast to suprabasal keratinocytes.

This concept corresponded well to the actin pattern described in the regenerating epidermis. At the wound margins, suprabasally located keratinocytes were seen to be strongly labelled by actin-antibodies. Strong immunoreactivity was also demonstrated within the most advanced epidermal cells at the tips of the regenerating epidermis. Gabbiani and Ryan (1974) postulated that during wound healing marginal keratinocytes develop a contractile microfilamentous apparatus. Whether the occurrence of strongly stained keratinocytes during epidermal repair is a consequence of an increase in actin-bearing microfilaments and/or only a redistribution of this filament system, remains unknown.

In addition to the origin of migrating epidermal cells, the mechanism of migration has been debated (Winter 1972). Epidermal migration may occur either as a coherent intact sheet (Weiss 1961) or as a movement of single cells, i.e. the slide of marginal cells over each other (Krawczyk 1971). This latter hypothesis seems the most likely in the context of our immunohistochemical findings. 67K-positive cells appeared to roll over the basal cells thus moving out of the epidermis into the serous exudate on the skin surface. This assumption was also supported by our transmission and scanning electron microscopical studies revealing an ameboid motion of epithelial cells. The lack of Dopa-positive melanocytes in the newly formed epidermis during the first 72 h also represents a strong argument against the concept of epithelial migration as an intact sheet (Ortonne, unpublished observation).

Epidermal repair by cellular migration is accompanied by the formation of desmosomes, hemidesmosomes and a basal membrane zone (Colson et al. 1971; Abell et al. 1973; Krawczyk and Wilgram 1973; Beerens et al. 1975; Brickman et al. 1977). The morphological sequence of the development of desmosomes and hemidesmosomes in our model was very similar to that previously presented by Krawczyk and Wilgram (1973). Using bullous pemphigoid autoantibodies, the presence of antigenic components of the lamina lucida at the wound margins was demonstrated (Harrist and Mihm 1979). Interestingly, no immunoreactivity was found in the centre of the wound lacking keratinocytes and the lamina densa alone was demonstrated by electron microscopy. Our observations support the hypothesis that the antigenic components of the junctional zone are in part of epithelial origin (Abell et al. 1973; Hintner et al. 1979; Woodley et al. 1979). PAS-positive substances were present in the centre of the wound. The PAS-positive basal membrane is supposed to derive from the dermis (Abell et al. 1973).

Possible antigenic changes in the intercellular spaces of the regenerating epidermis were also studied using pemphigus-autoantibodies. Expression of pemphigus antigen was not modified in the newly formed epidermis. The conclusion must be drawn that epidermal wound healing is not accompanied by identical antigenic changes as described in pathological skin conditions (Muller and Sutherland 1971; Muller et al. 1973).

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