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Ultrastructural studies of wound healing in mouse skin

II. Dermo-epidermal Interrelationships

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INTRODUCTION

We have described in a previous paper (Croft & Tarin, 1969) how, in wound healing, the epithelium cuts through the underlying connective tissue and separates off an area which forms the scab.

This paper is concerned with the events in the connective tissue in relation to this invasion and the morphological constitution of the scab.

MATERIALS AND METHODS

This work was conducted on the same specimens as those used for the previous paper (Croft & Tarin, 1969) and the time intervals for specimen selection were therefore identical. For details of histological and electron microscopical preparation the reader is referred to the previous text. Mallory's phosphotungstic acid haematoxylin stain (PTAH) and the periodic acid-Schiff reaction (PAS) were employed for the identification of fibrin (Pearse, 1961).

OBSERVATIONS

(*a*) *Light microscopy*

As with the study of epithelial behaviour, the light microscopic observations on connective tissue changes following skin wounding in the mouse were similar to those recorded by Ordmann & Gillman (1966) for wound healing in pig skin. The following account is therefore only a brief outline of the process.

Eight hours after wounding an area of intense eosinophilia developed in the superficial dermis 100–200 μm on either side of the incision. Surrounding this area, dermal cellularity was slightly increased. By 16 h after wounding, the eosiniphilic region was clearly necrotic and the cellularity of the surrounding dermis was further increased. The predominant cell types were polymorphonuclear leucocytes and mononuclear cells, the former being present in greater number. The polymorphonuclear leucocytes formed a band of cells which demarcated the living dermis from the area of necrosis. At this time epithelial invasion had begun and the cells passed between the band of leucocytes and the living connective tissue.

At 24 h and 32 h, dermal cellularity had increased progressively but the relative proportions of different cell types remained constant. Thus, by the time the invading epithelial spurs met (40 h) and severed the necrotic tissue from living dermis, there

was a closely packed mass of cells in the connective tissue immediately subjacent to the epithelium.

Between this time and 64 h, the predominant cell types in the dermal cell population changed so that there were now fewer polymorphonuclear leucocytes and more mononuclear cells. The superficial dermis at this time contained many dilated capillaries. By 6 d, the leucocytes disappeared completely and fibroblasts, monocytes, lymphocytes and macrophages could be recognised amongst the mononuclear cells

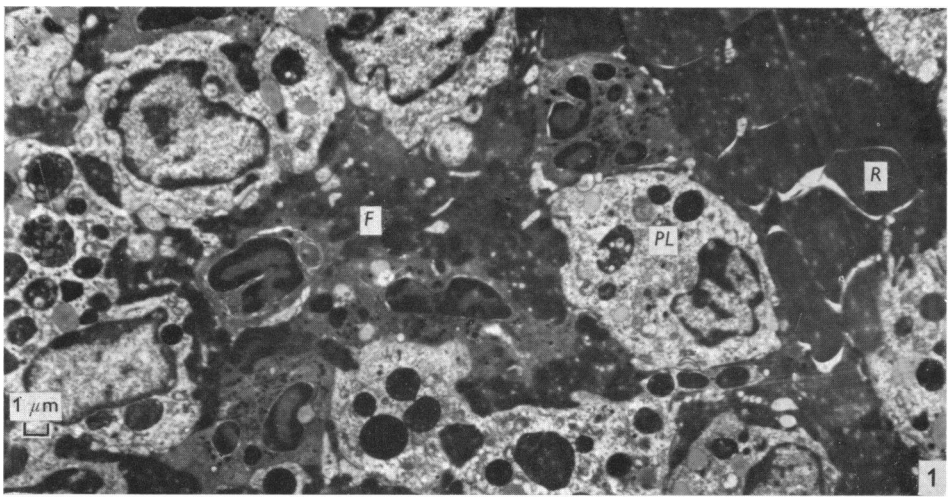
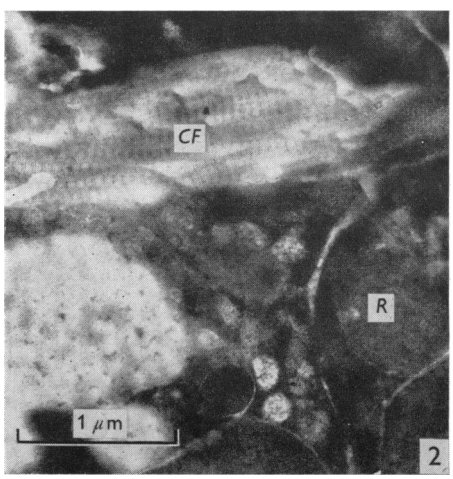


Fig. 1. 32 h wound. Showing ultrastructural features of the scab. Polymorphonuclear leucocytes (*PL*) can be observed in a meshwork of fibrin (*F*) containing red blood cells (*R*). ×3300.



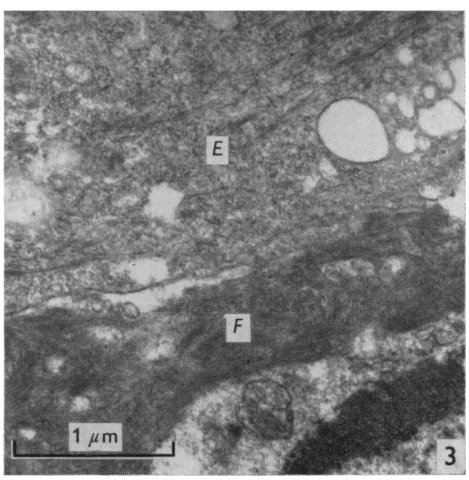


Fig. 2. 24 h wound. Higher power view of an area of the scab to illustrate the presence of collagen (*CF*) and red blood cells (*R*) ×21 000.

Fig. 3. 24 h wound. A portion of an epithelial cell (*E*) resting adjacent to dark fibrillar material (*F*). Ultrastructurally and histochemically this material has the characteristics of fibrin. ×21 000.

that remained. The dermal cells remained tightly packed and in close relationship to the epithelium.

On day 7, small amounts of intercellular substances were present and many spindle-shaped fibroblasts were seen aligned parallel to the skin surface. A day later the superficial dermal cells were separated by more intercellular material and van Gieson-

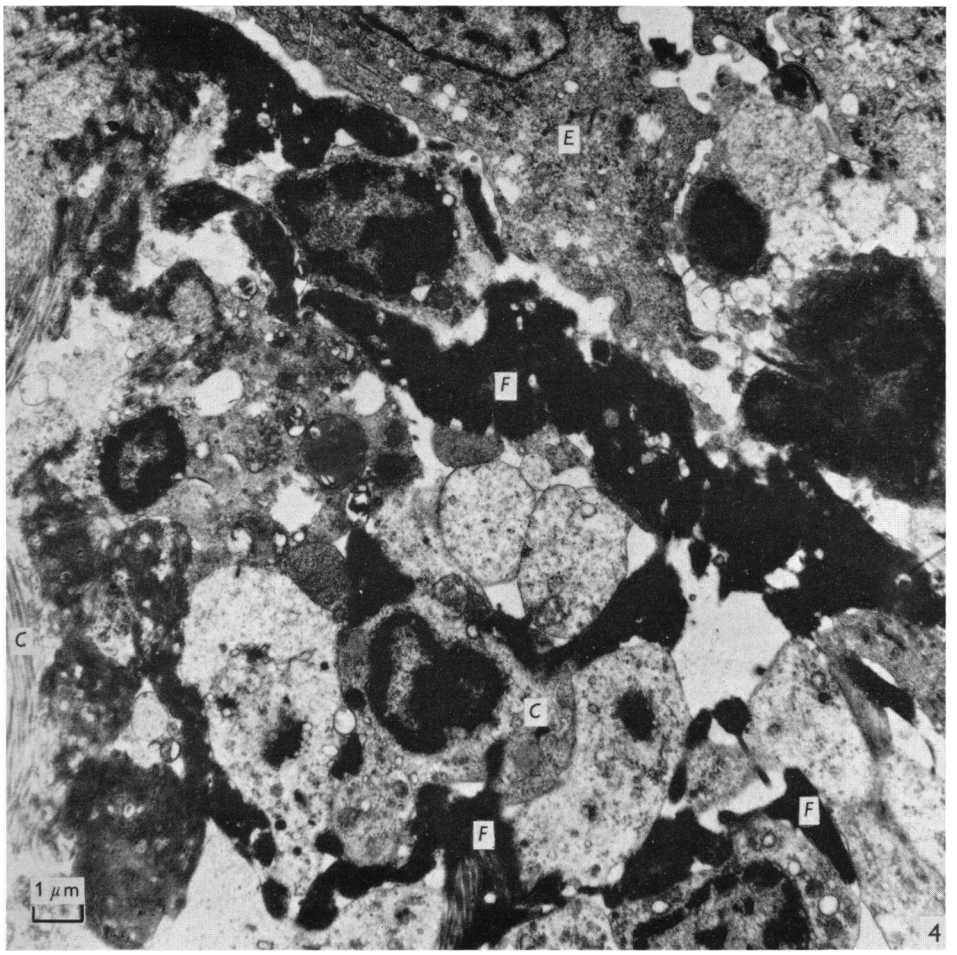


Fig. 4. 24 h wound. This illustrates the presence of a fibrin band (*F*) between epithelium (*E*) and connective tissue (*C*). Fibrin (*F*) can also be seen in the interstices between dermal cells. × 6600.

stained preparations showed the presence of fine collagen fibres presumed to be recently formed.

Days 9 and 10 showed diminishing cellularity associated with increase in the size and number of collagen fibres lying between the cells in the subepidermal region.

Electron microscopy

The epithelial invasion, following skin incision, undercut an area of necrotic tissue on either side of the incision (Croft & Tarin, 1969). This formed the scab and consisted of connective tissue overlain by a dead epithelium of normal thickness. Within the necrotic connective tissue of this region lay components identifiable as polymorphonuclear leucocytes, red blood cells, platelets, fibrin (Fig. 1) and collagen (Fig. 2).

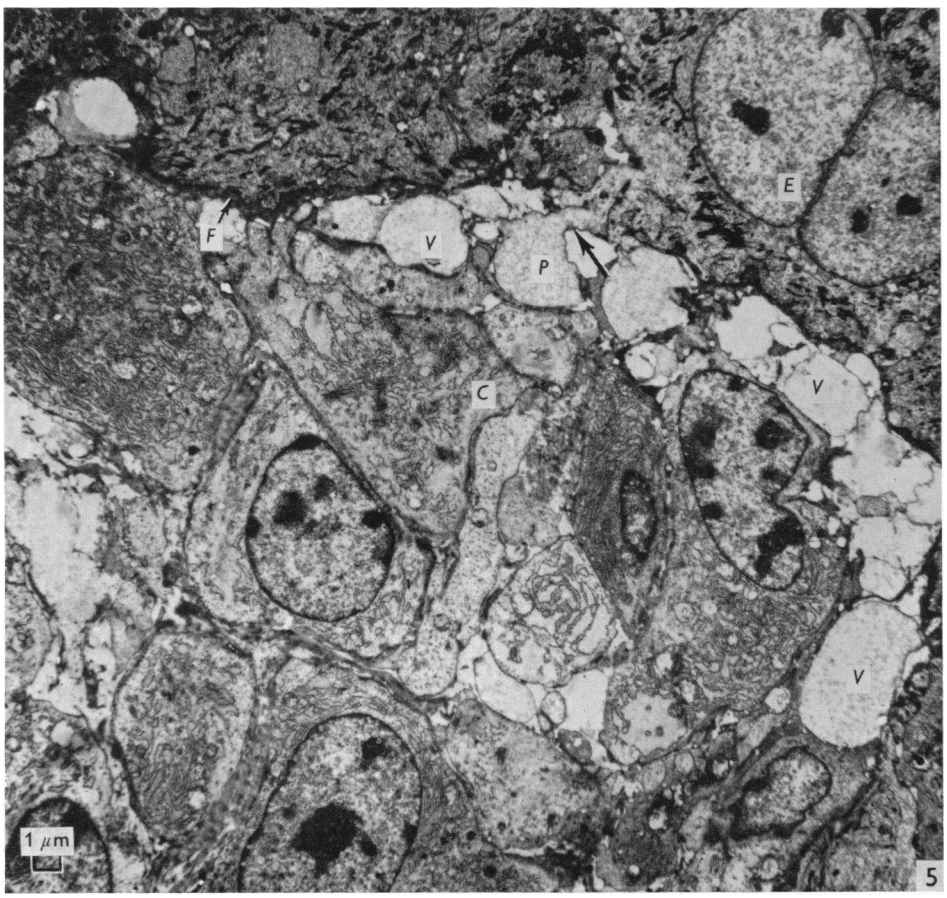


Fig. 5. 4 d wound. At the junction between connective tissue (*C*) and epithelium (*E*) pseudopodia (*P*) are observed. These contain granular material, are in continuity (arrow) with overlying epithelium, and are surrounded by membrane-bound vesicles (*V*). A small amount of fibrin (*F*) is still present. Note the number of closely packed dermal cells. × 3300.

On the basal aspect of the invading epithelial cells lay a mass of dark material (Fig. 3) across which the cells moved in their penetration into deeper tissues (Fig. 4). Electron microscopic examination of this substance showed it to be fibrillary in texture (Fig. 3) and the fine strands were tightly packed together to form a coagulum.

Optical microscopy showed that it was eosinophilic, stained dark blue-black with PTAH and gave a strong positive PAS reaction. Digestion with amylase did not alter the intensity of staining with PAS. On the basis of these staining properties and of its electron microscopic appearances this fibrillary material is presumed to be fibrin.

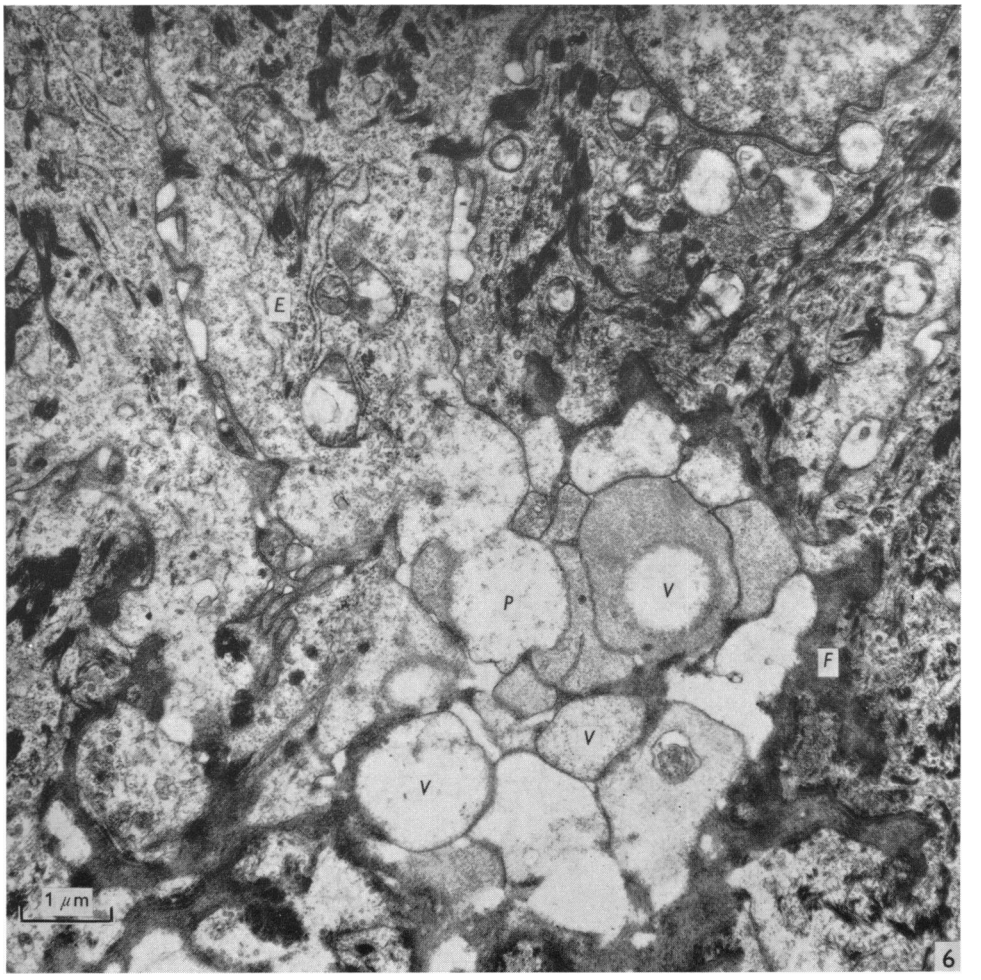


Fig. 6. 4 d wound. More detailed view of epithelial pseudopodia (*P*) conclusively establishing their connexion with the epithelial cells (*E*). The terminal portion of the pseudopod is bulbous and the proximal region has one or more constrictions. Membrane-bound circular profiles (*V*) are observed in the vicinity. These lack obvious continuity with overlying epithelium and contain varying amounts of course granular material. A small amount of fibrin is still present in relation to the vesicles. × 12 500.

In the first 24 h of wound healing the deposition of fibrin, following extravasation of blood from ruptured vessels, reached its peak and it was seen not only at the dermo-epidermal junction but also in the interstices between the superficial dermal

cells (Fig. 4). Subsequently the amount of this material decreased in quantity and its disappearance coincided with changes in the configuration of the dermal surface of the basal epithelial cells. Cytoplasmic projections extending from the epithelial cells into the adjacent fibrinous material were observed (Figs. 5, 6). These projections, longer than the pseudopods extended by actively invading epithelium (Croft & Tarin, 1969), each terminated in a bulbous portion and often showed a proximal constriction (Fig. 5). They were usually filled with amorphous granular material, but cytoplasmic organelles were never seen within them.

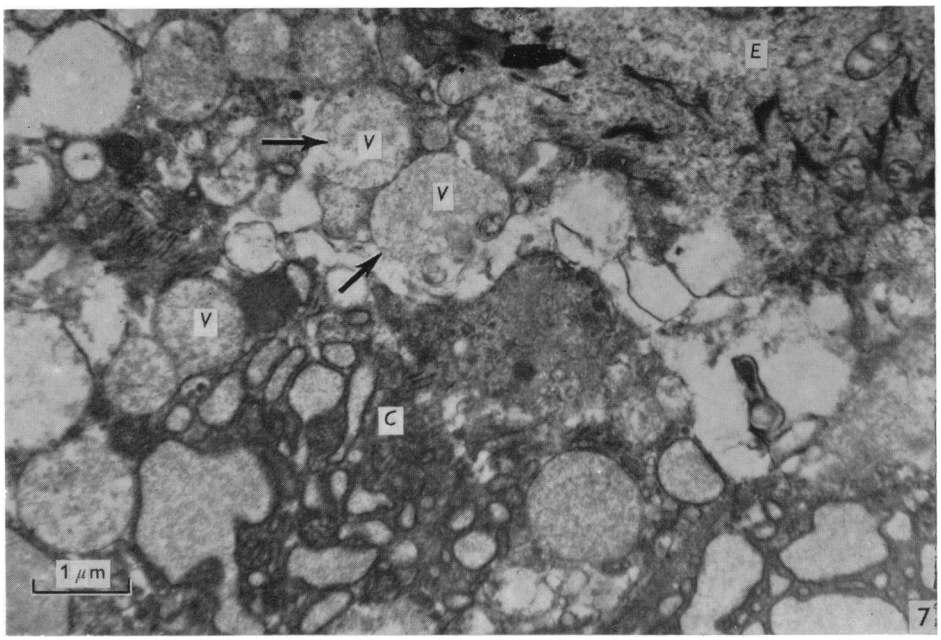


Fig. 7. 3 d wound. Further view of vesicles (*V*) and related epithelium (*E*) showing discontinuity or breakdown of vesicular membrane (see arrows). This may represent a mechanism for discharge of contents. × 12 500.

Immediately adjacent to these structures lay a number of circular profiles (designated 'vesicles'), some of which contained granular material while others were clear (Fig. 6). The membranes of many of these 'vesicles' were incomplete (Fig. 7), which indicated that they were possibly rupturing and releasing their contents.

The number of epithelial pseudopods and 'vesicles' seemed to be related to the amount of sub-epithelial fibrin in the vicinity. Where this was scanty more of these structures were seen. Also, as the amount of fibrin decreased in successively older wounds, the projections and vesicles were seen in more locations along the epitheliomesenchymal boundary. These changes were present in this region until all fibrinous material had disappeared and this process was usually completed by 4 d in small wounds with accurately apposed edges. After this time epithelial projections and 'vesicles' were rarely seen.

There followed a period during which dermal cells commonly came into contact with the basal surface of the epithelium (Fig. 8). In such cases the plasma membrane

of the dermal cell touched that of the epidermal cell at points along the line of apposition (Fig. 9). At these points the membranes of the adjacent cells were sometimes in direct contact without an intervening space, and at others were separated

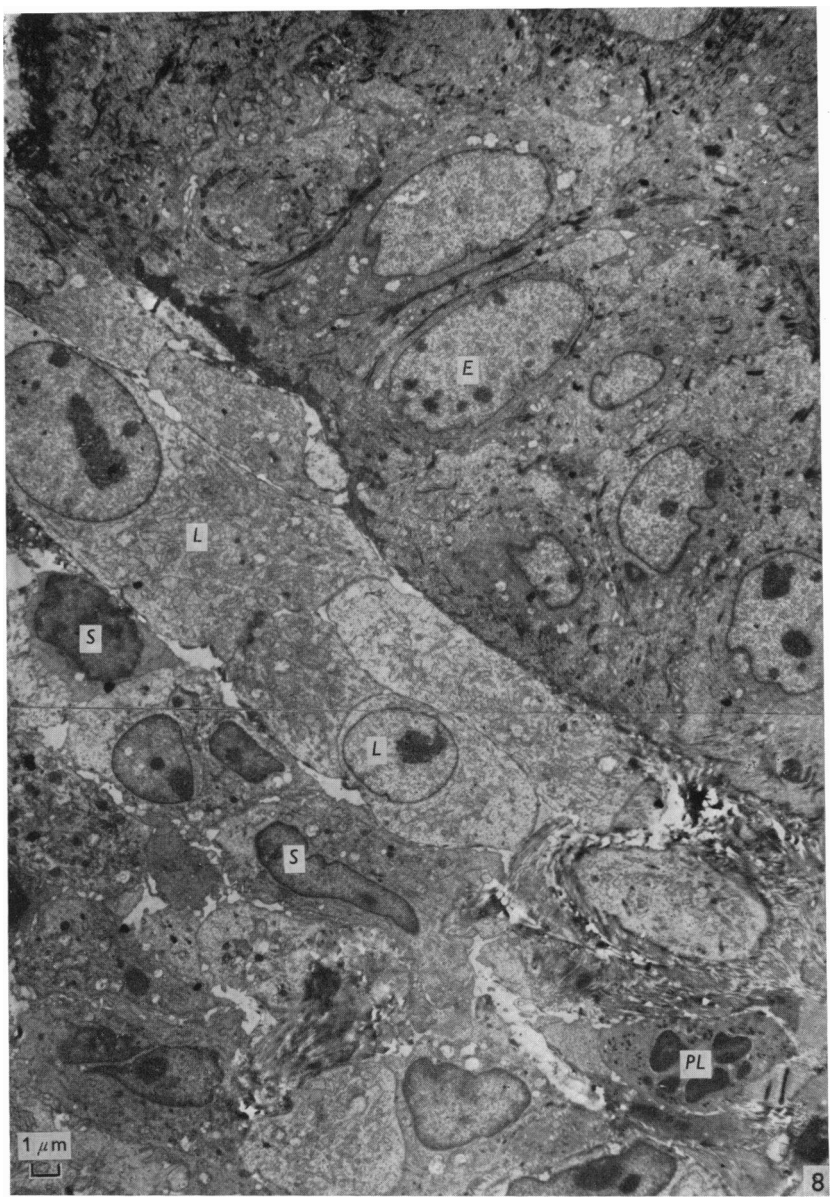


Fig. 8. 5 d wound. Dermal cells are in close contact with epithelium (*E*) undergong re-orientation. The dermal cells are irregularly arranged and closely packed. They can be grouped into three main cell types: polymorphonuclear leucocytes (*PL*), small dark cells (*S*) and large pale cells (*L*). The latter variety has prominent granular endoplasmic reticulum and nucleolus. It is the one consistantly seen in contact with epithelium at this stage. × 3300.

by a gap no larger than 50 μm. This close relationship between different cell types was usually seen between 3 and 7 d and was associated with the cessation of pseudopod projection by the epithelium. Thus the junction between epithelium and connective tissue became more regular and clearly defined.

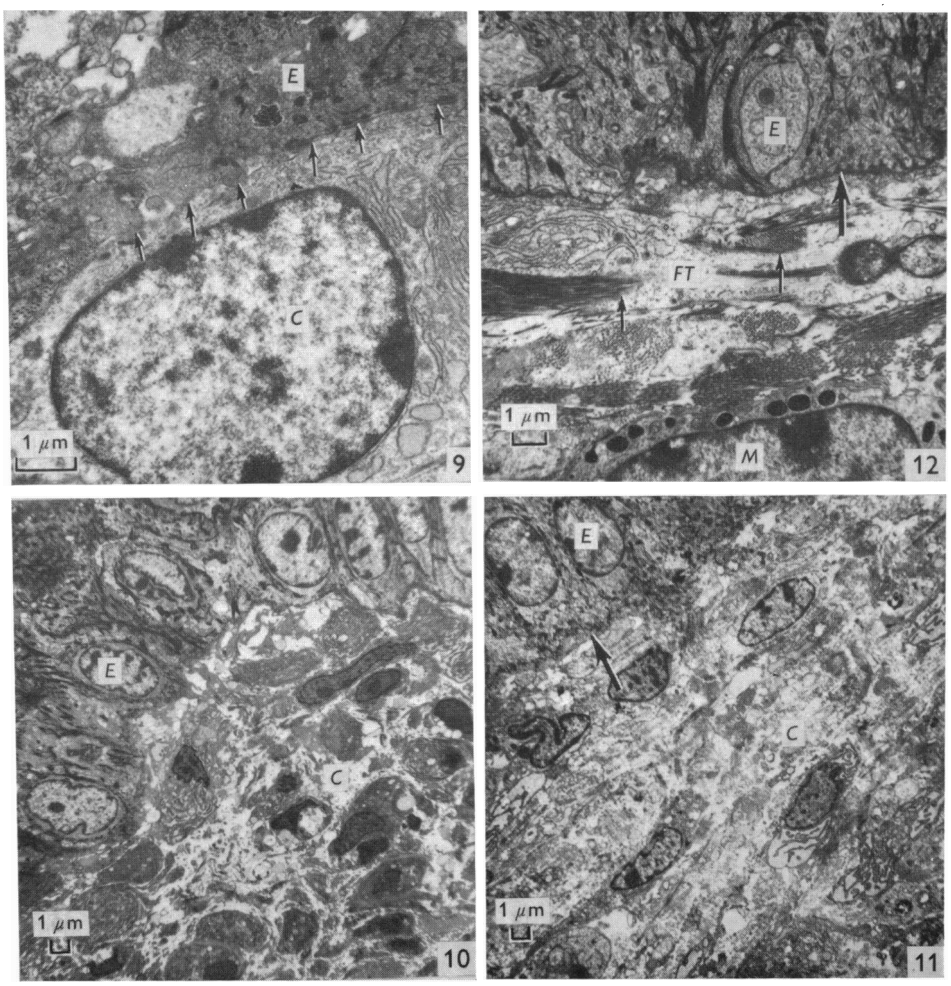


Fig. 9. 5 d wound. Closer view of intimate apposition (arrows) between an epidermal cell (*E*). and a connective tissue cell (*C*). Once again the dermal cell is pale and has prominent endoplasmic reticulum. The epidermal cell has reformed some hemidesmosomes but there is no basement membrane intervening between the two cells as yet. × 8300.

Fig. 10. 6 d wound. Dermal cells are separated by small amounts of intercellular substances. Their orientation is irregular. × 1500.

Fig. 11. 8 d wound. Dermal cells are now regularly arranged with fibroblasts orientated parallel to the dermo-epidermal junction (arrow). Intercellular materials have increased and there are more fibres relative to ground substance. × 1500.

Fig. 12. 9 d wound. This illustrates a reconstituted dermo-epidermal junction (arrow) and a mast cell (*M*) in the close vicinity. A fibroblast (*FT*) is observed orientated parallel to the basement membrane and collagen fibrils (arrows) are closely related to its membrane. × 4000.

From 3 to 5 d, the cells in the superficial dermis were closely packed (Figs. 5, 8) and consisted predominantly of immature forms which could be grouped into three main cell types (Fig. 8), as follows:

(*a*) Polymorphonuclear leucocytes.

(*b*) Cells with condensed nuclear chromatin and small amounts of dark cytoplasm containing scanty endoplasmic reticulum.

(*c*) Larger cells with pale nuclei, prominent nucleoli and abundant pale cytoplasm containing well developed endoplasmic reticulum. This third category of cells was invariably the one observed in close contact with the epithelium.

In specimens taken on 4 and 5 d polymorphonuclear leucocytes were less commonly observed. The other types of cell remained tightly packed together with the pale cells lying immediately adjacent to the epithelium. Because successive specimens were taken from different animals it was not possible to determine accurately which of the connective tissue cell types present in the later stages of wound healing arose from the groups of cells mentioned above.

Reconstitution of basement membrane began in limited areas along the dermo-epidermal junction from about 72 h onwards. In some places this process began at sites where local fibrin was still present. Here the basement membrane was formed superficial to the fibrin, close to the epithelium. Elsewhere the basement membrane was absent, permitting direct contact to occur between dermal and epidermal cells. It was presumed that in such areas basement membrane formation would occur later in healing, after contact had ceased, since by 7 d this structure was completely reconstituted. Further details of events in basement membrane formation are presented elsewhere (Croft & Tarin, 1969).

From 5 d onwards the dermal structure changed in that the cells became recognisable connective tissue cell types. This was associated with deposition of intercellular material, consisting of large amounts of amorphous ground substances in which lay small collagen fibrils (Fig. 10). The number of these fibrils increased between 6 and 10 d. Thus there was a progressive increase in the ratio of collagen fibrils to intervening ground substance and during this period collagen fibrils were frequently observed in close relationship to the cell surface of fibroblasts (Fig.12). This association has repeatedly been described by previous investigators (Fitton-Jackson, 1956; Ross & Benditt, 1961).

In the early stages of wound healing the closely packed dermal cells were irregularly arranged (Fig. 10). Later, as intercellular materials accumulated, the immature cells underwent differentiation to produce fibroblasts, monocytes and cells of the lymphoid series. The fibroblasts then became orientated with their long axes parallel to the skin surface (Fig. 11), and most of the collagen fibrils in the region of the healing wound were similarly aligned.

In the later stages of wound healing (9 and 10 d) a number of mast cells appeared in the superficial connective tissue close to the epithelium (Fig. 12). There was no evidence of discharge of granules from these cells and the reason for their presence in this region is unknown.

DISCUSSION

Ultrastructure of the scab

This ultrastructural investigation has confirmed the earlier claims of light microscopists (Ordmann & Gillman, 1966) that, in addition to coagulated blood, connective tissue components are present in the wound scab. These components consist not only of necrotic dermal cells but also of numerous collagen fibrils which still display the characteristic 64 μm periodicity.

The nature of the bond between the scab and the adjacent epithelium is one of simple apposition. The electron microscope demonstrates that there are no specific attachment sites between these structures. Subsequently, as previously described (Croft & Tarin, 1969), the scab is detached from the underlying tissues by the union of the invading epidermal spurs and their subsequent keratinisation.

It was also noted that there were large numbers of apparently viable polymorphonuclear leucocytes in the scab. The necrotic dermis which is to form the scab is not acellular as stated by Ordmann & Gillman (1966).

Removal of fibrin

Once the two invading epithelial columns have united the basal epidermal cells probably participate in the removal of the dense band of sub-epithelial fibrin. The following evidence supports this conclusion. First, large epithelial pseudopodial projections and associated 'vesicles' were only seen at the dermo-epidermal junction in specimens where the quantity of sub-epithelial fibrin was reduced. Secondly, following the complete removal of fibrin these structures were no longer observed. Thirdly, the relative paucity of dense fibrillary material in the immediate vicinity of the 'vesicles' and pseudopodia indicates that these structures were possibly involved in fibrinolysis. As they never contained cytoplasmic organelles, but only granular material, it seems unlikely that they were simple cellular protrusions.

The precise relationship of the 'vesicles' to the epithelial projections is at present unclear. It seems reasonable to presume that the 'vesicles' are of epithelial origin since, in the earlier stages of wound healing, they lay on the epithelial side of the dense band of fibrin. Later on, as fibrin decreased, they lay in the superficial connective tissue closely related to the dermo-epidermal junction. Whether these round bodies represented transversely sectioned pseudopodia, in continuity with epithelial cells outside the plane of section, or free-lying vesicles which had separated from the parent epithelium is, however, less certain. The second interpretation seems, to us, more attractive because longitudinal sections of the pseudopodial projections (Figs. 5, 6) showed them to have a bulbous end and constricted neck, indicating a possible site at which the connection might be severed. Further, in many of the 'vesicles' the bounding membrane was incomplete and some contained granular material while others were clear. It is therefore considered likely that these bodies rupture and release their contents into the surrounding tissues. Such an event is unlikely to be compatible with the vesicular structures maintaining connexion with the parent epithelial cells.

To conclude, on the basis of these observations it is tentatively proposed that the

epithelial cells participate in the removal of fibrin by pushing cytoplasmic projections into this substance. The projections probably constrict at their necks and separate from the epithelial cell to produce free-lying vesicles which rupture with lysis of adjacent fibrin. The removal of fibrin deeper in the connective tissue was accomplished by macrophages.

Remarkably similar appearances have been reported at the junction between epithelium and connective tissue during carcinogenesis (Tarin, 1967) and in certain established tumours (Ashworth, Sternbridge & Luibel, 1961; Hinglais-Guillaud, Moricard & Bernhard, 1961; Frei, 1962; Tarin, 1969). In carcinomas, however, tissue destruction involves collagen and other connective tissue components and occurs during active epithelial invasion. In wound healing, seemingly only fibrin is destroyed by this mechanism and this takes place after the two epithelial spurs have united. Furthermore, the invading epithelial column in wound healing does not produce vesicles in this manner and there is no extensive destruction of adjacent connective tissue (Croft & Tarin, 1969). This indicates that there may be fundamental differences in the mechanisms of epithelial invasion in wound healing and in carcinoma.

Alternatively, it could be argued that the connective tissue substratum in wounded skin differs markedly from that in neoplastic skin. In the former situation the epithelium follows the boundary between living and dead connective tissue whereas in the latter it passes through the midst of living, functioning dermis. The nature of the substratum may thus select the method used by the epithelium to pursue its course. Yet it remains surprising that the epithelium seems to be involved in the removal of fibrin in wound healing and that the method of accomplishing this seems to be morphologically similar to that observed in neoplastic epithelium invading connective tissue.

Contact between dermal and epidermal cells

The intimate contact we have observed between dermal and epidermal cells at some stages in the wound healing process is a marked and obvious feature occurring in many places along the dermo-epidermal junction. Ross & Odland (1968) reported somewhat similar findings, that epithelial pseudopodia came into contact with dermal cells, but did not indicate the extent of this contact along the dermo-epidermal junction. Nor did they note the association between epithelio-mesenchymal contact and the cessation of pseudopod production by the epithelium.

The significance of these observations is debatable. Clearly it is necessary to be cautious in making firm predictions about tissue behaviour purely on the basis of morphological observations. Nevertheless, we believe that the association of this contact with both the recent cessation of epithelial invasion, and the absence of any further pseudopod production by the basal epithelial cells, suggests that the dermis may be involved in controlling the process of epithelial penetration. In support of this proposition the following evidence is presented. First, in carcinomatous invasion there is no similar closely packed dermal cell reaction and the connective tissue cells present do not come into intimate contact with the neoplastic epithelium (Tarin, 1967). Although the number of cells in the connective tissue is increased, they mainly lie separate from one another and at some distance from the invading epithelial cells.

Secondly, connective tissue is known to exert some action in controlling epithelial invasion in other situations, such as implantation of the ovum (Kirby & Cowell, 1968). Here the limitation of epithelial penetration depends on the development of the decidual reaction in the maternal connective tissue. This reaction consists of the proliferation and enlargement of mesenchymal cells in the immediate vicinity of an ovum implanting in the uterine epithelium. The fully formed decidual mass consists of a tightly packed ball of cells which surrounds the implanting blastocyst. If the development of the decidual reaction is prevented or delayed, epithelial penetration continues into the uterine musculature and in some cases the wall of the uterus is virtually destroyed (Kirby & Cowell, 1968).

Further, more generally, connective tissue has been shown by several investigators to control the type of differentiation and rate of growth of its associated epithelium, both in embryos (Grobstein, 1953; Sengel, 1964; and see Grobstein, 1967, for review) and in adults (Billingham & Silvers, 1968). Close contact between the epithelial and connective tissue cells is essential for this control to be exerted.

Thus the intimate relationship between dermal and epidermal cells in wound healing is possibly an important factor in controlling the epithelial invasion and in re-establishing epithelial differentiation. In this connexion it is of interest that the dermal cells coming into contact with the epithelium possessed the characteristics known to be associated with active synthesis of proteins (viz: a prominent nucleolus and abundant rough surfaced endoplasmic reticulum).

Connective tissue replacement

In the later stages of wound healing the most significant changes were the deposition of amorphous ground substance between the dermal cells and the formation of collagen fibres. Our observations on these events were substantially in agreement with previous accounts of connective tissue formation (Ross & Benditt, 1961; Fitton-Jackson, 1956).

Of particular interest to those studying reconstruction of histological structure, however, is the regular orientation of dermal cells in the superficial dermis as collagen formation progressed. These cells became aligned with their long axes parallel to the skin surface. The factors responsible for this alignment could not be ascertained by our study but previous investigations (Weiss, 1939; Stearns, 1940*a b*) have suggested that tension forces in the tissue are at least partially responsible. It has also been claimed that the alignment of the cells controls the arrangement of collagen fibres they produce (Stearns, 1940*b*). Such an interpretation would be in agreement with our observations.

SUMMARY

In this paper the events at the dermo-epidermal junction and in the adjacent connective tissue during wound healing are presented.

The presence of dermal constituents, notably collagen, in the scab has been confirmed by electron microscopy.

Fibrin deposition at the dermo-epidermal junction and in the superficial dermis provided a scaffold for epithelial migration and its subsequent removal was probably effected in part by the activity of the basal epidermal cells. These cells produced

pseudopods, and possibly vesicles, which projected into the fibrin in areas where removal of this substance was believed to be in progress.

Close contact between dermal and epidermal cells was observed at the time when epithelial invasion ceased and it is suggested that this phenomenon is significant in controlling the extent of penetration. Subsequent events in connective tissue replacement consisted of increase in intercellular substances and alignment of fibroblasts parallel to the skin surface.

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