COMPARATIVE HISTOCHEMICAL OBSERVATIONS OF WOUND HEALING IN ADULT RATS AND CULTURED ADULT HUMAN EPITHELIUM

III. Alkaline and Acid Phosphatase*†

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Striking resemblances exist between the proliferating epidermis of wound healing *in vivo* and culture *in vitro*. These are of both a morphologic and biochemical nature. Previous papers (32, 33) have shown that glycogen, ribonucleic acid and thymonucleic acid metabolism may be included in the latter category. Alkaline phosphatase has repeatedly been demonstrated in areas where fibrous protein synthesis occurs, hair (21, 22) and collagen formation (10, 8). The relationship between this enzyme and *in vivo* wound healing has been the object of considerable study. This for the most part has centered about the problem of collagen formation. Normally mammalian epidermis and dermis contain only slight amounts of alkaline phosphatase located in the outer sheath of hair follicles, sebaceous glands and perivascular fibrocytes. However, during wound healing in rats Fell and Danielli (10) observed two peaks of alkaline phosphatase activity. The first associated with the formation of an eschar, was related to the concentration of polymorphonuclear leukocytes and probably liberated from them on disintegration. At this time the newly formed epithelium also showed activity in the nuclei. The second peak occurred at the time of collagen formation. They suggested "that the phosphatase connected directly or indirectly with the metabolic processes was more intimately concerned in the laying down of collagen". These observations were confirmed by Firket (11). Somewhat later Danielli and Fell (8) re-evaluated the problem in scorbutic guinea pigs where collagen formation is retarded. They observed a direct relationship between phosphatase activity and the degree of collagen formation. Bunting and White (5) however could find no such relationship in partially scorbutic guinea pigs. It has since been shown that freshly precipitated collagen fibers firmly bind alkaline phosphatase (15). In wounds however the local concentration of phosphatase is independent of the serum level (16).

No comments concerning the presence of the increased amounts of alkaline phosphatase in the skin during re-epithelialization could be found. This increase during wound healing could conceivably be a diffusion artefact from the overlying eschar or underlying dermis. The technic recently developed for successfully culturing human skin (24) offers a means of re-evaluating this problem. Epithelial explants to which a similar thickness of dermis is attached exhibit pure epithelial growth in culture. During the 9–10 days that the cells proliferate there is no growth of the fibrocytes in the underlying dermis. This permits evaluation of alkaline phosphatase activity in proliferating epithelium alone. The problem of separating diffusion effects from underlying growing fibroblasts is avoided.

The literature concerning acid phosphatase in skin is extremely sparse. Gomori (18) and Fisher and Glick (12) using the histochemical method of Gomori found none in human skin. Mescon (26) stated that a marked reaction occurs with the Gomori technic when unfixed frozen sections of human skin are used. No studies concerning its role in wound healing or cultured epithelium have been performed.

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MATERIALS AND METHODS

The technic of obtaining uniform burns in rats and the methods of culture used in human skin were described in detail in a previous paper (32).

Fixation: For alkaline phosphatase tissues were either fixed in 80% alcohol (24 hours) or prepared by freeze-drying. In the latter procedure, tissues were frozen at −80°C and maintained during lyophilizing at −35°C. For acid phosphatase, absolute acetone at 4° C for a period of 24 hours was employed in some cases and freeze-drying in the remainder.

Histochemical technic: Sites of alkaline phosphatase activity were detected according to the method independently devised by Gomori (17) and Takamatsu (31). Sections were incubated in a glycerophosphate substrate for periods of 6 and 24 hours without deparaffinization according to Goetsch *et al.* (13). Other sections were stained by the coupling azo dye technic of Seligman and Manheimer (30).

Acid phosphatase was stained according to Gomori's lead nitrate technic (18) with the modifications employed by Goetsch and Reynolds (14).

RESULTS

Rat burns

Fourteen rat burns were used to examine the sites of alkaline phosphatase activity during wound healing. In normal rat skin, activity is limited to a slight staining of the cells in the stratum granulosum, the dermal papillae and outer root sheath of the hair follicles. The dermis is negative except for the perivascular fibrocytes and sebaceous glands. The above areas are not sites of strong phosphatase activity as substrate incubation for 24 hours is required to elicit the reaction. During the first 2 days following burning there is a rapid increase in alkaline phosphatase at the wound site. This is limited to the disintegrating polymorphonuclear leukocytes that form the eschar and also infiltrate the dermis. Increased activity is also found in the epidermal cells that migrate over the dermis and under the eschar. The cellular location of this epithelial phosphatase varies with the method of fixation. Following the use of 80% alcohol the nuclei stain diffusely but after the freeze-dry method the activity is limited to the cytoplasm. During the 4th–10th days following burning there is a gradual accumulation of newly formed fibrocytes in the underlying dermis. The collagen fibers stain diffusely and with increasing intensity from the 6th to the 10th day. The overlying epithelium which now completely covers the wound shows no further increase in phosphatase activity. By the 12th day the dermal phosphatase activity has begun to regress and the epithelial layers are negative except for the stratum granulosum. These findings did not differ significantly with either the Gomori or Seligman technics for alkaline phosphatase determination. Following freeze-drying in 80% alcohol-fixed-material, epithelial staining was limited to the nucleus with the Gomori method and to the cytoplasm with the Seligman method.

Twelve rat burns were employed for studying the location of acid phosphatase

during wound healing. Tissues fixed in absolute acetone consistently failed to reveal any sites of acid phosphatase activity. Those wounds subjected to freeze-drying however were positive. In normal rat epithelium the keratinized layers and granules of the stratum granulosum are strongly stained following 6 hours of substrate incubation. After 24 hours, the remainder of the epithelial layers also stained diffusely, this is most evident in the basal layer. The dermis is entirely negative. During the first 4 days of wound healing the thickened epithelium at the edge of the burn contains slight amounts of acid phosphatase in the upper keratinizing layers. The epithelium that migrates over the dermal surface is always negative. This area of the wound remained phosphatase-free until epithelialization was complete and keratinization recommenced. At that time, the granules of the stratum granulosum and the keratin layers again revealed phosphatase activity. The polymorphonuclear leukocytes of the eschar and underlying dermis were rich sources of acid phosphatase; newly formed collagen however was entirely negative in contrast to the findings with alkaline phosphatase.

Cultured human skin

The alkaline phosphatase activity of 6 explants was examined following complete sectioning. At the time of culture, phosphatase activity was limited to a very slight staining of the stratum granulosum and outer root sheath of the hair follicles (Fig. 1). The dermis was entirely negative. Throughout the period of culture no increase in this slight activity was noted. The cells of the outwandering epithelium were entirely negative. The dermis also failed to reveal any

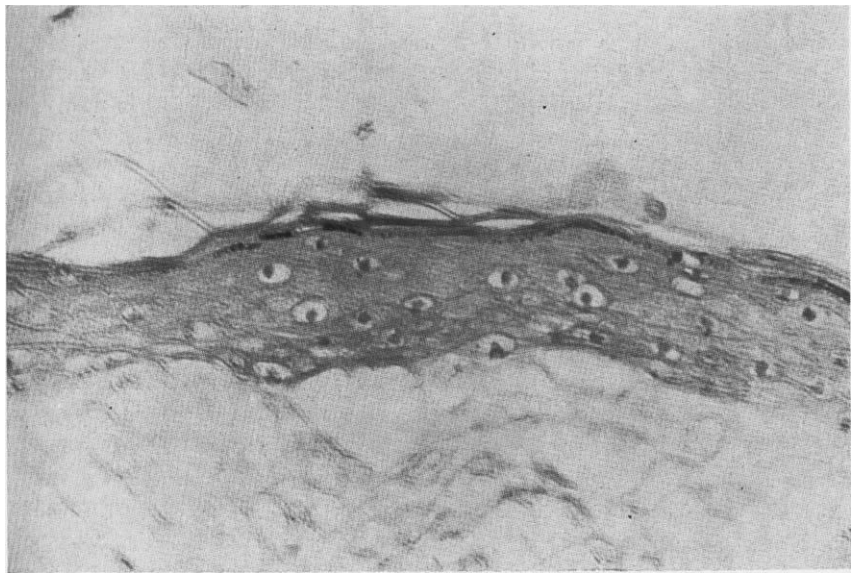

FIG. 1. Explant of human skin fixed on the fourth day following culture (80% alcohol) and stained by the Gomori method for alkaline phosphatase. The enzyme activity is restricted to the stratum granulosum.

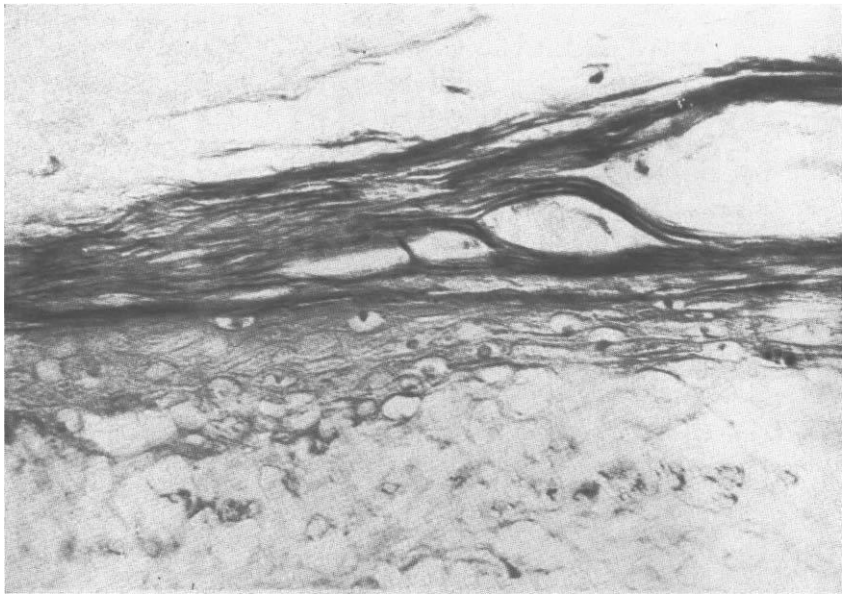

FIG. 2. Explant of human skin fixed on the fourth day following culture (acetone) and stained by the Gomori method for acid phosphatase. The enzyme activity is concentrated in the granules of the stratum granulosum and in the keratinized layers.

alkaline phosphatase during this period. Whole mount preparations were also negative either after freeze-drying or 80% alcohol fixation.

Eight explants were sectioned for determination of acid phosphatase. After 24 hours of incubation those which were fixed in absolute acetone remained negative. The explants subjected to freeze-drying however presented a distribution similar to that found in rat skin. The granules of the stratum granulosum were heavily stained as was the overlying keratin (Fig. 2). The remainder of the epithelium and the dermis was negative. During culture the outwandering cells were also negative. The acid phosphatase distribution was entirely restricted to those areas where keratinization occurred. Isolated epithelial cells of the outwandering layers when fixed and stained as whole mounts were also negative.

DISCUSSION

Alkaline phosphatase exists in normal epidermis in small quantities. The outer root sheath of hair follicles and sebaceous glands within the skin however are rich sources. The only cellular layer where it is consistently found is the stratum granulosum. At this site it appears to be associated with the keratohyalin granules. This observation has been confirmed in human skin (12, 29), guinea pig skin (4) and mouse skin (20). Thus the relationship between fibrous protein synthesis and alkaline phosphatase activity which exists in developing teeth (1), hair, bone and collagen also seems to be present in skin.

The phosphatase content of skin can be varied under experimental conditions. Injection of estrogens will increase the activity in the stratum granulosum of

guinea pigs (23). Mouse skin when painted with methylcholanthrene presented activity in the nuclei of the basal cells (2). During wound healing the epidermis reveals a pattern of phosphatase distribution similar to that seen in methylcholanthrene-treated animals. Commencing with cellular migration, alkaline phosphatase is observed in the lower layers of the epidermis. Its presence at this site persists throughout the period of collagen formation in the underlying wounded dermis. When the latter is completed and alkaline phosphatase disappears from the dermis a similar disappearance occurs in the lower layers of the newly formed epithelium. This relationship between phosphatase content of the dermis and the adjacent cellular layers of skin suggests the possibility of a diffusion artefact. Considerable evidence concerning phosphatase diffusion exists. When tissue sections with high alkaline phosphatase activity are super-imposed on already mounted sections of tissues with no activity and incubated, phosphatase is histochemically demonstrable in the latter (25). It was noted in this study that sections stained with the Gomori technic revealed the phosphatase activity chiefly in the nucleus regardless of the method of fixation. In contrast to this, localization by the azo-coupling reaction revealed the distribution to be limited to the cytoplasm of those cells in the lower layers. The subject of nuclear staining with the Gomori technic has been discussed by Goetsch *et al.* (13) and reviewed by Pearse (28). It has been concluded that nuclear staining is due entirely to diffusion. This is indirectly supported by biochemical analysis of isolated liver cell nuclei where little or no phosphatase activity was found (25, 27). Unfortunately investigations upon wound healing in scorbutic animals where the alkaline phosphatase content of the dermis is negligible make no mention of the epithelium.

The results obtained with cultured epithelium aid in determining the source of epithelial alkaline phosphatase during migration and proliferation. The underlying dermis *in vitro* unlike that in wound healing or in skin treated with methylcholanthrene exhibits no growth and hence has a negligible phosphatase content. Under these conditions it was found that no phosphatase was present in the out-wandering cells. This is strong evidence that the findings *in vivo* were due to diffusion either of the enzyme or its products of reaction during staining, from the underlying dermis. Evidence supports the view that diffusion occurs after cell death (9). If diffusion occurred prior to cell death then cultured explants would contain phosphatase since it is present in the media.

The observations of Biesele et al. (3) are different from the above. They noted that mouse embryo skin during culture does contain alkaline phosphatase in the nucleus. This did not vary in concentration during mitosis as measured with the microphotometric apparatus. The application of histochemical technics to cultured cells poses problems somewhat different from those encountered in tissue sections. Since the thinness of the cultured cells could constitute the limiting factor in whether a certain substance could be detected by a particular method.

The absence of alkaline phosphatase in the dermis is further proof against collagen formation in these cultures. It has already been shown that cultured

fibroblasts exhibit a strong alkaline phosphatase reaction, the intensity varying directly with the vigor of growth and mitotic index (34, 6, 7).

Acid phosphatase in both rat wound and cultured human epithelium was found to be entirely dependent on the method of fixation. Positive results were only obtained following freeze-drying. In these cases the activity bore a relationship to keratin formation being found in the keratohyalin granules of the stratum granulosum and the keratinized stratum corneum. No increase in activity was observed during epithelial proliferation *in vivo* or *in vitro*. In both cases the activity was suppressed during those periods when keratinization failed to occur.

SUMMARY AND CONCLUSIONS

Histochemically detectable alkaline phosphatase during wound healing in rats was elevated at the time of eschar formation and later coincident with the laying down of collagen. Throughout this period, the epithelium which normally contains alkaline phosphatase in the granules of the stratum granulosum also revealed activity in the lower layers. This was distributed in the cytoplasm when stained by the Gomori or Seligman technic following freeze-drying. After fixation in 80% alcohol the distribution was nuclear with the Gomori technic.

Human epithelium contained no alkaline phosphatase in the outwandering cells during the period of culture. This was verified on both sectioned explants and whole mounts. The dermis also remained phosphatase free in culture.

Acid phosphatase was only detected histochemically following freeze-drying. In normal skin it was limited to the granules of the stratum granulosum and the keratinized stratum corneum, none was found in the dermis. The migrating epithelium and newly formed dermis during wound healing was phosphatase free.

In culture human skin explants contained acid phosphatase in the stratum granulosum and stratum corneum. The outwandering cells and dermis when examined in whole mounts or sections were phosphatase free.

Alkaline and acid phosphatase content of epidermis are related to keratinization. During wound healing or culture the keratinization process of the migrating epithelium is suppressed and these enzymes are absent.

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