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Regeneration of Organized Epithelial Structure

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The role of connective tissue in facilitating and directing the growth of epithelia during adult life is uncertain. The basic processes associated with maintenance of epithelial structure and previous work concerning the role of mesenchyme in this process in the embryo and adult are reviewed. A series of experiments examining the role of connective tissue in facilitating epithelial growth and development in vitro and after transplantation in vivo is described. These confirm the requirement for dermal elements if normal structure is to be reestablished and point to the requirement of dermal, as opposed to deep, connective tissues for facilitation of the growth of adult epithelia in vivo. The in vitro experiments suggest the presence of diffusible dermally produced factors that facilitate epithelial growth.

It has been known for many years that dissociated specimens of skin or mucosa transplanted to suitable in vivo sites reorganize and regenerate functional tissues, although, in some circumstances, the finer details of normal structure may be lacking [1]. There have recently been many reports of the successful in vitro growth of epithelial cells, and in such cultures, cell proliferation and stratification occur. The conditions of culture may influence the degree to which normal epithelial maturation occurs, but even in the most successful systems, some features of normal epithelial histogenesis, particularly regionally specific features, are poorly expressed or absent. Interactions between epithelium and mesenchyme are essential to the normal development and histogenesis of epithelia during embryogenesis, and some evidence suggests an important role of epithelial-mesenchymal interactions during the continuous histogenesis of adult epithelial structures. Except in the laboratory, epithelia do not exist in isolation but are closely related to a supporting connective tissue. The question therefore arises as to the extent to which various features of normal epithelial histogenesis lacking in present epithelial culture systems are due to imperfect culture conditions and which are due to the absence of influences that would be normally derived in vivo from connective tissue. From the information currently available, this question may not be definitively answered, but some evidence appears to point to the necessity for connective-tissue influences for the full pattern of expression of epithelial synthesis and organization both in vivo and in vitro.

The first part of this paper presents a brief overview of the processes associated with the maintenance of normal epithelial structure and of previous work relating to the role of connective tissue in this process. The latter part summarizes the results of some recent investigations of the interaction between epithelia and the subepithelial connective tissues.

MAINTENANCE OF NORMAL EPITHELIAL STRUCTURE

The regionally various, stratified squamous epithelia covering or lining surfaces of the mammalian body are characterized by continuous replacement of structure. Cell proliferation pro-

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Abbreviations:
EDTA: Ethylenediaminetetraacetic acid
MCG: membrane-coating granule
MEM: minimum essential medium

duces cells that emigrate from the basal region to undergo various patterns of cell maturation before they are shed from the epithelial surface. Normally, the rates of cell formation and cell loss are balanced to produce a "steady state."

Ultrastructurally and biochemically, cell maturation is characterized by the appearance of the products of various specialized cell synthesis, such as keratins [2], keratohyalin [3], involucrin [4], membrane-coating granules (MCGs) [5], cell-surface antigens [6], and other components. The composition and appearance of these products may vary regionally: for example, keratin proteins differ from region to region [7], and the ultrastructural appearance of keratohyalin and MCGs varies between oral mucosa and skin [3,5].

Cell maturation is also associated with a changing cell morphology and, often, with patterns of cell positioning and packing that lead to a characteristic tissue architecture. A change from a cuboidal or columnar basal cell morphology to the large, flattened cells of the suprabasal strata is typical of most stratified epithelia. Alignment of suprabasal cells to form columnar units of structure is typical of most of the epidermis [8]. The epithelia of many regions normally form adnexal structures such as hair papillae and sebaceous glands, and in other regions such as the dorsum of tongue, complicated patterns of spatial organization and differential cell maturation are seen.

REGENERATION OF EPITHELIAL STRUCTURE IN VITRO

For varying periods of time, and under a very wide range of culture conditions, pure or essentially pure preparations of epithelial cells have been demonstrated to proliferate, stratify, and express various features of cell maturation. Under conditions of long-term culture, primary cultures, subcultures, and some cell lines may establish a steady state, with the rate of formation of cells approximately balancing the rate of cell loss from the epithelial surface. Different culture conditions (for example, the calcium concentration of the medium [9] or the presence of a diverse range of substances such as cAMP or dimethylsulfoxide) may differentially affect cell formation and cell maturation, but these basic features of epithelial histogenesis appear to be independent of specific influences of connective tissue.

The cellular architecture of epithelial tissue formed in vitro differs more or less markedly from a normal epithelium. Structure ranges from a poorly stratified epithelium with a flattened basal cell morphology, typical of epithelia cultured and submerged in medium and attached to plastic [10,11], to the markedly more normal architecture or epithelium grown on artificial collagen substrates at the medium-gas interface [12]. Development of regionally specific patterns of tissue architecture or of specialized epithelial structures in vitro does not appear to have been reported. Some maturation-related products of cell synthesis are expressed in culture. Involucrin synthesis and certain basic cell-surface antigens are expressed [4,13]. Membrane-coating granules are formed in epithelia maintained at the medium-gas interface, but not in submerged cultures [12]. Quantitative and minor qualitative differences between such products in vivo and in vitro do not appear to have been examined. Expression of the full range of regional differences in the type of keratins synthesized appears to be deficient in culture, but cultured cells are capable of reexpressing such differences on retransplantation to in vivo conditions [14]. Keratohyalin, a major product of most keratinizing epithelia, does not appear to be formed in any quantity by cultured epithelia.

THE ROLE OF CONNECTIVE TISSUE IN THE REGENERATION AND MAINTENANCE OF EPITHELIAL STRUCTURE IN VIVO

Epithelial-mesenchymal interactions play a vital role during the embryonic morphogenesis of many structures. The role of mesenchyme can be considered to be through (1) directive (or instructive) influences that act to specify epithelial phenotype, and (2) permissive (or facilitative) influences that provide an environment in which the established phenotype can be expressed [15]. The essential role of epithelial-mesenchymal interactions during the embryonic development of epidermal structure is well established, and the subject has been excellently reviewed by Sengel [16].

The role of subepithelial connective tissue in directing and facilitating the continuous histogenesis of epithelia throughout adult life is less clear. In an early series of experiments, Billingham and Reynolds [1] observed that the epithelial component of some transplanted recombinations of connective tissue and epithelium from skin and oral mucosa acquired features typical of the epithelium normally associated with the connective-tissue component. Other epithelia retained their original specificity following recombination. Several subsequent studies appear to confirm the ability of subepithelial connective tissue to remodulate the pattern of maturation of an associated epithelium [16]. Thus directive influences on epithelium of some adult subepithelial connective tissues appear to exist, and some epithelia appear to have the ability to remodulate in response to such influences. Some studies suggest that subepithelial connective tissue, as opposed to connective tissue in general, plays no special role in facilitating epithelial growth. Briggaman and Wheeler [18] observed that the growth of human epidermis on chick chorioallantoic membrane was inadequate but was facilitated in the presence of dermis and other connective tissues. We have undertaken a fairly extensive series of investigations to examine further the role of connective tissue in the regeneration of epithelial structure in vivo and in vitro, and the results of these investigations point to an important role of connective tissue in facilitating and directing epithelial growth.

MATERIALS AND METHODS

Tissues

Specimens of skin and oral mucosa were obtained from histocompatible neonate or adult C3H mice. Adult tissues were dissociated into epithelial and connective-tissue components using 2 m*M* EDTA [19]. Such specimens were recombined either as intact sheets or were further dissociated into cell suspensions using 0.1% collagenase. Suspensions of neonate skin cells were prepared by trypsin dissociation of whole dorsal neonate skin, from which pure epithelial fractions were prepared by centrifugation on Ficoll gradients [20].

Maintenance of Tissues in Vivo

Various recombinations of epithelial and connective-tissue sheets and of epithelial and connective-tissue cell suspensions were grown by transplantation to prepared sites, deep to the panniculus carnosus, in the flanks of recipient mice. Transplants were protected by placement of "hat-shaped" silicone or polyethylene chambers [19,20]. Specimens were typically grown for 3 weeks prior to examination.

Maintenance of Tissues in Vitro

Tissues were grown in culture under standardized conditions in Eagle's minimal essential medium (×4 MEM) with 17% fetal calf serum at 37°C in an atmosphere of 95% air and 5% CO_2 [20]. Epithelial cells were plated onto gels of collagen (type I) solubilized from mouse tail tendons and prepared in the form of a diaphragm in the lower part of a silicone chamber. Twenty-four hours after plating, medium was withdrawn from each chamber, and the collagen gel bearing the attached cells was supported on a Millipore filter at the medium-gas interface within a 3-cm culture dish. To examine the influence of connective tissue on the growth and maturation of such preparations, sheets of vital or killed (freeze-thawed) dermis were placed on the Millipore filter and closely apposed to the undersurface of the collagen diaphragm.

Examination of Specimens

Specimens were either (1) fixed in Bouin's solution and processed to wax for sectioning, or (2) frozen sectioned for light microscopy, or (3) fixed in glutaraldehyde, postosmicated, and resin-embedded for sectioning and examination by electron microscopy.

RESULTS

Re-Formation of Epithelial Structure by Neonate Epithelial and Connective-Tissue Cell Suspensions Transplanted to in Vivo Sites

Table I summarizes the results of examination of the epithelial component re-formed from transplantation of (1) pure neonate epithelial cells, (2) mixed neonate epithelial and dermal cells, and (3) precultured neonate epithelial and dermal cells. These data have been reported in detail elsewhere [20].

TABLE I. *Percentage of various types of neonate cell implants re-forming epithelial structures 21–29 days after implantation*

	Stratified epithelium	Appendages	Columns
Epithelial cells ($n = 87$)	77	3	0
Mixed skin cells ($n = 61$)	92	63	42
Cultured epi + fibi ($n = 37$)	90	47	0

Suspensions of epithelial cells transplanted without dermal cells re-formed a stratified epithelium that lacked hair follicles and the columnar pattern of organization typical of normal epidermis. A large proportion of transplanted primary suspensions of mixed epidermal and dermal cells re-formed epithelia (Fig. 1*a,b*) that developed hair follicles and showed a normal columnar pattern of organization. Mixed suspensions of epidermal and dermal cells cultured for various periods prior to transplantation similarly showed re-formation of an epidermis with development of hair follicles. The percentage of specimens showing re-formation of epithelia from mixed epidermal and dermal cell suspensions (92%) was somewhat higher than from epithelial cells alone (76%). Epithelial cells transplanted with connective-tissue cells of nondermal origin failed to form hair or a normal columnar structure.

Reorganization of Structure from Adult Epithelial and Connective-Tissue Components Transplanted to in Vivo Sites

Table II summarizes the results of examination of reorganization of epithelial structure in over 2000 transplants of var-

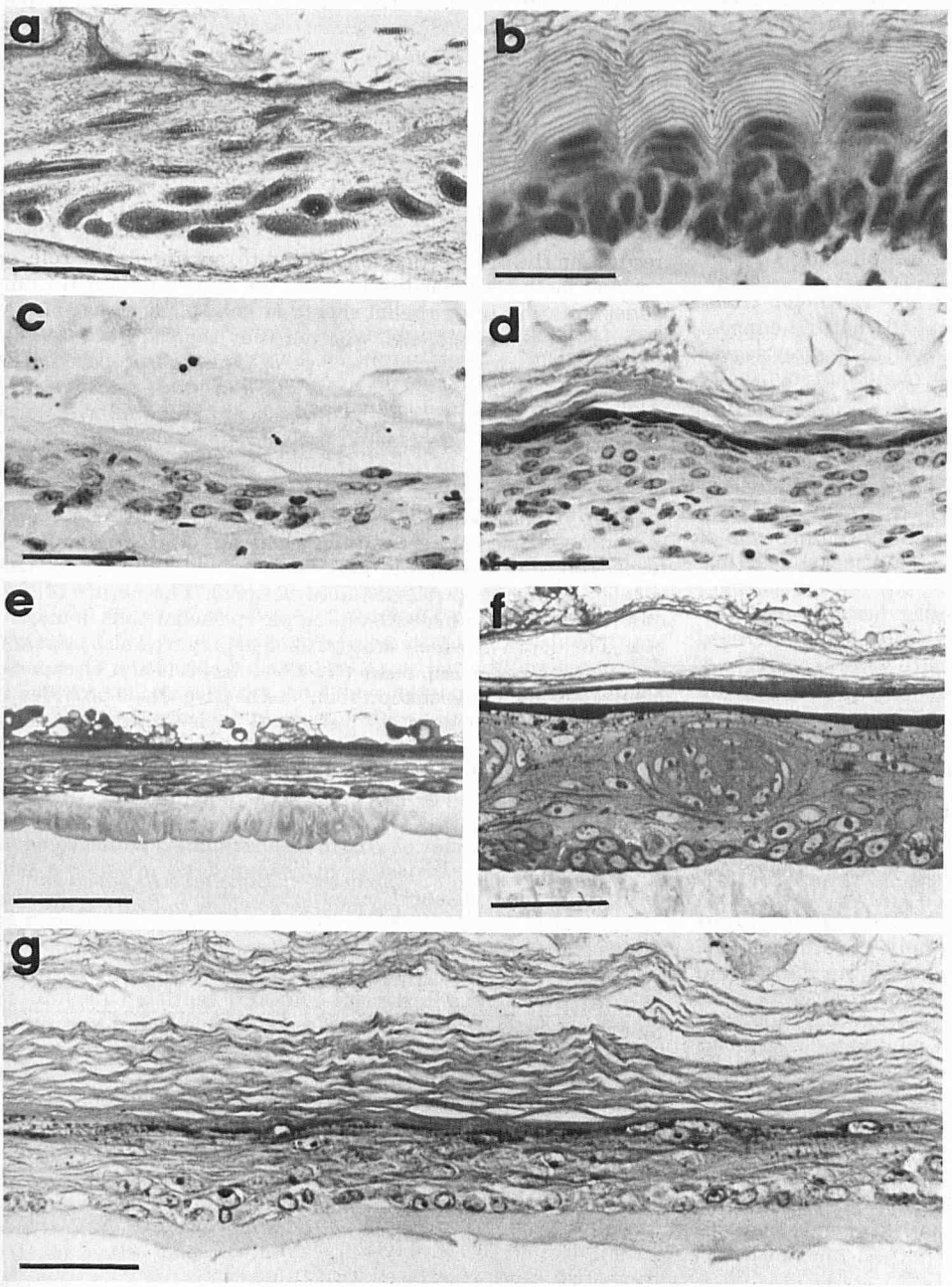

FIG 1. *a,* Tissue reorganized after 21 days in vivo from a suspension of mixed neonate dermal and epidermal cells. Hair follicle formation and a well-formed surface epidermal layer are seen (bar = 750 μm). *b,* Epidermis of a specimen comparable to (*a*) that has been frozen-sectioned and expanded in alkaline buffer to demonstrate the reorganization of a normal epidermal pattern of columnar alignment (bar = 30 μm). *c,* The epithelium re-formed 21 days after transplantation of a suspension of adult epidermal cells. The epithelium appears to be poorly organized and lacks a stratum granulosum and well-formed stratum corneum (bar = 50 μm). *d,* The epithelium re-formed 21 days after transplantation of a suspension of adult epithelial cells to a graft bed preseeded with dermal cells. The somewhat hyperplastic epithelium shows a well-formed stratum granulosum and stratum corneum (bar = 50 μm). *e,* Plastic embedded-specimen of the epithelium re-formed 7 days after plating neonate epidermal cells on collagen supported at the gas-medium interface in vitro. This specimen was grown in the presence of dead dermis. A stratified structure is formed, but there is a flattened basal cell morphology, and no stratum granulosum is apparent. The collagen gel is seen beneath the epithelium (bar = 40 μm). *f,* Same as (*e*), but grown in the presence of vital neonate dermis. The epithelium is well stratified, basal cell morphology is more normal, and basophilic masses are seen in the region of the stratum granulosum. A whorled arrangement of epithelial cells is seen in the midspinous region. The acellular nature of the supporting collagen is apparent (bar = 40 μm). *g,* Comparable specimen to (*f*) 15 days after plating, wax-embedded, stained with H&E, and demonstrating the formation of a granular layer and stratum corneum and the uniformity of the collagen gel (bar = 40 μm)

TABLE II. *Summary of results of examination of reorganization of epithelial structure in transplants of adult epithelia with and without subepithelial connective tissue (SCT) in vivo*

Transplant	Survival	Maturation	Special structures
Epithelial sheets	Rare	Poor	None
Epithelial cells	Poor	Poor	None
Epithelial sheets + SCT cells	Good	Fair	Occasional
Epithelial sheets + SCT	Good	Good	Good
Epithelial sheets + heterologous SCT	Good	Good	Various
Epithelial sheets + other CT	Rare	Poor	None

iously recombined adult epithelial and connective tissues. Epithelial sheets or cell suspensions transplanted without dermal elements seldom re-formed an epithelial structure. When an epithelium was reorganized from such transplants, it was typically poorly formed, lacking a stratum granulosum and well-formed stratum corneum (Fig. 1*c*).

Preseeding the transplant bed with dermal cells or recombination of epithelium with sheets of dermis prior to transplantation greatly facilitated epithelial survival. Epithelium was identified in almost all such transplants. Recombined with vital sheets of homologous connective tissue, epithelial maturation and spatial organization were essentially indistinguishable from that of the normal tissue [19]. With dermal cells, the maturation of the associated epithelium frequently appeared less complete, as judged by the prominence of the stratum granulosum and by stratum corneum staining (Fig. 1*d*). Mixed suspensions of adult epithelial and dermal cells occasionally formed epithelial structures resembling developing hair follicles. Epithelia transplanted in recombination with nonepithelially related connective tissues, such as muscle or tendon, showed no greater degree of survival or maturation than specimens transplanted directly to the deep connective-tissue bed.

Reorganization of Epithelial Structure in Vitro

Table III summarizes the results of examination of the structure and organization of epithelia re-formed 7 to 14 days after plating neonate epidermal cells on collagen in the absence of connective tissue or with living or dead neonate backskin dermis or adult footpad dermis.

Neonate epidermal cells grown on collagen at the medium-gas interface in the absence of connective tissue proliferated and stratified to re-form an epithelium with several cell layers. The most superficial cell layers formed an eosinophilic structure resembling a stratum corneum, but a stratum granulosum was not formed, the basal cells were flattened, and the epithelium was not very thick. This type of structure is termed *atrophic* in Table III. The epithelia re-formed in the presence of killed dermis apposed to the lower surface of the collagen diaphragm bearing the epithelium did not differ in structure from the control epithelia re-formed in the absence of connective tissue (Fig. 1*e*).

Epithelia re-formed in the presence of vital dermis (Fig. 1*f,g*) differed markedly in structure from epithelia re-formed in the absence of connective tissue. These epithelia were thicker and contained more layers of stratified cells at equivalent time periods, and the constituent cells appeared larger. Unlike the rather flattened basal cells of the control epithelia, basal cells were rounded and of an appearance more comparable with that seen in vivo. A thicker eosinophilic stratum corneum was formed, and dense, basophilic granules resembling keratohyalin were present in the cell layers immediately below the stratum corneum. Several specimens showed cell whorls in the midspinous region (Fig. 1*f*), and these were regularly spaced at intervals of about 200 μm. The significance of these structures is uncertain, but possibly they represent incomplete or abortive attempts to produce hair follicles.

Ultrastructurally, epithelia reorganized in the presence of living dermis showed electron-dense masses resembling keratohyalin in the superficial strata (Fig. 2*a*). Such structures were not seen in control specimens. The content of the stratum corneum cells of these specimens was more uniformly fibrillar than that of control specimens, which was more densely stained (Fig. 2*b,c*).

DISCUSSION

The results of the experiments described above indicate that within the in vivo transplant system used, (1) neonate epidermal cells fail to form hair or a normal columnar structure in the absence of skin connective-tissue cells, and (2) the presence of dermis or dermal cells, but not deep connective tissues, markedly facilitates the growth and reorganization of adult epithelial sheet or cells. The first finding is in keeping with general information about the role of specific mesenchyme in embryonic development [16] and with similar previous recombinant studies that indicate that dermal cells are necessary for the reorganization of hair [1,21]. The finding that subepithelial connective tissue, as opposed to deep connective tissue, is necessary for facilitation of epithelial growth and maturation differs from some previously published findings [17,18]. However, the findings reported here consistently point to this conclusion, since (1) epithelial sheets and cells from adult animals seldom survived and invariably failed to show normal maturation in the absence of dermis, (2) growth and normal maturation invariably occurred in the presence of vital dermal sheets, and (3) both survival and maturation of adult epithelia were greatly enhanced by the presence of dermal cells.

Many factors appear to influence the results obtained from transplantation of epithelial sheets or cell suspensions. These include the cell origin, e.g., whether they are from neonate or adult skin or transformed, the type of graft bed prepared, the period of time elapsed before sampling, and the degree to which the epithelial cell preparations may be contaminated with cells of dermal origin. These factors have not previously been systematically studied in detail. It appears that neonate cells and transformed cells have the ability for growth and organization on deep connective tissue [20,22,23] (Table I). We have observed that sheets of neonate epidermis survive under identical transplantation conditions as those in which adult epidermal sheets atrophy (unpublished observations). The nature of the difference between neonate and adult epithelial cells is uncertain. The depth at which the graft bed is prepared also appears to be of particular importance to the interpretation of experimental results. If the subepithelial connective tissue provides a specific growth-facilitating influence, it is uncertain what "depth" of tissue has this capacity. It is of interest that reports of long-term survival of transplanted epithelia have involved preparation of the graft bed superficial to panniculus carnosus [21,24], a region that may still possess dermal properties. Epi-

TABLE III. *Number of specimens of epithelial cells plated on collagen in vitro surviving and reorganizing epithelia in the presence of various dermal elements*

	Atrophic	Good basal cell morphology	Stratification	Keratohyalin
No CT ($n = 15$)	15	0	0	0
Dead neonate CT ($n = 36$)	35	1	1	0
Dead adult CT ($n = 7$)	7	0	0	0
Living neonate CT ($n = 34$)	12	22	23	13
Living adult CT ($n = 6$)	3	3	3	1

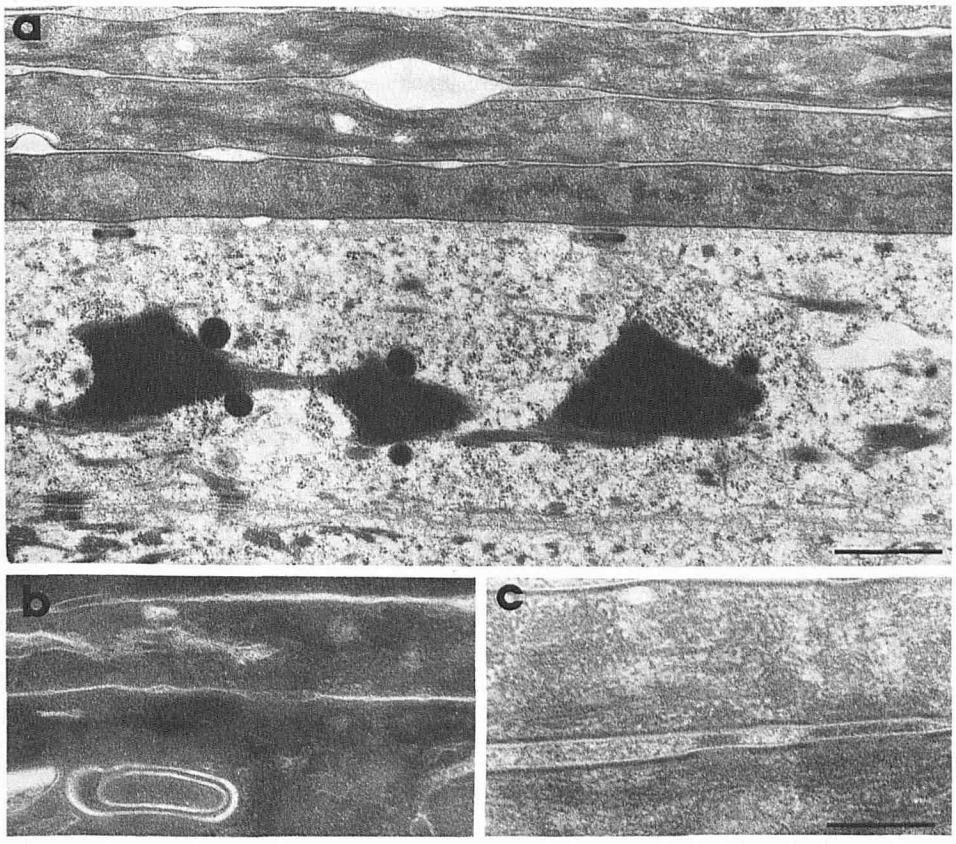

FIG 2. *a*, Electron micrograph of the stratum granulosum region of an epithelium re-formed in vitro in the presence of living dermis (bar = 1 μm). The stratum corneum of this specimen is seen at higher magnification in (*c*) and that of epithelium re-formed in the presence of dead dermis in (*b*) (bar = 0.4 μm).

thelia re-formed on a graft bed prepared deep to panniculus carnosus have been reported, with time, to atrophy [25] or to detach from the graft bed [1]. Experiments in which facilitation of epidermal growth by nondermal connective tissue was observed involved an interspecies graft system and only a short (10-day) period of observation [17].

The results obtained from in vivo transplantation experiments did not indicate the mechanism by which dermis facilitates epithelial growth. As has been previously suggested [26], dermis may stimulate epithelial growth by provision of a particular physical substrate or by synthesis of diffusible factors. With the series of in vivo experiments described here, some aspects of the deep connective-tissue matrix may differ from the matrix produced by dermal cells, and such differences could influence cell attachment and development of basal lamina. However, some specific product of dermal cells may have been lacking for epithelial growth, or alternatively, the deep connective tissue of the graft bed may produce factors inhibiting epithelial growth. In an attempt to distinguish such effects, the in vitro experiments were performed. The experimental conditions provided a standardized matrix for epithelial attachment and a uniform environment in other respects. The marked improvement in degree of epithelial organization and maturation resulting from the presence of vital dermis, even though the epithelium was separated from the dermis by a collagen gel of rather variable thickness, strongly suggests the requirement of diffusible dermal products for normal epithelial growth. Clumps of follicular cells are present in the epidermal suspensions prepared, and the presence of whorls of cells within the epithelia reorganized in the presence of connective tissue raises the possibility that the development of more complex structures such as hair follicles may also be facilitated by such factors.

Interactions between the epidermis and dermis are of interest as a biological problem and may also be of importance to an understanding of clinical problems such as the healing of deep and superficial wounds and reconstitution of surface coverage by grafting. The role of epithelial-mesenchymal interactions in the maintenance of normal epithelial structure and function in the adult has yet to be fully elucidated, but there are now several indications that it is an important one.

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