

CELLULAR INTERACTIONS IN MORPHOGENESIS OF EPITHELIAL MESENCHYMAL SYSTEMS

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The family of epithelio-mesenchymal systems consists of a variety of related forms. Common to all is the formation of epithelial-bounding membranes. These may be external to the mesenchyme as in skin, or internal to the mesenchyme as in ducted glands. We show how the formation of epithelial-bounding membranes can be accounted for in terms of the contact interactions between the cells. We have studied the behavior of epithelial outgrowths, dissociated epithelial cells, and dissociated mesenchymal cells on different noncellular and cellular substrata. The crucial observation is that neither mesenchyme nor epithelial cells can attach to, or move upon, the free surface of an attached epithelium. This observation and others, on the continuity within epithelial and mesenchymal associations, suggest that a discontinuity is obligatory at the epithelial surface, the epithelium necessarily forming bounding membranes.

MATERIALS AND METHODS

All the cells and tissues used (except HEP II cells) derived from human therapeutic abortions obtained locally. Lung and kidney fragments were obtained by finely dicing the organs with scalpels, discarding larger fragments over 1 mm in diameter in the course of saline washing to remove blood and debris. Lines of lung fibroblasts were obtained by trypsinizing the primary outgrowths obtained by culturing the fresh fragments for 5-7 days on plastic. These cells were subsequently passaged in the ordinary way. F10 medium supplemented with 10% fetal calf serum was used throughout. Hydrated

collagen lattices were prepared as described elsewhere (6). Time-lapse films were made using Wild equipment including $\times 6$ and $\times 10$ phase-contrast objectives, and lapse rates from 60 to 180 s.

RESULTS

We have investigated the behavior of dissociated epithelial and fibroblastic cells plated onto four different substrata: plastic, hydrated collagen lattices, fibroblastic lawns, and epithelia. 2×10^5 cells in 5 ml of routine medium were plated onto each substratum in a 50-mm dish. The behavior of the cells was recorded for up to 48 h by time-lapse microcinematography. In addition, the ability of kidney fragments placed on the substrata to produce epithelial outgrowths has been investigated by the same technique.

Plastic Substratum

DISSOCIATED FIBROBLASTS: Extension is initiated within 2-3 h of plating, Fig. 2 *a*. At first the cells exhibit ruffling membranes; later, as confluence is approached, the cells modulate to the bipolar spindle and form as previously described (5).

DISSOCIATED EPITHELIAL CELLS: Extension is initiated more rapidly; within 2 h of plating, few rounded cells are to be seen.

KIDNEY FRAGMENTS: Epithelial outgrowths are observed within 24 h of plating kidney fragments. The outgrowths expand over the next 4

days, remaining entirely epithelial Fig. 1 *b*. Around the 5th day, fibroblasts begin to emerge from the explant, moving on the substratum beneath the epithelium; they are never observed on the free, upper surface of the epithelium.

Hydrated Collagen Lattice Substratum (Reference No. 6)

DISSOCIATED FIBROBLASTS: The time course of the extension of fibroblasts is the same as on plastic. The cells extend immediately in the bipolar spindle form; however, ruffling membranes are rare (5) (Fig. 2 *b*).

DISSOCIATED EPITHELIAL CELLS: The cells remained rounded and failed to extend,

although the films registered their unsuccessful attempts to attach cytoplasmic processes to the substratum.

KIDNEY FRAGMENTS: Outgrowths extend especially rapidly on this substratum. It was surprising to observe that individual cells, detaching from the periphery of epithelia, rounded up and never extended again. This unique behavior was consistent, however, with that exhibited by dissociated epithelial cells plated onto this substratum (see above). Fig. 3 *a* shows a spreading epithelial sheet and 3 *b* individual epithelial cells still rounded after 36 h. The observations thus reveal an interesting divergence between the behavior of epithelia and that of dissociated epithelial cells. This difference is not manifest on other substrata.

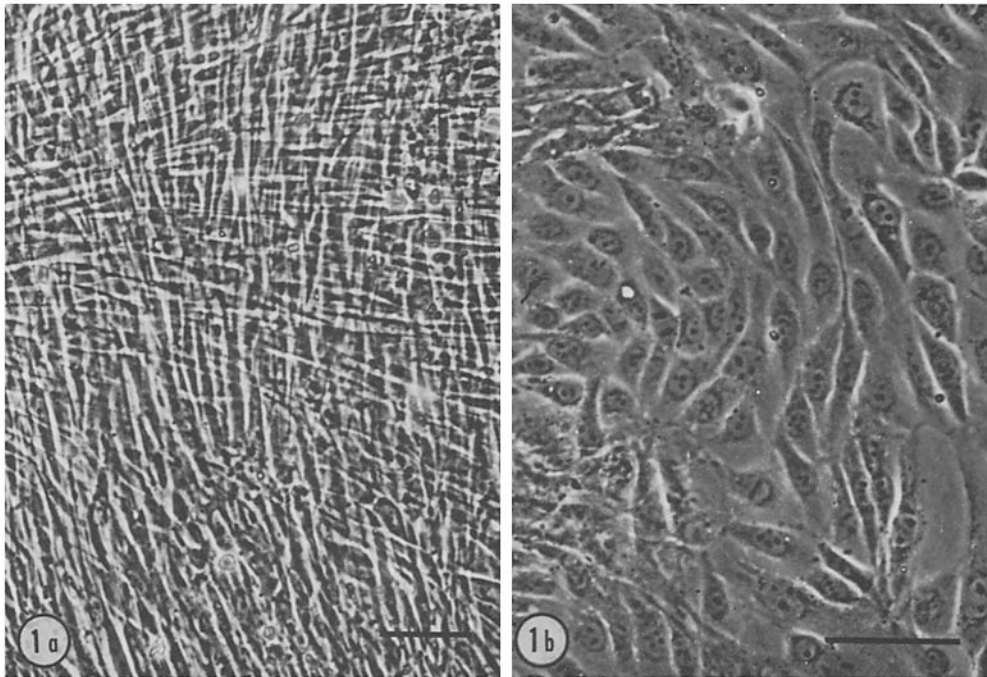


FIGURE 1 (*a*) Portion of a fibroblast lawn substratum illustrating features of the cellular arrangement in a dense fibroblast culture. The cells form continuous sheets in parallel array. The photograph gives the impression of a crisscross pattern. This pattern is generated by the migration of one parallel array over another (4). This phenomenon, which is not observed in the absence of collagen accumulation, is termed orthogonal multilayering. This term refers to the vertical stratification observed within the membranous tissue constructed by the cells and their secretions, and also expresses the observation that the cells in the overlying array are preferentially oriented orthogonally to the cells in the array beneath. The process need not stop at two layers; the extent of multilayering depends on the amount of postconfluent growth and the number of cells to be accommodated within the culture. $\times 120$. (*b*) Portion of an epithelium from a kidney explant. Continuous cell sheets free of intercellular spaces, and strict monolayering characterize most epithelia in culture. $\times 180$.

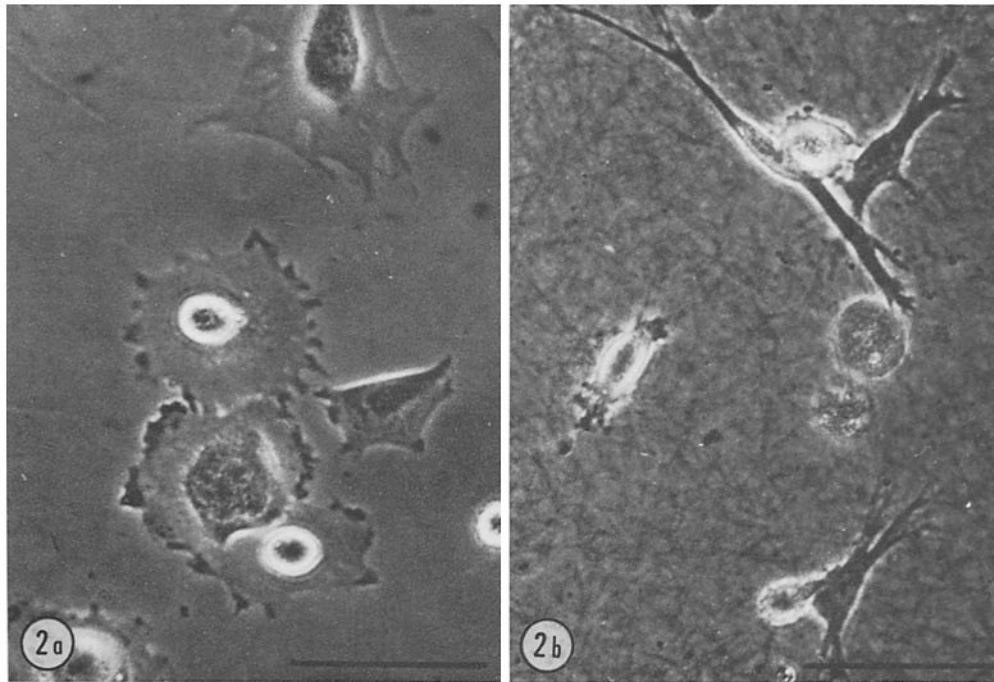


FIGURE 2 (a) Human embryo lung fibroblasts, 2 h after plating onto plastic. The cells have attached to the plastic by continuous cytoplasmic fringes. Over the next few hours they will extend and commence to move. $\times 260$. (b) Human embryo lung fibroblasts, 2 h after plating onto a hydrated collagen lattice. The fibrous nature of the substratum is apparent. The cells attach by two polar pseudopodia in contrast to 2 a. The cells are extending directly into bipolar spindles. $\times 260$.

Fibroblast Lawn Substratum

The substratum here consists of 10-day old, stationary cultures of human embryo lung fibroblasts (3). These are superconfluent cultures containing up to six monolayer equivalents (Fig. 1 a).

DISSOCIATED FIBROBLASTS: These cells extend within 3 h and disappear within the cell sheet. A further investigation was carried out to discover whether the proportion of plated cells that is incorporated into the substratum varied with the density of the fibroblastic lawns employed. The details of this experiment are presented in Table I. In all cases more than 95% of the plated cells were not recoverable in the medium after 24 h, a higher proportion indeed than attached to plastic control substrata. The experiments show that the capacity of fibroblastic sheets to accept more fibroblasts is not a function of cell density, nor does it matter whether the cells are growing or stationary.

DISASSOCIATED EPITHELIAL CELLS: These cells adhere to and spread out upon fibroblastic lawns.

EPITHELIA FROM KIDNEY FRAGMENTS: Outgrowths extend at about the same rate as on plastic. Whereas on the plastic the outgrowths are usually circular in outline and the cells isodiametric, the outgrowths on fibroblastic lawns extend more rapidly along the direction in which the fibroblasts are aligned beneath. Outgrowths are usually, therefore ellipsoidal in outline, and the individual cells within the epithelium are not usually isodiametric but similarly deformed.

Epithelial Substratum

This substratum was prepared by culturing kidney fragments in dishes for 4 days to obtain numerous large, island, epithelial outgrowths on the floor of a plastic dish.

DISSOCIATED FIBROBLASTS: The cells do not adhere and spread on these primary epithelia, nor do they interpose themselves between the epithelial cells. Fig. 4 was taken 24 h after plating; only the cells falling onto plastic have extended. The rounded fibroblasts lying on the free surface of

the epithelium are unattached; they roll around when the dish is moved (if they roll off onto the plastic they adhere and extend normally). Fibroblasts falling onto the plastic between the epithelial outgrowths eventually form a dense sheet. This sheet never encroaches upon the surface of the epithelia; indeed the continued expansions of the epithelial monolayers push back the fibroblast sheet, and pile-ups of fibroblasts are sometimes

observed at the junctions. Fibroblasts are strongly contact inhibited by the epithelia, but the reverse is not true in the situation described.

DISSOCIATED EPITHELIAL CELLS: Cells do not spread out on epithelia, but they may interpose themselves between the cells, if an opportunity arises and become permanently incorporated within the cell sheet. Precisely the same results are obtained with dissociated cells employing a recon-

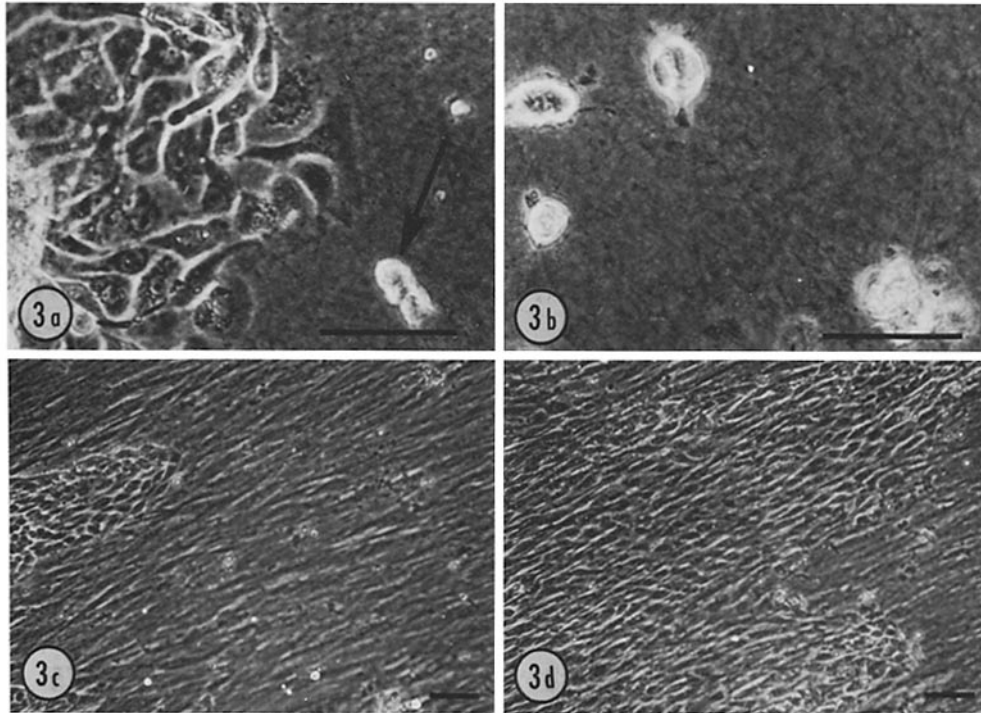


FIGURE 3 (a) From a 16-mm time-lapse sequence. Periphery of an epithelial outgrowth from a kidney fragment placed on a hydrated collagen lattice. The epithelium is able to expand over the substratum. A single cell that has broken free from the epithelium has rounded up (marked with the arrow). Such isolated epithelial cells are unable to utilize this substratum and remain rounded. This difference between the behavior of single cells and that of the collective suggests that the activities of the cells are concerted in a special way within the epithelium. $\times 185$. (b) Dissociated kidney epithelial cells 14 h after plating onto a hydrated collagen lattice. From a 16-mm timelapse sequence. Although the films reveal incessant surface activity, all attempts to anchor cytoplasmic processes are abortive, and the cells remain rounded. This contrasts firstly with the behavior of fibroblasts on the same substratum 3 b, and secondly with the behavior of similar epithelial cells on plastic and fibroblast lawn substrata. $\times 185$. (c) and (d) Two shots from a single 16-mm time-lapse sequence. 3 c was taken 48 h after placing kidney fragments on a fibroblast lawn; 3 d was taken a further 24 h later. The earlier photograph shows an epithelial outgrowth entering the field from the left. The later photograph shows the same outgrowth extending over most of the field. The fibroblasts in the substratum are extended along the diagonal from bottom left to top right. The epithelial migration follows this axis. The individual cells within the epithelium are somewhat elongated in the same direction. This illustrates the general finding that the migratory behavior of an epithelium is largely controlled by the pattern in the underlying cellular substratum. This observation suggests one way in which the mesenchyme could control the detailed patterning of the epithelial component in vivo. $\times 64$.

TABLE I
An Experiment to Determine the Plating Efficiency of Fibroblasts onto Fibroblast Sheets

Class	No. of fibroblasts in substratum (t = 0)	Final cell count (t = 8 h)		Plating efficiency %
		Cell sheet	Medium	
1	0 (bare plastic)	1.04×10^6	8.4×10^4	91.6
2	$5.7 \pm 0.2 \times 10^6$	1.9×10^6	6.8×10^4	93.3
3	$2.2 \pm 0.1 \times 10^6$	3.6×10^6	6.0×10^4	94
4	$9.8 \pm 0.4 \times 10^6$	—	5.5×10^4	95.4

In each case, 1.1×10^6 cells were pipetted into the dishes. After 8 h, cells in the cell sheet, and in the medium, were separately counted.

stituted epithelial substratum provided by a confluent culture grown from trypsinized kidney epithelial cells.

A similar finding, that the upper, free surface of a confluent culture of epithelial cells cannot be used as a substratum by other epithelial cells, derives from observations on cultures of HEP II cells. This line was derived from a human carcinoma. Growth continues after these cells have achieved confluence, as a result of which rounded cells, unable to adhere or spread, accumulate above the monolayer.

The formation of epithelial outgrowths from kidney fragments placed onto epithelia cannot be properly investigated because the underlying epithelium does not retain its integrity in the vicinity of tissue fragments.

DISCUSSION

The Epithelium and the Epithelial Cell

The behavioral characteristics of epithelia (1, 7) encourage the view that the activities of the component cells are concerted in some way to obtain the unitary behavior of the sheet as a whole. We infer that it is the result of this coordination that enables epithelia to utilize and colonize hydrated collagen lattice substrata, on which isolated epithelial cells are unable to make attachments and remain therefore nonfunctional.

Asymmetric Growth of Epithelia on Aligned Substrata

Ellipsoidal outgrowths of nonisodiametric cells are observed when kidney fragments are plated onto hydrated collagen lattice substrata and fibroblast lawns. The nonrandom alignment of the

collagen bundles in the former substrata has been previously described (6). Epithelial outgrowths appear to follow the alignment of the fibroblasts in parallel array in fibroblast lawn substrata. The way is clear to investigate the relevant possibility that branching of epithelial outgrowths is induced above discontinuities between arrays in the underlying fibroblastic sheet (4) and that a similar mechanism could operate in vivo.

The Polarity of Cells within an Epithelium

An epithelial cell lining a functional tubule possesses sides by which it attaches to like cells and two ends that are functionally specialized in different ways. One end is applied to the stromal connective tissue and contributes to the basement membrane; the other end faces the lumen of the tube. It is not known how this differentiation between the ends arises. The difference could depend upon a stable polarization of the subcellular architecture arising early in development before the morphogenetic events we are concerned with here. Alternatively, the differentiation might arise operationally, that is to say, the cell surface might remain undifferentiated until some part attached to the mesenchyme, after which the opposite pole differentiated. Thus, although it is observed that epithelia extend upon three of the substrata here used, we can only surmise but cannot prove that an epithelial cell would attach the same surface to one substratum as it would to another.

The Interaction between Epithelia and Fibroblastic Cells

The crucial finding concerning the cross interaction between epithelia and fibroblastic cells is that,

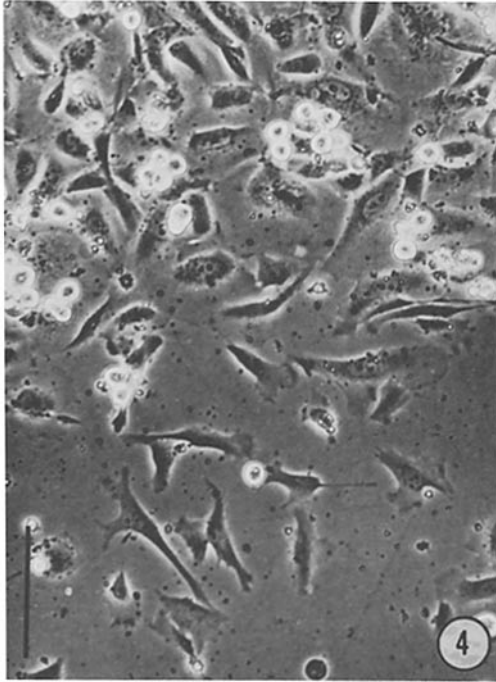


FIGURE 4 Photograph taken 24 h after plating dissociated fibroblasts onto a culture of kidney fragments and their associated epithelial outgrowths. The fibroblasts that have fallen onto the epithelial outgrowth that fills the left half of the field remain rounded; they roll around when the dish is tilted. The fibroblasts that have fallen out with the outgrowth on the right half of the field have attached to the plastic and extended ruffling membranes. This illustrates the inability of fibroblasts to use the free surface of an epithelium as a substratum for their attachment and extension. $\times 140$.

regardless of the nature of the substratum, the free surface of an attached and spreading epithelium is invariably nonadhesive to other cells be they fibroblastic or epithelial. Parallel observations on chick corneal, epidermal and gut epithelia have recently been reported (2).

The Formation of Epithelial-Bounding Membranes

Taking into consideration what is known about the continuity of the cellular associations involved, the observation that cells cannot associate with one surface of an epithelium accounts for the appearance of discontinuities in an epithelio-mesenchymal fabric: the epithelium is constrained to form bounding membranes. These bounding membranes may be external, investing the mesenchyme, or

internal in the form of vesicles or tubes (see Fig. 5). All members of the family of epithelio-mesenchymal systems display such patterns, which can be understood as a direct result of the interaction of the component cells alone.

Clearly, secondary factors must dictate whether external or internal membranes arise in a particular organ; also, they must dictate the pattern of branching in ducted glands, whether the arborizations are complex and close as in salivary gland or sparse as in mammary gland. Here, too, contact interactions may play a decisive role, and possible avenues of investigation are suggested by the present work.

SUMMARY

The free surface of an attached epithelium does not provide a suitable substratum for the attachment and locomotion of either fibroblastic or epithelial cells. This observation helps to explain the universal occurrence of epithelial-bounding membranes in epithelial mesenchymal systems.

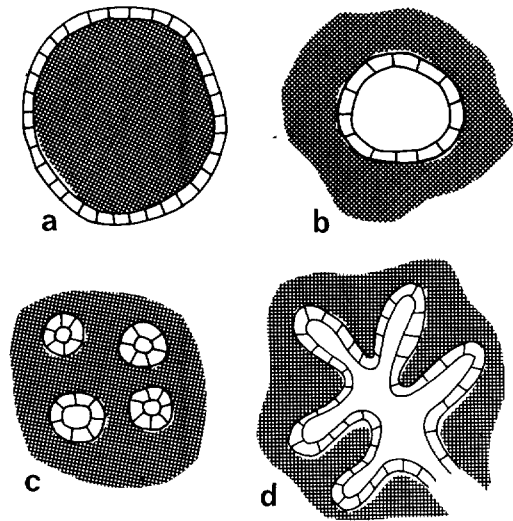


FIGURE 5 Diagram to illustrate the family of forms generated by epithelio-mesenchymal interactions (see text). Connective tissue hatched; epithelium drawn as sections through a pavement. (a) Epithelium forms an external-bounding membrane investing the connective tissue. (b) Epithelium forms an internal-bounding membrane invested by the connective tissue. (c) and (d) Two possibilities within the general scheme of internal, epithelial-bounding membranes. (c) Isolated vesicles or cysts, sebaceous glands in skin for example. (d) A continuous ducted tubular epithelial system as in ducted glands.

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