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MOVEMENTS OF EPITHELIAL CELL SHEETS *IN VITRO*

R. B. VAUGHAN* AND J. P. TRINKAUS
Department of Biology, Yale University, New Haven, Connecticut, U.S.A.

SUMMARY

Chick embryo epithelial cells, cultured *in vitro* on a plane glass surface, show ruffled membrane activity like that seen in fibroblasts. When similar epithelial cells come into contact, long-lasting adhesions form, which are associated with the cessation of membrane activity. Movement of epithelial cell sheets is correlated with adhesion to the substratum of some or all of the marginal cells. Such adhesion does not occur in the absence of membrane activity. Non-marginal cells ordinarily appear to be non-adherent to the substratum; occasionally, however, they become adherent when attachments to neighbouring cells in a sheet are broken. This behaviour is accompanied by marked membrane activity. Mobilization and orientation of marginal cells are the vital steps in movements of coherent cell sheets.

INTRODUCTION

Epithelial cells *in vivo* and *in vitro* tend to be arranged in the form of sheets of varying thickness, each cell being in close contact with other similar cells at its margins. Such cell sheets are known to be capable of active spreading movement *in vivo*, as for example in wound healing (Weiss, 1961) and in morphogenesis (Trinkaus, 1965). Under the artificial conditions of tissue culture also, they show active movement, as can be readily seen at the margins of a culture of skin tissue (Matoltsy, 1960).

A number of studies have been carried out on the zone of outgrowth which surrounds a living explant of tissues containing fibroblastic cells (Abercrombie & Heaysman, 1954; Abercrombie & Ambrose, 1958). The results of these studies have led to an understanding of some of the mechanisms underlying the movement of fibroblasts on a plane surface. Comparatively little work has been done along these lines in the case of epithelial cells.

Active movement by epithelial cells was discerned in early studies of wound healing (Barfurth, 1891; Eycleshymer, 1907). One of the earliest descriptions of the *in vitro* movement of cells other than protozoa or metazoan amoebocytes is that of Harrison (1910), who clearly described the movement of the growing nerve tip in the amphibian embryo. Later studies of the movements of epithelial cells *in vitro* (Holmes, 1914; Matsumoto, 1918; Ulenhuth, 1914; Hitchcock, 1939; Danes, 1949) led to the formulation of the following concepts: (*a*) sheets of epithelial cells will only move, and thereby spread, if the cells are presented with a solid substratum; and (*b*) movement

* Present address: Pathology Unit, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, W.C. 2

of the whole sheet of cells is due to 'amoeboid' activity of the cells at the free margin.

There has been little comment on the nature of the adhesions between the cells forming an epithelial sheet or on the effects of such adhesions on the movement of the cell sheet as a whole. Remarkably little is in fact known regarding the surface activities of any of the cells of a sheet other than those at the free edge. The present work was undertaken in an attempt to provide a clear description of the movements of epithelial cells, both individually and in the form of a coherent sheet. This is felt to be a matter of some importance, since the spreading and folding of epithelial cell sheets is a dominant feature of both wound healing and morphogenesis (Weiss, 1961; Trinkaus, 1965).

MATERIALS AND METHODS

The tissues cultured were all taken from 5-day-old chick embryos. Those used were skin and amnion, as they gave cultures containing very few non-epithelial cells. The tissues were prepared for culture as follows. After aseptic removal from the embryo, all tissues were placed in sterile Tyrode's solution at room temperature, in which they were cut into pieces 2–4 mm square. All tissues were then placed in sterile calcium- and magnesium-free Tyrode's solution containing 2·25% (w/v) Trypsin (Difco 1:250) and 0·75% (w/v) Pancreatin (Nutritional Biochemicals 5 XNF) for 15 min at 0–4 °C. From this solution they were transferred to ice-cold sterile Tyrode's solution containing 10% (w/v) horse serum (Difco TC liquid) in which the epidermis was separated from the rest of the skin, and as much mesenchymal tissue as possible removed from the amnion. Finally the tissues were transferred to pre-warmed (37 °C) culture medium. The culture medium used in all cases consisted of the following: medium 199 (Hanks base, Baltimore Biological Laboratories), 70%; 12-day chick embryo extract, 20%; horse serum (Difco TC liquid), 10%; streptomycin sulphate, 100 i.u./ml; penicillin G, 50 i.u./ml.

The tissues were cultured on 'Corning' glass coverslips, in chambers which permitted changing of the medium without disturbing the culture, as well as observation of the cultures with a high-power phase-contrast optical system (Rose, 1954; Sykes & Moore, 1959).

The explants were placed on the lower coverglass of a partly assembled culture chamber and incubated at 37 °C, in a moist chamber, in an atmosphere of 5% CO_2 and 95% air, for 3–4 h. At the end of this time the assembly of the culture chamber was completed and the chamber was carefully filled with culture medium. The cultures were maintained at 37 °C during filming. All observations were made and films taken using a Zeiss Standard Universal Microscope with Neofluar phase-contrast objectives. The chambers were inverted on the stage to give a good phase-contrast image. Total magnification varied from × 100 to × 800. Filming was carried out with an Arriflex 16 camera and intervalometer using Kodak plus-X negative 16 mm film. The exposure in all cases was 0·25 sec; the interval between exposures varied from 2 to 10 sec. Cultures were filmed at times varying from 12 to 48 h after their initial

explantation. Films were analysed by means of a Data Analyser projector (L-W Photo Inc. Calif.). Six films representing 20 cultures studied for periods varying from 4 to 72 h were analysed.

Because the film material was damaged in processing, illustrations to this paper are in the form of outline drawings made from projected stills from the films.

RESULTS

Movements of single isolated cells

On the rare occasions on which an isolated single cell was observed in motion, the leading margin of the cell appeared to be a very thin membrane in which considerable activity in the form of ruffles (Abercrombie & Ambrose, 1958) was seen. The ruffles were quite irregular in rhythm and showed a wave-like motion in the opposite direction to that in which the cell was moving. The shape of the ruffled membrane was quite irregular, and was liable to sudden change. The cells always appeared polarized and showed membrane activity of this type only at their leading ends. The ruffled membranes of these cells seem, in every way, like those of fibroblasts.

Movements of cell sheets

When the edge of an explant of epithelial tissue is observed within 3–4 h after explantation, the cells are seen to be arranged in a rather loose meshwork with apparently random orientation of the constituent cells and little membrane activity visible. After a variable length of time (12–24 h) the cells become active and move about in an apparently random manner. Adhesions between cells are frequently made and broken at this stage, sometimes lasting for only 20–30 sec. Over a period of 1–4 h the cells become more closely packed and adhere to one another along most of the cellular margin. They also become oriented in parallel, usually in a radial direction with respect to the main cell mass of the explant.

From this point on, the movement of the cell sheet outwards from the main cell mass appears to occur by one of two ways. All the cells along the free margin of the sheet may show membrane activity in the form of multiple small, short-lived, ruffled membranes which appear to adhere closely to the glass. This activity is correlated with a smooth outward movement of the whole free edge of the sheet (see Fig. 1). The second way, which appears to be much the commoner of the two, is by the formation of large localized outgrowths at intervals along the free margin. Each outgrowth may consist of anything from two to twenty cells. All around the periphery of such an outgrowth marked ruffled membrane activity is seen, being maximal at the tip of the outgrowth (Fig. 2).

The cells of the free edge between the outgrowths, which may number from one to ten or twelve, show little or no membrane activity. These cells come to be markedly stretched and tangentially oriented, in contrast to the cells of the outgrowth which are radially oriented with respect to the main cell mass. The sheet appears to move forward, in this instance, in association with active forward movement of one or more outgrowths. The outgrowths themselves appear to compete and those which attain

dominance lead the movement of the sheet, whereas the others retract into the free edge and disappear. Different outgrowths appear to become dominant at different times. As the sheet advances, the areas between outgrowths often become markedly concave, giving a scalloped appearance to the edge of the sheet.

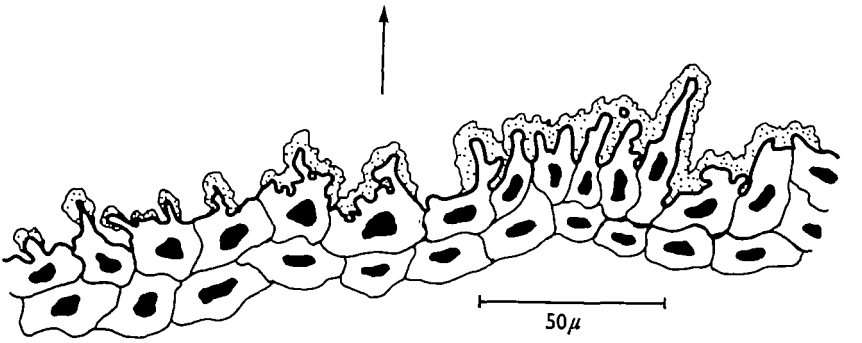

Fig. 1. Drawing of free edge of cell sheet. The stippled areas are those where ruffled membrane activity was seen. The arrow shows the direction of movement of the whole sheet. Membrane activity is distributed evenly along the whole margin.

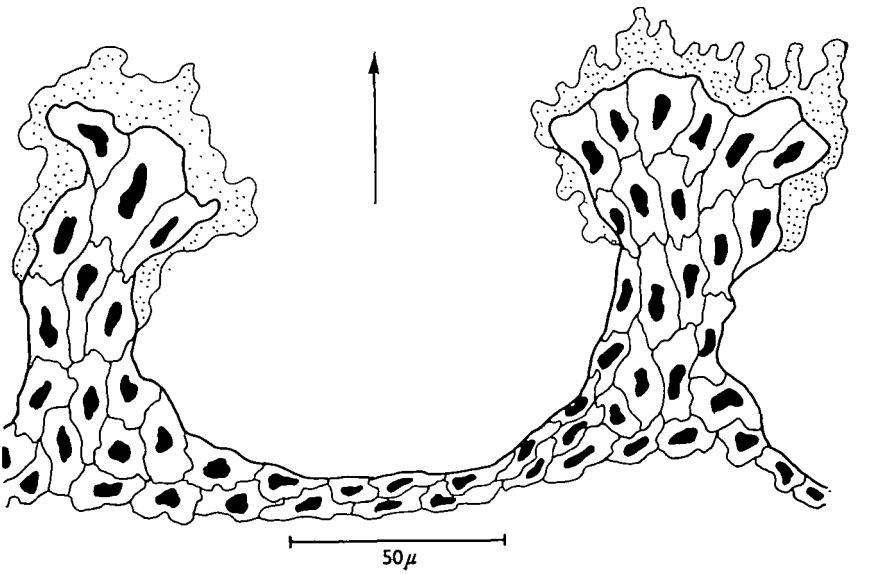

Fig. 2. Drawing of free edge of cell sheet. Stippled areas are those where ruffled membrane activity was seen. The arrow shows direction of movement of the whole sheet. Two large outgrowths are seen which show membrane activity at their tips and radial orientation of their constituent cells. Tangentially arranged cells are seen between them. No membrane activity is seen in these cells.

These appearances suggest that only the marginal cells forming the outgrowths are adherent to the glass. This is also suggested by observation of a retracting epithelial cell sheet. When a sheet retracts, as it will do if detached along part of its edge, or when subjected to localized heating, or occasionally after a change of medium, it can be seen that only those areas of the edge in which the cells show marked membrane

activity adhere to the glass. Such areas tend to become deformed and stretched out into long tongues of cells which may finally snap under the strain, leaving portions of cytoplasm and even whole cells or small groups of cells behind as the main cell mass retracts. Such areas of attachment or 'holdfasts' are invariably correlated with marked membrane activity on the part of the constituent cells.

It seems likely that only those cells at the free edge which show membrane activity are adherent to the glass and therefore capable of active movement. Possibly movement of the cell sheet as a whole is dependent upon the movement of these cells.

In the central part of a large explant the cells are at least three or four layers thick. In this area movement of cells over the surfaces of underlying cells may be discerned. The direction of such cell movement is very variable, different layers of cells moving, in the form of coherent sheets, in different directions. The overall tendency is for the movement to be centrifugal away from the main mass of the explant.

On moving out towards the edge of the zone of outgrowth the cell sheet is seen to become thinner until it is one cell thick as the free edge is neared. Close observation of this area, in a moving cell sheet, shows that the cells are closely adherent to each other all around their circumference, with no membrane activity discernible at their centrifugal ends. If the adhesions of a cell to any of its neighbours break down, however, marked membrane activity is seen to occur after a short space of time at the centrifugal end of the cell on the side of the break away from the free edge of the explant. Apart from the early stages of outgrowth when cell contacts are continually being made and broken as the coherent oriented sheet forms, two causes of such loss of cellular adhesion were commonly observed. One was divergent movement of cells. When this happened the cells were pulled apart, the appearances being exactly like those seen when cell attachments to glass were pulled off during retraction of a cell sheet. The second common cause of loss of intercellular adhesion was extreme radial tension in the cell sheet. The loss of adhesion almost always occurred close to the free edge. When the cells parted, often leaving fine strands of cytoplasm still joining them, the cells nearest the free edge continued to move. The cells still attached to the main mass of the explant retracted very rapidly and a large gap opened up, sometimes as large as one or two cell lengths. This rapid retraction again suggests that the cells behind the free edge are not adherent to the glass. After a brief pause, membrane activity was seen in the free anterior end of the retracted cells, which moved forward, closing the gap and in $\frac{1}{2}$–$1\frac{1}{2}$ h rejoined the free edge cells. The appearances often suggest that the cell is moving along the line of the thin cytoplasmic threads left when the break in the sheet occurred. It is worthy of note that the gap never seems to be closed by retrograde movement of the leading edge cell. Possibly, the already established ruffled membranes at the front of the free edge cells inhibit membrane activity at the rear ends of these cells (Weiss & Garber, 1952).

Occasionally, a loss of adhesion occurred between a particular cell and its neighbours. This occurrence was followed within a few seconds by marked membrane activity along the whole periphery of the cell which had become loose. After a period varying from 10 to 20 min the membrane activity became polarized and the cell moved towards the free edge of the cell sheet, over the surfaces of the other cells of the sheet. Freely

moving cells of this type usually moved out on to the glass, deforming as they did so the margin of the cells at the edge, over which they passed. Having reached the glass, the cell may initiate a large localized outgrowth of cells, leading the movement of the free edge, or gradually cease to show membrane activity and become a tangentially oriented, quiescent, leading-edge cell.

In older cultures (72 h onwards) the coherent sheet tended to break up at its margins and many small isolated groups of cells formed, suggesting a change in behaviour of the epithelial cells. This phenomenon has not been investigated further in the present work.

DISCUSSION

The movements described are very similar to those of fibroblasts under similar conditions (Abercrombie & Ambrose, 1958), except that adhesions formed between epithelial cells tend to be more stable. Membrane activity usually stops soon after contact is made with another epithelial cell, suggesting that contact inhibition is occurring (Abercrombie & Heaysman, 1954). In well-formed, actively spreading, epithelial sheets the strength of intercellular adhesions is demonstrated by the distortion of cell shape which occurs when such adhesions are broken. The fact that membrane activity appears only when the cells in a sheet lose their adhesions to neighbouring cells suggests again that contact inhibition may be operating under these conditions.

From the observations described it appears likely that the spreading of an epithelial cell sheet on glass is normally directed by those cells at the free edge which are adherent to the glass. Such cells appear to be those which show membrane activity. It seems probable that the cells behind the edge are being pulled passively along by virtue of their firm adhesion to the cells at the free edge. It must be noted, however, that proof of the adherence or non-adherence of the cells to the substratum is lacking. Studies utilizing a technique of the type recently described by Curtis (1964) should provide conclusive evidence on this point.

For epithelial cells, as for fibroblasts, it appears that contact inhibition is not an 'all or none' phenomenon. Cells in an epithelial sheet occasionally become free of intercellular adhesions and move over the surface of the sheet. Why such cells move towards the free edge of the sheet is not clear. Possibly the orientation of the movement is due to the underlying cells providing a form of contact guidance (Weiss, 1958) by virtue of their radial arrangement. This would not, however, account for the direction of movement towards the free edge (Trinkaus, 1965). It is worthy of note that, in all the cases observed, these unusual cells appeared near the base of a multicellular outgrowth of the free margin of a sheet in which the cells are markedly radially oriented.

Wound healing and morphogenesis involve movement of cells *en masse*, often in the form of coherent sheets. The observations reported in this paper support the view that contact inhibition is the mechanism which co-ordinates the movements of the constituent cells of a sheet.

The fact that cells can become detached from the body of a sheet and move over the surface of surrounding cells, later becoming leading marginal cells, shows that the adhesive properties of the cell surface are subject to rapid change. Such a capacity is a prerequisite for any mass movement of cells *in vivo*. It must be borne in mind that cell surfaces may be profoundly altered by constituents of the culture medium such as serum. This aspect of cellular adhesion was not investigated in the present work.

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