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Cell Biology

Cell density determines epithelial migration in culture

(wound healing/MDCK cell line)

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ABSTRACT **The dog kidney epithelial cell line (MDCK) has been shown to exhibit a density-correlated inhibition of growth at approximately 6.6×10^5 cells per cm^2. When a confluent monolayer at its maximal density was wounded by removal of a wide swath of cells, migration of the cell sheet into the denuded area occurred. Precise measurements of the rate of migration for 5 days showed that the cells accelerated at a uniform rate of 0.24 $\mu m \cdot hr^{-2}$ and, by extrapolation, possessed an apparent initial velocity of 2.8 $\mu m \cdot hr^{-1}$ at the time of wounding. The apparent initial velocity was considered to be the result of a brief (<10 hr) and rapid acceleration dependent on cell density. To verify this, wounds were made at different densities below the maximum. In these experiments, the cells did not migrate until a "threshold" density of 2.0×10^5 cells per cm^2 was reached regardless of the density at the time of wounding. At the threshold density, the cell sheet began to accelerate at the previously measured rate (0.24 $\mu m \cdot hr^{-2}$). Any increase in density by cell division was balanced by cell migration, so that the same threshold density was maintained by the migrating cells. Each migrating cell sustained the movement of the cell sheet at a constant rate of acceleration. It is proposed that an acceleration is, in general, characteristic of the vectorial movement of an epithelial cell sheet.**

The use of epithelial cultures to study the wounding response exploits the simplicity of working with a monolayer of a single cell type. Although there are drawbacks in attempting to correlate migration on plastic or glass with an *in vivo* system, it is still possible to make comparisons (1). The following experiments were carried out with the dog kidney epithelial cell line (MDCK), which has the properties of a transporting epithelium (2). It will be shown that the migration of an epithelial sheet can be divided into two stages: first, a brief (<10 hr) rapid acceleration that is dependent on a threshold cell density, and second, movement at a slower constant rate of acceleration, which is considered to result from an aggregate of the individual movements of each migrating cell.

MATERIALS AND METHODS

Cells of the MDCK cell line were obtained from the Naval Biosciences Research Laboratories (Berkeley, CA) at passage 54. When plated out, they grew as monolayers. The medium used was Waymouth's 752/1 (KC Biological, Lenexa, KS) supplemented with 10% (vol/vol) fetal bovine serum (GIBCO), 5 μg of insulin per ml, 0.20 mM (120 units/ml) of penicillin G, and 50 nM streptomycin sulfate (all from Sigma). Incubation was at 37°C in a 5% CO_2 atmosphere. A clone was developed by standard procedures from passage 55; for the experiments, cells from passages 58–64 were used. No changes in properties were observed in this range.

Wounding was carried out in tissue culture dishes (Corning) with a stiff razor blade (3). This left a rectangular wound, approximately 8 mm wide, with smooth, straight edges of cells. The edge of the wound was marked by the razor at its initial placement.

For the experiments begun at the maximal cell density, the times of wounding were staggered so that results at various time points could be collected at convenient hours. However, for those experiments carried out at intermediate cell densities, all of the dishes were wounded at the same time (i.e., over a period of 50 min for 45 dishes). During the experiments, the medium was changed daily. Two or three dishes were used for each time point and observations were made every 4 hr in the first 36 hr and every 6 hr up to 120 hr. Cells were fixed in 10% (vol/vol) buffered formalin and were stained with Harris-modified hematoxylin and Giemsa (both from Fischer Scientific, Fair Lawn, NJ).

Distances of migration were measured with an ocular graticule by light microscopy. Fifty measurements for each time point were made at random positions along the wound edge. An average was made after the five highest and the five lowest values were eliminated. These distances were plotted as the mean ± SD. The linear least-squares analysis assuming no error in the horizontal coordinates was used to fit the migration data. The slopes were compared for statistical significance by the *F* test (4).

RESULTS

MDCK cells plated at any concentration form a confluent monolayer at a stable density of 6.6×10^5 cells per cm^2. A monolayer at this maximal density that was fixed immediately after wounding shows the wound origin (Fig. 1*a*). In a similar dish 32 hr after wounding (Fig. 1*b*), the cells have migrated into the wound area and three regions can be described: the control region (C) of high density unaffected by cell migration; the wound region (W) that consists of the migrating cells at low density; and a narrow "gradient" region (G) of density intermediate between the densities of the other regions.

The distance traversed by the epithelium in the wound region can be described according to the equation for the motion of a body moving with a constant acceleration (5):

$$s = v_0 t + \tfrac{1}{2} a t^2$$

i.e.,

$$s/t = v_0 + \tfrac{1}{2} a t \qquad [1]$$

in which s = distance moved, v_0 = initial velocity, a = acceleration, and t = time. When s/t was plotted against t for the migration of MDCK cells into the wound (according to Eq. 1), straight lines resulted (Fig. 2), indicating that the cells accelerated at a constant rate. The final points of the uppermost curve in Fig. 2 showed signs of leveling off, but were still linear within experimental error. The slopes ($a/2$) of the curves from the three experiments yielded accelerations of 0.23, 0.29, and 0.21 $\mu m \cdot hr^{-2}$ for the upper, middle, and lower curves, respectively, which were not significantly different ($P > 0.05$). In addition, extrapolation of the upper curve to zero time

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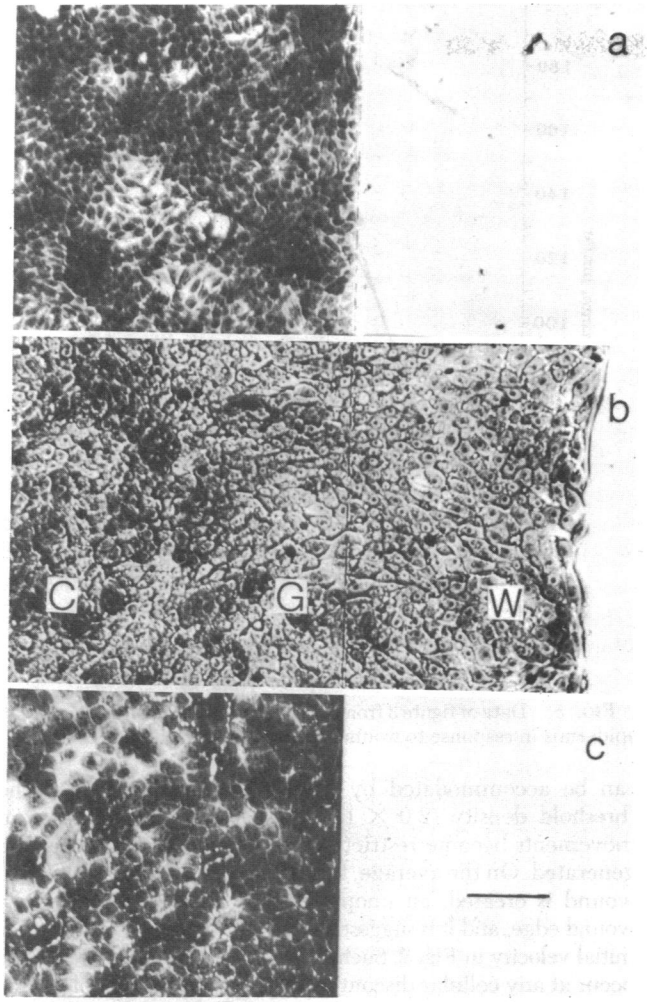

FIG. 1. Effect of density on the migration of MDCK cells. (*a*) MDCK epithelium at its maximal density (6.6×10^5 cells per cm^2) fixed immediately after wounding. (*b*) Epithelium as in *a*, but fixed 32 hr after wounding, showing the extent of migration. The original position of the wound edge is visible as a vertical line. Three regions (C, control; G, gradient; and W, wound) are delineated on the basis of density as a result of the migration. (*c*) MDCK cells wounded at only 1.3×10^5 cells per cm^2, showing no migration after 32 hr. All cells were fixed in buffered formalin and stained with hematoxylin and Giemsa. (Bar = 100 μm.)

yielded an "initial velocity" v_0 of 2.8 μm/hr; that is, the cells possessed a finite velocity at the instant of wounding. In that experiment, the cells were wounded after they reached their maximal density (6.6×10^5 cells per cm^2). In the other two experiments, wounds were made at two confluent but submaximal densities, 1.7×10^5 and 1.3×10^5 cells per cm^2 (Fig. 2, middle and lower curves, respectively). In these two experiments, the cells did not move until a threshold density was reached. For example, when the cells were wounded at 1.3×10^5 cells per cm^2, no migration of the cells into the wound had occurred even after 32 hr (Fig. 1*c*), the threshold density of cells still not having been achieved by the confluent epithelial sheet. When the movement did begin, the cell front showed the same acceleration, within experimental error, as in the other two experiments in which the cells were wounded at higher densities (Fig. 2).

The threshold density at which the cells began to migrate into the wound was determined graphically. As the cells advanced, they maintained a constant density within the wound region. However, the density in the control region continued to increase

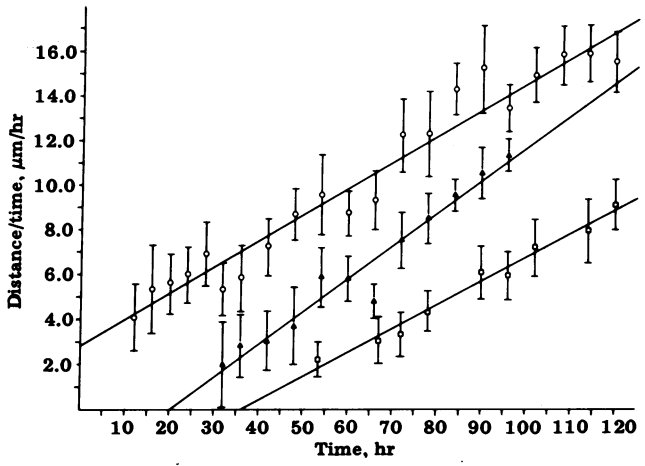

FIG. 2. Rate of migration of the wound edge of an MDCK epithelium plotted according to Eq. 1. Each set of points represents the migration response of the epithelial sheet to wounding at the different cell densities. ○, 6.6×10^5 cells per cm^2; ▲, 1.7×10^5 cells per cm^2; □, 1.3×10^5 cells per cm^2.

such that by 80 hr the maximal density was reached. For the two wounding experiments begun at submaximal cell densities, the cell density of the control region at a given time was plotted against the migration distance of the wound region corresponding to that time, obtained from Fig. 2. Although the two experiments were begun at different densities, migration did not occur until the same density was reached in both. This threshold density is 2.0×10^5 cells per cm^2, the value at which extrapolation of a smooth line through the points intercepted the ordinate at zero distance of migration. Fig. 3 is thus a general curve demonstrating that a threshold density of 2.0×10^5 cells per cm^2 is required before migration can begin.

If the movement of the cell sheet is density dependent, there should be no movement of isolated MDCK cells. The behavior of isolated cells and of small groups of two to five cells was examined by photographing the same field of cells at the beginning of the experiment and 28 and 51 hr later. Apart from cell division, no substantial displacement occurred during the entire experiment (Fig. 4). Time-lapse observations revealed small oscillatory movements (one to two cell diameters) with periods of 6–10 hr. From the results of Fig 2 and assuming no initial velocity, a cell would have traveled 620 μm in this time if it were part of an epithelial sheet.

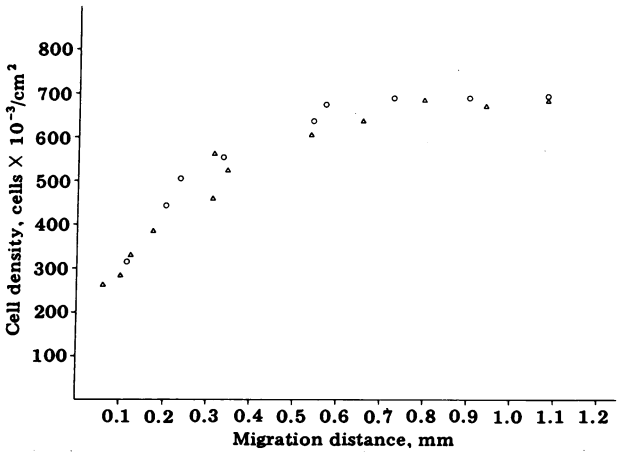

FIG. 3. Correlation of density with the onset of migration. Migration distances were obtained from the two wounding experiments in Fig. 2 that were begun at confluent, submaximal densities. △, 1.7×10^5 cells per cm^2; ○, 1.3×10^5 cells per cm^2.

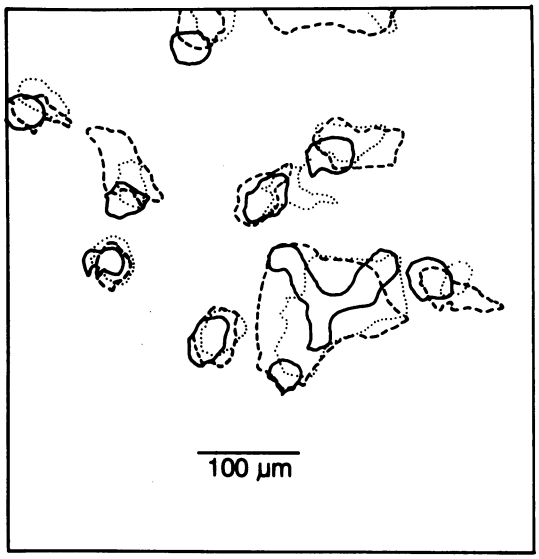

FIG. 4. Outlines of the positions of either isolated MDCK cells or small islands of cells with time: —, 0 hr; ·····, 28 hr; and - - -, 51 hr after observations were begun. Increases in the areas outlined are the result of cell division.

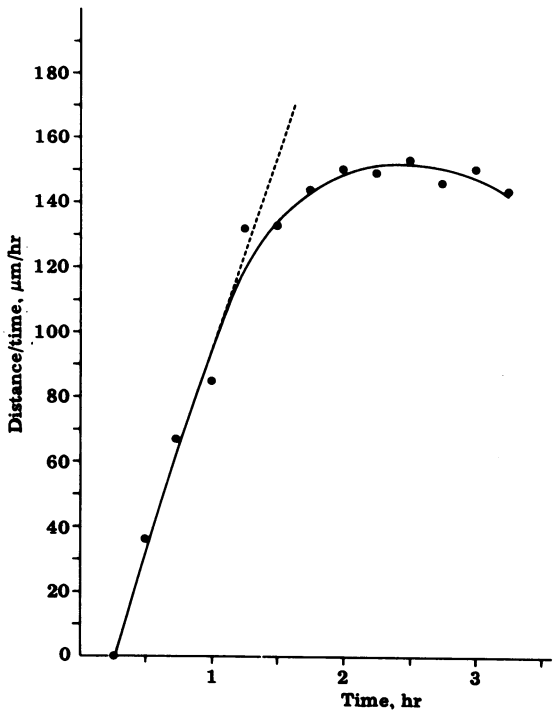

FIG. 5. Data of figure 3 from ref. 10 on the migration of amphibian epidermis in response to wounding, plotted according to Eq. 1.

DISCUSSION

Migration of epithelial cells has been studied previously both *in vivo* (6, 7) and *in vitro* (8, 9). The advantages of studying the migration of epithelial cells by wounding experiments is that the wounding itself acts as a trigger for migration and, because it can be initiated at a precise time and position, measurements of the migration and the factors that influence it can be analyzed. We have performed such wounding experiments and demonstrated that the epithelial cell sheet progresses into the wound at a constant rate of acceleration and that the movement into the wound began with an apparent "initial velocity." Moreover, we have demonstrated that the cell migration into the wound will begin only if the cells in the epithelial sheet have attained a certain threshold density.

The observation that the epithelial sheet possesses a velocity at the instant of wounding was unexpected. Common sense suggests that the cell migration would begin from rest because of the strong adhesion between cells in an epithelial sheet. We considered whether this velocity was the result of an impetus given the cells from mutual pressures caused by their crowding. These forces should be density dependent and would decrease if wounds were made at lower, but confluent, cell densities. Indeed, when cells were wounded at two different submaximal densities, the epithelium did not migrate until a certain time (characteristic for each experiment) had elapsed. Therefore, when cells are wounded at the maximal density, their motion may be accurately described as a brief (<10 hr), rapid acceleration (>0.5 $\mu m \cdot hr^{-2}$), followed by the slower, uniform acceleration seen in Fig. 2. Of course, the acceleration must eventually decrease as either the wound is closed (Fig. 5) or the terminal velocity is reached.

If the epithelium migrates only when its density is at or above the threshold density, then the epithelial cells at that density must possess some property of motion that they lack when isolated or at a subthreshold density. Evidence of this is that isolated MDCK cells barely moved over a period of 51 hr (Fig. 4). In addition, time-lapse observations showed that the cells were at rest or at most oscillating over distances of one to two cell diameters. It is assumed that the forces necessary to continue these random motions exist when the cells comprise an epithelium. At a subthreshold density, the movements of any cell can be accommodated by its neighbors. However, at the threshold density (2.0×10^5 cells per cm^2) these random movements become restricted by the mutual forces that are generated. On the average, these forces cancel out, but once a wound is created, an unopposed net force develops at the wound edge, and it is suggested that this force is observed as the initial velocity in Fig. 2. Such a density-dependent force should occur at any cellular discontinuity, such as the edge of a wide swath of cells or even after the removal of a single cell (11).

The subsequent migration of the cells, however, is not dependent on the density of the control region. The rates of acceleration for the three experiments presented here (0.23, 0.29, and 0.21 $\mu m \cdot hr^{-2}$) are the same within experimental error. The initial push given the cells at the time of wounding no longer plays a part in their subsequent migration. In other words, the property of migration originates in the mass of migrating cells (6, 7). Some authors maintain that an epithelial sheet moves by a traction developed at the leading edge of the cells (8, 9, 12). Our results do not agree with that description. If only those cells at the leading edge were pulling, then the acceleration should decrease as the wound region increases in width because the same force is being applied over a progressively larger number of cells. For the duration of all experiments, however, a constant rate of acceleration was observed (see Fig. 2). By each cell in the wound region contributing equally to the motion of the cell sheet, a constant acceleration can be maintained irrespective of the size of the wound region.

During an experiment, the velocity of the cell migration increased 10-fold from 3 to 32 $\mu m \cdot hr^{-1}$, yet the cell density in the wound region remained approximately 2.0×10^5 cells per cm^2, the threshold density at which the cells began to migrate. The tendency for cells to migrate and for their density to decrease is apparently balanced by their inability to migrate below this threshold density. Therefore, the threshold density is an optimal one for cell movement.

Some may find it tempting to confine or dismiss the acceleration as an artifact of cell culture. That it is not an artifact can be seen in the wounding experiments on the skin of amphibian

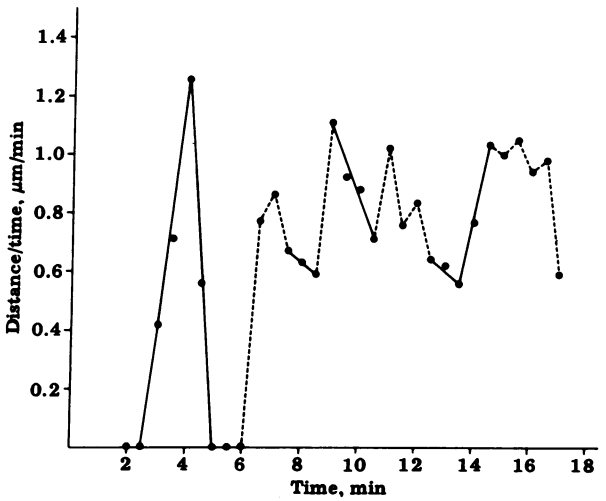

FIG. 6. Data of figure 3 from Abercrombie *et al.* (13) on the movement of embryonic chicken heart fibroblasts in culture, plotted according to Eq. 1. If three or more data points were approximately colinear, a solid line was drawn to represent a period of uniform acceleration or deceleration.

larvae (10). If the distance–time plot of figure 3 from Lash (10) is redrawn according to Eq. 1, then a constant rate of acceleration (112 $\mu m \cdot hr^{-2}$) is revealed (Fig. 5). Also, a deceleration occurred as the wound closed. Although the acceleration is ≈450 times greater than that of MDCK cells, the similarity is sufficient to suggest the generality of acceleration for epithelial movement. The same reexamination was applied to figure 3 of the results of Abercrombie *et al.* (13) on the movements of fibroblasts from primary cell cultures of embryonic chicken hearts. The fibroblasts advanced at a constant velocity (Fig. 6), although the cell motion showed short bursts of acceleration and deceleration.

It is not intended that this difference be interpreted as a distinction between epithelial cells and fibroblasts, but as one between collective and individual cell movements such that a net cellular migration will be an accelerating motion. The random movements of isolated MDCK cells become restricted when they are part of an epithelial sheet. A wound creates a discontinuity in the sheet, and these restricted movements are released and observed as a net pressure pushing the cells into the wound. The subsequent migration of the sheet is seen as the collective result of the random movements of its individual cells. As a result of being bound in an epithelium, they find the path of least resistance and migrate.

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