CELL MOVEMENT AND ADHESION IN THE DEVELOPING CHICK WING BUD: STUDIES ON CULTURED MESENCHYME CELLS FROM NORMAL AND TALPID$^3$ MUTANT EMBRYOS

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SUMMARY

Mesenchyme fragments from early wing buds of normal and talpid$^3$ mutant chick embryos were explanted for culture in plastic Petri dishes and the behaviour of individual cells as they moved out on to the plastic surface was studied by time-lapse ciné photography, followed by statistical analysis. Two parameters of cell movement were recorded: (1) the distances moved over measured 100-s intervals and (2) the length of time each cell spent at rest before moving on. The average speed of movement over the whole path tracked for each cell, inclusive of time at rest, was significantly greater in normal than in talpid$^3$ cells. There was no significant difference between normal and mutant cells in the average distance moved per 100-s step, equivalent to the speed over the whole path exclusive of time at rest, but the percentage of time spent at rest was significantly less in normal than in talpid$^3$ cells. This difference appears to be related to a difference in cell morphology, since it was observed that the mutant cells were more flattened than normals, with very extensive ruffled membranes and short spiky microvilli all round the cell periphery. The relation of these differences in cell morphology and behaviour in vitro to the production of the characteristically fan-shaped limb bud outgrowth and altered pattern of cartilage elements in the developing mutant limb bud is discussed.

INTRODUCTION

The problems of morphogenesis are ultimately problems about individual cells and their interactions - how by their activity they produce embryonic structures and how the activity of each is so integrated with the rest as to lead it to play its appropriate part in the development of a complex whole which may consist of several thousand cells. These problems appear clearly in the chick fore-limb bud, produced by ectodermal and mesodermal cells in particular regions of the trunk coming to proliferate, shift their relations with each other, change shape and finally differentiate, some dying, in such a way as to produce the developing form of the wing.

Almost all experimental studies on this classical embryonic system have entailed the removal, grafting or rearrangement of relatively large tissue pieces; the part played by individual cells is unknown, excepting what has been inferred from sections of fixed material. In our experiments we have studied the behaviour of individual cells

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in vitro as they move out on to plastic from cultured mesenchyme fragments, using normal embryos and also the talpid\textsuperscript{3} mutant, in which wing bud morphogenesis is dramatically altered to give a fan-shaped rather than the normal elongated form (Ede & Kelly, 1964). Though the behaviour of normal cells in culture cannot give, by itself, any certain information about their activity in the limb bud, we may expect that any marked differences observed between normal and mutant cells in vitro will be reflected in their morphogenetic activity in vivo and that the abnormal cell activity will lead or at least contribute to the production of the mutant limb form; per contra this will throw light on what happens in normal embryos.

**METHOD**

Eggs were obtained from matings of heterozygote talpid\textsuperscript{3} carriers maintained at the West of Scotland College of Agriculture, providing both mutant (\textit{ta}/\textit{ta}\textsuperscript{3}) and normal (+/+ and \textit{ta}/+) embryos. Wing buds were removed from stage 25 (Hamburger & Hamilton, 1951) embryos in Tyrode's solution and small mesenchymal explants were prepared for culture in Falcon plastic Petri dishes, using Eagle's Minimal Essential Medium (Flow Laboratories) supplemented with 10\% foetal bovine serum and antibiotics. Dishes were labelled with a code number only, and the origin of the explants was unknown to us until the film analyses (see below) had been completed. Ciné-films were made of the boundaries of the cell sheets moving out on to the plastic from the explants, with a lapse rate of 1 frame every 10 s, using Wild time-lapse equipment with a phase-contrast \times 10 objective. In some cases the optical shadow-casting technique of Hlinka & Sanders (1970) was used. Culture dishes were gassed continuously (5 \% CO\textsubscript{2}:95 \% air) in a small plastic chamber mounted on the stage, and the microscope was enclosed in a Perspex cabinet heated to a controlled temperature of 37.5 °C.

For analysis the films were projected (Specto analysing projector) on to tracing paper. Cells were selected for tracking only if they were situated at the edge of the cell sheet and were not in contact with other cells on all sides. Using one of its pair of nucleoli as a tracking point, we followed the path of each cell, marking (1) its new position at every 10th frame (100 s of culture) and (2) points at which it changed direction. Cells were followed until they (1) left the field, (2) underwent mitosis, (3) detached from the plastic (usually a prelude to mitosis) or (4) became stationary for several hours. Films were made from 8 normal and 8 talpid\textsuperscript{3} dishes; 94 normal cells and 87 talpid\textsuperscript{3} cells were followed.

**RESULTS**

**Random walk analysis**

The formula of Peterson & Noble (1972) predicts that if a cell is moving randomly the most probable distance ($R$) it will be from its starting point after $n$ steps of length $s_i$ is:

$$R = \left[ \sum_{i=1}^{n} (s_i)^2 \right]^{\frac{1}{2}}$$

The distance $s_i$ is measured between each detectable change in direction of cell movement. The authors suggest that to obtain comparisons of departure from randomness the ratio of the mean actual distance moved over the mean predicted distance might be calculated. We have called this value the Random Walk Coefficient (RWC); it is clear that an RWC of 1 would indicate a pattern of movement statistically indistinguishable ($P > 0.05$) from random motion.
Fig. 1. Tracings of cell paths used in the analysis of cell movement. A small segment of the path of a normal (A) and a talpid³ (B) cell has been enlarged to show the amount of time spent at rest. Each circle represents 100 s.
Almost all cells were observed to move radially away from the explant edge. This reflected in both normal and talpid\(^3\) having an average RWC greater than unity (normal = 1.56; talpid\(^3\) = 1.68). A \(\chi^2\) goodness of fit comparison between expected and observed values in both cases showed these values to be highly significant (maximum \(\chi^2\), in observations for one dish, of 640.80, d.f. = 13; minimum \(\chi^2\) of 45.88, d.f. = 6). Analysis of variance showed there was no significant difference between the

![Histogram giving the distribution of speeds of normal and talpid\(^3\) cells as well as the normal curve fitted to these distributions. Mean speeds were: normal, 16.55 \(\mu\)m/h; talpid\(^3\), 12.12 \(\mu\)m/h.](image)

means of \(ta^3\) and normal cells. In both cases the RWC increased, i.e. the cell movements became less random, the further the cell travelled from the edge of the cell sheet: cells that during the film moved less than 7 \(\mu\)m centrifugally appeared to move nearly at random with average RWC for normal = 1.03, talpid\(^3\) = 0.97; from 7 to 14 \(\mu\)m, average RWC values were normal = 1.69 and talpid\(^3\) = 1.65; beyond 14 \(\mu\)m, normal = 2.12, talpid\(^3\) = 2.38.

**Cell speed analysis**

When marking the nucleolar positions at 100-s intervals 2 parameters of cell movement were recorded: (1) the distances moved over the measured 100 s and (2) the
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length of time each cell spent at rest before moving on. These are illustrated in Fig. 1 which shows tracings of the movements of 6 cells, each from a different culture of either a normal or a talpid<sup>3</sup> explant. The smaller-scale tracks show only the first parameter, but the blown-up segments of two show the second also, each circle representing a rest of 100 s.

The average speed of movement over the whole path, i.e. inclusive of time at rest, is significantly greater, by analysis of variance, in normal than in talpid<sup>3</sup> cells (average speed: normal = 16·55 μm/h, talpid<sup>3</sup> = 12·12 μm/h; \( F = 10·71, P < 0·5 \% \)). Results within each group of normal and of talpid<sup>3</sup> dishes were homogeneous (normal: \( F = 2·50, P > 5·0 \% \); talpid<sup>3</sup> \( F = 1·66, P > 10·0 \% \)). There is no significant difference between normal and mutant cells in the average distance moved per 100-s step, equivalent to the speed over the whole path exclusive of time at rest (\( F = 0·67 \) and \( P > 25·0 \% \)), but the percentage of time spent at rest is significantly less in normal than in talpid<sup>3</sup> cells (normal = 75·5 \%, talpid<sup>3</sup> = 82·16 \%; \( F = 12·96, P < 0·5 \% \)).

In order to test the data against a normal fitted curve a computer programme was designed to draw a large number of random samples of cell speed (inclusive of time at rest) from the measured data. The means of these samples are distributed as in the histogram Fig. 2. The theoretical normal curve fitted to these histograms (Snedecor & Cochran, 1971, p. 70) shows them to be approximately normally distributed. Both are skewed slightly to the right (\( g_1 = 0·75 \) for normal cells and 0·54 for talpid<sup>3</sup>; in both cases the expected standard deviation is 0·02). The curve for normal cells is flatter than expected (\( g_2 = -2·67, \) expected s.d. = 0·05) while that for talpid<sup>3</sup> is much more sharply peaked than would be expected for a normal curve (\( g_2 = +37·25, \) expected s.d. = 0·05). The significance of these deviations is not obvious. The fact that the distributions are unimodal indicates that in both cases we are measuring single populations of cells, though since we have selected cells at the edge of the cell sheet, presumably the fastest moving cells in each case, this does not necessarily indicate that all cells within the explants are members of these populations.

Cell morphology

The statistical analysis reported above confirms and gives precision to differences which can be seen when the films are run. There are also qualitative differences in cell morphology, not recognizable in every case but in the majority, between normal and talpid<sup>3</sup> cells. These are best seen in motion, but some of these differences are shown in stills from the optical shadow-cast films, where a shadowed view of the cell gives an effect similar to stereo-scanning (Fig. 4, p. 313). Normal cells are typically elongated, with a ruffled membrane at the leading edge and attachments to the plastic and to other cells by long cytoplasmic extensions, chiefly at the trailing edge; these attachments break as the cell advances and the cytoplasm contracts into the cell body – in the time-lapse film it appears to snap back like elastic. Talpid<sup>3</sup> cells are much more flattened than normals and more extensive areas of the edge appear (when the cell is actually moving) to be involved in ruffled membrane production; there are few elongated cytoplasmic extensions produced, but many short spiky microvilli all round the cell periphery which attach to the plastic and to other cells.
DISCUSSION

Outgrowth from the explant

The non-linear random motion of cells at the origin of their measured paths mentioned in the results is well illustrated in Fig. 1. This could be a result of a higher chance of contact and consequent contact inhibition movements nearer the explant, though care was always taken that the cells were free of any contacts at the start of measurement. On the other hand, a classification of cells by distance moved away from the explant will automatically select for degree of randomness, since the less random cells move further, other things like velocity being equal. We could detect no difference between \textit{talpid}^3 and normal cells by our random walk analysis.

Relation between movement and adhesion on plastic

Ede & Agerbak (1968) suggested, on the basis of reaggregation experiments, that \textit{talpid}^3 cells were more adhesive to each other than normal cells and Ede & Flint (1975) have confirmed this by direct measurement. It is known that cultured cells adhere to plastic by their edges and their undersurfaces through local modifications of the cell surface—by ‘microvilli’ or ‘microspikes’ at the edges (Hodges & Muir, 1972; Tuffery, 1972; Witkowski & Brighton, 1971) and by smooth projections on the undersurfaces (Revel & Wolken, 1973). Our present direct observations are limited to the attachments at the cell edge, where there are many more fine short attachments in \textit{talpid}^3 cells, but the flattened appearance of the mutant cells strongly suggests that there are also more adhesions between the undersurface and the plastic than in normals. This excessive production of adhesion points may be the cause of the periods of rest, when the \textit{talpid}^3 cells are virtually immobilized, which leads to the overall reduction in speed of \textit{talpid}^3 cells compared with normal cells.

Cell movement and adhesion in the developing limb bud

Do corresponding characteristics appear in the limb bud and, if they do, what part do they play in morphogenesis? The activity of cells aggregating in suspension cultures, analysed in both normal and \textit{talpid}^3 by Ede & Flint (1975), is a step closer to the \textit{in vivo} situation. Here the mutant cells wrap around each other more closely and extensively than normal cells do, with contact between cytoplasmic microspikes. The behaviour and appearance of the cells in forming these aggregates does reflect their behaviour and appearance on plastic. In the wing bud itself, a scanning electron-microscope study by Ede, Bellairs & Bancroft (1974) suggests that corresponding characteristics also appear \textit{in vivo}. Undifferentiated mesenchyme cells are loosely arranged rather than closely packed as they are in the aggregates, so cytoplasmic extensions appear as elongated filopodia rather than microspikes. The cells appear to be in a state of considerable activity in the production of cytoplasmic processes, but whereas the normal cells tend to be elongated, with filopodia with long terminal arborizations arising chiefly at each end, \textit{talpid}^3 cells have shorter but more numerous filopodia which arise more generally around the cell periphery. In all 3 situations—on
plastic, in aggregates and in the limb bud – there appear to be more sites on the \textit{talpid}^3 cell surface involved in adhesion, and we have shown here that this affects movement as well as adhesion; if cell movement and adhesion do have a role in limb morphogenesis, these differences may be expected to lead to different forms of development.

The problem of whether cells are capable of moving when surrounded on all sides by other cells, either in monolayers or in solid masses, or whether they are immobilized in these situations by contact inhibition, has been investigated by many workers, e.g. by Armstrong & Armstrong (1973) who review much of the literature; it emerges from a wide variety of experiments that cells in such situations do retain their capacity for movement. Within the limb bud at least some small movements – shiftings or shufflings – must occur: e.g. the chondroblasts within each long bone rudiment become lined up so that in longitudinal section they appear oriented in rows at right angles to the long axis; in \textit{talpid}^3 the chondroblasts are not so oriented and are not lined up (Ede & Agerbak, 1968); the inference now seems clear that some movement is necessary to produce the orientation found in normal chondroblasts and that the restriction of movement in \textit{talpid}^3 cells due to their excessive adhesion to each other prevents it in the mutant.

There are two aspects of limb bud morphogenesis in which, as suggested by Ede & Agerbak (1968), cell motility and adhesion might play an important part: (1) in limb bud outgrowth, (2) in the formation of precartilage condensations. Both of them have a different form in the \textit{talpid}^3 mutant.

\textit{Limb bud outgrowth}

The increase in mass of the limb bud is produced by cell proliferation; the problem is to find what cell activity produces its distinctive elongated form. The distribution of mitosis within the bud will be important; Amprino (1965), Hornbruch & Wolpert (1970) and Ede, Flint & Teague (MS in preparation) have shown that there is a proximo-distal gradient, with the mitotic index highest distally. Mitolo (1971) has demonstrated, and O. K. Wilby (unpublished) has confirmed, with computer simulation that it is possible to obtain a somewhat limb-like outgrowth using only such controlled mitotic distributions, but the control would have to be complex and rigidly controlled. A much more simple and flexible control of outgrowth is obtainable if some distalward movement of cells is introduced. Stark & Searls (1973) showed that small blocks of wing mesenchyme implanted into early wing buds elongated as much as 16-fold in the proximo-distal direction during 3 days of growth, but grew very little transversely to that direction. They observed, like all other workers, no evidence for orientation of mitotic figures, but propose a ‘polarity of outgrowth’ to account for the elongation, influenced by the apical ectodermal ridge. In terms of cellular activity it would seem necessary to interpret this polarity of outgrowth in terms of cell movement.

Amprino (1965) and Hornbruch & Wolpert (1970) have emphasized the part the ectoderm might play, especially the apical ectodermal ridge, in moulding the growth of the underlying mesoderm; this again implies a movement of the mesenchyme cells in the process, though it might be a forced movement resulting from pressure built up
through cell proliferation, with space available only at the distal region, as Gould, Day & Wolpert (1972) have suggested.

Ede & Law (1969) have shown, using computer simulation, that a fair approximation to normal limb bud outgrowth can be obtained using a disto-proximal mitotic gradient and incorporating some distalward movement, and moreover that reducing the movement produces a shape very like that of the fan-shaped bud of the talpid\textsuperscript{3} mutant. Shaping by a constraining ectoderm is not necessary, but it is of course not excluded.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Camera lucida drawings of (A) stage 26 normal wing bud, (c) stage 26 talpid\textsuperscript{3} limb bud. Below these are silhouettes of photographed spills of (b) black ink and (d) treacle. The liquids were spilt on to a polished glass surface tilted at an angle of 1 or 2°.}
\end{figure}

Whether the cell movement is an active one, involving activity of cytoplasmic processes and with some directional element, or passive, our observations on talpid\textsuperscript{3} suggest that the extent of cell adhesion does play an important part in limb outgrowth. If distalward cell movement is essential for normal limb growth, reduction of movement would produce a fan-shaped outgrowth; in talpid\textsuperscript{3} the bud is fan-shaped, and we have shown that its mesenchyme cells are abnormally adhesive and that their motility is restricted. If the movement is passive, it will be a question of the cohesiveness of the mass of cells and hence the viscosity of the flowing system. As a simple analogy, tipping a quantity of thin oil on to a surface will give a long narrow stream, but tipping out a heavy syrup will give a broad stream which fans out as it advances (Fig. 3). If on the other hand, as Ede et al. (1974) suggest, there are indications that there is active cell movement within the limb bud, our results show that we should expect it to be reduced in the talpid\textsuperscript{3} mutant as a result of increased adhesion between the mesenchyme cells.
Precartilage condensations

The most striking morphogenetic event within the limb bud is the appearance of the condensations which prefigure the elements of the cartilaginous and subsequently the osseous skeleton. The formation of these condensations by close packing of the mesenchymal cells in these regions was first described by Fell (1925), whose account has been confirmed by several authors, e.g. Montagna (1945) and Saunders (1948), at the light-microscope level. The process has been investigated at the ultrastructural level in the chick leg bud by Thorogood & Hinchliffe (1975), who found that the pre-chondrogenic cells were characterized by large areas of surface contact in the early stages of condensation formation, when there was an increase of about 60% in the cell packing density. The close stage is a transient one, since it is followed by secretion of extracellular mucopolysaccharide matrix which forces the cells apart, and it may be that this is why close association of cells was not seen in the electron-microscope study by Searls, Hilfer & Mirow (1972). The apparent absence of close mesenchymal association in the study of the wing bud by Gould et al. (1972) is puzzling, but may be due to a fixation artifact since they did observe an increase in cell packing density of 30%.

The increased cell density in the condensation regions is undoubtedly real. No investigators have detected a local increase in mitosis that would account for the increased density and, though cell proliferation is still not entirely ruled out, it is most likely that it is produced by cell movement. Gould et al. (1972) suggest that the non-precartilage cells are dispersing while the precartilage cells remain steady, but Thorogood & Hinchliffe's (1975) observation of a 60% increase in packing, together with close association of the precartilage cells and reduction of intercellular spaces, supports the suggestion of Ede & Agerbak (1968) that there is a centripetal movement within the condensations. It is noteworthy that a number of observations on cartilage development in vivo and in vitro (e.g. Holtfreter (1968), Abbot & Holtzer (1966), Toole (1972), Drews & Drews (1972, 1973)) suggest that close cell packing involving changes in cell adhesiveness is a necessary step in chondrogenesis. In this case again, our observations on talpid3 support the hypothesis that cell adhesion and movement play an important part. If centripetal cell movement is essential for normal condensation formation, reduction of movement would lead to less well defined condensations, and this is what is found in the mutant. In talpid3 the edges of the condensations are indistinct, the cells merging with those of the surrounding mesenchyme and adjoining cartilage elements – e.g. the radius and ulna, and the metacarpals – tend to merge and form a single band (Ede & Agerbak, 1968). As we have shown, talpid3 mesenchyme cells are abnormally adhesive to each other; the centripetal movement is therefore likely to be impeded, producing the blurring of the edges and the failure of elements to separate properly that is observed.

Relation of cell adhesion and movement to other factors in morphogenesis

Though we have concentrated on these parameters in this investigation, other studies show that other cell activities are also affected in talpid3, notably cell death and mitosis; the former is reduced (Hinchliffe & Ede, 1967; Ede & Flint, 1972) and the
latter increased (Ede & Flint, 1972). These will also have effects – possibly synergistic with those of adhesion and movement – on morphogenesis, as described by Hinchliffe & Thorogood (1974) and Ede, Flint & Teague (in preparation). What may be even more interesting – their relation to each other at the cellular level – remains to be investigated.

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REFERENCES


Movement in normal and talpid³ cells


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Fig. 4. Frames taken from 16-mm time-lapse movie film of normal and talpid³ explanted limb mesenchyme cells (0-F). Corresponding tracings (A-C) of the cells are given at the top of the figure. Illumination was by optical shadow casting. A, D, normal. B, C and E, F, talpid³.
Movement in normal and talpid\textsuperscript{3} cells

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{\textbf{Movement in normal and talpid\textsuperscript{3} cells.} (A) Normal cell in culture. (B) Talpid\textsuperscript{3} cell in culture. (C) Normal cell in migration. (D) Talpid\textsuperscript{3} cell in migration. (E) Normal cell in division. (F) Talpid\textsuperscript{3} cell in division.}
\end{figure}