Behavioural transformation in chick yolk-sac cells

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SUMMARY

During the first 3 to 4 days of development in the chick, the extraembryonic part, the yolk-sac, expands to encompass the yolk mass. The yolk-sac, initially a two-layered epithelial sheet, is pulled out by the action of a specialized band of cells at its periphery. These attach to the overlying vitelline membrane and move out over it.

However, unincubated blastoderms are not attached to the vitelline membrane, and each comprises a loose assemblage of rounded cells, not an epithelial sheet.

The transformation of the unincubated blastoderm into an actively expanding epithelial sheet has been followed in culture, using hanging-drop cultures of fragments to observe large-scale behaviour, and disaggregated blastoderms to observe individual cell behaviour. The timing of events in culture accords well with related events in ovo, and the possibility that vitro changes are merely a response to culture conditions has been largely excluded.

0-6 h in ovo. No cells attached to the vitelline membrane. All cells loosely attached to one another.

0-6 h in vitro. Cells do not attach to the glass. The cells of disaggregated blastoderms are rounded and stationary.

6-10 h in ovo. Cells at the blastoderm periphery attach to the vitelline membrane inner surface, but expansion does not start. The cells remain loosely attached to one another.

6-10 h in vitro. Cells begin to stick to the glass surface. Cells from disaggregated cultures move around as individuals. They remain rounded with long, narrow filopodia. If two cells collide, the adhesion tends to be brief.

10+ h in ovo. The blastoderm starts to expand rapidly as a cohesive epithelial sheet, pulled by its specialized ‘edge’ cells. All yolk-sac cells become tightly attached to one another.

10+ h in vitro. Blastoderm fragments start to spread rapidly as flattened epithelial sheets. There is no sign of specialized ‘edge’ cells. Cells at the periphery of any fragment take on the role of the edge. Cells from disaggregated cultures flatten out on the glass with wide lamellae all round. When two cells collide, they now tend to stick permanently together.

The role of these changes in the mechanics of blastoderm expansion is briefly discussed.

INTRODUCTION

In the first 3 to 4 days of development after laying, the chick blastoderm expands to encompass the yolk mass. Most of the wide expanse of tissue required comprises the extra-embryonic yolk-sac, at first only two-layered: epiblast and hypoblast. New (1959) established that expansion is largely the result of the active centrifugal movement of a narrow marginal band of cells which use the vitelline membrane inner surface as a substratum for locomotion. These ‘edge cells’ are the only ones which attach to the vitelline membrane, and their organization and behaviour has

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been the subject of several investigations (Bellairs & New, 1962; Bellairs, 1963; Bellairs, Boyde & Heaysman, 1969; Downie & Pegrum, 1971).

However, in newly laid eggs, the blastoderm is not attached to the vitelline membrane. Vakaet (1962) found that fresh blastoderms attach after 5-6 h incubation, and I have found (Downie, 1971) that expansion normally begins after 8-12 h incubation, attachment occurring rather earlier. The exact onset of attachment and expansion is probably dependent on storage time and temperature, since Arora & Kosin (1968) have shown that metabolic processes are not halted in fertile hens' eggs stored at 18–20 °C, and Vakaet (1962) found that most blastoderms had attached after a week's storage at room temperature.

Clearly two important events occur during early incubation: (1) some blastoderm cells attach to the over-lying vitelline membrane, and (2) soon thereafter these cells start to migrate centrifugally as a coherent band across the vitelline membrane inner surface, making the blastoderm an increasingly wider sheet. This paper investigates the cell behaviour changes involved in these events. A future paper will discuss the ultrastructural changes.

Trinkaus (1963, 1969) has investigated rather similar events at the onset of Fundulus heteroclitus epiboly. In particular, Trinkaus (1963) disaggregated and cultured Fundulus blastoderms to investigate individual cell behaviour changes. A similar approach is adopted here.

MATERIALS AND METHODS

Chick blastoderm cells were observed either as outgrowths from fragments of whole blastoderms grown in hanging drop cultures or as single cells and clumps cultured after blastoderm disaggregation in Sterilin culture chambers. In all cases, cells were moving on the surface of acid-cleaned glass coverslips, in a medium of 90 % '199 (Morgan, Morton & Parker, 1950, with Hanks' salts) plus 10 % chicken serum.

For hanging drop cultures, whole blastoderms were removed into Hanks' balanced salt solution and washed free of yolk with a fine jet of Hanks'. Blastoderms were then cut into pieces as required.

Disaggregation of chick blastoderms up to 12 h incubation is a very simple matter. The blastoderms were removed into calcium- and magnesium-free Hanks' and the yolk washed off. Individual blastoderms were then immersed in 0-75 ml culture medium and passed 5 times through a narrow bore pipette (internal diameter 0-6-0-8 mm). The cells were centrifuged down for 45 sec at 500 rev/min and the supernatant, containing mainly yolk particles, removed. The cells were then resuspended in 0-5 ml culture medium and set up in Sterilin chambers.

Disaggregation of yolk-sac tissues from older embryos is more difficult since the cells adhere tightly together. Fortunately for these experiments, a suspension containing a few single cells amongst large numbers of clumps was adequate. This was obtained by the above procedure with a few additional passages through the fine pipette.
Behavioural transformation in chick yolk-sac cells

Fig. 1. Outgrowth of cells in hanging-drop cultures from orientated fragments of unincubated blastoderms. (A) The areas of blastoderm explanted. The dashed lines and letters correspond to those in (B). (B) Superimposed outline drawings of typical explants from unincubated blastoderms. The numbers indicate hours after explantation. The appearance of cells at the explant edges was similar all round. Rates of outgrowth from different edges were similar during the early stages, but appeared to differ later on. The significance of this, if any, is unclear.

Cultures were recorded by means of still phase-contrast photographs, or by time-lapse filming, with subsequent frame-by-frame analysis.

OBSERVATIONS

Hanging-drop cultures

Cultures of unincubated blastoderm fragments were originally grown to observe the development of normal ‘edge cells’. The fragments were either blastoderm quarters, or outer and central regions (Fig. 1A) with the normal outer edge clearly orientated. Unfortunately, no difference emerged between normal central and edge areas either in outgrowth pattern or rate. Typical outline outgrowths are shown in Fig. 1B.

What did emerge was a very clear age-difference. The first cells to emerge from the explant (Fig. 2A), 8 h after setting up, were rounded with narrow filopodia. The cell centre bulged with yolk granules, obscuring the nucleus. The mean area of the cell centre, excluding filopodia, was 482±4 ±116.2 μm² (19 cells; mean ± standard deviation). These cells jostled about rapidly, making mainly brief adhesions with one another (this is quantified later) and often changing shape greatly. The direction of movement was predominantly normal to the explant edge but uncoordinated, with cells moving at widely different rates and in different directions (Fig. 3A).
Cells at the edges of different explants.

(A) Rounded, filopodial cells emerging from an explant of unincubated blastoderm, about 8 h after explantation.

(B) Flattened, epithelial cells at the edge of an unincubated blastoderm fragment, about 20 h after explantation.

(C) Flattened, epithelial cells at the edge of an explant of 24 h epiblast soon after outgrowth emergence.

After longer periods in culture, the outgrowing cells changed dramatically. They flattened out (mean cell-centre area for 21 cells: $1318.5 \pm 333.1 \mu m^2$), making their nuclei clearly visible (Fig. 2B). The narrow filopodia became wide, flat lamellae, and the cells formed a close-knit typically epithelial sheet, rarely breaking apart from one another. Movement of cells at the edge of a sheet seemed co-ordinated, all the cells moving in the same direction, i.e. normal to the explant edge, at the same general rate (Fig. 3B).
Fig. 3. This shows the movements of cells at the edge of three different explants. The records are taken from time-lapse films. In each case the interval between records shown is 10 min (60 frames on the original films). The numbers represent the time in minutes since the start of the record. All three are to the same scale.

(A) 'Early' cells from an unincubated embryo, 8-10 h after explantation. Outlines of the whole cells are shown at the start, and the dots record the progress of the centres of these cells.

(B) 'Late' cells from an unincubated embryo, 20-22 h after explantation. The circles represent the nuclei of the cells at the start, and the dots record their centres as they move. The dashed lines show the positions of the leading lamellae of the explant at start and finish.

(C) Epiblast cells from a 24 h embryo, 6-7.5 h after explantation. Nuclei and lamellae are recorded as in (B).
This change might merely have been a response to culture conditions, but this seemed unlikely, since the outgrowth pattern of epiblast or hypoblast explants from 24 h embryonic yolk-sac was of the epithelial type from the start, never going through the rounded filopodial stage (Figs. 2C, 3C).

Clearly, during the first day of incubation, early blastoderm cells transformed into epithelial cells; and this change occurred not merely in the normal blastoderm edge, but apparently in all cells of the blastoderm. This phenomenon was further investigated using disaggregated blastoderms, to observe more closely the behaviour of individual cells before, during and after the transformation process.

Disaggregated cultures

General observations

The disaggregation procedure for early blastoderms outlined in Materials and Methods produces a suspension of single cells, small clumps and some free yolk.

The behaviour of cells from unincubated blastoderms, observed in time-lapse films, falls into three stages.

0–5 h. The cells remain rounded, with little or no attachment to the glass. If the culture is inverted, most cells fall off. This is not merely a reaction to the disaggregation procedure, since 24 h hypoblast or epiblast cells, subjected to a rougher disaggregation treatment, attach to and flatten on glass within 2 h.

5–10 h (Fig. 4A). After about 5 h incubation, individual cells attach to the glass by long filopodia, appearing identical to the cells at the edge of the early explant outgrowths in hanging-drop cultures. They move around rapidly with no particular orientation, frequently colliding with other cells or clumps. Adhesions so formed are usually of short duration.

10+ h (Fig. 4B). After 10–12 h clumps and single cells begin to flatten out, as in explant cultures. The movement of flattened single cells is still without apparent orientation, but those in clumps show co-ordinated epithelial spreading. When single cells collide with one another or with clumps, the adhesions tend now to be long-lasting or permanent. In confirmation of this, it is difficult to find single cells in later cultures; all have joined large spreading clumps.

Observations on disaggregated blastoderms confirm and extend the information from explant cultures. We find in addition an early non-adhesive stage, and a change in collision behaviour. The latter is particularly important since it determines whether or not the cells are behaving as epithelial cells.

Collision analysis

The relation between collision behaviour and the epithelial transformation described above has been quantified by following the durations of the adhesions formed after a large number of collisions, seen on time-lapse films of disaggregated cultures. The analysis is arbitrary in counting only certain categories of collision: only those between single cells and other single cells; or single cells and clumps, but not between two clumps. Start and finish of a
Fig. 4. Cultures from disaggregated unincubated blastoderms. (A) Cells after 6.5 h in culture. (B) Cells after 22 h in culture.
collision are also arbitrary. Filopodia or lamellae often come into brief contact and rapidly retract. These are not counted as collisions. A collision begins when the central dense areas of two cells come so close together that no space between is visible, and ends when a cell becomes completely free again. The analysis involves 207 collisions from 23 cultures. Eleven of these cultures were of unincubated blastoderms, 12 of embryos pre-incubated \textit{in ovo} for 10–12 h. The latter were necessary because of the scarcity of later collisions in cultures of unincubated blastoderms (most cells in such late cultures had become permanent members of clumps). The ‘real age’ in terms of incubation time of the pre-incubated cultures is rather dubious. Tests showed that the warming-up time of eggs in the incubator used was 2 h or more, though, of course, development begins before the full incubation temperature is reached. As a compromise, 1·5 h has been subtracted from the age of these cells in the analysis.

Figure 5 plots adhesion duration against the age of the cells at the start of a collision. The lapse interval in the films was 10 sec. ‘Finite’ adhesions ended during the duration of the film; ‘unbroken’ ones did not. Since some of the latter occurred near the end of a film, only ‘unbroken’ adhesions lasting a certain minimum time (100 min) are counted.

The results are fairly clear-cut. During the early stages, 0–7 h and 7·25–10 h, adhesions tend to be short-lived; 64·7 % and 55·8 % respectively lasting under 50 min. The number of ‘unbroken’ adhesions formed is small; 11·7 % and 23·1 %. After 10 h, however, ‘unbroken’ adhesions and ‘finite’ ones occur in almost equal proportion: in the period 10·25–12 h, 48·9 % are ‘unbroken’; from 12·25–14 h, 53·5 % are ‘unbroken’. After 14 h the numbers of ‘finite’ adhesions become rather small – only 31 %.

The analysis confirms the impression that after about 10 h incubation an epithelial transformation occurs, when cells, on colliding, tend to adhere permanently together, rather than move apart.

\textit{Could the epithelial transformation be a response to culture conditions?}

In a similar analysis of behaviour changes in \textit{Fundulus} early development, Trinkaus (1963) was reluctant to observe cells in culture longer than 6 h, for fear that culture conditions might alter behaviour. To eliminate this possibility, a timing experiment was carried out.

Cultures of disaggregated individual blastoderms, seven unincubated and seven pre-incubated for 6 h \textit{in ovo}, were filmed with an interval of 35 sec. The time of onset of transformation in each culture was assessed from the films. If transformation is the result of culture conditions, it should occur after the same time \textit{in vitro} in both sets of cultures. If transformation is a normal developmental event in these cells, its onset should be related to the total incubation time of the cells.

Table 1 shows the result of this experiment. Transformation occurs at the same total incubation time in both sets, and is therefore likely to be a normal developmental event.
Fig. 5. Collision analysis. Cells are grouped according to their age at time of collision. The histograms show the percentage of cells entering adhesions of different durations, for each time period.
Table 1. Transformation in dissociated early chick blastoderm cells

<table>
<thead>
<tr>
<th>Culture system</th>
<th>Number of cultures</th>
<th>Transformation time (h)*</th>
<th>Time in vitro (mean and range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells cultured in vitro throughout</td>
<td>7</td>
<td>12·5</td>
<td>12·5 (8·75–14·50)</td>
</tr>
<tr>
<td>Blastoderms incubated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 h in ovo before dissociation and in vitro culture</td>
<td>7</td>
<td>13·25</td>
<td>11·75 (9·25–13·75)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Corrected time†</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12·5</td>
<td>11·75 (8·75–14·50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7·25</td>
<td>(4·75–9·25)</td>
</tr>
</tbody>
</table>

* All times judged to nearest 0·25 h.
† Corrected time is total incubation time less 1·5 h warming-up time for the series incubated in ovo.

**DISCUSSION**

The work reported here examines changes occurring during the first dozen or so hours of development in the behaviour of chick yolk-sac cells. These changes are most easily observed in culture, using tissue fragments or disaggregated blastoderms, and appear to correspond well in time-scale with events normally occurring in ovo. The possibility that changes observed in vitro are merely a product of culture conditions has largely been excluded.

There are three stages (the times are approximate, and there is considerable individual variation):

0–6 h in ovo. No cells are attached to the vitelline membrane. All the cells are rather loosely attached to one another.

0–6 h in vitro. The cells do not attach to the culture dish, or move around actively in it.

6–10 h in ovo. Cells at the blastoderm periphery attach to the vitelline membrane inner surface, but expansion does not begin. All cells remain loosely adherent to one another.

6–10 h in vitro. Cells stick to the glass and move over it as individuals. Adhesions are short-lived. The cells appear rounded, with narrow filopodia.

10 + h in ovo. The blastoderm starts to expand rapidly as a cohesive epithelial sheet. The motive force for expansion comes from the centrifugal movement of a band of specialized ‘edge cells’ at the blastoderm periphery. All the cells of the yolk-sac (the extra-embryonic part of the blastoderm) become tightly attached to one another.

10 + h in vitro. On colliding, cells tend to stick permanently to one another. Individual cells flatten out, and clumps spread as flattened epithelial sheets.

We can ask two general questions about these events. First, how do they come about? The answers to this are the subject of current investigation. It seems likely that the cell surface, and intracellular structures such as microtubules and microfilaments are involved. Secondly, what is the role of these changes in the
unfolding of normal chick development? In his study on disaggregated *Fundulus* blastulae and gastrulae, Trinkaus (1963) was able to relate differences in cellular adhesivity (measured by their degree of flattening on glass) at different stages, to the onset of epiboly, a period of rapid epithelial spreading. A similar relationship is clear in the chick. Chick blastoderm expansion involves a 900-fold increase in tissue area in 3-4 days. This is achieved partly by proliferation, partly by increase in the area occupied by each cell — that is, by flattening out. During the early stages of expansion, this flattening is largely passive, due to the pull of the migrating edge cells; but active spreading may also be involved. Since the pull exerted by the ‘edge cells’ is considerable, the blastoderm cells must be stuck tightly together: hence the early development of a close-knit epithelial organization.

This work throws little light on the development of the specialized ‘edge cells’, unless to suggest that any early blastoderm cells in a peripheral position can behave as ‘edge cells’. Spreading of a clump occurs when cells at its periphery send out lamellae to attach to the surrounding substrate. Cells within the clump, like cells within a whole early blastoderm, may be inhibited from sending out processes by their neighbours — perhaps some form of ‘contact inhibition’. It was certainly impossible to find clear differences in spreading behaviour in culture between early blastoderm cells normally at the blastoderm periphery and those at the periphery of a blastoderm fragment, but normally surrounded by other cells.

Why should the embryo pass through a stage when its cells are rounded, immobile and loosely attached? There is no obvious answer other than that the early cells are immature. Lentz & Trinkaus (1971) find rather immature junctional complexes in *Fundulus* blastulae, the full complex developing during the rigours of epiboly. The work of Trelstad, Hay & Revel (1967) and Sanders & Zalik (1972a) on chick, and Sanders & Zalik (1972b) on *Xenopus* suggest that a low cell-cell adhesivity and immaturity of junctional complexes may be common in the early stages of vertebrate embryos in general. The relevance of this to blastoderm expansion in chick must await more detailed study of the ultrastructural development of chick yolk-sac cells.

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