Morphogenetic analysis of changing cell associations following release of 2-cell and 4-cell mouse embryos from cleavage arrest

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SUMMARY

Two-cell and four-cell mouse embryos were cultured in Cytochalasin D (CD) for 40-48 h. They were fixed for light and electron microscopy at various times after washing off the CD. Cleavage-arrested embryos in CD had well separated blastomeres but by 1 h from washing the embryos had compacted, in most cases without undergoing cell division. By 2 h after release from arrest one blastomere of the 2-cell arrested embryos had become crescent shaped and at 4–5 h the crescent-shaped blastomere had started to spread over the surface of the other rounded blastomere. This process continued until by 16-24 h from explantation to fresh medium one blastomere had almost completely engulfed the other. A similar process occurred in 4-cell arrested and released embryos. At this stage the embryos had accumulated fluid and become blastocyst-like vesicles. In 20% of 2-cell and 4-cell embryos one or two blastomeres underwent one cell division after release from arrest. Serial sections of these embryos lead to the conclusion that one or both progeny of the first cell to divide tended to be engulfed by the later dividing or non-dividing cell(s). These results are discussed in relation to the differentiation of ICM and trophectoderm in blastocysts.

INTRODUCTION

After three to four mitotic divisions early mouse embryos undergo compaction with the formation of tight and gap junctions between blastomeres (Ducibella & Anderson, 1975; Ducibella, Albertini, Anderson & Biggers, 1975; Magnuson, Demsey & Stackpole, 1977). Changes in the cell surface (Calarco & Epstein, 1973; Edidin, 1976) and in the distribution of microtubules and microfilaments (Ducibella & Anderson, 1975; Ducibella, Ukena, Karnovsky & Anderson, 1977) also occur. After the 32-cell stage, the embryo begins to accumulate fluid becoming a blastocyst with the formation of the inner cell mass (ICM) and outer trophectoderm.

Compaction and blastulation are Ca²⁺ dependent (Ducibella & Anderson, 1975, 1979) and can be prevented by inhibitors affecting the cytoskeleton (Granhholm, Brenner & Rector, 1979; Surani, Barton & Burling, 1980) and protein glycosylation (Surani, 1979) and also by antibodies against cell surface
molecules (Kemler, Babinet, Eisen & Jacob, 1977; Ducibella, 1979; Johnson et al. 1979).

Cytochalasin D (CD) is believed to exert its effect on cells primarily by inhibiting the polymerization of microfilament actin (Bray, 1979; Brown & Spudich, 1979; Lin & Lin, 1979; Lin, Tobin, Grumet & Lin, 1980). Cytochalasin allows chromosomal replication to proceed while arresting cytoplasmic division. It can therefore be used for the examination of processes of differentiation which depend on developmental age, nuclear:cytoplasmic ratio and number of chromosome replications, but which do not depend on number of cell divisions or cell interactions during early cleavage. The early differentiation of mouse embryos in the formation of the blastocyst seems to be such a process (Tarkowski & Wroblewska, 1967; Snow, 1973; Smith & McLaren, 1977; Granholm et al. 1979; Surani et al. 1980). Experiments using the cytochalasins on the embryos of ascidians (Whittaker, 1973, 1979; Satoh, 1979), amphibians (Cooke, 1973), nematodes (Laufer, Bazzicalupo & Wood, 1980) and mammals (Surani et al. 1980) indicate that cytoplasmic differentiation and morphogenesis can proceed in the absence of cell division. Thus cleavage-arrested embryos provide us with a simplified model with which to investigate the influence of cell interactions and cell position in development.

We have previously reported the formation of blastocyst-like structures by 2-cell mouse embryos released from arrest in CD (Surani et al. 1980). In this study we have carried out a cytological examination of cleavage-arrested embryos following their release from arrest and during their development into blastocyst-like structures. Our observations reveal rapid changes in cellular interactions and provide further evidence for the probable mechanism of formation of the ICM and trophectoderm.

MATERIALS AND METHODS

Animals

Embryos were obtained from an outbred strain of CFLP mice (Anglia Laboratories Ltd. U.K.) which were superovulated using 5 i.u. pregnant mare's serum (PMS) followed 42-48 h later by 5 i.u. human chorionic gonadotrophin (HCG) (Intervet, Milton). One or two females (approximately 6 weeks old) were caged with each F1 male (C57BL/CBA) and checked the following morning for copulation plugs. The day of the vaginal plug was counted as day 1 of pregnancy.

Recovery of embryos

Both 2-cell and 4-cell mouse embryos were flushed from the oviducts between 2 and 5 p.m. on day 2 of pregnancy about 45-48 h post HCG. All 2-cell and all 4-cell embryos were pooled and divided between CD-containing and control medium.
**Morphogenesis of embryos released from cleavage arrest**

**Culture medium**

Embryos were cultured in Brinster’s medium (Brinster, 1970) to which 4 mg/ml bovine serum albumen was added.

A stock solution of CD (Sigma, London) was prepared in 100% dimethyl sulphoxide at 2 mg/ml and stored at -20 °C. Two-cell embryos were incubated in Brinster’s medium containing 0.5 or 1.0 μg/ml CD for 19-21 or 44-48 h after which time they were approximately 68 or 90 h post HCG. By these times control embryos were uncompact ed 8-cell stage and fully compacted morulae to early blastocysts respectively. Four-cell embryos were incubated in medium containing 1.0 μg/ml CD for 41 h at which time control embryos were early blastocysts (about 90 h post HCG).

At the end of the period in CD a group of embryos was removed from both the control and experimental groups for immediate fixation. The remaining embryos in CD-containing medium were washed ten times in fresh Brinster’s medium and groups were fixed after 0.25-0.5 h, 1-2 h, 4-5 h and 24 h (2-cell arrested) or 2.5 h, 7 h and 24 h (4-cell arrested). A few control embryos were fixed at the same time as each experimental group.

**Preparation for light and electron microscopy**

Embryos were fixed in 3% glutaraldehyde, 0.5% paraformaldehyde in 0.05% sodium cacodylate buffer or 0.1 M phosphate buffer (pH 7.3). The osmolarity of the buffer was corrected to 270-290 m-osmole with sucrose. Embryos were post fixed in 1% OsO₄, block stained with 0.5 or 1% uranyl acetate, dehydrated in a graded series of ethanols and embedded in Spurr’s (1969) medium. Serial sections, 0.5 and 1.0 μm thick, were cut on an LKB or Reichert ultramicrotome and stained with 1% toluidine blue for light microscopy. Sections 50-100 nm thick were stained with a saturated solution of uranyl acetate in 50% ethanol followed by lead citrate (Reynolds, 1963). Thick sections were viewed and photographed using a Zeiss microscope and camera attachment. Thin sections were examined in a Philips EM300 or an AEI electron microscope.

**RESULTS**

Observations made on 2-cell cytochalasin-arrested and released embryos are summarized in Table 1. Cytochalasin-arrested embryos have well separated blastomeres (Fig. 1) and most become binucleate (Snow, 1973; Surani et al. 1980) as has been found for other cytochalasin-arrested cells (e.g. Carter, 1967). The nuclei of cytochalasin-arrested blastomeres have a much greater volume than those of normal blastomeres at the 2-cell and 4-cell stage. They are commonly oval in shape with usually four darkly staining spheroidal nucleoli.

Figure 2 shows a 2-cell embryo fixed 1 h after the start of washing in Brinster’s
<table>
<thead>
<tr>
<th>Incubation time after washing (h)</th>
<th>Nos. embryos observed at particular stage</th>
<th>Appearance</th>
<th>Appearance of concurrent controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-48</td>
<td>0</td>
<td>27</td>
<td>Late compacted morulae</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time in CD (h)</th>
<th>Age at HCG (h)</th>
<th>Time in CD (h)</th>
<th>Appearance</th>
<th>Appearance of concurrent controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90-94</td>
<td>4</td>
<td>Two well separated rounded blastomeres, binucleate, commonly with 3 or 4 condensed nucleoli. Microvilli in patches closer together, reduction in intercellular space. One blastomere particularly flattened on surface adjacent to second.</td>
<td>Appearance of concurrent controls</td>
</tr>
<tr>
<td>1</td>
<td>95-99</td>
<td>8</td>
<td>Embryos compacted. Junctions between adjacent blastomeres beginning to become crescent shaped and send out processes round second. (i) One of the two blastomeres extends round the second with an increase in area of cell contact. Second blastomere remains rounded while being engulfed by first. (ii) Blastomeres divided; 3 or 4 cells, 1 or 2 remain rounded, others spread over outside to surround them.</td>
<td>Appearance of concurrent controls</td>
</tr>
<tr>
<td>2</td>
<td>106-110</td>
<td>12</td>
<td>One cell completely or almost completely engulfed by others(s). Vacuoles present in cytoplasm. Presence of extracellular cavity in some embryos.</td>
<td>Appearance of concurrent controls</td>
</tr>
<tr>
<td>3</td>
<td>114-118</td>
<td>13</td>
<td>Embryo formed vesicle. One cell often completely engulfed and present in inside position when cells had divided. Some vesicles expanded.</td>
<td>Appearance of concurrent controls</td>
</tr>
<tr>
<td>4</td>
<td>150-154</td>
<td>16</td>
<td>50% formed irregular vesicles with cells containing fluid filled vacuoles.</td>
<td>Appearance of concurrent controls</td>
</tr>
</tbody>
</table>

Table 1. Morphology of cytochalasin D-arrested and released 2-cell embryos from plastic-embedded and sectioned material.
medium following 44-48 h in CD (90-94 h post HCG). The blastomeres are found to be in much closer association than in the CD-arrested 2-cell embryos. By 1–2 h after explantation to fresh medium embryos viewed in the dissecting microscope showed a morphological appearance similar to normal compacted embryos.

At an early stage after washing off the cytochalasin, adhaerens junctions and possible gap junctions and focal tight junctions were found between blastomeres and they continued to be found at all subsequent stages (Fig. 6). Two hours after release from CD arrest one of the two blastomeres had invariably become slightly crescent shaped. At 4–5 h after washing the crescent-shaped blastomere had extended cytoplasmic processes around the adjacent second blastomere (Fig. 5). The second blastomere remained rounded. By this stage the area of contact between the two cells had increased due to the spreading of one blastomere over the surface of the second. The number of presumptive focal tight and gap junctions had correspondingly increased.

Between 5 and 16 h after release from CD arrest vacuoles appeared in the cytoplasm of one or both blastomeres. The cytoplasmic processes of one blastomere continue to extend around the second so that 16–24 h after the start of washing one blastomere was sometimes almost totally engulfed by the other (Fig. 3). By 16 h after washing an extracellular cavity was found within the embryos which increased in size after 24 h and the washed vesiculated embryos resembled blastocysts (Fig. 4). They still contained only two cells in many cases (see below) and neither cell was solely restricted to the inside of the vesicle.

An understanding of the process of engulfment and vesicle formation was arrived at by the examination of a number of embryos at different stages in their formation (Table 1). The number of embryos which showed various stages in the process of engulfment (recorded in the table) amounted to 90% of those sectioned between 1 and 16 h after release from arrest.

Most of the 2-cell embryos washed after 44-48 h cytochalasin D incubation did not undergo cytoplasmic division. However, in about 20% of the embryos one or both of the blastomeres divided once and in these embryos also some blastomeres were engulfed by others (Fig. 7). In 11 of the embryos between 1 and 16 h from the start of washing some cells were arrested at metaphase or had divided following release from arrest. In seven of these cases serial semithin sections indicated that one or both of the progeny of the first cell to divide tended to be engulfed by the progeny of the later dividing cell, or by the cell which did not divide (Fig. 9). In two of the remaining embryos both of the 2-cell blastomeres had divided whilst in the other two engulfment had not proceeded far enough. In vesicles formed from CD-arrested embryos where cell divisions did occur after explanting to fresh medium an inside cell was often found (Fig. 8). Probable gap and focal tight junctions were found between the cells forming the walls of the vesicles.
Morphogenesis of embryos released from cleavage arrest

After embryos were incubated in CD for 41 h from the 4-cell stage they underwent similar morphological processes on release from CD as did 2-cell arrested embryos (Table 2). By 2.5 h from the onset of washing the embryos had compacted (Fig. 10) and one cell was commonly placed more centrally with the others spread over much of its surface. In embryos fixed 7 h after washing the outer blastomeres had the crescent shape seen in 2-cell embryos after washing off CD (Figs 11, 12). Their shape was less attenuated than in the 4–5 h and 16 h washed 2-cells. This is because in the 4-cell arrested and washed embryos there were three or more cells to cover the surface of the inner one. In some of the embryos at this stage one or two of the cells had divided or were in the process of doing so (Fig. 11). Again it seems that one or both of the progeny of the dividing cell tended to take up a more central position. Even at 7 h from release some embryos contained a small extracellular cavity (Fig. 1) and by 24 h the embryos were uniformly expanded vesicles.

In a further experiment, 2-cell and 4-cell embryos were incubated in Cytochalasin D for 65 h (~110–115 h post HCG). Towards the end of the period in CD-containing medium they started to pump fluid and formed irregular vesicles with large fluid-containing vacuoles in the cytoplasm.

DISCUSSION

Biochemical similarities have been found between 2-cell cleavage-arrested embryos and their concurrent controls (Surani et al. 1980; Pratt, Chakraborty & Surani, 1981). After embryos are released from CD arrest they compact rapidly, some blastomeres show active spreading over the surface of others and junctions are formed before the embryos become blastocyst-like vesicles. Thus the programme of morphogenesis is able to catch up with that of biochemical differentiation after the two have become out of phase.

Based on previous proposals (Steinberg, 1964, 1970; Sternfeld, 1979) a blastomere which is engulfed would be expected to have a more adhesive surface over which less adhesive blastomeres could spread. Since there is a

Figures 1–4

Fig. 1. Two-cell embryo arrested in cytochalasin D for 44 h. The blastomeres are not in close contact. MV, microvilli; N, nucleus; Z, zona pellucida.

Fig. 2. Two-cell embryo arrested in CD for 44 h and incubated for 1 h in fresh medium. The cells adhere closely. MV, patch of microvilli.

Fig. 3. Two-cell embryo arrested in CD for 44 h and incubated for 16 h in fresh medium. Note the cell process (P) of the outer cell (OC) which is extended around the inner cell (IC). The inner cell is pumping fluid to form a vesicle.

Fig. 4. Two-cell embryo arrested in CD for 44 h and incubated for 24 h in fresh medium. The embryo has formed an expanded vesicle which was indented during preparation for microscopy. C, cavity.
critical period in development after which compaction cannot occur (Johnson et al. 1979) it is probable that cytoplasmic differentiation proceeds to the phase of increased cell adhesion over a defined period. Differentiation may reach the 'adhesive membrane' phase in the prospective inner cell of the CD arrested and released 2-cell embryo in advance of the prospective outer cell.

It appears that the cell spreading seen in arrested and released embryos is an exaggerated form of a process found in normal embryos in which, due to a change in cell-cell adhesion, the outer cells of the normal compacting morula spread on one another (Ducibella & Anderson, 1975; Ducibella et al. 1977). As in the spreading shown here, spreading of the outer cells of normal compacting embryos involves a redistribution of microvilli (Calarco & Epstein, 1973; Ducibella & Anderson, 1975; Ducibella et al. 1977) which may be involved in adhesion as in other systems (Lin & Wallach, 1974; Ukena & Karnovsky, 1976). Inhibition of the synthesis of glycoproteins, which presumably mediate cell adhesion, has been shown to prevent compaction and blastocyst formation (Surani & Kimber, 1981; Surani, Kimber & Handyside, in preparation). A coordinate response from the underlying cytoskeleton must also be involved in the spreading process (Ducibella et al. 1977).

Differences in the adhesive properties and cytoskeletal mobilization between cells may also be responsible for the relocation of isolated inner cell masses (ICM) and embryonal carcinoma (ec) cells predominantly towards the inside of the embryo when these cells are aggregated with 8-cell embryos (Rossant, 1975; Stewart, 1980; Stewart & Kimber, unpublished). In analogy with CD-arrest embryos, normal 8-cell blastomeres send out cell processes to engulf ec cells (Stewart, 1980; Kimber & Stewart, unpublished). Small cell size does not appear to determine that a cell is engulfed since in 2-cell arrested embryos both cells are approximately equal in size, and not all types of ec cell are engulfed (Stewart, 1980).

**Figures 5–8**

Fig. 5. Electron micrograph showing an early stage in the spreading of one blastomere over the second. Two-cell embryo arrested in CD for 44 h and incubated for 4 h in fresh medium. MV, microvilli; P, cell process.

Fig. 6. Electron micrograph showing probable gap junction (GJ) as well as adhaerens junctions (AJ) between blastomeres of a 2-cell embryo after 44 h in CD followed by 23 h in fresh medium.

Fig. 7. Two-cell embryo which has undergone cell division after being explanted from CD to fresh medium. The outer cell (OC) is in the process of division while the inner cell (IC) is one of the progeny of the earlier dividing blastomere. 48 h CD arrest 16 h in fresh medium. P, process of outer cell.

Fig. 8. Electron micrograph showing part of a vesicle formed after 44 h arrest in CD from the 2-cell stage and 24 h after explanting to fresh medium. The cells divided after washing and one cell (IC) is occupying a position inside the vesicle. C, cavity.
Morphogenesis of embryos released from cleavage arrest

Cell division occurs in about 20% of embryos released from CD arrest. Sections of seven embryos indicated that the earlier dividing cell(s) tended to be engulfed by the later or non-dividing cell(s). The engulfed cells became localized on the inside in a similar position in the vesicle to the ICM of a normal blastocyst. It is relevant to note that in normal embryos the first blastomere to divide tends to contribute more cells to the ICM than later dividing blastomeres (Barlow, Owen & Graham, 1972; Kelly, Mulnard & Graham, 1978).

It has been shown that whether cells become ICM cells or trophectoderm probably depends partly on cell position (Tarkowski & Wroblewska, 1967; Hillman, Sherman & Graham, 1972; Rossant, 1975) and partly on their interaction with other cells (Barlow et al. 1972; Graham & Deussen, 1978; Kelly et al. 1978; Graham & Lehtonen, 1979) at the time of compaction. In this respect we have previously shown that vesicles formed after prolonged cleavage arrest in CD give rise only to trophectoderm derivatives (Surani et al. 1980). However, such vesicles synthesize all the polypeptides detected in intact blastocysts (Pratt, Chakraborty & Surani, 1981) indicating that some or all the arrested blastomeres synthesize polypeptide detected only in the ICM of normal embryos. The intracellular polarity of blastomeres observed at the 8-cell stage (Ducibella et al. 1977; Johnson, Pratt & Handyside, 1981) led to one explanation of this. In the absence of compaction and the development of intercellular communication it is suggested that each blastomere recreates the radial polarity of an entire embryo and therefore expresses properties of both ICM and trophectoderm (Johnson et al. 1981). Alternatively we suggest that, irrespective of polarization, each blastomere may be developmentally programmed to produce both sets of polypeptides as is probably the case in the cleavage-arrested embryos. The normal divergence of biochemical differentiation in the embryonic cell populations may only occur after compaction due to the influence of microenvironment and cell contact over a period of hours and depending on cell position. Definitive ICM and trophectoderm would then be formed.

Figures 9-12

Fig. 9. A three-cell embryo resulting from the division of one blastomere of a 2-cell embryo. 48 h arrest in CD 16 h in fresh medium. A and B, progeny of the divided cell.

Fig. 10. A four-cell embryo arrested for 41 h in CD followed by 7½ h in fresh medium. The inner cell (IC) is in the process of cell division.

Fig. 11. Four-cell embryos arrested for 41 h in CD and explanted to fresh medium for 2½ h. In the embryo on the left cell division is underway in one blastomere (arrowed). The orientation of the cleavage furrow appears to determine that one of the progeny cells will end up inside the embryo.

Fig. 12. Embryo arrested for 41 h in CD from the 4-cell stage and incubated for 7½ h in fresh medium. One cell (IC) is surrounded by the other cells. A cavity (C) has just started to appear.
<table>
<thead>
<tr>
<th>Time in CD (h)</th>
<th>Incubation time after washing (h)</th>
<th>Age at fixation HCG (h)</th>
<th>Nos. embryos observed at particular stage</th>
<th>Appearance</th>
<th>Appearance of concurrent controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>0</td>
<td>90</td>
<td>12</td>
<td>Four well separated blastomeres, binucleate. Densely staining spheroidal nucleoli</td>
<td>Early blastocysts</td>
</tr>
<tr>
<td>2 ½</td>
<td>—</td>
<td>6</td>
<td>6</td>
<td>Compacted 4-6 cells, one cell often almost surrounded by others</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>97</td>
<td>8</td>
<td>(i) Outer blastomeres crescent shape surrounding inner blastomere (ii) Some embryos with small extracellular cavity</td>
<td>Fully expanded blastocysts</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>114</td>
<td>8</td>
<td>Expanded vesicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>0</td>
<td>115</td>
<td>Irregular vesicles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
We therefore consider that the cell spreading seen after CD treatment and subsequent release is an exaggerated form of the cell behaviour found in the normal process of compaction and ICM formation. This suggests that division order, differential cell adhesiveness and directional deployment of the cytoskeleton are the central process in differentiation of ICM and trophectoderm. The last two may perhaps be the motive forces behind these processes. Cell position is presumably dependent on these factors and probably determines the subsequent differentiation of the cell types.

We would like to thank Mrs Sheila Barton for her expert help during the course of this work. S.J.K. is in receipt of a Training Fellowship from the Medical Research Council.

REFERENCES


SURANI, M. A. H. & KIMBER, S. J. (1981). The role of glycoproteins in the preimplantation


(Received 24 June 1980, revised 18 September 1980)