Distribution of microvilli on dissociated blastomeres from mouse embryos: evidence for surface polarization at compaction

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SUMMARY

Cells of mouse embryos develop a polarization of microvillous distribution at compaction. Cells of the 4-cell embryo show a uniform pattern of fluorescent-ligand binding and an even distribution of microvilli. Each cell of the early 8-cell embryo has a uniform distribution both of microvilli and of fluorescent ligand. During the 8-cell stage, there is a progressive increase in the incidence of cells which show microvilli restricted to a region normally on the exposed surface of the embryo. When late 8-cell embryos were disaggregated to single cells, and these sorted by pattern of fluorescent-ligand binding, each of the four patterns of staining related consistently to a characteristic distribution of microvilli as viewed by scanning electron microscopy. The 16-cell embryo possessed an inside population of uniformly labelled cells with a sparse microvillous distribution, and an outside population of cells, each of which had a microvillous pole.

INTRODUCTION

Cell position in the preimplantation mouse morula is thought to govern cell fate (Herbert & Graham, 1974). At the 8-cell stage, the embryo undergoes a process of compaction in which blastomere apposition increases, and cell outlines are no longer easily visible. This cellular reorganization and subsequent cell division results in some cells becoming completely surrounded by their neighbours. Later, those cells located in an internal position differentiate as inner cell mass while those located externally follow a different differentiative path, eventually developing into the trophectoderm of the blastocyst. The dramatic morphological change associated with compaction of the 8-cell embryo, and the dependence of cell fate on cell position make it pertinent to examine the morula stage for evidence of differences in surface and cytoplasmic organisation between the centre and the periphery of the embryo (Johnson, Pratt & Handyside, 1981). Distinctive differences of surface organization have recently been described by Handyside (1980) who labelled intact 8-cell embryos, and cells dissociated therefrom, with a variety of fluorescent ligands.

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to reveal a polarized pattern of surface binding. Surfaces which were normally part of the embryo periphery bound more ligand than the inner surfaces. Moreover in the 16-cell embryo two distinct populations of cells were found in which the pattern of labelling varied with the internal or external origin of cells. It was suggested that the poles of increased binding at the 8-cell stage, and the distinct sub-populations at the 16-cell stage, reflected at least in part the organization of microvilli on these cells.

Previous transmission (TEM) and scanning electron microscopic (SEM) studies of intact preimplantation mouse embryos (Calarco & Epstein, 1973; Calarco, 1975; Ducibella, 1977; Ducibella, Ukena, Karnovsky & Anderson, 1977; Van Blerkom & Motta, 1979) have indeed shown that blastomeres of 2- and 4-cell embryos have a uniform distribution of microvilli over their cell surface. In contrast, blastomeres of compacting 8-cell embryos developed a polarized microvillous topography with a dense localization of microvilli (microvillous pole) on the external face of each cell. This polarized microvillous distribution was also seen on the exposed cells of the compacted 16-cell embryo, but inside cells could not be examined. However, when fragments of morulae (Graham & Lehtonen, 1979; Lehtonen, 1980) or single cells isolated from morulae (Lehtonen & Badley, 1980) were examined, a restricted localization of microvilli was not described.

In this study, we re-examine the distribution of microvilli on both compacted and decompacted mouse embryos, and on single cells disaggregated from them. We show that blastomeres of the 8-cell embryo do develop a polarized microvillous distribution which corresponds to their polarized ligand-binding pattern, that this polarity is not lost upon embryo disaggregation and that in the 16-cell embryo two populations of cells with different microvillous patterns are present.

**MATERIALS AND METHODS**

**Embryo collection**

Female HC-CFLP mice (4–5 weeks; Hacking and Churchill) were superovulated with intraperitoneal injections of 5 i.u. of pregnant mare’s serum (PMS: Folligon, Intervet), followed after 44–48 h by 5 i.u. of human chorionic gonadotrophin (hCG: Chorulon, Intervet). Females were paired with HC-CFLP males, and vaginal plugs taken as an indication of mating.

Embryos were flushed from oviducts with phosphate-buffered medium 1 supplemented with 0·4% (w/v) bovine serum albumin (PBl + 0·4% BSA) (Whittingham & Wales, 1969) and cultured at 37 °C in medium 16 with 0·4% (w/v) BSA (Whittingham, 1971) in 5% CO₂ in air: 4-cell embryos were flushed at 54 h post-hCG; newly formed 8-cells were harvested at hourly intervals from cultures of 4-cell embryos; non-compacted, partially compacted and fully compacted 8-cells were sorted according to degree of cell flattening from a mixed population of cultured embryos, and predominated at 63, 65 and 68 h
Surface polarization of mouse blastomeres at compaction

post-hCG, respectively; 16-cell embryos were flushed from oviducts at 73 h post-hCG. Zonae pellucidae were removed by a 15 to 30 s incubation in prewarmed (37 °C) acid Tyrode's solution (pH 2.5) + 0.4% (w/v) polyvinylpyrrolidone (Nicolson, Yanagimachi & Yanagimachi, 1975) or by a 3- to 5-min incubation in 0.5% (w/v) solution of pronase (Calbiochem, Grade B; technique of Mintz, 1967) in citrate buffer at 37 °C.

Decompaction and disaggregation

Morulae were decompacted by incubation in calcium-free medium for 15 min. The reduced cellular apposition allowed the identification of individual cells.

Disaggregation into single cells was accomplished by pipetting embryos with a flame-polished micropipette after incubation in one of three different media (Handyside, 1980), all of which were pre-equilibrated for at least 30 min at 37 °C in the presence of 5% CO₂ in air. After disaggregation cells were immediately restored to medium 16 + 0.4% (w/v) BSA.

The disaggregation media were:

(I) Calcium-free medium: embryos were incubated for 20–30 min in calcium-free medium 16 + 0.6% (w/v) BSA. (Decompaed embryos were either labelled with fluorescent ligand or fixed immediately after incubation in calcium-free medium without further disaggregation).

(II) Cytochalasin D (CCD): embryos were incubated for 30 min in 0.5 μg/ml CCD (0.05% dilution of 1 mg/ml stock solution in dimethyl sulphoxide (Sigma) in medium 16 + 0.4% (w/v) BSA).

(III) Trypsin–EDTA: embryos were incubated in 0.5% (w/v) trypsin + 0.2% (w/v) EDTA (Gibco) in calcium-free medium 16. After 5 min, when decompaction appeared complete, the medium was drawn off and replaced with a large volume of PB1 + 0.4% (w/v) BSA.

Fluorescent labelling of cells

(a) Direct labelling

Fluorescein-conjugated concanavalin A (FITC-Con A; Miles Labs) was used routinely to assess the degree of cell polarization (Handyside, 1980; Ziomek & Johnson, 1980). Embryos or blastomeres were incubated for 15 min at room temperature in FITC-Con A (700 μg/ml in PBI + 0.4% (w/v) BSA) with 0.02% (w/v) sodium azide. Addition of azide inhibited the patching and pinocytosis of the FITC-Con A without affecting the pattern of polarized ligand binding (Handyside, 1980). FITC-Con A-labelled cells tended to agglutinate during fixation and processing. Hence in experiments in which cells were sorted according to the degree of polarized ligand binding, indirect immunofluorescence with antispecies antiserum was used. The fluorescent
Con A and indirect immunofluorescent labelling gave similar staining patterns (Handyside, 1980).

(b) Indirect labelling

Heat-inactivated rabbit anti-mouse species antiserum (RAMS) (Gardner & Johnson, 1975; Handyside, 1978) labelled the whole outer surfaces of all stages of preimplantation mouse embryos as judged by indirect immunofluorescence (Handyside, 1980). Individual blastomeres were incubated for 15 min at room temperature in 25 μl drops of whole serum (RAMS) diluted 1 in 15 in PB1 + 0·4 % BSA (w/v) followed by thorough washing in PB1 + 0·4 % (w/v) BSA, and a similar incubation in fluorescein-conjugated goat anti-rabbit IgG (FITC-GAR IgG; Miles Labs) diluted 1 in 15 in PB1 + 0·4 % (w/v) BSA.

Fluorescence microscopy

Fluorescent-labelled cells and embryos were washed extensively and mounted individually in drops of PB1 + 0·4 % (w/v) BSA + 0·02 % (w/v) azide under oil in wells of a tissue-typing slide (Baird & Tatlock). A Zeiss epifluorescence microscope, incident source HBO 200 with excitation filter system 427902 and barrier filter system 427903, was used to examine cells for FITC labelling. Cells were scored blind. Kodak Tri-X 35 mm film was used for both bright-field and fluorescence photography. After scoring, the coverslips were floated off under oil, and the cells recovered and pooled in groups according to their pattern of fluorescent labelling, before being fixed for SEM.

Scanning electron microscopy

Intact embryos and dissociated blastomeres were fixed in 6 % (v/v) glutaraldehyde (Sigma) in 0·1 M sodium cacodylate buffer at pH 7·4 for 60 min at 4 °C. Embryos and cells were then washed thoroughly in 0·1 M sodium cacodylate buffer at pH 7·4, and zonae pellucidae removed by incubation in pronase. Coverslips were washed with ethanol, dried for 1 h, and treated with poly-L-lysine (Sigma, Type 1-B) (1 mg/ml in H2O) for 15 min (Mazia, Schatten & Sale, 1975). After removal of poly-L-lysine, the coverslips were washed with 0·1 M-sodium cacodylate buffer before embryos or blastomeres were transferred to them. Cells were allowed to attach overnight at 4 °C before post-fixation in 1 % (w/v) osmium tetroxide in 0·1 M sodium cacodylate buffer for 1 h at room temperature. After dehydration through a graded alcohol series, embryos were dried from 100 % acetone via CO2 in a Polaron E3000 critical-point drying apparatus. Specimens were coated with a 40–50 μm thick layer of gold in a Polaron E5000 Diode sputtering system, and examined in a Cambridge Stereo-scan 600 electron microscope. Between 30 and 50 intact embryos, and 30 and 100 single cells were examined in each experiment which was repeated at least twice.
RESULTS

Intact embryos were always fixed before removal of zonae pellucidae as living embryos took approximately 2 h to recover their compacted appearance after acid Tyrode's or pronase treatment. The microvillous topography of embryos did not appear to vary with zona removal by acid Tyrode’s or pronase before fixation, or with pronase after fixation.

4-cell embryo

Both the intact 4-cell embryo (Fig. 1a) and its dissociated cells (Fig. 1b) labelled uniformly with FITC-Con A. In the SEM, blastomeres showed a uniform distribution of microvilli over their entire surface.

8-cell embryo

 Newly formed intact 8-cell embryos which had not yet compacted (Fig. 2a) and the dissociated blastomeres derived from them (Fig. 2b) were labelled uniformly with FITC-Con A, and were shown by SEM to have a uniform population of microvilli over the entire cell surface similar in density to those of the 4-cell embryo. Compact 8-cell embryos (Fig. 3) appeared heavily microvillous but with tracts of smoother membrane defining the areas of cell contact, and surrounding the microvillous pole of each cell. Decompaction (Fig. 4) or disaggregation (Fig. 5) in calcium-free medium showed that these embryos were comprised of individual cells with polarized fluorescent-ligand binding, and a restricted microvillous distribution which appeared to be directed towards the normally external surface of the embryo.

Non-compacted, partially compacted, and fully compacted embryos were sorted on the basis of degree of cell flattening from a population of 8-cell embryos. Each group was disaggregated individually, and the dissociated cells labelled by indirect immunofluorescence. Four ligand-binding patterns were identifiable (Fig. 5) (as classified in Ziomek & Johnson, 1980), regardless of whether embryos were disaggregated with trypsin, calcium-free medium or cytochalasin D. Those cells which showed uniform binding of ligand were scored as ring stained (Fig. 5a), and other cells with a polarized pattern of labelling were scored as three-quarters stained (Fig. 5b), half stained (Fig. 5c) or tightly polarized (Fig. 5d), depending on the size of the restricted area of intense fluorescence. The incidence of cells showing polarized ligand-binding increased with the progress of compaction (Table 1). Thus, whereas only 57% of the blastomeres isolated from embryos classified as non-compacted were polarized, 93% of those dissociated from fully compacted embryos exhibited polarized fluorescent-ligand binding. The cells dissociated and examined in these experiments were sorted according to their patterns of labelling into ring (Fig. 5a), three-quarter (Fig. 5b), half, (Fig. 5c) and pole (Fig. 5d) categories. Each sorted subpopulation was then prepared for examination by SEM,
with minimal losses. For example, of 167 cells showing a fluorescent pole and 83 having a ring stain, 119 and 82, respectively, were examined for their microvillous distribution (Table 2). The majority of cells in each of the four categories were found to have a microvillous distribution corresponding to their pattern of fluorescent-ligand binding (Table 2). For example, of the 99 fluorescent-polar cells which were scoreable by SEM for microvillous distribution, 91% had a restricted region (pole) of microvilli, 2% and 4% had microvilli occupying half and three-quarters of the cell surface, respectively, and only 2% showed a uniform distribution of microvilli.

**16-cell embryo**

The microvillous poles on the outside of the compacted 16-cell embryo (Fig. 6) are difficult to resolve unless the embryo is first decompacted (Fig. 7). The poles of intense fluorescent-ligand binding on the outside of decompacted embryos appear to correspond to the externally located microvillous poles seen in the SEM. Individual cells from completely disaggregated embryos showed either polarized or uniform fluorescent-ligand binding. After labelling by indirect immunofluorescence, dissociated cells were sorted into these two categories and examined by SEM. Cells which were polarized as judged by fluorescent-ligand binding consistently displayed dense microvillous poles (Fig. 8a) while cells ring stained by ligand binding had sparse microvilli distributed uniformly over their surfaces (Fig. 8b).

**DISCUSSION**

Intact 4-cell embryos and their dissociated cells show a uniform distribution of microvilli which is lost during the 8-cell stage. The development of an apically polarized microvillous distribution on individual cells parallels the

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Each figure includes a fluorescence micrograph with a bright-field comparison and a SEM micrograph. Bar = 10 μm for SEM. Bright-field and fluorescence micrographs × 300 for intact embryos; × 400 for single cells.

**Figures 1-4**

Fig. 1. (a) An intact 4-cell embryo and (b) an isolated 1/4 cell both show a uniform surface binding of fluorescent ligand and a uniform distribution of microvilli.

Fig. 2. (a) A newly formed 8-cell embryo and (b) dissociated cells from this stage, label uniformly with the fluorescent ligand, and have a uniform distribution of microvilli over their entire surface.

Fig. 3. The exposed surface of a compacted 8-cell embryo binds fluorescent ligand, and appears microvillous, but with non-microvillous areas adjacent to regions of cell apposition.

Fig. 4. A decompacted 8-cell embryo shows that each cell has an area of intense fluorescent-ligand binding which coincides with a microvillous pole on the normally exposed surface of the embryo. Note that fluorescent poles on some cells are not in focus.
Fig. 5. Dissociated cells of 8-cell embryos were labelled by indirect immunofluorescence, reharvested after scoring and sorted into (a) ring (b) $\frac{3}{4}$, (c) $\frac{1}{2}$ and (d) pole categories which were then examined by SEM. Each ligand-binding pattern related to a characteristic distribution of microvilli. Polarized blastomeres had microvilli concentrated at one pole of the cell, whereas non-polarized cells showed a uniform distribution of microvilli.

Table 1. Patterns of fluorescent-ligand binding shown by dissociated cells of 8-cell embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number of cells labelled</th>
<th>Pattern of fluorescence labelling</th>
<th>Polarized (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-compacted</td>
<td>465</td>
<td>Ring 32 32 177 42</td>
<td>57</td>
</tr>
<tr>
<td>Partially compacted</td>
<td>69</td>
<td>$\frac{3}{4}$ 3 4 6</td>
<td>87</td>
</tr>
<tr>
<td>Fully compacted</td>
<td>223</td>
<td>$\frac{1}{2}$ 12 20 5</td>
<td>93</td>
</tr>
</tbody>
</table>

* % polarized = \( \frac{\frac{1}{4} + \frac{3}{4} + \text{pole}}{\text{ring} + \frac{1}{2} + \frac{1}{4} + \text{pole}} \times 100 \)

† Code throughout tables: Ring = ring stained; $\frac{3}{4}$ = three-quarters stained; $\frac{1}{2}$ = half stained; Pole = tight pole; ? = unscoreable.
Table 2. Correlation of categories of polarization detected on individual cells from 8-cell embryos by fluorescent-ligand binding with those observed on the same cells by SEM

<table>
<thead>
<tr>
<th>Frequency of patterns of fluorescence labelling</th>
<th>Percentage of scorable cells (%)</th>
<th>Total no. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pole</td>
<td>167</td>
<td>119</td>
</tr>
<tr>
<td>Half</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>Three-quarters</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Ring</td>
<td>83</td>
<td>82</td>
</tr>
</tbody>
</table>

A total of 305 cells were examined for their pattern of fluorescence labelling. 21 of these cells were unscorable: 71% of the scorable cells were polarized. Discrepancies between the number of cells scored by pattern of fluorescence labelling and the total number of cells examined for microvillous distribution occur due to losses in the harvesting of cells after fluorescent labelling, and in their preparation for SEM. Debris and extreme juxtaposition caused some cells to be unscorable for microvillous distribution.

The distribution of microvilli corresponds to patterns of fluorescent-ligand binding described previously (Handyside, 1980; Ziomek & Johnson, 1980). When cells displaying each of the four characteristic staining patterns after labelling with a fluorescent ligand were sorted and prepared for SEM, they were found to exhibit the expected microvillous distribution in most cases. The discrepancies in the assessments of polarization between analysis by fluorescent labelling and analysis by SEM are only significant for cells scored as ring stained by the former, but three-quarter, half or polar by the latter technique. Such discrepancies could occur because cells underwent some polarization between scoring by fluorescence and fixation, because of the greater sensitivity of SEM, and also from errors in the reharvesting and sorting of cells after fluorescent labelling. It thus seems reasonable to conclude that the pole of intense fluorescence on a labelled cell can be explained adequately by a uniform density of ligand-binding sites in the cell membrane, with a non-uniform distribution of membrane (i.e. microvilli) over the cell surface (Handyside, 1980). The results
Fig. 6. A 16-cell embryo shows a uniform binding of fluorescent ligand over its surface which is predominantly microvillous.

Fig. 7. The outermost cells of a decompacted 16-cell embryo have apical regions of increased binding of fluorescent ligand which correspond to poles of microvilli (→) on the normally exposed surface. Cells with a uniform covering of microvilli (←) tend to be more internal.

Fig. 8. Dissociated cells of 16-cell embryos show either (a) a polarization of both fluorescent-ligand binding and distribution of microvilli or (b) a uniform binding of fluorescent ligand and an overall population of sparse microvilli.

do not of course preclude a non-uniform density of binding sites superimposed on this morphological polarity. The fact that antibody-treated cells did not differ in morphology from non-antibody-treated controls provides further evidence to that already cited by Handyside (1980) that ligand binding does not itself induce polarization. More non-polarized than polarized cells were recovered in the SEM (Table 2). Since microvillous poles were seldom seen in the area of attachment of the cells to the coverslip, it is possible that polarized cells attach only weakly by their microvillous poles, and that this population of cells is lost during preparation. Alternatively, cells might never attach by their microvillous poles. The percentage recovery of cells varied between experiments, and
successful recovery may depend on the cleanliness of coverslips, the time between poly-L-lysine removal and the placement of cells, and the amount of turbulence generated in the solution around individual coverslips.

Our results are consistent with, and extend, those obtained previously. Examination by SEM (Calcarco & Epstein, 1973; Ducibella et al. 1977; Van Blerkom & Motta, 1979) and TEM (Ducibella, 1977; Ducibella et al. 1977) of compacted intact 8-cell embryos suggested a polarized distribution of microvilli which is even more striking on our dissociated 1/8 cells. Previous studies with fragments of embryos showed that the microvillous density of 8-cell embryos varied to some extent (Graham & Lehtonen, 1979) but that the late 4- and late 8-cell embryos have approximately the same microvillous density (Lehtonen, 1980). Only occasionally was a polarized distribution of microvilli noted (Graham & Lehtonen, 1979), although inspection of published micrographs suggests that its presence was more common. The failure to detect polarization consistently was probably due to the age of the 8-cell embryos, and may also have arisen from the abnormal flattening of embryo fragments into a monolayer under oil prior to fixation. Furthermore, visualization of polarization is enhanced by analysis of isolated cells, or by decompacting couplets and embryo fragments before fixation.

Whereas the compacted 8-cell embryo consists of polarized cells, the 16-cell embryo has two distinct populations of cells, polarized and non-polarized. Both SEM studies and the fluorescent labelling experiments of Handyside (1980) suggest that the polarized cells are in the outer position. The finding of distinct inner and outer subpopulations of this type is consistent with, but not a demonstration of, the conservation of polarity at division from 1/8 to 2/16. The results thus offer support for the polarization hypothesis recently propounded to explain the generation of positional differences within the morula (Johnson et al. 1981).

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