Molecular differentiation of inside cells and inner cell masses isolated from the preimplantation mouse embryo

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SUMMARY

Clusters of inside cells (ICs) were isolated immunosurgically from morulae and early cavitating blastocysts, and cultured for varying periods in vitro. The ICs responded to their changed position by transformation to a blastocyst-like vesicle and also showed a correlated synthesis of trophodermal-marker polypeptides. Both changes were inhibited by a-amanitin, indicating that transcriptional events are involved in this response to changed position. Inner cell masses (ICMs), isolated immunosurgically from expanded blastocysts, did not undergo a morphological transformation to a blastocyst-like structure and did not show a corresponding and complete change in synthesis of trophodermal-marker polypeptides. Elements of the change in polypeptide activity were, however, present, suggesting an ineffective response to position by the ICMs.

INTRODUCTION

Each blastomere of the eight-cell mouse embryo evidently retains developmental totipotency (Kelly, 1977). At some point shortly thereafter, cells embark on a restricted course of development in response to positional cues (Johnson, 1979). It is proposed that inside cells (ICs) at the morula stage develop into the inner cell mass (ICM) of the blastocyst and outside cells (OCs) into the trophoderm (Tarkowski & Wroblewska, 1967; Hillman, Sherman & Graham, 1972). The time at which inside and outside positions are first recognized by embryonic cells has been investigated by use of a set of intrinsic cell markers. The two tissues of the 3½-day blastocyst may be differentiated from each other by their synthesis of tissue marker polypeptides (Van Blerkom, Barton & Johnson, 1976). When whole embryos at earlier developmental stages are analysed, they are also found to synthesize some of these marker polypeptides, and, moreover, there appears to be a relationship between the position of the cells within the morula or early blastocyst and the type of polypeptides that they synthesize (Handyside & Johnson, 1978). Thus, inside cells do not synthesize the trophodermal-tissue polypeptides but do synthesize ICM-tissue polypeptides (Handyside & Johnson, 1978). It seems likely from these results that cells recognize position, as reflected by their molecular differentiation, at least as early as the morula stage.

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Whilst populations of inside and outside cells taken from the morula and early blastocyst may be differentiated from each other, their development along a restricted lineage is not irreversible since, on their isolation and culture in vitro, clusters of either inside or outside cells can give rise to blastocysts (Johnson, Handyside & Braude, 1977; Handyside, 1978a, b). Furthermore, blastocysts derived from aggregates of ICs will implant in utero to yield both normal egg cylinders and placental tissues (Handyside, 1978b). Final commitment to ICM formation at least does not appear to occur until late in blastocyst expansion (Johnson et al. 1977; Handyside, 1978a, b; Surani, Torchiana & Barton, 1978; Spindle, 1978; Hogan & Tilley, 1978), after which time ICMs do not contribute to the trophectoderm lineage either in vivo (Rossant, 1975; Handyside, 1978b) or in short-term cultures in vitro (Handyside, 1978a, b).

The spatially defined patterns of polypeptide synthesis which occur in response to positional cues within the morula could be regulated either via transcriptional control through selective gene expression or via post-transcriptional control through, for example, selection of cytoplasmic mRNA. Indeed a transition with time from post-transcriptional to transcriptional control could provide a molecular explanation for the apparent period of 'reversible' differentiation prior to commitment. The experiments described in this paper have attempted to discriminate between these alternatives.

**MATERIALS AND METHODS**

1. **Embryos**

Outbred 4- to 6-week-old CFLP mice (Anglia Laboratory Animals) were superovulated, mated and embryos recovered at 84, 96 or 120 h post-HCG. Dead or retarded embryos at each time were discarded; 84 h embryos were separated into morulae and early blastocysts. Zonae were removed by a 10 sec exposure to acid tyrode's solution, and the embryos left for 30-60 min in phosphate-buffered medium 1 (PB1) before use.

2. **Immunosurgery and culture**

Immunosurgical recovery of clusters of inside cells (ICs) from morulae and early cavitating blastocysts (84 h post-HCG) and of ICMs from fully expanded 96 or 120 h blastocysts was carried out by procedures shown by several techniques to yield cell clusters uncontaminated by outer cells, as described in detail elsewhere (Handyside, 1978a; Handyside & Barton, 1977). ICs or ICMs were cultured in RPM1 + 10% foetal calf serum supplemented with glutamine in an atmosphere of 5% CO2 in air. Some embryos were cultured for varying periods of time in these conditions prior to immunosurgery as described in the text. In some experiments, α-amanitin (11 μg/ml, Boehringer Mannheim) was included both in the medium in which the immunosurgery was performed and in the medium for culture and labelling of embryos (Braude, 1979a).
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3. Labelling of embryos and electrophoretic separation of polypeptides

Embryos, or immunosurgically isolated parts thereof, were placed in 0.1 ml of a protein-free medium 16 (Whittingham, 1971) containing 10 μl of [35S]-methionine (~ 1000 Ci/mmole, Radiochemical Centre, Amersham) for 4 h as described previously (Handyside & Johnson, 1978). Labelled embryos were harvested into lysis buffer, frozen and thawed 3×, and applied to cylindrical gels for isoelectric focusing. The gels were then laid on gradient SDS polyacrylamide slab gels for electrophoresis according to molecular size (O'Farrell, 1975; as described in detail in Handyside & Johnson, 1978). Gels were fixed, impregnated with PPO (Laskey & Mills, 1975), dried, and exposed to preflashed Fuji Rx film or Kodak RP45 film (Bonner & Laskey, 1974). Films were coded and analysed 'blind' as described in detail in Handyside & Johnson (1978) and Braude (1979a). Six trophectodermal-marker polypeptides designated 2, 3, 4, 6, 13 and 16 and described previously (Handyside & Johnson, 1978) were used as markers of trophectodermal differentiation (see also legend to Fig. 1).

RESULTS

Inside cells (ICs) isolated from late morulae and early cavitating blastocysts (84 h post-HCG) and cultured for 0, 8, 16 or 24 h, showed signs of fluid accumulation in individual cells by 16 h and had formed blastocyst-like vesicles within 24 h, thus confirming previous observations (Handyside, 1978a). The patterns of incorporation of [35S]methionine into the six trophectodermal-marker polypeptides indicated in Fig. 1 are summarized in Fig. 2A, B. For comparison, Fig. 2C includes a summary of the patterns obtained for intact embryos 84 and 96 h post-HCG. It will be observed that after 24 h, followed by 4 h in label, the ICs (84/24/4 in Fig. 2A, B) show the same profile as intact blastocysts (96/4 in Fig. 2C) and this correlated with their blastocyst-like morphology. Intact embryos recovered at 84 h post-HCG were also cultured for 24 h, subjected to immunosurgery and the ICMs labelled directly for 4 h. The polypeptide pattern obtained is summarized in Fig. 2D. Further groups of embryos were left in vivo until 108 h post-HCG, and after recovery their ICMs were immunosurgically isolated and labelled directly for 4 h (Fig. 2D). The ICMs from intact embryos, in contrast to those cultured in isolation, do not acquire or increase their capacity to synthesize trophectoderm-marker polypeptides.

Blastocysts recovered from the uteri at 96 h post-HCG were subjected to immunosurgery and the ICMs recovered were placed in culture for 0, 8, 16, 24 or 36 h prior to 4 h of labelling. At no time during the incubation did the cells of the isolated ICMs show evidence of fluid accumulation, and blastocyst-like vesicles did not form, thus confirming the results of Handyside (1978a). The polypeptide synthetic profiles of the cultured ICMs are summarized in Fig. 3. Some trophectodermal-marker polypeptides show indications of increased synthesis; however, none is as uniformly pronounced as in cultured ICs and synthesis of all is greatly retarded over that observed for ICs.
Fig. 1. Fluorograph of [³⁵S]methionine-labelled polypeptides synthesised by ICs derived from mouse embryos (84 h post-HCG) and separated by two-dimensional electrophoresis. Horizontal separation is by iso-electric focusing over the pH range indicated. Vertical separation is by SDS gradient acrylamide electrophoresis giving separation by molecular weight (as indicated by 1, 5 and 10 x 10⁴ M.W.). The position of the polypeptide markers for trophectoderm are indicated and numbered 2, 3, 4, 6, 13 and 16 (Van Blerkom et al. 1976; Handyside & Johnson, 1978). (b) similar to (a) but after labelling performed after 24 h culture of the ICs.
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Fig. 2. Diagram to indicate relative synthesis under different conditions of the six tissue-specific polypeptides (2, 3, 4, 6, 13, 16) indicated in Fig. 1. Each small block represents analysis of 1 gel for 1 polypeptide. □ = no detectable synthesis; ▸ = relatively weak synthesis compared to 96 h post-HCG blastocyst; ■ = comparable synthesis to that observed in 96 h post-HCG blastocyst; □ = not scorable.

Panel A represents data from ICs isolated from early cavitating blastocysts (84 h post-HCG), cultured in vitro for 0–24 h and labelled over a 4 h period. The numbers at left represent in hours (age post-HCG/culture period/labelling period). Panel B is the same for ICs isolated from morulae (84 h post-HCG). Numbers at left as for A. Panel C represents data for intact early blastocysts (84 h post-HCG) or expanded blastocysts (96 h post-HCG) after incubation in label for 4 h. Numbers at left represent in hours (age post-HCG/labelling period). Panel D represents data for ICMs derived from early blastocysts (84 h post-HCG) which were cultured intact for 24 h either in vitro or in vivo (108 h at recovery), immunosurgically dissected, and then the ICMs incubated directly in label for 4 h. Numbers at left as for A. Number of gels analysed = 28 for A, 6 for B, 12 for C, 8 for D.
Intact embryos (84 h post-HCG) and ICs derived immunosurgically from them were also cultured for either 8 or 24 h in 11 μg/ml α-amanitin. The results of this culture are summarized in Table 1. Prolonged culture in α-amanitin resulted in death of ICs, although ICMs in intact embryos were not prevented from forming nor were they killed, as has also been reported by Braude (1979b). Inspection of cultures revealed that death of the ICs occurred between 12 and 14 h after isolation of the ICs in the presence of α-amanitin, that is prior to the overt morphological transition to the blastocyst in control cultures. The polypeptide synthetic profiles of ICs incubated for 12 h in the presence of α-amanitin, the last four of which were in the presence of labelled methionine, are shown in Fig. 4.
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Fig. 4. Diagram, details as for Fig. 2, comparing data for ICs isolated from early cavitating blastocysts (84 h post-HCG) and labelled directly (0 + 4), or after incubation in vitro for 8 h in the absence or presence of α-amanitin (11 μg/ml). Numbers at left as for Fig. 2A. Number of gels analysed = 12.

Table 1. Effect of incubation in 11 μg/ml α-amanitin on development in vitro of intact 84 h post-HCG embryos, or of the ICs derived therefrom

<table>
<thead>
<tr>
<th>Time (h) and nature of treatment</th>
<th>Number of embryos showing indicated morphology at the end of incubation</th>
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<tbody>
<tr>
<td></td>
<td>Morulae</td>
</tr>
<tr>
<td>ICs (84 h post-HCG)</td>
<td></td>
</tr>
<tr>
<td>8 h</td>
<td></td>
</tr>
<tr>
<td>α-Amanitin</td>
<td>485</td>
</tr>
<tr>
<td>Untreated†</td>
<td>90</td>
</tr>
<tr>
<td>24 h</td>
<td></td>
</tr>
<tr>
<td>α-Amanitin</td>
<td>2</td>
</tr>
<tr>
<td>Untreated†</td>
<td>3</td>
</tr>
<tr>
<td>Intact embryos (84 h post-HCG)</td>
<td></td>
</tr>
<tr>
<td>8 h</td>
<td></td>
</tr>
<tr>
<td>α-Amanitin</td>
<td>1</td>
</tr>
<tr>
<td>Untreated†</td>
<td>0</td>
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<tr>
<td>24 h</td>
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<tr>
<td>α-Amanitin</td>
<td>0</td>
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<tr>
<td>Untreated†</td>
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* As assessed both by direct observation and immunosurgical isolation.
† All controls done in parallel with test experiments.
DISCUSSION

The ICs isolated from morulae and early blastocysts (84 h post-HCG) and cultured in vitro, showed a change in their polypeptide synthetic profile that corresponded temporally to their change in morphology. The acquisition of marker polypeptides occurred in the same sequence as that reported for intact differentiating morulae but with a lag of approximately 12–18 h on absolute embryonic time (Handyside & Johnson, 1978). The observation that ICs left within trophoderm, in vivo or in vitro, over the same time period did not undergo these changes in synthetic profile (Fig. 2D) suggests that this response was abnormal and was elicited by the changed environment of the ICs.

The sensitivity to α-amanitin of ICs isolated in vitro compared with those retained within trophoderm suggests that transcriptional processes are involved in a complete response to altered position. It is not clear, however, that the very earliest molecular changes were totally blocked (Fig. 4). Those species of polypeptide (2, 3, 6, 16), which showed a partial resistance to α-amanitin, are identical to those observed in the incomplete synthetic response of isolated ICMs (Fig. 3), and are also seen weakly in some fluorographs of freshly isolated ICMs or ICs indicating either their synthesis at a low level or very rapid activation of their synthesis during the 4 h labelling period in response to positional change. These considerations make it impossible to exclude a post-transcriptional control mechanism for some of the earlier events of the IC transformation to a blastocyst. However, both these results and those using α-aminatin on intact blastocysts (Braude, 1979b), make it highly unlikely that post-transcriptional controls play the sole and major role in controlling blastocyst formation (Johnson, 1979). Perhaps a clearer resolution of this problem will come from analysis of the in vitro translates of mRNA isolated from ICs at various times after their immunosurgical recovery (Braude & Pelham, 1979).

In contrast to ICs, ICMs isolated from fully expanded blastocysts (96 h post-HCG) did not acquire the complete set of trophodermal-marker polypeptides over the same time course (compare Fig. 2A with Fig. 3). Neither did they show evidence of a morphological transformation to a blastocyst. However, elements of molecular differentiation in a trophodermal direction were detected, since synthesis of the earliest trophodermal-marker polypeptides seen in the ICs was also seen in some ICMs, although less evident and later in time of appearance. The ICMs thus do appear to respond to their altered position by developing trophodermal-marker polypeptides, but do so more slowly and less completely than the ICs. Whilst synthesis of all the trophodermal-marker polypeptides appears to be diagnostic for mature trophoderm, synthesis of only some is not in itself an adequate criterion for the diagnosis of trophodermal cells. The less complete molecular response of the ICM to positional change could be interpreted as an attempt to unscramble and reverse differentiation by some or all of the cells in the ICMs. ICMs cultured...
Differentiation of cells isolated from mouse embryo in vitro for prolonged periods show only limited and probably abnormal differentiation (Hogan & Tilly, 1978) and it has not been established that 24–36 h culture in vitro is compatible with normal ICM function on relocation within a trophectodermal vesicle. It is possible that cultured ICMs are abnormal because of their incomplete or confused molecular responses to positional change in these early stages of culture. This result is consistent with models for commitment which do not invoke a special, quantal molecular mechanism but rather a progressive accumulation of differentiative changes (Johnson et al. 1977; Johnson, 1979).

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REFERENCES


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