Cytoplasmic projections of trophoderm distinguish implanting from preimplanting and implantation-delayed mouse blastocysts

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Summary. A scoring scheme was devised to characterize visually the morphological differentiation of whole-mount, unfixed mouse blastocysts. Embryos were recovered from groups of intact mice (implanting embryos) and mice ovariectomized on Day 3 of pregnancy (implantation-delayed embryos) every 3 h from 18:00 h on Day 4 until 12:00 h on Day 5. Blastocyst differentiation was assessed according to the presence of a zona pellucida, the appearance of the outer margin of trophoderm cells, the visibility of the blastocoele and the relative size of the inner cell mass. The results obtained indicate that, during this period, implanting and implantation-delayed mouse blastocysts lose the zona as well as exhibit rounded trophoderm cells, an enlarged inner cell mass and an increasing opacity of the blastocoele. In contrast, the trophoderm cells of implanting blastocysts only exhibit extensive cytoplasmic projections, probably due to remodelling of the intracellular cytoskeleton. Growth of the inner cell mass appeared to precede the other morphological changes in the majority of blastocysts, and thus might be a prerequisite for further differentiation. The rate of blastocyst differentiation and the survival of embryos were adversely affected by the condition of delayed implantation, induced by ovariectomy. This study suggests that the appearance of cytoplasmic projections from trophoderm cells is central to the control of blastocyst implantation.

Keywords: blastocyst; morphology; trophoderm; implantation; mouse

Introduction

Uterine signals which influence embryonic development before implantation can be inferred from the phenomenon of delayed implantation. The signals which initiate or terminate (or both) delayed implantation are thought generally to be expressed by altered uterine secretions. However, there is little agreement as to whether the uterus secretes an inhibitory substance during the delay of implantation or whether it secretes an activating substance when delay is terminated (McLaren, 1973). An important possibility, noted by Edwards (1980), is that an activating substance is a prerequisite for blastocyst differentiation, regardless of whether a period of delay occurs. There are several observations that support this conclusion. First, blastocysts retained in the oviduct fail to differentiate trophoblast (Tutton & Carr, 1984) and embryos transferred to oviducts of immature mice become blastocysts and enter a form of diapause (Papaioannou, 1986). Second, differentiation of hatched blastocysts in vitro requires the presence of serum (Cole & Paul, 1965), serum-derived, high molecular-weight protein fractions (fetuin) (Rizzino & Sherman, 1979) or extracellular matrix macromolecules (Armant et al., 1986). Comparisons between primiparous and

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post-partum matings provide further evidence that uterine secretions function in normal blastocyst development. That is, embryo development is retarded by about 24 h following post-partum matings (with removal of litter) (Menke & McLaren, 1970) whereas the protein concentration in uterine flushings from mice undergoing lactational delay is only one-half of that from mice in which delay has been induced by ovariectomy (Aitken, 1977).

The embryonic response to delayed implantation has been characterized primarily in terms of metabolism but cellular proliferation and cell surface structures have also been examined. Essentially, the embryo reduces its energy demands (Nieder & Weitlauf, 1984) by reducing, but not eliminating, macromolecular synthesis (Weitlauf & Greenwald, 1968; Sherman & Barlow, 1972; Weitlauf, 1982, 1985) with a corresponding overall decline in O2 consumption (Menke & McLaren, 1970). Mitotic activities (McLaren, 1968) in polar and mural trophoderm as well as inner cell mass decline and ultimately cease but do so at differing rates (Copp, 1982). Alterations in the concanavalin-A binding and surface charge of the cell surface of trophoderm in response to delay have been reported (Wu & Chang, 1978; Wu, 1980; Nilsson et al., 1973), but not confirmed (Chavez & Enders, 1982). However, Dolichos biflorus lectin (DBA) binding sites on the trophoderm cell surface of mouse blastocysts in delay and their disappearance following reactivation have also been observed (Chavez & Enders, 1982).

The present study was carried out to determine the morphological stage at which blastocyst differentiation is interrupted under the conditions of delayed implantation. It was reasoned that this approach might offer insight into the nature of the uterine signals which mediate embryo quiescence during delay and into the cellular processes within the embryo which are affected by this interruption. To do so, a scoring scheme was devised to characterize visually (by light microscopy) the morphological differentiation of whole-mount, unfixed blastocysts from mice undergoing implantation or entering delay of implantation.

Materials and Methods

Animals. Virgin CD1 female mice at 6–8 weeks of age, obtained from Charles River, Quebec, Canada, were mated randomly with CD1 males. Females were separated from males on Day 1 of pregnancy, the day of finding a vaginal plug. Approximately one-half of the pregnant mice were ovariectomized bilaterally on the afternoon of Day 3, under pentobarbitone sodium anaesthesia (Somnotol, M.T.C. Pharmaceuticals, Mississauga, Canada; 2 mg/25 g body weight injected intraperitonally), to induce delay of implantation. All ovariectomized mice were injected subcutaneously with 0.5 mg progesterone (McLaren, 1971) (Sigma, St Louis, MO, USA) in sesame oil immediately after surgery and 24 h later.

Embryo collection. Mice were killed by cervical dislocation in groups of 3–7 animals every 3 h from 18:00 h on Day 4 of pregnancy until 12:00 h on Day 5. Embryos were recovered from individual mice by flushing excised uteri with M1 medium (Miller & Schultz, 1983) containing 2% (w/v) bovine serum albumin (Pentex, Miles Scientific, Kankakee, IL, USA) into square watch glasses (Baxter CanLab, Winnipeg, Canada). To enhance recovery of blastocysts clumped in implantation crypts, the following technique was employed. A 27-gauge needle, connected to a 1-ml syringe filled with flushing medium, was inserted into the transected cervical end of the excised uterine horn. Gentle pressure, sufficient to cause the uterine horn to balloon, was applied to the syringe piston. The needle and syringe were then removed and inserted into the ovarian end of the uterine horn; the horn was then flushed with 0.5 ml medium. Embryos were recovered from flushings, washed once in medium and transferred to a small drop of medium on a glass slide. To prevent evaporation, a glass cover slip was lowered over the droplet to rest upon dabs of silicone grease placed at four corners about the droplet. A Zeiss IM35 inverted microscope was used to examine and photograph the embryos.

Statistical analysis. Totals of 157 implanting embryos from 33 intact mice and 147 implantation-delayed embryos from 37 ovariectomized mice were analysed (see ‘Results’) separately. In consideration of the subjective nature of assessment, the photographs of embryos were examined and scored in random order. To determine for each morphological feature whether the percentage distribution of scores differed significantly between times of examination, the R × C table described by Snedecor & Cochran (1967 p. 250) was used. To determine for each score whether the percentage of embryos assigned that score, relative to the total collected at that time of examination, differed significantly between times of examination, equal probabilities for all classes using the average percentage were assumed (Snedecor & Cochran, 1967 p. 231).
Results

A total of 424 embryos was recovered and examined. Of these, 120 were not included in subsequent assessment of blastocyst development (Table 1). Half (57) were eliminated because they appeared abnormal or degenerate; for example, zonae containing dense floccular material or embryos with extruded blastomeres or 1–8 blastomeres of variable sizes were observed. The remaining 63 were considered difficult to score either because both the inner cell mass and blastocoele were obscured from view or because the embryo had not differentiated to the blastocyst stage (see below). The proportion of embryos from implantation-delayed animals which were abnormal or degenerate was significantly ($\chi^2 = 25.02$, $P < 0.05$) greater than those from implanting animals. There was, however, no significant difference ($\chi^2 = 1.61$, $P > 0.05$) between groups of embryos in regard to scoring ambiguities. The total number of embryos scored for each time of recovery is given in Table 2.

Table 1. Summary of embryos recovered between 18:00 h on Day 4 and 12:00 h on Day 5 of pregnancy from mice undergoing implantation and delayed-implantation

<table>
<thead>
<tr>
<th>Animals (no.)</th>
<th>No. of embryos</th>
<th></th>
<th>Degenerate in appearance</th>
<th>Scoring ambiguities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovered</td>
<td>Scored</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Implanting (33)</td>
<td>193</td>
<td>157</td>
<td>9</td>
<td>27*†</td>
</tr>
<tr>
<td>Implantation-delayed (37)</td>
<td>231</td>
<td>147</td>
<td>48</td>
<td>36‡§</td>
</tr>
</tbody>
</table>

*Includes 26 embryos for which neither inner cell masses nor blastocoeles were visible.
†Includes 1 nascent blastocyst.
‡Includes 13 morulae or nascent blastocysts.
§Includes 2 embryos for which neither inner cell masses nor blastocoeles were visible.

Table 2. Summary of numbers of embryos scored for each time of recovery between 18:00 h on Day 4 and 12:00 h on Day 5 of pregnancy

<table>
<thead>
<tr>
<th>Animals</th>
<th>18:00</th>
<th>21:00</th>
<th>24:00</th>
<th>03:00</th>
<th>06:00</th>
<th>09:00</th>
<th>12:00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implanting</td>
<td>21 (3)</td>
<td>39 (6)</td>
<td>20 (5)</td>
<td>32 (7)</td>
<td>14 (4)</td>
<td>20 (5)</td>
<td>11 (3)</td>
</tr>
<tr>
<td>Implantation-delayed</td>
<td>16 (7)</td>
<td>22 (4)</td>
<td>34 (7)</td>
<td>21 (4)</td>
<td>26 (7)</td>
<td>15 (4)</td>
<td>19 (4)</td>
</tr>
</tbody>
</table>

Values in parentheses are the number of animals.

An irregular outer margin was observed on trophectoderm cells of implanting blastocysts recovered on Day 5 (see Fig. 1). To examine the possibility that this 'roughness' was due to fragments of uterine epithelium adhering to the trophectoderm, several implanting blastocysts were incubated with 0.4% trypan blue in phosphate-buffered saline for 10 min. Stained material which
was floccular in nature and probably intracellular in origin was observed to adhere to some, but not all, implanting blastocysts (Fig. 2).

The scoring scheme summarized in Table 3 was developed from a preliminary examination of embryos recovered from intact mice. By application of this scheme, each embryo was given a 4-digit number in which each digit, left-to-right represented the score assigned for morphological features 1 through IV (summarized in Table 3). Photomicrographic examples of embryos illustrating this scoring scheme are presented in Fig. 1. There is a maximum of $2 \times 3 \times 4 = 24$ possible scores; however, 42 of these are not feasible due either to inherent relatedness between scores of morphological features (e.g. -- 42) or to the arbitrary restriction of the analysis to include full blastocysts only (i.e. the score 1132 would describe a nascent blastocyst). Among implanting blastocysts, the score assigned most frequently (48/157) was 2343 with 38 occurring at 03:00 h on Day 5 or later. The second most frequently assigned score was 1111 (27/157), all of which were observed before 03:00 h on Day 5. Among implantation-delayed blastocysts, the most frequently observed score was 1111 (80/147). From Fig. 3, it is clear, based upon the percentage distribution of scores at early times compared to those at later times, that the score 1111 represents an early stage of blastocyst differentiation whereas 2343 represents a later stage. Of the remaining 28 feasible scores, several were either without representatives or were represented by no more than 2 blastocysts (i.e. < 1.5%
Fig. 2. Implanting embryos stained with 0·4% trypan blue in phosphate-buffered saline (reprinted from Fujichrome colour slide). Trypan blue-stained regions (arrowheads) are absent from some blastocysts (arrow). × 150.

of total). Without exception, all embryos scored as 3 for trophoderm (i.e. irregular outer margin) were also 2 for zona pellucida (i.e. zona absent). In contrast, no embryo scored 3 for trophoderm was scored 1 for inner cell mass (i.e. thin disc under trophoderm). Other rarely observed combinations were 1 for trophoderm (i.e. cells elongated) and 3 or 4 for blastocoele (i.e. residual blastocoele or blastocoele not visible) and secondly, 2 for zona pellucida (i.e. absent) and 1 for inner cell mass (i.e. a thin disc). Lastly, only 2 blastocysts (both implantation-delayed) were scored 1 for zona pellucida (i.e. zona present) and 3 or 4 for blastocoele (i.e. residual blastocoele or blastocoele not visible) (see Fig. 1).

**Time dependencies of morphological features**

*Zona pellucida.* The proportions of embryos from implanting and implantation-delayed animals possessing a zona pellucida at the time of examination depended significantly (implanting, $\chi^2 = 92·6$, $P < 0·05$; implantation-delayed, $\chi^2 = 34·4$, $P < 0·05$) upon the time at which the embryos were examined. At the earliest time investigated (18:00 h), 21/21 implanting embryos had a zona whereas by 24:00 h the majority had lost the zona and after 06:00 h all embryos examined were without one (Fig. 3). In contrast, at most times of examination, the majority of implantation-delayed embryos possessed the zona.

**Table 3.** Summary of scoring scheme used for morphological assessment of mouse blastocysts

<table>
<thead>
<tr>
<th>Morphological feature</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>I Zona pellucida</td>
<td>Present</td>
</tr>
<tr>
<td>II Trophoderm</td>
<td>Outer margin smooth, cells apparently flat and elongated</td>
</tr>
<tr>
<td>III Blastocoele</td>
<td>Comprises majority of blastocyst volume</td>
</tr>
<tr>
<td>IV Inner cell mass</td>
<td>Thin disc stretched under trophoderm</td>
</tr>
</tbody>
</table>
Fig. 3. Relationship between the percentage of embryos assigned indicated scores for zona pellucida (a), troph loderm (b), blastocoele (c) and inner cell mass (d), according to the scheme summarized in Table 3, and the time of recovery. Implanting blastocysts are represented by open bars and implantation-delayed blastocysts by stippled bars.

Trophoderm. The percentage distribution of trophoderm scores of implanting embryos depended significantly upon time of examination (χ² = 112.4, P < 0.05). Specifically, the proportions of embryos exhibiting the first two types (i.e. smooth outer margin with flat or rounded cells; Table 3) decreased significantly with time (Score 1, χ² = 251; Score 2, χ² = 64.9). By contrast, the proportion exhibiting the third type of appearance (i.e. irregular outer margin) increased significantly (χ² = 189, P < 0.05) with time. Implanting embryos exhibiting this feature first appeared between 18:00 and 21:00 h on Day 4. By 03:00 h on Day 5, the vast majority were scored similarly. At any given time of examination, the majority of embryos were scored as 1 or 3. It appears probable, therefore, that a trophoderm with a smooth outline and rounded cells is probably an intermediate phase between 1 and 3. A rounding of trophoderm cells has been observed in implanting rat blastocysts in situ, as early as Day 5, but more commonly on the afternoon of Day 6 (Enders & Schlafke, 1967).

The percentage distribution of implantation-delayed embryos exhibiting each type of trophoderm did not depend significantly (χ² = 11.6, P > 0.05) upon time of examination. At all times studied, the majority exhibited trophoderm with a smooth margin and elongated cells. Only 18 out of 147 embryos were scored as having rounded trophodermal cells. Lastly, and most notably, no delayed-implanting embryo was scored as having trophoderm cells with irregular outer margins.

Blastocoele. The percentage of implanting embryos in which a visible blastocoele comprised the majority of the blastocyst's volume decreased significantly (χ² = 144, P < 0.05) over time. The distribution of blastocoele scores 2, 3 and 4 did not depend significantly (χ² = 18.2, P = 0.1) upon time of examination although the percentage of embryos exhibiting each type increased significantly with time (Score 2, χ² = 75.8; Score 3, χ² = 60.4; Score 4, χ² = 68). Overall, 40% of
implanting embryos were scored as having a dominantly visible blastocoele (Score 1) and in 34% the blastocoele was not visible (Score 4).

In contrast to implanting embryos, the percentage distribution of implantation-delayed embryos exhibiting each blastocoele appearance did not differ significantly ($\chi^2 = 15.0, P > 0.05$) over time. Approximately 80% of embryos were scored as having a dominantly visible blastocoele and another 15% were scored as having blastocoele and inner cell mass approximately equal in contribution to the blastocyst volume. The remaining blastocysts from implantation-delayed animals (8/147 or 5%) were scored as having a residual blastocoele visible or no blastocoele visible.

**Inner cell mass.** The inner cell mass appeared as a thin disc stretched under the trophectoderm in implanting and implantation-delayed embryos in significantly ($\chi^2 = 27.7, P < 0.05$; $\chi^2 = 41, P < 0.05$, respectively) decreasing proportions over time. By 03:00 h on Day 5, this type of inner cell mass was no longer observed in implanting embryos. By contrast, in implantation-delayed embryos, approximately 30–50% of all embryos recovered exhibited this type of inner cell mass. The percentage distribution between scores 2 and 3 did not differ significantly over time in implanting and implantation-delayed embryos, although the percentage of embryos exhibiting each type increased significantly with time.

**Discussion**

The present study represents a systematic examination of the gross morphology of blastocysts from mice undergoing implantation and from those in which implantation has been interrupted by ovariectomy. The results demonstrate that, in mice, blastocyst differentiation involves growth of the inner cell mass followed by loss of the zona pellucida and differentiation of trophectoderm. Furthermore, under conditions of delay, blastocyst differentiation is interrupted after a rounding up of trophectoderm cells but before the appearance of an irregular outer margin on these cells. In addition it appears that delay of implantation, induced by ovariectomy in mice, is associated with retarded development and increased mortality of preimplantation embryos.

Irregular cell extensions on the surface of mouse trophoblast cells of implanting blastocysts were observed by Bergstrom & Nilsson (1976) using scanning electron microscopy. These cytoplasmic extensions or projections probably correspond to the irregular outer margin of trophectoderm cells described in the present paper. However, this irregularity or 'roughness' might also be due to fragments of uterine epithelium adhering to the trophectoderm. In an attempt to evaluate the latter possibility, implanting embryos were stained with trypan blue. The floccular nature of the stained material did not appear consistent with the morphology of the trophectoderm outer margin as seen in this study. It was concluded therefore that the irregularity of the cell surface represents cytoplasmic projections. Notably, 'tongues' of trophoblast cytoplasm have been observed *in situ* to extend between groups of epithelial cells in mice (Potts, 1968). Furthermore, protrusions of trophoblast cells, extending from the cell surface adjacent to the endometrium and interdigitating or extending adjacent to the endometrium and lining Reichert's membrane, have also been observed by electron microscopy on Day 6 of pregnancy in rats (Enders & Schlafke, 1967) and mice (Bevilacqua & Abrahamsen, 1988). Thus, the morphological feature observed uniquely in regard to implanting blastocysts probably represents projections of trophoblast cytoplasm. Eruption of the trophectoderm cell surface into cytoplasmic projections, probably due to remodelling of the intracellular cytoskeleton, might then be a prerequisite for blastocyst implantation.

As noted by Bergstrom (1971), Dickson (1963, 1966a, b) clearly concluded that optical opacity of the mural and abembryonic trophectoderm of mouse blastocysts resulted from giant cell transformation. In Dickson's original study (1963) and in the present study, some implantation-delayed blastocysts were partly or completely optically opaque (8 of 134 and 8 of 147, respectively). Therefore, either giant cell transformation occurs in some blastocysts during delay of implantation or other factors account for opacity of the blastocoele. However, as also noted by Bergstrom
(1971), opacity precludes identification of individual cells, making it difficult to determine whether the cells are, in fact, giant. Nevertheless, partial or complete opacity was usually associated in this study with rounded trophectoderm cells or trophectoderm cells exhibiting cytoplasmic projections, suggesting that opacity does result from some aspect of trophectoderm differentiation.

The systematic approach used in the present study permits the following additional observations.

1. An intact zona pellucida and elongated trophectoderm cells (i.e. before rounding) were usually associated with well-expanded blastocoeles, suggesting that these features are morphogenetically related.

2. The sequence of morphological changes in a majority of blastocysts appeared to be (a) growth of the inner cell mass, (b) rounding of trophectodermal cells, loss of the zona pellucida, collapse (or partial obscurity) of the blastocele and (c) in implanting blastocysts, development of extensive cytoplasmic projections by trophectodermal cells. That growth of the inner cell mass usually precedes zona loss is probable since only 2 blastocysts (1 implanting and 1 implantation-delayed) were scored 1 for inner cell mass in the absence of a zona. That this growth also precedes the other morphological changes is apparent from Fig. 3; for the majority of implanting and implantation-delayed blastocysts, the shift in scores from 1 to 2 (or 3, 4) occurred earliest for inner cell mass (i.e. 21:00 h and 06:00 h, respectively). Thus, the growth of the inner cell mass which apparently precedes the appearance of cytoplasmic projections on trophectoderm might be a prerequisite for the latter but not a direct cause since growth of the inner cell mass also occurs in implantation-delayed blastocysts (Fig. 3; Copp, 1982).

Notably, blastocyst hatching and trophoblast proliferation in vitro are adversely affected by the absence of a viable inner cell mass (Ansell & Snow, 1975).

This study has also revealed that the rate of blastocyst differentiation and the viability of preimplantation embryos are adversely affected by the condition of delayed implantation, induced by ovariectomy. From Fig. 3 it is apparent that, from 21:00 h on Day 4 onwards, a smaller proportion of implantation-delayed blastocysts, relative to implanting ones, had lost the zona as well as exhibited growth of the inner cell mass and differentiation of the trophectoderm. Furthermore, substantial numbers of morulae and nascent blastocysts were recovered from implantation-delayed animals (Table 1). Delayed implantation, induced by ovariectomy, was also associated with a statistically greater incidence of abnormality and degeneration (Table 1). The recovery of degenerating embryos with large blastomeres suggests that loss of viability occurs, in some cases, before the blastocyst stage. These observations suggest that the milieu of oviduct and uterus were detrimentally affected by ovariectomy on Day 3, possibly due to tissue trauma at ovariectomy or to insufficient or incomplete hormone replacement. Notably, a requirement of progesterone, during early pregnancy in rodents, for normal embryonic development and embryo survival has been demonstrated (Psychoyos & Prapas, 1987; Rider et al., 1987). These observations suggest that environmental factors may influence and, perhaps, control embryonic development in vivo to an extent not revealed by culturing embryos in vitro. These observations also indicate that the morphological criteria used in the present study are probably relevant to the more critical problem of assessing biological viability following embryo manipulations (see Shea, 1981).

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References


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