In vitro studies on the control of trophoblast outgrowth in the mouse

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SUMMARY

When placed in serum-free medium on reconstituted collagen surfaces re-implantation mouse embryos are capable of producing characteristic trophoblast outgrowths. Previously this pattern of differentiation has been considered to be essentially dependent on the presence of serum macromolecules. Such activity is expressed only at the late blastocyst stage and is qualitatively different from the adhesive interactions between blastomeres earlier in development. The development of the properties responsible for outgrowth is intrinsic to the blastocyst, being independent of stimulation by exposure either to the uterine environment or to whole serum. The significance of these observations related to implantation control in vivo is discussed.

INTRODUCTION

Although the basic requirements for implantation of the mouse embryo have now been established (McLaren, 1969) the nature of the initial attachment and interactions between the blastocyst and the uterus is largely unknown. In vitro techniques can be used to investigate this problem more directly than is possible in the intact animal. Such methods have shown that mouse blastocysts cultured in various serum-containing media attach and spread on the surface of the culture vessel (Cole & Paul, 1965). This attachment and outgrowth is considered to be an expression of the same activity which leads to implantation in utero (Bryson, 1964; Gwatkin, 1966a, b; Menke & McLaren, 1970; McLaren & Menke, 1971).

On the basis of in vitro studies of outgrowth, Gwatkin (1966a, b) suggested that implantation may be dependent upon changes in the amino acid content of the uterine fluid. However, he later (1969) found that the amino acid pattern during experimental delay of implantation was identical to that at normal implantation. In the same study it was shown that the total protein content of the uterine fluid increased just before implantation, indicating an alternative possibility that a uterine protein factor, having growth-promoting effects on the

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blastocyst, could be responsible for the initiation of implantation. This might correlate with the findings of Menke & McLaren (1970) and McLaren & Menke (1971), who found that the metabolic activity of blastocysts produced by culture in serum-free medium from the 8-cell stage was lower than that of blastocysts recovered directly from the uterus. This low level of activity could be stimulated to higher levels by the addition of serum to the medium once the blastocyst stage had been reached. Since outgrowth also only occurred in serum-containing medium, it was concluded that metabolic activation, by factors contained in serum and possibly also produced in the oestrogen-sensitized uterus, was necessary for trophoblast outgrowth in vitro and in vivo.

The presence of serum in the medium is known to be important for the survival of many cell types in vitro. One of its functions in this respect is apparently to provide a protein coat on the surface of the culture vessel on which cells can attach and spread. It has been suggested that this physical role of serum may be at least as important as its nutritional contribution (Trinkhaus, 1969; Witkowski & Brighton, 1972). Previous conclusions based on studies of blastocyst outgrowth using serum-containing medium, which indicate specific nutrient requirements or metabolic activation of the blastocyst for outgrowth, do not take into account this potentially dual role of serum. We have now examined the capacity for outgrowth of embryos recovered from the uterus at various times and cultured in serum-free medium on glass, plastic and reconstituted collagen surfaces. In this way it has proved possible to separate the physical and metabolic requirements for trophoblast outgrowth and to assess the possible effects upon it of exposure to the uterine environment.

MATERIALS AND METHODS

Embryo collection

Embryos were collected from spontaneously ovulating hybrid mice kept on a 16 h light/8 h dark schedule. The middle of the dark period was taken as the approximate time of ovulation and embryos were recovered at selected intervals after this time (see Table 1). Where necessary the zona pellucida was removed either mechanically with a pipette of slightly smaller diameter than the blastocyst (Tarkowski, 1961) or enzymically with a 0.5% solution of pronase in Earle’s balanced salt solution (Mintz, 1962).

Collagen preparation

Reconstituted collagen was prepared by a method of acid extraction of rat tail tendons (Elsdale & Bard, 1972). Rat tail tendons were extracted with 0.5M acetic acid for 24 h and the resulting viscous solution was dialysed for 4 days against two changes of Earle’s basal medium or Earle’s balanced salt solution (B.S.S.). During dialysis the pH was kept at 4, by the addition of hydrochloric acid, in order to prevent gelation. This stock solution was then stored at 4°C until required. Immediately before use a known volume of collagen was pipetted into
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a sterile tube kept cool on ice and sufficient medium of \( \times 10 \) concentration was added to give a normal concentration. This mixture was then titrated against 0.142M sodium hydroxide to pH 7 when it was dispensed into culture dishes as described below.

Culture methods

Collagen cultures were carried out in glass well slides (Butterworth). Collagen solution was pipetted into the well to fill one third and allowed to gel forming a pad. The well was then filled to two-thirds with culture medium and finally topped up with liquid paraffin gassed with 8% CO\(_2\) in air. Embryos were introduced with a pipette so as to lie on the collagen surface and the well was sealed with a coverslip. The medium overlying the collagen pad was that formulated by Whittingham (1971), consisting of a balanced salt solution supplemented with bovine serum albumin at 4 mg/ml.

For comparison some embryos were grown in a similar manner but with 2% agar (Difco Noble agar) made up in Earle’s B.S.S. replacing the collagen. In this case the overlying medium was Earle’s B.S.S. supplemented with 10% foetal calf serum (Flow Laboratories).

The possibility that collagen might ‘condition’ the medium and so exert a metabolic effect on blastocyst outgrowth was tested by culturing embryos in a small glass saucer fabricated from a sliver of coverslip and placed with medium on a collagen pad prepared as described above. In this way embryos were cultured in the presence of collagen although not in direct contact with it.

Routine culture of 8-cell embryos prior to transfer to collagen was carried out either in small glass chambers or in commercially produced tissue culture chambers made from plastic specially treated to facilitate cell adhesion (Sterilin Ltd). Embryos were cultured in these chambers in small drops of Whittingham’s medium under liquid paraffin gassed with 8% CO\(_2\) in air. Similar culture conditions were used to test the ability of pre- and post-zona loss blastocysts to form outgrowths in the absence of whole serum.

In all cases the culture chambers, after setting up, were enclosed in a Perspex box which was gassed with 8% CO\(_2\) in air and incubated at 37°C. Cultures were examined twice daily, the Perspex chambers being of a suitable design to allow examination under a binocular microscope without the need for opening and re-gassing.

RESULTS

The results obtained are summarized in Tables 1 and 2. All blastocysts recovered 84 h after the estimated time of ovulation and placed, after zona removal, on a collagen surface in Whittingham’s medium showed trophoblast outgrowth (groups A, B). This outgrowth usually started on day 7 (about 148 h post-ovulation) and rapidly produced a large spread of trophoblast cells
Fig. 1 (A) Blastocyst 160 h post-ovulation cultured from 84 h stage in serum free medium. No trophoblast outgrowth.
(B) Blastocyst 150 h post-ovulation cultured from 84 h stage in serum free medium on a collagen surface. Note the onset of trophoblast outgrowth (arrowed).
(C) Same blastocyst as Fig. (B) after a further 20 h culture. The trophoblast (T) is now well spread.
(D) Blastocyst 195 h post-ovulation cultured from 84 h stage in serum free medium on a collagen surface. Extensive trophoblast outgrowth with cells pushing out long pseudopodia. The inner cell mass (I) remains as a disorganized group of cells on the upper surface.
Table 1. Effect of substratum and method of zona removal on outgrowth

<table>
<thead>
<tr>
<th>Group</th>
<th>Stage of recovery</th>
<th>No. of embryos</th>
<th>Method of zona removal</th>
<th>Medium</th>
<th>Substratum</th>
<th>Time of outgrowth</th>
<th>No. with outgrowth (Day 8)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Day 4, blastocysts (82–84 h)†</td>
<td>28</td>
<td>Pronase</td>
<td>Whittingham's</td>
<td>Collagen</td>
<td>Days 7–8</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>Day 4, blastocysts (82–84 h)</td>
<td>20</td>
<td>Mechanical</td>
<td>Whittingham's</td>
<td>Collagen</td>
<td>Days 7–8 onset ca. 148h†</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>C</td>
<td>Late day 4, pre-zona loss (93 h)</td>
<td>36</td>
<td>Mechanical</td>
<td>Whittingham's</td>
<td>Collagen</td>
<td>Day 6, onset ca. 136h</td>
<td>31</td>
<td>86</td>
</tr>
<tr>
<td>D</td>
<td>Day 4, blastocysts (82–84 h)</td>
<td>18</td>
<td>Mechanical</td>
<td>Whittingham's</td>
<td>Glass</td>
<td>Days 8–9</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>E</td>
<td>Day 4, blastocysts (82–84 h)</td>
<td>43</td>
<td>Pronase</td>
<td>Whittingham's</td>
<td>Glass</td>
<td>Day 8</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>F</td>
<td>Day 4, blastocysts (82–84 h)</td>
<td>22</td>
<td>Mechanical</td>
<td>Whittingham's</td>
<td>Plastic</td>
<td>Day 8</td>
<td>1</td>
<td>4.5</td>
</tr>
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<td>G</td>
<td>Day 4, blastocysts (82–84 h)</td>
<td>20</td>
<td>Pronase after culture to 105 h stage</td>
<td>Whittingham's</td>
<td>Glass</td>
<td>—</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>Day 4, blastocysts (82–84 h)</td>
<td>35</td>
<td>Mechanical</td>
<td>Whittingham's</td>
<td>Glass saucer on collagen</td>
<td>—</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>Late day 4, Post-zona loss</td>
<td>23</td>
<td>—</td>
<td>Whittingham's</td>
<td>Glass</td>
<td>—</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>J</td>
<td>Day 4, blastocysts (82–84 h)</td>
<td>37</td>
<td>Pronase</td>
<td>Earle's B.S.S. &amp; 10% foetal calf serum</td>
<td>Agar</td>
<td>—</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

* Day of vaginal plug = day 1. † Hours after the estimated time of ovulation.
Table 2. **Behaviour of embryos, pre-cultured from eight-cell stage, on collagen surfaces in Whittingham’s medium**

<table>
<thead>
<tr>
<th>Group</th>
<th>Stage of recovery</th>
<th>No. of embryos</th>
<th>Method of zona removal</th>
<th>Stage when placed on collagen*</th>
<th>Time of outgrowth</th>
<th>No. with outgrowth (Day 8)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>Day 3, 8-12 cell (57 h)</td>
<td>22</td>
<td>Pronase just before transfer to collagen</td>
<td>Day 4, late morulae (81 h)</td>
<td>Days 7-8</td>
<td>20</td>
<td>91</td>
</tr>
<tr>
<td>L</td>
<td>Day 3, 8-12 cell (57 h)</td>
<td>13</td>
<td>Pronase just before transfer to collagen</td>
<td>Day 5, expanded blastocyst (105 h)</td>
<td>Days 7-8</td>
<td>12</td>
<td>92</td>
</tr>
<tr>
<td>M</td>
<td>Day 3, 8-12 cell (57 h)</td>
<td>28</td>
<td>Pronase when recovered</td>
<td>Immediately after zona removal</td>
<td>Day 8</td>
<td>25</td>
<td>89</td>
</tr>
</tbody>
</table>

* Culture prior to transfer to collagen was carried out in microdrops under paraffin.
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(Fig. 1 B, C, D). Blastocysts recovered at 93 h (group C), just before zona loss, started outgrowth on day 6 (about 136 h) and also gave a high percentage outgrowth (Tables 1, 2).

A high percentage of embryos recovered from the oviduct at the 8-cell stage and cultured to the late morula stage (group K) or to the expanded blastocyst stage (group L) before zona removal and transfer to collagen produced outgrowths similar to that of uterine blastocysts. As with blastocysts recovered from the uterus at 84 h, this started on day 7 irrespective of the time of transfer to collagen (cf. groups K, L and M). Zona-free 8-cell embryos cultured immediately on collagen surfaces developed normally until the late blastocyst stage before attaching and spreading (group M). To confirm this observation groups of 8-cell stage blastomeres, obtained by mechanical disaggregation of 19 whole embryos, were seeded on a collagen surface. Again these showed no affinity for the gel at this stage although they did show great affinity for each other and reaggregated to produce normal blastocysts and trophoblast vesicles.

Blastocysts cultured in Whittingham’s medium on glass or plastic surfaces usually remained ‘free-floating’ (Fig. 1a) and showed outgrowth in only a very small number of cases by day 8. Such outgrowths were usually slight, consisting of a small halo of cytoplasm or a few narrow pseudopodia. If these cultures were continued for a further 2–4 days it was found that the blastocysts, although collapsed, remained apparently healthy and sometimes produced slight outgrowth in up to 20% of cases. Occasionally some of these collapsed masses fused to form larger masses, an observation possibly indicative of the breakdown of the tight junctions normally present between the trophoblast cells.

When blastocysts were cultured in the presence of collagen, but without being in direct contact with it (group H), the rate of outgrowth was negligible, as in Whittingham’s medium alone, suggesting that the effect of the collagen on outgrowth is physical rather than metabolic. The importance of the physical characteristics of the substrate in permitting outgrowth was emphasized by absence of outgrowth on agar surfaces, even in the presence of whole serum (group J).

No difference in outgrowth potential on collagen was detected between blastocysts from which the zona was mechanically removed (groups B and C) and those exposed to pronase (groups A, K and L), indicating that exposure to a zona lytic factor does not facilitate outgrowth. The low rate of outgrowth on glass (group D) and plastic (group F) was not improved by exposure to pronase (cf. group E). To exclude the possibility that exposure to a zona lytic factor could only increase trophoblast adhesiveness when acting on a mature trophoblast surface, 84 h blastocysts were cultured overnight to 105 h (expanded blastocysts) before zona removal (group G). Again, however, there was no significant outgrowth. Furthermore, blastocysts recovered from the uterus after natural loss of the zona showed no greater ability to produce outgrowth on glass in serum-free medium than did blastocysts recovered before zona loss (group I). Thus exposure to the in utero zona lysin, proposed by Mintz (1971) as
the agent promoting 'stickiness' of the trophoblast and thereby initiating implantation, does not facilitate outgrowth on glass.

**DISCUSSION**

Previous observations on blastocyst outgrowth *in vitro* have suggested a control of implantation dependent on a uterine factor which stimulates blastocyst metabolic activity (Menke & McLaren, 1970) or on the provision of a nutrient factor necessary for further blastocyst development (Gwatkin, 1969). Mintz (1971) has also implicated a uterine factor in the initiation of implantation, although in a somewhat different manner. She postulated that the immediate trigger for implantation is an increase in trophoblast adhesiveness brought about by the action of a uterine zonalytic enzyme. This, she suggests, also acts as an implantation initiation factor (IIF) either by having an enzymic effect on the trophoblast surface and exposing adhesive groups or by acting as a linking molecule between the trophoblast and the epithelium.

The present results demonstrate that one of the main requirements for outgrowth is the provision of a physically suitable surface. Given such a surface, e.g. reconstituted collagen, blastocyst outgrowth can occur in the absence of serum macromolecules in a simple medium known not to stimulate blastocyst metabolic activity (Menke & McLaren, 1970). In addition, embryos cultured from the 8-cell stage, and therefore isolated from the uterine environment, are as capable of outgrowth as uterine blastocysts. Thus it would appear (a) that outgrowth, and by analogy implantation, is not necessarily dependent on an exogenous metabolic activator produced by the uterus or provided by serum *in vitro* and (b) that changes in the trophoblast necessary for outgrowth are intrinsic to the embryo. The outgrowth of blastocysts in a serum-containing medium is therefore probably a consequence of the protein coating produced on glass surfaces by serum macromolecules and it is not necessarily associated with the increased metabolic activity observed in such media, as previously suggested (Menke & McLaren, 1970). The possibility that the uterus exerts a control over trophoblast maturation by producing a repressor which inhibits the intrinsic changes in the trophoblast until it is removed (Nilsson, 1970) cannot, however, be eliminated. This could possibly be tested by assessing the effect of uterine secretion, collected at different times, on outgrowth on collagen in the absence of complicating serum factors.

Trinkhaus (1969) has suggested that increased spreading activity on the part of cells is indicative of an increase in their adhesiveness. If this idea is accepted, the ability of the trophoblast to undergo intrinsic changes in its capacity for outgrowth makes Mintz's (1971) hypothetical IIF superfluous. This is supported by the fact that trophoblast adhesiveness, expressing itself as outgrowth, did not increase following exposure either to the enzymic effects of a known zona lysis (pronase) *in vitro* or to the uterine environment at the peak of the supposed zona lysis activity. Further, it might be expected that if uterine IIF acted as a
linking molecule on the trophoblast surface it might have a similar linking action between trophoblast and glass, as serum macromolecules appear to have, and thereby promote outgrowth. This was not found to be the case.

Prior to the late blastocyst stage the embryonic cells show no affinity for the collagen surface even if the zona is removed. They do, however, have a great affinity for each other as shown by the reaggregation of dissociated 8-cell-stage blastomeres seeded on collagen. Evidently as development proceeds there are qualitative changes in the nature of the adhesive properties of the embryonic cells, the adhesiveness involved in blastomere interaction being of a different specificity to that displayed by the trophoblast at the time of implantation. These changes provide further evidence of the specialized differentiation of the trophoblast and do not take place until the embryo is sufficiently developed to exploit the maternal resources which become available to it at implantation.

Recently the suggestion has been made that cell adhesion results from an enzyme/substrate interaction between a glycosyl transferase at one cell surface and an incomplete oligosaccharide at the surface of another (Roseman, 1970; Bossman, 1971). Thus the different nature of the adhesive properties of the early blastomeres and the trophoblast could be explained in terms of the activity of different transferases at different times in development. Alternatively it is conceivable that the changes in the trophoblast leading to attachment involve a decrease in the negative charge which the trophoblast probably carries (Clemetson, Moshfeghi & Mallikarjuneswara, 1971) enabling it to approach and adhere to the negatively charged endometrium. Such changes could be brought about by an alteration in the composition or configuration of the cell surface so as to remove or conceal charged groups, e.g. the end-groups of sialic acid, known to contribute to cell surface negative charge. One theory recently forwarded is that implantation is initiated by an increase in the K⁺ content of the uterine fluid leading to a decrease in the negative membrane potential of the trophoblast (Clemetson, Kim, Mallikarjuneswara & Wilds, 1972). However, it can be seen from the present observations that the adhesiveness of the trophoblast can increase without changes in the Na⁺/K⁺ ratio in the medium. It seems more likely, therefore, that the effect of increased K⁺, if any, would be more important in terms of its effect on epithelial charge.

The two requirements for trophoblast outgrowth in vitro - those of adhesive maturation and the provision of a suitable surface for the expression of this outgrowth - suggest a potential mechanism for implantation control at the cell surface level. It is only when these two requirements are simultaneously fulfilled that outgrowth can occur and implantation in utero could be similarly controlled. In vivo, it is the uterine epithelium which has to provide the 'suitable surface'. At most times the epithelium may be functionally similar to agar or uncoated glass to which the eggs cannot attach even if matured. Given maturation of the trophoblast, which appears intrinsically when the embryo is ready for its next phase of development, the precise onset of implantation could be controlled by
changes in the epithelial properties perhaps to provide a surface functionally
similar to collagen. It seems very likely that the hormonally controlled ultra-
structural changes of the luminal surface at the time of implantation (Potts,
1969; Nilsson, 1970) provide such a surface.

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REFERENCES


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