Development of a placental blood circulation in rat embryos in vitro

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SUMMARY

Rat embryos explanted with their membranes at head-fold stage (9½ days gestation) formed an allantoic placenta which enlarged in culture and developed a foetal blood circulation. Embryos explanted at early somite stages (10½ days) also formed a growing allantoic placenta but only after removal of most of the ectoplacental trophoblast. Assays of total protein in the embryo and placenta suggested that, in the absence of a maternal blood circulation to the placenta, embryo and placenta compete for the respiratory and nutritional resources obtained through the yolk-sac.

INTRODUCTION

It has been shown that rat embryos of head-fold stage (9½ days gestation) can be grown in vitro to the 25-somite stage (equivalent to 11½ days gestation) at rates of growth and differentiation indistinguishable from those in vivo (New, Coppola & Cockroft, 1976a). Older embryos grow more slowly (Robkin, Shepard & Tanimura, 1972; Cockroft, 1973, 1976) probably because they require the support of a chorio-allantoic placenta which is lacking in culture. Attempts to culture this placenta with the explanted embryo have previously been unsuccessful. It has either failed to acquire a blood circulation or, if a circulation was already present at the time of explantation (i.e. if the embryo was older than about the 17-somite stage), it has failed to develop further. In both types of culture, the internal tissues of the placenta soon became necrotic. A blood circulation has occasionally been observed in the placenta of cultured mouse embryos but only in a small proportion of all the embryos explanted (New & Stein, 1964; Hernandez-Verdun & Legrand, 1971, 1975).

Using improved culture methods, we have now obtained growth of the chorio-allantoic placenta in a high proportion of explanted rat embryos, with the development of a functional placental blood circulation.

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MATERIALS AND METHODS

Rat embryos of CFHB strain were explanted with their membranes at the head-fold (9½ days post coitum) and early somite (10½ days) stages as described previously (New, 1966, 1971). Reichert's membrane was torn open and most of it removed but the visceral yolk-sac was left intact. The whole explanted conceptuses will be referred to in this paper as 'explants' and the term 'embryo' restricted to the part of the explant that develops into the foetus. 'Placenta' will be used to indicate the chorio-allantoic placenta, not the yolk-sac.

In some of the explants, most or all of the ectoplacental cone (trophoblast) was removed with watchmaker's forceps; the development of these explants was compared with others from the same litters cultured with the cone intact.

The improvements in culture methods, as compared with those used in previous studies of placental growth in vitro, were (i) incubation of the explants in roller bottles, (ii) an improved culture medium, and (iii) a reduced oxygen concentration for the pre-somite stages of development (New, Coppola & Cockroft, 1976b). The explants were incubated at 38 °C in homologous serum in cylindrical bottles rotated continuously at 40–50 rev/min for 44–48 h. The bottles were of 30 ml capacity, each containing four 9½-day explants in 4 ml serum or four 10½-day explants in 6 ml serum. The serum was prepared from blood centrifuged immediately after extraction (Steele, 1972; Steele & New, 1974) and heat-inactivated (56 °C for 30 min) before being added to the culture bottles. The bottles containing 9½-day embryos were gassed with 10 % O₂/5 % CO₂/85 % N₂ for the first 24 h, followed by 20 % O₂/5 % CO₂/75 % N₂; those containing 10½-day embryos with 20 % O₂/5 % CO₂/75 % N₂ for the first 24 h, followed by 95 % O₂/5 % CO₂.

At the end of the culture period the explants, and particularly the placentas, were examined under a dissecting microscope. Some of the explants were then sectioned at 8 μm and stained with haematoxylin and eosin for histological examination; the others were separated into embryonic and placental components and assayed for protein content by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Embryos explanted at 9½ days

At the beginning of incubation (12.00 noon) the embryos were at the head-fold stage and the allantois, chorion and ectoplacental cone were still separate (Fig. 1). The ectoplacental cone consisted of inner small cells and outer giant cells. Twelve embryos were cultured with the cone intact and 12 after it had been completely removed, exposing the chorion.

The results are summarized in Table 1. All the 24 embryos developed to 23–26 somites, rotated to the ventrally-concave foetal position and had a vigorously
Fig. 1. Head-fold (9½-day) embryo with membranes and ectoplacental cone, as explanted. Broken line indicates line of cut for the explants in which the ectoplacental cone was removed. al, allantois; ch, chorion; r.m., Reichert’s membrane; e.c., ectoplacental cone.

Fig. 2. Placenta of a 9½-day embryo explanted with the ectoplacental cone, after 48 h in culture. The placenta, which had a functioning blood circulation, contains a much-folded chorion and blood capillaries embedded in allantoic mesenchyme. The spherical mass at the bottom contains giant cells and necrotic material derived from the ectoplacental cone.

beating heart and blood circulation to the yolk-sac at the end of the 48 h culture period. In all the explants except one, the allantois, chorion and ectoplacental cone fused to form a placenta, and large umbilical vessels could be seen in the allantoic stalk. Because the allantois was surrounded by yolk-sac it was occasionally difficult to determine whether a blood circulation was present in the placenta, but in at least eight of the explants with ectoplacental cone and nine without the cone a placental circulation was clearly seen.

The placenta of the explants grown with ectoplacental cone typically appeared
in section as in Fig. 2. A much-folded chorion was evident, interspersed with blood capillaries embedded in allantoic mesenchyme. The placenta was cone-shaped, unlike the normal disc-shaped placenta found in embryos of equivalent age in vivo. A spherical mass of giant cells and degenerating cells was usually loosely attached over a small part of the external surface and the remainder was covered with a single layer of cells resembling parietal yolk-sac which had probably grown over from the cut edges of Reichert's membrane. In some explants, the mass of giant cells had become completely detached and the 'parietal layer' covered the whole placental surface.

No difference was noted in the placental histology of the embryos grown without the ectoplacental cone except that the giant cells were of course absent.

Four explants from each group were separated into embryo, allantoic placenta and yolk sac + amnion and compared for protein content. The differences were insignificant (Table 1).

**Embryos explanted at 10½ days**

At explantation, the embryos had 7–15 somites and a beating heart. In many of them, blood had begun to circulate in the yolk-sac. The allantois, chorion and ectoplacental cone had already fused and the allantois contained blood islands but had not yet acquired a blood circulation. The chorion was folded and covered externally by a thick layer of giant trophoblast cells. Between the chorion and giant cells were clusters of small trophoblast cells and spaces containing maternal blood. Twenty-four explants were cultured with the ectoplacental cone (trophoblast) intact and 24 after removal of most of the cone.

The results are summarized in Table 1 and Figs. 3–6. Nearly all the embryos developed normally to 33–37 somites and early in the culture a blood circulation was established in the yolk-sac which persisted for most or all of the remaining culture period. Development of the embryo was similar in the explants with and without the ectoplacental cone but major differences were found in the placenta.

In the explants with the ectoplacental cone, the layer of giant cells thickened further and the adjacent tissues became necrotic (Fig. 4). There was a tendency for the cone to round up, but it was less pronounced than in the 9½-day explants and the cone remained attached to the chorion over a large area. The chorion and allantois showed varying degrees of degeneration. Only a few of the explants in this group established a placental blood circulation; in the others, blood capillaries had failed to develop or had become occluded. The allantoic stalk was usually thin, without continuous umbilical blood vessels, and in some explants it disappeared entirely leaving embryo and placenta unconnected.

In the explants without ectoplacental cone, the placenta enlarged up to about 2 mm diam. and a blood circulation was established which persisted to the end of the culture period. Under the dissecting microscope, the placenta was often observed to pulsate with each heart beat and blood could be seen flowing along the umbilical vessels and through the capillaries underlying the whole placental
Rat placenta in vitro

Fig. 3. Diagram of placental development in culture of 10½-day embryos. (A) Explant with ectoplacental cone intact, and (B) placental region of the same after 48 h culture; the layer of giant cells has thickened, most of the remaining placenta has become necrotic and a large blood vessel bypasses the umbilical vessels at the embryonic end of the allantoic stalk. (C) Explant with most of the ectoplacental cone removed, and (D) placental region of the same after 48 h in culture; chorionic vili have formed with blood capillaries connected to the embryonic blood circulation by a large umbilical vein and artery in the allantoic stalk.

Surface. The placenta was usually smaller than that of equivalent age in vivo, but similar in shape. In section, the chorionic vili were found to have formed a labyrinthine structure with numerous blood capillaries connected to a large umbilical artery and vein (Figs. 5, 6). Most of the placenta appeared healthy, with many cells in mitosis, even after 48 h of culture.

Examination of serial sections showed that the connexions of the umbilical blood vessels with the rest of the embryonic circulation closely resembled that described for rat embryos in vivo at 12–12½ days gestation (Monie & Khemmani, 1973). The single umbilical artery of the allantoic stalk was supplied by the right and left primary umbilical arteries from the dorsal aorta, and the secondary umbilical arteries were forming. The single umbilical vein, on entering the body from the stalk, divided into a degenerating right branch and a large left branch which extended forward to enter the sinus venosus. In those explants cultured with the ectoplacental cone where a placental circulation had failed to develop, a connexion was established between the umbilical vein and artery in the allantoic stalk which bypassed the placenta.

Two experiments were made to determine the amount of protein synthesis in
Figs 4–6. For legend see facing page.
Table 1. Development of placental blood circulation, and protein content of embryo, placenta and membranes in rat conceptuses explanted at 9½ and 10½ days of gestation and grown in culture for 44–48 h

<table>
<thead>
<tr>
<th>Age (d)</th>
<th>No. of embryos with Umbilical vessels</th>
<th>Placental circ.</th>
<th>Protein* (µg) Mean ± s.E. in Embryo</th>
<th>Yolk-sac + amnion</th>
<th>Allantoic placenta</th>
<th>Whole conceptus</th>
</tr>
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<tbody>
<tr>
<td>9½</td>
<td>12 + cone 11</td>
<td>8†</td>
<td>190 ± 7</td>
<td>170 ± 8</td>
<td>49 ± 3</td>
<td>409 ± 5</td>
</tr>
<tr>
<td></td>
<td>12 – cone 12</td>
<td>9†</td>
<td>183 ± 7</td>
<td>154 ± 2</td>
<td>47 ± 1</td>
<td>384 ± 8</td>
</tr>
<tr>
<td>10½</td>
<td>12 + cone 1</td>
<td>1</td>
<td>630 ± 50</td>
<td>396 ± 28</td>
<td>1026 ± 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 – cone 11</td>
<td>11</td>
<td>525 ± 47</td>
<td>434 ± 29</td>
<td>959 ± 74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 + cone 3</td>
<td>3</td>
<td>576 ± 32</td>
<td>251 ± 8</td>
<td>93 ± 6</td>
<td>920 ± 40</td>
</tr>
<tr>
<td></td>
<td>12 – cone 12</td>
<td>11</td>
<td>508 ± 22</td>
<td>234 ± 8</td>
<td>136 ± 13</td>
<td>878 ± 34</td>
</tr>
</tbody>
</table>

* Protein determinations were made on two-thirds (48) of the explants. See text for numbers of protein determinations in each experiment.
† These are minimum figures, indicating only the number of placentas in which a blood circulation could be seen clearly.

the embryonic and placental components of the explants (Table 1). In the first experiment, eight explants cultured with the ectoplacental cone were compared with eight cultured without the cone. The embryo and the embryonic membranes (including the placenta) of each explant were assessed separately. The results indicated more protein in the membranes, but less in the embryo, of the explants without the cone. A second experiment with 12 explants in each group gave similar results and further information was obtained by determining the protein content of the placenta separately from that of the yolk-sac and amnion. *t* tests showed that placental protein was significantly higher in the explants without the cone than in those with cone ($P < 0.01$) while embryo protein was significantly less ($P = 0.02$).

Figs. 4–6. Placentas of embryos explanted at 10½ days and grown for 48 h in culture.
Fig. 4. The embryo was explanted with the ectoplacental trophoblast intact. After culture, a thick layer of giant cells surrounds a mass of necrotic material. Above are degenerate chorion, allantoic mesenchyme and blood islands. No functional blood capillaries are present.
Fig. 5. The embryo was explanted with most of the ectoplacental trophoblast removed. After culture, a placenta has formed with a functional blood circulation and the section shows branches of the umbilical artery (*ua*) extending into the chorionic villi.
Fig. 6. Part of the placental ‘labyrinth’ of a similar embryo to that of Fig. 5. Note the numerous foetal capillaries (*fc*) lined with endothelium and separated mostly by chorionic tissue. Only a few giant cells (*g*) are present, at the bottom right.
DISCUSSION

The formation of the rat placenta from the embryonic membranes in vivo is described by Steven & Morriss (1975). An early account of the histology of the developing placenta was given by Bridgman (1948a, b) and more recently the ultrastructural changes have been investigated by Jollie (1964) and Davis & Glasser (1968). The similar ultrastructural development of the mouse placenta is described by Hernandez-Verdun (1974) with particular reference to the development of the cellular and syncytial layers separating maternal and foetal blood. Hernandez-Verdun & Legrand (1971, 1975) have also studied, by light and electron microscopy, the changes in the chorionic and ectoplacental trophoblast of mouse embryos explanted at head-fold and early somite stages and grown in culture.

Although the placental growth and development of our rat embryos in culture is still limited, it appears to follow many of the same steps as in vivo including the initial extension of the allantois to join the chorion, the progressive folding of the chorion to form a placental labyrinth, and the differentiation of capillaries and umbilical vessels in the allantois to provide a blood circulation. An obvious difference is the lack of any maternal blood circulation or maternal blood spaces.

The behaviour of the ectoplacental cone is interesting. Our observations agree with those of Hernandez-Verdun & Legrand (1971) that when explants with the ectoplacental cone are made at head-fold stage, the giant cells frequently become partially or completely detached, apparently as the result of an extension of the parietal yolk-sac and Reichert membrane. Such explants usually develop a placental blood circulation. But in our explants at early-somite stage (10½ days) it was essential to remove the ectoplacental cone at the outset for a placental circulation to develop. If the cone were left intact, although there might be some rounding up of the giant cells, they remained attached to the rest of the placenta and inhibited further development. This inhibition may result simply from the thick mass of giant cells acting as a barrier to diffusion of respiratory gases and nutrients between the culture medium and remainder of the placenta. But it is possible that the giant cells may also be more actively destructive by secreting cytolytic agents that in vivo would be directed towards the maternal tissues. A secretion of cytolytic agents by trophoblast has been suggested by Kirby (1963) from studies on ectopically implanted blastocysts, and by Sherman & Salomon (1975) from observations on trophoblast growing on monolayers of uterine cells in vitro.

No improvement in growth of the embryo followed the development of a placenta with a blood circulation. In fact these embryos showed a small but significant reduction in the amount of protein synthesized when compared with those of explants in which the placenta had failed to develop. This suggests that, in the absence of a maternal blood circulation in the placenta, the growing placenta may compete with the embryo for the respiratory and nutritional resources obtained through the yolk-sac and distributed by the foetal blood.
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REFERENCES


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