Growth and proliferation in mouse parietal yolk sac during whole embryo culture

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Summary. Use of the culture techniques for postimplantation rodent embryos, modified by explanting Day 9 or Day 10 embryos with the trophoblast cells removed but the remainder of the parietal yolk sac left intact, resulted in significant expansion of Reichert's membrane, accompanied by a marked increase in the numbers of the adherent parietal endoderm cells which synthesize the membrane. The surface area of the parietal endoderm/Reichert's membrane complex roughly doubled during culture, and the combined protein content of the cells and their basement membrane was also raised after incubation. Parietal endoderm cell numbers, calculated from area and cell density measurements on flattened membranes, increased by 54–190%, depending on the age of the embryo.

Introduction

The parietal yolk sac of the mid-gestation mouse fetus consists of a parietal endoderm and a trophoblast layer, with Reichert's membrane in between. It surrounds the conceptus and is closely apposed to the visceral yolk sac which is internal to it. The tissue has been studied mainly as a convenient system for investigating basement membrane synthesis (Minor *et al.*, 1976a, b; Hogan, 1980; Hogan *et al.*, 1980). Although in conventional histological preparations parietal endoderm cells appear to be similar to each other, it has been shown by scanning electron microscopy and other means that there are striking regional and temporal variations in the morphology of the cells, and time-lapse video recordings of whole mount preparations in tissue culture have shown that the motile activity of the cells depends on their position and the age of the embryo (Cockroft, 1986). The above studies demonstrated that parietal endoderm cells on their basement membrane survive for prolonged periods in culture, and that they continue to synthesize basement membrane, but there has been no previous unequivocal evidence that parietal endoderm cell numbers increase *in vitro*. Normal relations between the parietal and visceral endoderm cells could be important for such proliferation to happen, and so the present study was undertaken using a modified whole embryo culture technique (New, 1978). It has previously been shown that some expansion of the rat parietal yolk sac *in vitro* can be achieved by use of a higher than normal oxygen concentration, and with dilution of the culture serum (Ellington & New, 1980).

Materials and Methods

The mice used were of the P.O. (Pathology Oxford) strain, and they were killed by cervical dislocation on Day 9 or Day 10 of pregnancy, Day 1 being that on which a vaginal plug was observed. The uteri were torn open in phosphate-buffered saline (PBS) and the conceptuses removed and transferred to PB1 medium (Whittingham & Wales, 1969), modified by the inclusion of glucose (1 g/l) instead of lactate, and fetal calf serum (10% v/v) in place of bovine serum albumin. Explantation was completed as described by New (1971), except that, for embryos to be cultured with intact Reichert's membrane, the trophoblast was carefully peeled off leaving Reichert's membrane, with its internally adherent parietal endoderm cells, as an intact layer overlying the visceral yolk sac. For some embryos, however,

Reichert's membrane was torn open, exposing the visceral yolk sac to the medium. The maximum and minimum diameters of the visceral yolk sac or of the parietal endoderm/Reichert's membrane complex for each embryo were measured before culture. (Because the parietal and visceral yolk sacs are closely apposed to each other, and the outermost (parietal) layer is very thin, the surface areas of the two membranes are virtually identical in a particular specimen, and so the measurements may be used interchangeably.) The surface areas of the parietal endoderm/Reichert's membrane or of the visceral yolk sac were calculated using the formula πab , where a and b are respectively the maximum and minimum diameters of the membranes.

Two embryos with an intact Reichert's membrane and two with it open were placed in each culture vessel, a 50-ml polypropylene centrifuge tube (Falcon; Beckton Dickinson, Oxford, U.K. or Costar; L. H. Engineering Co., Stoke Poges, Bucks, U.K.) containing 5 ml pure rat serum prepared by centrifugation immediately after withdrawal from the donor animal, and heat-inactivated (30 min at 56°C) and gassed before use (Steele & New, 1974; New *et al.*, 1976). Day-9 embryos were gassed initially with 10% O₂/5% CO₂/85% N₂, which was changed to 20% O₂/5% CO₂/75% N₂ after 18 h of culture. Day-10 embryos were gassed with 10, 20, 40 or 95% O₂ throughout culture, each with 5% CO₂, and N₂ to make up the balance (if any). At the end of the culture period, which was 17 h for Day-10 embryos, or 30 h for Day-9 embryos, heart beats and visceral yolk sac blood circulations were recorded and the yolk sac dimensions were measured again. After removal of the extraembryonic membranes, somite counts were made and crown-rump lengths were measured. In some instances protein contents of the embryos and of the parietal endoderm/Reichert's membrane were determined by the method of Lowry *et al.* (1951).

To confirm that cell proliferation was taking place during culture, the number of parietal endoderm cells on the membranes of cultured and uncultured embryos was determined by measuring the areas of the membranes and estimating cell densities. To do this, the entire conceptus with intact Reichert's membrane was fixed for 10 min in formal saline at room temperature, the Reichert's membrane (with adherent parietal endoderm cells) was removed and cuts were made in it to allow it to be spread out substantially flat on a microscope slide and mounted in Aquamount (BDH; Poole, Dorset, U.K.). The outline of the membrane was traced using a drawing tube attached to a Wild M5 dissecting microscope, and cell counts on four fields, each of 30276 μm^2 , were made for each membrane, using a drawing tube attached to a Zeiss RA microscope. Thus it was possible to estimate the cell density and the total number of cells on each membrane.

Results

Representative embryos explanted on Day 10 are illustrated in Fig. 1. Development of such embryos was marginally better in 40% O₂ than in 20% O₂, and considerably better than in 10% O₂ (Table 1). Increase in the surface area of Reichert's membrane was similar with all three oxygen concentrations, at 86–88%, compared with an increase of up to 112% in the visceral yolk sacs of

Fig. 1. Day-10 mouse embryos: (a) at explantation, (b) after 17 h culture with an intact parietal endoderm/Reichert's membrane, and (c) after 17 h culture with Reichert's membrane opened. Considerable expansion of the intact Reichert's membrane has taken place, but not quite as much as of the visceral yolk sac of the embryo in which it was opened.

Table 1. Development of Day-10 mouse embryos cultured for 17 h with a closed or open parietal endoderm/Reichert's membrane (PE/R) with 10, 20 or 40% O₂

O ₂ conc.	PE/R	No.	Heart beat at end	VYS circ. at end	PE/R or VYS				Crown-rump (mm)	Embryo protein (µg)	PE/R protein (µg)
					Initial area* (mm ²)	Final area* (mm ²)	% increase	Somites			
Uncultured embryos		23	—	—	24.1 ±1.2	—	—	21.9 ±0.8	2.17 ±0.09	106 ±9	8.5 ±0.9 (6)
10%	closed	12	10	6	24.0 ±1.8	44.1 ±4.1 (10)	88	26.6 ±0.7	3.18 ±0.18	154 ±14	17.3 ±1.7 (6)
10%	open	12	12	11	23.7 ±1.4	45.5 ±2.9 (11)	92	28.3 ±0.1	3.90 ±0.30	207 ±9	—
20%	closed	12	12	8	24.1 ±1.6	44.4 ±2.5	86	28.1 ±0.7	3.33 ±0.18	195 ±17	21.5 ±4.2 (6)
20%	open	12	11	11	24.1 ±1.5	50.0 ±2.0	112	29.3 ±0.6	3.76 ±0.18	253 ±13	—
40%	closed	12	11	6	24.3 ±1.5	45.5 ±2.9	88	29.4 ±0.7	3.46 ±0.17	230 ±22	19.9 ±1.7 (6)
40%	open	12	10	10	24.2 ±1.9	50.0 ±3.2	110	30.3 ±0.5	3.74 ±0.11	275 ±18	—

Values are mean ± s.e.m.
 Numbers in parentheses indicate the no. of embryos used for a particular measurement. When no such figure is shown, all the embryos in that group were used.
 VYS = visceral yolk sac; circ. = circulation.
 *See text for formula.

Table 2. Estimates of cell numbers in the parietal endoderm/Reichert's membrane (PE/R) complex uncultured and 17 h-cultured Day-10 mouse embryos

Oxygen conc. %	PE/R				No. of PE cells/ membrane	% increase	Somites	Crown-rump length (mm)
	No.	Initial area (mm ²)	Final area (mm ²)	% increase				
Uncultured embryos	6	29.4 ± 1.5	—	—	58 800 ± 3900	—	25.5 ± 0.3	2.72 ± 0.09
20	6	28.4 ± 0.9	51.4 ± 2.2	81	95 000 ± 6400	62	31.7 ± 0.4	3.93 ± 0.05
40	6	28.6 ± 0.9	53.2 ± 1.5	86	90 700 ± 5900	54	32.5 ± 1.0	3.87 ± 0.18

Values are mean ± s.e.m.

embryos cultured with Reichert's membrane opened. There was also a significant increase, up to 2.5-fold, in the protein content of the parietal endoderm cells + Reichert's membrane, although this was marginally less in the embryos cultured with 10% O₂ than with higher oxygen concentrations. On average the parietal endoderm cell number in Day 10 embryos cultured with 20% or 40% O₂ increased by 62% ($P < 0.001$) and 54% ($P < 0.002$) respectively, compared with matched, uncultured controls (Table 2).

Because development at the highest oxygen concentration used was marginally better than with the lower oxygen tensions, a further series of experiments was done to compare development in 95% O₂ with that in 40% O₂ and 20% O₂. Also, because it was thought that the quantity of serum might be limiting development, only two embryos, one with an open and one with a closed Reichert's membrane, were placed in each culture tube, which contained 4 ml serum. The results (Table 3) show that development with the lower oxygen concentrations is similar to that obtained previously, and with 95% O₂ it is inferior, but not markedly so, except for the protein content of the parietal endoderm/Reichert's membrane. The greatest expansion in the area of Reichert's membrane in this case was 101%, obtained with 20% O₂, and so was almost as great as the visceral yolk sac expansion of the embryos cultured with open Reichert's membranes (110%).

The results for Day-9 embryos are shown in Table 4. The embryos cultured for 30 h with intact parietal endoderm/Reichert's membrane showed an approximate doubling in surface area during culture, together with a 3-fold increase in parietal endoderm cell number. However, although they maintained a heart beat and visceral yolk-sac circulation throughout culture, the embryos in these explants were obviously severely constricted by the extraembryonic membranes; there was much greater expansion of the visceral yolk sacs in the embryos of this age cultured with open Reichert's membrane.

Discussion

These results demonstrate that significant increases in parietal endoderm cell number take place in whole embryo culture, together with expansion of the underlying basement (Reichert's) membrane. For Day-10 embryos, the expansion of conceptuses with intact Reichert's membrane is almost as great as for those with it open, whereas Day-9 embryos expand far less with a closed than with an open Reichert's membrane, perhaps because of the longer culture period (30 h instead of 17 h).

Although there have been no previous studies on the parietal yolk sac of the mouse in whole embryo culture, there is some pertinent work on the rat embryo. Payne & Deuchar (1972) cultured rat embryos with the parietal yolk sac (including trophoblast cells) in place, and found higher embryonic protein contents and leucine incorporation than when these tissues were removed,

Table 3. Development of Day-10 mouse embryos cultured with increased medium quantity for 17 h with closed or open parietal endoderm/Reichert's membrane (PE/R) with 20, 40 or 95% O₂

O ₂ conc. (%)	PE/R	No.	Heart beat at end	VYS circ. at end	PE/R or VYS				Somites	Crown-rump (mm)	Embryo protein (µg)	PE/R protein (µg)
					Initial area (mm ²)	Final area (mm ²)	% increase					
Uncultured embryos		12	—	—	24.5 ± 1.2	—	—		21.7 ± 0.7	2.20 ± 0.09	101 ± 14	9.5 ± 2.4
20%	closed	6	5	3	24.9 ± 1.9	50.0 ± 3.4	101		29.3 ± 0.6	3.50 ± 0.14	208 ± 15	22.2 ± 2.3
20%	open	6	6	6	25.1 ± 1.6	51.8 ± 1.9	110		30.0 ± 0.4	3.90 ± 0.12	301 ± 14	
40%	closed	6	5	5	25.7 ± 1.4	46.5 ± 2.2	81		30.7 ± 0.7	3.40 ± 0.22	243 ± 39	20.8 ± 2.5
40%	open	6	6	4	23.9 ± 2.6	50.7 ± 5.6	113		30.0 ± 0.6	3.67 ± 0.22	283 ± 42	
95%	closed	6	4	4	24.9 ± 1.0	39.9 ± 2.1	61		29.3 ± 0.7	3.20 ± 0.14	206 ± 26	14.8 ± 1.8
95%	open	6	6	4	24.3 ± 2.1	42.9 ± 3.6	78		30.0 ± 0.9	3.48 ± 0.21	249 ± 35	

Values are mean ± s.e.m.

Table 4. Development of Day-9 mouse embryos cultured for 30 h with closed or open parietal endoderm/Reichert's membrane (PE/R)

	No.	Heart beat at end	VYS circ. at end	PE/R				No. of PE cells/membrane	% increase	Somites	Crown-rump length (mm)
				Initial area (mm ²)	Final area (mm ²)	% increase					
Uncultured embryos	8	8	8	7.2 ± 0.8	—	—		6989 ± 697	—	7.0 ± 0.4	1.00 ± 0.05
Closed PE/R	8	8	8	7.0 ± 0.6	14.3 ± 1.4	107		20 316 ± 2297	191	21.4 ± 1.3	2.56 ± 0.11
Open PE/R	8	8	8	7.2 ± 0.8	32.2 ± 1.9	379		—	—	27.6 ± 0.6	3.00 ± 0.09

Values are mean ± s.e.m.

although the most normal morphology and expansion of the extraembryonic cavities were obtained under the latter conditions. However, in both cases development was poor compared with that previously obtained with embryos of similar age cultured under standard conditions (New *et al.*, 1973). Ellington & New (1980) cultured Day-11 rat embryos for 24 h with intact parietal yolk sacs (i.e. without removing the trophoblast). At best they obtained a 30% increase in yolk sac area, using 50% serum/50% Hanks as the culture medium, and using a gas phase which increased from 20% to 95% O₂ during culture. Ellington & New (1980) stated that only 25–35% of the surface of the conceptus was covered with trophoblast at explantation, and that the trophoblast extended to cover 50–75% of the surface by the end of culture. Presumably it was this thick trophoblast layer which necessitated such high oxygen concentrations and it may also have physically impeded expansion of the conceptus. In the mouse, the trophoblast forms a continuous sheet completely enveloping the conceptus at the stages studied in this paper, and early attempts to culture embryos with this layer intact met with no success, even at high oxygen tensions. Dilution of the serum was also found to be deleterious. However, with the trophoblast removed, the intact Reichert's membrane appears to offer little impediment to the passage of oxygen, and the optimum oxygen concentration with closed or open Reichert's membrane is quite similar (present study).

Very little is known about the normal differentiation and proliferation of the parietal endoderm *in vivo*. The apparent paucity of dividing cells in the tissue and the morphology of cells in the marginal zone between visceral and parietal layers has led to the suggestion that parietal cells are recruited from the visceral endoderm (Hogan & Newman, 1984). Circumstantial evidence in favour of this hypothesis is the observation that, under experimental conditions, visceral endoderm cells can acquire a parietal phenotype and synthesize large amounts of laminin and type IV procollagen—proteins characteristic of Reichert's membrane (Hogan & Tilly, 1981). Further evidence that visceral cells retain the ability to transform to the parietal type comes from blastocyst injection experiments (Gardner, 1982); injected Day-6 or Day-7 visceral endoderm cells contribute almost exclusively to the parietal endoderm in the resulting chimaeras. However, to obtain further information on the normal growth process in the parietal endoderm, it is desirable to have an *in-vitro* system such as the one described here, in which Reichert's membrane expands and the parietal endoderm cell numbers increase. Presumably the reason why such growth occurs in whole embryo culture, but not in cultures of the parietal endoderm/Reichert's membrane alone, is that the normal relations between visceral and parietal layers are important. The tension induced in Reichert's membrane by the expansion of the conceptus may also be significant; in cultures of parietal endoderm cells on isolated pinned-out Reichert's membrane, where expansion of the membrane does not take place, clumps of cells appear for which there is apparently no room on Reichert's membrane (Cockroft, 1986). The presence of the trophoblast does not appear to be necessary for growth and proliferation of the rest of the parietal yolk sac.

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