Morphometric analysis of the visceral yolk sac endoderm in the rat *in vivo* and *in vitro*

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Summary. Rat embryos together with their visceral yolk sac were grown *in vitro* during the early period of organogenesis from days 9.5 to 11.5 and the ultrastructural morphology of the visceral yolk sac cells was compared with that *in vivo* at the beginning and at the end of the culture period. Identical areas of the visceral yolk sac endodermal cells were analysed morphometrically. A measure of the functional activity was obtained by comparison of the volume density and surface density of the vacuolar system. At 9.5 and 11.5 days, the values obtained were virtually identical for yolk sacs *in vivo* and *in vitro*. At 9.5 days, the volume and surface density of the endocytotic vacuoles in the embryonic endoderm was significantly lower than in the visceral yolk sac endoderm.

It is concluded that the digestive function of the yolk sac is almost certainly identical in culture and *in vivo* and that the cells of the embryonic endoderm do not take a significant part in embryonic nutrition.

Introduction

Preplacental embryonic nutrition is achieved by the uptake and catabolism of maternal macromolecules which constitute the so-called histiotroph. This process takes place in the extra-embryonic membranes. Histiotroph arises from a variety of sources, but chiefly from the breakdown of proliferated endometrial cells, uterine secretion and extravasated maternal blood (Amoroso, 1952). In eutherian mammals, it is the only form of nutrition available to the embryo before the establishment of a chorio-allantoic placenta which transfers material directly from the maternal to the embryonic circulation across a 'placental barrier'. In the rat the chorio-allantoic placenta begins to function at about 11.5 days of gestation, but histiotrophic nutrition continues alongside this until term. The principal site for the final digestion of rat histiotroph is the visceral layer of the yolk sac epithelium (Beck, Lloyd & Griffiths, 1967) and this membrane persists as a functional entity until full term.

Rat embryos can now be grown routinely with their visceral yolk sac during much of the period of organogenesis (i.e. from Days 9.5 to 11.5) in specially prepared rat serum using the roller-tube culture technique (see New, 1978, for review). The rate and quality of embryonic growth and differentiation *in vitro* is virtually identical to that *in vivo* (Cockroft, 1976), but it has not been shown that embryonic nutrition is identical *in vivo* and *in vitro*.

In the present studies (part of a joint project with the University of Keele), a detailed morphometric analysis was carried out at 9.5 and 11.5 days of gestation to see whether yolk sac activity is truly identical in culture and *in vivo*.

Materials and Methods

Animals

Male and female Wistar rats were placed together in the evening and vaginal smears were examined for the presence of spermatozoa on the following morning. Rats showing a positive smear were presumed to have mated within 2 h of midnight (24:00 h) on the previous night, conceptuses thus being 0.5 days old at 12:00 h (noon).

Embryos

In vitro. The uterus was quickly removed from 9.5-day-pregnant rats (early head-fold stage) and aseptically transferred to a dish containing Hank's Balanced Salt Solution (Flow Laboratories Ltd., P.O. Box 17, Irvine, Scotland). It was then carefully opened with forceps to expose pear-shaped masses of decidual tissue from which the embryos and their yolk sacs, with a little adherent trophoblast and the ectoplacental cone, were dissected out in fresh Hank's Solution under a dissecting microscope. The outer layer of the yolk sac (Reichert's membrane with attached trophoblast and parietal endoderm) was opened but the visceral yolk sac, amnion and ectoplacental cone were left intact. Between 10 and 30 min after removal from the mother these specimens were fixed in glutaraldehyde (see below). The conceptuses (egg cylinders) prepared in this fashion constituted the in-vitro series because, in contrast to the rapidly fixed in-vivo embryos, they were prepared in Hank's solution and remained in this fluid for up to 30 min. This procedure was identical to that adopted when embryos were cultured for 48 h. Fixation was carried out at the point when incubation in serum would begin in the 11.5-day explanted specimens.

To obtain 11.5-day specimens, similarly prepared 9.5-day egg cylinders were placed in a sterile glass bottle which contained 1 ml of immediately centrifuged heat-inactivated rat serum per embryo (New, 1978). There were 4–6 egg cylinders in each bottle. The bottles were stoppered and placed horizontally on rollers in an incubator at 37°C at 40–50 rev./min for 48 h (New, 1978). For the first 24 h of the culture period, the embryos were gassed with a gas mixture containing 5% O₂–5% CO₂–90% N₂. This was replaced with 20% O₂–5% CO₂–75% N₂ between 24 and 36 h of culture and with 40% O₂–5% CO₂–55% N₂ between 36 and 48 h of culture.

In vivo. Specimens were obtained by killing rats at 9.5 or 11.5 days *post coitum*. The sterile uterus was immediately transferred to a dish containing heat-inactivated rat serum at 37°C. In this fluid conceptuses were rapidly dissected out and placed in 3% glutaraldehyde. Unlike the in-vitro preparations, the in-vivo embryos were never in contact with Hank's solution.

Tissue processing

All specimens were eventually fixed in 3% glutaraldehyde in phosphate buffer (pH 7.2–7.4) for 3 h at 4°C, washed twice in sucrose buffer (6.82 g sucrose in 100 ml phosphate buffer, pH 7.2–7.4) at a 5-min interval and left overnight in sucrose buffer at 4°C. The tissue was then post-fixed in 2% osmium tetroxide for 1 h at 4°C, dehydrated in graded ethanol solutions at room temperature and embedded in araldite.

Tissue orientation

To compare the endocytotic activity of the yolk sac epithelium *in vivo* and *in vitro*, identical areas of the yolk sacs were sectioned at both stages of gestation (Text-fig. 1).

Text-fig. 1. Diagrams of rat embryos at (a) 9.5 and (b) 11.5 days, showing the area of the visceral yolk sac endoderm and embryonic endoderm studied *in vivo* and *in vitro*. Reichert's membrane with attached trophoblast and parietal yolk sac were removed.

Microscopy

Semithin sections (about 1 μm thick) were cut and stained with toluidine blue, and examined under the light microscope to identify the orientation of the yolk sac cells.

Ribbons of ultrathin sections (70 nm thick) were cut from each of the tissue blocks, collected on 200 micromesh copper grids and stained with uranyl acetate and lead citrate. Electron micrographs were taken using a JEOL 100S electron microscope at an accelerating voltage of 80 kV. Micrographs were taken at 7000 magnification and printed to a final magnification of 21 000.

Stereological analyses

Volume density (V_v) is the volume of a profile of a particular interest expressed as a proportion of its containing volume and is calculated (Williams, 1977) as points over the object profile/total points applied to the micrograph.

In the present studies, measurements were made on photomicrograph prints. The volume density of the endocytotic vacuoles was expressed as a percentage of the total cytoplasmic volume obtained by 'point counting' analysis of electron photomicrographs as developed by Weibel (1969). The intersections of a squared lattice marked on a transparent screen which was superimposed onto each photomicrograph provided the points. The size of the test lattice used was 1 cm^2 . The procedure was greatly accelerated by using a quantitative analyser (MOP AMO2) and a table top computer (Apple II).

Surface density (S_v) relates the surface area of a cell compartment made up of any specific organelle type to the total cell volume in which it is contained. It is expressed as $S_v = 2 \cdot L_v$ where L equals the total length of horizontal lines placed in an overlay upon a photomicrograph and I the number of times the lines cut the contours of interest (Williams, 1977). Estimates of membrane area of the endocytotic vacuolar compartment were obtained by counting the number of intersections on the vacuolar membrane. The results are expressed as μm^2 per μm^3 of the cytoplasmic volume.

Sampling procedure

A sample large enough to ensure reasonable levels of accuracy for the measurements of volume density and surface density was obtained by drawing 'Cumulative Mean Plots'

(Williams, 1977). The sample size required to achieve and remain within $\pm 10\%$ of the final cumulative mean was taken as the 'Minimal Sample Size' (MSS) for that particular measurement.

Three pregnant rats were used in each experimental group, two conceptuses were taken from each rat and two sections were taken from each conceptus. Photomicrographs of yolk sac endodermal cells were obtained by a random sampling procedure which covered the entire depth of the endodermal cells appearing on the ultrathin sections (Text-fig. 2). Point counting was done on each photomicrograph (see above) and a graph was plotted to show the cumulative means for volume density and surface density. Each of the plots showed that the cumulative mean falls and remains within 10% of the final value used in our calculations after analysis of 11–14 micrographs (Text-fig. 3). In order to be quite certain of obtaining a representative sample for

Text-fig. 2. Diagram showing the procedure of random sampling of the visceral yolk sac. The first photomicrograph is produced by positioning a random portion of the ultrathin section at the top left-hand corner (a) of the area of the copper grid illustrated and subsequently photographing random fields such as b, c and d (stippled).

Text-fig. 3. Graph showing the determination of the minimum sample size required for morphometric analysis. This particular plot is for the volume density (V_v) of type (a) endocytotic vacuoles in the rat visceral yolk sac endoderm. The minimum sample size (arrow) (i.e. the number of micrographs required to bring the V_v estimate permanently within 10% of the final value) is 11 micrographs (see text).

both the volume density and surface density, however, about 25–27 micrographs (i.e. well over the 11–14 minimum) were measured from each area. Data from each embryo were analysed separately. The mean values for each embryo were then combined with those from other embryos to give the overall means and standard errors quoted. Mathematical comparison of the mean results for the in-vivo and the in-vitro systems was made by using the Student's *t* test.

Results

Ultrastructure of visceral yolk sac endoderm cells

Day 9.5 in vitro. The ultrastructural appearance of the yolk sac cells is illustrated in Pl. 1, Fig. 1. The cells were columnar and contained a moderate amount of rough endoplasmic reticulum and Golgi apparatus. The apical plasmalemma was characterized by the presence of microvilli and was separated from the convoluted lateral plasmalemma by junctional complexes. The most striking feature of the cells was the population of endocytotic vacuoles which tended to occupy most of the apical cytoplasm.

Several morphological forms of the endocytotic vacuole were apparent (Pl. 1, Figs. 1 and 2): (a) small vacuoles located in the apical region of the cell at the base of the microvilli; (b) larger vacuoles varying from completely empty forms to those containing lightly flocculent material. These are situated somewhat deeper in the cytoplasm than are the small vacuoles; (c) vacuoles containing flocculent material and a homogeneous electron-dense area; and (d) vacuoles consisting entirely of electron-dense material.

By culturing rat embryos and the visceral yolk sac in the presence of electron-opaque markers (iron dextran or ferritin), it was shown that all these vacuoles are involved in endocytosis (Gupta, Gulamhusein & Beck, 1979).

Day 9.5 in vivo. The visceral yolk sac endodermal cells *in vivo* looked almost identical to those *in vitro* except that the completely empty (type b) endocytotic vacuoles were observed only rarely compared to the in-vitro series.

Day 11.5 in vitro and in vivo. The cells were a little less tall than at 9.5 days but were otherwise similar morphologically (Pl. 2, Figs 4–6). A clearly discernible Golgi apparatus was present together with moderate amounts of rough endoplasmic and some smooth reticulum. The endocytotic vacuoles were again the most striking feature located in the apical region of the cytoplasm. The four types seen in 9.5-day yolk sac were again distinguishable and each form took up marker macromolecules.

Ultrastructure of embryonic endoderm

At 9.5 days, the visceral layer of the yolk sac is continuous with the embryonic endoderm (see Text-fig. 1) and the latter is widely exposed to the culture medium. It was therefore of interest to see if the embryonic endodermal cells take part in endocytosis. By Day 11, the embryo becomes dorsally convex and invaginated into its yolk sac so that the embryonic endoderm is no longer a surface structure. Morphometric analyses were, therefore, carried out on the embryonic endoderm cells only at 9.5 days. The ultrastructural appearance is illustrated in Pl. 1, Fig. 3. The cells were squamous with very few microvilli. There were hardly any endocytotic vacuoles although a fair amount of rough endoplasmic reticulum and Golgi apparatus could be seen.

Morphometric results

The morphometric measurements for the visceral yolk sac cells and the embryonic endodermal cells are presented in Table 1.

There were no statistically significant differences between yolk sac or embryonic endoderm

Table 1. Results of morphometric analysis of the rat embryo endoderm

Area of the egg cylinder	Day 9.5		Day 11.5	
	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>
<i>Visceral yolk sac endoderm</i>				
Volume density	15.82 ± 0.51	16.61 ± 0.59	10.68 ± 0.21	11.20 ± 0.64
Surface density	0.58 ± 0.02	0.58 ± 0.02	0.49 ± 0.05	0.51 ± 0.02
<i>Embryonic endoderm</i>				
Volume density	2.26 ± 0.12	2.20 ± 0.18	—	—
Surface density	0.21 ± 0.01	0.18 ± 0.02	—	—

Each value is the mean ± s.e.m. for 6 embryos.

fixed *in vivo* and those prepared and grown *in vitro*. However, volume density and surface density values were significantly different ($P < 0.001$) for visceral endodermal cells at Days 9.5 and 11.5 and for visceral yolk sac endoderm and embryonic endoderm at Day 9.5. The values for the embryonic endoderm were much lower than any specimen of the visceral yolk sac endoderm.

Discussion

As experienced by many others (Fantel, Greenway, Juchau & Shepard, 1979; Freeman, Beck & Lloyd, 1981) using the technique of New (1978) whole mammalian embryo culture allows the embryo to grow as quickly in culture as in the uterus. However, one must assess whether the various 'placental' processes, such as the breakdown of histiotroph, the level of oxygenation and the methods of energy production, function in a similar manner *in vivo* and in culture before one can extrapolate from the in-vitro to the in-vivo situation. Based on the morphometry of the vacuolar system the present study provides evidence that the rate of endocytosis and digestion of macromolecules in the visceral yolk sac endodermal cells is similar *in vitro* and *in vivo* at 9.5 and 11.5 days although the extent of the vacuolar system at 11.5 days is significantly less than at 9.5 days. The important point is that measurement of the vacuolar system shows it to be similar *in vivo* and *in vitro* both at 9.5 and at 11.5 days. The lower measurements demonstrated on Day 11.5 could be due to the fact that the chorio-allantoic placenta would be just beginning to form *in vivo*.

PLATE 1

Fig. 1. Electron micrograph of the rat visceral yolk sac endoderm at 9.5 days *in vitro*. A large variety of endocytotic vacuoles (en.v) can be seen. ×2520.

Fig. 2. A higher magnification of the visceral yolk sac endoderm at 9.5 days *in vitro*, showing the supranuclear region. Note the presence of different types of vacuoles. Types of vacuoles (see text) are designated as a, b, c and d. ×12 600.

Fig. 3. Electron micrograph of the embryonic endoderm at 9.5 days *in vitro*. Note the presence of a few microvilli and endocytotic vacuoles. ×12 600.

PLATE 2

Fig. 4. Electron micrograph of the visceral yolk sac endoderm at 11.5 days *in vitro*. ×2520.

Fig. 5. Electron micrograph of the visceral yolk sac endoderm at 11.5 days *in vitro*. ×12 600.

Fig. 6. A higher magnification of the visceral yolk sac endoderm at 11.5 days *in vivo*. ×12 600.

PLATE I

(Facing p. 244)

PLATE 2

The significantly lower volume density and surface density values of the vacuoles present in the embryonic endoderm when compared to the visceral yolk sac endoderm at 9.5 days shows that the embryonic endoderm cells probably do not play a significant part in the uptake and breakdown of macromolecules, and by implication in embryonic nutrition.

The suggestion that endocytotic function is identical in culture and *in vivo* during the yolk sac phase of embryonic nutrition leads to more confidence in applying the technique of New (1978) to other problems in development. The system could be used to test certain teratogens to show whether they are having a direct effect on the conceptus or require the presence of the maternal organism to activate the teratogenic agent (as in cyclophosphamide; see Fantel *et al.*, 1979).

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