A stimulatory effect of the fluid from preimplantation rabbit blastocysts upon luteinization of monkey granulosa cell cultures

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Summary. Blastocyst fluid was aspirated from Day 6½–7 rabbit blastocysts and was added to cultures of granulosa cells obtained from preovulatory follicles of untreated rhesus monkeys or from follicles of monkeys treated with PMSG. The stimulation of progesterone secretion was measured and equated with that produced by hCG. The hCG-like activity was also measured in a radioreceptor assay using ¹²⁵I-labelled hCG and porcine granulosa cells.

In 8 out of 10 experiments with cultured cells from untreated monkeys, addition of 20% blastocyst fluid from Days 6–9 of culture stimulated progesterone secretion by 2- to 6-fold. Similar findings were obtained in 5 experiments with cultures from PMSG-treated monkeys except that the blastocyst fluid was added from Days 0 to 6 of culture. The granulosa cells in such cultures underwent morphological luteinization. Compared to a standard of purified hCG the blastocyst fluid contained about 0.76–2.5 ng hCG-like activity/ml which was non-dialysable. The radioreceptor assay indicated the presence of 0.5–2.5 ng hCG-like material/ml.

Introduction

The observation of Fuchs & Beling (1974) that blood progestagen concentrations in the pregnant rabbit are significantly higher than those in the pseudopregnant rabbit after only 5 days of gestation indicates either that the conceptus may be secreting a substance which stimulates the corpus luteum (CL) to secrete progesterone or that the blastocyst itself could be secreting the progestagen. Implantation in the rabbit occurs 7 days *post coitum* and so the stimulus for such secretion must come from the preimplantation blastocyst. Haour & Saxena (1974) observed that preimplantation rabbit blastocyst fluid contained a substance(s) which could inhibit the binding of labelled hCG to membranes of bovine luteal cells, and concluded that the blastocyst could secrete an hCG-like gonadotrophin which could act locally to stimulate luteal progestagen secretion before implantation. Fujimoto, Euker, Riegel & Dukelow (1975), using a non-specific LH antiserum, were also able to demonstrate a substance in blastocyst fluid which could inhibit binding of iodinated LH to the antiserum. However, Holt, Heise, Wilson & Keyes (1976) and Sundaram, Connell & Passantino (1975) were unable to detect any hCG-like activity in rabbit blastocyst fluid when using in-vitro assays with rat ovarian and Leydig cells, respectively. Because of these discrepancies we decided to assay the luteotrophic activity of fluid from rabbit preimplantation blastocysts by using cultures of monkey granulosa cells which are known to respond to small doses of gonadotrophins (Channing, 1970, 1974; Channing & Kammerman, 1973) with an increase in progesterone secretion and morphological luteinization. Monkey granulosa cells can respond to gonadotrophins of a wide range of species including reptiles, sheep, pigs, monkeys and man (Channing, Licht, Papkoff & Donaldson, 1974; Channing, 1974).

Materials and Methods

Collection of blastocyst fluid

Rabbit blastocysts were obtained by two methods from 16 mature New Zealand White rabbits. Some rabbits were mated at oestrus and 6–7 days later the rabbits were killed with sodium pentobarbitone and the uteri were removed. The blastocysts were located and either flushed out or left *in situ* and the blastocyst fluid was aspirated by a 23-gauge needle and 1-ml syringe and immediately placed on ice and centrifuged at 1000 *g* to remove cells and debris. In the second method, oestrous rabbits were induced to ovulate by an intravenous injection of 100 µg ovine LH (NIH-LH-S18) dissolved in 1 ml phosphate-buffered saline. The rabbits were immediately inseminated artificially with 1×10^8 spermatozoa collected from males of proven fertility. On the 7th day after the LH injection the females were tranquillized with an intravenous injection of 3 mg acepromazine maleate (Ayerst, New York, U.S.A.) and anaesthetized with 75 mg sodium pentobarbitone (Abbott, North Chicago, Illinois, U.S.A.). The uterine horns were exposed by laparotomy and blastocyst fluid was harvested from the expanded blastocysts by aspiration through the uterine wall with a 23-gauge needle attached to a 1-ml syringe. The blastocyst fluid was immediately transferred to sterile tubes on ice and centrifuged to remove cells and debris. All the blastocyst fluid samples were frozen at -20°C until used in monkey granulosa cell cultures or in the radioreceptor assay.

Monkey granulosa cells

Collection. Eleven (11) rhesus monkeys were used as a source of granulosa cells for the assay of hCG-like activity. The housing and care was as described previously (Channing, 1970). The granulosa cells were from preovulatory follicles of untreated monkeys and from follicles of monkeys treated with PMSG. The 8 untreated monkeys were observed for two consecutive menstrual cycles and, during the third menstrual cycle, they were bled daily from Day 5 until Days 9 to 12 (Day 1 = first day of menstruation). A rapid radioimmunoassay of oestrogen was carried out on each serum sample as originally described by Hotchkiss, Atkinson & Knobil (1971) and modified and validated by Channing & Coudert (1976). On the day of the oestrogen 'surge' (>200 pg oestrogen/ml serum) the monkey was subjected to laparotomy and the granulosa cells were harvested from the largest follicle present (Channing & Ledwitz-Rigby, 1975). Only follicles 4 to 11 mm in diameter were used. In 4 out of 8 animals examined intact oocytes were recovered which were immature and contained a germinal vesicle or had a polar body.

The 3 treated monkeys were injected subcutaneously with PMSG as outlined previously (Channing, 1974; Channing & Ledwitz-Rigby, 1975): 1000 i.u. were given on Days 1 and 2, 500 i.u. on Days 3, 4 and 5, and 200 i.u. on Days 7–9. The monkeys were bilaterally ovariectomized on Day 10 of the treatment schedule. At this time, the ovaries were enlarged and contained numerous 3–10 mm follicles. Blood oestrogen was measured by radioimmunoassay (Channing & Coudert, 1976) daily during the treatment period. Granulosa cells were aspirated from the follicles and cultured. Such granulosa cells secrete low basal levels of progesterone and are highly responsive to exogenous gonadotrophin (hCG, LH and FSH) added from the start of culture (Channing & Ledwitz-Rigby, 1975). The PMSG treatment was used because large numbers of granulosa cells could be harvested from each monkey.

Culture. Aliquots of $0.1\text{--}0.5 \times 10^6$ granulosa cells were cultured in Microtest Plates (Falcon Plastics, Oxnard, California, U.S.A.) in a volume of 0.2 ml medium 199 with 25 mM-Hepes buffer, 50 µg Gentamicin/ml and 15% (v/v) serum from a fasting male monkey. This serum contained <1 ng hCG-like material/ml (measured by radioreceptor assay: Channing, 1974), <60 pg oestrogen/ml and 1–2 ng progesterone/ml (measured by radioimmunoassays: Channing & Coudert, 1975). This culture medium was designated as Medium W. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 2 days and frozen for later progesterone analysis by radioimmunoassay. At the end of the culture period, the cells were fixed in 10% aqueous formalin and stained with haematoxylin and eosin or oil red O and haematoxylin. Blastocyst fluid (usually 20% by volume in culture Medium W) or an hCG standard (1, 10, 100 ng/ml culture medium) was added at the beginning of culture (PMSG-treated follicles) or at Day 5 or 6 of culture (untreated

follicles) and at each medium change until the end of the culture period (usually Day 12). The standard of hCG (12 000–14 000 i.u./mg) was kindly donated by Dr Robert Canfield, Columbia University, New York.

Progesterone assay

Progesterone in the spent culture medium was measured, without prior extraction, by using an antiserum against 11-hydroxyprogesterone conjugated to BSA (No. 325) and the radioimmunoassay previously described and validated for this laboratory (Channing & Ledwitz-Rigby, 1975; Channing, Tsai & Sachs, 1976). To use progesterone secretion rate as an approximate quantitative assay of hCG-like activity, the progesterone secretion rate was first normalized to give a per cell per day value before being expressed as a percentage of the control (secretion rate observed in the absence of hormones) value so that results of experiments with different control secretion rates could be compared.

Vacuum dialysis

A pool of blastocyst fluid obtained from 3 rabbits was placed in a vacuum dialysis apparatus and dialysed overnight against 50 ml Eagle's medium. The blastocyst fluid was then reconstituted to its original volume in Medium W and passed through a 0.22 millipore filter. Aliquots of 20 μ l reconstituted dialysate were added to 400 μ l culture medium and the mixture (5% blastocyst fluid) was added on Days 6 and 8 of culture to 3 replicate cultures of granulosa cells obtained from a pre-ovulatory monkey follicle.

Radioreceptor assay

The hCG-like material present in the rabbit blastocyst fluid was quantitated by a radioreceptor assay which employed (per tube) 5 ng (in 100 μ l) 125 I-labelled hCG (20–30 μ Ci/ μ g), porcine granulosa cell suspension in 100 μ l 100 mM-Tris, pH 6.5, and 100 μ l test solution or standard hCG in 100 μ l 100 mM-Tris, pH 6.5, plus 0.1% BSA. The reaction mixture (total volume 0.3 ml) was mixed by shaking for 1 h at 37°C followed by centrifugation, 3 washes in 3 ml 100 mM-Tris containing 0.1% purified pigskin gelatin (Eastman, New York, U.S.A.), pH 6.5, and counting of the cell pellet in an autogamma counter.

Details of the preparation of the porcine granulosa cells and the radioreceptor assay have been published elsewhere (Sakai, Engel & Channing, 1977). Non-specific binding was determined by examining the amount of 125 I-labelled hCG bound in the presence of excess unlabelled hCG (1 μ g) and was usually <10% of total binding. Binding to the glass tubes used in the assay represented <1% of total binding and was subtracted from each value. The coefficient of variation of three hCG standard curves was 7.8% on a log-logit slope comparison. The lowest detectable level of hCG-like material in this assay was 0.4 ng/ml. The 'control' solution was 100 μ l 100 mM-Tris, pH 6.5, plus 0.1% BSA and the binding in this solution did not differ from that with a solution containing 0.9% NaCl, 100 mM-Tris and 0.1% BSA (pH 7).

Results

Blastocyst fluid samples obtained from LH-treated artificially inseminated rabbits exerted a small but significant 2- to 6-fold stimulation in progesterone secretion in cultures of granulosa cells from pre-ovulatory or PMSG-treated monkeys (Table 1). Rabbit serum obtained from 3 rabbits at the same time as the blastocyst fluid had no detectable stimulatory action upon progesterone secretion (Table 1). There was considerable variation in the ability of blastocyst fluid obtained from different rabbits to stimulate progesterone secretion. For example, two samples of blastocyst fluid (from different rabbits and not shown in Table 1) exerted a 3296% and 2090% stimulation in replicate cultures with one batch of granulosa cells. In another culture experiment two additional samples of blastocyst fluid exerted a 1190% and <300% stimulation over a 3-day period. Variability in control progesterone secretion rate between replicate cultures in the same experiment was only 20%. It was possible to make approximate potency estimates of the hCG-like activity in different batches of blastocyst fluid: by using 10 cultures from untreated, preovulatory monkeys as an assay system, the approximate

maximal potency, expressed as ng native hCG/ml, was estimated as 0.6, 0.6 and 2.5 for blastocyst fluid from mated untreated rabbits, and 1.0, 0.7, 0.8, 0.6, 0.8, 0.5 and 0.5 for blastocyst fluid harvested from LH-treated artificially inseminated rabbits. The mean value for these 10 estimates was 0.86 ng hCG/ml. When 5 cultures of cells obtained from PMSG-treated monkeys were used for the assay system the potencies were 0.12, 0.12, 0.5, 10 and 2.5 (mean = 2.6) ng hCG/ml.

Table 1. Effect of rabbit blastocyst fluid (BF) and hCG upon progesterone secretion by cultures of monkey granulosa cells from (a) preovulatory follicles of untreated monkeys and (b) follicles of PMSG-treated monkeys

Days in culture	Addition to culture	Progesterone secretion rate				
		Control (pg/cell/day)		With blastocyst fluid or hCG (% of control)		
		Mean $\pm$ s.e.m.	<i>n</i>	Mean $\pm$ s.e.m.	<i>n</i>	Significance
(a)	6	24 $\pm$ 11	6	267 $\pm$ 67	9	$P < 0.01$
	8	22 $\pm$ 13	6	308 $\pm$ 63	8	$P < 0.01$
	10	13 $\pm$ 11	6	198 $\pm$ 29	6	$P < 0.01$
	6	24 $\pm$ 11	6	1057 $\pm$ 501	4	—
	8	22 $\pm$ 13	6	3512 $\pm$ 1808	4	—
	10	13 $\pm$ 11	6	19673 $\pm$ 1300	3	—
	6	57 $\pm$ 11	4	47 (38, 55)	2	—
	8	62 $\pm$ 6.6	4	34 (28, 40)	2	—
	10	57 $\pm$ 6.1	4	26 (20, 32)	2	—
(b)	2	1.27 (1.24, 1.30)	2	117 (114, 119)	2	—
	4	0.84 (0.98, 0.70)	2	130 (102, 158)	2	—
	6	0.35 (0.33, 0.36)	2	96 (80, 111)	2	—
	2	0.81 $\pm$ 0.27	8	556 $\pm$ 132	8	$P < 0.01$
	4	0.59 $\pm$ 0.15	8	1587 $\pm$ 348	8	$P < 0.01$
	6	0.40 $\pm$ 0.003	8	1239 $\pm$ 351	8	$P < 0.01$
	2	1.27 (1.24, 1.3)	2	1116 (1013, 1219)	2	—
	4	0.84 (0.98, 0.70)	2	739 (641, 836)	2	—
	6	0.35 (0.33, 0.36)	2	510 (451, 568)	2	—
	2	1.27 (1.24, 1.30)	2	2309 (2283, 2334)	2	—
	4	0.84 (0.98, 0.70)	2	4262 (4120, 4401)	2	—
	6	0.35 (0.33, 0.36)	2	11210 (10120, 12300)	2	—

The hCG or blastocyst fluid was added at Days 5, 6 and 8 of culture for (a) and Days 0, 2, 4 and 6 of culture for (b).

Blastocyst fluid which had been vacuum dialysed overnight in Eagle's medium retained its ability to stimulate progesterone secretion: a 5% solution brought about a 449 $\pm$ 66% ($n = 3$) stimulation over 3 days of culture. In the same experiment 1.0 ng hCG exerted a 331% stimulation of progesterone secretion.

The blastocyst fluid also stimulated morphological luteinization of the granulosa cells (Pl. 1, Figs 1, 2 and 3). The effect was most evident after 4 days of exposure of the cells to the fluid. At a concentration of 20%, blastocyst fluid increased the accumulation of small dense granules and lipid droplets in granulosa cells examined by phase-contrast microscopy and after oil red O staining. Addition of the 0.9% NaCl or phosphate-buffered saline plus 0.1% BSA (pH 7.4) 'control' solution did not cause these morphological changes.

The results of the radioreceptor assay of the blastocyst fluid from individual rabbits are shown in Table 2. The hCG-like activity was often barely detectable and close to the limit of sensitivity of the assay. The range of potency estimates from the radioreceptor assay approximated that obtained from the monkey granulosa cell culture assay.

Photographs showing the luteinizing effect of (b) 5% and (c) 20% rabbit blastocyst fluid and (d) 10 ng hCG compared with controls (a) on granulosa cells from monkey preovulatory follicles. The substances were added after 7 days of culture. $\times 350$

Fig. 1. Cells exposed to blastocyst fluid and hCG for 1 day

Fig. 2. Cells exposed for 2 days.

Fig. 3. Cells exposed for 4 days.

Table 2. Estimation of hCG-like activity in rabbit blastocyst fluid by a radioreceptor assay

Rabbit providing fluid (100 µl/assay)	1	2	3	4	5	6	7
hCG-like activity (ng/ml)	0.68 (0.49, 0.87)	0.99 (0.48, 1.5)	2.0 (1.8, 2.2)	2.5*	1.78*	1.85*	1.16*

* Insufficient quantities of fluid for duplicate assays.

The progesterone content of two pools of blastocyst fluid was measured and found to be 0.125 and 0.280 ng/ml. This is insignificant compared to the progesterone levels of >2 ng/culture consistently found in the medium from cultured granulosa cells.

Discussion

These observations support the concept that there may be a gonadotrophin in the preimplantation rabbit blastocyst and confirm and extend the earlier observations of Haour & Saxena (1974) and Fujimoto *et al.* (1975). The inability of Sundaram *et al.* (1975) to observe any activity in testis cells may have been due to the method of harvest, because freezing the entire blastocyst before separating the fluid may release proteolytic enzymes, or the gonadotrophin may be labile in the testis assay system. The latter explanation may also be the reason why Holt *et al.* (1976) failed to detect blastocyst fluid activity with an ovarian assay. Alternatively, the longer culture period used in the present studies may be better for demonstrating small amounts of a luteotrophic substance. The evidence derived from the radioreceptor assays for hCG activity in blastocyst fluid strengthens that from the granulosa cell cultures.

While these data do not prove that there is a discrete gonadotrophin secreted by the rabbit blastocyst before implantation, they are strongly indicative. The finding that the activity is non-dialysable suggests that the molecular weight of the material may be >10 000, and it thus resembles LH and hCG which have molecular weights of 30 000 and 38 000, respectively. The alteration of progesterone secretion in the cultures by other blastocyst components, which are also non-dialysable, or which are bound to large molecules, cannot be ruled out.

The fact that we have demonstrated a biologically active hCG-like activity in rabbit blastocyst fluid gives added physiological significance to the concept of a preimplantation gonadotrophin which could start the rescue of the corpus luteum. Saxena, Hasan, Haour & Schmidt-Gollwitzer (1974) have detected, with a radioreceptor assay, a rise in chorionic gonadotrophin in the blood of women before implantation and Wiley (1974) has demonstrated hCG-like activity in mouse morulae by a histo-immunofluorescence technique. The observation of Dickmann (1976) that blastocysts secrete steroid hormones in early stages of implantation lends support for a role of an embryonic gonadotrophin which could act locally to stimulate this steroidogenesis.

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