Characterization of long-term monolayer cultures of human granulosa cells from follicles of different size and exposed *in vivo* to clomiphene citrate and hCG

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Summary. To determine the effects of prolonged hCG treatment *in vitro* upon granulosa cells from follicles of various sizes previously exposed to clomiphene citrate and hCG *in vivo*, progesterone and relaxin concentrations of spent media were correlated with light microscopic and ultrastructural characteristics. Intact, freshly dispersed cells were characterized by numerous lipid droplets, elliptical mitochondria with tubular or lamellar cristae, moderate rough-surfaced endoplasmic reticulum (RER), sparse smooth-surfaced endoplasmic reticulum (SER), and few Golgi. After 10–24 days in culture, 2 morphologically distinct cell types, ‘granulosa-type’ and ‘luteal-type’, were noted at the light microscopic level. Ultrastructurally, lipid droplets decreased in number, mitochondria became pleomorphic, RER became more prominent and dilated, and Golgi became more widely dispersed. Tubular SER became abundant and annular nexuses became more numerous after hCG treatment *in vitro*. Granulosa cells generated from all follicles responded to hCG treatment with significantly increased progesterone secretion after 4 days in culture. Relaxin was not detectable in any sample of medium. This study shows that human granulosa cells from 15–25-mm follicles retain their differentiated function of progesterone secretion in long-term culture and recover responsiveness to hCG *in vitro*, as demonstrated by enhanced progesterone secretion and development of prominent SER and increased annular nexuses.

Introduction

In an attempt to study hormonal regulation of cellular function, human granulosa cells from follicular and luteal sources have been compared morphologically and hormonally. Electron microscopic studies of granulosa lutein cells *in situ* have revealed abundant tubular smooth-surfaced endoplasmic reticulum (SER), stacks of rough-surfaced endoplasmic reticulum (RER), large pleomorphic mitochondria, moderately extensive Golgi complexes, non-extractable lipid droplets and annular nexuses (Adams & Hertig, 1969; Crisp, Dessouky & Denys, 1970). Comparisons with follicular granulosa cells have recognized the transformation into granulosa lutein cells *in vivo* as the appearance of the prominent SER (Christensen & Gillim, 1969). Granulosa cells isolated from healthy preovulatory follicles secrete progesterone *in vitro* for at least 6 days under basal conditions (McNatty, Makris, DeGrazia, Osathanondh & Ryan, 1979; Batta, Wentz &

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Channing, 1980). Progesterone secretion by luteal cells derived from the corpus luteum of the menstrual cycle has been stimulated by gonadotrophins in short-term (Williams, Roth, Marsh & LeMaire, 1979; Richardson & Masson, 1980) and long-term (Goldsmith, Essig, Sarosi, Beck & Weiss, 1981) culture systems. However, in the study of Goldsmith *et al.* (1981), chorionic gonadotrophin did not stimulate relaxin secretion characteristic of the human luteal cell of pregnancy (O'Byrne *et al.*, 1978).

Granulosa cells aspirated from follicles after exposure *in vivo* to the 'LH surge' present a transition phase between the follicular cell and the fully transformed luteal cell. In short-term studies *in vitro*, LH stimulated progesterone secretion by granulosa cells aspirated from follicles of diameter 5–19 mm from women in whom LH levels had previously risen above a surge level of 60 mi.u./ml. However, the same dose of LH had no effect on similarly treated granulosa cells from follicles > 20 mm in diameter over the same 24-h period (Bieszczad, McClintock, Pepe & Domino, 1982). The purpose of our study was to characterize long-term cultures of granulosa cells from follicles of various sizes, previously exposed to clomiphene citrate and hCG *in vivo*, and to assess the effect of hCG treatment *in vitro* upon these granulosa cells. Our approach was to examine light microscopic and ultrastructural properties of these granulosa cells *in vitro* in conjunction with measurements of the concentrations of progesterone and relaxin in the media under basal conditions and after exposure to hCG.

Materials and Methods

Source of follicles. Three women participating in our in-vitro fertilization programme (Quigley, 1983) were treated with 50 mg clomiphene citrate on cycle Days 5–9 inclusive (one patient underwent 2 treatment cycles). Follicular diameters were followed with daily ultrasonographic measurements beginning on Day 10. On the day that the largest follicle reached at least 20 mm, each patient received an i.m. injection of 2500 i.u. hCG (Serono Laboratories, Randolph, MA, U.S.A.). After securing informed consent, granulosa cells were harvested from 15–25-mm follicles aspirated for oocyte retrieval via laparoscopy 36 h after hCG injection, approximately 2–4 h before ovulation would be expected to occur spontaneously (Edwards & Steptoe, 1975). The method of oocyte retrieval entailed puncture of the preovulatory follicle, removal of follicular fluid and agitation of the follicle wall to free mural granulosa cells. To locate the oocyte, all 'cumulus-type' masses of granulosa cells were segregated. These masses of granulosa cells were cultured *in vivo* with the oocyte or were separated from the granulosa cells used in our experiments. Oocytes were incubated and fertilized as previously reported (Quigley, 1983). Embryos were transferred into the donor's uterus by transcervical catheter 48 h after insemination at the 2–4-cell stage. Pregnancy was detected by serum β -hCG radioimmunoassay and later confirmed by ultrasonographic examination in each of the 3 patients. Follicle and culture characteristics are detailed in Table 1.

Incubation of granulosa cells. Long-term monolayer cultures of human granulosa cells were established by modification of the method of Goldsmith *et al.* (1981). Throughout the 24 days of each experiment, granulosa cells remained separated according to the specific follicle of origin. Clumps and sheets of granulosa cells were removed from follicular fluid by centrifugation at 600 g for 10 min and incubated for 10 min in Earles Balanced Salt Solution without Ca or Mg (EBSS*) containing 0.2% collagenase and 0.01% deoxyribonuclease. The larger clumps of cells were dispersed by repeated pipetting through a Pasteur pipette for 5 min. The cell suspension was centrifuged at 600 g for 10 min and the pellet was resuspended in Medium EBSS*. Granulosa cells were counted with a haemocytometer and plated on a glass cover-slip in a 35-mm plastic Petri dish (Falcon, Division of Becton, Dickinson and Co., Cockeysville, MD, U.S.A.) at a concentration of 1.0×10^5 viable cells. These glass cover-slips had been sequentially washed with 50% HNO₃, water, 1% NaEDTA, distilled-deionized water and 70% ethanol. The number of granulosa cells recovered from each follicle ranged from 2.0×10^5 to 50.0×10^5 , with a mean $\pm$ s.e.m. of $16.6 \times 10^5 \pm 14.6 \times 10^5$.

The granulosa cells were maintained *in vitro* for up to 24 days in Medium 199, supplemented with 10% fetal calf serum, 100 units penicillin/ml, 100 µg streptomycin/ml and 2 mM-L-glutamine (Grand Island Biological Company, Grand Island, New York, U.S.A.). From each of 9 follicles, 1–6 plates remained untreated and 1–6 plates were exposed to 100 i.u. hCG/ml (NICHED, Bethesda, MD: Batch CR-121) throughout the experiment. Culture media were changed every 2 days and spent media were stored at -20°C until assayed for progesterone and relaxin by RIA. Morphological changes were assessed at 2-day intervals by phase-contrast microscopy. Viability was monitored by trypan blue exclusion at the start and end of each experiment. After 24 days in culture, a dissecting microscope ($\times 80$) was used to estimate the relative proportion of 'granulosa type' and 'luteal type' cells on each plate, as described in 'Results'. Cells not fixed for *en bloc* electron microscopy were then moved from plates using 0.25% trypsin (Sigma Chemical Co., St Louis, MO, U.S.A.: 230 units/mg) in Medium EBSS* and counted with a haemocytometer.

Electron microscopy. Electron microscopic studies were performed on Follicles 2L₁ (dispersed cells, Days 2, 10 and 24), 2R₁ (dispersed cells, Days 2 and 24) and 3L₁ (dispersed cells, Days 10 and 24). Samples of dispersed cells were centrifuged at 600 *g* for 10 min and immediately fixed in 2.5% glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4. Cell samples obtained after 2, 10 or 24 days of culture were fixed on the plates or removed from the plates with 0.25% trypsin in Medium EBSS*, incubated at 37°C for 15 min and centrifuged at 600 *g* for 10 min. After washing with Medium EBSS*, the pellets were fixed as above. All samples were rinsed in 0.1 M-sodium cacodylate buffer, osmicated in 1% osmium tetroxide in cacodylate buffer, dehydrated in a graded series of ethanol and embedded in Spurr resin (Electron Microscopy Sciences, Ft Washington, PA). Gold-to-silver sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100B electron microscope.

Radioimmunoassays. Concentrations of progesterone were determined by radioimmunoassay (Kendall, Richards & Shih, 1983). Duplicate 25 µl samples of media from cultured cells were extracted once with 3 ml petroleum ether (b. pt $42\text{--}60^{\circ}\text{C}$) with an average efficiency of 82%. The ether extracts were dried under nitrogen and redissolved in phosphate buffer (400 µl to 2 ml). Aliquants were taken for RIA. The sensitivity of the RIA was 6 pg. Serial dilutions of medium samples gave progesterone values with a mean coefficient of variation of 4.9%. Recoveries of 75 pg and 150 pg progesterone added to progesterone-free culture medium were 89% and 92%, respectively. The interassay coefficients of variation for these standard samples were 24% (mean $\pm$ s.d. = 80.2 ± 19.0 pg, $n = 37$) and 16% (178.0 ± 28.5 pg, $n = 48$). The interassay coefficients of variation were 15% (3.23 ± 0.50 ng/ml, $n = 21$) for plasma samples from men and 12% (63.70 ± 7.50 ng/ml, $n = 40$) for plasma from pregnant women.

Relaxin was measured by an homologous porcine radioimmunoassay (Kendall, Richards, Shih & Farris, 1982; Kendall *et al.*, 1983) incorporating modifications needed to determine concentrations of the hormone in media from cells in culture. Six NIH-RXN-P1 standards ranging from 62.5 to 200 pg/100 µl buffer were incubated with 400 µl culture medium, 100 µl R6 antiporcine relaxin serum (final dilution 1 : 50 000) and 100 µl ^{125}I -labelled porcine relaxin at 4°C for 48 h. The sensitivity of the RIA was 100 pg. The assay of 122, 163 and 816 pg of relaxin added to relaxin-free culture medium gave values of 99%, 102% and 72%, respectively. Culture medium blanks gave non-detectable values. The detection of relaxin in human menstrual corpora lutea has previously been documented (O'Byrne *et al.*, 1978).

All culture medium samples from a given experiment were assayed in the same progesterone and relaxin RIAs to avoid interassay variability. All RIA values were calculated by a W.H.O. programme developed by Malan & Edwards (1981).

Statistics. Progesterone concentrations in media were analysed by multiple and stepwise linear regressions using BMDP programs 1R and 2R (BMDP Statistical Software, 1983), respectively. Independent variables were patient cycles, follicular size, day of culture and hCG treatment. Significance was defined as $P < 0.05$.

Results

Culture characteristics

The granulosa cells survived well in monolayer culture for up to 24 days. Viability was not affected by hCG (100 i.u./ml), remaining between 80 and 90% throughout each experiment. When examined with inverted phase microscopy after 2 days in culture, the granulosa cells appeared stellate with prominent nuclei and intracytoplasmic granules. Cytoplasmic processes seemed to bridge these 'fibroblast-like' cells (Pl. 1, Fig. 1). By 6 days in culture, this 'luteal-type' cell with fibroblast-like morphology was accompanied by a second cell type with a smooth round-to-ovoid shape and a higher nucleus-to-cytoplasm ratio (Pl. 1, Fig. 2). Two morphologically distinct populations were apparent after 10 days in culture: (1) elongated 'luteal-type' cells (Pl. 2, Fig. 3) with granular cytoplasm in close juxtaposition and (2) round-to-polygonal 'granulosa-type' cells with increased nucleus-to-cytoplasm ratio (Pl. 2, Fig. 4). These latter occurred in rosette-like formations resembling Call-Exner bodies (Blaustein, 1977). The area between these cells was spanned by very fine cellular processes (Pl. 3, Fig. 5). Although a few areas of the plates exhibited a predominance of a single cell type, the 'luteal-type' and 'granulosa-type' cells were most often intermingled (Pl. 3, Fig. 6). The plating characteristics established by Day 10 of culture continued throughout the remainder of the experiments. Both types of cells remained in monolayer with only a few areas of cells piling upon one another. The major portion of most plates was occupied by the 'luteal-type' cells as indicated in Table 1.

Table 1. Characteristics of follicular sources of granulosa cells and culture profiles after 24 days

Clinical evaluation of follicles aspirated for granulosa cells						In-vitro characteristics of granulosa cells after 24 days			
Follicle designation		Follicle diam. (mm)	Outcome			Average approximate % of plates covered by 'granulosa-type' cells (number of plates)		No. of cells after 24 days/no. at start of culture ($\pm$ s.e.m.)	
Patient cycle	Ovarian follicle		Oocyte retrieved	Oocyte and embryo transferred	Pregnancy achieved	Control	100 i.u. hCG/ml	Control	100 i.u. hCG/ml
1	L ₁	25	Yes	Yes	Yes	40 (4)	30 (4)	6.0 ($\pm$ 0.3)	4.3 ($\pm$ 0.1)
1	R ₁	15	No	—	—	90 (1)	100 (1)	7.1 (—)	6.9 (—)
2*	L ₁	23	No	—	—	10 (4)	5 (4)	3.8 ($\pm$ 0.2)	4.1 ($\pm$ 0.3)
2	R ₁	22	Yes	Yes	No	5 (3)	10 (3)	2.5 ($\pm$ 0.1)	3.5 ($\pm$ 0.5)
3	L ₁	18	Yes	Yes	Yes	5 (4)	5 (4)	1.9 ($\pm$ 0.3)	2.1 ($\pm$ 0.3)
3	R ₁	22	Yes	Yes		50 (4)	60 (4)	—	—
4*	L ₁	15	Yes	Yes	(Yes)†	90 (1)	80 (2)	5.9 (—)	3.6 ($\pm$ 1.2)
4	L ₂	15	Yes	No		5 (1)	5 (1)	1.8 (—)	4.4 (—)
4	R ₁	18	Yes	Yes		20 (6)	30 (6)	3.8 ($\pm$ 0.5)	3.0 ($\pm$ 0.4)

* These were two successive treatment cycles in the same patient.

† A fourth follicle, 20 mm in diameter, was also aspirated during this patient cycle and resulted in a fertilized oocyte and embryo transfer. Granulosa cells from this follicle were not cultured.

After 24 days, 'granulosa-type' cells covered approximately 90% of the plates generated from two 15-mm follicles (1R₁ and 4L₁) but only 5% of a third 15-mm follicle (4L₂) as noted in Table 1. In contrast, the 18–25-mm follicles produced plates in which the 'granulosa-type' cells constituted only 5–60% of the cell population. The estimation of plating distribution between 'granulosa-type' and 'luteal-type' cells did not warrant statistical comparison. However, there appeared to be a tendency for a predominance of 'granulosa-type' cells to plate from the smaller follicles.

Under basal conditions and after hCG treatment, the number of cells removed from the plates after 24 days surpassed the number plated (Table 1). An aliquant of buffer calculated to contain 1.0

PLATE I

Light photomicrographs of human granulosa cells harvested from follicles exposed *in vivo* to clomiphene citrate and hCG.

Fig. 1. After 2 days in culture. 'Fibroblast-like' cells are seen. $\times 950$.

Fig. 2. After 6 days in culture. 'Fibroblast-like' cells and smooth-surfaced round-to-ovoid cells, with a higher nucleus-to-cytoplasm ratio, are adherent to the plates. $\times 950$.

(Facing p. 282)

Light photomicrographs of the appearances of the 2 types of cells after 10 days in culture.

Fig. 3. 'Luteal-type' cells (LTC) comprising a lawn of closely apposed elongated cells. $\times 950$.

Fig. 4. 'Granulosa-type' cells (GTC) with rounded-to-polygonal contours, occurring in rosette-like formations resembling Call-Exner bodies (CEB). $\times 950$.

Fig. 5. Very fine processes extending between 'granulosa-type' cells (GTC). $\times 1900$.

Fig. 6. 'Luteal-type' cells (LTC) and 'granulosa-type' cells (GTC), in close proximity. $\times 950$.

Transmission electron micrographs of freshly isolated granulosa cells from follicles previously exposed *in vivo* to clomiphene citrate and hCG.

Fig. 7. A dispersed cell. The predominant feature is non-extractable lipid droplets (L). Points of exocytosis of lipid droplets (E) are coupled with membranous infoldings (MI). $\times 12\,000$.

Fig. 8. Annular nexuses (AN) noted in these granulosa cells after dispersion. $\times 20\,000$.

Fig. 9. Perinuclear cytoplasm. Smooth-surfaced endoplasmic reticulum is scarce in these cells while rough-surfaced endoplasmic reticulum (RER) is found amidst free polyribosomes (FP). Note perinuclear Golgi (G) and bacilliform mitochondria (M). $\times 22\,000$.

Transmission electron micrographs of untreated cells after 10 days of culture.

Fig. 10. Non-extractable lipid droplets (L) are relatively unchanged. The rough-surfaced endoplasmic reticulum (RER) has become more prominent and dilated, but smooth-surfaced endoplasmic reticulum remains scarce. Mitochondria (M) have become more pleomorphic with branching. Golgi (G) is now dispersed throughout the cytoplasm. $\times 14\,000$.

Fig. 11. Microfilaments (MF) have become more noticeable. $\times 18\,000$.

Fig. 12. Golgi apparatus (G) is easily identified. $\times 31\,000$.

Fig. 13. Prominent dilated RER is noted after 10 days of culture. $\times 52\,000$.

Fig. 14. Transmission electron micrograph of a cell exposed to hCG *in vitro* for 10 days. The main difference between this treated cell and those depicted in Plate 5 (10 days untreated) is the increase in the amount of smooth-surfaced endoplasmic reticulum (SER). Note rough-surfaced endoplasmic reticulum (RER) enveloping mitochondria (M), characteristic of active protein synthesis and branching of SER from RER (arrow). $\times 54\,000$.

Transmission electron micrographs after 24 days in culture under basal conditions.

Fig. 15. Low-power view. Non-extractable lipid droplets (L) have decreased in number compared to those in treated and untreated earlier cultures. The relative proportions of endoplasmic reticulum and the bizarre branching mitochondria (arrow) closely resemble those of Day-10 cells not exposed to hCG *in vitro*. $\times 5000$.

Fig. 16. Annular nexus (AN) resembling those of Day 10 cells untreated. $\times 12\,000$.

Fig. 17. High-power view. The bristled pinocytotic vesicles (BPV), the dilated rough-surfaced endoplasmic reticulum (RER) and the bizarre mitochondria (M) are similar to those of Day-10 cells not exposed to hCG. $\times 26\,000$.

Fig. 18. Sparse smooth-surfaced endoplasmic reticulum (SER) scattered within the cells. $\times 24\,000$.

Transmission electron micrographs of granulosa cells exposed to hCG *in vitro* for 24 days.

Fig. 19. The 2 striking changes between these cells and those not exposed to hCG *in vitro* are the enhancement of abundant smooth-surfaced endoplasmic reticulum (SER) and the increase in the number of annular nexuses (AN). $\times 19\ 000$.

Fig. 20. Note tubular cristae (arrow) within mitochondria (M). $\times 53\ 000$.

Fig. 21. SER is seen in close juxtaposition to rough-surfaced endoplasmic reticulum (RER). $\times 34\ 000$.

$\times 10^5$ viable cells had been originally distributed to each culture dish, but 1.8×10^5 to 7.1×10^5 viable cells were retrieved by trypsinization at 24 days. There was no significant difference between control plates (mean $\pm$ s.e.m. = $4.1 \times 10^5 \pm 0.4 \times 10^5$ cells/plate) and hCG-stimulated cultures ($3.7 \times 10^5 \pm 0.3 \times 10^5$ cells/plate).

Fine structure of isolated and cultured cells

The predominant feature of freshly isolated granulosa cells from these follicles was non-extractable lipid droplets (Pl. 4, Figs 7, 8 & 9). The cells exhibited multiple infoldings of the cell surface and exocytosis of lipid droplets. SER was limited, but RER and free polyribosomes were scattered throughout the cells. Golgi were limited to perinuclear locations. Elliptical and bacilliform mitochondria exhibited tubular and lamellar cristae. Annular nexuses were present. After 2 days in culture, little ultrastructural modification had occurred. By 10 days in culture (Pl. 5, Figs. 10, 11, 12 & 13), the relative proportion of non-extractable lipid droplets remained the same. However, RER had become more prominent and dilated and Golgi had become more widely dispersed. Pleomorphism and branching now characterized the mitochondria. Microfilaments were abundant. hCG effected an increase in the amount of SER as evident in Pl. 6, Fig. 14.

As shown in Pl. 7 (Figs 15, 16, 17 & 18) and Pl. 8 (Figs 19, 20 & 21), Day-24 cells under basal conditions and those of hCG exposure differed from earlier cultures by a decrease in non-extractable lipid droplets and more prominent microfilaments. The trend of enhancement of SER by gonadotrophin continued at 24 days of culture. Furthermore, the number of annular nexuses increased with hCG exposure.

Hormonal studies

Progesterone was detected in the media of all plates containing granulosa cells throughout the 24-day culture period. Under basal conditions, progesterone levels declined over the first 10 days and gradually plateaued. In accord with observations previously noted for luteal cells by other authors (Dennefors, Sjogren & Hamberger, 1982b), we found a wide variation in basal levels of progesterone which was not related to follicular diameter (Table 2). For instance, on Day 2 the

Table 2. Progesterone levels (ng/ml) in media, reflecting secretion over the 2 days of culture before collection

Follicle	Day of culture							
	Day 2		Day 4		Day 10		Day 24	
	Control	100 i.u. hCG/ml	Control	100 i.u. hCG/ml	Control	100 i.u. hCG/ml	Control	100 i.u. hCG/ml
1L ₁	2324 ± 481.0	1625 ± 139.0	2238 ± 387.0	3430 ± 420.0	128 ± 15.8	723 ± 199.0	200 ± 33.3	329 ± 32.5
1R ₁	5041	4268	1674	3752	64	110	28	32
2L ₁	455 ± 143.4	489 ± 39.7	541 ± 115.8	1107 ± 178.1	28 ± 4.0	516 ± 48.4	20 ± 3.6	33 ± 4.6
2R ₁	325 ± 113.1	419 ± 72.4	548 ± 130.2	635 ± 65.9	76 ± 15.5	468 ± 19.0	5 ± 1.7	10 ± 1.4
3L ₁	3.1 ± 0.4	4.3 ± 0.6	4.3 ± 0.7	3.8 ± 1.5	2.8 ± 0.4	5.3 ± 0.9	11.7 ± 1.1	19.9 ± 3.6
3R ₁	4.0	3.6	1.8	4.7	3.2	3.4	—	—
4L ₁	230 ± 31.5	465 ± 150.5	671 ± 175.0	1020 ± 146.0	177 ± 79.0	182 ± 27.5	8 —	40 ± 4.4
4L ₂	108	241	176	368	59	348	103	168
4R ₁	1543 ± 162.1	2029 ± 349.9	1825 ± 201.0	2121 ± 272.3	584 ± 63.6	1465 ± 107.7	340 ± 40.0	840 ± 134.3

Values are mean $\pm$ s.e.m. The number of culture plates generated from each follicle corresponds to the value given in Table 1 with the exception of Follicle 4L₁ control plates (Days 2, 4 and 10: 2 plates each; Day 24: 1 plate).

mean $\pm$ s.e.m. progesterone values for granulosa cells generated from two 18-mm follicles varied from 3.1 ± 0.4 ng/ml medium (Follicle 3L₁) to 1543 ± 162.1 ng/ml medium (Follicle 4R₁). The values for 2 larger follicles were 2324 ± 481.0 ng/ml (Follicle 1L₁, 25 mm) and 455 ± 143.4 ng/ml (Follicle 2L₁, 23 mm).

As seen in Table 3, treatment with 100 i.u. hCG/ml did not elicit a significant effect on progesterone secretion by 2 days of culture. However, by 4 days of culture a significant difference ($P < 0.05$) was noted in granulosa cells from all follicles. This significant difference between stimulated and control plates continued throughout the 24 days of culture. Granulosa cell progesterone secretion from 2 representative follicles is depicted in Text-fig. 1. The progesterone response to hCG was coupled with enhancement of SER and increase in annular nexuses at 10 and 24 days of culture. In no case was there evidence of increased steroidogenesis which was not associated with increased SER. Responsiveness to hCG noted after 4 days of culture was not related to the size of the follicles from which granulosa cells had been aspirated. Follicular size did not significantly affect progesterone production by the granulosa cells *in vitro* under basal or stimulated conditions.

Table 3. The effect of hCG on progesterone secretion from granulosa cells after 2, 4, 10 and 24 days of culture

Day	Regression coefficient (r)	Standard error of r	t values	P (2-tail)
2	-11.9	194.8	-0.06	0.9500
4	467.4	154.4	3.02	0.0040
10	410.5	90.0	4.55	0.0001
24	170.4	69.1	2.46	0.0184

Multiple linear regression BMDP 1R (1983).

Text-fig. 1. Effect of hCG on progesterone secretion from human granulosa cells aspirated from 2 large preovulatory follicles identified in Table 1. Cells were cultured under basal conditions or incubated with 100 i.u. hCG/ml. Each bar represents the mean ($\pm$ s.e.m.) value for culture media from at least 4 culture dishes.

Relaxin was not detected in any sample of culture media, regardless of follicular source, follicular size or exposure to hCG.

Clinical outcome

The limited number of follicles prevented statistical comparisons between the clinical outcomes of oocytes and follicular size or granulosa cell culture characteristics at 24 days. However, as depicted in Table 1, oocytes were retrieved from 15–25-mm follicles. All oocytes from 18 to 25-mm follicles and 1 of 2 oocytes from 15-mm follicles cleaved normally and were transferred to the donor uterus. During the treatment cycle in which pregnancy was achieved, all 3 patients had at least one oocyte from an 18–25-mm follicle transferred.

Discussion

These studies demonstrate that human granulosa cells exposed *in vivo* to clomiphene citrate and hCG can be maintained as progesterone-secreting cells in a long-term monolayer culture system. Furthermore, granulosa cells from 15–25-mm follicles recovered responsiveness to hCG after 4 days in culture, evident in enhancement of progesterone secretion and ultrastructural modifications of SER and annular nexuses. However, these cells in culture did not develop the specialized lutein function of relaxin secretion, characteristic of the corpus luteum of pregnancy (O'Byrne *et al.*, 1978).

Prolonged exposure *in vitro* to hCG was required before enhancement of progesterone production was observed. The biochemical basis for this unique time course remains to be elucidated, but may be related to acclimatization of the cells to culture conditions, to removal from follicular fluid or to steroidogenic blockade before progesterone production. Regardless of the mechanisms involved, our results seem to complement earlier short-term studies of isolated granulosa cells pooled from preovulatory follicles. After a 2-h incubation, 100 i.u. hCG/ml significantly increased tissue levels of cAMP but only "tended to stimulate" progesterone production (Dennefors, Nilsson & Hamberger, 1982a). When these experiments were repeated using granulosa cells excised from women 24 to 30 h after i.m. administration of 9000 i.u. hCG, an increase in basal progesterone production *in vitro* was noted. Once again, however, progesterone enhancement was not significant over the 2-h incubation period (Dennefors, Hamberger & Nilsson, 1983). The cells from preovulatory follicles exposed to the LH surge *in vivo* and those from preovulatory follicles exposed to exogenous hCG *in vivo* responded to 100 i.u. hCG/ml *in vitro* with increased tissue cAMP formation. Our long-term monolayer cultures of granulosa cells exhibited a clear progesterone response to 100 i.u. hCG/ml only after 4 days. Although we first observed a corresponding increase in SER and annular nexuses at 10 days of culture, these ultrastructural changes may have occurred as early as 4 days as suggested by earlier monolayer cultures of pooled granulosa cells (Suzuki *et al.*, 1981).

As elegantly demonstrated by McNatty & Sawers (1975), the ability of human granulosa cells to secrete progesterone *in vitro* is predetermined by the follicular microenvironment from which they were removed. Initial exposure to FSH followed by the addition of oestradiol promotes proliferation of the granulosa cells and an increase in LH receptors. It is the "final entry" of LH into the follicle that initiates progesterone secretion. The apparent discrepancy between our present findings and those of Bieszczad *et al.* (1982) concerning the progesterone responsiveness of cells from small compared to large preovulatory follicles may actually be a function of the length of stimulation *in vitro*. In the 24-h incubation studies (Bieszczad *et al.*, 1982), only granulosa cells from follicles <20 mm responded to ovine LH (1 µg/ml) with enhanced progesterone production, and the authors suggested that the inability of LH to affect progesterone secretion by cells from larger follicles might be due to a maximal binding of LH to the receptors at the time of culture or to desensitization. Our studies of 15–25-mm follicles tend to support the former hypothesis during the first 2 days of culture, but are not consistent with a continued down regulation. In fact, our cultures exhibit prolonged responsiveness to hCG *in vitro* of all luteinized granulosa cells, consistent with

the anatomical and functional maintenance of the human corpus luteum observed during pregnancy (Crisp *et al.*, 1973). Throughout the 280-day gestation period, the corpus luteum is thought to secrete both progesterone and relaxin in the presence of hCG (Quagliarello, Szlachter, Steinetz, Goldsmith & Weiss, 1979), despite failure to detect significant hCG receptors between 6 and 34 weeks of pregnancy (Rao, Griffin & Carman, 1977). Perhaps, as in ovine luteal cells in monolayer culture (Suter & Niswender, 1983), LH/hCG receptors may be recycled without increasing in number. As an alternative hypothesis, LH/hCG may increase its own receptors in our cultured granulosa cells in a manner similar to the effect of LH/hCG upon granulosa cells of preovulatory follicles in the rat (Richards, 1978; Ireland & Richards, 1978). Quantitation of LH/hCG receptors at the time of cell dispersion and at the conclusion of future experiments will clarify this issue. It is also possible that the preovulatory exposure of the granulosa cells *in vivo* to clomiphene citrate and hCG in our study may have effected responsiveness to hCG *in vitro* and, hence, may invalidate extrapolation to normal follicles before ovulation.

At the light microscopic level, 2 morphologically distinct populations of cells could be identified. However, because of the relative scarcity of the 'granulosa-type' cells, separation of these two cells must precede ultrastructural comparisons. Since progesterone secretion under basal or stimulated conditions was not affected by the predominance of cell types in culture, the functional difference between the 2 types of cells remains unknown. Our electron microscopic observations are limited to the 'luteal-type' cell. Some of the ultrastructural changes of granulosa cells undergoing luteinization (Adams & Hertig, 1969; Christensen & Gillim, 1969) appeared in this *in-vitro* system, especially under the influence of hCG: the cells possessed non-extractable lipid droplets and developed SER and gap junctions which extended along processes into adjacent cells as annular nexuses. However, even after up to 24 days in culture, the SER never achieved the abundance seen in granulosa lutein cells *in situ*.

In conclusion, we have shown that human granulosa cells obtained from follicles exposed *in vivo* to clomiphene citrate and hCG retain the specialized function of progesterone secretion *in vitro* for up to 24 days. After a time lag of 4 days, these cells recover the ability to respond to hCG with enhanced progesterone secretion, SER and annular nexuses. Therefore, this *in-vitro* system may be useful in studying, in more detail, the retained endocrine function of progesterone production. Furthermore, this approach may be used to investigate prolonged differentiated functions of other ovarian cells.

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