RELATIONSHIP BETWEEN THE ENDOCRINE ENVIRONMENT WITHIN THE GRAAFIAN FOLLICLE AND THE SUBSEQUENT RATE OF PROGESTERONE SECRETION BY HUMAN GRANULOSA CELLS IN VITRO

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SUMMARY

The steroidogenic potential of granulosa cells harvested from human Graafian follicles containing varying concentrations of pituitary and steroid hormones was examined. The mitotic activity and production of progesterone by granulosa cells in vitro was found to be correlated with their hormonal environment at the time of harvesting. Only cells from follicles containing some FSH and high concentrations of oestradiol underwent spontaneous mitosis in vitro. However, mitosis could be induced by adding FSH and high concentrations of oestradiol to the culture, provided that the concentration of LH was low. Cells harvested from follicles containing LH, FSH and high concentrations of oestradiol secreted significantly more progesterone than cells from follicles which did not contain all three hormones.

It is suggested that after the initiation of follicular development by FSH, a long period of exposure (8–10 days) to both FSH and oestradiol is necessary before the maximum biosynthetic capacity of granulosa cells is achieved; this synthetic potential is then only realized under the influence of LH and prolactin. Premature exposure to LH inhibits both the mitotic activity and the steroidogenic potential of these cells.

INTRODUCTION

The granulosa cells within the Graafian follicle do not have direct access to a blood supply but are bathed in a follicular fluid which contains most serum proteins, variable amounts of gonadotrophins and high concentrations of steroids (Edwards, 1974; McNatty, Hunter, McNatty & Sawers, 1975). This hormonal micro-environment may be of importance in determining the subsequent function of these cells in the corpus luteum.

There is considerable evidence in mammals to suggest that the steroidogenic potential of granulosa cells in vitro is related to the stage of the ovarian cycle at which they were harvested (Channing, 1969a; 1970). Recently it has been shown that a precise sequence of hormonal changes occurs in the follicular fluid of the developing human Graafian follicle, and it has been suggested that the sequence in which these changes occur is of considerable importance for the growth of that follicle and the secretory activity of its granulosa cells both before and after ovulation. In order to obtain direct evidence on this point, follicular fluid and granulosa cells were obtained from human Graafian follicles of varying sizes throughout the menstrual cycle. The concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), oestradiol and progesterone were measured in the follicular fluid, and cells from each follicle were cultured to assess their steroidogenic potential.
MATERIALS AND METHODS

Subjects

Follicles were obtained from the ovaries of 55 women (aged between 21 and 48 years), at various stages of the menstrual cycle, who were undergoing hysterectomy for various gynaecological conditions. The indications for surgery were stage 0 carcinoma of the cervix (4), menorrhagia due to fibroids (7), endometriosis (4), cystic glandular hyperplasia (1) and chronic pelvic pains or dysmenorrhea (39). Those with stage 0 carcinoma of the cervix had regular menstrual cycles (21–32 days) and were considered to be endocrinologically normal. The menstrual cycles of the group with menorrhagia varied in length from 21–34 days and ovulation had occurred in 50% of them. The remaining women were considered to be in the follicular phase as assessed from the date of the last menstrual period and the presence of a proliferative endometrium.

Dating the menstrual cycle

The stage of the menstrual cycle was assessed from the presence or absence of a corpus luteum at operation, endometrial histology and the pituitary and steroid hormone concentrations in peripheral plasma immediately before oophorectomy (McNatty et al. 1975). The menstrual cycle was divided into six phases: early follicular, patient still menstruating; mid-follicular; late follicular; early luteal; mid-luteal and late luteal.

Collection of follicular fluid for radioimmunoassay

Follicular fluid and granulosa cells were obtained from whole ovaries or wedge biopsies in the 55 subjects described above.

Ovarian tissue was collected in chilled Medium 199 containing Hanks' salts with Hapes [2-(N-2-hydroxyethylpiperazin-N'-y1)ethanesulphonic acid] buffer (20 mmol/l), gentamicin (50 µg/ml) and amphotericin-B (2.5 µg/ml) (Flow Laboratories, Irvine, Scotland). All of the follicles which were > 4 mm in diameter (138) were dissected from the ovaries within 2 h of the operation. The diameter of each isolated follicle was measured and the antral fluid aspirated through a 27G needle into a 500 µl Hamilton syringe. The fluid was frozen at −20°C until assayed.

The radioimmunoassays for LH, FSH, oestradiol and progesterone in follicular fluid were described by McNatty et al. (1975).

Techniques for culturing granulosa cells

The method of collecting and culturing human granulosa cells was a modification of that described by Channing (1969b). The granulosa cells were harvested from each of the follicles and each culture chamber contained cells from individual follicles. The collapsed follicle after removal of the follicular fluid was slit open and the granulosa cells were scraped into culture medium with the aid of a platinum loop. The remainder of the follicle was fixed in Bouin's aqueous fixative for subsequent histological examination to ensure that the basement membrane was still intact. A sample of the suspension of granulosa cells was counted using a haemocytometer and cell viability was determined with either nigrosin (Paul, 1972) or lissamine green (Channing, 1969b). The remaining cell suspension was sub-divided into aliquots each containing a minimum of $5 \times 10^4$ 'live cells' and layered on 18 mm² no. 3 cover-slips which had previously been washed in acetone, dried with lens tissue and then heated to 200°C. These cover-slips were placed in a tissue culture box containing 21 19·0 mm² compartments (Flow Laboratories). Cultures were carried out in triplicate whenever possible although the low viabilities (< 10%) of some cell preparations meant that some studies had to be carried out on single cultures. Each
culture received 1 ml Medium 199 containing antibiotics and calf serum (20 %, v/v) and was then incubated at 37 °C using air as the gas phase. Each culture was maintained for 2–30 days and the culture medium was replaced daily and stored frozen at −20 °C until assayed for progesterone. At the end of culture the cover-slips were removed, extensively rinsed in medium without serum and fixed with Smearfix (Raymond A. Lamb, London). The cells on the cover-slips were stained with haematoxylin and eosin and the cell numbers were determined by counting the number of cells within 0-12 mm² at 23 uniformly spaced sampling points within the 18 mm² of the cover-slip boundaries. A PDP-9 computer, mechanical microscope and Quantimet television system were used (Green, 1974).

Radioimmunoassay for progesterone in the culture medium
The technique was described by Neal, Baker, McNatty & Scaramuzzi (1975). Progesterone antiserum (RI-4) was raised in a rabbit against progesterone-11α-hemisuccinate conjugated to bovine serum albumin and the specificity was similar to that previously reported (Dighe & Hunter, 1974). The assays were conducted in duplicate at two dilutions and the minimum detectable level of progesterone was 50 pg/ml.

Pituitary and steroid preparations
The gonadotrophins which were added to the cultures were: human LH (Hartree IRC-2, 24.vi.69) containing 7550 units LH/mg and < 25 units FSH/mg; human FSH (Butt CPDS/6) containing 2200 units FSH/mg and 90 units LH/mg and human FSH (MRC 73/519) containing 2200 units FSH/mg and 8-8 units LH/mg. These immunological potencies were assessed using the following standards: LH, MRC 68/40 assumed 77 units/ampoule; FSH, MRC 68/39 assumed 32-8 units/ampoule (MRC National Institute for Biological Standards and Control).

Concentrations of LH and FSH are expressed as mu./ml. The gonadotrophins were diluted in culture medium and stored at −20 °C until used in the cultures. Oestradiol at a concentration of 10 µg/ml and dibutyryl cyclic AMP (dbcAMP) at 465-4 µg/ml were made up in the culture medium and 0-1 ml was used for each culture. The final concentration of oestradiol (1 µg/ml) present in the culture medium was similar to that found in actively developing human Graafian follicles while that for dbcAMP (46-5 µg/ml) was the maximum dose which did not cause cellular necrosis.

Determination of the biological activity of the gonadotrophins
Human granulosa cells were cultured in medium containing calf serum and the progesterone secretion was compared with that achieved by cells cultured in a variety of media each of which contained human serum with known endogenous concentrations of LH and FSH. Human sera used for cell culture were obtained either from hypophysectomized patients (supplied by Professor A. P. M. Forest) containing low but detectable levels of gonadotrophins or from pooled batches (supplied by Flow Laboratories) containing higher levels of gonadotrophins. The biological activity of LH and FSH preparations added to the cultures containing 20 % calf serum were examined by measuring the daily progesterone production by granulosa cells in response to varying amounts of LH, FSH or LH + FSH.

RESULTS
Viability of granulosa cells before and during culture
The mean viability of human granulosa cells at harvesting was 37·0 % (range 4-4–100-0 %, n = 138). There were no apparent relationships between cell viability and stage of cycle, follicle size, or hormone concentrations in follicular fluid at the time of harvest. The greatest
proportion of live cells were found in follicles harvested soon after oophorectomy. There were no significant differences in the assessment of viability when either nigrosin or lissamine green vital stains were used ($P > 0.2$).

Granulosa cells remained viable in culture for at least 16 days, although after 20 days the number remaining on the cover-slips declined rapidly. The mitotic index (number of cells present after 10 days, divided by the number of live cells at the commencement of culture) was related to the environment to which the cells had been subjected in the follicle in vivo (Table 1); only cells harvested from follicles with FSH and high concentrations of oestradiol in the follicular fluid increased in number (Table 1). When granulosa cells from any stage of the cycle were grown in culture medium containing <1 mu. LH/ml, mitosis only occurred in the presence of added FSH (30 mu./ml) and oestradiol (1 μg/ml), and the mitotic index was $6.6 \pm 1.9$ (s.e.m., $n = 10$).

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Mitotic index refers to the average number of cells remaining at the end of 10 days of culture divided by the number of live cells at the commencement of culture. The symbols + or $-\text{LH}$ or FSH indicate the presence or absence of detectable amounts of these hormones ($\text{LH} > 2.8 \mu\text{g/ml}, \text{FSH} > 1.3 \mu\text{g/ml}$) in the follicular fluid from which the cells were harvested. For oestradiol, $+0_{c}$ indicates a concentration > 250 ng/ml. No. of observations in parentheses.

**Biological activity of the hormones**

Calf serum which was added to the culture medium contained immunological LH activity equivalent to 0.7 ng NIH-LH-S14/ml, or <0.4 mu. MRC 68/40/ml (FSH was not determined). However, this calf serum stimulated progesterone production by human granulosa cells in vitro in a manner comparable to a human serum preparation containing 1.8 mu. LH/ml and 1.7 mu. FSH/ml (Fig. 1).

The immunoactive human FSH and LH concentrations in calf serum were comparable to the lowest levels of FSH and LH found at any stage of the human menstrual cycle. Progesterone and oestradiol were undetectable in calf serum (<50 and <6 pg/ml respectively).

The production of progesterone by granulosa cells in response to the addition of LH, FSH, or LH + FSH was variable. The doses of gonadotrophins which induced the maximum steroidogenic response in all follicles were tested on granulosa cells harvested from the smallest follicles (4 mm diameter) and the results are shown in Fig. 2. There was no significant change in production of progesterone when the concentration of LH was increased from 1.8 to 30 μu./ml provided that the dose of FSH was constant. Similarly there was no increase in the production of progesterone when FSH was increased to 30 μu./ml when the LH dose was constant. However, with increased concentrations of FSH the steroidogenic response to LH was enhanced (Fig. 2). There was no significant increase in the production of progesterone when the concentration of both LH and FSH were increased from 30 to 90 μu./ml ($P > 0.5$) (paired $t$-test).

In all subsequent gonadotrophin stimulation experiments, the concentrations of LH and FSH were 30 μu./ml; there were no significant differences in response between FSH CPDS/6 and FSH 73/519. The concentrations of LH and FSH added to the culture were within the
normal physiological ranges at the time of the mid-cycle ‘surge’ (e.g. 25–60 mu./ml) but about three times higher than the levels in follicular fluid (McNatty et al. 1975).

**Effect of various hormone concentrations in the follicular fluid on the production of progesterone by granulosa cells in vitro**

Since there were no differences in the way granulosa cells grew or secreted progesterone when harvested from patients with stage 0 carcinoma of the cervix or menorrhagia, the results from all follicles were pooled. The variation in the daily production of progesterone between replicate cultures for all treatments was 4·9 % (range 0·0–19·6 %, n = 315).

The daily production of progesterone during 10 days of culture after various treatments in relation to the hormone concentrations in follicular fluid at the time of harvesting is shown in Fig. 3. Cells harvested from follicles containing LH, FSH and high concentrations of oestradiol secreted maximum amounts of progesterone into the culture medium, and were not influenced by the addition of LH, FSH, LH + FSH, LH + oestradiol or dbcAMP (Fig. 3e). By contrast cells from follicles lacking FSH, LH or oestradiol secreted significantly less progesterone (P < 0·001), but were stimulated by the daily addition of LH + FSH, dbcAMP, or LH + FSH + oestradiol (P < 0·01) (see Fig. 3f). The response to dbcAMP was similar to that of LH + FSH and was significantly less effective than LH + FSH + oestradiol but more effective than LH, FSH, FSH + oestradiol or oestradiol alone with one exception (see Fig. 3f) (P < 0·01 and P < 0·01 respectively). The steroidogenic response by granulosa cells in vitro to the daily addition of LH + FSH or dbcAMP was related to the hormone environment from which the cells had been harvested. For example cells previously exposed to FSH + oestradiol secreted significantly more progesterone each day than those which had been exposed to FSH + LH or FSH alone and cells previously exposed to the latter hormones secreted significantly more than those previously exposed to LH or no hormone (P < 0·01, P < 0·01 respectively, paired t-test).

The maximum production of progesterone by a granulosa cell was about 5 pg/day and this
Fig. 2. Production of progesterone by granulosa cells in vitro in response to varying amounts of LH and FSH. Production of progesterone per cell per day when the following concentrations of gonadotrophin were present (or added) in culture medium containing 20% serum from hypophysectomized patients: (×) 0-6 and 0-4 mu. LH and FSH/ml; (▲) 1-8 and 1-7, 4-4 and 1-7, 16-4 and 1-7 or 30-0 and 1-7 mu. LH and FSH/ml; (●) 4-0 and 30-0 mu. LH and FSH/ml; (△) 30-0 and 30-0 mu. LH and FSH/ml, and (○) 90-0 and 90-0 mu. LH and FSH/ml. The cells were harvested from 5 early and mid-follicular phase follicles (all 4 mm in diameter) and each experiment is the mean result of duplicate cultures.

was achieved within 1–2 days in culture if cells had been harvested from follicles containing LH, FSH and oestradiol. By contrast, cells harvested from follicles devoid of any hormone never achieved the maximum daily production during 10 days in culture, even after the addition of LH + FSH + oestradiol (Figs. 3a and f).

Fig. 3. Average daily production of progesterone by human granulosa cells during 10 days of culture after various treatments in relation to the hormone concentrations present in the follicular fluid. The average results (+1 s.e.m.) for the individual treatments are represented by the lines indicated. The average results of a number of different treatments were not significantly different from each other (P>0.05, paired t-test), consequently they are represented by the same line. The levels of hormones in the follicular fluid are given at the top of each figure: + LH > 2-8 mu./ml, − LH < 2-8 mu./ml; + FSH > 1-3 mu./ml and − FSH < 1-3 mu./ml; + oestradiol (O2) > 250 ng/ml and − O2 < 250 ng/ml. The hormone treatments given in the culture media are shown on the figures. The number of experiments is shown in parentheses. DBC, Dibutyryl cyclic AMP.
Fig. 3. For legend see facing page.


**DISCUSSION**

The most interesting aspect of this study is the clear demonstration that the ability of human granulosa cells to undergo mitosis or secrete progesterone in culture is predetermined by the hormone environment of the follicle from which they were harvested. Therefore, this gives added point to our previous studies (McNatty et al. 1975) which noted a critical sequence of hormonal changes that take place within the follicular fluid before ovulation.

It seems that the mitotic activity of the granulosa cells in the intact follicle is stimulated by the presence of FSH and high concentrations of oestradiol in follicular fluid, an ability that is retained in culture. However, the presence of LH in the follicular fluid, or the addition of LH to the culture medium, impairs this mitotic activity (Delforge, Thomas, Roux, Carneiro de Siqueira & Ferin, 1972; McNatty et al. 1975).

The biosynthetic activity of the granulosa cells in culture is also influenced by their prior hormonal environment. Progesterone secretion was greatest in cells harvested from follicles that contained high concentrations of FSH, LH and oestradiol. Similarly, the addition of these three hormones to the culture medium was able to stimulate progesterone secretion if the cells were obtained from hormone-deprived follicles. These findings are consistent with those reported by Goldenberg, Bridson & Kohler (1972) for porcine granulosa cells.

It is of interest to relate these findings to the sequence of hormonal changes taking place in the Graafian follicle during the course of follicular maturation (McNatty et al. 1975). During the early follicular phase FSH and low concentrations of oestradiol were present in a large proportion of small antral follicles. By the mid-follicular phase there were few small follicles with FSH, but some of the larger ones contained high concentrations of both FSH and oestradiol. Just before ovulation, the largest follicle contained high concentrations of LH in addition to FSH and oestradiol. It is tempting to conclude that the initial appearance of FSH may be essential for follicular growth (Baird, Baker, McNatty & Neal, 1975); subsequent exposure to FSH and oestradiol stimulates both the mitotic activity of the granulosa cells and an increase in the number of receptors for LH (Zeleznik, Midgley & Reichert, 1974; Channing, 1975) which prepares them for eventual steroid secretion. The final entry of LH into the follicle inhibits further mitosis and together with prolactin (McNatty, Sawers & McNeilly, 1974) initiates progesterone secretion.

There seems to be good agreement between the time course of these events in vivo and in vitro. For example, FSH treatment in vivo takes 6–10 days to mature a follicle (Brown, Evans, Adey, Taft & Townsend, 1969; Gemzell & Johansson, 1971; Bertrand, Coleman, Crooke, Mcnaughton & Mills, 1972) and granulosa cells harvested from small early follicular phase follicles (containing FSH) achieve their maximum biosynthetic capacity in vitro after 8–10 days if LH, FSH and oestradiol are added to the culture each day. Although it is necessary to add LH to assess the steroidogenic potential of granulosa cells in vitro, LH does not enter the follicle in appreciable amounts until the mid-cycle LH peak in plasma (McNatty et al. 1975). These studies suggest, therefore, that a relatively long period, 8–10 days, of continual exposure to FSH and oestradiol is required before the maximum biosynthetic capacity of the granulosa cells is achieved; this synthetic potential is then only realized and sustained in the presence of low physiological amounts of gonadotrophin and prolactin (McNatty et al. 1974).

During the luteal phase, Graafian follicles secrete little or no oestradiol (Baird & Fraser, 1975), presumably because of insufficient gonadotrophic support (Baird et al. 1975). These follicles have low concentrations of oestradiol in their follicular fluid (Sanyal, Berger, Thompson, Taymor & Horne, 1974; Baird & Fraser, 1975; McNatty et al. 1975) but they may contain LH or LH + FSH. Granulosa cells harvested from these follicles do not undergo mitosis in culture and furthermore they secrete only small amounts of progesterone.
Thus it appears that granulosa cells within luteal phase follicles lacking oestradiol are unable to proliferate or achieve their maximum biosynthetic capacity. These findings also suggest that the premature appearance of LH in the follicular fluid of a Graafian follicle could arrest mitosis and interfere with the ability of granulosa cells to achieve their maximum biosynthetic potential. It appears that only the actively maturing follicle receives the critical sequence of hormones that will allow its granulosa cells to proliferate and achieve their maximum biosynthetic potential after ovulation.

Our studies have shown that it is only granulosa cells from the actively maturing follicle whose steroidogenic potential is related to the stage of the ovarian cycle; Channing (1969a, 1970) had made similar observations for the horse, pig and rhesus monkey. However, most follicles recovered throughout the menstrual cycle were presumably not active because they were devoid of gonadotrophin(s) and/or oestradiol, and the steroidogenic potential of their granulosa cells was unrelated to the stage of the cycle.

These studies strongly suggest that the hormone environment of the Graafian follicle influences both the mitotic activity and biosynthetic potential of its granulosa cells, so that endocrine events occurring some considerable time before ovulation may dictate the subsequent activity of the corpus luteum.

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