The endocrine environment of the human oocyte

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Introduction

Follicular fluid is a unique body fluid containing a large number of biochemical components, some in extremely high concentrations, whilst in the middle of this relatively isolated biological compartment lies the arrested oocyte awaiting the signals to resume meiosis and progress towards timely ovulation. Delineation of these signals poses a challenging task. While there have been numerous reports on the concentrations of various steroids and peptides in follicular fluid after both natural and stimulated follicular growth and, similarly, the relationship between various components of oocyte maturation and the perceived signal has been studied in depth, the precise chronology of the endocrine changes relative to the sequence of oocyte maturation has not been systematically evaluated. Ideally the changing endocrine profile of the late preovulatory follicle would be best investigated by repeated frequent sampling (say every 2 h) of a single follicle. Unfortunately in the human such a longitudinal study would be difficult and so as an alternative approach we have considered the pattern of simultaneous endocrine changes in a cross-sectional group of follicles timed with respect to a common event (the start of the endogenous LH surge). The study of human follicular fluid is relatively simple because of the large volumes (2–8 ml) present in a preovulatory follicle but precise timing of the endogenous LH surge is more difficult due to the problems of frequent blood sampling. An alternative although slightly less precise method is to measure LH in frequent urine collections.

Finally, if the primary reason for aspirating the follicle was to obtain an oocyte for in-vitro fertilization, then it becomes unethical to attempt to obtain follicular fluid before 20–25 h from the start of the LH surge since it is unlikely that sufficiently mature fertilizable oocytes would be obtained. Therefore, to study the chronobiology of human follicular fluid from before the onset of the spontaneous LH surge until the time of ovulation, two sources of patients are required. For the early follicular fluid, patients coming into hospital for laparoscopic sterilization were recruited whilst later follicular fluids were obtained during the course of an in-vitro fertilization performed in unstimulated or natural cycles.

Materials and Methods

Collection of follicular fluids

Follicular fluid was aspirated from the dominant follicle either just before the start of the expected LH surge or at defined times after the start of the surge but before spontaneous follicular rupture (ovulation). For the early follicles,

i.e. those just before or within 20 h of the start of the LH surge, the largest, presumptively dominant follicle was aspirated during the course of a diagnostic laparoscopy, or at the time of laparoscopic sterilization. These subjects had given consent for the aspiration of follicular contents, and had submitted to twice daily blood sampling in order to determine whether the LH surge had begun by the time of aspiration. Blood samples were assayed for LH and oestradiol concentrations. Follicles classed as before the LH surge were obtained from women who had high circulating concentrations of oestradiol in association with a large (> 17 mm diameter) follicle, but in whom there was no increase in plasma LH by the time of the laparoscopy. It is difficult to assess exactly how many hours before the LH surge these individuals were because it has been shown that aspiration of the dominant follicle can produce temporal alterations in the timing of the expected surge (Lenton *et al.*, 1985). The follicles after the start of the LH surge were obtained from women in whom plasma oestradiol concentrations were still high, although a clear increase in LH had occurred within 10 or 20 h of the time of operation. No attempt was made to classify these follicles more closely because the monitoring blood samples had only been obtained twice a day.

Later (i.e. 20–35 h) follicular fluids were obtained during the course of oocyte collection for the purpose of in-vitro fertilization. Neither these, nor the above laparoscopy subjects had received any form of hormonal stimulation, and all gave a history of regular, apparently ovulatory cycles. In the in-vitro fertilization patients follicular aspiration was performed as an out-patient procedure using ultrasonography, and aspirations were more accurately timed with respect to the start of the spontaneous LH surge. This was achieved by 4–5-h urine collections, equally spaced over the 24 h which were then assessed for LH content using LH dipsticks (Ovustick: Medimar Laboratories, 17 The Queensway, Chalfont St Peter, Bucks S19 8NB, U.K.). These patients were also being scanned daily, and the start of the LH surge was defined as the time of the beginning of the first 4-h urine collection with detectable LH levels, provided that semi-quantitative LH levels continued to rise serially over the next 12–24 h. It was appreciated that the onset of the urinary LH surge detected in this way lagged behind the plasma LH surge by a few hours (E. A. Lenton, S. Amos, G. Lanjou & I. D. Cooke, unpublished observations) but this delay was well within the age bands selected for grouping the follicular fluids. It was therefore considered that, for the purpose of the study presented here, follicular fluids which have been grouped into 10-h age bands were equivalent regardless of whether the surge was detected by urinary or plasma LH measurements.

Finally, a small group of 4 follicular fluids which were assessed as being more than 40 h from the start of the LH surge is included. One of these follicles was from a patient who regularly demonstrated luteinized unruptured follicles, but in the other 3 patients follicular rupture had been artificially blocked by the administration of indomethacin (Indocid: Thomas Morson Pharmaceuticals, Hoddesdon, Herts EN11 9BU, U.K.) (100–200 mg in divided doses for 24–48 h). These follicles are referred to as 'post-ovulatory' follicles. In addition, 2 of the 8 follicles in the 35–40-h group were also obtained after indomethacin administration (Murdoch & Dunn, 1983).

Follicular fluids were stored frozen at -20°C until assayed for the steroids oestradiol, androstenedione, testosterone and progesterone and the peptides prolactin, LH, FSH and inhibin. Concentrations of cAMP were also measured.

Selection of normal follicular fluids

The follicular fluids discussed here were selected from a larger series of follicular fluid samples on the basis of the following criteria.

- (1) The onset of the LH surge (particularly applicable to the urinary monitoring) had to be clear and unequivocal.
- (2) All follicles had to be assayed for at least 6 of the 8 endocrine measures under discussion (in fact for 86% of the follicular fluids all 8 measurements were available).
- (3) The numbers of follicular fluids had to be equal in each age group and this number was arbitrarily set at 8 per group except for the oldest follicles in the 'greater than 40 h' group, for which only 4 follicular fluids were available.
- (4) All follicles had to be demonstrably active preovulatory follicles (oestradiol concentrations > 2000 ng/ml or progesterone concentrations > 5000 ng/ml, and luteinized granulosa cells evident).

Samples from 44 follicles form the basis of the data presented here. In each of the figures, the respective follicular fluid concentrations have been shown individually, but in the same sequence throughout, in order to demonstrate the extent of the variability within and between age groups, and between different hormone measurements on the same fluid. The mean concentrations at each interval are, however, given in Table 1, relative to the equivalent concentration in the peripheral circulation at mid-cycle.

Hormone measurement

All steroid measurements were made using commercially available direct (i.e. non-extraction) radioimmunoassay kits. Follicular fluids were first diluted (v/v) in appropriate hormone-free serum as follows: oestradiol 1:10 000, progesterone 1:1000, androstenedione 1:250 and testosterone 1:25. The radioimmunoassay kits were obtained from Steranti Research Ltd, St Albans, Herts AL1 1TA (oestradiol, ER-155 and androstenedione, ER-750 and testosterone ER-355) and from RIA UK Ltd, Armstrong Estate, Tyne & Wear NE37 1PP (progesterone, Gamma-B). Sensitivities at the above dilutions and inter-assay coefficients of variation were 100 ng/ml and 5.1% (oestradiol), 600 ng/ml and 11.0% (progesterone), 10 ng/ml and 7.0% (androstenedione) and 2 ng/ml and 15.0% (testosterone).

The peptides LH, FSH and prolactin were measured using monoclonal antibody-based immunoradiometric assays (Majacalone) from Serono Diagnostics Ltd, 21 Woking Business Park, Woking, Surrey GU12 5JY. Follicular

fluids were again diluted 1:2 (v/v) with control (hormone-free) serum to avoid any error due to the slightly different protein spectrum in follicular fluid compared with plasma/serum. Sensitivities and interassay coefficients of variation were 0.3 u/l and 3.9% (LH); 0.3 u/l and 6.8% (FSH) and 12 mu/l and 5.2% (prolactin).

The inhibin measurements were performed with a previously described radioimmunoassay (McLachlan *et al.*, 1986a) using inhibin of M_r 58 000 and 31 000 as antigen and tracer respectively.

Cyclic AMP in the follicular fluids was measured using a competitive protein binding assay as described by Brown *et al.* (1971), utilizing [3 H]cAMP. Sensitivity was 5 pmol/ml and intra- and inter-assay coefficients of variation were 15 and 17% respectively.

Results and Discussion

Oestradiol and progesterone in follicular fluid

Concentrations of oestradiol and progesterone in human follicular fluid have been described in follicles from different stages of the cycle (McNatty *et al.*, 1975), in follicles of different sizes (Westergaard *et al.*, 1986), after the spontaneous LH surge (Edwards *et al.*, 1980; Testart *et al.*, 1983) and after an ovulatory injection of hCG in otherwise unstimulated (Bomsel-Helmreich *et al.*, 1979) or mildly hyperstimulated (Templeton, 1985) women. In addition there are numerous reports of follicular fluid steroid concentrations following hyperstimulation with exogenous gonadotrophins for in-vitro fertilization. Although most authors are in agreement about the order of magnitude of the steroid concentrations, only the studies of Bomsel-Helmreich *et al.* (1979), Testart *et al.* (1983) and Templeton (1985) are able to give any indication of the qualitative nature of the concentration changes over the 35 h from the start of the LH surge until the point of ovulation. Even these studies suffer from the disadvantages of either small numbers (Testart *et al.*, 1983) or that the changes follow administration of hCG to mimic the LH surge, rather than the spontaneous LH surge itself (Templeton, 1985). Firstly, it is not clear whether hCG can in fact mimic exactly all of the follicular changes induced by a spontaneous LH surge and, secondly, hCG given in this way to assist timing of ovulatory events must precede the spontaneous LH surge and therefore the follicles are being exposed to hCG at an earlier time than they would have been exposed to endogenous LH. Such differences may be irrelevant from the point of view of subsequent oocyte recovery for in-vitro fertilization, but they will undoubtedly distort the natural sequence of events that would occur around the start of a spontaneous surge.

The qualitative changes in the concentrations of oestradiol and progesterone from the spontaneous follicles in our study are shown in Fig. 1 while mean concentrations are given in Table 1. As already stated, each follicular fluid sample was allocated to an age group, based on the time from the onset of the LH surge. Within groups however, they have been ranked in order of descending oestradiol concentrations.

Oestradiol concentrations in the late preovulatory follicle just before or about the time of the LH surge are slightly higher than found by others (Bomsel-Helmreich *et al.*, 1979; Testart *et al.*, 1983; Templeton, 1985) and then fall slowly during the next 15 h, more rapidly between 15 and 30 h, and then more gradually again as the follicle ages. Concentrations recorded in the 'post-ovulatory' follicles (> 40 h) are lower than the lowest concentrations recorded by others, but without indomethacin blockade it is extremely likely that these follicles would already have ovulated and thus would not have been measurable in other studies. From the work of Bomsel-Helmreich *et al.* (1979), it is clear that the large oestrogenic follicle is the healthy preovulatory follicle and the one that is destined to ovulate.

Progesterone concentrations in the early follicles before the LH surge are of the same order of magnitude as oestradiol concentrations at this time (Table 1), and this again confirms the work of others (Bomsel-Helmreich *et al.*, 1979; Templeton, 1985). However, within a very short time of the start of the LH surge, progesterone concentrations rise rapidly and continue to do so for about the first 25 h. At this point the rate of increase slows down, and concentrations seem to plateau at around 10 000–12 000 ng/ml. This would be the time at which aspiration for in-vitro fertilization

Fig. 1. Individual concentrations of oestradiol and progesterone in 44 follicular fluids obtained before or at different times after the start of the spontaneous LH surge (see text). The follicular fluids have been grouped into 7 age bands, but within each group fluids have been arbitrarily ranked in order of descending oestradiol concentrations. Throughout these and subsequent figures, the order of the follicular fluids is the same. Follicles aspirated at >40 h from the start of the surge were those for which ovulation had failed to occur (indomethacin blockade in 3 and spontaneous luteinized unruptured follicle in 1). All follicular fluids were obtained during natural, unstimulated cycles.

would ideally take place and these progesterone concentrations are very similar to those recorded at this time by others (18 500 ng/ml: Edwards *et al.*, 1980; 12 000 ng/ml: Carson *et al.*, 1982; 10 000 ng/ml: Testart *et al.*, 1983; 9400 ng/ml: Templeton, 1985).

Although the number of 'post-ovulatory' follicles was small, it would seem that, in these follicles, progesterone concentrations have increased sharply (to 17 500 ng/ml), although oestradiol concentrations have only fallen slightly. It may be of significance that plasma progesterone concentrations have also been shown to undergo a sudden increase at this time (Hoff *et al.*, 1983; Djahanbakhch *et al.*, 1984). These changes in circulating progesterone concentration have been taken to indicate ovulation, but if they are related to the late increase in progesterone synthesis seen in the 'post-ovulatory' follicle, then it is more likely that they reflect a time-dependent change in progesterone synthesis rather than follicular rupture *per se*. In view of the accelerating change in the oestrogen:progesterone ratio to which an oocyte retained within these follicles will be exposed, it will be of great interest to study the fertility potential of oocytes recovered from late or 'post-ovulatory' follicles, particularly as luteinized unruptured follicles are not uncommon in infertile women.

Table 1. Mean concentrations ($\pm$ s.e.m.) of selected steroids and peptides in peripheral plasma, and in follicular fluid before and at intervals after the start of the spontaneous LH surge: all follicles were obtained during unstimulated cycles

	Before LH surge (n = 8)	Hours from LH surge					Average mid-cycle peripheral plasma conc.
		0–10 h (n = 8)	10–20 h (n = 8)	20–30 h (n = 8)	30–40 h (n = 16)	> 40 h* (n = 4)	
<i>Steroids</i>							
Androstenedione (ng/ml)	1280 ± 16	190 ± 40	180 ± 30	200 ± 50	170 ± 22	150 ± 40	6.0
Testosterone (ng/ml)	110 ± 10	45 ± 4	52 ± 12	39 ± 4	28 ± 2	37 ± 8	0.6
Oestradiol (ng/ml)	3730 ± 360	3580 ± 270	2790 ± 230	1450 ± 80	810 ± 45	450 ± 60	0.3
Progesterone (ng/ml)	2900 ± 380	5410 ± 710	8650 ± 260	9880 ± 580	12200 ± 530	17400 ± 350	1.0
<i>Peptides</i>							
LH (U/l)	2.4 ± 0.4	2.4 ± 0.4	7.6 ± 1.3	15.2 ± 1.5	13.6 ± 1.1	7.4 ± 0.5	38.0
FSH (U/l)	1.8 ± 0.3	2.0 ± 0.3	3.3 ± 0.5	4.9 ± 0.3	6.4 ± 0.6	8.1 ± 2.4	12.5
Inhibin (U/mg)	9.9 ± 1.3	13.5 ± 2.5	11.3 ± 1.3	9.1 ± 0.8	9.3 ± 1.0	6.9 ± 2.3	—
Prolactin (U/l)	390 ± 30	280 ± 13	360 ± 30	330 ± 20	390 ± 32	400 ± 6.0	180
<i>cAMP</i>							
Mean conc. (nmol/l)	61 ± 12	50 ± 8	33 ± 15	14.5 ± 5.0	10.6 ± 4.0	5.8 ± 0.7	
Follicles with detectable levels (%)	100	100	50	38	19	12	

*Ovulation (follicular rupture) was artificially delayed (by giving indomethacin) in 3 out of the 4 women whose follicular fluids were not recovered until > 40 h from the start of the spontaneous LH surge.

Finally, it should be noted that the magnitude of the changes in progesterone synthesis are much greater than those shown by oestradiol. From a biological point of view it becomes important to consider the following questions. (i) Do the falling oestradiol concentrations reflect metabolism, sequestration or secretion into the general circulation? (ii) What is the function of the relatively enormous (compared with the amounts required by the secretory endometrium or implicated in LH feedback in the luteal phase) progesterone concentrations? (iii) Are there obvious sequelae for the follicle cells exposed to these large steroid levels? For example is progesterone free in follicular fluid or bound (see Fleming & McGaughey, 1982)? (iv) At follicular rupture does the 50 μ g or so of progesterone and 5 μ g of oestradiol (assuming a follicle with a volume of 5 ml) that is suddenly released into the peritoneal cavity have any physiological function?

Androstenedione and testosterone

Androstenedione, testosterone or total androgen concentrations in human follicular fluids have been described (Edwards *et al.*, 1980; Brailly *et al.*, 1981; Hillier *et al.*, 1981; Carson *et al.*, 1982; Testart *et al.*, 1983; Templeton, 1985; Westergaard *et al.*, 1986) but there is little uniformity in the actual concentrations recorded, nor is there a consensus on the qualitative changes to be expected after the LH surge (see Westergaard *et al.*, 1986). In general it is considered that all small follicles are androgenic regardless of whether they are destined to ovulate or become atretic. Since granulosa cell number and proliferation rates are variable amongst small androgenic follicles it must be assumed that their intrafollicular milieu reflects thecal cell steroid synthesis. However, the two-cell theory of follicular steroidogenesis dictates that FSH acting on the granulosa cells of small follicles is able to stimulate aromatization of thecal-derived androgens to oestrogens. FSH and oestradiol within the follicle synergize to promote granulosa cell proliferation and thus more oestradiol production. High oestrogen concentrations co-existing with low androgen concentrations are considered to reflect healthy follicles (McNatty, 1982). Androgen synthesis by the theca interna is

Fig. 2. Individual concentrations of androstenedione and testosterone in 44 follicular fluids obtained before or at different times after the start of the spontaneous LH surge. (See legend of Fig. 1 for details.)

thought to be regulated by LH and even though the exact sequence of change at the time of the LH surge is not well established, the end result is a change in the status of the follicle from oestrogenic to progestagenic.

Analysis of the follicular fluids from the present series for both androstenedione and testosterone gave the results shown in Fig. 2 and Table 1. Having established that all of the follicles in this series were oestrogenic (and therefore presumably healthy as opposed to atretic) it is difficult to reconcile our finding of very high androstenedione values before the LH surge with the data of others. Apart from McNatty *et al.* (1979a) and Westergaard *et al.* (1986) who reported mean androstenedione concentrations of 692–1050 ng/ml in healthy preovulatory follicles in the late follicular phase, and in selected highly oestrogenic follicles from the series of Hillier *et al.* (1981) and Westergaard *et al.* (1984), other groups consider mean androstenedione concentrations of 268 ng/ml (Brailly *et al.*, 1981) and 321 ng/ml (Templeton, 1985) appropriate for follicles not yet exposed to LH-hCG. After an endogenous surge or hCG injection, androstenedione concentrations fall to a mean of 68 ng/ml (Brailly *et al.*, 1981), 95 ng/ml (Templeton, 1985) or 28 ng/ml (Edwards *et al.*, 1980). In hyperstimulated women, concentrations after the post-ovulatory stimulus were clearly higher at 300 ng/ml (Carson *et al.*, 1982) and 500 ng/ml (Kerin *et al.*, 1985).

Aside from the quantitative differences between the data presented here and those in some other reports, it is also important to consider the qualitative changes. Assuming that the data on the follicular oestradiol and progesterone concentrations (Fig. 1) correctly indicate active preovulatory follicles, as was suggested from the plasma LH and oestradiol measurements made to establish the stage of the cycle, then the clearly elevated androstenedione concentrations before the LH surge which declined rapidly with the start of the surge must have some biological significance. It is reassuring that similar changes are seen in intrafollicular testosterone concentrations, although, as would be expected for a steroid that is further down the synthetic pathway, the changes are not so acute. The changes in follicular oestradiol (Fig. 1) are seen to be lagging a few hours behind those of

testosterone, again supporting the concept of a steroid cascade moving from cholesterol through progesterone to androstenedione ($\pm$ testosterone) within the theca. The androgen is then transferred to the granulosa in order to complete the synthetic pathway, androstenedione to testosterone to oestradiol.

It should be noted that intrafollicular concentrations of androstenedione and testosterone are much lower than those of oestradiol. This supports the concept that they are merely intermediary in the pathway to oestradiol. If the dramatic decline in androstenedione shown here can be verified (and see Dieleman *et al.*, 1983a, for the cow), then the implications for the control of the switch in follicular biosynthesis from oestrogens to progesterone are numerous. It is already established that even after natural cessation of oestrogen synthesis, granulosa cells still have the ability to secrete the steroid provided they are supplied with androgen substrate (Hillier *et al.*, 1981). It would therefore appear that the regulatory system does not reside at the level of aromatase (i.e. the conversion of testosterone to oestradiol). By implication then, it is the depletion of utilizable substrate that causes the decline in follicular oestradiol secretion. From our data it would seem that the depletion of androgen is a rapid and early event and this tends to suggest that it is regulated within the thecal cell layer rather than within the follicle by the granulosa cell (Dieleman *et al.*, 1983a). There are a number of possibilities such as an LH-induced inhibition of androstenedione synthesis or alternatively a blockade of androstenedione transport across the basement membrane. This latter possibility seems quite likely as plasma androstenedione (and testosterone) concentrations decline rather more slowly but within same sequence androstenedione–testosterone–oestradiol than do intrafollicular levels (Djahanabakhch *et al.*, 1984; Hoff *et al.*, 1983). McNatty *et al.* (1979b) and Channing (1980) amongst others consider that the major portion of the peripheral plasma oestradiol concentration is a consequence of thecal cell and not granulosa cell synthesis and secretion.

Prolactin concentrations

Little has been written on the concentration of prolactin in follicular fluid. McNatty *et al.* (1975) observed that prolactin was detectable in almost all follicular fluids (unlike FSH or LH) and that concentrations were significantly lower in the late follicular phase compared with all other stages of the cycle. Concentrations were, however, similar to expected plasma concentrations (although not to the actual plasma concentrations measured which were obtained during the stress of surgery). Cooke *et al.* (1983) observed that prolactin concentrations in follicular fluids from the late follicular phase were somewhat higher than equivalent preoperative plasma concentrations but correlated closely with the plasma levels ($r = 0.85$, $P < 0.001$, $n = 22$). In the present study different reagents (utilizing a monoclonal antibody) were used to measure prolactin. Concentrations in this assay are lower than those obtained with more familiar radioimmunoassays, but prolactin concentrations in follicular fluid were easily detectable (Fig. 3) and were relatively constant over the period of the LH surge. These concentrations are about twice the normal plasma concentrations at that time of the cycle (Table 1), which suggests some form of selective uptake or concentration of prolactin within the follicle. It is unlikely that there is an intrafollicular source of prolactin since follicular fluids from patients treated with bromocriptine (Parlodel: Sandoz Products Ltd, Feltham, Middlesex TW13 4EP, U.K.) have undetectable follicular fluid prolactin concentrations (Cooke *et al.*, 1983).

FSH concentrations

FSH is considered to be of crucial importance in the process of recruitment and selection of the dominant follicle due to its ability to stimulate aromatase activity in the granulosa cell which converts the follicular microenvironment from androgenic to oestrogenic (Westergaard *et al.*, 1986). Furthermore, FSH is capable of stimulating aromatase activity *in vitro*, even after the LH

Fig. 3. Individual concentrations of prolactin, FSH and LH in 44 follicular fluids obtained before or at different times after the start of the spontaneous LH surge. (See legend of Fig. 1 for details.)

surge (Hillier *et al.*, 1981). Despite these observations few studies have measured FSH concentrations directly (McNatty *et al.*, 1979a, 1979b; Testart *et al.*, 1983; Templeton, 1985) but when this has been done concentrations were found to be low (relative to plasma levels) even several hours after the start of the LH/hCG surge.

Recent availability of monoclonal antibody based FSH assays with improved sensitivity (0.4 u/l) has prompted us to reconsider the qualitative and quantitative uptake of FSH by the follicle (Fig. 3; Table 1). Mean concentrations are low before and for some 10 h after the start of the LH surge, but then a gradual increase occurs such that by the time of expected ovulation, intrafollicular levels are about 50% of plasma values. These values agree closely with those of Testart *et al.* (1983) after a natural LH/FSH surge, and Templeton (1985) in whose study hCG was given instead of allowing a natural surge when it might be expected that endogenous peripheral FSH concentrations would not be elevated. In the earlier studies of McNatty *et al.* (1975) the sensitivity of the assays used was much lower (about 1.3 mU/ml), but despite this FSH was detectable in a proportion of follicles examined, and the mean levels recorded are almost indistinguishable from the current ones once allowance is made for inclusion of undetectable samples in their group means.

LH concentrations

LH concentrations in the follicles of the present study are shown in Fig. 3 and Table 1. The qualitative change across the LH surge is similar to FSH, and whilst concentrations are initially quite low, values start rising between 10 and 20 h to peak at 20–30 h. Plasma LH concentrations are also maximal at about this time (Djahanbakhch *et al.*, 1984) although follicular fluid levels only ever attain about 40% of plasma levels. Despite the use here of a highly sensitive LH assay (0.4 u/l) no immediate increase in follicular fluid LH was seen at the start of the LH surge. In fact levels at this time were uniformly low as described by others (McNatty *et al.*, 1975; Testart *et al.*, 1983).

An interesting aspect to the appearance of LH within the follicle is the observation that, from about 20 h after the LH surge, the basement membrane of the follicle starts to deteriorate (Bomsel-Helmreich *et al.*, 1979) and morphological and functional luteinization becomes apparent (Dieleman *et al.*, 1983b). It is at about this same time that LH (and FSH) start to appear within the follicle, and it is also the time when oestradiol concentrations are declining most rapidly. Perhaps, as suggested by Carson *et al.* (1986), entry of LH into the follicle is normally restricted, and it is only following an ovulatory dose of hCG (or LH) that follicular permeability is significantly increased and follicular LH and FSH concentrations rise gradually towards but never reaching the concentrations outside the follicle (Dieleman *et al.*, 1983a).

Relationship between FSH and inhibin

The concentration of inhibin compared with the equivalent concentration of FSH in the 44 follicular fluids is shown in Fig. 4. Inhibin concentrations are highest in the follicles shortly after the start of the LH surge and then decline slightly. Although peak inhibin concentrations are seen at a time when oestradiol is maximal (McLachlan *et al.*, 1986b), suggesting a granulosa cell source, the relative decline in the two compounds is quite dissimilar. However, over the period when inhibin concentrations are declining, intrafollicular FSH concentrations are rising slowly such that there is an inverse relationship between these two peptides (although the present data show this relationship rather poorly, a larger series of follicular fluids analysed for both hormones did give a significant inverse correlation: E. A. Lenton and R. I. McLachlan, unpublished observations).

Recent developments in understanding on inhibin physiology have suggested that the β -subunits of inhibin A and inhibin B combine to form β -dimers (FRP and activin) (Vale *et al.*, 1986; Ling *et al.*, 1986) which are active in stimulating the biosynthesis and release of FSH from pituitary cells in culture. The inhibin radioimmunoassay used here employed an antibody against inhibin of M_r 58 000 and showed < 5% cross-reactivity with bovine FRP. Furthermore, free α - and β -subunits showed no cross-reactivity in the assay. Consequently it is unlikely that the inhibin detected in follicular fluid could represent interfering β -dimers or free α -subunit which, in the cow, is not found in follicular fluid (Miyamoto *et al.*, 1986).

Some indirect evidence for the presence of β -dimers in these follicular fluids was, however, obtained when an attempt was made to confirm the radioimmunoassay concentrations by bioassay. After charcoal–dextran extraction (to remove the high amounts of steroid present) radioimmunoassayable inhibin concentrations also fell, suggesting that inhibin was being absorbed by the charcoal. In contrast, bioassayable concentrations paradoxically appeared to rise, suggesting that overall inhibin/ β -dimer ratio had been modified by charcoal extraction. For this reason no discussion of the function of inhibin in follicular fluid, or its relationship to FSH, will be complete until an assay is also available to measure the β -dimer concentrations.

Relationship between LH and progesterone

Whereas the decrease in oestradiol concentration in follicular fluid has been attributed to changes in aromatase activity or, the explanation more currently favoured, to depletion of androgen substrate, it has been tentatively assumed that the increase in progesterone was a consequence

Fig. 4. Individual concentrations of inhibin in 44 follicular fluids related to simultaneous FSH concentrations obtained before or at different times after the start of the spontaneous LH surge. (See legend of Fig. 1 for details.)

of direct LH stimulation. From a visual comparison of the progesterone profile (Fig. 1) and the LH profile (Fig. 3) and remembering that these are exactly the same follicular fluids, it is obvious that the changes in progesterone are well advanced before any LH is detectable in the follicular fluid. Other explanations must therefore be sought. It is possible that LH does not reach the follicular fluid until uptake by the granulosa cell layer is saturated (Dieleman *et al.* (1983a). Whilst remaining a possibility, this seems somewhat unlikely since most small proteins that can easily enter the follicle are found in significant concentrations in follicular fluid (Anderson *et al.*, 1976). Another possibility is that testosterone or oestradiol can selectively inhibit the intrafollicular conversion of cholesterol to progesterone (Batta *et al.*, 1980; Veldhuis *et al.*, 1983). For such a mechanism to regulate progesterone secretion, testosterone and/or oestradiol concentrations would need to decline before and not contemporaneously with the increase in progesterone. Although it is clear that there is an inverse relationship between testosterone and possibly oestradiol, and progesterone (Figs 1 & 2), this mechanism appears insufficiently precise to account solely for the increase in progesterone associated with the onset of the LH surge.

One further possibility (and there may well be numerous others) that has been examined in the present study is the relationship between changes in follicular hormone concentrations and cAMP (see Moor *et al.*, 1975).

Cyclic AMP in follicular fluid

cAMP has been postulated as the second messenger involved in the transmission of LH-mediated events (see review by Marsh, 1976). Certainly the morphological changes associated with luteinization in the granulosa cells can be induced by cAMP (Soto *et al.*, 1986). In many species cAMP has also been considered to have a role in oocyte maturation (see later discussion). Follicular fluid cAMP concentrations have been measured after hyperstimulation for in-vitro fertilization and significantly lower values are found in follicles yielding fertilized or cleaved oocytes than in those oocytes which did neither (Tarlitzis *et al.*, 1985). Apart from these data little appears to be known about in-vivo concentrations of cAMP in human follicles.

Fig. 5. Individual concentrations of cAMP in 44 follicular fluids related to simultaneous FSH concentrations obtained before or at different times after the start of the spontaneous LH surge. (See legend of Fig. 1 for details.)

cAMP was measured in all the follicles in the present study as shown in Fig. 5 and Table 1 but was only detected (at a concentration > 5 pmol/ml) in some of the fluids. It appeared that the percentage of follicles with detectable levels was related to the time since the onset of the LH surge. In the early follicles (up to 10 h from the LH surge) 100% of the fluids had measurable cAMP concentrations. This figure declined to 50% (between 10 and 20 h), to 38% (between 20 and 30 h) and to 19% (between 30 and 40 h). Mean concentrations also declined (Table 1) although the degree may well have been underestimated since all fluids in which cAMP was not detectable were assumed to have a concentration of 5 pmol/ml for the purpose of calculating the mean.

The marked change in intrafollicular cAMP concentrations from high levels present in all follicles even before the start of the LH surge to low/undetectable concentrations in the majority of follicles older than 20 h was unexpected, but seems too clear to be fortuitous. The reason for the progressive reduction in cAMP in older follicles is presently unknown although it could be due to an increase in phosphodiesterase activity. There was no phosphodiesterase activity present in an early follicular fluid, but a systematic check on follicles of all ages has not yet been performed.

The relationship of cAMP to LH

As mentioned above, cAMP is thought to mediate the actions of LH. Certainly ovarian levels of cAMP rise rapidly in response to hCG (Dennefors *et al.*, 1982). In the rabbit this response appeared to be biphasic (Goff & Major, 1975) but even so concentrations had fallen markedly by 8 h (ovulation in the rabbit occurs at 10–12 h). Comparison of the qualitative changes in cAMP and LH shown here (Figs 3 & 5) suggests an inverse relationship between the two compounds. However, this would not be surprising if cAMP was indeed the second messenger, transmitting the LH stimulus to the granulosa cells within the follicle shortly after the start of the LH surge. An essential part of this hypothesis would be that cAMP should be secreted by the thecal cells and then rapidly move across the basement membrane to reach the granulosa cells (and of course the oocyte and cumulus cells).

There is good evidence that theca cells respond more directly to LH/hCG with secretion of cAMP than do granulosa cells (Dennefors *et al.*, 1982; Fletcher *et al.*, 1983) and indeed that any cAMP detected in intact follicles in culture is a result of transmission from the thecal compartment (Weiss *et al.*, 1976; Fletcher *et al.*, 1983). Degradation of secreted cAMP is presumed to occur (by membrane-bound phosphodiesterase) but it is postulated (Moor & Seamark, 1986) that sufficient cAMP may remain to stimulate granulosa cells to release more cAMP which serves to amplify the signal. The data presented here strongly support a thecal derivation of secretion; (i) magnitudes of the follicular changes are similar to those of androstenedione and testosterone, which mainly derive from the theca; (ii) the rapidity of the changes precedes any intrafollicular alteration in LH; and (iii) there is evidence that thecal cAMP production becomes refractory to continued stimulation by

hCG (Dennefors *et al.*, 1983) in the same way that thecal androstenedione and testosterone synthesis are terminated after the LH surge. The remarkable similarity between the intrafollicular profiles of testosterone and cAMP (Figs 2 & 5) reinforces the impression that both compounds are produced by the same source (thecal cells), responding to the same signal (LH) and are following a similar time dependent course (see Moor *et al.*, 1975).

The endocrine environment and oocyte maturation

Briefly, oocyte maturation can be considered to consist of three separate and independently regulated processes, namely nuclear, cytoplasmic and cumulus maturation. These components of the maturation process have been extensively reviewed elsewhere (McGaughey, 1983; Tsafirri, 1985; Yoshimura & Wallach, 1987) but discussion of the endocrine environment of the oocyte would be incomplete without an attempt to set the above endocrine changes in the context of meiotic maturation.

There are subtle differences in the regulation of oocyte maturation in amphibians and most domestic species compared with man, not least of which is the timing of various aspects of maturation. In the human these events are, for nuclear maturation; approximately 25 h for germinal vesicle breakdown (GVBD), 28–30 h for extrusion of the first polar body and 32 h for arrest in metaphase II; for cytoplasmic maturation; approximately 30–40 h for migration of cortical granules within the oocyte and for cumulus maturation; approximately 25 h for expansion and 25–30 h for breakdown of intercellular communications. Crude though these timings are, they do illustrate two important features, the first is that the earliest visible signs of oocyte maturation are not perceived until about 25 h, and secondly that all aspects of maturation are proceeding independently but within a closely similar time course. Thus any intrafollicular signals associated with the initiation of maturation would need to be present by 25 h. In fact by this time oestradiol concentrations have already fallen markedly and progesterone concentrations have plateaued at a high level, and so the oestradiol:progesterone ratio which, before the LH surge, was approximately 1:1 will by now have reached 1:15. Cytoplasmic maturation is considered to be a consequence of changes in steroid balance within the follicle (Osborn & Moor, 1983) which are indirectly attributable to the LH surge.

From about 25 h, FSH begins to enter the follicle in increasing amounts. FSH suppresses intercellular coupling (Moor *et al.*, 1981) and is thought to promote the secretion of glycosaminoglycans (GAGs) from granulosa cells (Yanagishita *et al.*, 1981). These proteins are probably involved in maturation of the cumulus.

cAMP has long been considered to be a likely candidate as the second messenger, or distant activator for LH (Moor & Seamark, 1986) but equally it is well known that high intra-oocyte cAMP levels inhibit resumption of meiosis (Eppig & Downs, 1984). It has been assumed that the oocyte takes up cAMP from its surrounding cumulus cells, and therefore with advancing cumulus maturation intercellular communications decline, allowing intra-oocyte cAMP concentrations to fall and nuclear maturation to commence (Dekel & Beers, 1978). Unfortunately for this hypothesis, nuclear maturation in the sheep (Moor & Heslop, 1981) occurs even when cAMP levels are high and before signs of breakdown in intercellular communication. Furthermore, GVBD in the sheep follicle is not inhibited by cholera toxin (a potent stimulator of cAMP) until concentrations become so high that there is a direct toxic effect (Crosby *et al.*, 1985). An alternative hypothesis is that cAMP stimulates production of a maturation inhibitor from cumulus cells, and it is the inhibitor rather than cAMP which is transferred to the oocyte (Freter & Schultz, 1984).

Whether or not a reduction in oocyte cAMP is obligatory for the resumption of meiosis in mammals, it appears, at least in the human, that follicular fluid cAMP levels decline spontaneously several hours before the expected time of GVBD. Whether this decline is due to degradation (by phosphodiesterase), efflux from an increasingly 'leaky' follicle or desensitization (down regulation) of synthesis and production, as in the case of testosterone, is not known. In any event, it seems

likely that nuclear maturation starts in response to a release from inhibition, such as when oocytes are removed prematurely from the follicle following a minimum but mandatory period within the follicle (Eppig, 1978). Factors associated with inhibition may be numerous and synergistic (e.g. OMI or MIS: Westergaard *et al.*, 1984; Winer-Sorgen *et al.*, 1986; hypoxanthine: Downs & Eppig, 1986) and of course both cAMP and testosterone itself have been implicated (Rice & McGaughey, 1981; Racowsky, 1983; Yoshimura & Wallach, 1987).

Conclusions

The present discussion of some of the endocrine changes occurring within the follicle over the last 40 h before ovulation is far from comprehensive. For example, no data on the important prostaglandin changes have been included. However, it is hoped that the approach described here, whereby the start of the LH surge is assessed by frequent urine or plasma measurements, and this assessment of the 'age' of the follicle corroborated by the relative ratio of oestradiol to progesterone concentrations, will serve as a useful model in which to assess the dynamic equilibrium existing within the human follicle. Information obtained using this model should allow the components of the intrafollicular milieu to be categorized with respect to their functional role within the follicle. For example, some compounds such as collagenase may have primarily a facilitatory role whilst others such as cAMP may have a direct signalling role. The chronological changes in these compounds could then in turn be correlated with morphological and functional changes in the granulosa, basement membrane, and most importantly, the oocyte itself. Eventually it may be possible to go further and locate the biochemical abnormality in specific types of infertility (e.g. the luteinized unruptured follicle syndrome) or when the oocyte fails to undergo normal embryo development, with the ultimate goal of providing rational methods of treatment for these conditions.

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