Role of gonadotrophins and ovarian steroids in the development of mouse follicles *in vitro*

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A whole follicle culture system has been used to investigate the actions of gonadotrophic hormones, oestrogen and progesterone in the regulation of follicular development and steroidogenesis. Recombinant human FSH was required for the growth of preantral follicles and for Graafian morphogenesis, whereas recombinant LH was ineffective. While pure FSH was sufficient for growth and morphogenesis, production of oestrogen was greater when androstenedione or LH was present in combination with FSH, confirming that there is a two-cell mechanism for oestradiol production in the mouse follicle. When an antiserum to oestrogen or to progesterone or an oestrogen receptor antagonist were added to the culture medium, there was no significant effect on either follicular growth or oestradiol production. Thus, physiological concentrations of oestradiol are not needed for follicle development, although a role cannot be completely ruled out. In conclusion, the obligatory role of FSH was demonstrated. It appears to be sufficient for follicle development even in the absence of LH, and the paracrine or autocrine effects of oestradiol and progesterone, if any, appear to be minor in the mouse ovary.

Introduction

Ovarian follicles consist of layers of granulosa and thecal cells enveloping a central oocyte and, as such, represent the fundamental unit for ovarian function, namely, the production of eggs and hormones. When cultured whole, follicles are able to develop and produce oestradiol in a manner that closely resembles growth and secretion *in vivo* (Gosden *et al.*, 1993) and are able to produce fertile oocytes (Spears *et al.*, 1994). Culture of whole follicles provides an opportunity to investigate the actions of gonadotrophic hormones and the role of paracrine and autocrine factors at each stage in a controlled environment. The opportunity to investigate follicular biology *in vitro* has been taken in this study to explore the value of this model for investigating gonadotrophin requirements for growth and to gain new information about the follicular endocrinology of an important laboratory species: the mouse.

Much of our current understanding of follicle biology rests on studies of rat ovaries, either *in vivo* or using isolated follicular cells. It has become clear that there is a two-cell mechanism for oestrogen biosynthesis in which neither the granulosa cells nor the theca cells are independently capable of producing normal amounts of oestrogen (Dorrington *et al.*, 1975; Armstrong *et al.*, 1979). The gonadotrophic hormones, LH and FSH, have complementary effects: LH stimulates the production of androgens by theca cells which are aromatized in the granulosa layer under the influence of FSH. This model

holds in a number of species, although exceptions are known in which cell autonomous production of oestradiol occurs, such as in the pig (Evans *et al.*, 1981), which makes it important to clarify the biology of such an important experimental model as the mouse.

This paper reports the actions of pure gonadotrophins on follicles growing *in vitro*. It also tests the hypothesis that FSH alone is sufficient to support follicle growth, whereas LH is needed to stimulate maximal oestradiol output. This study also investigated steroid action on follicle growth and confirmed earlier assumptions about the role of progesterone, while throwing doubt on the role of oestrogen as a paracrine mediator.

Materials and Methods

Animals

Twenty-four-day-old C57BL/6 × CBA/Ca F₁ hybrid, female mice were housed in a temperature- and light-controlled room on a 14 h light:10 h dark photoperiod and were provided with food and water *ad libitum*. Female hypogonadal mice (*hpg/hpg*) produced by mating heterozygous parents were selected on the basis of their phenotype at postpubertal ages and were maintained under similar conditions.

Isolation and culture of follicles

The mice were killed by cervical dislocation and their ovaries were removed to watchglasses containing Leibovitz L-15

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medium (Gibco-BRL, Irvine) and 3 mg BSA ml⁻¹ (Fraction V, Sigma, Poole) at 37°C. Preantral mouse follicles were isolated by microdissection at a diameter of 185 ± 15 µm using fine needles and pipetted individually into 96-V-well microtitre plates (Bibby-Sterilin Ltd, Stone) in 20 µl of α -minimal essential medium (Gibco-BRL, Renfrew) under 50 µl mineral oil, according to the method of Boland *et al.* (1993), and based on an earlier method developed by Nayudu and Osborn (1992). The medium was supplemented with 10 µg human transferrin ml⁻¹ (Sigma) and 5% serum from hypogonadal mice. Gonadotrophins were added to some of the wells at a concentration of either 1 or 5 iu ml⁻¹. These were either human pituitary FSH (NIH-FSH, National Hormone and Pituitary Program, Bethesda, MD), which contained approximately 1% (w/v) LH as an impurity, pure recombinant FSH (rFSH) or recombinant LH (rLH) (Serono Laboratories, Geneva). Follicles were transferred to new wells with fresh media each day: media from the previous day were then frozen for future measurement of oestradiol. No attempts were made to stimulate ovulation with LH, although this culture system is known to allow the development of potentially fertile oocytes (Boland *et al.*, 1993; Spears *et al.*, 1994).

Measurement of oestradiol

After follicle transfer, a sample of medium from the incubation of the previous day was removed from each well (see above), frozen, and later analysed immunoenzymatically for oestradiol using an ELISA method (Serono Diagnostics, Woking). The inter- and intra-assay coefficients of variation were both ≤ 5% and the sensitivity was ≤ 18 pmol l⁻¹.

Follicle growth and morphology

Follicle growth was monitored by measuring follicle diameter on each day of culture using a precalibrated ocular micrometer at × 40 magnification. Data presented include measurements from all follicles still viable on the final day of culture. About 20% of follicles burst or became atretic, usually early in the culture period (probably due to damage on the day of dissection). Measurements from such follicles were not included in the analyses. No differences were found among the percentages of such follicles across treatments.

In Expts 1, 3 and 4, the DNA accumulation by follicles was also assessed using a fluorescent dye, Hoechst 33258 (bisbenzimidazole; Sigma), by measurement in a microtitre plate fluorometer (Dynatech, Billingham) (Boland and Gosden, 1994).

Preovulatory follicles were classified as having (i) a diameter ≥ 400 µm; (ii) a large antral cavity; (iii) an eccentrically placed oocyte-cumulus complex; (iv) no visible signs of atresia (that is, dark patches of dead granulosa cells).

Experiment 1: DNA content of developing follicles

Forty-four follicles were dissected for each experiment, four of which were frozen immediately and stored at -20°C for DNA analysis. The remaining 40 follicles were cultured in control medium as described above (day 0). On each successive

day of culture (days 1–5), four representative, non-atretic follicles were also frozen for DNA analysis.

Experiment 2: effects of gonadotrophic hormones on follicle growth and steroidogenesis

Follicles were cultured in media containing either human pituitary FSH (NIH-FSH, with 1% (w/v) LH contamination), or rFSH or lacking FSH. The effects of culturing follicles in NIH-FSH (1 iu ml⁻¹) and rFSH (5 iu ml⁻¹) were compared (Expt 2a). Follicles were then cultured in the presence of NIH-FSH, rLH only (1 or 5 iu ml⁻¹) or in the absence of gonadotrophins (Expt 2b). Follicles were then cultured with rFSH (1 or 5 iu ml⁻¹), plus or minus supplements of androstenedione (1 µg ml⁻¹; Expt 2c).

Experiment 3: effect of oestrogen on follicle development

Anti-oestrogen antibodies were used to neutralize oestrogens secreted by the cultured follicles. The high titre antiserum was raised by immunizing castrated Suffolk cross-bred sheep with oestrone-6-carboxymethyl-oxime conjugated to egg albumin (Land *et al.*, 1982). No significant crossreactivity of the antibody occurs with either progestagens or androgens (Webb *et al.*, 1985).

In an additional experiment, follicles were cultured with a potent, competitive and highly specific oestrogen receptor antagonist, ICI 162,780 (Wakeling *et al.*, 1991). The molar concentration of antagonist in the medium was approximately 1000 times greater than the concentration of oestradiol accumulated by control follicles in the medium in 24 h (4.95 ± 0.4 nmol l⁻¹).

Fifty preantral follicles were dissected for each experiment. All follicles were cultured with 1 iu NIH-FSH ml⁻¹. In addition, ten follicles were cultured with 3% anti-oestradiol antiserum from day 2 of culture, and another ten follicles were incubated with 5 µmol oestrogen receptor antagonist l⁻¹. A further ten follicles were incubated with FSH and diethylstilbestrol (DES, 1 µg ml⁻¹; Sigma). DES was chosen as it did not crossreact with the antibody but did give a specific oestrogenic effect. As the oestradiol antibody and antagonist were diluted in sheep serum and alcohol, respectively, ten control follicles were cultured with medium containing 3% sheep serum, and another ten follicles served as a vehicle control with 1 µl ethanol ml⁻¹ in addition to the standard supplements. Treatments commenced 24 h after the beginning of culture (day 1); any degenerating follicles were discarded at this time.

Experiment 4: effect of progesterone on follicle development

For this experiment, an anti-progesterone antiserum was used to neutralize progesterone secreted by the cultured follicles. The high titre antiserum was raised in a sheep by injecting progesterone 11 α -hemisuccinyl-BSA (Flint *et al.*, 1974). The crossreactivity of this antibody with either androgens or oestrogens was negligible. Twenty preantral follicles were dissected for each experiment. Ten follicles were

Fig. 1. Changes in the DNA content (□) and diameter (●) of mouse follicles grown from the preantral to preovulatory stage over a period of 5 days *in vitro* in the presence of pituitary FSH.

cultured with 3% antiserum from day 1, and ten were cultured in control medium containing normal sheep serum and the standard supplements.

Histological preparations

Cultured follicles were prepared for examination by light microscopy after fixing overnight in 5% (w/v) paraformaldehyde. Follicles were then embedded in LR White resin (Taab, Aldermaston) and 2 μm sections were cut and stained with haematoxylin and eosin.

Statistical analysis

Each experiment was repeated three times, with the exception of Expt 1 which was performed in duplicate. Probability values (*P*) between the different groups were determined by the unpaired Student's *t* test and analysis of variance.

Results

Experiment 1: DNA content of developing follicles

During the culture period, approximately 80% of the follicles developed from the mid-preantral to large preovulatory stage with a fully expanded antral cavity and without visible signs of atresia. The DNA content of the developing follicles increased linearly 20-fold with increasing follicle diameter over the 5 days of culture ($r = 0.85$; $P < 0.001$) (Fig. 1). In view of the high correlation, follicle diameter alone was used in subsequent experiments for monitoring growth, although DNA content was measured at the end of the culture period in some experiments, to verify that expansion in follicle size reflected an increase in the number of cells.

Experiment 2: effects of gonadotrophic hormones on follicle growth and steroidogenesis

These studies demonstrated the roles of gonadotrophic hormones on follicle growth and steroidogenesis. Pituitary FSH

(1 iu) and rFSH (5 iu) produced almost identical rates of growth (both producing maximal stimulation), although these units are not strictly comparable (Expt 2a; Figs 2 and 3). Indeed, Graafian morphology can be obtained in the presence of 1 iu rFSH ml⁻¹ alone (A. A. Murray, unpublished). Oestradiol production, which was increased by days 4 and 5, was nevertheless substantially and significantly lower with the pure recombinant hormone preparation than with the NIH-FSH which has some degree of LH contamination.

In the absence of gonadotrophins, follicles grew initially at the same rate as in medium containing FSH, but were significantly lagging by day 2 ($P < 0.05$) (Expt 2b; Fig. 4). Three to four days later they were shrunken and presumably atretic. At no time did they form an antral cavity. Follicles grown in rLH only (1 or 5 iu) fared little better since they remained preantral, and hardly changed in size after day 2. Follicles cultured in pituitary FSH had produced substantial amounts of oestradiol by day 5, whereas concentrations remained close to the limits of detection in the other groups (Fig. 4).

Addition of androstenedione to medium containing rFSH produced an increase in follicular growth throughout the culture period ($P < 0.05$). A more marked increase was obtained in respect of oestradiol production compared with unsupplemented medium ($P < 0.01$) (Expt 2c; Fig. 5). The production of oestradiol by control follicles treated with rFSH was, in Expt 2c, much higher than in Expt 2a (Fig. 2), indicating considerable variability between cultures and the necessity to include controls in every experimental replicate.

Experiment 3: effects of anti-oestrogen antiserum and oestrogen receptor antagonist

In contrast with Expt 2, neither the anti-oestrogen antiserum (data not shown) nor the oestrogen receptor antagonist (Fig. 6) affected the rate of follicle growth in Expt 3. In both cases, the DNA contents on day 5 were similar to that of the controls. Supplementation of DES to the medium containing FSH did not affect the final sizes of follicles: $393 \pm 5 \mu\text{m}$ and $381 \pm 7 \mu\text{m}$, for controls and DES-treated follicles, respectively.

Oestradiol production showed the characteristic increase on days 3–5 but the amounts produced per follicle were unaffected by the presence of the oestrogen receptor antagonist (Fig. 6). No measurements of production rates in the presence of anti-oestrogen antiserum were made because of the possibility of antibody interference in the assay.

Experiment 4: effects of anti-progesterone antiserum

Follicles grew at similar rates in medium with or without this antiserum (Fig. 7), and the DNA contents on day 5 did not differ significantly ($655 \pm 22 \text{ ng}$ per follicle and $660 \pm 18 \text{ ng}$ per follicle, respectively). Similar numbers of follicles grew to antral sizes in the medium containing antiserum and they produced comparable amounts of oestradiol as they did in control medium (Fig. 7).

Discussion

Studies of whole follicles isolated *in vitro* demonstrate more clearly than in any other model that the follicle is a

Fig. 2. Comparison of the effects of 5 iu rFSH ml⁻¹ (□) and pituitary 1 iu NIH-FSH ml⁻¹ (◆) on the rates of growth and 24 h production of oestradiol (inset) by mouse follicles grown *in vitro* for 5 days from the preantral stage.

Fig. 3. Photomicrograph of a mouse follicle cultured in rFSH alone. Scale bar represents 100 µm.

developmental unit. The follicle is able to grow, differentiate and produce oestradiol in the absence of other ovarian cell types, provided it is stimulated by FSH in a nutrient medium. These studies confirm and extend a large body of evidence showing a differential role of the gonadotrophins, and demonstrate for the first time that the 'two-cell theory' of oestradiol production applies in the mouse as in several other species (Hillier *et al.*, 1995).

Follicles were unable to develop fully in the absence of FSH and degenerated at the stage when an antrum is incipient, similar to the *in vitro* results of Nayudu and Osborn (1992) and

Hartshorne *et al.* (1994) and to follicle development in transgenic mice lacking FSH (Kumar *et al.*, 1997). LH was unable to stimulate Graafian morphogenesis. Therefore, the culture system provides a useful model for investigating the physiology of the poorly understood process of atresia. When pure FSH was used to stimulate follicle development to the preovulatory stage, only small quantities of oestradiol were produced, compared with control groups in that same experiment in which LH was also present. A high degree of variation in oestradiol concentrations has been found consistently from culture to culture, suggesting that comparisons across cultures

Fig. 4. Comparison of the effects of medium containing FSH (×), 1 iu rLH (□), 5 iu rLH (△) or no gonadotrophins (◆) on the rates of growth and 24 h production of oestradiol (inset) by mouse follicles grown *in vitro* for 5 days from the preantral stage.

Fig. 5. Comparison of the effects of addition of androstenedione with recombinant FSH (□) and recombinant FSH alone (◆) on the rates of growth and 24 h production of oestradiol (inset) by mouse follicles grown *in vitro* for 5 days from the preantral stage.

are of relatively little value: control groups are needed within each experiment for valid comparisons. The oestradiol concentration decreased when follicles cultured in serum from hypogonadal animals were compared with those cultured in serum from hypophysectomized mice (N. Spears, unpublished). This finding suggests either that serum concentrations of LH that are below the limits of detection in hypogonadal mice (H. M. Charlton, personal communication) can stimulate steroidogenesis, or the involvement of other endocrine factors (for example, hypoadrenocorticism in hypophysectomized mice) (Ben-Chetrit, 1996). The results *in vitro* confirmed those

of hypogonadotrophic patients and animals, which have shown that FSH must be combined with LH for the synthesis of normal amounts of follicular oestradiol (Eshkol and Lunenfeld, 1967; Couzinet *et al.*, 1988; Mannaerts *et al.*, 1991, 1994; Karnitis *et al.*, 1994; Schoot *et al.*, 1994; Balasch *et al.*, 1995; Zelinski-Wooten *et al.*, 1995). Cortvrindt *et al.* (1996) have demonstrated the ability of rFSH to support oocyte growth and maturation and oestradiol production from a similar follicle culture but in which the follicular membrane is not kept intact.

The effects of incubation with pituitary FSH suggest that trace quantities of LH are sufficient to stimulate production of

Fig. 6. Variations in the rate of growth and production of oestradiol (inset) by mouse follicles grown *in vitro* for 5 days from the preantral stage with (□) and without (◆) a highly potent and specific oestrogen receptor antagonist (ICI 182,780).

Fig. 7. Variations in the rate of growth and production of oestradiol (inset) by mouse follicles grown *in vitro* for 5 days from the preantral stage with (□) and without (◆) anti-progesterone antibody.

oestradiol, which presumably depends on augmented androgen synthesis in the theca layer. Treatment with LH alone was unable to promote oestradiol production. Paracrine effects may account, in part, for the oestradiol production of follicles cultured in rFSH alone: FSH stimulates inhibin production by granulosa cells which then acts on the thecal cells to stimulate increased LH-induced release of androgens and P450 aromatase

and cytochrome P450 17 α -hydroxylase (Gray *et al.*, 1995, 1996; Hillier *et al.*, 1995; Smyth *et al.*, 1995).

Supplementing the medium with androstenedione increased oestradiol on each of the days tested, but failed to give a proportionate increase on day 5. The weak response of preovulatory follicles may be a sign that aromatization was already maximally stimulated or that the effects of androgens

on oestradiol production vary at different developmental stages (Harlow *et al.*, 1988; Hillier *et al.*, 1988). The better growth obtained by addition of androstenedione to pure FSH indicated a possible stimulatory role for androgens, which was the subject of a separate investigation (Murray *et al.*, 1997).

In theory, the production of progesterone by granulosa cells could provide an additional loop in the process of steroidogenesis. For example, antibodies to this hormone inhibited oestrogen synthesis when granulosa and theca cells were co-cultured, presumably because progesterone had been converted to androgens in the theca (Lui and Hsueh, 1986). The present results provided no evidence that this hypothetical mechanism plays any physiological role, as follicle growth was unaffected when the addition of progesterone antibody substantially reduced the amount of progesterone available. This should not be surprising, as growing follicles produced undetectable amounts of progesterone in this culture system until the preovulatory stage (N. Spears, A. A. Murray, V. Allison, N. I. Boland and R.G. Gosden, unpublished), similar to the situation found *in vivo* before the mid-cycle LH surge (Zelevnik and Resko, 1980).

The ability of ovarian steroids to influence the development of follicles through paracrine or autocrine mechanisms is controversial, and immunoneutralization of follicular steroids or competitive inhibition of oestrogen binding to their receptor in culture provides an opportunity to test this theory. Clearly, large doses of oestrogens can stimulate follicular growth in hypophysectomized rats (Williams, 1940; Richards, 1980; Hsueh, 1986). Furthermore, disruption of the oestradiol receptor by gene targeting produces a dearth of antral follicles and anovulation in homozygous mice (Lubahn *et al.*, 1993), although it is not clear to what extent this effect is due to intraovarian effects. However, there is experimental evidence *in vivo* and *in vitro* that high concentrations of oestrogen are not mandatory for preovulatory development in some species (Schoot *et al.*, 1992; Mannaerts *et al.*, 1994; Boland and Gosden, 1994; Zelinski-Wooten *et al.*, 1994), while in others, inhibitory effects of oestrogen have been described (Dierschke *et al.*, 1994). In the presence of either anti-oestrogen antibodies or a highly potent and reversible receptor antagonist (Wakeling *et al.*, 1991), follicles grew at a normal rate *in vitro* and appeared morphologically indistinguishable from controls. The antagonist did not affect the production of follicular oestradiol, which appears to rule out any major self-priming effect on aromatization or cellular proliferation. As further evidence that any autocrine effects of oestrogen are minor or absent in mice, supplementation of oestradiol in the absence of gonadotrophins in the culture medium was unable to support normal follicle growth to the antral stage (N. Spears, A. A. Murray, V. Allison, N. I. Boland and R. G. Gosden, unpublished). This result is consistent with the findings of Wang and Greenwald (1993), who administered synthetic oestrogen each day to hypophysectomized mice and were unable to obtain full development. In conclusion, follicular development in this species under the conditions studied does not require the concentrations of oestrogens normally found in gonadotrophin-stimulated follicles. However, it should be noted that oocytes were not examined at the end of this study and, therefore, the possibility cannot be excluded that oestrogens stimulate oocyte development.

By implication, the main actions of oestrogen in the oestrous cycle are probably extra-follicular, although this study does not completely rule out a local role for oestrogen, such as the stimulatory effect of oestrogen on rat granulosa cell proliferation (Rao *et al.*, 1978). Oestrogen receptors are present in the granulosa cells and oocyte, but their abundance appears to vary between species and, consequently, extrapolations from one to another may be invalid (Wu *et al.*, 1992; Kawashima and Greenwald, 1993; Moudgal *et al.*, 1996). However, it remains possible that, despite a large excess of the oestrogen receptor antagonist, trace amounts of oestradiol had bound to the cognate receptor and that these were sufficient to maintain follicular development or, alternatively, that oestrogen was acting other than through its receptor.

These studies demonstrate the value of follicle growth *in vitro* for investigating mechanisms of follicle development and secretion. Since follicles can be grown from early preantral to the preovulatory stage, it is possible to explore the mechanisms of follicle development at almost every stage. In these studies, it has been possible to demonstrate the overriding importance of FSH during follicle development, and that LH has little role beyond increasing the production of oestradiol, until ovulation. Antibodies were used to investigate the role of steroid metabolism on follicle development. Similarly, the system can be used to explore paracrine-autocrine mechanisms (for example, see Smyth *et al.*, 1994). Finally, the results of gonadotrophin deprivation have shown that FSH appears to be an essential survival factor: in the absence of FSH, follicles became clearly atretic. Therefore, the culture system can be valuable either as a model of follicular atresia, or for investigating the role of paracrine factors.

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