Partial purification from bovine follicular fluid of a factor of low molecular mass with inhibitory effects on the proliferation of bovine granulosa cells *in vitro* and on rat follicular development *in vivo*

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Bovine follicular fluid was aspirated from follicles of 2–20 mm in diameter, charcoal-treated to remove steroids and then separated into low and high molecular mass fractions. The low molecular mass (< 10 kDa) fraction was purified on a Sephadex G-25 chromatography column with formic acid as the eluent. Seven peaks were isolated and assayed for biological activity in cultures of bovine granulosa cells at concentrations of 10, 100 and 1000 ng ml⁻¹. One peak (peak 4) inhibited ($P < 0.001$) the proliferation of granulosa cells when measured by cell counting and by [³H]thymidine incorporation (33–37% inhibition). This peak inhibited proliferation of granulosa cells from both small (< 2 mm) and medium (2–10 mm) follicles, but not large (> 10 mm) follicles. The inhibitory effect of peak 4 was not due to a toxic effect on cells. Administration of peak 4 to rats did not affect liver or kidney masses but did decrease uterine (25%, $P < 0.01$) and ovarian (35%, $P < 0.01$) masses. Peak 4 also caused a reduction in the number of large follicles (65%, $P < 0.01$) but increased the number of small follicles (55%, $P < 0.01$). We have named the inhibitory factor associated with peak 4, granulosa cell-inhibitory factor (GCIF). The results presented suggest that GCIF may be a factor secreted by dominant follicles that inhibits the development of subordinate follicles.

Introduction

While ovulation of one or more viable oocytes is central to normal mammalian reproduction, knowledge of the control of ovulation and of folliculogenesis on which ovulation depends is limited. It is only in recent years that the processes whereby some follicles are selected for ovulation while others regress and undergo atresia are becoming clear (Ireland and Roche, 1987; Spicer and Echternkamp, 1986). For many years it was thought that ovulation was strictly under the control of gonadotrophins, the gonadal steroids, and interactions between these two groups of compounds. However, in spite of the fact that many follicles are subjected to the same endogenous gonadotrophic environment and theoretically should all be capable of ovulating, only a tiny minority do so (Spicer and Echternkamp, 1986). This observation, coupled with variation in follicular response to standard exogenous doses of gonadotrophins has led to the belief that, while these hormones play a central role in folliculogenesis, their action at the ovary is modulated by intra-ovarian mechanisms (Hammond *et al.*, 1981; Ledwitz-Rigby *et al.*, 1981; Driancourt, 1991). It has been suggested that this intra-ovarian control may be exerted in part by inhibin (Findlay *et al.*, 1981) or by variations in local steroid concentrations (Leung and Armstrong, 1980).

Several other factors, such as luteinizing inhibitor (Channing *et al.*, 1980), follicular-regulating protein (DiZerega *et al.*, 1982), oocyte-maturation inhibitor (Lintern-Moore and Moore, 1979), luteinizing stimulator (Youglai, 1972) and fibroblast and epidermal growth factors (Hammond, 1981) have also been found in follicular fluid, and some of these factors may have a role in the intra-ovarian control of ovulation.

The administration of steroid-free ovine follicular fluid reduces the number of large preovulatory follicles in acutely hypophysectomized ewes, suggesting that follicular fluid contains a factor(s) that has a direct action at the ovary (Cahill *et al.*, 1984). This paper reports the partial purification of a factor from bovine follicular fluid of low molecular mass that has the ability to inhibit the proliferation of bovine granulosa cells *in vitro* and to modulate rat ovarian follicular dynamics *in vivo*.

Materials and Methods

Fractionation of bovine follicular fluid

Bovine ovaries were collected from a local abattoir and maintained on ice before aspiration of follicular fluid from normal follicles of 2–20 mm diameter. The follicular fluid was centrifuged for 15 min at 600 *g* and the supernatant removed and stored at –20°C. After thawing, steroids were removed from the supernatant by treatment with dextran-activated

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charcoal (Sigma, Poole, Dorset, UK) at a concentration of 5 mg ml^{-1} overnight at 4°C . The charcoal was removed by centrifugation at 1000 g for 30 min and the follicular fluid separated into high and low molecular mass fractions by passing it through a hollow fibre unit (Amicon Ltd., Stonehouse, Glos.) with a molecular mass cut off of 10 kDa. The $< 10 \text{ kDa}$ fraction (low molecular mass) was lyophilized and then reconstituted in 5% (v/v) formic acid before being run (flow rate: 0.1 ml min^{-1}) on a Sephadex G-25 superfine (Pharmacia LKB Biotechnology AB, Uppsala) gel filtration column (column volume: 113 ml, $1.2 \text{ cm} \times 100 \text{ cm}$). Absorbance was monitored at 280 nm and the eluate collected in 1.5 ml fractions. Fractions corresponding to each peak were pooled and lyophilized before testing for biological activity. The amount of peptide equivalent material in each peak was determined by absorbance measurements at 260 and 280 nm for concentrations $> 1 \text{ mg ml}^{-1}$ (Johnstone and Thorpe, 1982) and at 205 nm for concentrations $< 1 \text{ mg ml}^{-1}$ (Scopes, 1974).

Experiment 1: investigation of the effects of peaks collected from follicles 2–20 mm in diameter on granulosa cells from follicles 2–20 mm in diameter

The peaks isolated by column chromatography were assayed (modified from Henderson and Franchimont, 1981) for their effects on the proliferation of bovine granulosa cells. The granulosa cell pellet remaining after separation of follicular fluid was resuspended in Minimum Essential Medium (MEM; GibcoBRL, Life Technologies Ltd, Paisley) and washed twice before plating. The granulosa cells were plated in 12-well tissue culture plates (Costar Europe Ltd, Badhoevedrop) at a concentration of $20 \times 10^4 \text{ cells ml}^{-1}$ of medium. Plates were prepared in duplicate from each pool of granulosa cells. Estimation of cell proliferation was based on haemocytometric counting and [^3H]thymidine incorporation, carried out on separate sets of duplicate plates. MEM supplemented with 10% fetal calf serum (FCS), and 1% of each of the following solutions, 50 000 iu penicillin ml^{-1} , 50 mg streptomycin ml^{-1} , 0.25 mg fungizone ml^{-1} , 29.2 mg L-glutamine ml^{-1} and a 50 $\times$ MEM-non-essential amino acid solution without glutamine (all from GibcoBRL, Life Technologies) was used as the culture medium and culture was carried out in a 5% CO_2 in air atmosphere at 37°C . After 24 h the culture medium was aspirated, replaced with fresh medium and the chromatographically separated and lyophilized peaks of follicular fluid added. Each peak from several chromatographic separations was tested on any one pool of cells. The peaks were reconstituted in MEM and added randomly to culture wells at concentrations of 10, 100 and 1000 ng of peptide equivalent material MEM ml^{-1} . One peak, peak 4, was also tested at concentrations of 0.1 and 1 ng ml^{-1} . At the same time [^3H]thymidine (methyl- ^3H]thymidine, 1 mCi ml^{-1} , Amersham International plc, Amersham, Bucks) was added ($2 \mu\text{l}$) to each culture well. All plates were maintained in culture for a further 24 h at which time the granulosa cells were harvested and counted. This protocol of allowing cells to settle for 24 h and then adding peak material for another 24 h was chosen because it was clear from preliminary growth curves that the maximum proliferation rate of granulosa cells occurred between 24 and 48 h after seeding.

The culture medium was removed from the plates before haemocytometric cell counting. The cells were then trypsinized using 2.5% (w/v) trypsin (GibcoBRL) for 30 min at 37°C . MEM (2 ml) with 10% FCS was then added to the wells. Duplicate ($20 \mu\text{l}$) samples were removed from each well and counted twice using a haemocytometer chamber. For radiolabelled counting, 2 ml of 10% (w/v) trichloroacetic acid containing 5 mg calf thymus DNA ml^{-1} was added to each of the culture wells. The entire contents of each well were then filtered through individual glass microfibre filters and the filters dried overnight. The filters were then put into scintillation vials (L.I.P., Ballybane Industrial Est., Galway) and 5 ml of scintillation cocktail (Ready Protein, Beckman Instruments, Sands Industrial Estate, High Wycombe, Bucks) added before counting for 1 min in a beta counter.

Experiment 2: investigation of the effects of peak 4 collected from follicles 2–20 mm in diameter on granulosa cells from small ($< 2 \text{ mm}$), medium (2–10 mm) or large (10–20 mm) follicles

Follicles were measured and categorized as small ($< 2 \text{ mm}$ diameter), medium (2–10 mm diameter) or large (10–20 mm diameter). Granulosa cells from each follicle size class were maintained separately. Peak 4 (the only peak to inhibit proliferation of granulosa cells) from the G25 fractionation of follicular fluid was tested for *in vitro* biological activity on each pool of granulosa cells using the bioassay described for Expt 1. In Expt 2, proliferation of granulosa cells was monitored using the incorporation of [^3H]thymidine as described for Expt 1.

Experiment 3: investigation of the effects of peak 4 on the number of rat follicles and ovarian, uterine, liver and kidney masses in vivo

The effect of peak 4 on follicular function *in vivo* was tested in rats. Mature female rats (Sprague-Dawley, Bioresources unit, Dublin University) were housed at 20°C and given food and water *ad libitum*. Vaginal smears were taken until three consecutive normal 4 day oestrous cycles were monitored. Rats were then randomly allocated to two treatment groups and two control groups. Peak 4 was administered subcutaneously to the treatment groups, at concentrations of 6 μg and 24 μg of peptide equivalent material in 1.0 ml saline (0.9% (w/v) sodium chloride) for three consecutive days beginning on the day of dioestrus. One control group received 1 ml of saline for three days in a similar manner to the treatment groups. Injections at four sites along the flank of the animal were given at 10.00 h each day. A second control group received no injection. On the day of metoestrus the rats were killed by cervical dislocation and the liver, kidney, uterus and ovaries were removed and weighed immediately and the ovarian follicles counted. Follicles protruding from the surface of the ovary were defined as large follicles while all others were defined as small follicles.

Statistical analyses

Data from all experiments were analysed by analysis of variance (Steel and Torrie, 1960) followed, where appropriate, by a two-tailed Dunnett's test to compare each treatment with

Fig. 1. Elution profile of a G-25 Sephadex chromatographic separation of the low molecular mass fraction from bovine follicular fluid.

Table 1. The recovery of peptide equivalent material from 10 mg of the fraction of low molecular mass from bovine follicular fluid following fractionation on a Sephadex G-25 column

Peak number	Elution volume (ml)	Peak volume (ml)	Peptide equivalent (mg)
1	33.80	7.00	0.14
2	67.60	8.60	0.26
3	77.80	11.00	0.40
4	85.40	9.00	1.07
5	96.60	12.20	0.47
6	113.60	21.00	4.04
7	136.00	18.60	0.81

controls. Where the treatment $\times$ week (that is, replicates) interaction term was significant a revised analysis of variance table was constructed, in which a pooled treatment $\times$ week interaction term was used as the corrected error term to test for treatment effects.

Results

Experiment 1: the effects of peaks collected from follicles 2–20 mm in diameter on granulosa cells from follicles 2–20 mm in diameter

Gel filtration chromatography of the low molecular mass (< 10 kDa) fraction of follicular fluid on Sephadex G-25 resulted in a consistent elution profile over several chromatographic separations. The profile (Fig. 1) consisted of seven distinct peaks. An average of 72% of the peptide equivalent material loaded (based on 260/280 absorbance) was recovered following gel chromatography and the elution volumes, peak volumes and peptide equivalent content of all seven peaks isolated are shown (Table 1).

The effect of each of the seven peaks isolated from the low molecular mass fraction of follicular fluid on the proliferation of granulosa cells was measured by haemocytometric counting and expressed as a percentage of number of control cells

(Fig. 2). Each peak had its own control value and these values ranged from 44 to $68 \times 10^4 \text{ ml}^{-1}$. Since cells were seeded at $20 \times 10^4 \text{ ml}^{-1}$, it is clear that cell proliferation occurred in all cases. Data for [^3H]thymidine incorporation are not shown but were similar to the haemocytometric data. Peaks 3 and 4 were the only peaks with a significant effect on granulosa cell proliferation. Peak 3 stimulated cell proliferation as measured by haemocytometer cell counting ($P < 0.01$) and [^3H]thymidine incorporation ($P < 0.01$) with the greatest stimulatory effect caused by the lowest concentration of Peak 3 (10 ng ml^{-1}). Peak 4 significantly inhibited cell proliferation in each case ($P < 0.01$), when measured both by haemocytometric cell counting and [^3H]thymidine incorporation (see Figs 2 and 3), the greatest inhibitory effect was observed with 10 ng ml^{-1} . The experimental protocol was changed in one experiment to check whether the effect of peak 4 on cell proliferation was due to either a toxic effect or inhibition of cell attachment. At the end of culture the medium from each well (discarded in other experiments) was combined with the cells from that well; cells were stained with trypan blue and live and dead cells counted. The cell numbers ($\times 10^{-4}$) and the percentages of dead cells, respectively, in the various treatments (six to eight replicates per treatment, means $\pm$ SEM) were as follows: control: 38.4 ± 2.4 and 0.8 ± 0.2 ; $10 \text{ ng peak 4 ml}^{-1}$: 20.2 ± 2.0 and 1.1 ± 0.6 ; $100 \text{ ng peak 4 ml}^{-1}$: 19.6 ± 2.9 and 1.7 ± 0.7 and $1000 \text{ ng peak 4 ml}^{-1}$: 22.6 ± 2.2 and 1.1 ± 0.4 . Total numbers of cells for all peak 4 treatments were different from the control treatment ($P < 0.01$) but percentages of dead cells did not differ between treatments ($P > 0.1$). Examination under a microscope of the culture medium aspirated at the end of culture indicated that very few floating or detached cells were present.

Experiment 2: the effects of peak 4 collected from follicles 2–20 mm in diameter on granulosa cells from small ($< 2 \text{ mm}$), medium (2–10 mm) or large (10–20 mm) follicles

Peak 4 inhibited the proliferation of granulosa cells from small ($P < 0.01$) and medium-sized ($P < 0.01$) follicles but had no effect on granulosa cells from large follicles (Fig. 4).

Experiment 3: the effects of peak 4 on the number of rat follicles and ovarian, uterine, liver and kidney masses in vivo

Administration of peak 4 to cyclic female rats had no effect on liver and kidney masses ($P > 0.10$, data not shown). In contrast, administration of peak 4 caused a decrease in the number of large ovarian follicles (Fig. 5, $P < 0.01$) and a concomitant increase in the number of small follicles (Fig. 5, $P < 0.01$). Associated with the decrease in the number of large follicles, peak 4 also caused a significant decrease in ovarian and uterine masses ($P < 0.01$, Fig. 6).

Discussion

The results reported here demonstrate clearly that bovine follicular fluid contains a low molecular mass fraction (peak 4) with the following major biological effects: (1) at very low concentrations it markedly inhibits granulosa cell proliferation

Fig. 2. Effect of various peaks from the G-25 chromatographic separation of the low molecular mass fraction from bovine follicular fluid on the proliferation of bovine granulosa cells as measured by haemocytometric cell counting. Peptide equivalent concentrations of the various peaks are shown as follows: 0 ng ml⁻¹: □; 10 ng ml⁻¹: ▨; 100 ng ml⁻¹: ▩; 1000 ng ml⁻¹: ■. Values are means ± SEM based on the following numbers of replicates: four replicates for peaks 1, 2, 3, 5 and 7; 14 replicates for peak 4 and eight replicates for peak 6. Mean numbers of control cells for the various peaks varied from 44 to 68 × 10⁴ ml⁻¹.

in vitro; (2) it selectively inhibits the proliferation of granulosa cells from small and medium-sized follicles, but has no effect on cells from large follicles and (3) administration to cyclic female rats inhibits the formation of large follicles, increases the number of small follicles and reduces uterine and ovarian masses without affecting the masses of the liver or kidneys. We propose to name the factor responsible for this activity granulosa cell inhibitory factor (GCIF). The effect of GCIF is not a toxic effect because treatment with GCIF did not increase the proportion of dead cells in culture.

Attempts to destroy the inhibitory effect of GCIF on granulosa cell proliferation by protease digestion with trypsin were unsuccessful (unpublished data) suggesting that the active material is not a peptide. However, it is impossible to draw definite conclusions from this work since many small peptides are resistant to digestion with proteases such as trypsin.

At least two factors reported to date resemble GCIF; one such factor is that isolated by Kigawa *et al.* (1986) from pig follicular fluid. This factor, estimated by the authors to have a M_r of 1–3 kDa, inhibited oestradiol and progesterone secretion both by rat granulosa cells *in vitro* and by the ovaries of hypophysectomized diethylstilboestrol-treated immature rats *in vivo*. The method of separation of the low molecular mass fraction of pig follicular fluid using a G-25 Sephadex column

and acetic acid instead of formic acid was similar to the procedure described here for the purification of peak 4 from bovine follicular fluid and resulted in a similar elution profile.

Another factor that resembles GCIF closely is that reported by Chakravorty *et al.* (1993) which they named granulosa cell mitostatic protein (GCMP). These authors found that this low molecular mass (< 10 kDa) fraction from rat follicular fluid inhibited granulosa cell proliferation *in vitro* by up to 60%. Further purification of this < 10 kDa fraction using electrophoretic techniques yielded two peaks of inhibitory activity with molecular masses of 0.9–1.3 and 1.7–2.3 kDa. They suggested that the lower molecular mass factor is a degradation or monomeric form of the larger GCMP.

It is probable that the molecular mass of GCIF is < 5 kDa, the exclusion limit of the G-25 column because GCIF eluted well after the void volume of the column. This gives GCIF a similar molecular mass to that of the GCMP of Chakravorty *et al.* (1993). However, some caution is necessary in assigning molecular masses to low molecular mass compounds based on elution position on Sephadex gel filtration columns, as it has been shown that low molecular mass compounds applied to such columns show elution profiles that are inconsistent with their molecular masses owing to nonspecific binding

Fig. 3. Effect of various concentrations of peak 4 extracted from bovine follicular fluid for 24 h on proliferation of bovine granulosa cells as measured by haemocytometric cell counting and [³H]thymidine incorporation into acid-precipitable material. Values are means $\pm$ SEM. (a) Effect of peak 4 on number of cells at concentrations from 0.1 to 10 ng ml⁻¹; four replicates per treatment. (b) Effect of peak 4 on number of cells at concentrations from 10 to 1000 ng ml⁻¹; 20 replicates per treatment. (c) Effects of peak 4 at concentrations from 10 to 1000 ng ml⁻¹ on [³H]thymidine incorporation; 10 replicates per treatment. Treatments with * and ** are significantly different ($P < 0.05$, $P < 0.01$, respectively) from the control treatment.

Fig. 4. Effects of peak 4 extracted from bovine follicular fluid on the proliferation of granulosa cells from small (< 2 mm, □), medium (2–10 mm, ▨) and large (10–20 mm, ■) bovine follicles as measured by [³H]thymidine incorporation into acid-precipitable material. Values are means $\pm$ SEM based on nine replicates. * Significantly different ($P < 0.01$) from the 0 concentration of peak 4 for small and medium follicles: there were no significant differences between the effect of different concentrations of peak 4 on large follicles.

Fig. 5. Effect of administration to rats of peak 4 extracted from bovine follicular fluid on the number of large (□) and small (■) follicles per rat. Values are means $\pm$ SEM based on 9–12 rats per treatment. * Significantly different ($P < 0.01$) from the corresponding saline control treatment.

interactions with the Sephadex matrix (Eaker and Porath, 1967; Williams, 1972).

Follicular-regulatory protein, first isolated by DiZerega *et al.* (1982), inhibits the incorporation of [³H]thymidine by

granulosa cells *in vitro* (Ono *et al.*, 1988) in a manner similar to that reported here for GCIF. However, follicular-regulatory protein is a different factor from GCIF since it has a molecular mass of 12–15 kDa, which is significantly higher than the probable molecular mass of GCIF (< 5 kDa).

Fig. 6. Effect of administration to rats of peak 4 extracted from bovine follicular fluid on ovarian and uterine masses. Values are means $\pm$ SEM based on 9–12 rats. * Significantly different ($P < 0.01$) from the saline control treatment.

Follicular growth in cattle occurs in two or three waves throughout the cycle (Ireland and Roche, 1987). During the dominance phase, normally one follicle grows to preovulatory size suppressing the growth of subordinate follicles in its cohort (Ireland and Roche, 1987). This inhibition by dominant follicles was previously thought to be exerted through the negative feedback of ovarian steroids on the hypothalamic–pituitary–ovarian axis. However evidence suggests that the dominant follicle produces a specific inhibitor(s) to the subordinate follicles (McNatty and Sawyers, 1975; DiZerega *et al.*, 1982; Driancourt, 1991) which operates at the ovary.

McNatty and Sawyers (1975) found that cauterization of large follicles was associated with a compensatory increase in the mitotic index of granulosa cells in the remaining follicles. These authors found that this increase in mitotic index could not be accounted for solely by changes in gonadotrophin activity and they postulated that cauterization removed an endogenous inhibitor(s) of granulosa cell mitosis present in the follicular fluid of large follicles. The GCIF described here may be one of the factors involved in this inhibition. This hypothesis is supported by the susceptibility of granulosa cells from small and medium follicles to inhibition by GCIF. The *in vivo* data from rats presented strongly supports this hypothesis, since GCIF decreased the number of large follicles by 50%

while at the same time significantly increased the number of small follicles. Evidence that production of GCIF is greatest in large dominant follicles would lend even more support to this hypothesis.

The large increase (100% over controls) recorded in the number of small follicles indicates that GCIF does not stop the formation of small follicles but instead appears to prevent their further development to large follicles. GCIF may also directly stimulate formation of small follicles. It is also possible that inhibition of large follicle growth by GCIF removes some factor (not GCIF) produced by such follicles which is inhibitory to the formation of small follicles. The decrease in uterine mass caused by GCIF administration is almost certainly due to decreased oestradiol production by ovaries possessing few large follicles. Uterine mass in the rat has long been known to be oestrogen-dependent (Richardson, 1966). The possibility that some of the effect of GCIF seen *in vivo* could be due to an inhibitory action on gonadotrophin secretion from the pituitary should not be excluded.

The form of the dose–response shown here in which increasing concentrations of GCIF resulted in decreasing granulosa cell inhibition is interesting. One possible explanation is that contamination with material of a stimulatory nature from the adjacent peak 3 is responsible. However, this explanation is probably not the sole reason, since further studies on the purification of GCIF indicate that this type of dose response is not lost even in highly purified material (unpublished data), suggesting that the form of the dose–response curve may also be an inherent property of the active material, possibly involving the down-regulation of GCIF receptors. It is interesting to speculate that a very useful property of a factor produced by a dominant follicle to suppress competing follicles would be the ability to inhibit the granulosa cells of the competing follicle on both ovaries at very low concentrations coupled with an inability to inhibit granulosa cells at high concentrations such as might be found in the dominant follicle producing it.

Studies on the purification and chemical identification of GCIF and on the effects of immunization against this factor on follicular growth patterns and ovulation rates in sheep are currently in progress.

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