The establishment of follicular dominance in co-cultured mouse ovarian follicles

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An *in vitro* model of dominant and subordinate ovarian follicles was developed to allow a closer investigation of the phenomenon of follicular dominance. Preantral mouse ovarian follicles were cultured either alone or in pairs. Pairs of follicles were either in direct contact or in shared medium, but without physical contact. The experiments showed that where contact was allowed to develop between follicles one follicle invariably became dominant, while the other would grow and develop little during the culture period. In contrast, there was no effect of co-culture on follicle development in the absence of contact between the follicles. There was, therefore, no evidence of secretion of a diffusible factor by a dominant follicle that could affect the development of neighbouring follicles. After 6 days of co-culture with contact, histological examination of the subordinate follicle showed that it was healthy, in spite of remarkably little growth during culture. In a further experiment, the subordinate follicle was separated from the dominant one after 2 days of co-culture (when a significant difference in size had already developed), and cultured alone. These 'released' follicles exhibited a spurt of growth during the remaining culture period, attaining a size and appearance indistinguishable from those of controls by the end of culture. This confirms that the dominant follicle, while depressing the growth of its neighbour, is not inducing irreversible atresia in the subordinate follicle in this model. The *in vitro* model will allow a more detailed study of direct influences of dominant–subordinate follicle interactions, and should increase our knowledge of a poorly understood phenomenon.

Introduction

Most mammals control their litter size primarily by regulating the number of oocytes released during ovulation. The oocytes are ovulated from preovulatory, or Graafian, follicles. The mechanism by which each species ensures that the number of follicles appropriate to uterine capacity develop to the Graafian stage is not well understood. From a large group of developing early-antral follicles, a species-characteristic number is selected, establishing dominance over the remaining, now subordinate, follicles in the cohort (Faddy *et al.*, 1983; Hirshfield, 1991). These dominant follicle(s) proceed to the preovulatory stage; subordinate follicles failing to grow terminate development in atresia.

Two main theories have been proposed to explain the mechanism of follicular dominance (see Fortune 1994, for review). One states that dominant follicles exert an active and direct influence on subordinate follicles, for example through secretion of an inhibitory factor (Ireland, 1987). Alternatively, the process could be an indirect one, acting via negative

feedback mechanisms. According to this theory, it is assumed that dominant follicles secrete a substance which then acts on the hypothalamic–pituitary system to decrease FSH concentrations. The fall in FSH compromises the development of the subordinate follicles, while those that are slightly more developed or less FSH dependent survive the decline (Zelevnik and Hillier, 1984; Baird, 1987). Neither theory explains how the initial imbalance of dominant versus subordinate follicles is established, nor how any system can reliably lead to the development of only one Graafian follicle each cycle in women and yet 6–8 Graafian follicles in mice.

Investigations into the regulation of dominance have been hampered by the difficulty of studying the phenomenon *in vivo*. Almost all of the work has been confined to large domestic mammals, and has relied heavily on ultrasonography (e.g. Savio *et al.*, 1990). We therefore decided to use a recently developed method for culturing whole mouse ovarian follicles to investigate the phenomenon of dominance in mice, *in vitro*. The culture system allows the development of single primary, preantral follicles to the Graafian stage, at which time they can be ovulated and the oocytes fertilized (Boland *et al.*, 1993; Spears *et al.*, 1994). The aims of the study were first to see whether dominance occurred *in vitro*, and second to investigate the possibility that dominant follicles secrete a diffusible factor(s) that suppresses growth or induces atresia in neighbouring follicles.

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Materials and Methods

Animals

The mice in these experiments (C57BL/6 × CBA/Ca) were provided with food and water *ad libitum* and housed in a temperature- and light-controlled room under a photoperiod of 14 h light:10 h dark.

Follicle isolation

Ovaries from 24-day-old F_1 mice were aseptically removed and placed in watchglasses of Leibovitz L-15 medium (Gibco-BRL, Paisley), supplemented with 3 mg BSA ml^{-1} , fraction V (Sigma, Poole, Dorset). Individual preantral follicles measuring $190 \pm 10 \mu\text{m}$ in diameter were dissected from mouse ovaries using fine, sharp needles. Each follicle was removed from the ovary with a small clump of thecal–stromal tissue attached (not included in the diameter measurement), since denuded follicles fail to develop fully in this system. Follicles showing signs of atresia (that is, dark or shrunken) or with eccentrically sited or misshapen oocytes were discarded.

Follicle culture

Follicles were cultured in 96-well, flat-bottomed microtitre plates (Bibby Sterilin Ltd, Stone, Staffs) in 100 μl droplets of alpha minimal essential medium (Gibco-BRL) under 75 μl mineral oil (Sigma; $d = 0.84 \text{ g ml}^{-1}$) in 5% CO_2 in air at 37°C. Medium was supplemented with 10 mg human transferrin ml^{-1} (Sigma), 1 iu hFSH ml^{-1} (biological potency = 5760 iu ml^{-1}) and 5% serum obtained from adult female F_1 mice. Follicles were measured in watchglasses containing Leibovitz, as described above, and transferred to a new well with fresh medium every 2 days. The day of dissection was designated day 0.

Histology

At the end of the culture period, representative follicles were fixed in 4% (w/v) paraformaldehyde overnight, and then embedded in either wax or plastic. Wax sections were stained with acridine orange and examined by fluorescence microscopy using a filter for blue excitation (wavelength = 450–490 nm), to examine the nuclei of granulosa cells and oocytes for early signs of apoptosis. Plastic sections were counterstained with haematoxylin and eosin for routine histological examination by light microscopy.

Experiment 1

Follicles were placed one or two per well (6–12 wells per culture, repeated for three cultures). Where two follicles were cultured in each well, they were placed either side-by-side, and gently pushed together (see Fig. 1a), or at opposite ends of the well. Follicles were checked regularly to ensure a lack of contact between follicles, where appropriate. In all co-cultures, great care was taken to choose pairs of follicles of similar size and appearance under the dissecting microscope. Cultures were maintained for 6 days.

Fig. 1. Photomicrographs of a pair of follicles co-cultured with contact between the follicles on (a) day 0 of culture, (b) day 1 of culture and (c) day 2 of culture. Scale bars represent 40 μm .

Experiment 2

Three groups of follicles were cultured, two per well (4–12 wells per culture, repeated for four cultures). The first two

Fig. 2. Growth patterns (mean $\pm$ SEM) of control follicles cultured alone (○; $n = 22$) and follicles co-cultured without contact (●; $n = 27$).

groups were as described above (pairs side-by-side, or not in contact). In the third group, initially cultured in contact, the larger of the two follicles was removed on day 2 of the experiment as in the initial dissection described above. The smaller follicle was then returned to culture for 4 subsequent days.

Statistical analyses

Results were compared using ANOVA and Student's *t* test, as appropriate.

Results

Experiment 1

The development and growth of follicles co-cultured without physical contact was indistinguishable from those of follicles cultured alone (Fig. 2). Where contact was allowed between follicles, they rapidly developed close and extensive adhesion (Fig. 1b, c). With few exceptions (2 out of 27), only one of the two follicles developed to the antral stage, with the other growing little (Fig. 3). The development of the larger (dominant) follicles was, however, significantly less than that of the control follicles (Fig. 3). A thin, shared thecal layer developed between the conjoined follicles, which invariably

Fig. 3. Growth patterns (mean $\pm$ SEM) of dominant (x) and subordinate (▲) (both $n = 27$) follicles co-cultured in contact, compared with that of control follicles cultured alone (○; $n = 22$).

assumed a spherical shape. The difference in size between the two follicles was statistically significant by day 2 of culture. Histological examination after day 6 of culture revealed that the smaller follicle appeared healthy: there were few apoptotic cells visible either by light (Fig. 4) or fluorescence microscopy, according to the criteria of Byskov (1974), with substantially fewer than 5% of pyknotic nuclei visible in any cross-section.

Experiment 2

Removal of the larger follicle at day 2 of culture was a difficult process, often resulting in the death of the smaller follicle (< 25% successful). Although conjoined follicles developed approximately spherically, the remaining follicle regained a spherical shape within minutes of the removal of the larger follicle. The smaller follicles rapidly increased their growth rate compared with smaller follicles that remained in contact with a larger one. By day 4 they were indistinguishable from the larger co-cultured follicles in contact, and by day 6 they had caught up with the development of control follicles, often attaining Graafian development (Fig. 5). As in Expt 1, both types of histological examination revealed healthy follicles with few apoptotic cells.

Fig. 4. Photomicrographs of two sections through a pair of follicles co-cultured with contact (d: dominant follicle; s: subordinate follicle). Plastic sections were stained with haematoxylin and eosin. Scale bars represent 40 μm .

Discussion

The mouse ovary is small and compact with a high ratio of follicular to stromal mass. Accordingly, many follicles are in contact with each other, the more so as they grow. In ovaries of this type, interactions and competition between neighbouring follicles may be physiologically important. In the ovaries of Old World primates and ruminants, however, there is abundant stromal tissue and follicle densities are lower. Interactions are less likely to occur as a result of contact, although longer-range interactions, either via the pituitary gland or within the ovaries, are possible. The present results support the hypothesis that dominant follicles can suppress the growth of neighbouring follicles with which they are in contact, in mice. They provide no evidence to support the existence of diffusible substances secreted by growing follicles affecting others in the same cohort. Whether there are interactions between different stages of follicular development has not been addressed in this study. Nor do the results rule out the possibility of long-range intraovarian interactions in other species. The hypothesis that dominance is achieved by an indirect, negative feedback mechanism as a result of reduced FSH secretion cannot be tested by this model, although it is highly likely that an indirect mechanism plays an important role in the regulation of dominance *in vivo*.

When follicles were co-cultured in contact, close and extensive adhesion developed between the pair, and one follicle rapidly appeared to become dominant, inducing subordinate development in the other. Surprisingly, in the experiments described here, subordinate follicles usually survived to the end of the culture period, when histological examination indicated

that they were not atretic, possibly due to the high FSH concentrations used in the cultures. Subordinate follicles were able to escape the effect of the dominant follicles, if separated after 2 days of co-culture. This may be a further indication that these subordinate follicles were not atretic: Hirshfield (1989) found that atresia in rats *in vivo* was irreversible, although Hay *et al.* (1979) reversed early sheep follicle atresia *in vitro*. The distinction may be a matter of the degree of atresia rather than a species difference. If a contact-mediated effect occurs *in vivo*, it is possible that it results only in the cessation of growth of neighbouring follicles, as has been found here. Atresia of the subordinate follicles may then occur owing to the subsequent lowering of FSH concentrations (following negative feedback on the hypothalamic–pituitary system owing to oestrogen and/or inhibin release by the dominant follicles).

Dominant follicles, while attaining antral development, grew less well than did the individually cultured follicles. When follicles were cultured in contact, the two rapidly developed an extensive area of contact. This two-follicle unit became spherical, achieving a minimal surface area to volume ratio. Consequently, the surface area available to each follicle for exchange of metabolites or products of metabolism (for example glucose or lactate) may be compromised (Boland *et al.*, 1993, 1994), if only at the interface between the conjoined follicles. Alternatively, the growth of the larger follicle may be constrained if the two-follicle unit is unable to exceed a diameter of about 500 μm . While individually cultured mouse follicles would not be expected to grow beyond 450–500 μm , rat follicles cultured using the same method failed to grow much beyond this size, although it falls slightly short of the full size of rat Graafian follicles *in vivo* (Smyth *et al.*, 1994). It is possible, therefore, that

Fig. 5. Growth pattern (mean $\pm$ SEM) of subordinate follicles separated on day 2 of culture ($\blacksquare$; $n = 11$), compared with that of subordinate ($\blacktriangle$) or dominant ($\times$) follicles that remained in contact for 6 days. The growth pattern of control follicles cultured alone is also shown ($\circ$).

the culture system used imposes a constraint on the upper size limit of the cultured follicles. Although these experiments do not allow us to determine the mechanism of growth retardation in the two-follicle unit as a whole, the more intriguing phenomenon is the differential growth of pairs of apparently identical follicles that become progressively diverse in size as the culture proceeds. Further work is needed to investigate the mechanism for this process, and its physiological relevance.

Developing antral follicles have been described as undergoing first recruitment (at the early antral stage) and subsequently selection (at a late antral stage) (Fortune, 1994). These steps are equivalent to the gonadotrophin-responsive and gonadotrophin-dependent follicles described by Scaramuzzi *et al.* (1993). Descriptions of dominance in large mammals usually refer to the final selection of one or two late antral follicles from the cohort or group of follicles that has developed to the mid-antral stage. In mice, antral development takes only 4–6 days, with a final

selection of 4–8 follicles: it is not clear whether the effect reported here occurs during recruitment and/or selection, or even if the distinction is relevant in mice. It should be remembered that in ovaries with rapidly growing follicles preovulatory follicles will normally be found alongside less-developed antral follicles.

The suggestion that dominant follicles secrete substances that directly affect the development of other antral follicles was made some years ago, but there has been little evidence either to support or refute the theory. diZerega *et al.* (1982) proposed the existence of one such factor, the follicle regulatory protein, although further studies apparently did not confirm this role (Fortune, 1994). Support for the idea of the secretion of inhibitory factors by dominant follicles has come from studies of follicular fluid extracts that have been found to affect the development of large ovarian follicles (ovine: Campbell *et al.*, 1991; bovine: Hynes *et al.*, 1993). Campbell *et al.* (1991) suggest that high FSH concentrations may override the inhibitory effect of such a factor, possibly because the factor acts by modulating an FSH-dependent process. The concentration of FSH used in these cultures was chosen on the basis of optimal growth and oestradiol production *in vitro* (Nayudu and Osborn, 1992), but its physiological relevance is unknown. It is possible that the amount of FSH used in the present study is high, and therefore masks the effect of any diffusible inhibitory factor secreted by the dominant follicle. Further studies are needed to test whether follicles co-cultured without contact in low concentrations of FSH differentiate into dominant and subordinate types.

Since multilaminar preantral follicles are frequently found in small clumps in mouse ovaries, a contact-mediated mechanism could play a role, together with other direct and/or indirect effects of the dominant follicle, in regulating the ovulation rate. As a result, only one member of each clump would develop to the Graafian stage. It is not yet known whether follicles can affect only the development of others that are in direct contact: if the effect can be exerted via interstitial tissue or intermediate follicles to other nearby follicles, the phenomenon would clearly have a much greater potential, with dominant follicles possibly able to influence the development of a group of follicles in close proximity. Such a system could be one way, alongside others, in which mammals can regulate the number of follicles being selected for growth and development at each ovulatory cycle.

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