Effects of Androgens on Early Development of Mouse Follicles in Organ-Cultured Ovaries

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Abstract: *Ovaries from 4-day-old mice were organ-cultured and the effects of progesterone, androstenedione and estradiol-17β on early follicular development were investigated. Both androstenedione (40–1,000 ng/ml) and estradiol-17β (8–1,000 ng/ml) promoted proliferation of granulosa cells in developing follicles, while progesterone showed no remarkable effect. Cyproterone acetate (4,000 ng/ml), an androgen receptor antagonist, partially inhibited the granulosa cell proliferation induced by androstenedione (40 ng/ml). Dihydrotestosterone (8–1,000 ng/ml), which is not converted to estrogens, also induced proliferation of granulosa cells. These results suggest that androgens directly promote proliferation of granulosa cells in early developing mouse follicles in vitro.*

Key words: *Androstenedione, Granulosa cell, Mouse, Organ culture, Ovary.*

After birth, mouse oocytes increase in volume with an arrested nucleus at the diplotene stage of the first meiotic prophase, and ovarian follicles develop as granulosa cells proliferate within them. Oocyte growth is initially accompanied by a thickening of the flattened primitive granulosa cells to a cuboidal shape, and then by proliferation of granulosa cells. As the granulosa cells proliferate, a thecal layer appears on the surface of the follicle [1, 2]. Before the follicles develop their thecal layer, negligible amounts of steroid hormones are produced in the ovaries. During thecal formation, the follicles begin steroid hormone synthesis [3, 4], and then develop markedly. In later developmental stages, the follicles produce progestins, androgens and estrogens by gonadotropic stimulation [5, 6], and these steroids are thought to regulate follicular development in ovaries [7–9].

In organ-cultured mouse ovaries, oocyte growth proceeds normally, though proliferation of granulosa cells is severely retarded [10] unless stimulated by exogenous FSH [11, Ikeda *et al.*, unpublished data], and the organ culture system of ovaries is thought to allow the effects of steroid hormones on the proliferation of granulosa cells to be examined in a highly controlled environment. In practise, the effects of estrogens on the early development of follicles have been examined in organ-cultured mouse ovaries and estrogens promoted proliferation of granulosa cells in follicles of ovaries from both 4-day- [12] and 8-day-old mice [11]. Kent [13] reported that pregnenolone, testosterone and estradiol-17β stimulated the growth of small follicles in organ-cultured ovaries from 15-day-old mice. In that experiment, however, follicular development was assessed without respect to the growth of oocytes which is one of the major factors determining follicular size in the early stages.

Using the organ culture system of mouse ovaries, we examined the effect of steroid hormones on the proliferation of granulosa cells with respect to oocyte growth in early developing follicles. Ovaries from 4-day-old mice were cultured and the effects of three steroid hormones, progesterone, androstenedione and estradiol-17β, on follicular development were examined. Since androstenedione stimulated early development of the follicles, we further examined whether androgens, like their derivatives estrogens, have a direct mitogenic activity on the granulosa cells in ovaries.

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Materials and Methods

Collection and culture of ovaries

Ovaries were obtained from 4-day-old female ICR mice. The mice were killed by cervical dislocation and their ovaries were removed under a dissection microscope. The ovaries were pooled in 10 mM HEPES-buffered Waymouth's medium (MB752/1, Sigma Chemical Co., St. Louis, MO) containing 0.08 mg/ml kanamycin (Sigma), and washed twice in basic culture medium. The basic culture medium was Waymouth's medium containing 50 μg/ml sodium pyruvate and 5% fetal calf serum (Filtron Ltd., Victoria, Australia). Each group of two or three ovaries was cultured on a filter paper (pore size; 3 μm) floating on the medium in an organ culture dish (#3037, Falcon, Becton Dickinson Labware, Lincoln Park, NJ) under a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 4 days.

Progesterone (Nacalai Tesque, Inc., Kyoto, Japan), androstenedione (Nacalai Tesque), estradiol-17β (Sigma) or 5α-dihydrotestosterone (Sigma) were dissolved in absolute ethanol at various concentrations so that addition of 10 μl of it into 1.2 ml of the culture medium made final concentrations of 0 (control), 8, 40, 200, 1000 and 5,000 ng/ml. The steroids were added into the medium just before use.

In the experiment using the androgen receptor antagonist, ovaries were cultured in the basic culture medium containing 5 μl of concentrated solution of cyproterone acetate (Sigma) in addition to 40 ng/ml androstenedione prepared as described above. The final concentrations of cyproterone acetate were 400 and 4,000 ng/ml.

Histological examination

Ovaries from 4- and 8-day-old mice and the organ-cultured ovaries were fixed in 4% (para) formaldehyde in phosphate buffered saline, dehydrated and embedded in methacrylate resin JB-4 (Polysciences, Inc., Warrington, PA). Serial sections of 3 μm were made and reacted with periodic acid-Schiff and stained by Ehrich's hematoxylin. We focused on follicles including a growing oocyte with a diameter of 40 μm or more, because an increase in the number of granulosa cells surrounding smaller oocytes is only slight under all circumstances [14]. All of the healthy follicles without any degenerative signs of oocytes and granulosa cells were chosen from the sections, and examined as follows. One section containing an oocyte having a maximum diameter was chosen from the serial sections of each follicle containing a growing oocytes with a diameter of 40 μm or more. In the section, the oocyte diameter (not including zona pellucida) was measured by an ocular micrometer, and the number of surrounding granulosa cells was recorded.

Statistical analysis

In each experimental group, 4 to 12 ovaries were cultured, and the number of granulosa cells was counted for all of the follicles that contained oocytes of 40.0–49.5, 50.0–59.5 and 60.0–69.5 μm in diameter. The mean number of granulosa cells was calculated for each experimental group and analyzed by Student's *t*-test.

Results

Ovaries from 4-day-old mice contained primordial, primary and a small number of secondary follicles. Of these, most of the follicles were primordial follicles containing 15–20 μm oocytes. Only 1.2% of the examined oocytes were 40.0–49.5 μm in diameter. Such oocytes were found in the secondary follicles enclosed by two layers of granulosa cells (Fig. 1A). The mean diameter $\pm$ SD of the examined oocytes was 18.6 $\pm$ 4.0 μm (n=400) in the ovaries. In ovaries from 8-day-old mice, the largest follicles contained 2–3 layers of granulosa cells and the oocytes in the follicles exceeded 60 μm in diameter.

In the cultured ovaries, the effects of steroid hormones on the follicles were examined in all of the follicles containing oocytes over 40 μm in diameter. No apparent differences in general patterns of oocyte growth were observed among the groups, and some oocytes grew to over 60 μm in diameter in each experimental group (Fig. 1). Progesterone increased the number of granulosa cells slightly in the follicles containing oocytes 50.0–59.5 μm in diameter, although it had no effect on the follicles containing 40.0–49.5 μm oocytes (Fig. 2A). Androstenedione over 40 ng/ml increased the number of granulosa cells significantly (Figs. 1C and 2B). Estradiol-17β also showed a proliferative effect on granulosa cells in the follicles containing oocytes of 50.0–59.5 μm in diameter (Figs. 1D and 2C). However, no effects were observed in the follicles containing oocytes of 60.0–69.5 μm.

To examine whether the effect of androstenedione was exhibited via the androgen receptors in granulosa cells, the following two experiments were conducted. In the first experiment, cyproterone acetate was added to the medium containing androstenedione. Cyproterone

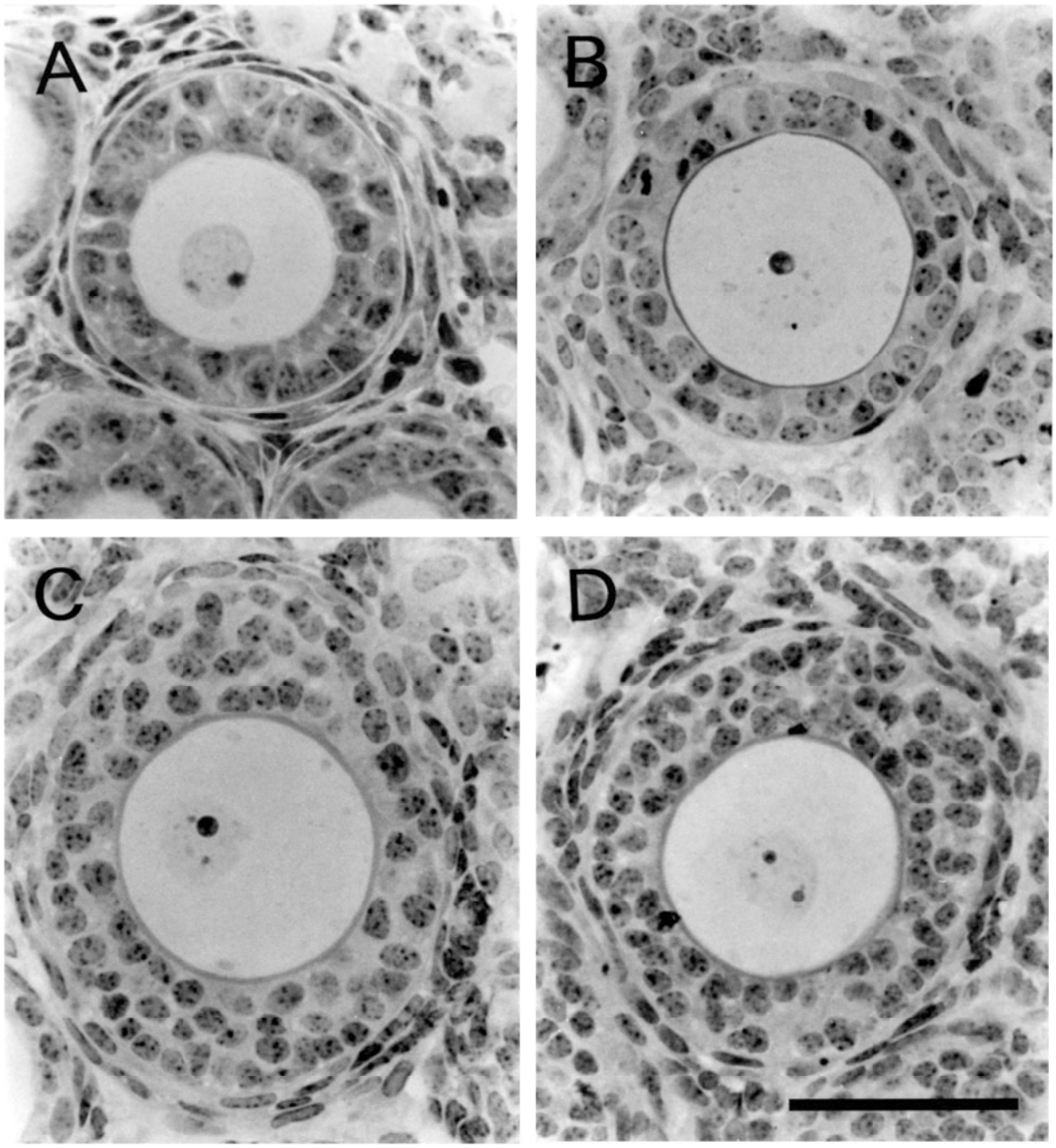

Fig. 1. Sections of early developing mouse follicles. A secondary follicle containing two layers of granulosa cells and an oocyte of 45 μm diameter in an ovary from a 4-day-old mouse (A). After 4 days of culture, oocytes grew in each experimental group, although no apparent difference of oocyte growth was observed among the groups. Developing follicles in organ-cultured ovaries in control medium (B), and in the medium containing androstenedione (1,000 ng/ml) (C) and estradiol-17β (8 ng/ml) (D). The bar represents 50 μm.

acetate at 4,000 ng/ml inhibited the increase in number of granulosa cells due to androstenedione in follicles containing oocytes of 40.0–49.5 and 60.0–69.5 μm in diameter (Fig. 3). In the second experiment, examining the effects of dihydrotestosterone, proliferative effects similar to those of androstenedione on granulosa cells in organ-cultured ovaries were noticed (Fig.4).

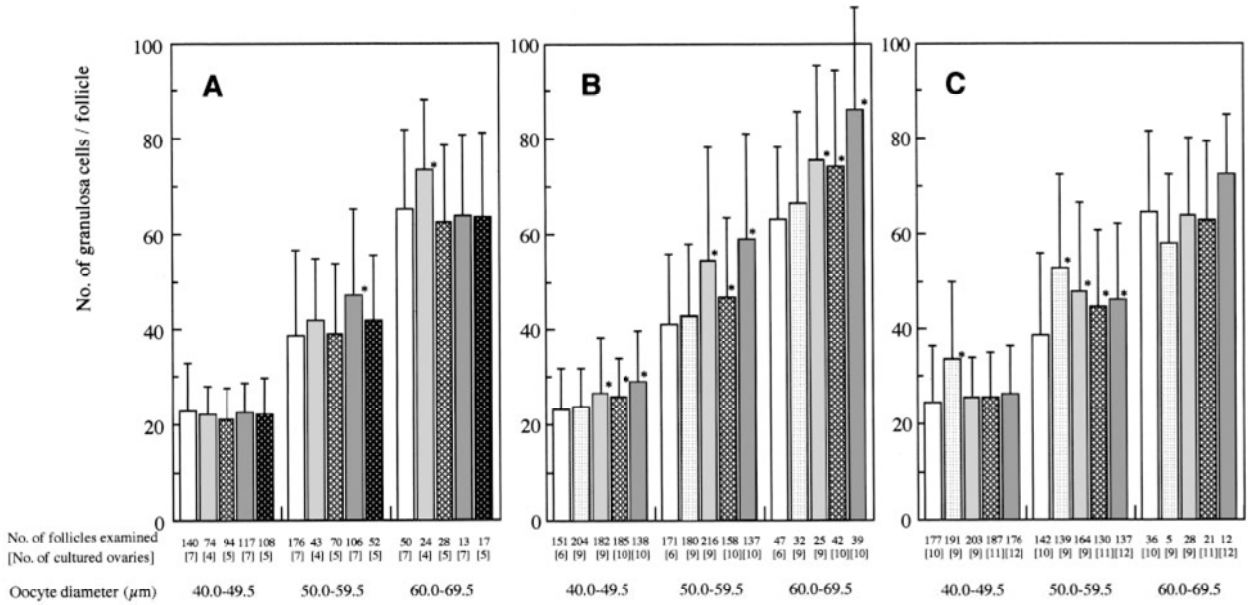

Fig. 2. Effects of progesterone (A), androstenedione (B) and estradiol-17β (C) on the proliferation of granulosa cells in early developing follicles in organ-cultured mouse ovaries. Ovaries were collected from 4-day-old mice and cultured for 4 days. Steroid hormones were added to the culture medium at various concentrations (0, 8, 40, 200, 1,000 and 5,000 ng/ml). Number of granulosa cells was counted in each developing follicle. Columns with a vertical bar represent the mean ± SD, and the asterisks show significant differences to the 0 ng/ml control group ($P<0.05$).

Discussion

In organ-cultured mouse ovaries, it has been reported that progesterone (1 and 0.5 μg/ml) had no significant effect on the development of follicles with three or more layers of granulosa cells [13]. On the other hand, estrogen showed a proliferative effect on granulosa cells in cultured ovaries from 4-day- [12], 8-day- [11], and 15-day old-mice [13]. In this study, both of these observations reported by others were confirmed using our culture systems, and moreover, it was found that androstenedione itself is a potent stimulator of proliferation of granulosa cells.

Proliferation of granulosa cells was induced by testosterone in cultured ovaries from 15 day-old mice [13]. Androstenedione also showed a significant proliferative effect on granulosa cells in the follicles in our experiment. However, the responses of the ovaries to exogenous androgens might be different between 15-day-old mice and 4-day-old mice. Both testosterone [15, 16] and androstenedione [17] are produced from progesterone by thecal cells and converted to estrogens by aromatases in granulosa cells. The synthesis of androstenedione begins at 7 days of age in mice (D6D2F$_1$ and C3H/HeH × 101/H F$_1$) with ovaries containing follicles with 1–2 layers of granulosa cells surrounded by a clearly defined thecal layer [3, 4]. Synthesis of estradiol-17β starts at a similar age in mice, and is stimulated by the combination of FSH and LH [3]. In organ-cultured ovaries, Terada, *et al.* [18] reported that ovaries from as young as 3 day-old mice (WB × C57BL/6) converted exogenous androgens to estrogens in the absence of FSH. The follicles of 4-day-old ICR mice used in our study were considered to be at least in part responsible for converting androgens to estrogens. By extension, we cannot deny the possibility that in the culture, androstenedione was converted to estrogens and thus exerted a proliferative effect on the granulosa cells.

However, it is equally possible that androstenedione stimulated the proliferation of granulosa cells in a direct action. To test this possibility, two experimental paradigms were used. In the first experiment, cyproterone acetate was used. Cyproterone acetate inhibits the binding of androgens to their receptors, which blocks translocation of the androgen-androgen receptor complexes to the nucleus [19]. In organ-cultured mouse ovaries, cyproterone acetate (4,000 ng/ml) inhibited the proliferation of granulosa cells by androstenedione.

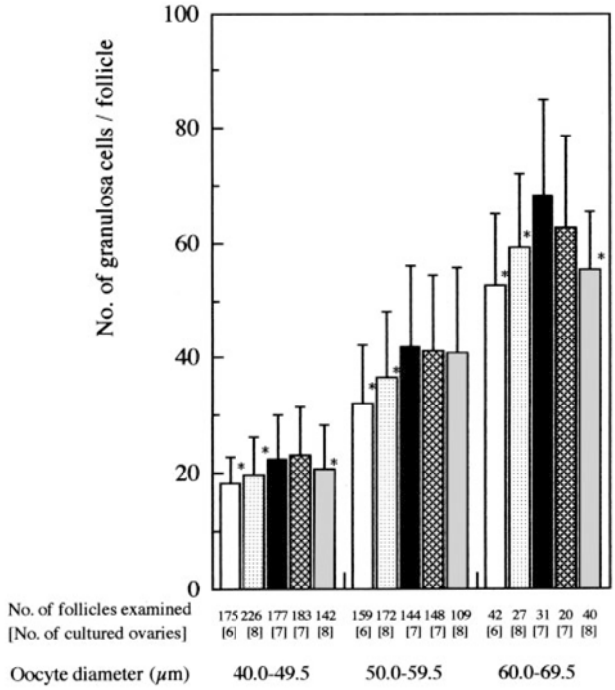

Fig. 3. Effects of an androgen-receptor antagonist, cyproterone acetate (CA), on the proliferation of granulosa cells stimulated by androstenedione in early developing follicles in organ-cultured mouse ovaries. Ovaries were collected from 4-day-old mice and cultured for 4 days. CA was added to the culture medium containing 40 ng/ml androstenedione (□ none, ▨ 4,000 ng/ml CA, ■ 40 ng/ml androstenedione, ▩ 40 ng/ml androstenedione + 400 ng/ml CA, and ▤ 40 ng/ml androstenedione + 4,000 ng/ml CA). Number of granulosa cells was counted in each developing follicle. Columns with a vertical bar represent the mean ± SD, and the asterisks show significant differences to the 40 ng/ml androstenedione group ($P<0.05$).

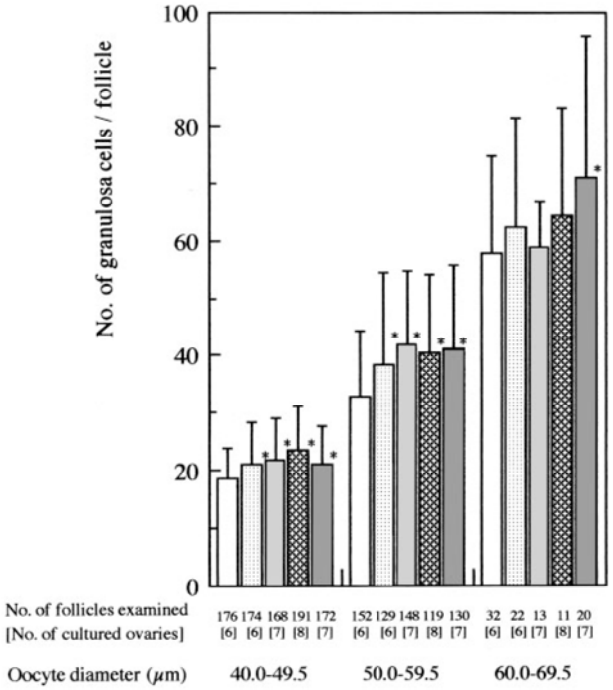

Fig. 4. Effects of dihydrotestosterone on the proliferation of granulosa cells in early developing follicles in organ-cultured mouse ovaries. Ovaries were collected from 4-day-old mice and cultured for 4 days. Dihydrotestosterone was added to the culture medium at various concentrations (□ 0, ▨ 8, ▤ 40, ▩ 200, and ■ 1,000 ng/ml). Number of granulosa cells was counted in each developing follicle. Columns with a vertical bar represent the mean ± SD, and the asterisks show significant differences to the 0 ng/ml control group ($P<0.05$).

Cyproterone acetate, without androstenedione slightly increased the number of granulosa cells in the present experiment. Since cyproterone acetate shows androgenicity at high concentrations (10^{-5} M: approximate 4 μg/ml) [20], the proliferative effects on granulosa cells are thought to be attributed to such characteristics. In the second experiment, dihydrotestosterone was added into the culture medium. It has been reported that dihydrotestosterone exhibits the androgenic effect by binding to the androgen receptor, although it is not converted to estrogens [21]. Dihydrotestosterone, similar to androstenedione, promoted the proliferation of granulosa cells in this experiment. These results suggest that androstenedione can directly promote a proliferative effect on granulosa cells in early developing mouse follicles. Perhaps the stimulatory effect of androstenedione initially found in this study was produced as a result of the direct action of androstenedione as well as conversion of some portion of it into estrogens.

Recently, developmentally regulated androgen receptors were discovered in the granulosa cells of developing rat and marmoset follicles [22–24], and it has been suggested that androgens could have a paracrine action in controlling follicular development. However, the effect of androgens on follicular development has been unclear. Based on the *in vitro* culture experiments of preantral follicles (the mean diameter 185 μm) from 24-day-old mice, Spears *et al.* [25, 26] recently suggested the possibility that the stimulatory effect of androgens on follicle development is due to their direct action on the follicles. Our results are in good agreement with their results, and extend the possibility to much smaller mouse follicles.

Acknowledgments

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