Involvement of Meiotic Maturation of Oocytes in Promoting Granulosa Cell Proliferation

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Abstract. The effect of porcine oocytes on the proliferation of rat granulosa cells was studied in a heterologous culture system. Morphologically high quality oocytes showed a more potent stimulatory effect on DNA synthesis in granulosa cells than low quality oocytes. This stimulatory effect of porcine oocytes was enhanced by FSH and cAMP, but was unaffected by both transforming growth factor- β and tumor necrosis factor- α . Maturation of porcine oocytes *in vitro* attenuated the stimulatory effect of FSH and cAMP on granulosa cell DNA synthesis. In addition, rat oocytes also exhibited a similar facilitatory effect on DNA synthesis in rat granulosa cells. These results suggest that a soluble factor(s) secreted from oocytes, the secretion of which is dependent on their meiotic progression, can stimulate proliferation of rat granulosa cells in a heterologous culture system. The molecular nature of the factor(s) remains to be elucidated.

Key words: Oocyte, Granulosa cell, FSH, TGF- β , TNF- α .

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A specific role of the oocyte in the regulation of cumulus cell function has been established *in vitro* in several species of mammals [5, 27, 28, 36]. Cumulus cell expansion is induced *in vitro* by follicle stimulating hormone (FSH) mediated by a cAMP-dependent mechanism. Ovarian follicle development involves the interaction between growth and maturation of the oocyte and proliferation of the surrounding somatic cells in a concerted manner. Oocyte growth has been stimulated by the c-kit ligand that is produced by granulosa cells [14, 25]. Subsequently, maintenance

of the oocyte in the dictyorate stage of the first meiotic prophase is dependent on the balance between intracellular factors, such as cAMP, purine, and calmodulin, and intercellular communication with cumulus cells [12]. Granulosa cells have also been known to produce putative inhibitors and activators for the meiotic progress of mammalian oocytes, the identity of which have not been clearly established [6, 19].

According to studies by El-Fouly *et al.* [11], the removal of oocytes from Graafian follicles in rabbit ovaries induces luteinization of granulosa cells, and also impairs their ability to undergo cumulus expansion and to synthesize hyaluronic acid in

response to FSH or cAMP. Moreover, the existence of oocyte-derived-factors which are capable of acting on granulosa cells in different species has been proved in heterologous culture systems [26, 31, 33]. Soluble factors produced by the oocyte have been known to affect other parameters for cell differentiation in surrounding granulosa cells. Vanderhyden and co-workers [36, 37] have reported that when the oocyte is removed from the murine oocyte cumulus complex (COCs), the amount of estradiol production is decreased, whereas that of progesterone synthesis is enhanced. This effect can be reversed by the addition of the oocyte-conditioned-media. In addition, an oocyte-derived high-molecular weight factor inhibits FSH, which stimulates the production of tissue type plasminogen activated by cumulus and granulosa cells [7]. There are also some experimental data showing the stimulatory effect of the oocyte on the proliferation of granulosa cells and the maintenance of the structural organization of the ovarian follicle [18]. DNA synthesis is more active in granulosa cells closest to the oocyte than in those cells close to the basement membrane [18]. Furthermore, Vanderhyden *et al.* [35] have reported the existence of a mitogenic effect of murine oocyte-secreted factors on granulosa cells from preantral and antral follicles, but the identity of this factor(s) has not been fully identified. Dong *et al.* [9] also demonstrated that growth/differentiation factor-9, which is a member of the transforming growth factor superfamily and is expressed specifically in the oocyte [24], is required for early ovarian folliculogenesis. Although FSH plays a central role in controlling both differentiation and proliferation of granulosa cells, its actions are known to be regulated by intra-ovarian steroids and non-steroidal factors, acting as autocrine or paracrine modulators [17]. Furthermore, Bley *et al.* [2, 3] have demonstrated that in a rat granulosa cell culture system FSH exerted cAMP-mediated comitogenic effects like insulin-like growth factor-I (IGF-I) and sex steroids.

In our experiment, a heterologous coculture system was chosen for studying the interaction between FSH and porcine oocyte-derived factors in the regulation of granulosa cell proliferation in the rat.

Materials and Methods

Rat granulosa cell cultures

Ovaries were obtained from 24 to 25 days old female Sprague-Dawley rats after three days of diethylstilbesterol (DES) treatment (subcutaneous silastic implants containing 5 mg DES). Granulosa cells were prepared and cultured as previously described [1]. The purified granulosa cell layer was collected from the top of the Percoll solution and was suspended in DMEM: F 12 (1:1) modifying the concentration of bicarbonate (2.2 g/liter, pH 7.4). Cells were seeded on plastic 96-well plates precoated with rat tail collagen and the initial plating density was 3×10^5 viable cells/cm². The plated cells were maintained at 37 C with 5% CO₂. The media were changed to remove non-attached cells after two hours, and fresh media containing several samples, e.g. FSH, transforming growth factor- β (TGF- β) and tumor necrosis factor- α (TNF- α), for testing of their activity were added.

Hormones and chemicals

TGF- β from porcine platelets was purchased from R & D system (Minneapolis, MN), recombinant mouse TNF- α from Genzyme Diagnostics (Cambridge, MA), and radioactive [³H]-thymidine (4Ci/ml) from DuPont NEN Research Products (Boston, MA). DES, hyaluronidase, collagenase, Hoechst, and all other reagents were obtained from Sigma chemical Co. (St. Louis, MO). Collagen was prepared from rat tail.

Isolation of rat oocytes

Rat oocytes were isolated from ovaries of immature (25 days old) females. Ovaries were minced and incubated in porcine follicle fluid with pregnant mare's serum gonadotropin (pFF-PMSG) (5 IU/ml) containing collagenase (2.5 mg/ml) and 400 U/ml DNase. After 20 minutes incubation at 37 C under constant shaking, 11% BSA was added, and the mixture was filtered through a 200 μ m nylon mesh. The filtrate was centrifuged at 200 $\times$ g for 2 min and the pellet was suspended in pFF-PMSG with EGTA (2.4 mM). The suspension was centrifuged for 3 min, and the pellet was resuspended in pFF-PMSG. When the suspension was vortexed for 2 min, the zona pellucida free-oocytes were observed in the medium.

Isolation and in vitro maturation culture of porcine oocytes

Porcine ovaries were collected at a local slaughterhouse. The removed ovaries were transported to our laboratory at 30°C and washed three times with pre-warmed saline solution. Cumulus oocyte complexes were manually collected from 2 to 3 mm follicles with an 18 gauge needle fitted to a 5 ml syringe. Oocytes were classified as (A): cumulus oocyte complexes surrounded by 3 or more layers of high quality cumulus cells with homogenous cytoplasm, and (B) oocytes partially denuded or having a heterogenous cytoplasm [22]. A part of the oocytes were cultured for 24 hours in the presence or absence of a protein synthesis inhibitor cycloheximide (CHX). These oocytes were added to rat granulosa cell cultures to assess the effect of oocytes on DNA synthesis in granulosa cells.

Assays for DNA synthesis

Granulosa cells were cultured in 96-well plates in the presence of different hormones and growth factors. Tritiated thymidine (4 mCi/ml) was added to the cultures 2 hours later in glass hollow fibers with a multi-well cell harvester (Nunc, Denmark), and the radioactivity of [³H]-thymidine incorporated into DNA was measured for the determination of DNA synthesis in the culture after 72 hours stimulation.

Statistical analysis

Statistical analysis of the results was performed by one-way analysis of variance (ANOVA). Results are expressed as the mean $\pm$ SEM of triplicate cultures.

Results

Effects of porcine oocytes on DNA synthesis in rat granulosa cells

Rat granulosa cells were cocultured with freshly isolated porcine oocytes. Oocytes were classified in two groups according to the morphological appearance as described in Materials and Methods: high quality oocyte (group A) and poor quality oocyte (group B). As shown in Fig. 1, oocytes stimulated granulosa cell DNA synthesis in proportion to the number of oocytes in culture.

Fig. 1. Effects of porcine oocytes on DNA synthesis in rat granulosa cells. Granulosa cells were cocultured with increasing numbers of porcine oocytes. Oocytes were classified into two groups: (A) high quality oocytes and (B) poor quality oocytes. Twenty-four hours after plating, [³H]-thymidine incorporation was performed for 24 hours. Poor quality oocytes (group B) showed a lower stimulatory effect than high quality oocytes (group A).

Group B exhibited a lower potency in their stimulatory effect compared to group A ($P < 0.01$).

Effects of FSH and cAMP on DNA synthesis in granulosa cells

In Fig. 2, effects of FSH and cAMP on DNA synthesis in granulosa cells were examined in two cultures, one was coculture of granulosa cells with porcine oocytes and the other was granulosa cells alone. As shown in Fig. 2A, DNA synthesis in granulosa cells with porcine oocytes was increased in proportion to the amount of FSH added to the culture until its concentration reached an optimum (60 ng/ml). The addition of FSH did not affect DNA synthesis in granulosa cells without porcine oocytes in culture. Addition of either FSH or cAMP to the culture increased DNA synthesis by about two folds in granulosa cells cultured with porcine oocytes, while they did not affect DNA synthesis in granulosa cells cultured alone (Fig. 2B).

Fig. 2. Effects of FSH and cAMP on DNA synthesis in granulosa cells. (A) Granulosa cells were cultured with or without porcine oocytes (15 oocytes/well) in increasing concentrations of FSH. (B) Granulosa cells were cultured in control medium (control), or in media containing FSH or dibutyryl-cAMP. Twenty-four hours after plating, [³H]-thymidine incorporation was performed for 24 hours. The results are mean $\pm$ SEM of triplicate determinations (* P <0.05, ** P <0.01 vs. control).

Effects of TGF- β and TNF- α on DNA synthesis in granulosa cells

As shown in Fig. 3, addition of neither TGF- β nor TNF- α (5 and 10 ng/ml, respectively) affected DNA synthesis in granulosa cells irrespective of the presence of porcine oocytes.

Effects of maturation culture of oocytes on DNA synthesis in granulosa cells

DNA synthesis was increased most when the granulosa cells were cocultured with freshly collected porcine oocytes (Fig. 4). The maturing oocyte was the least effective in stimulating DNA synthesis in granulosa cells, while the CHX treatment partially restored its efficiency.

Effects of rat oocytes on DNA synthesis in rat granulosa cells

In the homologous culture system in which rat oocytes were added to rat granulosa cells, DNA synthesis was increased, as it was when porcine oocytes were added to the heterologous system (Fig. 5). Both FSH and TGF- β increased DNA synthesis in rat granulosa cells irrespective of the presence of rat oocytes. Simultaneous application of FSH and TGF- β synergistically increased the DNA synthesis by around two folds.

Fig. 3. Effects of TGF- β and TNF- α on DNA synthesis in granulosa cells. Granulosa cells were cultured with or without oocytes (15 oocytes/well) in control medium, or in media containing TGF- β (5 ng/ml) or TNF- α (10 ng/ml). Twenty-four hours after plating, the incorporation was carried out for 24 hours. The results are mean $\pm$ SEM of triplicate determinations (** P <0.01 vs. control).

Fig. 4. Effects of oocyte maturation on DNA synthesis in granulosa cells. Granulosa cells were cocultured with increasing numbers of freshly isolated oocytes (FIO), maturing oocytes (IVM), and oocytes treated with cycloheximide (CHX) (10 μ g/ml) for 24 hours. CHX was added to prevent spontaneous maturation, and later it was washed out. [3 H]-Thymidine incorporation values were mean $\pm$ SEM of triplicate cultures.

Discussion

In both the homologous and heterologous systems, we were able to demonstrate that oocytes could promote cell proliferation in granulosa cells. In addition, this effect of oocytes appeared to depend on the meiotic maturation of them. Porcine oocytes can be easily obtained, and their meiotic processes can be controlled partially *in vitro*. The defined culture system for rat granulosa cells used in this study has been extensively characterized in terms of the distinct reactions against hormones and growth factors [1–3, 30]. This inter-species system may offer substantial advantages over other models requiring micromanipulation to separate the oocyte from the cumulus oophorus, and the use of serum supplemented in the culture media. The stimulatory effects of freshly isolated porcine oocytes were revealed more clearly when oocytes were of a high morphological quality. Vanderhyden and co-workers [35] have reported that mouse oocytes promote proliferation of granulosa cells from both preantral and antral follicles. They have also found out that oocyctomy resulted in a decrease of follicle cell proliferation, and that addition of medium

Fig. 5. Effects of rat oocytes on DNA synthesis in rat granulosa cells. Granulosa cells were cultured with or without rat oocytes (25 oocytes/well) under control condition (control), with FSH (20 ng/ml), TGF- β (5 ng/ml), and a combination of them. Twenty-four hours after plating, [3 H]-thymidine incorporation was performed for 24 hours. Values are mean $\pm$ SEM of triplicate culture (* P <0.05, ** P <0.01 vs. control).

conditioned by oocytes restored follicle cell proliferation at least to the level that the intact oocyte follicle promotes.

Even with a different experimental scheme, the same result was drawn in immature rat granulosa cells cultured under defined conditions in either a homologous or heterologous system. Furthermore, we have demonstrated that DNA synthesis in rat granulosa cells stimulated by porcine oocytes was increased two-fold by adding FSH to the culture. A similar effect was also observed in culture after addition of gonadotropin with sex steroids, IGF-I or TGF- β [2, 3] in the same experimental model, and it indicates that physiological concentrations of FSH are required for its mitogenic effect. The effects of the oocyte on granulosa cell proliferation was enhanced by FSH, and it could be reproduced by cAMP. This result suggests that the oocyte factor(s) acts either independently or downstream from FSH induced cAMP production.

Both TGF- β and TNF- α are known to be present in oocytes in heterologous and homologous coculture systems [8, 16, 23], and they are also present in oocytes to modulate rat granulosa cell DNA synthesis [10, 21]. Moreover, TGF- β 1 has been known to reproduce the effect of the soluble factor(s) elicited by the oocyte in the synthesis of

hyaluronic acid stimulated by FSH in cumulus cells [27, 32]. We have demonstrated, however, that the growth factor and cytokine are not responsible for the stimulatory action of oocytes in our heterologous culture system.

Salustri *et al.* [28] have reported a decrease in stimulating activity by oocytes for hyaluronic acid synthesis in reculture. In this study, the decrease in stimulatory activity of recultured oocytes could be prevented by the addition of cycloheximide, a protein synthesis inhibitor and known blocking agent of meiotic maturation of porcine oocytes [20]. It is therefore suggested that the decrease of stimulatory activity of the oocyte was associated with progression of meiosis. Previous studies have reported that the secretion of paracrine factors by the oocyte is regulated in the developmental

process [4, 13, 29, 34]. Furthermore, Eppig *et al.* [15] have recently reported that mature oocytes suppress production of luteinizing hormone receptor mRNA in murine granulosa cells. The ability of the active factor(s) produced by the GV stage oocyte might be a consequence of its chemical nature and/or its active degradation by granulosa cells. This ability would be consistent with the expected transient nature of the signal mediated by this putative factor and the restriction of its range of cumulus cells [15].

In conclusion, our results indicate that a soluble factor from oocytes, the secretion of which is dependent on meiotic progression, can stimulate proliferation of rat granulosa cells in a heterologous culture system.

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