ORIGINAL RESEARCH

Luteal macrophage conditioned medium affects steroidogenesis in porcine granulosa cells

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Received 1 August 2010; accepted 5 April 2011

SUMMARY
The purpose of the study was to examine the effect of luteal macrophage conditioned medium (LMCM) on progesterone and estradiol production by cultured granulosa cells. Porcine granulosa cells were cultured for 48 h with or without LMCM in the absence or presence of 100 ng/ml LH, FSH or prolactin. Progesterone and estradiol concentrations were measured by radioimmunoassay. Granulosa cells were analyzed histochemically and immunocytochemically for the activity and presence of Δ5, 3β-hydroxysteroid dehydrogenase (3β-HSD), respectively. LMCM stimulated basal and LH-, FSH- or prolactin-induced progesterone secretion. Similarly, LMCM augmented basal and stimulated activity of 3β-HSD in the examined cells. In contrast, LMCM decreased LH- and prolactin-induced estradiol secretion but increased FSH-induced estradiol secretion. These data demonstrate the clear stimulatory effect of LMCM on granulosal progesterone production. It is concluded that substances secreted by macrophages modulate gonadotropin effect on follicular progesterone secretion in a paracrine manner *via* 3β-HSD activity. *Reproductive Biology* ***2011*** *2: 117 – 134.*

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Key words: granulosa cells, luteal macrophage conditioned medium (LMCM), gonadotropins, Δ5, 3β-hydroxysteroid dehydrogenase

INTRODUCTION

Although gonadotropins, and to some extent prolactin, are considered to be predominant regulators of follicular development [6, 37] it is now clear that there is a wide range of paracrine factors modulating gonadal function. These factors may amplify or attenuate the effects exerted by gonadotropins [23, 24]. The participation of the immune system in the regulation of ovarian functions has been recognized. Leukocyte populations are present within the ovary and they constitute potential *in-situ* modulators of ovarian functions [9, 10]. Macrophages form an ovarian leukocyte population, which is well characterized in many species [39], and infiltrate the ovary in a cyclic manner. Ovarian macrophages were found in theca and stroma cells with maximum levels reported before the preovulatory LH surge. In addition, their presence was demonstrated in granulosa cells (GC) of atretic follicles [15]. Macrophages have also been found in mid and late luteal corpora lutea (CL) of pigs [21, 40]. They express over one hundred various membrane receptors on their surface, which enables them to interact with both surrounding tissues and other immune cells [11]. Macrophages secrete a diverse range of cytokines, including interleukin (IL) -1, -6, -10 and -12, interferon-α (INF-α), tumor necrosis factor- α (TNF-α), and granulocyte macrophage-colony stimulating factor (GM-CSF). These cytokines have been identified in the ovary of many species and are known to impact many aspects of ovarian functions including follicle growth and differentiation, ovulation, CL formation and luteal function [7, 28, 29, 32]. Despite the extensive research on communication between the endocrine and immune systems, there is little information concerning the nature of this dialogue. In this experiment, we investigated whether the hormonal activity of granulosa cells is regulated by products secreted by luteal macrophages. We examined the effect of luteal macrophage conditioned medium (LMCM, a mixture of macrophage products) on basal and pituitary hormone-stimulated progesterone and estradiol secretion by granulosa cells isolated from porcine medium follicles. Moreover, the effect of LMCM on

the localization and activity of Δ5, 3β-hydroxysteroid dehydrogenase (3β-HSD), a key steroidogenic enzyme, was also investigated in these cells.

MATERIALS AND METHODS

Animals

Porcine ovaries were obtained from Polish landrace sows at a local slaughterhouse and placed in a cold phosphate-buffered saline (PBS; pH 7.4, Laboratory of Sera and Vaccines, Lublin, Poland) containing penicillin (100 IU/ml; POLFA Tarchomin SA, Tarchomin, Poland). Ovaries were transported to the laboratory within 30 minutes and rinsed twice with sterile PBS supplemented with antibiotics. To study the possible interactions between granulosa cells and luteal macrophages, granulosa cells were collected from the same ovaries as corpora lutea. In each experiment, ten ovaries from five animals were selected for cell isolation. Assuming that each ovary yielded 3-5 follicles as well as CL, the total number of follicles and CL in one experiment varied from 30 to 50. The phase of the estrous cycle was determined according to the established morphological criteria [19]. Medium follicles (6-8 mm in diameter) and CL from the midluteal phase (10-14 days after ovulation) were selected for cell cultures. This procedure has been chosen to minimize the variability between tissues and animals.

Granulosa cell isolation and cell culture

The isolation of GC was performed according to the technique developed in our laboratory [20]. Granulosa cells were scraped from the follicular wall with round tip-ophthalmologic tweezers. After collection, GC were washed several times in PBS and recovered by low speed centrifugation (90×g for 10 min). Cell viability was tested by the trypan blue exclusion test (mean± SD: 91%±4%). The cells were seeded in 24-well culture plates (Nunc, Kalmstrup, Denmark) at an initial density of 5×10^5 cells/ml (for immunocytochemistry or histochemistry) or in 6-well culture plates (Nunc, Kalmstrup, Denmark) at an initial density of 9×10^5 cells/ml (for Western blot). Control cultures were carried in M199 medium supplemented with 5% calf serum and penicillin (100 IU/ml), in a humidified atmosphere of 95% air :5% CO_2 at

37ºC, in the presence or absence of LH, FSH (100 ng/ml, a gift from NIH, Bethesda, MD, USA) or prolactin (100 ng/ml, ovine, Sigma Chemical Co., St. Louis, MO, USA). Experimental cultures were carried out in M199 medium supplemented with LMCM, 1:1 (v/v) prepared previously, with the addition of hormones listed above. After 48 h of culture, all media were collected and stored at -20ºC for radioimmunoassay of estradiol and progesterone. All experiments were performed in quadruplicate (four wells) in three separate cultures (n=3 independent experiments). In the immunocytochemistry and histochemistry experiments, each well was equipped with a round coverslip.

LMCM preparation

LMCM was prepared from CL of the midluteal phase (10-14 day after ovulation) originating from the same ovaries that were used for GC isolation [18]. Briefly, luteal tissue was minced mechanically and exposed to trypsinization for 10 min with 6-7 ml of 0.25% trypsin (Laboratory of Sera and Vaccines, Lublin, Poland) in PBS at 37ºC. Subsequently, the luteal cells were separated by decantation and the procedure was repeated three times. Finally, the cells were spun and resuspended in M199 medium (Laboratory of Sera and Vaccines, Lublin, Poland) supplemented with 5% calf serum to yield a suspension of 3.5×10^5 cells/ml medium. Cell viability was assessed by trypan blue exclusion test (mean± SD: 89%±4%). Cells were grown in 6-well plates (Nunc, Kalmstrup, Denmark) for 2 h in a humidified atmosphere containing 5% CO_2 in air. This allowed us to separate the luteal cells from luteal macrophages due to the ability of the latter to attach rapidly to the bottom well. Following these 2 h, the unattached cells (luteal cells) were removed and the remaining adherent cells (luteal macrophages) were cultured for 24 hours. Then, the media were collected and treated with charcoal (10 min, 4ºC) to remove endogenous steroids. After final centrifugation (1600×g, 15 min, 4ºC), the LMCM was stored at -20ºC for further experiments with granulosa cells.

Identification of macrophages

Macrophages were identified by: 1/ their rapid attachment to bottom wells, 2/ their typical macrophage morphology visualized

by May-Grunwald Giemsa staining, 3/ histochemical test for non-specific esterase, characteristic for macrophages, 4/ pinocytosis using Oil red test, and 5/ phagocytosis using Latex granule test (Sigma Chemical Co., St. Louis, MO, USA; [5]).

Cytokine content in LMCM

The Cytometric Bead Array (Human Inflammation kit, CBA; BD Biosciences) was used to study cytokine content in LMCM. To simultaneously detect IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α in LMCM, human inflammation kit was used according to the manufacturer's instructions.

Flow cytometric analysis was performed using a FACScan cytometer (FACSCaliburTM flow cytometer, BD Biosciences). Data were acquired and analyzed using CBA software (BD Biosciences). The sensitivities of the CBA for IL-1β, IL-6, IL-8, IL-10, TNF-α and IL-12p70 were 5.0, 17.5, 52.7, 2.5, 7.3, and 10.7 pg/ml, respectively. The concentrations of IL-1β, IL-6, IL-8, IL-10, TNF-α and IL-12p70 in LMCM were computed in pg/ml medium and were expressed as means±SEM.

Immunocytochemistry

The cells were fixed in 2% paraformaldehyde, permeabilized with 0.1% TritonX-100 in Tris-buffered saline (TBS; 0.05 M Tris-HCl plus 0.15 M NaCl, pH 7.6). To quench endogenous peroxidase activity, cells were treated for 30 min with 0.1% H_2O_2. Non specific binding was blocked by incubation for 30 min with 5% normal goat serum. Then, cells were incubated overnight at 4ºC with a polyclonal anti-mouse 3β- HSD (gift from prof. A. H. Payne from Stanford University) at a dilution 1:1000. Afterwards, the cells were intensively washed in TBST (TBS plus 0.1% Tween 20) and incubated for 1.5 h at room temperature (RT) with biotinylated goat anti-rabbit antibodies (1:300; Vector Lab., Burlingame CA, USA) followed by washing with TBST and incubated at RT for 1 h with avidin-biotin-peroxidase complex (1:1:100; Strept ABC complex/HRP, DAKO/AS, Glostrup, Denmark). The color reaction was performed using Stable DAB solution (Research Genetics, Inc., Huntsville AL, USA) for 4 min. For negative control the primary antibody was omitted. The

intensity of 3β-HSD immunostaining varied among the cells and was evaluated as weak (+), moderate (++) or strong (+++) after visual examination of cytoplasmic localization of the antigen.

Western blot analysis

After 48 h of incubation, GC were washed twice with ice-cold saline, and then proteins were extracted with 50 µl of radioimmunoprecipitation assay buffer (RIPA; Thermo Scientific, Inc. Rockford IL, USA) in the presence of a protease inhibitor cocktail (Sigma Chemical Co., St Louis, Missouri, USA). Protein concentration was determined with Bradford reagent (Bio-Rad Protein Assay; Bio-Rad Laboratories GmbH, München, Germany) using bovine serum albumin as a standard. Aliquots (50 µg protein) of cell lysates were solubilized in a sample buffer consisting of 62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol (Bio-Rad Laboratories, GmbH, München, Germany) and heated for 3 min at 99.9°C. After denaturation, samples were separated *via* 12% SDS-polyacrylamide gel electrophoresis under reducing conditions according to Laemmli [27]. Separated proteins were transferred onto a nitrocellulose membrane using a wet blotter in the Genie Transfer Buffer (20 mM Tris, 150 mM glycine in 20% methanol, pH 8.4) for 90 min at a constant voltage of 135 V. After overnight blocking with 5% non-fat milk in TBS, 0.1% Tween 20 (dilution buffer) at 4°C with gentle shaking, the membranes were treated with the primary antibody (for details see immunohistochemistry subchapter: anti 3β-HSD, dilution 1:8 000) for 1.5 h at room temperature (RT). The membranes were washed and incubated with a secondary antibody conjugated with the horseradish-peroxidase labeled goat anti-rabbit IgG (Vector Labs., Burlingame, CA, USA; dilution 1:1000) for 1 h at RT. The presence of 3β-HSD was visualized as follows: the nitrocellulose membrane was incubated in a substrate solution containing 3'3'-diaminobenzidine (0.5 mg/ml; Sigma) in TBS. The membrane was dried and then scanned using an Epson Perfection Photo Scanner (Epson Corporation, CA). Molecular masses were estimated by reference to standard proteins (Standards Low Range; Bio-Rad

Laboratories, GmbH, München, Germany). Western blot analysis was repeated three times.

Histochemistry

In order to demonstrate the activity of Δ5, 3β-HSD in granulosa cells, a histochemical test was used [13]. Shortly, the cells were incubated in a medium containing dehydroepiandrosterone (DHEA: Sigma Chemical Co., St. Louis, MO, USA) as a substrate dissolved in dimethylformamide (DMF: Serva Feinbiochemica, GmbH, Heidelberg, Germany), nitro blue tetrazolium (NBT: Sigma Chemical Co., St. Louis, MO, USA) as dye and NAD (Boehringer Mannheim GmbH, Mannheim, Germany) as the cofactor of the reaction. Histochemical reaction resulted in forming formazan deposits (dark blue granules in the cytoplasm of GC). In each well, 3×100 cells were investigated microscopically by two independent blinded evaluators. The percentage of cells exhibiting one of the three intensity grades: weak (+), moderate (++) and strong (+++) and reflecting the strength of the histochemical reaction was calculated. In order to rule out a nonspecific reaction, parallel monolayers were incubated in the control medium without the substrate.

Radioimmunoassay

Samples of the culture medium were analyzed for estrogen and progesterone content using radioimmunoassay. Estrogen medium concentration was assessed using 2,4,6,7-^{3}H-estradiol (New England Nuclear, [22]) as a tracer (spec. act. 140 Ci/mmol) and antibodies raised in a rabbit against estradiol-17β-6-oxime-BSA (a gift from prof. Brian Cook, University Glasgow, Scotland). The antibodies cross-reacted with estradiol (100%), estrone (66%) and estriol (only 2.1%). The cross-reactivity of other steroids was lower than 0.1%. The assay sensitivity was 5 pg/tube. Coefficients of variation within and between assay were 3.5% and 7.5%, respectively. Progesterone was assessed using 1,2,6,7-^{3}H-progesterone (Amersham, Denmark; [1]) as a tracer (spec. act. 96 Ci/mmol) and antibodies raised in a sheep against 11β-hydroxyprogesterone-BSA (a gift from prof. B. Cook, University Glasgow, Scotland). The progesterone antibodies cross-reacted with pregnenolone (1.9%), corticosterone (1.5%), 17α-hydroxyprogesterone (0.8%), testosterone (0.12%) and androstendione (0.02%). The cross-reactivity of other steroids was lower than 0.1%. The assay sensitivity

was 50 ng/tube. Coefficients of variation within and between assays were 5% and 9.8%, respectively.

Statistical analysis

All radioimmunological data are expressed as means±SEM from three independent experiments (n=3). Each experiment was performed in quadruplicate, resulting in twelve observations. Significant differences in the steroid medium concentration between the control and LMCM-treated cells were assayed by Student t-test. $P<0.05$ were considered statistically significant.

RESULTS

Pro-inflammatory cytokine medium concentration

Concentrations of cytokines in LMCM were as follows - IL-1β: 175.3±0.5; IL-6: 2±0.3; IL-8: 42.0±1; IL-10: 2.2±0.3; IL-12p70: 2±0.3, TNF-α: 126.6±0.6 pg/ml.

Morphology of granulosa cells *in vitro*

All granulosa cells grown for 48 h formed a monolayer attached to the culture dishes. However there were differences in the rate of formation of GC monolayer either in control or LMCM-treated cells. The cells growing in the M199 medium (control cells, without LMCM) required a longer time to form a monolayer. These cells formed only a few short processes compared with numerous and long processes formed by those cultured in the presence of LMCM.

Immunolocalization of 3β-HSD

The presence of 3β-HSD was detected in all granulosa cell cultures (tab. 1). 3β-HSD was localized only in the cytoplasm. A strong immunoreaction was observed in FSH- and LH-treated granulosa cells. In contrast, prolactin reduced the intensity of 3β-HSD immunoreaction. LMCM increased 3β-HSD immunostaining in basal and prolactin-treated granulosa cells, but did not amplify the effect of pituitary hormones.

Table 1. Evaluation of the intensity of 3β-HSD immunocytochemical reaction

	Control	LH	FSH	Prl
CM	+ +	+ + +	+ + +	+
LMCM	+ + +	+ + +	+ + +	+ +

3β-HSD immunoreactivity was designated as weak (+), moderate (+ +) and strong (+ + +). 3β-HSD: Δ5, 3β-hydroxysteroid dehydrogenase, CM: control medium, LMCM: luteal macrophage conditioned medium, Prl: prolactin

To provide further evidence that granulosa cells express 3β-HSD protein and to confirm the specificity of the antibody used, Western blot analysis was performed. The presence of 3β-HSD was demonstrated in all control and LMCM-treated cells (fig. 1).

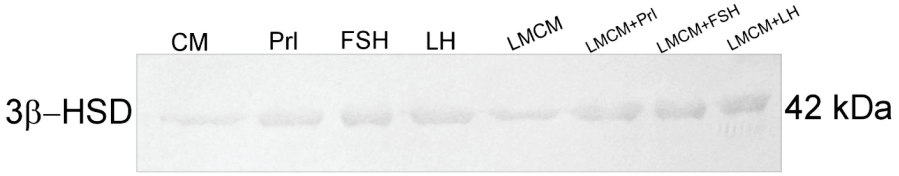

Figure 1. Representative Western blot analysis of Δ5, 3β-hydroxysteroid dehydrogenase (3β-HSD) protein expression levels in granulosa cell lysates. Control and treated granulosa cells were incubated in control medium (CM) or luteal macrophage conditioned medium (LMCM); Prl: prolactin.

Activity of 3β-HSD

After 48 h of culture, control and prolactin-treated granulosa cells exhibited similar low activity of 3β-HSD (strong color intensity: 10% of cells; fig. 2). LH and FSH increased the basal enzyme activity (strong color intensity: 40%). Sixty percent of granulosa cells treated with LMCM exhibited either strong or moderate activity of 3β-HSD. LMCM further increased 3β-HSD activity in granulosa cells supplemented with LH (strong color intensity: 90% of cells), FSH (strong color intensity: 90% of cells) and prolactin (strong color intensity: 20% of cells).

Progesterone and estradiol medium concentration

LMCM stimulated basal (1.9-fold) and gonadotropin-stimulated (1.6-fold) progesterone secretion by porcine granulosa cells. The

highest increase (3-fold) in progesterone secretion was observed after concomitant treatment with LMCM and prolactin ($p<0.05$, fig. 3).

LMCM did not affect basal estradiol secretion by porcine granulosa cells. In contrast, LMCM decreased LH or prolactin-stimulated estradiol secretion (1.5-fold). In turn, in FSH-treated cells, LMCM increased (1.1-fold) estradiol secretion ($p<0.05$; fig. 3).

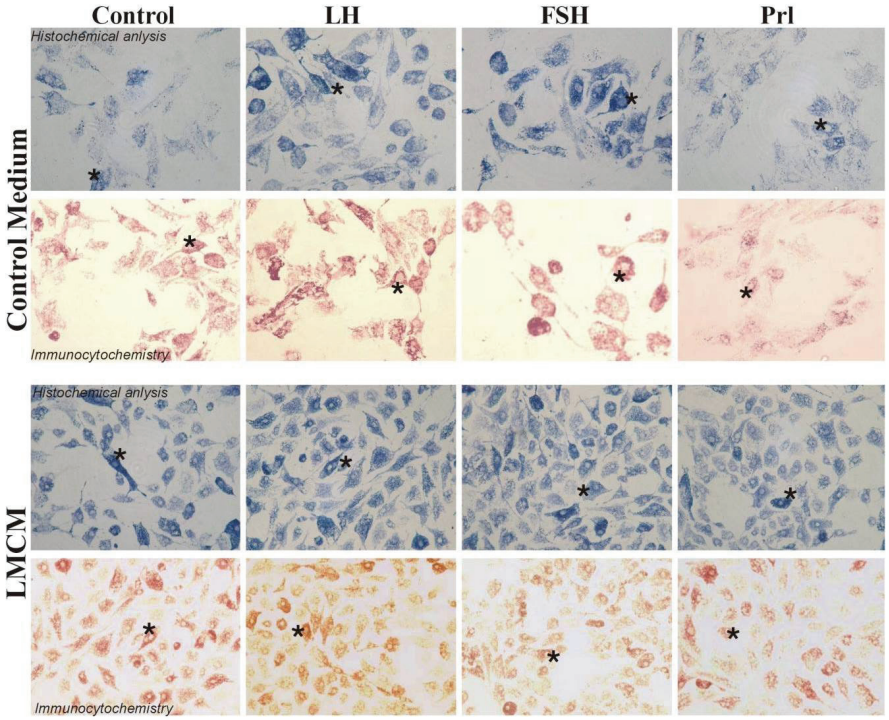

Figure. 2. Histochemical analysis of Δ5, 3β-hydroxysteroid dehydrogenase (3β-HSD) activity and immunocytochemical localization of 3β-HSD in porcine granulosa cells *in vitro*. The first column shows granulosa cells without addition of gonadotropins or prolactin (Prl), the next columns show granulosa cells stimulated by LH, FSH and prolactin, respectively. Granulosa cells were incubated in control medium (CM) or luteal macrophage conditioned medium (LMCM). Asterisks indicate an exemplary positive staining (magnification ×100 – photos CM; ×40 - photos LMCM).

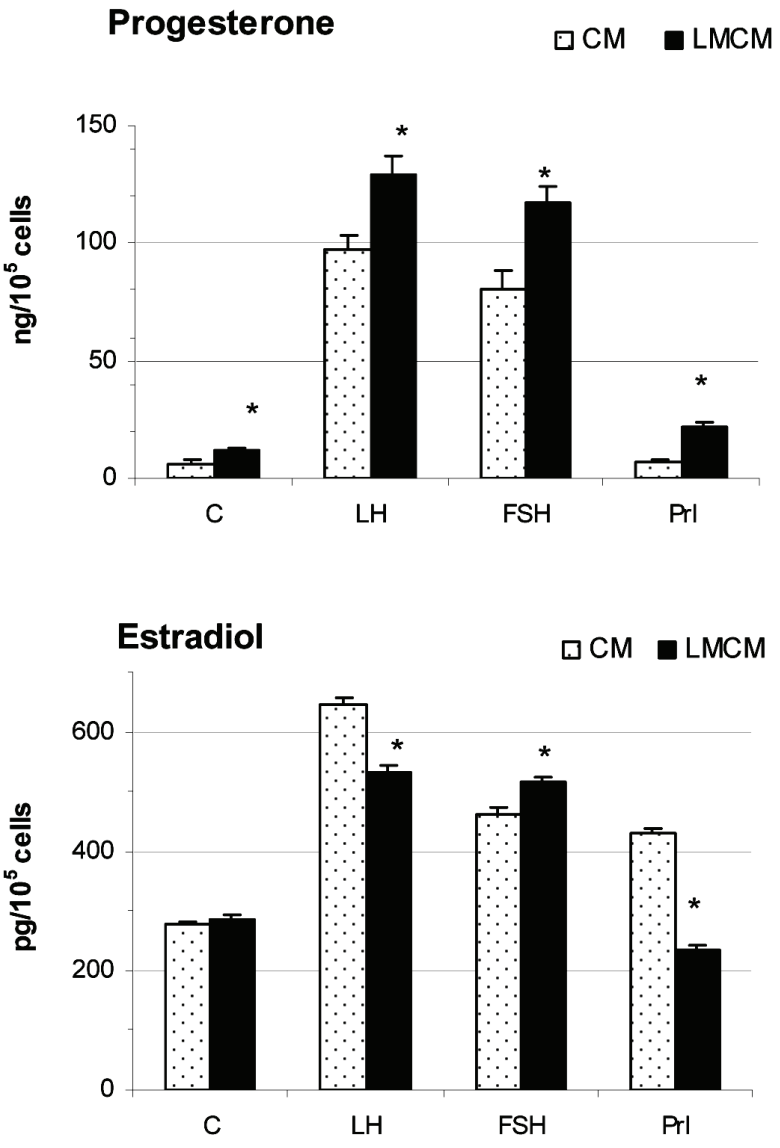

Figure. 3. Progesterone and estradiol (mean±SEM) secretion by granulosa cells cultured for 48 h (n=3 independent experiment). The open bars represent cells cultured in control medium (CM), the shaded bars represent cells cultured in luteal macrophage conditioned medium (LMCM). The statistical differences between control and LMCM-treated cells were determined by Student t-test. Asterisks indicate statistically significant differences (*$p<0.05$).

DISCUSSION

The results of the study demonstrate that LMCM modulated progesterone and estradiol secretion by porcine granulosa cells. LMCM appeared to play an important role in the mechanism that regulates the activity of 3β-HSD. Our data indicate that LMCM may be either stimulatory or inhibitory depending on the presence of other regulators of ovarian function.

Macrophages constitute major a cellular component of the immune system within the ovary, and macrophage-generated cytokines have been implicated in the physiology and pathology of the ovary [30]. Resident macrophages may act *in situ* as regulators of ovarian function involved in paracrine intraovarian interactions. Follicular atresia is a key phenomenon by which the ovary eliminates follicles that will not ovulate, and is apparently initiated by apoptotic death of granulosa cells followed by degeneration of the oocyte at the last stages of atresia [3]. It is thought that ovarian macrophages use phagocytosis to remove apoptotic granulosa and luteal cells [25] thereby contributing to the processes of follicular atresia and luteolysis, respectively.

In the mouse ovary, macrophages are present over the course of the estrous cycle. The number of macrophages appearing in the corpora lutea and atretic follicles suggest a role for macrophages in CL differentiation and follicular atresia. The pattern of their distribution during proestrus and metestrus within micro-environmental compartments suggests a functional association with the events of ovarian development [31]. Macrophages may play a role in the control of dynamic expression of LH receptor (LHR) in different compartments of the ovary. It has been shown that the acquisition of LHR in theca cells represents a key event in ovarian physiology, because it may influence selection of developing follicles. IL-1, a product of macrophages, inhibits FSH-induced development of LHR in undifferentiated granulosa cells [17]. Therefore, it is possible that thecal macrophages serve as intraovarian centers for the inhibition of premature follicular luteinization during follicular development. Macrophages through their multifunctional cytokines may also play a role in ovulation [8].

Macrophage conditioned medium is a mixture of various macrophage secretory products partially characterized in our study.

We found that IL-1 and TNF-α were present in LMCM in higher concentrations than other cytokines. The secretory products present in LMCM may affect granulosa cell function dependently on the amount and activity of each particular component of the mixture. In the present study, we demonstrated that granulosa cells treated with LMCM flattened and easily formed the monolayer, while cells cultured without LMCM grew and flattened more slowly. Katabuschi et al. [26] showed the positive effect of macrophages on granulosa cell proliferation ratio using gonadotropin-primed immature female rats and osteopetrotic mice, a model defective in monocyte- macrophage lineage cells. Moreover, Fukumatsu et al. [14] demonstrated that co-culture of rat granulosa cells with macrophages had a stimulatory effect on granulosa cells proliferation. These findings suggest that macrophages located in the developing follicles participate in promotion of granulosa cell growth through a paracrine mechanism.

We also demonstrated that LMCM increased basal progesterone secretion by porcine granulosa cells. This stimulatory effect of LMCM was even more pronounced in the presence of gonadotropins or prolactin. Our results are consistent with those of Baratta et al. [4] who had reported that TNF-α and IGF-1 increased progesterone production by granulosa cells isolated from large mature bovine follicles. In contrast, peritoneal macrophages inhibited gonadotropin-induced progesterone secretion by cultured rat granulosa cells [34]. It is possible that the macrophage-induced effects are species dependent.

The increased secretion of progesterone demonstrated in LMCM-treated porcine granulosa cells may result from LMCM effect on 3β-HSD activity. In fact, we found that LMCM exerted stimulatory effect on basal and gonadotropin/prolactin-stimulated activity of the enzyme. Similarly, LMCM increased the basal and prolactin-induced expression of 3β-HSD protein. Due to the strong expression of the enzyme in LH- and FSH-treated cells, the immunoexpression of 3β-HSD was not further augmented by LMCM in this study. It seems that the cellular presence of an enzyme may not entirely reflect its activity [38]. Thus, to confirm the LMCM effect on progesterone secretion, in addition to steroid content measurement we have analyzed immunolocalization as well as activity of 3β-HSD.

There are several reports suggesting that cytokines, especially IL-4, may regulate the expression of 3β-HSD gene in animals and

humans [36]. It has been shown that IL-4 and IL-13 induce 3β-HSD type I gene expression in ZR-75-1, T47-D, MDA-MB-231 breast cancer cell lines as well as in normal human mammary and prostate epithelial cells in primary culture [16]. Unfortunately, the content of IL-4 and IL-13 in LMCM was not determined in this study.

In contrast to its effect on progesterone secretion, LMCM did not change the basal estradiol secretion by porcine granulosa cells. However, LMCM decreased LH- and prolactin-induced and increased FSH-induced estradiol secretion by the cells. A stimulatory effect of cytokines on estradiol secretion was shown in granulosa cells derived from immature rat ovaries [33]. Shores and Hunter [35] also described a stimulatory effect of macrophage products on estradiol secretion by porcine theca cells. On the other hand, cytokines decreased basal and FSH-stimulated estradiol secretion by human and rat granulosa cells [2, 12]. From data collected in this study it appears that LMCM had a modulatory effect on basal and stimulated estradiol secretion. This effect depends probably on the species and maturational status of an animal.

The presented results showed the stimulatory effect of macrophages' secretory products on the activity and immunolocalization of 3β-HSD as well as on progesterone secretion by porcine granulosa cells. The study provided evidence that LMCM substantially modulated granulosa cell steroidogenesis reflecting complex intra-ovarian interactions between the immune and endocrine systems.

ACKNOWLEDGEMENTS

This research was supported by UJ/IZ/BW/49/ 2006 grant.

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