***In Vitro* Differentiation of Bovine Granulosa Cells Obtained from Small Antral Follicles**

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Abstract. The present study was undertaken to investigate whether bovine granulosa cells obtained from small antral follicles could differentiate into luteal-like cells *in vitro*. Granulosa cells obtained from small antral follicles (3–5 mm in diameter) were cultured up to 9 days in the presence or absence of insulin (2 $\mu\text{g}/\text{ml}$), forskolin (10 μM) and insulin (2 $\mu\text{g}/\text{ml}$) plus forskolin (10 μM). On days 1, 3, 5, 7 and 9 of culture, spent media were harvested for hormone assays. Progesterone production in the granulosa cells incubated with insulin plus forskolin increased continuously during the culture period. On the other hand, estradiol-17 β production in the granulosa cells sharply decreased on day 3, and then continued to decrease. The size of granulosa cells cultured with insulin plus forskolin for 9 days was significantly larger than that of granulosa cells at the beginning of culture ($P < 0.001$), and the size was similar to that of large luteal cells. Collectively, these data suggest that bovine granulosa cells obtained from small antral follicles can differentiate into large luteal-like cells during culture in the presence of insulin and forskolin.

Key words: Bovine, Granulosa cells, Small follicles, Luteinization.

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The corpus luteum of several mammalian species contains two distinct luteal cell types, small and large cells [1–3]. Previous studies indicated functional differences in responses to various agents between these types of cells. It has been demonstrated that bovine and ovine small cells are extremely sensitive to added LH, while large cells from both species are relatively insensitive to LH [4, 5]. Bovine large luteal cells are also insensitive to PGF $_2\alpha$ and phorbol ester, the activator of phospholipid-dependent calcium-activated protein kinase, although these agents stimulate progesterone production by small cells [4]. These results were obtained from studies that employed enzymatically dissociated luteal cells separated by density gradient [6], elutriation centrifugation [7]

or flow cytometry combined with density gradients [4]. These methods yield luteal cells with variable degrees of homogeneity (60–95%).

Recently, Meidan *et al.* [8] have demonstrated a different approach to obtain separated luteal cells. They cultured the bovine theca and granulosa cells collected from large, preovulatory follicles. Theca and granulosa cells cultured in the presence of insulin plus forskolin could be differentiated into small and large luteal-like cells, respectively. This new method can be considered as a simple and convenient way to investigate the functions of small and large luteal cells. However, it is not easy to obtain preovulatory follicles at any given time, because not many cows processed in local abattoirs have ovaries with preovulatory follicles.

Thus, this study was undertaken to investigate whether bovine granulosa cells obtained from small antral follicles, which can be easily obtained at a

local abattoir, differentiate into luteal-like cells *in vitro*. Luteinization was assessed by morphological and functional criteria.

Materials and Methods

Cell preparation

Bovine ovaries were obtained at a local abattoir within 30 min of slaughter and transported to the laboratory in 0.9% (w/v) NaCl at 30–37 °C in a thermos flask. Granulosa cells in follicular fluid were aspirated from small antral follicles (3–5 mm in diameter) with an 18-gauge needle fixed to a 10-ml disposable syringe. After the removal of oocytes and cumulus cells with a fine glass pipet under a dissecting microscope, granulosa cells were filtered through metal meshes (150 $\mu\text{m} \times 3$, 80 $\mu\text{m} \times 1$). The granulosa cells were centrifuged (200 $\times$ g, 5 min at 4 °C), decanted, and resuspended in Dulbecco's Modified Eagle's Medium (DME; Sigma Chemical Co., St. Louis, MO, USA, #D1152; with 25 mM HEPES and 0.1% BSA) three times. The cells were then centrifuged again and resuspended in a suitable volume of culture medium (see below), counted with a hemocytometer and assessed for viability by trypan blue dye exclusion. The culture medium was DME/Ham's Nutrient Mixture F12 (1:1; Sigma #D8900) containing 10% calf serum (Sigma, #C6278), 20 $\mu\text{g}/\text{ml}$ gentamicin (Gibco BRL, Gaithersburg, MD, USA, #600-5750AD), 1 μM testosterone (Sigma, #T1500) and 0.5 mM ascorbic acid (Wako Pure Chemical Industries, Ltd, Osaka, Japan, #013-12061).

Cell culture and experimental design

Granulosa cells were cultured in culture media with or without 10 μM forskolin (Research Biochemicals International, Natick, MA, USA, #70-0501-05), 2 $\mu\text{g}/\text{ml}$ insulin (Sigma, #I4011) and insulin (2 $\mu\text{g}/\text{ml}$) plus forskolin (10 μM) at the concentration of 2×10^5 viable cells/ml/well (37.5 °C, 5% CO_2 in air) for 9 days. The culture medium was replaced with fresh media with or without the above supplements on days 1, 3, 5, 7 of culture. At the time of each replacement, spent media and cells were harvested and kept at -20 °C until the progesterone, estradiol-17 β and DNA assays.

Enzyme immunoassay

Concentrations of progesterone and estradiol-17 β in the culture media were determined directly with an enzyme immunoassay (EIA), that was modified from the original method [9]. For the progesterone determination, progesterone 3- (O-carboxymethyl) oxime linked to peroxidase type VI through a 6-aminocaproic acid spacer as a tracer (Sigma, #P3659; 1 : 120,000 final dilution) and anti-progesterone serum (OK-1) were used. The final antibody dilution was 1 : 200,000. The standard curve ranged from 0.39 ng/ml to 100 ng/ml and the effective dose for 50% inhibition (ED_{50}) of the assay was 12.0 ng/ml. Cross-reactivities of OK-1, validated by comparing the inhibition of binding of peroxidase-labeled progesterone to OK-1, were 100% progesterone, 7.1% 5 α -pregnanedione, 2.4% pregnenolone, 1.0% 17 α -hydroxy-progesterone, 0.7% 20 α -hydroxy-progesterone, 1.2% deoxycorticosterone, 0.3% corticosterone, < 0.1% estradiol-17 β , 0.1% testosterone and < 0.1% androstenedione. The intra- and inter-assay coefficients of variations were 4.7% and 14.3%, respectively. For estradiol-17 β measurement, 17 β estradiol 17-hemisuccinate linked to peroxidase type VI through a 6-aminocaproic acid spacer was used as a tracer (1 : 20,000 final dilution). Anti-estradiol serum was purchased from Medical System Service Kanagawa Co. Ltd. (Kanagawa, Japan). The final antibody dilution was 1 : 40,000. The standard curve ranged from 0.8 pg/ml to 200 pg/ml and the ED_{50} of the assay was 23.2 pg/ml. The intra- and inter-assay coefficients of variations were 2.4% and 2.7%, respectively.

DNA estimation and calculation of cell number

To estimate DNA content, cells were detached by three freeze-thaw cycles using liquid nitrogen and a 40 °C water bath, and then sonicated briefly. DNA was estimated fluorometrically as described by Labarca and Paigen [10]. Calf thymus DNA (Sigma, #D1501) was used as standard sample and the standard curve ranged from 0.625–40 $\mu\text{g}/\text{ml}$. Cell number was ascertained from the relationship 1.4×10^5 cells/ μg DNA.

Measurement of cell sizes and microscopic observations

Sizes of granulosa cells were measured at the beginning of culture, and after 9 days of culture with insulin plus forskolin and luteal cells. At day

9 of culture, cells attached to the culture dish were washed with PBS 3 times and then detached by treatment with PBS containing 0.05% trypsin (Sigma, #T0646) and 0.02% EDTA at 37.5 C for 15 min. The cells were then washed with PBS immediately to remove trypsin and EDTA and used for observations. Luteal cells were prepared by dispersing the tissue of the corpus luteum at an early luteal stage by enzymatic treatment with 0.05% collagenase (Sigma, #C0130) and 0.005% deoxyribonuclease (Sigma, #D5025) at 37.5 C for 20 min. The luteal phase was classified by macroscopic observation of the ovary as described by Okuda *et al.* [11]. Granulosa cells from small antral follicles were obtained as described above. The cell diameters were measured from photographs of the above cells ($n = 86-97$). Each cell diameter was measured 4 times in different directions and the mean of these values was taken as the cell size.

Statistical analysis

Steroid concentrations in culture media and cell sizes were assigned for 2-way analysis of variance (ANOVA). When ANOVA revealed a significant effect, the values were compared by Student's *t*-test or the Cochran-Cox test. Differences were considered significant when $P < 0.05$.

Fig. 1. Progesterone production in granulosa cells obtained from small antral follicles during culture in the presence or absence of insulin (I; 2 μ g/ml) or forskolin (F; 10 μ M) and insulin plus forskolin (I+F). Results represent the mean $\pm$ SEM of 6 experiments. Values with different superscripts are significantly different ($P < 0.05$ or lower).

Results

Progesterone production

The induction of progesterone secretion in granulosa cells obtained from small antral follicles by different supplements is illustrated in Fig. 1. On the first day of culture, granulosa cells secreted a small amount of progesterone, and there was no difference among granulosa cells cultured with the different supplements ($13.3 \pm 2.88-21.8 \pm 1.65$ ng/ 10^5 cells). Although progesterone secretion from granulosa cells cultured with the supplements increased during the culture period, that in the granulosa cells without any supplements didn't change. In the granulosa cells cultured with insulin, progesterone production reached a maximum on day 3 of culture (15.8 ± 1.12 ng/ml) and then decreased gradually. Progesterone secretion from the cells cultured with forskolin reached to a plateau (37.7 ± 4.92 ng/ml) on day 5 of culture. On the other hand, progesterone production in granulosa cells incubated with insulin plus forskolin increased continuously during the culture period. The highest level of progesterone was observed in granulosa cells cultured with insulin plus forskolin on day 9 of culture (63.5 ± 6.29 ng/ 10^5 cells).

Estradiol-17 β production

Estradiol-17 β production in the granulosa cells

Fig. 2. Estradiol-17 β production in the granulosa cells obtained from small antral follicles during culture in the presence or absence of agents as specified in Fig. 1. Results represent the mean $\pm$ SEM of 6 experiments. Values with different superscripts are significantly different ($P < 0.01$).

during the culture period is shown in Fig. 2. Although the granulosa cells produced a large amount of estradiol (37.0 ± 2.5 – 139.0 ± 14.3 pg/ 10^5 cells) on the first day of culture, the ability to produce estradiol- 17β sharply declined from day 1 to day 3 of culture (1.4 – 4.5% of day 1), and then continued to decline gradually.

Cell size and microscopic morphology

The size of granulosa cells cultured for 9 days in the presence of insulin plus forskolin was compared with the size of granulosa cells at the beginning of culture and with the size of large

luteal cells (Table 1). The mean cell size of cultured granulosa cells (18.0 ± 0.4 μm) was similar to that of large luteal cells (19.7 ± 0.3 μm), and significantly larger than that of granulosa cells at the beginning of culture (8.0 ± 0.21 μm ; $P < 0.001$). The microscopic morphologies of these cells are shown in Fig. 3.

Discussion

Previous studies demonstrated that the profile of steroidogenesis in granulosa cells dramatically changes when the luteinization of granulosa cells begins. Progesterone production begins a few hours before ovulation in granulosa cells [12], and the estradiol- 17β content of the bovine preovulatory follicle rises until the LH surge and then declines sharply when luteinization of the follicular cells begins [13, 14]. Furthermore, bovine luteal cells at the early luteal stage had only a limited capacity to aromatize exogenous androgen [15]. In the present study, progesterone release from the granulosa cells increased during culture. The highest progesterone production was observed in the granulosa cells cultured with insulin plus forskolin. On the other hand, estradiol- 17β production in the

Table 1. Size of granulosa cells and large luteal cells

Cell size (μm)		
GC (day 0) ¹⁾	GC (day 9) ²⁾	LLC ³⁾
7.96 ± 0.21^a	18.02 ± 0.4^b	19.74 ± 0.3^b

¹⁾ Granulosa cells at the beginning of culture.

²⁾ Granulosa cells after 9 days of culture in the presence of insulin (2 $\mu\text{g}/\text{ml}$) and forskolin (10 μM).

³⁾ Large luteal cells from the corpus luteum at the early luteal stage.

Results represent the mean $\pm$ SEM of 86–97 cells. Values with different superscripts are significantly different ($P < 0.001$).

Fig. 3. Microscopic morphologies of granulosa cells and luteal cells. GC (day 0): Granulosa cells obtained from small antral follicles at the beginning of culture; GC (I+F, day 9): Granulosa cells after 9 days of culture in the presence of insulin and forskolin; LC: Large (arrows) and small luteal cells from the corpus luteum at the early luteal stage ($\times 308.6$).

granulosa cells sharply decreased during the culture period, although the culture media contained 1 μ M testosterone as a substrate for estradiol production. Moreover, granulosa cells cultured for 9 days in the presence of insulin plus forskolin were morphologically quite similar to large luteal cells. Based on these data, one could assume that bovine granulosa cells obtained from small antral follicles can differentiate into large luteal-like cells during culture *in vitro*, and that the cultured granulosa cells can be used as a model of large luteal cells.

Meidan *et al.* [8] showed that bovine granulosa cells obtained from preovulatory follicles differentiated into large luteal-like cells during culture in the presence of insulin plus forskolin. In the present study, granulosa cells obtained from small antral follicles could also be luteinized when they were incubated with insulin plus forskolin. It is well demonstrated that insulin or insulin-like growth factor-I (IGF-I) stimulates proliferation and progesterone production in granulosa cells [16–23] *in vitro*. In addition, forskolin induces increased intracellular cyclic AMP concentration via activation of adenylate cyclase. Hence, insulin and/or IGF-I and other factors that induce changes in intracellular cyclic AMP concentration, such as LH and FSH, may have important roles in the induction of luteinization of granulosa cells *in vitro*.

It is of interest to note that some of the large luteal-like cells (which were alive) were obviously smaller than other cells. In the present study, androstenedione production by granulosa cell culture was negligible (data not shown), indicating no contamination by theca cells. Hence, it is inferred that the small cells contained in the large luteal-like cell population were not derived from theca cells.

Alila *et al.* [24] indicated that 14% of bovine small luteal cells bound to a monoclonal antibody that was specific for granulosa cell surface antigens between day 4 and day 6 of the estrous cycle, and that the percentage of small cells bound to the granulosa cell antibody decreased as the luteal stage proceeded. In addition, Jarry *et al.* [25] demonstrated that some of the small luteal cells of the corpus luteum at the early stage secreted oxytocin, although oxytocin had been believed to be produced only in large luteal cells. These reports suggest that some of the bovine small luteal cells may enlarge as the corpus luteum ages. Thus, the small cells observed in the large luteal-like cell population may enlarge during further incubation. However, the functions of the small cells observed in the large luteal-like cell population remain to be defined.

In summary, the present study provides a simple method for obtaining purified large luteal-like cells. These luteinized granulosa cells can be used to investigate the functions of large luteal cells.

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References

1. Corner GW. On the origins of the corpus luteum of the sow from both granulosa and theca interna. *Am J Anat* 1919; 26: 117–183.
2. Koos RD, Hansel W. The large and small cells of the bovine corpus luteum: Ultrastructural and functional differences. In: Schwartz NB, Hunzicker-Dunn M (eds.), *Dynamics of Ovarian Function*. New York: Raven Press; 1981: 197–203.
3. McNutt GW. The corpus luteum of the ox ovary in relation to the oestrous cycle. *J Am Vet Med Assoc* 1924; 65: 556–597.
4. Alila HW, Dowd JP, Corradino RA, Harris WV, Hansel W. Control of progesterone production in small and large bovine luteal cells separated by flow cytometry. *J Reprod Fertil* 1988; 82: 645–655.
5. Farin CE, Sawyer HR, Niswender GD. Analysis of cell types in the corpus luteum of the sheep. *J Reprod Fertil* 1989; Suppl 37: 181–187.
6. Rodgers RJ, O'Shea JD. Purification, morphology and progesterone production and content of three cell types isolated from the corpus luteum of the sheep. *Aust J Biol* 1982; 35: 441–455.
7. Harrison LM, Kenny N, Niswender GD. Progesterone production, LH receptors, and oxytocin

- secretion by ovine luteal cell types on Days 6, 10 and 15 of the oestrous cycle and Day 25 of pregnancy. *J Reprod Fertil* 1987; 79: 539–548.
8. **Meidan R, Girsh E, Blum O, Aberdam E.** In vitro differentiation of bovine theca and granulosa cells into small and large luteal-like cells: morphological and functional characteristics. *Biol Reprod* 1990; 43: 913–921.
  9. **Prakash BS, Meyer HHD, Schallenberger E, Van de Wiel DFM.** Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. *J Steroid Biochem* 1987; 28: 623–627.
  10. **Labarca C, Paigen K.** A simple, rapid and sensitive DNA procedure. *Anal Biochem* 1980; 102: 344–352.
  11. **Okuda K, Kito S, Sumi N, Sato K.** Study of the central cavity in the bovine corpus luteum. *Vet Rec* 1988; 123: 180–183.
  12. **Hay MF, Moor RM.** Distribution of $\Delta 5-3\beta$ -hydroxysteroid dehydrogenase activity in the graafian follicle of the sheep. *J Reprod Fertil* 1975; 43: 313–322.
  13. **Hansel W, Convey EM.** Physiology of estrous cycle. *J Anim Sci* 1983; 57 (Suppl 2) : 404–424.
  14. **Fortune JE, Sirois J, Quirk SM.** The growth and differentiation of ovarian follicles during the bovine estrous cycle. *Theriogenology* 1988; 29: 95–109.
  15. **Henderson KM, Moon YS.** Luteinization of bovine granulosa cells and corpus luteum formation associated with loss of androgen-aromatizing ability. *J Reprod Fertil* 1979; 56: 89–97.
  16. **Savion N, Lui GM, Laherty R, Gospodarowicz D.** Factors controlling proliferation and progesterone production by bovine granulosa cells in serum-free medium. *Endocrinology* 1981; 109: 409–420.
  17. **Holtorf AP, Furuya K, Ivell R, McArdle CA.** Oxytocin production and oxytocin messenger ribonucleic acid levels in bovine granulosa cells are regulated by insulin and insulin-like growth factor-I: Dependence on developmental status of the ovarian follicle. *Endocrinology* 1989; 125: 2612–2620.
  18. **McArdle CA, Hortolf AP.** Oxytocin and progesterone release from bovine corpus luteal cells in culture: Effects of insulin-like growth factor I, insulin, and prostaglandins. *Endocrinology* 1989; 124: 1278–1286.
  19. **Saumande J.** Culture of bovine granulosa cells in a chemically defined serum-free medium: the effect of insulin and fibronectin on the response to FSH. *J Steroid Biochem Mol Biol* 1991; 38: 189–196.
  20. **Gong JG, McBride D, Bramley TA, Webb R.** Effects of recombinant bovine somatotrophin, insulin-like growth factor-I and insulin on the proliferation of bovine granulosa cells in vitro. *J Endocrinol* 1993; 139: 67–75.
  21. **Hsueh AJW, Adashi EY, Jones PBC, Welsh TH.** Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocr Rev* 1984; 5: 76–127.
  22. **Adashi EY, Resnick CE, D'Ercole AJ, Svoboda ME, Van Wyk JJ.** Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocr Rev* 1985; 6: 400–420.
  23. **Schams D, Koll P, Li CH.** Insulin-like growth factor-I stimulates oxytocin and progesterone production by bovine granulosa cells in culture. *J Endocrinol* 1988; 116: 97–100.
  24. **Alila HW, Hansel W.** Origin of different cell types in the bovine corpus luteum as characterized by specific monoclonal antibodies. *Biol Reprod* 1984; 31: 1015–1025.
  25. **Jarry H, Hornschuh R, Pitzel L, Wuttke W.** Demonstration of oxytocin release by bovine luteal cells utilizing the reverse hemolytic plaque assay. *Biol Reprod* 1992; 46: 408–413.