Effects of Culture Conditions on The Expression of Cyclin B1 Protein during the First Meiotic Maturation in Bovine Immature Oocytes

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ABSTRACT

Cyclin B1 is known to reflect the M-phase promoting factor (MPF), a universal regulator of G2/M-phase transition, activity during the process of oocytes maturation. To investigate whether culture condition affects the maturation rate and the expression of cyclin B1 protein, bovine immature oocytes are stimulated and cultured according to the following protocols: Experiment 1: denuded oocytes (denude) only, COC only, denuded oocytes + granulosa cells (denude + GCs) and COC + GCs; Experiment 2: no-activation (control), 7% ethanol for 5 min and 10 μl/ml ionomycin for 5 min at immediately before maturation. The maturation rates of denude and no-activation group were significantly lower in both experiments ($P<0.05$), respectively. Co-culture or stimulation method in bovine immature oocytes culture increases the cyclin B1 expression significantly in both experiments ($P<0.05$). Based on these results, culture condition affects the maturation rate and the expression of cyclin B1 protein during the first meiotic maturation in bovine immature oocytes.

(Key words : Bovine oocyte, Activation, Co-culture, First meiotic maturation, Cyclin B1 protein)

INTRODUCTION

In most animal species, oocytes are formed during fetal life and are naturally arrested at the diplotene of the first prophase, which corresponds to the G2 phase of the cell cycle. Meiotically arrested oocytes are referred to as immature oocytes. The resumption of meiosis in these oocytes is known as oocyte maturation and entails a G2-to-M transition. The mitotic cyclins are synthesized at a constant rate throughout the cell cycle (Evans *et al.*, 1983) and destroyed at each cell division.

One of the most important factors in oocyte maturation is cumulus cells. Without these cells, the maturation rate of the immature oocytes was reduced significantly (Zhang *et al.*, 1995). On the other hand of oocyte maturation, M-phase promoting factor (MPF) is another important factor. The increase in MPF activity is strongly correlated with cyclin B1 synthesis (Hample and Eppig, 1995; Winston, 1997). Two lines of evidence suggest that elevated cyclin synthesis may be responsible for MPF-induced maturation: the treatment of oocytes with protein synthesis inhibitors prevents the increased MPF activity as well as polar body extrusion. The injection of cyclin B1 mRNA into oocytes accelerates the rate of maturation.

The studies showed that MPF activity appears shortly before germinal vesicle breakdown (GVBD), reached a peak in metaphase-I (MI) oocytes, decreased dramatically during transition from MI to metaphase-II (MII) and regains its maximal level again in MII oocytes (Hashimoto and Kishimoto, 1988; Choi *et al.*, 1991). And Marangos and Carroll (2004) reported that GVBD in mouse oocytes is sensitive to cyclin B1 abundance and that the changes in distribution of cyclin B1 contribute to progression through MI using a cyclin B1-green fluorescent protein (GFP) fusion protein.

Recently, to increase the maturation rate, several culture systems have been developed such as co-culture or activation method (Minamihashi *et al.*, 1993; Zhang *et al.*, 1995). Under the hypothesis that the accumulation of cyclin B1 protein in cytoplasm is affected by different culture systems, we investigated the effects of culture conditions on the expression of cyclin B1 protein during the first meiotic maturation in bovine immature oocytes.

MATERIALS AND METHODS

Oocyte Collection

Bovine oocytes were aspirated from 2~6 mm sized follicles on abattoir-recovered ovaries. The collected cumulus-oocytes complexes (COC) were isolated and cultured in medium containing 100 μM 1-isobutyl-3-methylxanthine (IBMX; Sigma Aldrech Chemical Co., USA)

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to maintain meiotic arrest. Resumption of meiosis was initiated by placing oocytes into IBMX-free medium. The maturation medium used for all experiments was TCM-199 supplemented with 10% FBS, 0.2 mg/ml FSH (F-2293, Sigma, USA), 0.5 mg/ml E2 (E-8875, Sigma, USA), and 1 mg/ml EGF (E-4127, Sigma, USA). The oocytes were cultured at 39℃ in humidified 5% CO_2 incubator.

Experimental Designs

Experiment 1: The germinal vesicle (GV) stage oocytes were cultured under following conditions for 18 hr (Park, 2004) oocytes without cumulus cells (denude) only, COC only, denuded oocytes + granulosa cells monolayer (denude + GCs, 1×10^5 cells/ml), and COC + GCs. The follicular aspirates from large follicles were centrifuged for 10 min at 600 ×g and the cell pellet was resuspended with TCM-199. The resuspended cells were layered in 3 ml 50% Percoll solution (Sigma, St. Louise, MO) and centrifuged for 5 min at 600 ×g to pellet the blood cells. Purified granulosa cells were aspirated from the interphase (1 to 2 ml) and washed twice with TCM-199 (Dirnfeld *et al.*, 1997). Immatured bovine oocytes were treated with hyaluronidase (1 mg/ml, type IV-S, Sigma, USA) for 3 min, and the cumulus cells surrounding oocyte were gently removed by pipetting.

Experiment 2: The denuded oocytes were treated with following protocols; no-activation (control), 7% ethanol for 5 min, and 10 μl/ml ionomycin for 5 min at immediately before maturation. Following 18 hr of maturation, the cumulus cells surrounding oocyte were removed by hyaluronidase treatment. The maturation rate was scored by first polar body extrusion.

Western Blot Analysis

After maturation, the oocytes (50 oocytes/treatment) were homogenized and lysed in 0.5 ml of RIPA buffer (150 mM NaCl, 1% ethylphenylpolysthylenglycol, 0.5% sodium deoxcholate, 0.1% SDS, 50 mM Tris pH 8.0) with freshly added 1mM phenylmethylsulphonylfluoride (PMSF, Sigma, Missouri, USA) for 30 min on ice. The lysates were centrifuged at 13,000 ×g for 15 min at 4℃, and the supernatants were stored at 70℃. Equal amount of total protein were separated by SDS-PAGE and then transferred to support Westran®PVDF (Schleicher & Schuell, New Hampshire, USA) by applying 100 V-1 hr with a plate electrode apparatus (Mini Trans-Blot Cell; Bio-Rad, USA). The blots were blocked for 2 hr in 5% non-fat dry milk/TBST (0.2 M NaCl, 0.1% Tween-20, 10 mM Tris-HCl pH 7.4). Subsequently, the blots were incubated with antibody against cyclin B1 (1:500; Santa Cruz Biotechnology, C-17) in TBST and then the blots were also incubated in anti-rabbit IgG (1:2,000; Amersham Pharmacia Biotech, Freiburg, Germany) in TBST. The blots were washed several times with TBST after each step. The bound antibody was detected with an enhanced chemi-luminescence (ECL) system (Amersham Pharmacia Biotech).

The image was scanned with Gel Documentation System (Gel Doc 1000, Bio-Rad Hercules, CA, USA) and relative densities were analyzed using Multi-analyst fingerprinting program (Version 1.1). The relative densities of the bands were expressed as arbitrary absorbance units per area.

Statistical Analysis

The statistical analysis for maturation rate was performed with Chi-square test. The statistical analysis for the differences of cyclin B1 protein expression among treatment groups was performed using Kruskal-Wallis test. Statistical significance was given when P value was less than 0.05.

RESULTS

Experiment 1

As shown in Table 1, the maturation rate of denude group was significantly lower than that of COC, denude + GC, and COC + GC group after 18 hr of *in vitro* maturation ($P<0.05$). The expression pattern of cyclin B1 protein in each group was shown in Fig. 1. Co-culture with GC monolayer groups (denude + GC and COC + GC) were shown significantly higher cyclin B1 protein level than denude and COC groups ($P<0.05$). Interestingly, the cyclin B1 protein level of COC group was similar to that of control group, although the maturation rate of COC group was significantly highercompared to that of control group.

Experiment 2

As shown in Table 2, the maturation rate of no-activation (control) group was significantly lower than that of activated (ethanol and ionomycin) groups ($P<0.05$). And the expression of cyclin B1 protein was

Table 1. The maturation of germinal vesicle stage oocytes cultured with or without CC and/or GC

	No. of oocytes	
	GV stage	MII stage (Mean%±SD)
Denude	161	61(17.5±2.9)[a]
COC	165	89(53.9±1.2)[b]
Denude + GC	163	85(52.1±3.5)[b]
COC + GC	154	85(55.2±6.3)[b]

Three replications were performed at each group.
COC: cumulus-oocyte-complexes; GC: granulosa cell
[a,b] Values with different superscript differ significantly ($P<0.05$).

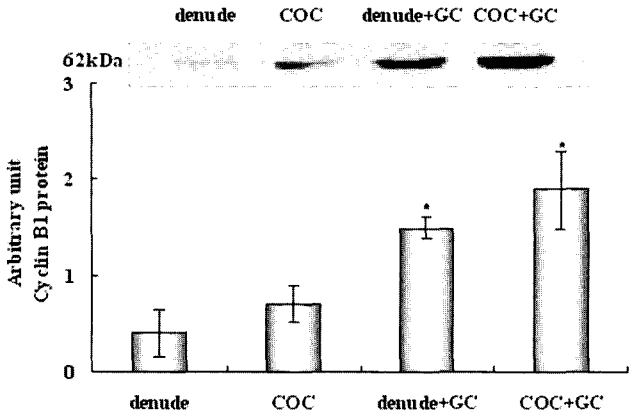

Fig. 1. The expression of cyclin B1 protein in the oocytes cultured with or without cumulus cells and/or granulosa cells were analyzed by western blotting. The relative densities of the bands were expressed as arbitrary absorbance units.
Results are expressed as mean ± SD.
COC: cumulus-oocyte-complexes; GC: granulosa cell
* Significantly differ from the denude and COC groups ($P<0.05$).

Table 2. The maturation rate of denuded oocytes with or without activation immediately before the onset of maturation

	No of oocytes	
	GV stage	MII stage (Mean%±SD)
Control	170	50(29.2±6.1)[a]
Ethanol	213	135(64.3±7.7)[b]
Ionomycin	232	160(69.7±10.2)[b]

Three replications were performed at each group.
[a,b] Values with different superscript differ significantly ($P<0.05$).

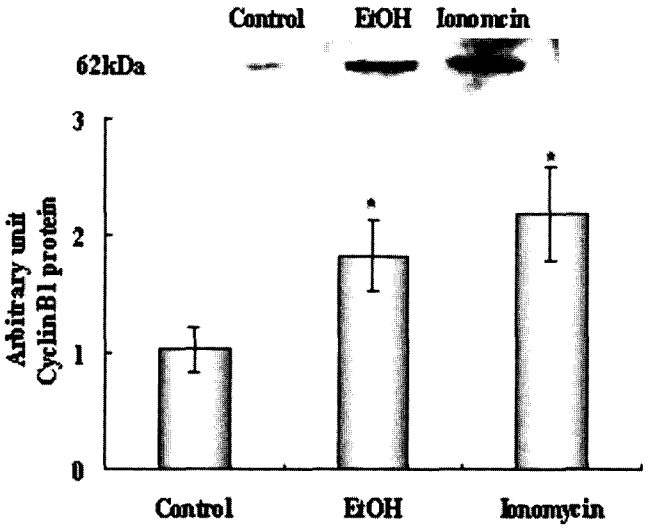

Fig. 2. The expression of cyclin B1 protein in the oocytes with or without activation was analyzed by western blotting. The relative densities of the bands were expressed as arbitrary absorbance units.
Results are expressed as mean ± SD.
* Significantly differ from the control group ($P<0.05$).

significantly higher in activated groups compared to that of control group ($P<0.05$, Fig. 2).

DISCUSSION

It is well known that the cumulus cell is prerequisite for the maturation of oocytes. In mammals, cumulus cell was known to play an important role in nuclear maturation, in cytoplasmic maturation, in male pronucleus (PN) formation and in early embryonic development (Fukui, 1990; Janssenswillen *et al.*, 1995). And also it was reported that supplementation of maturation media with GC improved cytoplasmic maturation of oocytes (Mattioli *et al.*, 1988).

In this experiment, co-culture with follicular cells increases the maturation rate and the accumulation of cyclin B1 protein in cytoplasm. While the denuded oocytes showed significantly lower maturation rate, the denuded oocytes cultured with GC monolayer during the first meiotic maturation improved the maturation rate. It can be suggested that GC monolayer plays a role in maturation of denuded oocytes.

Within the follicle, GC, cumulus cell, and oocytes were found to be metabolically coupled through gap junctions providing physical pathways for intracellular communications (Rabahi *et al.*, 1991; Levesque and Sirard, 1996), each of which plays important roles during oocytes growth and maturation (Buccione *et al.*, 1990). In this study, the level of cyclin B1 protein was significantly increased in the oocytes cultured with GC monolayer. Although the difference was not statistically significant, the cyclin B1 protein expression of COC group was higher than that of denude group. Interestingly the maturation rate and cyclin B1 protein level of COC group was not matched. It can be postulated that the presence of cumulus cells may not affect to an accumulation of cyclin B1 protein during first meiotic maturation of bovine immature oocytes, however, the cells have a pivotal role to oocyte maturation. According to above result, granulosa cells may support the synthesis and accumulation of the cyclin B1 protein in the cytoplasm of oocytes.

The mature oocytes arrest at metaphase of meiosis II by virtue of a calcium-sensitive activity named cytostatic factor (CSF), which stabilizes MPF activity (Masui, 1974; Bogliolo *et al.*, 2000; Kim and Chung, 2004). Normally calcium-elevating agents, such as ethanol and ionomycin, have been used to increase matured MII stage oocytes activation, but little is known about the role of these agents during the first meiotic maturation.

In this study, GV stage oocytes activated with ethanol and ionomycin were significantly higher maturation rate than in control group. And the expression of cyclin B1 protein was significantly higher in both acti-

vated groups than in control group. Although the exact mechanism remains to be elucidated, calcium activation may be associated with bovine immature oocytes maturation.

Based on these results, co-culture with GCs or stimulation of oocytes affects the maturation rate and the expression of cyclin B1 protein during the first meiotic maturation in bovine immature oocytes.

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