Effect of KIT Ligand on the Viability of Porcine Primordial Follicles In Vitro

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Abstract: *In mammals, the mechanisms regulating the initiation of follicle development remain poorly understood. We previously reported that testosterone induced the activation of porcine primordial follicles after 7 days of culture. Herein, the effect of KIT ligand (KL) on the viability, activation and development of porcine primordial follicles was examined. Ovarian strips containing primordial follicles were cultured for 7 days in a medium supplemented with KL (10, 100, 250 or 500 ng/ml). The percentage of degenerated follicles was significantly decreased (39 $\pm$ 2%) in the 100 ng/ml KL-treated group compared to the control (56 $\pm$ 1%), whereas almost all of the primordial follicles degenerated at higher concentrations of KL. No primordial follicles developed in culture at any concentration of KL. When ovarian strips were cultured in medium supplemented with testosterone (10^{-6} M) + KL (100 ng/ml), primordial follicles developed to early secondary follicles (5 $\pm$ 3%), however the total percentages of developing follicles in the testosterone group (21 $\pm$ 5%) and the testosterone + KL group (28 $\pm$ 5%) were not significantly different. These results suggest that KL does not promote primordial follicle activation, although it does promote follicle viability in culture.*
Key words: *In vitro culture, KIT ligand, Pig, Primordial follicle, Testosterone*

Introduction

In mammals, the mechanisms regulating the initiation of follicle development remain poorly understood [1–3]. Many kinds of molecules that regulate primordial follicle activation have been reported. KIT ligand (KL) [4–6], basic fibroblast growth factor [7], leukemia inhibitory factor [8], insulin [9, 10] and bone morphogenetic protein 4 [11] have been shown to promote the primordial to primary follicle transition in rodents. The KIT receptor tyrosine kinase and KL are well-documented molecules that are involved in the activation of primordial follicles in rodents [1, 12]. KL is produced by granulosa cells [13], and KIT is present in oocytes [14, 15]. In mice bearing spontaneous mutations of *Steel*, the gene encoding KL [16], follicular development is impaired [4]. Similarly, in mice bearing a mutation of the gene encoding *KIT* [17] or treated with an antibody against KIT [5], folliculogenesis is impaired. Although the role of KL is controversial in the follicle activation process in the mouse [18, 19], pig [15] and rabbit [13], KL might not be required for follicle activation. KL is predominantly a survival factor for primordial germ cells, protecting them from apoptosis [20], and it stimulates granulosa cell proliferation in the presence of oocytes [21].

Recently we reported that testosterone positively regulates the activation of primordial follicles derived from prepubertal pigs [22]. Testosterone at 10^{-6} M induced the primordial follicle transition to the intermediate and primary stages after 7 days of culture. Furthermore, testosterone-treated primordial follicles developed to the antral stage after 2 months of xenografting.

The objective of the present study was to determine the effect of KL on the activation and development of cultured porcine primordial follicles. We histologically examined the effect of KL on the activation and development of porcine primordial follicles and then the effect of KL in combination with testosterone in cultured ovarian tissues.

Materials and Methods

Collection of ovarian cortical strips containing primordial follicles

Ovaries were collected from 6-month-old crossbred gilts at a slaughterhouse in Kobe, Japan. Since pigs reach puberty at 6–7 months, ovaries without corpus luteum were collected from prepubertal gilts. The ovaries were washed once in 0.2% (w/v) cetyltrimethylammonium bromide (Wako Pure Chemical Industries, Osaka, Japan) and Dulbecco's phosphate buffered saline (PBS) supplemented with 0.1% (w/v) polyvinyl alcohol (PVA; Sigma, MO, USA) 3 times. Cortical strips, from randomly selected ovaries, with a thickness of 0.5 mm (approximately) were dissected with surgical blades (No.11; Feather Safety Razor, Osaka, Japan). The strips were examined under a dissection microscope and then under an inverted microscope, and strips containing primary and secondary follicles were avoided. Primordial follicles were identified as those having oocytes with diameters within a range of 30–35 μm and oocytes containing a large spherical nucleus surrounded by small lipid droplets [23]. Ovarian strips (approximately 2 mm × 1 mm × 0.5 mm) that contained primordial follicles were selected and cut into two pieces (each approximately 1 mm × 1 mm × 0.5 mm). One part was fixed immediately for histological examination to assess the follicle number and morphology, and the other part was washed 3 times and immersed in HEPES-buffered TCM-199 (pH 7.4; Nissui Pharmaceutical, Tokyo, Japan) before culture. The HEPES-buffered TCM-199 contained 25 mM HEPES (Sigma), 10 mM NaHCO$_3$, 0.1% PVA, and 0.08 mg/ml kanamycin sulphate (Sigma) in TCM 199.

In vitro culture

In each experiment, cortical strips (1 mm × 1 mm × 0.5 mm) were cultured on a cellulose acetate floating membrane filter (pore size 0.45 μm and diameter 25 mm; Advantec, Toyo Roshi, Tokyo, Japan) in an organ culture dish (# 3037; BD Falcon, NJ, USA) for 7 days under a humidified atmosphere of 5% CO$_2$ and 95% air at 38.5°C. The basic culture medium was alpha minimum essential medium (α-MEM; Invitrogen, NY, USA) supplemented with 0.08 mg/ml kanamycin sulphate, 2.2 mg/ml NaHCO$_3$, 50 μM 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan), 0.1 mg/ml sodium pyruvate (Sigma), 1% (v/v) Insulin-Transferrin-Selenium (ITS; Invitrogen) and 5% (v/v) bovine plasma (Nippon Biotest Laboratories, Tokyo,

Japan) after heat inactivation. During the culture period, half of the volume of the total medium was changed every 2 days. In the experimental groups, the basic culture medium was supplemented with 0, 10, 100, 250 or 500 ng/ml (w/v) of KL (recombinant mouse stem cell factor # 455-MC; R&D Systems, MN, USA). In the combination experiment, the basic culture medium was supplemented with 100 ng/ml KL with or without 10^{-6} M testosterone (Nacalai Tesque). Testosterone was dissolved in absolute ethanol, and 1 μl of the solution was added to each 1 ml of culture medium to obtain the final concentration just before use. As the vehicle, 1 μl of ethanol was added to the control groups.

Assessment of follicular development

Ovarian cortical strips before and after culture were fixed in 3% (w/v) paraformaldehyde in PBS, then dehydrated, embedded in methacrylate resin (JB-4; Polysciences, IL, USA), serially sectioned into 5-μm sections, and stained with hematoxylin and eosin. The numbers of follicles at different stages were recorded. The follicles were counted in every section in which an oocyte nucleus was seen. Double counting in adjacent sections was avoided. The follicles were classified into four categories according to the number and morphology of granulosa cell layers: primordial follicles with a single layer of flattened granulosa cells surrounding the oocyte, intermediate follicles with a single layer containing a mixture of flat and cuboidal granulosa cells, primary follicles with a single layer of cuboidal granulosa cells and secondary follicles with more than one layer of cuboidal granulosa cells or the beginning of a second layer of granulosa cells. Degenerated follicles were classified by the following staining properties of the oocyte and granulosa cells: follicles having oocytes with pale cytoplasm, dark pyknotic nucleus, shrunken or extensive cytoplasmic vacuolations, and/or a disintegrated granulosa cell layer. The diameters of the oocytes were measured by taking the average of two perpendicular measurements to the nearest 1 μm with an ocular micrometer (Nikon, Tokyo, Japan) attached to an inverted microscope.

Data and statistical analysis

All the data were analyzed by the Graph Pad InStat (Version 3.0) statistical analysis program (GraphPad Software, CA, USA). The quantitative results are presented as the mean ± S.E.M. The data were analyzed using ANOVA followed by Tukey-Kramer multiple comparisons among multiple groups when appropriate. For statistical comparisons between two
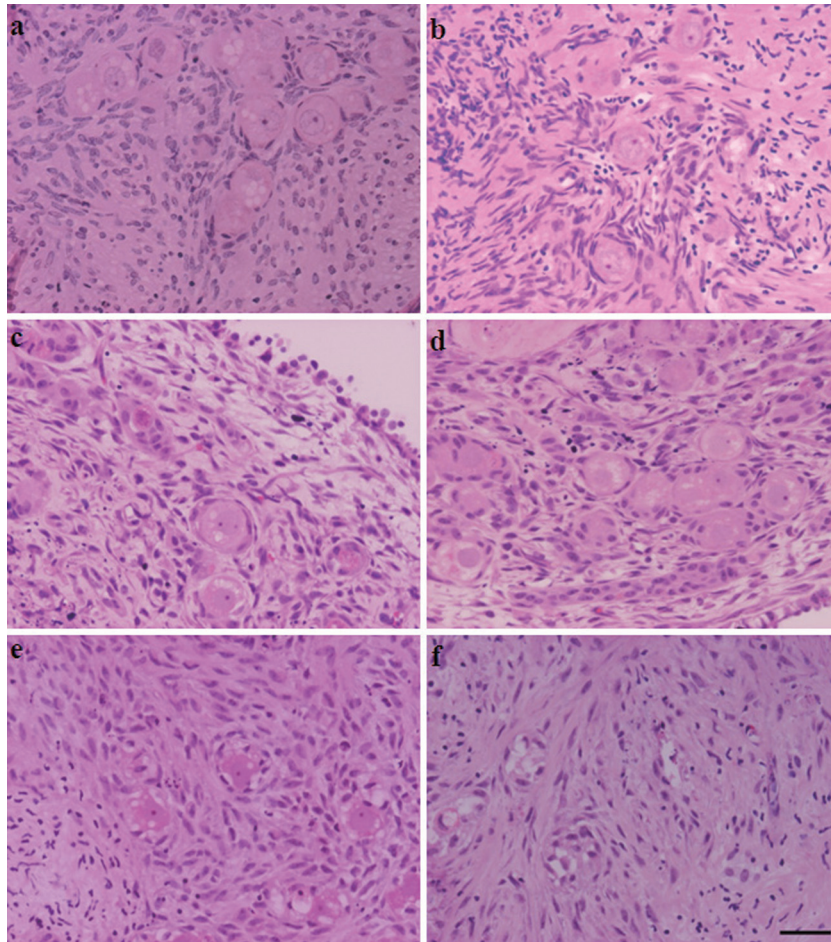
Fig. 1. Histological sections of porcine ovarian strips before (a) and after culture with KL at the concentration of 0 (b: control), 10 (c), 100 (d), 250 (e) or 500 ng/ml (f) for 7 days. The percentages of healthy intact primordial follicles were higher in the 100 ng/ml KL-treated group (d) than in the other cultured groups. There were no developing follicles in any cultured group. Scale bar represents 40 μm.

groups, the *t* test was used. All percentile data were transformed to arc-sin values before analysis. Differences with *P* < 0.05 were considered statistically significant.

Results

Effect of KL on primordial follicle activation

Histological examination confirmed that dissected porcine ovarian strips contained only primordial follicles before culture (Fig. 1a). Each oocyte contained a large nucleus and cytoplasmic lipid droplets. The percentage of degenerated follicles was 14 ± 3% before culture (Table 1). After 7 days of culture, the strips had changed slightly due to the appearance of degenerated somatic cells (Figs. 1b-f) and an increase in the percentage of degenerated follicles (Table 1). There was no significant difference in the percentages of degenerated follicles between the control and the 10 ng/ml KL-treated groups (Figs. 1b, 1c and Table 1), whereas the percentage was significantly decreased by 100 ng/ml KL (39 ± 2%, Fig. 1d). However, the mean numbers of follicles were decreased by 250 and 500 ng/ml KL (Table 1). At these concentrations of KL, most of the follicles were degenerated (Figs. 1e and 1f). No developing follicles were observed in any strip treated with any concentration of KL.

Table 1. Distribution of different types of follicles in porcine ovarian strips after culture with KIT ligand (KL)

Treatment*	Day of culture	No. of tissues examined	No. of follicles/tissue	No. (%) of follicles**				
				Primordial	Intermediate	Primary	Secondary	Degenerated
None	0	8	36 ± 8[a]	31 ± 7 (86 ± 3)[a]	0 ± 0	0 ± 0	0 ± 0	5 ± 2 (14 ± 3)[a]
Control								
0 ng/ml KL	7	8	33 ± 5[a]	14 ± 1 (44 ± 1)[b]	0 ± 0	0 ± 0	0 ± 0	18 ± 3 (56 ± 1)[b]
10 ng/ml KL	7	8	36 ± 3[a]	15 ± 4 (42 ± 3)[b]	0 ± 0	0 ± 0	0 ± 0	20 ± 4 (58 ± 3)[b]
100 ng/ml KL	7	8	32 ± 3[a]	20 ± 2 (61 ± 2)[c]	0 ± 0	0 ± 0	0 ± 0	12 ± 1 (39 ± 2)[c]
250 ng/ml KL	7	8	21 ± 3[a]	2 ± 1 (10 ± 3)[d]	0 ± 0	0 ± 0	0 ± 0	19 ± 2 (90 ± 3)[d]
500 ng/ml KL	7	8	10 ± 3[b]	0 ± 0	0 ± 0	0 ± 0	0 ± 0	10 ± 3 (100 ± 0)[d]

*Ovarian strips (approximately 1 mm × 1 mm × 0.5 mm) containing primordial follicles were cultured in medium containing 0, 10 100, 250 or 500 ng/ml KL for 7 days. **Each value represents the mean ± S.E.M. [a–d] Values with different superscripts within the same colum differ significantly ($P < 0.05$).

Table 2. Oocyte growth and follicular development in porcine ovarian strips after culture with KIT ligand (KL) and testosterone

Treatment*	Day of culture	No. of tissues examined	No. of follicles/tissue	No. (%) of oocytes on the basis of diameter			No. (%) of follicles**				
				≤35 μm	36–55 μm	56 μm ≤	Primordial	Intermediate	Primary	Secondary	Degenerated
None	0	4	35 ± 5[a]	31 ± 4 (88 ± 2)[a]	0 ± 0	0 ± 0	31 ± 4 (88 ± 2)[a]	0 ± 0	0 ± 0	0 ± 0	4 ± 1 (12 ± 2)[a]
Vehicle (Control)	7	4	36 ± 3[a]	15 ± 2 (41 ± 2)[b]	2 ± 1 (5 ± 2)[a]	0 ± 0	17 ± 1 (46 ± 1)[b]	0 ± 0	0 ± 0	0 ± 0	19 ± 2 (54 ± 1)[b]
KL	7	4	35 ± 7[a]	16 ± 3 (46 ± 7)[b]	6 ± 3 (16 ± 2)[b]	0 ± 0	22 ± 4 (62 ± 2)[c]	0 ± 0	0 ± 0	0 ± 0	14 ± 3 (38 ± 2)[c]
Testosterone	7	4	41 ± 5[a]	18 ± 3 (44 ± 4)[b]	13 ± 2 (32 ± 3)[c]	0 ± 0	22 ± 4 (55 ± 5)[bc]	6 ± 2 (14 ± 4)[a]	3 ± 1 (7 ± 2)[a]	0 ± 0	10 ± 1 (24 ± 4)[d]
Testosterone + KL	7	4	36 ± 6[a]	12 ± 4 (35 ± 9)[b]	13 ± 3 (37 ± 4)[c]	2 ± 1 (5 ± 3)	17 ± 5 (49 ± 9)[b]	3 ± 1 (9 ± 2)[a]	5 ± 1 (14 ± 2)[a]	2 ± 1 (5 ± 3)	8 ± 2 (23 ± 4)[d]

*Ovarian strips (approximately 1 mm × 1 mm × 0.5 mm) containing primordial follicles were cultured with 100 ng/ml KL in combination with 10^{-6} M testosterone. **Each value represents the mean ± S.E.M. [a–d] Values with different superscripts within the same colum differ significantly ($P < 0.05$).

Effect of KL in combination with testosterone on primordial follicle development

To examine the effect of KL on primordial follicle activation and development in combination with testosterone, prepubertal porcine ovarian strips were cultured with KL (100 ng/ml) and testosterone (10^{-6} M) for 7 days (Table 2). Ovarian strips before culture contained only primordial follicles (Fig. 2a). After culture, there were no developing follicles in the KL-treated group, however the percentage of primordial follicles (62 ± 2%) was significantly higher than in the vehicle-treated control (46 ± 1%; Table 2, Figs. 2b and 2c). On the other hand, intermediate and primary follicles were observed in the ovarian strips treated with testosterone, irrespective of KL treatment (Figs. 2d and 2e). Furthermore, in the testosterone-alone and testosterone + KL groups, the percentages of degenerated follicles were reduced compared to the control (Table 2), and the percentages of total

developing follicles including intermediate, primary and secondary follicles, were increased to 21 ± 5% and 28 ± 5%, respectively.

Discussion

The recruitment of primordial follicles into the growth phase may involve both stimulatory and inhibitory mechanisms. Although the exact mechanisms that regulate the activation of primordial follicles remain unelucidated, the study of rodents has shown that KIT and KL are involved in this process [6, 20]. The role of KIT has been demonstrated by inhibiting experiments using its specific antibody, ACK2. Yoshida *et al.* [5] injected mice with ACK2 at various times during the first 2 weeks after birth and reported that the neutralization of KIT caused disturbances in initial follicle recruitment. KIT has been reported to express a stimulatory effect on primordial follicle activation in different animal species
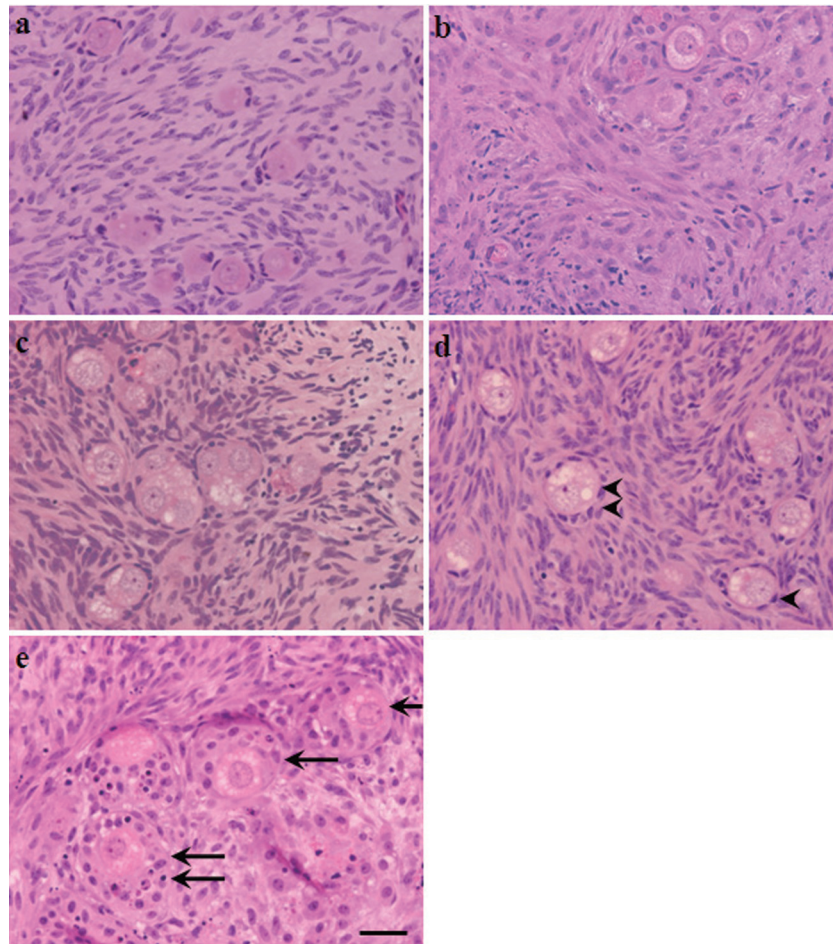
Fig. 2. Histological sections of porcine ovarian strips before and after culture with KL and testosterone. The ovarian strips before culture contained healthy primordial follicles (a). 10^{-6} M testosterone treatment (d) yielded intermediate follicles (*arrowhead*) or early primary follicles (*double arrowheads*). The ovarian strips treated with 10^{-6} M testosterone + 100 ng/ml KL (e) yielded both primary (*arrows*) and early secondary follicles (*double arrows*). In the ovarian strips treated with KL 100 ng/ml (c) and in the control treated with vehicle (b), the primordial follicles had not initiated development. Scale bar represents 40 μm.

including rats [6], mice [24, 25] and sheep [26] in culture conditions. In the present study, 100 ng/ml KL increased the number of viable porcine primordial follicles while reducing the number of degenerated follicles after 7 days of culture. This result suggests that KL at this concentration maintains the follicle viability during culture, and it is in agreement with previous results for the pig [15], rabbit [13], human [27, 28] and mouse [18]. Although much higher concentrations of KL induced degeneration of primordial follicles, KL did not support primordial follicle activation at any concentration in the present study. Carlsson *et al*. [28]

showed that KL (10 and 100 ng/ml) did not affect the follicular activation in cultured human ovarian tissues, although the number of viable follicles increased. In our previous study, the treatment of primordial oocytes from prepubertal pigs with KL (50 and 100 ng/ml) for 3 days *in vitro* followed by transplantation to severe combined immunodeficient (SCID) mice for 2 months did not induce oocyte growth or follicular development [15]. Hutt *et al*. [13], reported that KL (50 and 150 ng/ml) had no effect on rabbits' primordial follicle development.

KL at 100 ng/ml yielded the highest percentages of viable primordial follicles in the 7-day culture performed

in the present study. We used commercially available mouse recombinant KL instead of porcine KL. It is reasonable to think that high concentrations of mouse KL are required to activate porcine primordial follicles. However, KL at concentrations of 250 and 500 ng/ml induced follicle degeneration in our culture conditions for unknown reasons.

Porcine primordial follicles cultured with testosterone developed to the antral stage 2 months after xenografting [22]. Testosterone-containing culture medium in the present study yielded intermediate and primary follicles, a result that is in agreement with our previous report. In the medium containing both testosterone and KL, some follicles developed to the early secondary stage, although the total percentage of developing follicles was not significantly different from that produced by culture in testosterone alone. This result suggests that there is no specific effect of KL on primordial follicle activation. On the other hand, testosterone alone decreased the degeneration of primordial follicles indicating two possible roles for testosterone: primordial follicle activation and/or promotion of follicle viability. Testosterone may directly affect the primordial follicle activation via the androgen receptor in oocytes [22], and it may also induce expression of KL, thereby maintaining follicle viability. Further studies are needed to explore this idea that testosterone induces KL action.

In summary, in the culture conditions in the present study, 100 ng/ml KL decreased the degeneration of porcine primordial follicles in prepubertal pigs during a 7-day culture. Some primordial follicles developed to the early secondary stage in the medium containing both KL and testosterone, although KL did not induce the activation of porcine primordial follicles.

Acknowledgements

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