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Kit Ligand Actions on Ovarian Stromal Cells: Effects on Theca Cell Recruitment and Steroid Production

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ABSTRACT Factors that control recruitment of theca cells from ovarian stromal-interstitial cells are important for early follicle development in the ovary. During recruitment, theca cells organize into distinct layers around early developing follicles and establish essential cell–cell interactions with granulosa cells. Recruitment of theca cells from ovarian stromal stem cells is proposed to involve cellular proliferation, as well as induction of theca cell-specific functional markers. Previously, the speculation was made that a granulosa cell-derived "theca cell organizer" is involved in theca cell recruitment. Granulosa cells have been shown to produce kit-ligand/stem cell factor (KL). KL is known to promote stem cell proliferation and differentiation in a number of tissues. Therefore, the hypothesis was tested in the current study that granulosa cell-derived KL may help recruit theca cells from undifferentiated stromal stem cells during early follicle development. The actions of KL were examined using adult bovine ovarian fragment organ culture and isolated ovarian stromal-interstitial cells. In organ culture KL significantly increased the number of theca cell layers around primary follicles. Experiments using purified stromal-interstitial cell cultures showed that KL stimulated ovarian stromal cell proliferation in a dose-dependent manner. Stromal cell differentiation into theca cells was analyzed by the induction of theca cell functional markers (i.e., androstenedione and progesterone production). Bovine ovarian stromal cells produced low levels of androstenedione (5–40 ng/μg DNA) and progesterone (5–30 ng/μg DNA) in vitro that were approximately 20-fold lower than theca cells under similar conditions. Treatment with KL did not affect ovarian stromal cell androstenedione or progesterone production. Interestingly, hormones such as estrogen and hCG did stimulate stromal cell steroid production. The results in this study suggest that granulosa cell-derived KL appears to promote the formation of theca cell layers around small (i.e., primary) ovarian follicles. KL directly stimulated ovarian stromal cell proliferation but alone did not induce functional differentiation (i.e., high steroid production). Therefore, KL is proposed to promote early follicle development by inducing proliferation and organization of stromal stem cells around small follicles. Observations suggest that KL may act as a granulosa-derived "theca cell organizer" to promote stem cell recruitment of ovarian stromal cells in a manner similar to the way that KL promotes hematopoietic and lymphoid stem cells in bone marrow and the thymus. *Mol. Reprod. Dev. 55:55–64, 2000.*
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Key Words: kit ligand; KL; stem cell factor; theca; stroma; recruitment; follicle; ovary; mesenchymal-epithelial; menopause

INTRODUCTION

Early ovarian follicle development involves active RNA synthesis within the oocyte, the formation of the zona pellucida, and the proliferation of granulosa cells. Granulosa cells differentiate from a flattened to squamous morphology and begin to express granulosa cell functional markers. Although less understood, another critical aspect of early follicle development is the organization and differentiation of theca cells around the follicle. Theca cells organize around early stage follicles and provide structural support for the developing follicle. In addition theca cells help establish mesenchymal-epithelial cell interactions between theca cells and granulosa cells that are essential for later stages of follicular development. In the 1970s, granulosa cells were proposed to produce a "theca cell organizer" that acts on adjacent stromal stem cells to recruit theca cells (Midgley et al., 1974; Peters, 1979). Such a factor was proposed to promote theca cell organization around the developing follicle. In addition, this factor may promote proliferation and functional differentiation of theca

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cells from ovarian stromal stem cells. Identification of such a factor would be useful for understanding the mechanisms that control early follicle development in the ovary. This study establishes that kit ligand (KL) may be such a "theca cell organizer."

Kit ligand (KL) and its receptor c-kit are essential for oocyte migration during embryonic development (Bennett, 1956; Mintz and Russell, 1957; Mintz, 1960; McCoshen and McCallion, 1975; Matsui et al., 1990; Orr-Urtreger et al., 1990; Keshet et al., 1991) and follicular development in the adult ovary (Besmer et al., 1993; Manova et al., 1993; Packer et al., 1994). KL and c-kit are the products of the Steel (Sl) and White Spotting (W) loci in mice, respectively (Chabot et al., 1988; Geissler et al., 1988; Copeland et al., 1990; Flanagan and Leder, 1990; Huang et al., 1990; Williams et al., 1990; Witte, 1990; Zsebo et al., 1990). A number of mutations at Sl or W have been described that cause sterility due to defects in oocyte migration or follicular development. Ovaries in mice carrying steel panda (Sl^{pan}), steel t (Sl^{t}), and steel contrast (Sl^{con}) mutations contain large numbers of follicles that arrest at early stages of follicular development (Kuroda et al., 1988; Huang et al., 1993; Bedell et al., 1995). Follicular development is arrested in these mutant mice at the stage that theca cells normally organize around the follicles. Although follicular development appears to have initiated, no theca cells are apparent around these arrested follicles. These observations suggest that KL may be important for theca cell recruitment during early follicle development.

Granulosa cells in developing follicles produce KL (Manova et al., 1993; Motro and Bernstein, 1993) that can act on theca cells and oocytes. Both theca cells and developing oocytes express the receptor c-kit (Manova et al., 1990; Horie et al., 1993; Manova et al., 1993; Motro and Bernstein, 1993). In vitro KL has a variety of effects on isolated oocytes including oocyte expansion and maintenance of meiotic arrest (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991; Packer et al., 1994; Ismail et al., 1996). In addition, KL can directly stimulate proliferation and functional differentiation (i.e., androstenedione production) of theca cells (Parrott and Skinner, 1997). In situ and immunohistochemical studies have shown scattered expression of the receptor c-kit in ovarian stromal-interstitial cells adjacent to developing follicles (Manova et al., 1993; Motro and Bernstein, 1993; Lammie et al., 1994). Studies in bone marrow and thymus have shown that KL may induce proliferation and functional differentiation of several stem cell populations (de Vries et al., 1991; Ogawa et al., 1991; Matsuzaki et al., 1993). Therefore, granulosa cell-derived KL is also proposed to directly stimulate proliferation and/or differentiation of ovarian stromal stem cells to become theca cells. The possibility that granulosa cell-derived KL acts on ovarian stromal-interstitial cells suggests that KL may be a "theca cell organizer" during early follicle development.

Bovine Ovary Fragment Organ Culture

Bovine ovaries were obtained from adult cycling heifers and delivered on ice by Golden Genes (Fresno, CA). Ovaries were chosen that contained large areas of stromal tissue and that lacked a corpus luteum. Ovaries were dipped in 70% ethanol to augment sterilization techniques and to remove the outer layer of surface epithelium (OSE). Thin strips of stromal tissue containing small numbers of primary follicles were microdissected from the cortex of the ovaries. Each strip was cut into small fragments (≈2–4 mm thick) and immediately placed in culture. A method similar to that developed in Dr. Gerald Cunha's laboratory at the University of California at San Francisco for organ culture of seminal vesicle and bulbourethral gland was used to culture fragments of bovine ovaries (Cooke et al., 1987; Shima et al., 1990; Alarid et al., 1994). Bovine ovarian fragments were cultured on 12-mm-diameter floating filters (0.4 µm Millicell-CM, Millipore, Bedford, MD) in 0.5 ml Dulbecco's Modified Eagle's Medium (DMEM)-Ham's F-12 medium (1:1, vol/vol) containing 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO), 0.1% albumax (Gibco) and 0.05 mg/ml L-ascorbic acid (Sigma) in a 16-mm diameter well of a four-well culture plate (Nunc plate, Applied Scientific, South San Francisco, CA). 5X ITS-X (5 µg/ml insulin, 2.75 µg/ml sodium transferrin, 3.35 µg/ml sodium selenite, 1 µg/ml ethanolamine; Gibco BRL, Gaithersburg, MD) was included in the medium to provide sufficient insulin levels for organ cultures (Cooke et al., 1987; Shima et al., 1990; Alarid et al., 1994). Medium was supplemented with penicillin, streptomycin, and gentamycin to prevent bacterial contamination. Each ovarian fragment was placed in a small drop of medium (approximately 10 µl) on the floating filter. Eight ovarian fragments per floating filter were cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 5 days. This organ culture method allowed sufficient nutrient exchanges from the medium and sufficient gas exchange from the humidified chamber to the ovaries. Cultures were treated with no factor (control), recombinant human kit-ligand (KL, 100 ng/ml; R&D Systems, Minneapolis, MN) or follicle stimulating hormone (FSH, purified ovine oFSH-20, 200 ng/ml; National Pituitary Agency, Baltimore, MD) and human chorionic gonadotropin (hCG, 200 ng/ml; 4010 IU/mg, Calbiochem, La Jolla, CA). After 2 days fresh media was added and the cultures were retreated. On culture day 5, media was collected and ovarian fragments were placed in histochoice tissue fixative (Amresco, Solon, OH). Ovarian fragments cultured under these conditions appear healthy and do not show any signs of necrosis.

Histology

In order to examine the potential recruitment of theca cells from the surrounding stroma, fresh or cultured bovine ovarian fragments were embedded in paraffin, sectioned (5-µm sections), and stained with hematoxylin and eosin. Ovarian fragments were chosen

that contained large areas of stromal tissue and no obvious follicles when analyzed under a dissecting microscope (3× magnification). These ovarian fragments consisted of at least 95% stromal tissue with some primary follicles. No antral follicles were observed in any ovarian fragment. All of the follicles appeared to be primary follicles because of the presence of at least one layer of cuboidal granulosa cells around the oocyte. These follicles are at early stages of follicular development and have an average diameter of 42 µm. Theca cells that had been recruited from surrounding stromal stem cells were apparent as organized cellular layers around these primary follicles. Follicles were analyzed at 400× magnification under light microscopy and photographed with a Kodak DCS 420 digital camera (Eastman Kodak, Rochester, NY). Cultured ovarian fragments from each treatment were found to contain approximately 1–3 primary follicles. The total number of follicles did not vary with treatment. Follicles were analyzed "blinded to treatment" by two independent investigators and values compared. At the largest follicle diameter, thickness of the theca cell layer was determined and normalized to total follicle diameter generally follicles with nuclei present were used. Data were expressed as percent theca cell thickness per follicle in order to correct for variation of theca cell layers from follicle to follicle. Approximately 1000 total follicles were examined. Average follicle diameter did not change after treatment due to variation in the initial size of the follicles. Individual follicles increased in overall size after KL treatment.

Growth Assays

Cell growth was analyzed by quantitating (^{3}H) thymidine incorporation into newly synthesized DNA. Strips of stromal-interstitial tissue that lacked follicles were dissected from ovarian cortex. Stromal cells were dispersed from these small fragments of stromal-interstitial tissue by digestion with 2 mg/ml collagenase (Sigma) and 0.5 mg/ml DNAse (Sigma) in Hanks balanced salt solution without Ca^{++} or Mg^{++} at 37°C for 2 hr. Stromal-enriched cells were plated at subconfluent densities (less than 1 million cells/cm^2) in 0.5 ml Dulbecco's Modified Eagle's Medium (DMEM) containing 0.1% calf serum. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hr the cells were treated with no growth factor (control), 10–50 ng/ml KL, or 50 ng/ml epidermal growth factor (EGF) as a positive control. Cells were plated for 24 hr then treated for an additional 20 hr. After treatment 0.5 ml DMEM containing 2 µCi (^{3}H)thymidine was added to each well, and the cells were incubated for 4 hr at 37°C followed by sonication. The quantity of (^{3}H)thymidine incorporated into DNA was determined as previously described (Skinner and Fritz, 1986). Data were normalized to total DNA per well using an ethidium bromide procedure, described previously (Roberts and Skinner, 1990). Under these subconfluent culture conditions, approximately 0.5 µg DNA was detected per well. Values of (^{3}H)thymidine incorporation were generally greater than 2×10^3 cpm/µg DNA. Data were expressed as percent control (untreated cells) and presented as the mean ± SEM of triplicate determinations from four different experiments.

Steroid Assays

Androstenedione and progesterone production by bovine ovarian stromal cells were examined during days 0 to 3 of culture. Stromal cells were isolated as described above and plated at confluent densities (at least 3 million cells/cm^2) in Ham's F12 media containing 0.1% bovine serum albumin (BSA). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Stromal cells were immediately treated with no factor (control), KL (10–50 ng/ml, R&D Systems), estradiol (E, 10^{-7} M, Sigma) or human chorionic gonadotropin (hCG, 100 ng/ml; 4010 IU/mg, Calbiochem). After 3 days media were collected and assayed for androstenedione and progesterone using the RSL ^{125}I androstenedione kit and the ImmunoChem ^{125}I progesterone kit, respectively (ICN, Costa Mesa, CA). The sensitivities of the steroid assays are 0.01 ng/ml for androstenedione and 0.01 ng/ml for progesterone. Data were normalized to total DNA per well using an ethidium bromide procedure, described previously (Roberts and Skinner, 1990). Under these confluent culture conditions approximately 2–5 µg DNA was detected per well. Androstenedione and progesterone levels were 2–10 ng/µg DNA and 2–9 ng/µg DNA respectively. There was no theca cell contamination in these stromal cell cultures as described in the Results section.

Statistical Analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute Inc., Cary, NC). Effects of KL or gonadotropins (FSH + hCG) on theca cell thickness were analyzed by a one-way analysis of variance (ANOVA). Observed significance probabilities of 0.05 (Prob > F) or less were considered evidence that an ANOVA model fits the data. Primary follicles were counted on at least two sections from each treatment in three different experiments. Significant differences between mean percent theca cell thickness for each treatment were determined using the Tukey-Kramer HSD test (honestly significant difference) which protects the significance tests of all combinations of pairs (Kramer, 1956; Tukey, 1991). Effects of KL, EGF, and hormones (i.e., estradiol and hCG) on stromal cell growth and steroid production were analyzed by a one-way analysis of variance (ANOVA) as described above. Significant differences between control (untreated) and treated (growth factor or hormone) means were determined using the Dunnett's test which guards against the high alpha-size (Type I) error rate across the hypothesis tests (Dunnett, 1955). These multiple comparisons tests are recommended for multiple comparisons with control (Dunnett's) or multiple

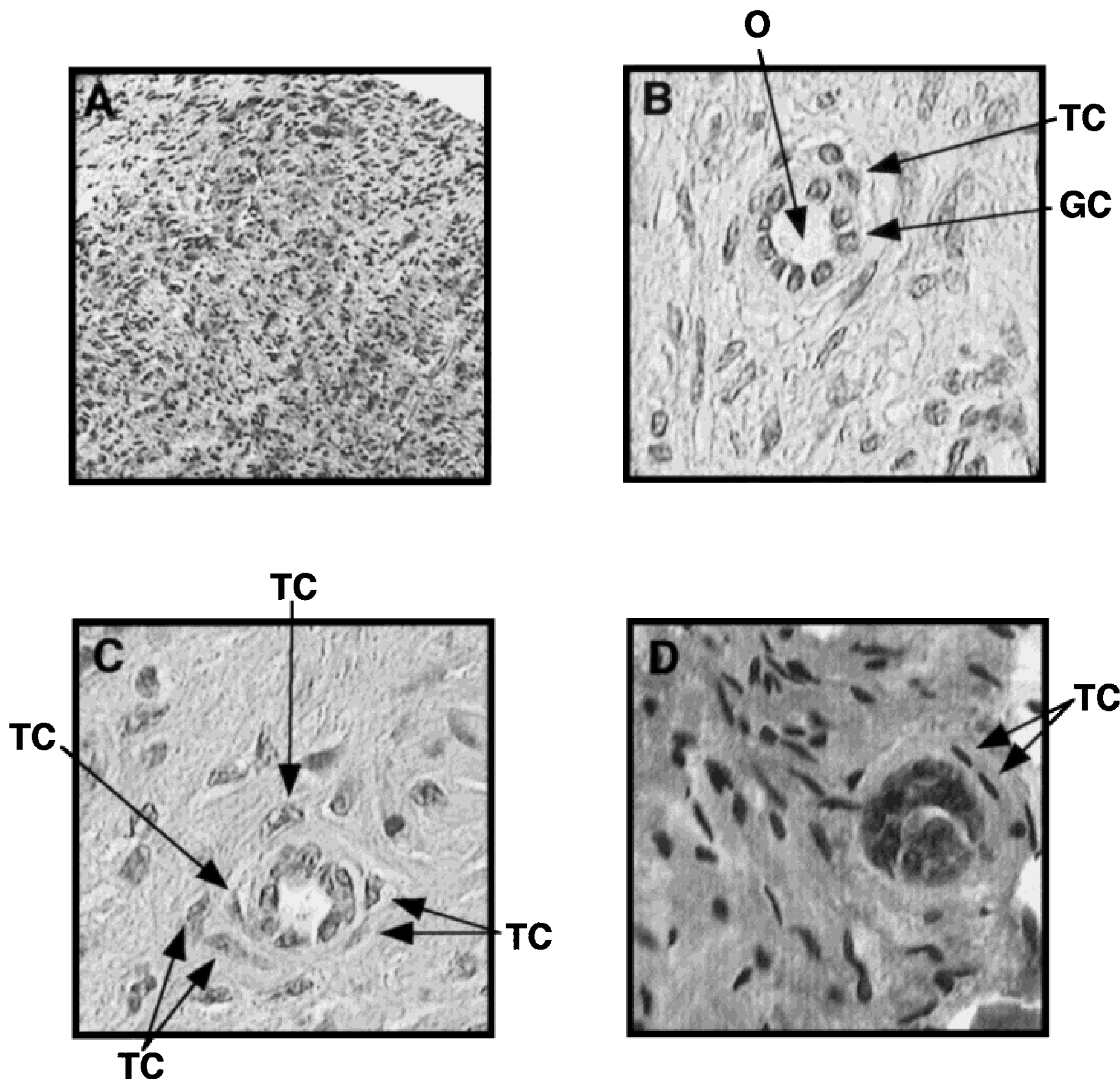

Fig. 1. Bovine ovarian fragment organ culture. Small fragments (approximately 2–4 mm) of adult bovine ovaries were isolated that contained >95% stromal-interstitial cell and some primary follicles. (**A**) Representative fragment composed mostly of stromal cells (magnification approximately 100×). Ovarian fragments were cultured on floating filters for 5 days. (**B**) Representative cultured fragment containing a primary follicle. The oocyte (O) is surrounded by a single layer of granulosa cells (GC) and some theca cells (TC). (**C**) Representative cultured fragment treated with kit-ligand. The theca cell layers (TC) appear thicker. (**D**) Representative cultured fragment treated with gonadotropins (F + hCG). Magnification is approximately 300× for (B), (C), and (D). Overall diameter of primary follicles did not significantly vary between treatments.

comparisons of all pairs (Tukey-Kramer HSD) (Hsu, 1996).

RESULTS

Ovary Fragment Organ Culture

Fragments of adult bovine ovarian cortex were cultured on floating filters in order to examine the effects of KL on the recruitment of theca cell layers around primary follicles. The bovine ovary fragments that contained >95% stroma and some primary follicles were dissected into 2–4-mm fragments and cultured. Figure 1 shows a representative example of a follicle from control and KL-treated ovarian fragments. Within each treatment primary follicles were characterized in terms of total diameter and thickness of the theca cell layers. Theca cell thickness was normalized to total follicle diameter for each follicle. The total diameter of primary follicles varied between 40 and 50 µM. Average total diameter of primary follicles was not affected by treatment with KL or gonadotropins (F + hCG) (Fig. 2A). The slight reduction seen by KL was not statistically significant. However, treatment with KL significantly increased the percentage of theca cell thickness ($P < 0.01$, Fig. 2B). Theca cell layers constituted up to

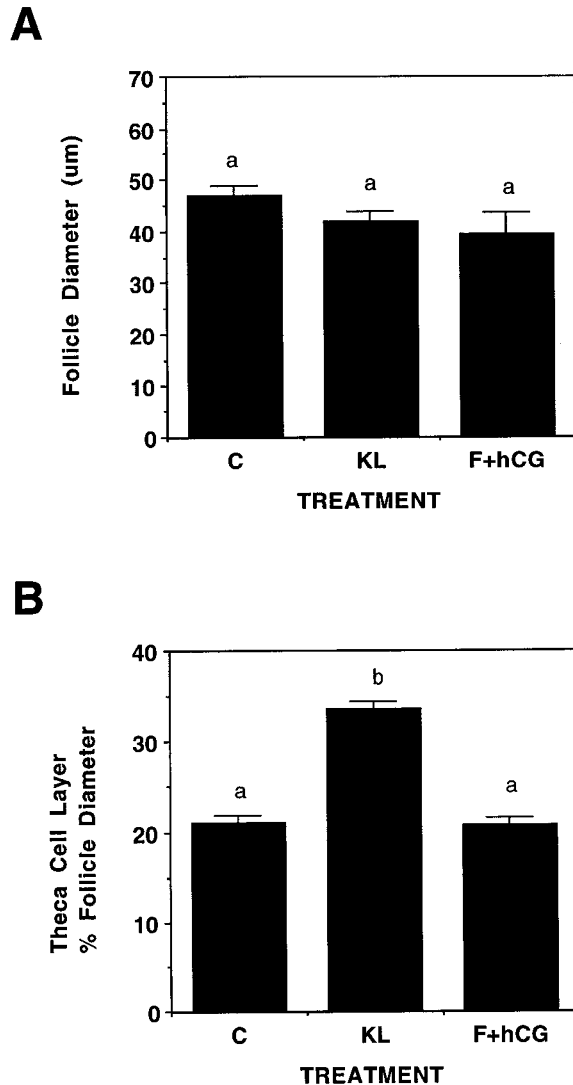

Fig. 2. Effects of kit-ligand (KL) on the thickness of theca cell layers around primary follicles. Bovine ovarian fragments were cultured on floating filters for 5 days. Ovarian fragments were sectioned and stained with hematoxylin and eosin for morphological analysis. (**A**) Total follicle diameter did not vary among treatments (μM ± SEM). (**B**) Thickness of theca cell layers was determined and data were expressed as percent of total follicle diameter (percent diameter ± SEM). C, control; KL, kit-ligand; F + hCG, follicle stimulating hormone and human chorionic gonadotropin. Data are presented as the mean ± SEM of at least 8 follicles from five different experiments. An analysis of variance was performed and significant differences were determined using the Tukey-Kramer HSD test. Bars with different superscript letters are statistically different ($P < 0.01$).

35% total follicle diameter in KL-treated cultures compared to 20% in control- and gonadotropin-treated cultures. These effects of KL on theca cell thickness in primary follicles suggested that KL may promote the recruitment of theca cells from undifferentiated stromal cells. Alternatively, KL may promote proliferation of already recruited theca cells. The establishment of theca cell layers is a critical aspect of early follicle development (Peters, 1979). Therefore, KL actions on ovarian stromal cells and theca cells may be important for the development of early stage follicles in the ovary.

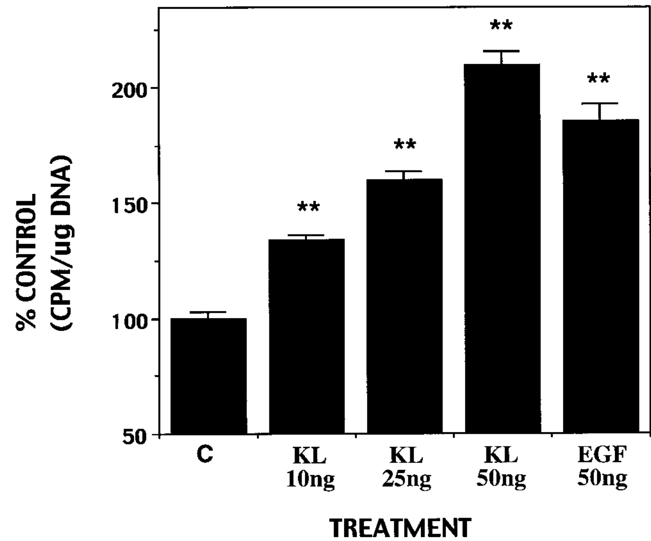

Fig. 3. Effects of KL on ovarian stromal cell (^{3}H)thymidine incorporation into DNA. Bovine ovarian stromal cells were cultured at subconfluent densities (growth permissive) in the absence of growth factor for 24 hr. Then cells were treated as indicated for 20 hr followed by a 4-hr incubation with (^{3}H)thymidine. Counts per min (CPM) of (^{3}H)thymidine incorporated into DNA was determined and normalized to the total DNA per well. Values were generally greater than 2×10^3 cpm/μg DNA. Data are expressed as percent control (untreated cells) and are presented as the mean ± SEM of triplicate determinations from four different experiments. An analysis of variance was performed and significant differences from control cells were determined using the Dunnett's test. Bars with asterisks (**) differ from control ($P < 0.01$). C, control untreated cells; KL, kit ligand treatment at indicated amounts per ml; EGF, epidermal growth factor.

Ovarian Stromal Cell Proliferation

Recruitment of theca cells from undifferentiated stromal cells involves the proliferation and differentiation of ovarian stromal-interstitial cells to become theca cells. Granulosa cell-derived KL may potentially be involved in theca cell recruitment. Therefore the ability of KL to stimulate stromal cell proliferation was examined. Bovine ovarian stromal-enriched cells were cultured and treated with no factor (control), recombinant human KL (10–50 ng/ml) or EGF (50 ng/ml) as a positive control. KL significantly stimulated ovarian stromal cell growth in a dose-dependent manner ($P < 0.01$, Fig. 3). This action of KL on ovarian stromal cell proliferation is similar to the actions of KL on other stem cells such as mast cells and primitive hematopoietic cells (de Vries et al., 1991; Ogawa et al., 1991; Matsuzaki et al., 1993; Galli et al., 1994). KL induces proliferation of stem cells as an initial step in stem cell recruitment. The ability of KL to stimulate ovarian stromal cell growth suggests that KL may potentially influence theca cell recruitment by stimulating stromal cell proliferation around developing follicles.

Ovarian Stromal Cell Steroid Production

Another aspect of theca cell recruitment from undifferentiated stromal stem cells involves the induction of theca cell functional markers. One of the most important functional markers of theca cells that has been identified is steroid production. During early follicular development theca cells differentiate and produce increasing amounts of androstenedione followed by progesterone. Stromal-interstitial cells that surround developing follicles have also been shown to produce low amounts of steroids (i.e., approximately 2–500 fold lower than theca cells) (McNatty et al., 1979). Therefore the functional differentiation of theca cells from adjacent stromal cells can be observed by a dramatic increase in steroid production. The hypothesis was tested that KL promotes theca cell recruitment by inducing high levels of androstenedione and progesterone production in ovarian stromal-interstitial cells. Confluent stromal cell cultures were treated with no factor (control), KL (50 ng/ml), estradiol (E, 10^{-7} M) or human chorionic gonadotropin (hCG, 100 ng/ml). Androstenedione and progesterone accumulation were determined by RIA from days 0–3 of culture. Under control conditions, approximately 10–15 ng/ml androstenedione and 5–15 ng/ml progesterone were produced by ovarian stromal cells. These levels of ovarian stromal cell steroid production are approximately 5% of bovine theca cell steroid production under similar culture conditions. Interestingly, KL did not affect stromal cell androstenedione or progesterone production (Figs. 4 and 5). The observation that KL stimulates theca cell (Parrott and Skinner, 1997) but not stromal cell androstenedione production helps to confirm the purity of these stromal cell cultures. The inability of KL to induce steroid production by ovarian stromal cells suggests that KL may not induce early theca cell functional differentiation. However, steroid production may be induced later in theca cell development and other early theca cell functional markers not analyzed may be induced by KL in the initial stages of follicle development. Preliminary experiments to examine 3β-hydroxysteroid dehydrogenase (3βHSD) levels in the cells showed low levels that were not reproducibly detected in these early stage follicles (data not shown).

The inability of KL to induce stromal cell androstenedione or progesterone production was not due to the inability of stromal cell cultures to respond and produce steroids. Treatment with estradiol resulted in a small but significant increase in androstenedione and decrease in progesterone production ($P < 0.01$, Figs. 4 and 5). Treatment with hCG also stimulated androstenedione production ($P < 0.01$) but had no effect on progesterone (Figs. 4 and 5). The regulation of ovarian stromal cell steroid production by luteinizing hormone (LH) and hCG has been described (Craig, 1967; Dennefors et al., 1980; Barbieri et al., 1984; Brook and Clarke, 1989; Nagamani et al., 1992; Thompson and Adelson, 1993) but the effects of estradiol have not been previously examined. Both estradiol and hCG stimulated ovarian stromal cell androstenedione production to approximately 140% of control. Estradiol decreased stromal cell progesterone production to approximately 60% of control. The observation that hCG stimulates theca cell (Roberts and Skinner, 1990) but not ovarian stromal cell progesterone production helps confirm the purity of these stromal cell cultures.

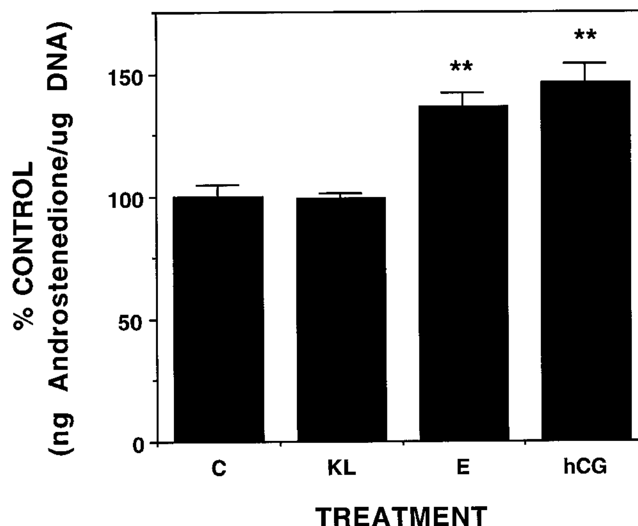

Fig. 4. KL does not alter ovarian stromal cell androgen production. Bovine ovarian stromal cells were cultured at confluent densities (non-growth-permissive) in the absence of serum. Androstenedione accumulation in the culture medium was determined during days 0–3 of culture. Cells were treated with no factor (C), 50 ng/ml kit-ligand (KL), 10^{-7} M estradiol (E) or 100 ng/ml human chorionic gonadotropin (hCG). Androstenedione accumulation was determined by RIA and normalized per µg DNA. Values ranged from 10 to 40 ng/µg DNA. Data are expressed as percent control and are presented as mean ± SEM of triplicate determinations from four different experiments. An analysis of variance was performed and significant differences from control cells were determined using the Dunnett's test. Bars with asterisks (**) differ from control ($P < 0.01$).

DISCUSSION

Interactions between mesenchymal-derived theca cells and epithelial granulosa cells are essential for follicular development in the ovary. However, ovarian follicles do not contain differentiated theca cells during the early stages of folliculogenesis. When a primordial follicle starts to develop, its oocyte begins to synthesize RNA (Oakberg, 1968; Moore and Lintern-Moore, 1974; Moore et al., 1974) and pregranulosa cells enlarge to become squamous granulosa cells. Theca cells are recruited from adjacent ovarian stromal stem cells soon after primordial follicles initiate development (Midgley et al., 1974; Peters, 1979). This recruitment of theca cells is essential for follicular development to continue (Peters, 1979). Theca cells organize into distinct theca cell layers around the follicle. Organization of theca cells around the follicle provides structural integrity and helps to establish mesenchymal-epithelial cell interactions between theca cells and granulosa cells (Skinner, 1990; Skinner and Parrott, 1994). Recruitment of

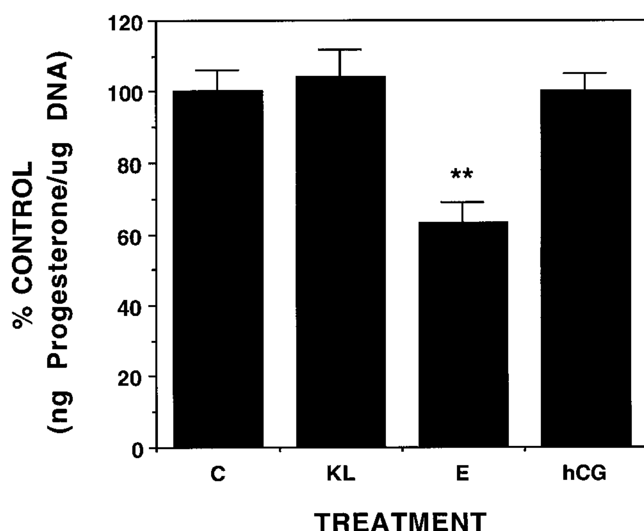

Fig. 5. KL does not alter ovarian stromal cell progesterone production. Bovine ovarian stromal cells were cultured at confluent densities (non-growth permissive) in the absence of serum. Progesterone accumulation in the culture medium was determined during days 0–3 of culture. Cells were treated with no factor (C), 50 ng/ml kit-ligand (KL), 10^{-7} M estradiol (E) or 100 ng/ml human chorionic gonadotropin (hCG). Progesterone accumulation was determined by RIA and normalized per µg DNA. Values ranged from 5 to 30 ng/µg DNA. Data are expressed as percent control and are presented as mean ± SEM of triplicate determinations from four different experiments. An analysis of variance was performed and significant differences from control cells were determined using the Dunnett's test. Bars with asterisks (**) differ from control ($P < 0.01$).

theca cells may in part be induced by a granulosa cell-derived "theca cell organizer" that acts on adjacent stromal stem cells to proliferate and differentiate into theca cells (Midgley et al., 1974; Peters, 1979). This study suggests that KL may act as a "theca cell organizer" in the ovary.

Fragments of adult bovine ovaries were cultured that contained at least 95% stromal cells and some primary follicles. These tissue fragments provided a useful model system to study the potential recruitment of theca cells from stromal cells around small primary follicles. Ovarian fragments were cultured on floating filters as organ cultures. This method has the advantage of preserving the overall structure of the organ. Normal cell-to-cell contacts and extracellular matrix (ECM) interactions are not disturbed. As a result, these organ cultures permit the structural organization of theca cells around small follicles to be observed. When bovine ovarian fragments were cultured in the presence of KL, theca cell layers appeared thicker around primary follicles (Figs. 1 and 2). Organization and thickening of theca cell layers around early developing follicles is a critical step for normal follicular development. Follicles that do not develop proper theca cell layers have been proposed to become atretic and degenerate (Peters, 1979). The ability of KL to promote the formation of theca cell layers around primary follicles in vitro suggests that KL may be involved in establishing theca cell layers in vivo. In the rodent, KL is produced by granulosa cells (Motro et al., 1991; Manova et al., 1993; Motro and Bernstein, 1993) and the receptor c-kit is expressed in stromal tissue around small follicles (Manova et al., 1990; Horie et al., 1993; Motro and Bernstein, 1993). Bovine ovarian cells have a similar expression pattern of KL and c-kit (Parrott and Skinner, 1997). The actions of granulosa cell-derived KL on theca cell recruitment are proposed to be mediated by direct actions on adjacent stromal-interstitial stem cells.

The direct actions of KL on stromal cells were examined by analyzing two processes that occur during theca cell recruitment: (1) cellular proliferation and (2) steroid production (i.e., functional differentiation into theca cells). KL stimulated stromal cell proliferation in vitro (Fig. 3). This result is significant since one of first aspects of theca cell recruitment is proposed to be proliferation of stromal stem cells adjacent to developing follicles. The ability of KL to stimulate stromal cell proliferation suggests that granulosa cells may promote stromal cell proliferation around developing follicles by producing KL. Other factors from granulosa cells may also promote stromal cell proliferation during theca cell recruitment. This is one of the first studies to examine the actions of KL or any other granulosa cell-derived factor on ovarian stromal-interstitial cells. The actions of other granulosa cell-derived factors on ovarian stromal cells remain to be elucidated.

KL apparently did not induce the functional differentiation of purified ovarian stromal cells to become theca cells. Androstenedione production and progesterone production were used as theca cell functional markers since differentiated theca cells produce large amounts of these steroids. Normally ovarian stromal cells produce 2–500 fold less steroids than differentiated theca cells (McNatty et al., 1979). In this study, bovine ovarian stromal cells produced approximately 20–fold less steroids than was previously shown for bovine theca cells (Parrott and Skinner, 1997). KL had no effect on ovarian stromal cell androstenedione or progesterone production. The inability of KL to induce androstenedione or progesterone suggests that KL may not induce theca cell functional markers in stromal cells. However, it remains possible that steroid production may not be the most appropriate functional marker for early theca cell differentiation. Early developing theca cells may not produce high levels of steroids. KL may promote functional differentiation of theca cells by inducing expression of other early theca cell genes. A detailed analysis of the effects of KL on early theca cell differentiation will require the identification of other early theca cell functional markers. These could include the LH receptor and 3βHSD.

Although hormones such as estrogen and LH do not induce recruitment of theca cells from stromal cells, hormonal regulation of stromal cell functions including steroid production has been reported (Dennefors et al., 1980; Barbieri et al., 1984; Barbieri et al., 1986; Snowden et al., 1989; Nagamani et al., 1992). Ovarian stromal

cells have been shown to produce low levels of androstenedione and progesterone in the human, mouse and rat (Craig, 1967; Serment et al., 1969b; Laffargue and Serment, 1973; Guraya and Uppal, 1978; McNatty et al., 1979; Barbieri et al., 1984; Brook and Clarke, 1989; Nagamani et al., 1992; Thompson and Adelson, 1993). The low levels of stromal cell steroid production are probably not physiologically significant in young cycling animals. However, older animals whose ovaries contain negligible follicles are proposed to be more affected by ovarian stromal cell steroid production (Serment et al., 1969b; Laffargue and Serment, 1973; Dennefors et al., 1980; Snowden et al., 1989; Nagamani et al., 1992; Thompson and Adelson, 1993). In humans ovarian stromal cells are the primary source of androgen during perimenopausal and postmenopausal life. Cancers of the ovary and endometrium have been proposed to be influenced by ovarian stromal cell androgen production (Nagamani et al., 1992; Thompson and Adelson, 1993). These and other studies have established that the hormonal regulation of ovarian stromal cell steroid production may be important for many normal and pathologic conditions in humans. This study established that bovine ovarian stromal cells produce androstenedione and progesterone that are regulated by estrogen and hCG (an LH agonist). Adult bovine ovarian stromal cells can be isolated in large quantities. Therefore these cells may provide a useful model system for studying perimenopausal and postmenopausal ovarian stromal cell function.

Observations from the current study on ovarian stromal cells are influenced by the purity of stromal cell cultures. Since the hypothesis was tested that stromal cells are recruited to become theca cells, potential theca cell contamination needs to be considered. Potential theca cell contamination was assessed morphologically and biochemically. First ovarian stromal tissue strips were microdissected under a microscope to remove any follicles that may contribute to theca cell contamination. Second the effects of KL, estrogen and hCG on purified stromal cells were compared to the previously shown effects of these factors on purified theca cells. The profile of steroid production in response to KL, estrogen and hCG was different between bovine theca cells and bovine ovarian stromal cells. KL stimulated theca cell androstenedione production but had no effect on stromal cell androstenedione production. Estrogen had similar effects on theca cell and stromal cell steroid production except that progesterone production was more dramatically inhibited by estrogen in stromal cells. hCG was a potent stimulator of theca cell progesterone production, but had no effect on ovarian stromal cell progesterone production. These results demonstrate that theca cells respond differently than ovarian stromal cells. Taken together these observations confirm the purity of bovine ovarian stromal cell cultures.

Ovarian stromal cell function has been studied for several decades (Craig, 1967; Serment et al., 1969a, 1969b; Balboni, 1970, 1973; Guraya, 1971; Laffargue and Serment, 1973; Hughesdon, 1978; McNatty et al., 1979; Dennefors et al., 1980; Barbieri et al., 1984, 1986; Brook and Clarke, 1989; Nagamani et al., 1992; Vilain et al., 1992; Thompson and Adelson, 1993; Karlan et al., 1995). It is well accepted that the recruitment of theca cells from adjacent stromal stem cells is a critical aspect of early follicle development in the ovary (Peters, 1979). However, the factors that mediate the organization and differentiation of theca cells from stroma are not known. This study suggests that KL may be important for the recruitment of theca cells during follicular development in the ovary. KL was shown to increase the number of theca cell layers around primary follicles in adult bovine ovarian fragment organ cultures. Ovarian stromal cell proliferation was stimulated by KL but androstenedione production and progesterone production were unaffected. In contrast, estrogen and hCG influenced the low levels of stromal cell androstenedione and progesterone production. The ability of hormones to regulate ovarian stromal cell steroid production suggests that these cells may be a useful model system to study human perimenopausal and postmenopausal stromal cell function. KL may be a granulosa cell-derived "theca cell organizer" proposed by Midgley (Midgley et al., 1974; Peters, 1979) that helps recruit theca cells during early follicle development. This study identifies that KL may be one of the first factors to be involved in the recruitment of theca cells. A detailed understanding of the mechanisms of theca cell recruitment will require the identification of other early theca cell functional markers. It is anticipated that other granulosa cell derived factors will likely be involved in the process of theca cell recruitment and early follicle development.

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