BIOLOGY OF REPRODUCTION 38, 790–797 (1988)

Bovine Thecal Cells Secrete Factor(s) That Promote Granulosa Cell Proliferation[1]

JAMES J. BENDELL,[2] DEREK K. LOBB, ANDREW CHUMA,
MATHIAS GYSLER, and JENNIFER H. DORRINGTON

Banting and Best Department of Medical Research
and
Department of Obstetrics and Gynaecology
University of Toronto
Toronto, Ontario, Canada M5G 1L6

ABSTRACT

To determine if soluble factors (other than steroids) secreted by bovine thecal cells may be involved in local regulation of follicular development, we examined the effects of thecal cell secretory products on the growth of granulosa cells obtained from the same follicles. DNA synthesis (assessed by the incorporation of ^{3}H-thymidine) by granulosa cells plated on coverslips and cocultured with, but not directly in contact with, thecal cells in organ culture dishes in a serum-free medium was 5-fold greater than controls. The effect of the thecal cell-secreted products on DNA synthesis by granulosa cells was significantly higher than the maximum response produced by epidermal growth factor (EGF). Thecal cell-conditioned medium stimulated ^{3}H-thymidine incorporation into the DNA of granulosa cells and a normal rat kidney cell line in a dose-dependent manner. The increases in ^{3}H-thymidine incorporation into granulosa cell DNA subsequently lead to an increase in cell number. Preliminary characterization studies using ultrafiltration membranes indicated that the mitogenic factor was retained in the >10,000 molecular weight fraction. The activity was stable to heating at 90°C for 5 min and was not extracted in ether. The thecal cell-generated growth factor may act as a paracrine regulator of granulosa cell growth, thus providing the dominant follicle with autonomy over other follicles in the cohort.

INTRODUCTION

In the adult female mammal, the follicle selected to ovulate is the fastest growing functional unit in the body. The increase in size of the follicle is due to the accumulation of fluid in the follicular antral cavity and the proliferation of granulosa cells in the layers surrounding the antrum (Goldenberg et al., 1972). It has been proposed that the events taking place during the rapid phase of growth prior to ovulation result from the interactions between thecal cells and granulosa cells and that these enable the dominant follicles to acquire eminence over other follicles which subsequently undergo atresia (Dorrington et al., 1984). The developmental advantages acquired by the dominant follicle may be due to its ability to synthesize locally those factors required for differentiation and morphogenesis. The concept of intrafollicular regulators was developed initially from the observations that steroids synthesized by the follicle could act locally on cells within the same follicle. For example, testosterone synthesized by the follicle serves as a substrate for aromatization and also acts as a hormone to influence follicle-stimulating hormone (FSH)-induced steroidogenesis in granulosa cells (Armstrong and Dorrington 1976; Goff et al., 1979). Similarly, estrogen synthesized from androgens within the follicle acts on granulosa cells to modulate FSH actions in addition to its extragonadal actions on the hypothalamic-pituitary axis and the uterus (Knecht et al., 1984).

In this paper, we have investigated the possibility that soluble factors secreted by bovine thecal cells may be involved in the local regulation of granulosa cell proliferation. This work stems from the earlier observations of McNatty et al. (1980) that coculture of human granulosa cells with thecal or stromal tissue leads to an increase in the number of granulosa cells. Subsequently, Makris et al. (1983) showed that extracts of porcine thecal tissue and thecal cell-conditioned medium supplemented with calf serum contain a protein-like factor that stimulates the pro-

Accepted November 24, 1987.
Received October 14, 1986.

[1] Presented at the 19th annual meeting of the Society for the Study of Reproduction, July 14–17, 1986, Ithaca, New York, Abstract No. 78. This work was supported by MRC Canada.

[2] Reprint requests.

liferation of 3T3 cells and granulosa cells. In the present study, we have developed a coculture system in which thecal cells and granulosa cells do not come into direct contact and have demonstrated that thecal cell-secretion products stimulate the growth of granulosa cells in the absence of serum components.

MATERIALS AND METHODS

Calf serum, trypsin, penicillin, streptomycin, and Fungizone were purchased from Gibco, Grand Island, NY. Bovine serum albumin (BSA); collagenase, type I; soybean trypsin inhibitor, type I; and DNAase were obtained from Sigma Chemical Company, St. Louis, MO. Heparin was purchased from Organon Canada Ltd., Toronto, Ontario. Culture dishes were acquired from Falcon Plastics, Los Angeles, CA, and 24-well plates were Linbro brand from Flow Laboratories, McLean, VA. ^{3}H-Thymidine was obtained from New England Nuclear, Boston, MA.

Preparation of Granulosa and Thecal Cells

Ovaries were collected at a local abattoir from heifers less than 20 min after slaughter. The ovary containing the dominant follicle was removed from each animal and placed in medium A (Hanks' Buffered Salt Solution containing 0.1% BSA, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.6 μg/ml Fungizone). All tissue was kept at 4°C during the 45-min trip to the laboratory.

Under sterile conditions, the dominant follicle was removed from the ovary, punctured by a hypodermic needle, and drained of follicular fluid. Follicular health was assessed by the criteria of McNatty et al. (1984), with a healthy preovulatory follicle being no less than 10 mm in diameter, possessing a vascular pink to red theca, and showing no debris in the follicular fluid. The follicle was flushed several times with medium A (supplemented with 15 U heparin/ml), cut into hemispheres, and gently scraped with a rubber policeman to remove the granulosa cells. The granulosa cells were transferred to medium A, and the follicles, reduced to basement membrane and theca, were rinsed and transferred to a clean dish.

The granulosa cell fraction was centrifuged at 50 × *g* for 5 min, decanted, gently agitated by passage through a Pasteur pipette, and resuspended in culture medium containing 10% calf serum. The cells in the suspension were then counted by a hemocytometer and assessed for viability by trypan blue dye exclusion. The culture medium used was Eagle's Minimum Essential Medium (MEM) with Earle's salts and 0.1 mM of each of the following amino acid supplements: L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine, and glycine. The medium also contained 4 mM glutamine, 2.5 g/l sodium bicarbonate, 1.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin, and 0.6 μg/ml Fungizone).

The thecal layer was removed from the follicular sections with fine forceps under 6 × magnification and incubated for 30 min in 0.25% trypsin in culture medium at 37°C to dislodge any remaining granulosa cells. The theca was then chopped, agitated in medium A, allowed to sediment, and the supernatant was discarded. After a 45-min digestion in 3 mg/ml collagenase with 24 μg/ml DNAase, the tissue fragments were centrifuged at 50 × *g* for 5 min, passed 20 times through a Pasteur pipette, and resuspended in culture medium containing 10% calf serum. Aliquots of thecal cell preparations were counted and tested for viability with trypan blue dye exclusion.

Cocultures of Thecal and Granulosa Cells

All cells were cultured at 37°C in a humidifed atmosphere of 5% CO_2 and 95% air to maintain the medium at pH 7.4. Granulosa cells (approximately 3 × 10^5/coverslip) were plated onto 18 × 18 mm glass coverslips placed in 10-cm dishes and maintained in culture medium plus 10% calf serum for the first 24 h, then in culture medium plus 0.1% calf serum for a further 72 h.

Approximately 3 × 10^5 thecal cells were divided into aliquots and placed in organ culture dishes in 1.0 ml of culture medium plus 10% calf serum. After 24 h, the confluent cells were washed with serum-free culture medium three times over an 8-h period. Subsequently, the cells were cultured in 2.0 ml of serum-free culture medium for 48 h. At this time, the granulosa cell coverslips were washed with serum-free culture medium and placed in the organ culture dishes so that they rested on the middle ledge, but were not in contact with thecal cells (see Fig. 1). A further 1.0 ml of serum-free culture medium was added to each dish to ensure that the granulosa cells were immersed in the medium. After 18 h, the granulosa cell coverslips were individually placed in 35-mm dishes and incubated with 2.0 μCi/ml ^{3}H-thymidine in serum-free culture medium for 4 h. The amount of ^{3}H-thymidine incorporated into DNA was determined as described below.

Granulosa Cell Growth Assay

Thecal cells plated in 10-cm dishes were cultured in medium containing 10% calf serum for 48 h, washed with serum-free medium three times over an 8-h period, and then incubated for 48 h with serum-free culture. The conditioned medium was collected and centrifuged at 1400 × *g* for 5 min to remove cellular debris.

Granulosa cells (1.5×10^5/well) in 24-multiwell culture plates were incubated for 24 h in culture medium containing 10% calf serum, followed by 72 h in culture medium containing 0.1% calf serum. The medium was removed, and the cells were washed with culture medium and subsequently treated for 18 h with graded dilutions of the thecal cell-conditioned medium. The conditioned medium was replaced with 0.5 ml culture medium containing 1 μCi of ^{3}H-thymidine, and the cells were incubated for 4 h. The amount of ^{3}H-thymidine incorporated into DNA was determined as described below.

To determine if the increases in ^{3}H-thymidine into DNA resulted in an increase in cell proliferation, the numbers of cells in each culture dish were counted for 6 days after treatment with epidermal growth factor (EGF) or bovine thecal cell-conditioned medium. Bovine granulosa cells (2.4×10^5/dish) in 3.0 ml 10% calf serum were plated into 60-mm tissue culture dishes. After 48 h, the medium was replaced with 0.1% calf serum, and the cells were maintained in culture for a further 72 h. At this time the cells were washed with medium and were cultured in 0.1% calf serum containing 10 ng EGF/ml or 50% bovine thecal cell-conditioned medium. Whereas bovine granulosa cells remained viable over the 22 h of culture in the absence of serum and ^{3}H-thymidine incorporation into DNA could be demonstrated under these conditions, it was necessary to supplement the medium with low levels of serum (0.1%) to maintain cell survival over the period required to demonstrate an increase in cell number (Savion et al., 1981). On the day of treatment (time zero) the number of cells/dish were counted; thereafter, cells were counted every 48 h over a 6-day period. The plating efficiency was 50%. Culture medium was changed every 48 h and the cells were retreated with EGF or conditioned medium. The cells were prepared for counting in a hemocytometer by washing the monolayers three times with 3 ml Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate-buffered saline and incubating for 20 min with 600 μl 0.25% trypsin in Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate-buffered saline. Soybean trypsin inhibitor (1 mg in 10μl) was added, and the cells were gently flushed from the plate with a Pasteur pipette. Counts were obtained from duplicate cultures at each time point with 8–12 separate aliquots being counted from each culture.

Normal Rat Kidney (NRK) Cell Line Growth Assay

Confluent cultures of NRK cells were subcultured with 0.25% trypsin and replated at 10% confluence into 24-well Linbro plates. The cells were maintained for 24 h in culture medium containing 10% calf serum, then for 72 h in culture medium containing 0.1% calf serum. The cells were washed in serum-free medium and treated with thecal cell-conditioned medium; the growth assay was conducted as described for granulosa cells.

Determination of ^{3}H-Thymidine Incorporation into DNA

After incubation for 4 h with ^{3}H-thymidine, the medium was removed and replaced with 0.5 ml of 0.25% trypsin solution in Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate-buffered saline. The plates then were frozen at −20°C after a 15-min incubation at 37°C. After thawing, the trypsin incubations were sonicated in the culture wells with 15 pulses from the needle probe of a Sonifer cell disruptor (Branson Sonic Power Co., Danbury, CT). Duplicate aliquots (100 μl) were filtered on Whatman DE-81 filter paper discs on a Millipore filter system. DNA remains bound to the diethylaminoethyl (DEAE)-cellulose paper (Litman, 1968). The filters were washed twice with 2 ml H_2O and counted in 5 ml aquasol (modification of the method described by Griswold et al., 1976).

The DNA content of the sonicated samples was measured by the method of Karsten and Wollenberger (1977) as modified by Louis and Fritz (1979). An aliquot of each sonicate was made up to 100 μl with buffer B (20 mM NaCl, 5 mM disodium ethylenediaminetetraacetate, 0.02% sodium azide, and 10 mM tris (hydroxymethyl)aminomethane-HCl, pH 7.8). Aqueous heparin, 50 μl of 10 IU/ml stock solution, was added and incubated for 30 min at room temperature. Subsequently, 0.1 ml of a freshly made 1:400

dilution of a 1 mg/ml stock solution of ethidium bromide (both in buffer B) was added to each sample. The samples were read immediately in a spectrophotofluorometer with excitation at 350 nm and emission at 585 nm. Duplicate samples of calf thymus DNA up to 2.0 μg were used to establish the standard curve for the calculation of the DNA content of the samples.

The effectiveness of the trypsin-sonication procedure to release DNA was established by comparing this with other methods of rupturing the cells. Cells disrupted by the standard trypsin-sonication procedure gave 3656 ± 160 cpm/filter, trypsinization followed by homogenization gave 3566 ± 542 cpm/filter, and trysinization-homogenization followed by sonication gave values of 3491 ± 416 cpm/filter. Control cell-free media containing ^{3}H-thymidine had background binding of 148 ± 34 cpm/filter disc.

Characterization

Pooled thecal cell-conditioned medium known to have growth promoting activity was divided into four aliquots. One aliquot was extracted twice with two volumes of diethyl ether to remove any steroids that may have been synthesized during the culture period. The diethyl ether was washed twice with an equal volume of MEM prior to use. Fresh culture medium was washed with ether in an identical fashion for use as a control. Both samples of ether-treated medium were heated to 40°C under N_2 to remove residual traces of ether, and then were gassed with 95% air and 5% CO_2 before use.

Other aliquots of conditioned medium were heated at either 90°C for 5 min or 100°C for 20 min. Fresh culture medium was treated similarly. The samples of medium were allowed to cool and were gassed with 95% air and 5% CO_2 before use.

Conditioned medium was concentrated and separated into two fractions by passage through a 10,000 molecular weight exclusion ultrafiltration membrane (Amicon Corp. Danvers, MA) followed by passage of the filtrate through a 1000 molecular weight exclusion membrane. The volumes of the two molecular weight fractions, 1000 to 10,000 and greater than 10,000, were then restored to their starting volumes with fresh culture medium. The fractions of conditioned medium were tested for their ability to promote the growth of NRK cells in comparison to the starting material.

Statistical Analysis

The data are presented as the mean ± SEM of determinations from three replicate cultures within each treatment group and are derived from a single experiment. Observations were confirmed in two or more independent experiments. Mean values were compared using the Student's *t*-test. The Student's *t*-test and Two-way Analysis of Variance (see Fig. 3) were performed with "Statworks" software on a Macintosh Plus. The data presented in Figures 1 and 4–6 are expressed as the cpm ^{3}H-thymidine incorporated into DNA, normalized for total μg DNA. The short-term treatment of the cells did not result in a significant increase in DNA content between treatment groups within a given experiment. The data were expressed per μg total DNA to normalize for differences in numbers of cells plated in various experiments and differences in plating efficiencies.

RESULTS

Following initial plating in 10% serum, thecal cells were maintained in the center wells of double-welled organ culture dishes for 48 h in serum-free culture medium to allow secretory products to accumulate. Granulosa cells obtained from the same follicles as the thecal cells were grown on coverslips for the same length of time and were then placed in the organ culture dishes as shown in Figure 1. The amount of ^{3}H-thymidine incorporated into DNA by the granulosa cells cocultured with thecal cells for 24 h was 5-fold greater than in similarly prepared granulosa cells cultured in medium alone ($p<0.001$) (Fig. 2). Since Gospodarowicz and Birdwell (1977) have shown that EGF effectively stimulates the proliferation of bovine granulosa cells, EGF was used as a positive control. The incorporation of ^{3}H-thymidine into DNA by granulosa cells on coverslips incubated in medium containing a maximally effective concentration of EGF (10 ng/ml) was significantly less than that by granulosa cells in the coculture system ($p<0.05$) (Fig. 2). Over a 6-day period of culture with EGF (10ng/ml), the number of granulosa cells increased ($p<0.001$) (Fig. 3). The ability of growth factors to cause an increase in granulosa cell number over prolonged periods of time in culture has been studied in detail by Savion et al. (1981). The addition of 50% bovine thecal cell-conditioned medium also caused an increase in cell number over a 6-day culture period ($p<0.001$) (Fig. 3). This experiment established that the stimulation of ^{3}H-thymidine incorporation into DNA was a valid index of cell proliferation. The effect of the conditioned medium on the growth of granulosa cells was not due to the nutritive effects of the proteins in the medium since albumin, added at a concentration of 14 μg/ml to be comparable to the amount of secreted protein, had no effect.

Subsequently, various amounts of thecal cell-conditioned medium were added to granulosa cells established in monolayer cultures. After treatment of the granulosa cells with conditioned medium for 18 h, the incorporation of ^{3}H-thymidine into DNA over a 4-h period was determined. As shown in Figure 4, 25% thecal cell-conditioned medium caused a significant increase in ^{3}H-thymidine incorporation into DNA ($p<0.05$), and there was a dose-dependent increase up to 100% conditioned medium.

To determine if the growth-promoting activity present in the thecal cell-conditioned medium acted exclusively on granulosa cells or if its action was more general, the effect on NRK cells was also studied. As shown in Figure 5, the ^{3}H-thymidine incorporation into the DNA or NRK cells was increased by the addition of thecal cell-conditioned medium, and the dose-response curve was similar to that obtained with bovine granulosa cells.

The growth-promoting activity of thecal cell-

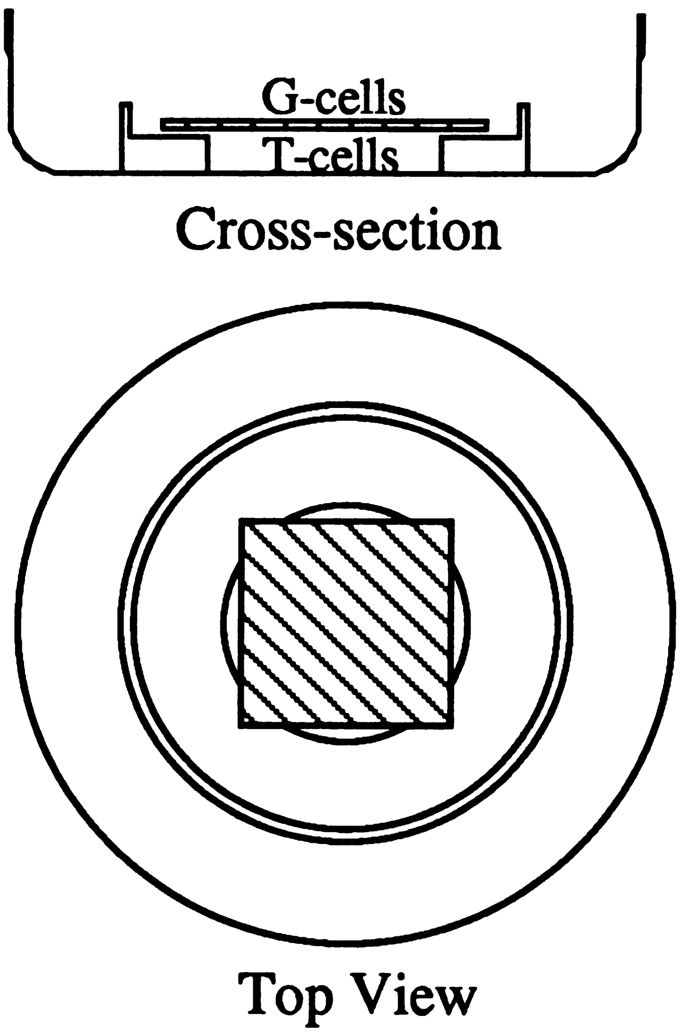

FIG. 1. Schematic diagram of the double-welled culture dishes used for the coculture of thecal cells *(T-cells)* and granulosa cells *(G-cells)* to indicate the position of the respective cell monolayers. Thecal cells are plated in the central well, whereas granulosa cells are cultured on 18 X 18 mm coverslips *(hatched area)* and placed on the shoulder of the central wells, so that they are submerged in conditioned medium.

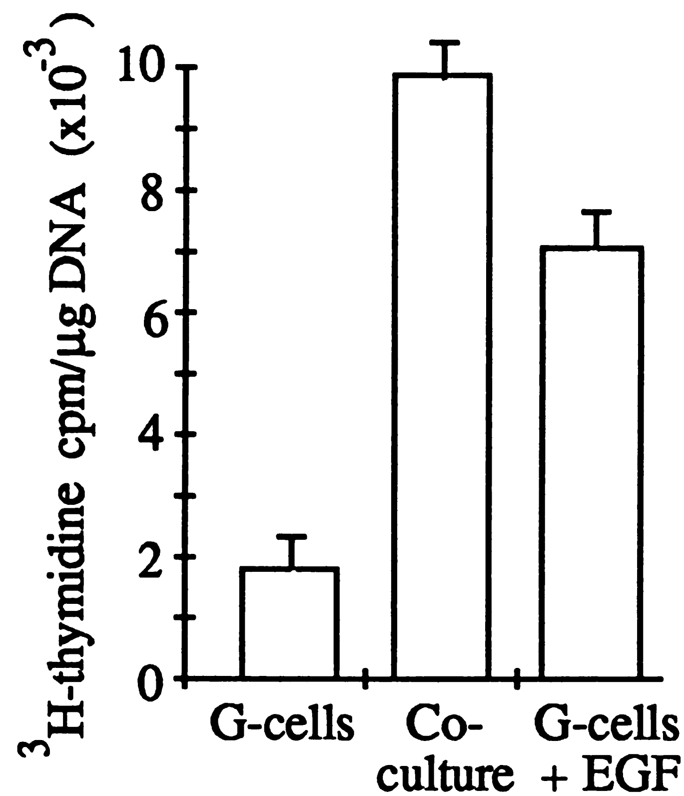

FIG. 2. Influence of thecal cells in coculture on the ^{3}H-thymidine incorporation into granulosa cell DNA. Granulosa cell cultures established on coverslips for 72 h were placed in organ culture dishes (see *Fig. 1*) containing culture medium alone (G-cells), in conditioned medium generated by thecal cells cultured in the same well (coculture), or in culture medium containing 10 ng epidermal growth factor (*EGF*)/ml. After 18 h, the coverslips were removed and the cells were incubated with 2.0 μCi ^{3}H-thymidine/ml for 4 h. The granulosa cells were removed from the coverslips with trypsin, sonicated, and the ^{3}H-thymidine incorporation into DNA was measured. The effects above control were significant for both coculture ($p<0.001$) and EGF treatment ($p<0.01$). There was also a significant difference between the coculture group and the G-cells + EGF group ($p<0.01$). Each value is the mean of triplicate cultures ± SEM.

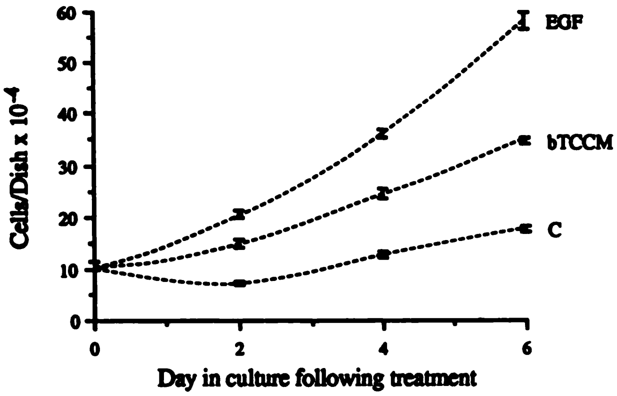

FIG. 3. The ability of epidermal growth factor (*EGF*, 10 ng/ml) and 50% bovine thecal cell-conditioned medium (*TCCM*) to increase granulosa cell number. Cells were maintained in 10% serum for 48 h, after which they were well-washed and maintained in culture medium for 3 days in 0.1% serum. On Day 5 (Time zero of treatment), the medium was replaced with fresh 0.1% serum, and the cells were treated with EGF or conditioned medium. At Time zero and every 48 h subsequently, the cells were removed by trypsinization in 0.25% trypsin and were counted in a hemacytometer.

conditioned medium on NRK cells was unchanged after extraction with ether. Ether-extracted culture medium also did not influence significantly the basal level of ^{3}H-thymidine incorporation into DNA. The growth-promoting activity was stable to heating at 90°C for 5 min but was destroyed when heated at 100°C for 20 min. When conditioned medium was concentrated through an ultrafiltration membrane, the growth factor(s) was retained in the greater than 10,000 Dalton fraction (Fig.6).

DISCUSSION

The main conclusion that can be drawn from the data presented in this paper is that bovine thecal cells secrete a factor that promotes the proliferation of granulosa cells, as assessed by increased incorporation of ^{3}H-thymidine into DNA and increased cell number.

In the past, thecal cells have been recognized as the main androgen-synthesizing cells in the ovary

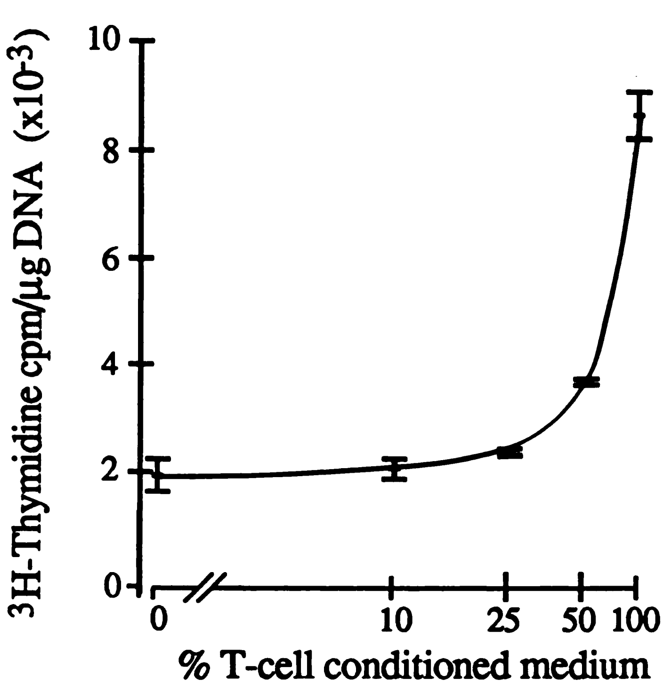

FIG. 4. Dose-dependent effect of thecal cell-conditioned medium on the ^{3}H-thymidine incorporation into granulosa cell DNA. Granulosa cells were established in culture for 96 h prior to treatment with graded dilutions of conditioned medium generated by cultured thecal cells. After 18 h of treatment, the medium was replaced by 0.5 ml culture medium containing 1 µCi ^{3}H-thymidine. The medium was discarded 4 h later, and the amount of ^{3}H-thymidine incorporated into DNA was measured. Each value is the mean of triplicate cultures ± SEM and is representative of 3 separate experiments.

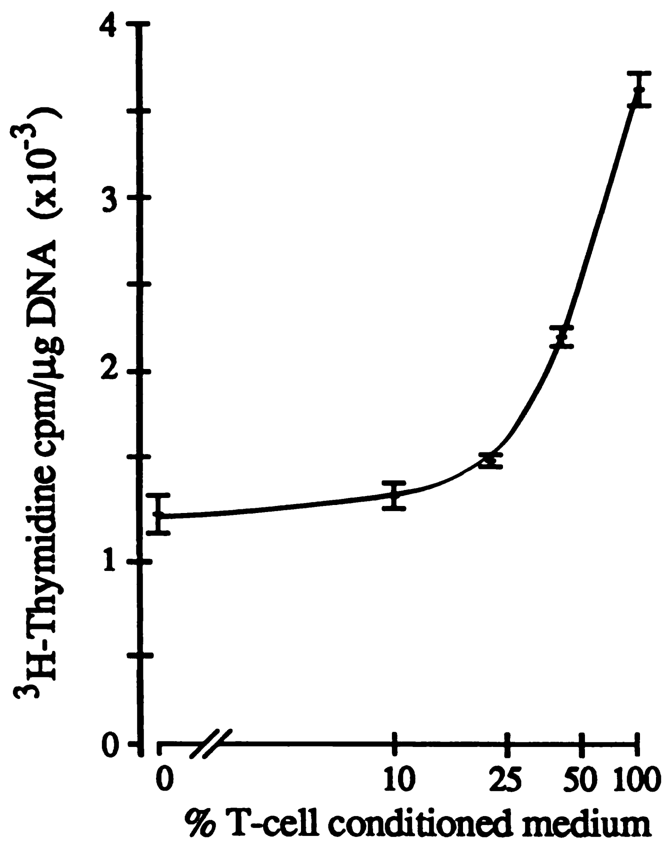

FIG. 5. Dose-dependent effect of thecal cell-conditioned medium on the ^{3}H-thymidine incorporation into normal rat kidney cell DNA. NRK cells were plated at 10% confluence and were maintained in culture, treated with thecal cell-conditioned medium and assayed for ^{3}H-thymidine incorporation into DNA as described for granulosa cells in the legend to Figure 4. Each value is the mean of triplicate cultures ± SEM.

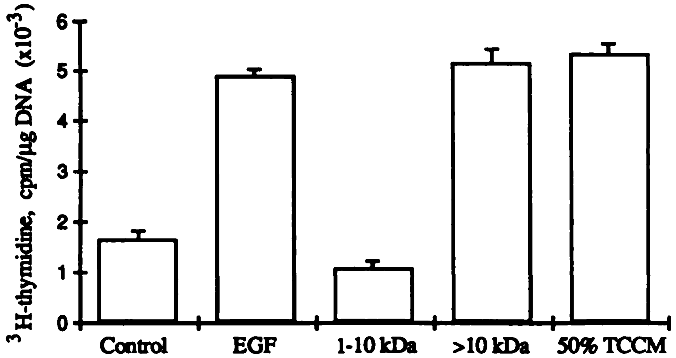

FIG. 6. The ability of the greater than 10,000 molecular weight fraction and the 1000 to 10,000 molecular weight fraction to stimulate the incorporation of ^{3}H-thymidine into normal rat kidney cell DNA. The two fractions were obtained by passing concentrated thecal cell-conditioned medium through molecular weight exclusion ultrafiltration membranes. The fractions were restored to the initial volume of the conditioned medium and tested for their growth-promoting activity on NRK cells as described in *Figure 5*.

(Erickson et al., 1985). Granulosa cells are deficient in the enzymes that convert progesterone to androstenedione and therefore depend upon the thecal cells to produce the androgen substrate for estrogen production. Whereas the regulation and steroidogenic activities of thecal cells has been described in detail, their ability to secrete other soluble products and the physiological significance of these products have received little attention.

To test the possibility that thecal cell-derived factors may influence granulosa cell functions, the coculture system was devised to enable thecal cells and granulosa cells to be cultured in the same medium without having direct contact. Since granulosa cells proliferate and acquire differentiated functions during follicular development, we initially investigated possible effects of thecal cell-secreted products on granulosa cell proliferation. Using the coculture system, the granulosa cell cultures grown on coverslips can be removed readily for quantification of ^{3}H-thymidine incorporation into DNA. The coculture system provides an additional advantage in that the thecal cell-conditioned medium does not require freezing and storage before use that may affect activity. Using this coculture system, we demonstrated that the conditioned medium generated by thecal cells had growth-promoting activity, as indicated by the enhanced incorporation of ^{3}H-thymidine into granulosa cell DNA. The increase in the ^{3}H-thymidine incorporation into DNA of granulosa cell cultures in the presence of thecal cell-conditioned medium was greater than that achieved in the presence of EGF. EGF is a potent mitogen for the proliferation of human and bovine granulosa cells (Gospodarowicz et al., 1977) and was employed at 10^{-9} M, a level above that required for maximal effect.

The growth factor has not been characterized; however, some of its properties are known. The growth-promoting activity was not ether-extractable, showing that it was not due to a steroid secreted by thecal cells. This is consistent with previous findings that androgens and estrogens are ineffective in stimulating the proliferation of bovine granulosa cells. Granulosa cells contain receptors for several growth factors, including somatomedin-C, EGF, FGF, and PDGF (Hsueh et al., 1984), and furthermore EGF, FGF and somatomedin-C have been shown to be potent mitogens for granulosa cell proliferation (Savion et al., 1981). It is reasonable to consider, therefore, that the thecal cell-derived growth factor is the same as, or has sequence homology with, those factors that are known to influence granulosa cell proliferation. It is also possible that several growth factors act in concert to produce the observed response. The resistance of the growth factor to heating at 90°C for 5 min is somewhat intriguing, even though the activity was destroyed by heating at 100°C for 20 min. However, EGF-like growth factors have been shown to be resistant to heating (Carpenter and Cohen, 1979), while other peptide mitogens—such as FGF (Gospodarowicz et al., 1978) and chrondrocyte growth factor (Kasper et al., 1982)—are heat labile under less stringent conditions. The growth-promoting activity was retained in the greater than 10,000 molecular weight fraction after concentration through an ultrafiltration membrane, which is consistent with the view that the factor is a protein or bound to a binding protein.

Granulosa cell proliferation is essential for normal follicular development and oocyte maturation; consequently, the growth factors involved in this process are critical components in the maintenance of fertility in the adult female. Both granulosa cells and thecal cells have been considered as possible sources of follicular growth factors. In this regard, porcine granulosa cells have been shown to produce somatomedin-C, and this factor may act as an autocrine regulator of growth in addition to its well-recognized role as a local modulator of FSH-induced granulosa cell-differentiated functions (Hammond et al., 1985).

Makris et al., (1983) showed that porcine thecal cell cytosol contained a growth factor that stimulated the proliferation of 3T3 and granulosa cells. The presence of a growth factor in a tissue does not necessarily indicate the source of synthesis, since the factor may have been taken up from the circulation in vivo and retained in the cytosol. Porcine thecal cell-conditioned medium was found to contain mitogenic activity; however, it was stated that the activity was highly variable, and the presence of calf serum in the conditioned medium, which may contribute mitogens or mitogen precursors, made interpretations difficult. The latter observations did not distinguish between the possibility of a thecal cell factor that acts directly on granulosa cells as opposed to a thecal cell product that modifies a component in serum which is then responsible for the stimulation of granulosa cell growth. The major contribution of the present studies is that the thecal cell has been established as the source of a secreted growth factor that can stimulate the proliferation of granulosa cells. This is not due to the uptake of the factor from serum and its subsequent release into conditioned medium, since the thecal cells secreted the growth factor at a constant rate throughout 6 days of culture.

The demonstration of the production of growth-promoting activity by thecal cells and its paracrine action on granulosa cell growth adds further credence to the hypothesis that the dominant follicle completes its developmental program as a result of thecal cell-granulosa cell interactions (Dorrington et al., 1984). The cell-cell interactions that take place in the developing follicle can be compared to those that occur in the structuring of other tissues, such as the uterus, prostate, and mammary gland, in which "inducer molecules" produced by mesenchymal cells promote epithelial morphogenesis and differentiation (Cunha et al., 1983).

ACKNOWLEDGMENTS

We would like to express our thanks to the Management of Canadian Dressed Meats for allowing us to collect the bovine ovaries used in these experiments. We would like to thank Heather McKeracher for excellent technical assistance.

REFERENCES

Armstrong DT, Dorrington JH, 1976. Androgens augment FSH-induced progesterone secretion by cultured rat granulosa cells. Endocrinology 99:1411–14

Carpenter G, Cohen S, 1979. Epidermal growth factor. Annu Rev Biochem 48:193–216

Cunha GR, Chung LWK, Shannon JM, Taguchi O, Fujii H, 1983. Hormone-induced morphogenesis and growth: role of mesenchymal-epithelial interactions. Recent Prog Hrom Res 39:559–98

Dorrington JH, McKeracher H, Garzo G, Skinner MK, 1984. Granulosa cell-theca cell interactions during follicular development. In: Labrie F, Proulx L (eds.), Excerpta Medica. Amsterdam: Elsevier Science Publishers, pp. 807–10

Erickson GF, Magoffin DA, Dyer CA, Hofeditz C, 1985. The ovarian androgen producing cells: a review of structure/function relationships. Endocr Rev 6:371–99

Goff AK, Leung PCK, Armstrong DT, 1979. Stimulatory action of follicle-stimulating hormone and androgens on the responsiveness of rat granulosa cells to gonadotropins in vitro. Endocrinology 104:1124–29

Goldenberg RL, Vaitukaitis JL, Ross GT, 1972. Estrogen and follicle stimulating hormone interactions on follicle growth in rats. Endocrinology 90:1492–98

Gospadarowicz D, Bialecki H, Greenburg G, 1978. Purification of the fibroblast growth factor activity from bovine brain. J Biol Chem 253:3736–43

Gospodarowicz D, Ill CR, Birdwell CR, 1977. Effects of fibroblast and epidermal growth factors on ovarian proliferation in vitro. I. Characterization of the response of granulosa cells to FGF and EGF. Endocrinology 100: 1108–20

Griswold MD, Mably ER, Fritz IB, 1976. FSH stimulation of DNA synthesis in Sertoli cells in culture. Mol Cell Endocr 4:139–49

Hammond JM, Baranas JLS, Skaleris D, Knight AB, Romanus JA, Rechler MM, 1985. Production of insulin-like growth factors by ovarian granulosa cells. Endocrinology 117:2553–55

Hsueh AJW, Adashi EY, Jones PBC, Welsh TH, 1984. Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr Rev 5:76–127

Karsten U, Wollenberger A, 1977. Improvements in the ethidium bromide method for direct fluorimetric estimation of DNA and RNA in cell and tissue homogenates. Anal Biochem 77:464–70

Kasper S, Ivorsley IG, Rowe JM, Shiu RPC, Friesen HG, 1982. Chondrocyte growth factor from the human pituitary gland. J Biol Chem 257:5226–30

Knecht M, Darbon JM, Ranta T, Baukal AJ, Catt KJ, 1984. Estrogens enhance the adenosine 3',5'-monophosphate mediated induction of follicle-stimulating hormone and luteinizing hormone receptors in rat granulosa cells. Endocrinology 115:41–49

Litman RM, 1968. A deoxyribonucleic acid polymerase from Micrococcus luteus isolated on deoxyribonucleic acid-cellulose. J Biol Chem 243:6222–33

Louis BG, Fritz IB, 1979. Follicle-stimulating hormone and testosterone independently increase the production of androgen-binding protein by Sertoli cells in culture. Endocrinology 104:454–60

Makris A, Klagsbrun MA, Yasumizu T, Ryan KJ, 1983. An endogenous ovarian growth factor which stimulates BALB/3T3 and granulosa cell proliferation. Biol Reprod 29:1135–41

McNatty KP, Heath DA, Henderson KM, Lun S, Hurst PR, Ellis LM, Montgomery GW, Morrison L, Thurley DC, 1984. Some aspects of thecal and granulosa cell function during follicular development in the bovine ovary. J Reprod Fertil 72:39–53

McNatty KP, Makris A, DeGrazia C, Osathanondh R, Ryan KJ, 1980. Steroidogenesis by recombined follicular cells from the human ovary in vitro. J Clin Endocrinol Metab 51:1286–92

Savion Lui G, Laherty R, Gospodarowicz D, 1981. Factors controlling proliferation and progesterone production by bovine granulosa cell in serum-free medium. Endocrinology 109:409–20