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Rat thecal/interstitial cells produce a mitogenic activity that promotes the growth of granulosa cells

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Summary

To test the hypothesis that the cells outside the basal lamina of the follicle secrete paracrine factors that influence the cells on the inside of the follicle, two ovarian cell populations were obtained from diethylstilbestrol-treated rats. Granulosa cells were obtained by extrusion from the follicles and an ovarian cell preparation, termed thecal/interstitial, was derived from the granulosa cell-depleted ovaries. Light microscopy showed that each cell population in culture had distinctive morphologies. Electrophoretic examination of the radiolabeled proteins secreted by the two ovarian cell preparations revealed that each secreted unique protein components into the culture medium. Rat thecal/interstitial cell-conditioned medium promoted [³H]thymidine incorporation into normal rat kidney cell line (NRK) DNA and into bovine granulosa cell DNA. The growth-promoting activity was stable to heating at 70°C for 5 min whereas native fibroblast growth factor (FGF) lost its activity, showing that the factor was not characteristic of FGF. To further characterize the growth-promoting activity thecal/interstitial cell-conditioned medium was concentrated and the proteins separated by size exclusion high performance liquid chromatography. The growth-promoting activity eluted with an apparent molecular weight between 15 000 and 25 000. The finding that thecal/interstitial cells in culture secrete growth-promoting factors suggests that those cells that are in close proximity to the granulosa cells may secrete protein factors that diffuse into the follicular antrum and influence granulosa cell proliferation.

Introduction

The steroidogenic and gametogenic processes that occur in the mammalian ovary require the presence of the gonadotrophins, luteinizing and follicle-stimulating hormone. Even though LH and FSH are essential for follicular maturation, it has

become increasingly apparent that intraovarian regulatory mechanisms involving locally produced soluble factors may also play an important role in mediating cell-cell interactions in the follicle.

The role of follicular steroids as mediators of thecal/interstitial-granulosa cell interactions has been well defined. For example, in the rat, the thecal/interstitial cells produce androgens that serve both as substrates for aromatization by the granulosa cells, as well as augmenting FSH-induced functions (Armstrong and Dorrington,

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1976; Goff et al., 1979). Granulosa cell-derived estradiol-17 β appears to act in an autocrine fashion by synergizing with FSH to influence granulosa cell-differentiated functions (Knecht et al., 1984). The role of non-steroidal factors synthesized within the ovary that may be involved in regulating the growth and differentiation of the oocyte has also received considerable attention. These non-steroidal factors include prostaglandins, plasminogen activator, inhibin, arginine-vasopressin, oxytocin, vasoactive intestinal peptide and growth factors (Hsueh et al., 1984). Our working hypothesis is that follicular maturation involves the progression to a stage at which a paracrine self-amplifying system is established between thecal and granulosa cells of the follicle. The development of this system of cell-cell interaction involving the local production of soluble factors could enable the dominant follicle to become autonomous and acquire eminence over other follicles that become atretic.

To test this hypothesis we have examined the possibility that rat thecal/interstitial cells may secrete factors that influence granulosa cell proliferation. The conditioned medium generated by thecal/interstitial cells cultured in serum-free medium contained a potent mitogen for both a normal rat kidney cell line and bovine granulosa cells. The growth factors that conferred mitogenic activity were separated by HPLC size exclusion chromatography and eluted with apparent molecular weights between 15 000 and 25 000.

Materials and methods

Animals

Immature Wistar CrI:(W1)BR rats were obtained from Charles River Canada (Montreal, Que., Canada) and maintained with their mothers under conditions of controlled light and temperature. Female 21-day-old rats were treated daily for 4 days with 1 mg diethylstilbestrol (DES) in 0.1 ml sesame oil by s.c. injection.

Preparation of cell cultures from the rat ovary

(a) *Granulosa cells.* Rats were killed at 25 days of age, and the granulosa cells were recovered from the ovaries by puncturing the follicles with a

fine needle, as described previously (Dorrington et al., 1975). Cells (approximately 100 μ g protein) were plated into 24-multiwell Falcon tissue culture plates (Falcon Plastics, Los Angeles, CA) as 1.0 ml aliquots in culture medium consisting of Eagle's minimum essential medium (MEM) with Earle's salts and 0.1 mM 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/liter NaHCO₃, 1.5 mM HEPES and antibiotics, 50 U/ml penicillin, 50 μ g/ml streptomycin and 0.6 μ g/ml Fungizone (Gibco, Grand Island, NY). The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air to maintain the medium at pH 7.4. The cells were allowed to attach to the surface of the plate for 3 h before the addition of dibutyl cyclic adenosine monophosphate ((Bu)₂cAMP) to the existing culture medium.

(b) *Rat thecal / interstitial cells.* Following granulosa release the remaining ovarian tissue was washed once in Hanks' balanced salt solution (HBSS) then chopped into approximately 0.25 mm² sections (Mickle McIlwain Tissue Chopper, Laboratory Eng. Co., Gomshall, Surrey, U.K.). Tissue fragments were washed once in HBSS to remove residual granulosa cells and allowed to settle under gravity. The supernatant was discarded and the remaining tissue was digested in HBSS containing 1 mg collagenase/ml and 5 μ g DNase/ml (Sigma, St. Louis, MO) at 37°C for 45 min. Digested ovarian sections were centrifuged at 30 \times g for 5 min and further dispersed by 20 passages through a Pasteur pipette. Remaining undigested tissue that consisted of large cell aggregates and vascular elements was removed by sedimentation under gravity for 10 min. The supernatant containing small cell aggregates and single cells was centrifuged at 30 \times g for 5 min, resuspended and plated in a 150 mm tissue culture dish (Falcon) in culture medium containing 10% calf serum and maintained at 37°C as described above. After 24 h the confluent thecal/interstitial-enriched preparation was trypsinized (0.25% trypsin in HBSS) and subcultured at a split ratio of 1:4 into 150 mm tissue culture dishes in culture medium containing 10% calf serum. After 48 h confluent thecal/interstitial cell subcultures were

washed 3 times with serum-free culture medium over an 8 h period, after which serum-free conditioned medium was collected every 48 h. Conditioned medium was centrifuged at $5000 \times g$ for 15 min at 4°C and stored at -20°C . As required, conditioned medium was concentrated 100-fold via passage through a 10 000 MW cut-off ultrafiltration membrane (Amicon, Danvers, MA).

To determine the proportion of steroidogenic cells in the subcultured thecal/interstitial cells, the cultures were treated with 0.5 mM $(\text{Bu})_2\text{cAMP}$ from the time of plating to stimulate the activity of 3β -hydroxysteroid dehydrogenase. 90% of the cells showed positive staining for 3β -hydroxysteroid dehydrogenase using the method of Sharpe and McNeilly (1980).

Normal rat kidney (NRK) cell line growth assay

Stock confluent cultures of NRK cells were trypsinized (0.25% trypsin in HBSS) and plated into 24-well culture plates in 10% serum at a density of approximately 10 000 cells per well. The medium was removed after 24 h and the cell cultures were washed with culture medium and replenished with medium plus 0.1% serum, after a further 72 h the cultures were washed and maintained in serum-free culture medium containing graded dilutions of thecal/interstitial cell-conditioned medium. After 18 h of treatment with conditioned medium, the medium was removed and replaced with 0.5 ml culture medium containing $1 \mu\text{Ci}$ [^3H]thymidine (New England Nuclear, Boston, MA) and the cells were incubated for 4 h. The medium was aspirated, 0.5 ml of a 0.25% trypsin solution in Ca^{2+} , Mg^{2+} -free Dulbecco's phosphate-buffered saline was added to the cells and the plates were incubated at 37°C for 15 min and then frozen at -20°C . After thawing, the trypsin digests were sonicated in the culture wells using 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 system. DNA remains bound to the DEAE-cellulose paper (Litman, 1968). The filters were washed twice with 2 ml H_2O and counted in 5 ml Aquasol (Amersham, Oakville, Ont.) (modification of the method described by Griswold et al., 1976). The radioactivity on the filters was unchanged when the filters were washed

4 times with either water, 5% Na_2HPO_4 or 10% trichloroacetic acid.

To determine if increases [^3H]thymidine into DNA subsequently resulted in an increase in cell proliferation, cell numbers were estimated. NRK cells were plated in 35-mm tissue culture dishes in medium containing 10% calf serum for 24 h. The medium was removed from triplicate plates and cell cultures were trypsinized to remove them from the plates and the cells were counted on a haemocytometer. This number of cells is referred to as the zero time control. Other cell cultures were maintained in culture medium alone or in culture medium supplemented with 25% thecal/interstitial cell-conditioned medium for a further 6 days, with medium changes every 2 days. After 6 days the number of cells in each plate was counted.

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-HCl, pH 7.8). Heparin, 50 μl of 10 IU/ml in buffer B 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 in a spectrophotofluorometer with excitation at 350 nm and emission at 585 nm. Calf thymus DNA was used as a standard.

Bovine granulosa cell growth assay

Ovaries were collected at a local abattoir in medium A (HBSS containing 50 $\mu\text{g}/\text{ml}$ gentamicin) on ice. Under sterile conditions the dominant follicle was removed from the ovary and punctured by a hypodermic needle to drain off the 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 USP 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, centrifuged at $50 \times g$ 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 using a haemocytometer and the viability (30–40%) was determined by trypan blue dye exclusion. Aggregates of granulosa cells contained viable cells whereas single cells were usually not viable. Aliquots containing 50 000 viable cells were plated in 24-multiwell culture plates and incubated for 24 h in 1 ml culture medium containing 10% calf serum, followed by 72 h in culture medium containing 0.1% calf serum. Medium was removed, the cells were washed with culture medium and treated for 18 h with various concentrations of rat thecal/interstitial cell-conditioned medium. The medium was then replaced with 0.5 ml culture medium containing 1 μ Ci [3 H]thymidine, and the cells were incubated for 4 h. The amount of [3 H]thymidine incorporated per μ g DNA was then determined as described above.

Gel electrophoresis

Primary cultures of thecal/interstitial cells were maintained in medium plus 10% calf serum in 24-multiwell culture plates for 48 h. After 24 h half of the wells were trypsinized, as described above and cells were subcultured in medium plus 10% calf serum for a further 24 h. Subsequently, both primary and subcultured thecal/interstitial cell and granulosa cell cultures were washed with methionine-free culture medium and 0.5 ml of the same medium containing 5 μ Ci [35 S]methionine (New England Nuclear, Boston, MA) was added to each well. After 24 h the medium was removed, centrifuged at $5000 \times g$ and frozen at -20°C .

Electrophoresis was performed on 5–15% polyacrylamide gradient slab gels with the use of the Laemmli SDS buffer system (Laemmli, 1970). Samples were reduced with mercaptoethanol prior to electrophoresis. Gels were fluorographed using diphenyloxazole in acetic acid as previously described (Skinner and Griswold, 1983).

Size exclusion high pressure liquid chromatography (HPLC)

Size exclusion chromatography of concentrated medium from thecal/interstitial cells was accom-

plished with an HPLC apparatus (Beckman). The column utilized was a series of TSK (Toyo-Soda) 7.5 mm \times 30 cm columns including one SW-4000, two SW-3000 and one SW-2000. The series of columns was equilibrated and eluted at 0.5 ml/min with 1 M ammonium acetate, 0.5 M Na_2SO_4 , pH 4.5. Fractions were collected, dialyzed for 48 h at 4°C , frozen at -70°C and lyophilized. Standardization of the column for molecular weights was done with thyroglobulin (660 kDa), catalase (240 kDa), bovine serum albumin (68 kDa) ovalbumin (42 kDa) and chymotrypsinogen (25 kDa).

Results

The rat granulosa cell population obtained by the procedure described has been biochemically characterized in previous publications (Dorrington et al., 1983). Other investigators using similar methods have described the morphology of these cells (Amsterdam et al., 1981). Fig. 1A shows a light micrograph of the granulosa cell culture after 48 h and it is apparent that the cultures represent a homogeneous cell population.

The cell preparation referred to as thecal/interstitial cell is more difficult to define precisely. In essence, the preparation initially plated consists of the ovarian cells remaining after the removal of the bulk of the granulosa cells by extrusion into the medium, followed by repeated agitation of the minced ovarian tissue after chopping. Whereas granulosa cell aggregates can be seen in the cultures in the early stages, the numbers diminish with time in primary culture and are rarely identified after subculture of the cells. Fig. 1B shows a light micrograph of the thecal/interstitial cells after 48 h in primary culture. The morphology is similar to that of thecal cells in the intact follicle.

To determine more precisely the degree of granulosa cell contamination in the thecal/interstitial cell preparation, the granulosa cells and thecal/interstitial cells isolated from the same ovaries were treated with and without $(\text{Bu})_2\text{cAMP}$ (0.5 mM) from the time of plating and were cultured under identical conditions. $(\text{Bu})_2\text{cAMP}$ rather than FSH was used to stimulate aromatase activity since trypsinization, subculturing of the cells and increasing time in culture may compro-

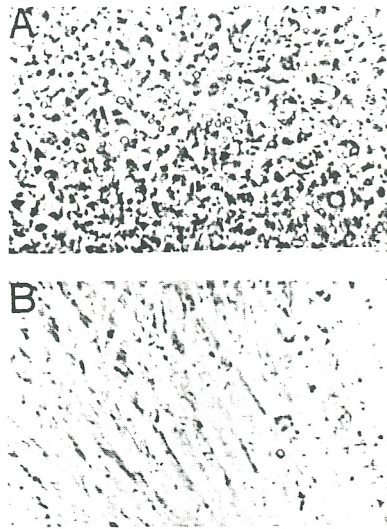


Fig. 1. Light micrographs of (A) untreated granulosa cells and (B) untreated thecal cells after 48 h in culture (magnification 250 \times).

mise FSH receptors. After 4 days in culture the cells were incubated with [1β - ^3H]testosterone for 2 h and the release of $^3\text{H}_2\text{O}$ determined as an index of aromatase activity (Gore-Langton and Dorrington, 1981). The data were as follows, control granulosa cells 3340 ± 53 dpm/mg protein, and (Bu) $_2$ cAMP-treated granulosa cells, $138\,580 \pm 5800$ dpm/mg protein, control thecal/interstitial cells, 1986 ± 82 dpm/mg protein, and (Bu) $_2$ cAMP thecal/interstitial cells, 3960 ± 162 dpm/mg protein.

The two ovarian preparations were further biochemically characterized by examining the respective profiles of secreted radiolabeled proteins by electrophoresis on SDS-polyacrylamide gradient slab gels (Figs. 2 and 3). The profile of granulosa cell-secreted radiolabeled proteins shown in Fig. 2 is similar to that published earlier, showing that fibronectin (220 000 Da) is a major protein. The thecal/interstitial cell preparation secreted radiolabeled proteins that are quite distinct from those secreted by granulosa cells. After subculture of the thecal/interstitial cells, the profile of secreted radiolabeled proteins remained essentially the same (Fig. 3).

Having characterized the thecal/interstitial cell preparation as indicated, their ability to secrete growth-promoting activity was assessed using

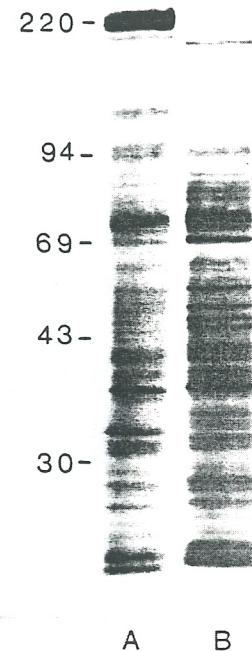


Fig. 2. Fluorogram of electrophoretic profiles of granulosa cell-secreted proteins. Radiolabeled proteins were obtained from control (A) and FSH-treated cultures (200 ng/ml NIH FSH-15) (B), after 72 h of culture.

NRK cells that respond to a range of growth factors. As shown in Fig. 4, conditioned medium obtained from subcultured thecal/interstitial cell cultures stimulated the growth of NRK cells in a dose-dependent manner. The addition of 25% rat thecal/interstitial cell-conditioned medium caused an increase in NRK cell number over a 6-day culture period. At the onset of the treatment period $60\,000 \pm 12\,000$ cells were present in each well, after 6 days in culture in serum-free medium $57\,300 \pm 11\,000$ cells remained, whereas in cultures treated with 25% rat thecal/interstitial cell-conditioned medium there was an increase in the number of cells to $80\,000 \pm 6200$ /well.

Subsequently, the effects of the conditioned

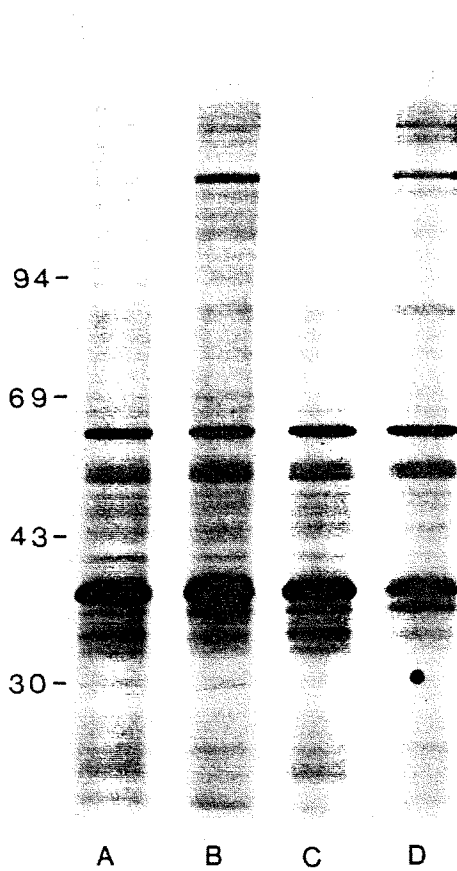


Fig. 3. Fluorogram of electrophoretic profiles of thecal/interstitial cell-secreted proteins. Radiolabeled proteins obtained from control (A) and $(\text{Bu})_2\text{cAMP}$ -treated (0.1 mM) (B) cells of primary cultures are similar to those of subcultured control (C) and $(\text{Bu})_2\text{cAMP}$ -treated (0.1 mM) (D) cells 48 h after subculture. This is representative of four similar fluorograms.

medium on the growth of bovine granulosa cells was assessed to determine if the growth-promoting activity could play a role in the local regulation of granulosa cell proliferation. Rat granulosa cells were not used in these experiments since rat granulosa cells only have a limited ability to proliferate *in vitro* under conditions tested to date (Epstein-Almog and Orly, 1985). At the onset of the treatment period with conditioned medium each well of bovine granulosa cells contained 0.9–1.0 μg DNA/well. There was no increase in the DNA content of the cultures after treatment

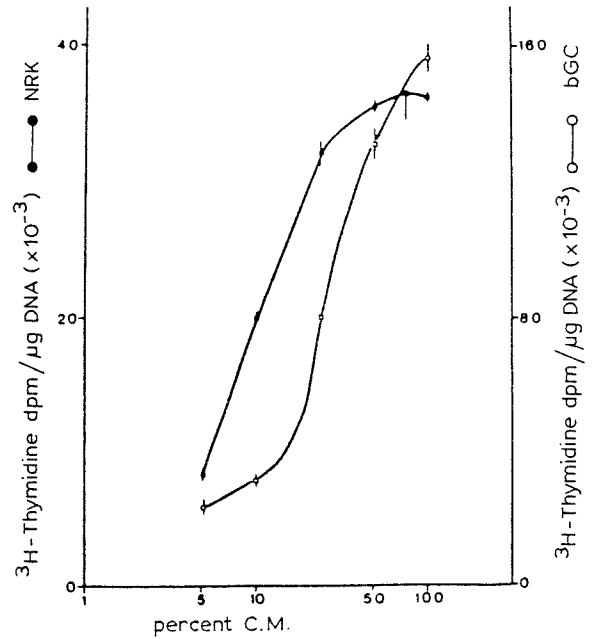


Fig. 4. Dose-dependent effect of thecal/interstitial cell-conditioned medium on the $[^3\text{H}]$ thymidine incorporation into NRK cell DNA and bovine granulosa cell (bGC) DNA. Both NRK cells and granulosa cells were maintained in culture for 96 h prior to treatment with graded dilutions of conditioned medium generated by rat thecal/interstitial cells. After 18 h treatment the medium was replaced with 0.5 ml culture medium containing 1 μCi $[^3\text{H}]$ thymidine. The medium was discarded 4 h later and the amount of $[^3\text{H}]$ thymidine incorporated into DNA was counted. Each value is the mean of triplicate cultures \pm SEM.

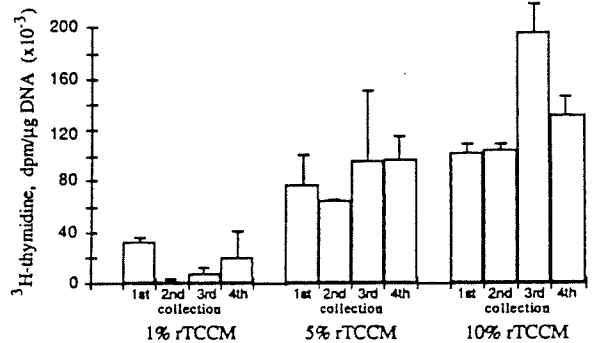


Fig. 5. Effect of time in culture of subcultured thecal/interstitial cells on the production of growth-promoting activity. Conditioned medium was collected over four 48 h collection periods and tested for its ability to promote $[^3\text{H}]$ thymidine incorporation into NRK cell DNA as described in the legend to Fig. 4. Each value is the mean of triplicate cultures \pm SEM.

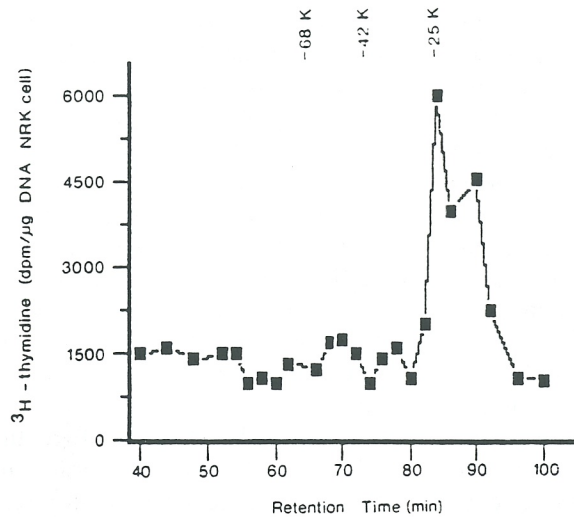


Fig. 6. Size exclusion high pressure liquid chromatography of concentrated thecal/interstitial cell-conditioned medium. Mitogenic activity in individual fractions was determined using NRK cells and represented as [^3H]thymidine dpm/ μg NRK cell DNA. The void volume was at 40 min retention time and total volume at 100 min retention time. Molecular weight standards eluted at the retention times indicated.

with conditioned medium for 18 h. As shown in Fig. 4, conditioned medium from rat thecal/interstitial cells promoted the growth of bovine granulosa cells in a dose-dependent fashion.

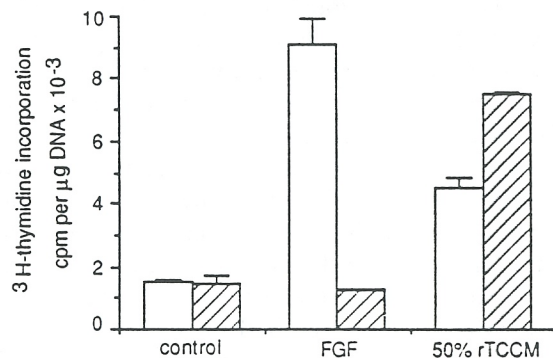


Fig. 7. The stability of mitogenic activity in conditioned medium was examined in an NRK cell growth assay. Medium with and without FGF (10 ng/ml) and thecal/interstitial cell-conditioned media was heated to 70°C for 5 min and the ability to stimulate DNA synthesis was tested as in Fig. 4. Hatched columns represent heat-treated medium. The values are [^3H]thymidine incorporation per μg DNA of triplicate cultures \pm SEM.

Fig. 5 shows the effect of time in culture of the subcultured cells on the production of the growth-promoting activity. The production of this growth factor persisted over the four 48 h collection periods.

Thecal/interstitial cell-conditioned medium was concentrated and applied to an HPLC size exclusion column. The mitogenic activity of the eluted fractions was assessed using the NRK cell line growth assay. The activity eluted in two peaks with apparent molecular weights between 15 000 and 25 000 (Fig. 6).

The mitogenic activity of thecal/interstitial cell-conditioned medium when assayed on NRK cells was increased after heating at 70°C for 5 min. In contrast, heat-treated medium containing FGF was reduced to control values (Fig. 7).

Discussion

The rat ovary is the most widely used and best understood model system for the study of follicular development (Dorrington et al., 1983; Hsueh et al., 1984). Granulosa cells isolated from immature, DES-treated rat ovaries acquire intracellular proteins, including the steroidogenic enzymes, aromatase, cholesterol side-chain cleavage and 3β -hydroxysteroid dehydrogenase when treated with FSH in culture (Dorrington et al., 1983). Cell surface receptors for LH, prolactin and growth factors (IGF-1, EGF) are also induced as granulosa cells differentiate. In addition to acquiring cellular proteins, granulosa cells secrete a number of proteins that have been identified, such as fibronectin (Skinner et al., 1985), proteoglycans (Mueller et al., 1978), plasminogen activator (Canipari and Strickland, 1986) and IGF-1 (Hammond et al., 1985). An analysis of the electrophoretic profile of radiolabeled proteins revealed that the granulosa cells secreted a wide range of different proteins, some of which could be used as markers to distinguish the granulosa cell population from other cells present in the ovary.

Whereas the composition of the thecal/interstitial cell preparation is not known, it appears to be free of granulosa cells at the time of collection of conditioned medium, as assessed by aromatase activity and the absence of granulosa cell marker proteins, e.g. 220 and 75 kDa. Under the light

microscope the thecal/interstitial cells had the morphological characteristics of thecal cells in situ (Erickson et al., 1985). Vascular elements for the most part were discarded in the undigested portions of the ovarian tissue. Small islands of endothelial cells were seen occasionally, but these cells did not constitute more than 1–2% of the total population. The production of the growth-promoting activity by the cultures was maintained at a constant level through four 2-day collection periods. This combined with the constancy of the thecal/interstitial cell protein profiles, both before subculture and with increasing time in culture implies that the integrity of the cultures was maintained and that no minor component (e.g. fibroblasts) was gaining dominance with time in culture.

The establishment of a cell culture of ovarian cells distinct from granulosa cells allowed us to pursue the concept that granulosa cells may be influenced in part by paracrine regulators. Possible soluble factors that may be involved in cell communication in the ovary include steroids and peptides. The role of androgens produced by thecal cells in influencing granulosa cell metabolism set the precedence in establishing the current concepts of cell–cell interaction in the follicle. Estrogens are known to be mitogenic for granulosa cells in vivo (Goldenberg et al., 1972). However, estrogens are not mitogenic for granulosa cells in vitro. Because granulosa cells of the developing follicle are one of the most rapidly proliferating cell types in vivo and in vitro growth cannot be directly attributed to known endocrine factors, we postulate that locally produced intrafollicular regulators of growth are involved. As a first step in pursuing this idea we collected conditioned medium from thecal/interstitial cell cultures under serum-free conditions and found that this stimulated [³H]thymidine incorporation into NRK cell DNA. In determining the physiological relevance of the thecal/interstitial cell growth-promoting activity, rat granulosa cells were not used since conditions under which they will respond to growth factors have not been established. This is not the case for bovine granulosa cells since they do respond to epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin and IGF-1 in vitro (Savion et al., 1981). When rat thecal/in-

terstitial cell-conditioned medium was applied to bovine granulosa cells under serum-free conditions [³H]thymidine incorporation into DNA was stimulated, the dose-response curve being similar to that for NRK cells. Peptides from porcine follicular fluid previously have been shown to stimulate granulosa cell proliferation in vitro, but it was not clear if these peptides were produced locally or are taken up from the serum (Hammond and Yoshida, 1983). Furthermore, Makris et al. (1983) have found that thecal tissue homogenates contain a mitogenic factor for porcine granulosa cells.

In the studies described here, thecal/interstitial cells were deprived of serum and conditioned medium was collected under serum-free conditions. The constant release of growth-promoting activity over an 8-day period suggests that the results cannot be attributed to the retention by the cells or the extracellular matrix of growth factors taken up from the serum present in the medium after subculture. These observations indicate that the thecal/interstitial cells are the source of the growth-promoting activity. In parallel studies on the bovine follicle, thecal cell layers dissected out free of granulosa cells and interstitial cells and also cultured under serum-free conditions secreted a component(s) that stimulated the growth of bovine granulosa cells and NRK cells (Lobb et al., 1986).

Both bovine granulosa and NRK cells respond to FGF with increased growth, and the growth-promoting activity generated by thecal/interstitial cells is in the correct molecular weight range for FGF. The heat stability of the thecal/interstitial cell factor argued against this, however, since native FGF when heated at 70 °C for 5 min lost its ability to stimulate [³H]thymidine incorporation into the DNA of NRK cells. EGF and IGF-1 are also mitogens for granulosa cell proliferation, however, both of these native peptides have lower molecular weights (i.e. 6 and 7 kDa) than that of the growth-promoting activity from thecal/interstitial cells. Whether the factor is a precursor molecule for EGF or IGF-1 or a different peptide will be determined when it is purified.

Growth factors are known to be peptides that exert their effects on neighbouring cells and possibly only those cells in close proximity are exposed

to sufficiently high concentrations to be responsive. In this paper we have shown that thecal/interstitial cells produce a growth-promoting activity that may act locally within the follicle. Thecal/interstitial cells are not the only source of growth factors in the ovary as shown by the ability of granulosa cells to secrete IGF-1 which is a potent mitogen for granulosa cell proliferation (Savion et al., 1981; Hammond et al., 1985). Cell growth is a multifactorial process (Pledger, 1985) and it is likely that the growth factors produced by both granulosa cells and thecal/interstitial cells will interact to promote follicular development.

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