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Ovarian Thecal/Interstitial Cells Produce an Epidermal Growth Factor-Like Substance*

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ABSTRACT. An ovarian thecal/interstitial cell preparation was obtained by enzymatic digestion of immature rat ovaries depleted of granulosa cells. A component in thecal/interstitial cell-conditioned medium inhibited specific binding of [^{125}I]iodo-epidermal growth factor ([^{125}I]EGF) to its cell surface receptor, as determined with an EGF radioreceptor assay. Thecal/interstitial cell secreted proteins also stimulated growth of an EGF-dependent cell line. Granulosa cells isolated from the same ovaries did not produce detectable levels of an EGF-like substance, as determined by both EGF RRA and EGF growth assay. Fractionation of thecal/interstitial cell secreted proteins by size exclusion HPLC resulted in a 35K component being detected by both the EGF RRA and the growth assay. An EGF-like substance was also detected when thecal/interstitial cell secreted proteins were fractionated by reverse phase HPLC. Several biochemical properties of the EGF-like substance examined were different from those of authentic mouse EGF. However, the biological activities of the EGF-like substance and mouse EGF were similar in their ability to promote the growth of an EGF-dependent cell line. In addition to the production of an EGF-like substance, the thecal/interstitial cell preparation also produced an EGF inhibitory activity. This thecal/interstitial cell secreted product was fractionated from the EGF-like substance and inhibited the ability of EGF to stimulate cell growth.

EGF receptors are present on granulosa cells, and EGF is known to influence both the growth and differentiation of these cells. Observations indicate that ovarian thecal/interstitial cells produce an EGF-like substance that may act as a paracrine factor to regulate granulosa cell growth and differentiation. In addition, an EGF inhibitory activity is produced that also may have a role in regulating follicular cell growth. (*Endocrinology* **121:** 1892–1899, 1987)

THE OVARIAN follicle is one of the most rapidly proliferating normal tissues known *in vivo*, and granulosa cell growth accounts for the majority of this follicle cell expansion (1). The proliferation of granulosa cells is necessary for the development of the follicle and to establish the microenvironment required to maintain and control oocyte maturation (2). The factors responsible for promoting the growth of the granulosa cell population are, therefore, critical for the maintenance of fertility in the female. In spite of their important role in normal ovarian function these factors have not been completely elucidated. One of the initial factors found to stimulate follicle development and granulosa cell growth *in vivo* was estrogen (3). However, estrogen does not act as a mitogen for granulosa cells *in vitro* (4). Therefore, the effects of estrogen on follicular growth *in vivo* appears to be indirectly mediated, which is supported by the observation that estrogens indirectly mediate the proliferation of a number of tissues (5). Several growth factors have been shown to directly stimulate granulosa cell growth *in vitro* (6), including fibroblast growth factor (7, 8) and insulin-like growth factor (9). In addition to the *in vitro* stimulation of cell proliferation, growth factors have also been shown to effect the differentiated functions of granulosa cells. For example, IGF known to be produced by porcine granulosa cells (10) and promote the growth of granulosa cells (9) also augments FSH-induced aromatase activity and stimulates progesterone production (11, 12).

Epidermal growth factor (EGF) (13) stimulates the growth of a number of cell types (14) and is a potential ovarian growth factor. Granulosa cells contain EGF receptors (15) and respond directly to EGF through stimulation of cell growth *in vitro* (7, 8). Although EGF can stimulate the proliferation of granulosa cells isolated from a number of species *in vitro* (8), the physiological significance of EGF in the ovary has not been directly demonstrated. EGF also has the ability to influence granulosa cell function and differentiation (review in Ref. 6). EGF generally has been shown to have inhibitory actions on estrogen biosynthesis, particularly in combi-

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nation with agents that stimulate estrogen production (15, 16). In contrast, EGF has stimulatory effects on progestin biosynthesis (15). Therefore, EGF has the ability to regulate granulosa cell function, growth, and differentiation.

Growth factors in general, act short range by diffusion to neighboring cell populations, whose response depends upon the presence of the appropriate receptors (17). Since granulosa cells contain receptors for a number of growth factors, we have investigated the possibility that the cells (*e.g.* thecal and interstitial) that surround the granulosa cell layers may generate growth factors that have a role in regulating granulosa cell growth and differentiation. The current study was designed to provide insight into the physiological significance of the effects of EGF on granulosa cells through an examination of the possible local production of an EGF-like substance.

Materials and Methods

Cell culture and medium collection

Immature Wistar Crl: (W1) BR were obtained from Charles River Canada (Montreal, 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 diethylstilbesterol in 0.1 ml sesame oil by sc injection. Animals were killed at 25 days of age, and granulosa cells were recovered from the ovaries by puncturing the follicles with a fine needle (18). Granulosa cells were plated in Eagle's Minimum Essential Medium 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 glycerine. The medium also contained 4 mM glutamine, 2.5 g/liter $NaHCO_3$, 1.5 mM HEPES, and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin, and 0.6 μg/ml fungizone). The cells were cultured at 37 C in a humidified atmosphere of 5% CO_2 and 95% air to maintain the medium at pH 7.4. Fibroblasts, elongated and flattened cells, were seen occasionally in the cultures, but did not proliferate under these culture conditions. Thecal cells were not apparent biochemically, as indicated by undetectable levels of androgens in the medium.

Thecal/interstitial cells were isolated from the ovaries after the release of granulosa cells. The remaining ovarian tissue was washed once in Hank's Balanced Salt Solution (HBSS) and then chopped into approximately 0.25-mm^2 sections. These ovarian sections were washed again in HBSS and allowed to settle under gravity to remove any newly released granulosa cells. The supernatant was discarded, and tissue fragments were digested in HBSS containing 1 mg/ml collagenase and 5 μg/ml DNase (Sigma) at 37 C for 25 min to remove any adhering granulosa cells. Digested ovarian tissue was then gravity sedimented, and the supernatant was discarded. The resulting pellet was dispersed, and any remaining sections were removed by gravity sedimentation. The supernatant containing single cells and small cell aggregates was centrifuged, and the pellet was harvested. Cells were then plated in Minimum Essential Medium, supplemented as described above, containing 10% calf serum and grown to confluence. Unless otherwise designated, cells were then subcultured and plated at 25% confluence. After 3–4 days of culture subcultured cells were confluent and washed for 24 h with serum-free medium. The cells were then cultured for up to 2 weeks in serum-free medium with 48-h medium collections. These cell cultures will be referred to as thecal/interstitial cell cultures. Androgen production by these cultures increased in response to hCG, as determined with an androgen RIA using conditioned medium. Cells were viable throughout the culture period, as assessed with trypan blue exclusion; however, after 2 weeks of serum-free medium collection, cells generally started to detach from the culture plates.

Freshly collected serum-free medium was centrifuged at 1000 × *g* for 15 min at 4 C to remove cell debris. When required, medium was frozen and stored at −20 C. Conditioned medium was concentrated 100-fold by ultrafiltration with an Amicon system (Amicon Corp., Lexington, MA) using membranes of 2,000 and 10,000 mol wt exclusion limits. The amount of protein present in concentrated conditioned medium (>10,000 mol wt) was generally between 0.2 and 0.7 mg/ml.

Size exclusion HPLC

Size exclusion chromatography of concentrated medium from thecal/interstitial cells was accomplished with a HPLC apparatus (Beckman, Fullerton, CA). The column used was a series of TSK (Toyo-Soda) 7.5-mm × 30-cm columns, including one SW-4000, two SW-3000, and one SW-2000. The column was equilibrated and eluted at 0.5 ml/min with 100 mM Na_2SO_4, 50 mM phosphate, 1 mM triethylamine, and 0.5% (vol/vol) ethylene glycol, pH 7.0. Fractions were collected (2 min) and stored at −20 C before being used. Standardized of the column for mol wt was done with thyroglobulin (660 K), catalase (240 K), BSA (68K), ovalbumin (42 K), and chymotrypsinogen (25K).

Reverse phase HPLC

Reverse phase chromatography was performed on an analytical Vydac-C4 column (Hesperia, CA). The column was equilibrated in 0.1% (vol/vol) trifluoroacetic acid, 0.5% (vol/vol) ethylene glycol, and 1 mM triethylamine pH 2.0. Acidified samples were loaded and eluted at 0.5 ml/min with a linear gradient from 25–60% acetolnitrile. Fractions were collected (2 min) and dried in a Speed-Vac apparatus (Savant Instruments, Hicksville, NY). Dried samples were then reconstituted and stored at −20 C before being used.

EGF RRA and growth assay

The presence of EGF was determined with a RRA by the ability of a substance to displace [^{125}I]iodo-EGF from an EGF receptor preparation, as previously described (19). Samples were incubated 1–2 h on confluent human fibroblasts in six-well cluster dishes with 2 ng [^{125}I]iodo-EGF (~ 60,000 cpm/ng) in the absence or presence of excess unlabeled EGF (500 ng). Similar results were obtained when samples were conincubated with [^{125}I]iodo-EGF or a preincubation with sample was used. Incubation of cells at 4 C *vs.* 37 C also gave similar results.

Generally, at 37 C coincubation with sample and [^{125}I]iodo-EGF was used. Specific binding was determined, and the percent inhibition of [^{125}I]iodo-EGF binding was assessed.

Growth of an EGF-dependent cell line, MK cells, was performed as previously described (20). Samples were incubated in the presence or absence of EGF on MK cells cultured at 37 C for 72 h. Cell number was then determined to assess EGF growth stimulation. EGF assays were performed by the Vanderbilt University, Reproductive Biology Research Center, Tissue Culture Core Laboratory, directed by Dr. G. Carpenter.

Results

Serum-free conditioned medium was obtained from cultured granulosa cells and thecal/interstitial cells. Conditioned medium was concentrated 100-fold by ultrafiltration and fractionated into a greater than 10,000 mol wt fraction and a 2,000–10,000 mol wt fraction. Granulosa cell secreted proteins will be designated GSP, while thecal/interstitial cell secreted proteins will be designated TSP. Displacement of [^{125}I]iodo-EGF in a RRA was used to initially detect the presence or absence of an EGF-like substance in GSP and TSP fractions. This EGF RRA is sensitive to different salt concentrations and buffers, such that a displacement of less than 10–15% is not considered significant and is probably an artifact of the assay (19). Neither the 2–10K nor the more than 10K GSP fractions had a significant displacement in the EGF RRA (Fig. 1). Therefore, under the conditions used with this EGF assay granulosa cell secreted proteins contained no detectable EGF-like activity. However, although the 2–10K TSP fraction had no displacement, the more than 10K TSP fraction did contain a substance that significantly inhibited the binding of [^{125}I]EGF to the EGF receptor (Fig. 1). Therefore, the thecal/interstitial cell secreted protein preparation that was greater than 10K contained an EGF-like substance that displaced EGF from its receptor. A minimum estimate of the amount of EGF-like material in TSP from the RRA is 100 pg EGF/μg TSP.

To confirm the presence of an EGF-like substance in TSP, an EGF growth assay was used. This assay relies on a cell line, MK cells, which is dependent on EGF for growth and is sensitive to less than 0.01 ng EGF (20). TSP (50 μg) did stimulate the growth of this EGF-dependent cell to the same extent as 0.01 ng EGF (Fig. 2). Higher concentrations of EGF (3 ng) stimulated cell growth approximately 20-fold (Fig. 2). TSP in combination with 3 ng EGF significantly reduced the ability of EGF to stimulate cell growth (Fig. 2). Therefore, in addition to the presence of an EGF-like substance, TSP also contains a component that inhibits the actions of EGF. Due to the presence of this inhibitory substance an accurate quantitation of the amount of EGF-like activity in TSP cannot be obtained with the growth assay. In support of this observation higher protein concentrations of TSP were not found to stimulate cell growth in the EGF growth assay, but did displace [^{125}I]iodo-EGF in the RRA. Further characterization of the EGF-like substance will require fractionation of the TSP. GSP had undetectable levels of growth-promoting activity in the EGF growth assay.

Experiments were done to determine whether the EGF activity present in TSP may be due to a cell culture artifact. A control fibroblast cell line (3T3 cells) was plated in 10% calf serum, grown to confluence, then subcultured and grown again to confluence. The calf serum was then removed, and serum-free conditioned

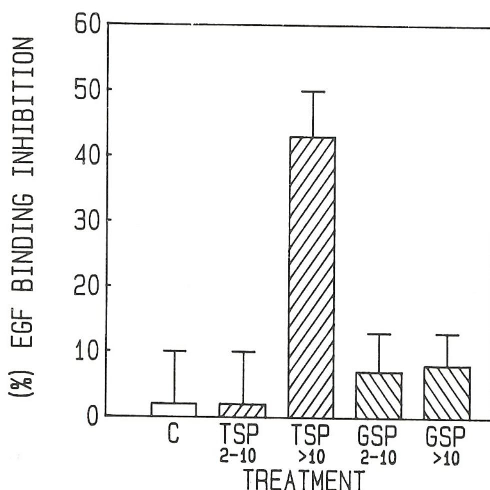

FIG. 1. EGF RRA on TSP and GSP. The percent binding inhibition of [^{125}I]iodo-EGF in a RRA was determinated with no treatment [control (C)] or 10 μg 2–10K TSP, 50 μg more than 10K TSP, 8 μg 2–10K GSP, and 30 μg more than 10K GSP. The mean ± SEM for duplicate samples in three separate experiments are presented.

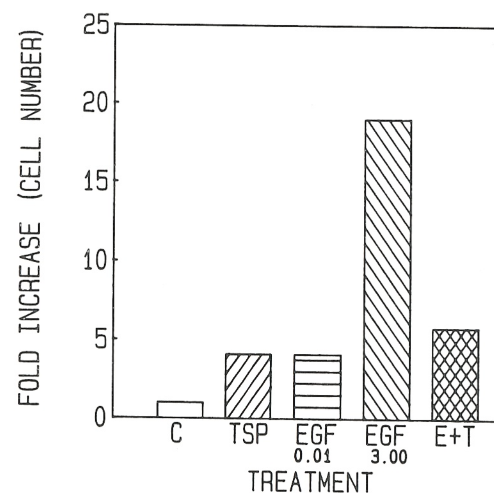

FIG. 2. EGF growth assay on TSP and EGF. The fold increase in MK cell number was determined after no treatment [control (C)] or 50 μg TSP, 0.01 ng EGF, 3 ng EGF, and a combination of 3 ng EGF plus 50 μg TSP (E + T). The mean of a triplicate determination is presented (coefficient of variation, 10–15%) for a representative of three separate experiments.

medium was collected for 1 week. The serum-free conditioned medium was then concentrated 100-fold and termed control fibroblast secreted proteins (CFSP). The manipulation of the cells and culture conditions used to obtain CFSP were identical to that used to obtain TSP. No detectable EGF activity was present in CFSP at several concentrations of protein with either the EGF RRA or EGF growth assay (Table 1). This observation indicates that the culture conditions used did not generate the EGF activity detected in TSP. Thecal/interstitial cells could not be plated efficiently in the absence of serum, so primary cell cultures were obtained by plating cells at a high density for 24 h in 10% calf serum. The serum was then removed, and serum-free conditioned medium was collected every 48 h for 6 days. The conditioned medium was then concentrated and assayed for the presence of EGF activity, as described in *Materials and Methods*. Quantitation of the amount of EGF activity from TSP isolated from primary and subcultured cells is shown in Table 1. Approximately the same amount of EGF activity was present in TSP isolated from primary cultures as was detected in subcultured cells. This observation indicates that the EGF activity present in TSP from subcultured cells is not an artifact of the subculture. Subcultured cells were routinely used, since larger quantities of thecal/interstitial cells and TSP can be obtained. The amount of EGF activity present in TSP determined with the RRA was significantly higher than the amount detected with the growth assay. The reduced level detected in the growth assay is probably due to the growth inhibitor(s) present in unfractionated TSP (Fig. 2).

Gel filtration of TSP was performed on a HPLC size exclusion column. Fractions were collected and analyzed for the presence of EGF-like activity. A substance of approximately 35K molecular mass had EGF growth activity (Fig. 3). These same fractions were found to

TABLE 1. Quantitation of EGF activity with an EGF RRA (EGF-RRA) and an EGF growth assay (EGF-growth)

Sample	Protein (μg)	EGF-RRA (ng EGF)	EGF-growth (ng EGF)
TSP (subculture)	50	5.5 ± 0.5	0.05 ± 0.01
TSP (primary)	36	3.1 ± 0.3	0.03 ± 0.01
CFSP (subculture)	35	ND	ND
	70	ND	ND
EGFa-GFP	0.8		0.11 ± 0.02
	1.6		0.85 ± 0.03
	6.5	4.2 ± 0.4	
	13	7.8 ± 0.6	

Standard curves were constructed for each assay with mouse EGF, and the presence of EGF (nanograms of EGF) was determined in samples including TSP from subcultured cells, TSP from primary cultured cells, CFSP from subcultured cells, and an EGF-like activity present in a gel filtration column peak (EGFa-GFP). The mean ± SEM are given triplicate determinations on different samples. ND, Nondetectable levels.

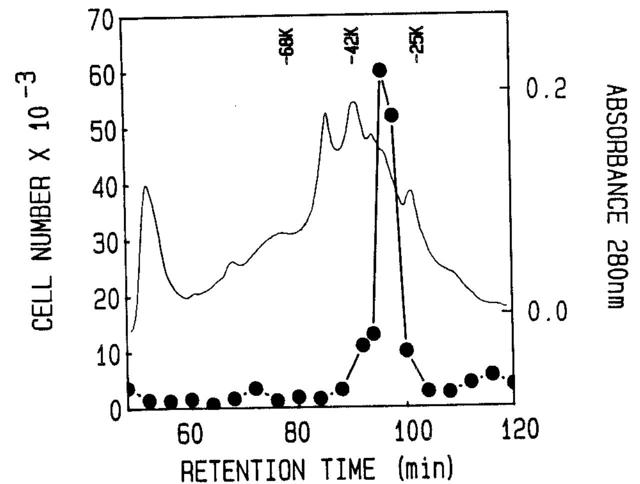

FIG. 3. Size exclusion HPLC on TSP. Collected fractions were analyzed for the ability to promote MK cell growth (●–●), represented as cell number × 10^{-3}, and protein elution was monitored at 280 nm. Shown is a representative profile of four separate experiments which all gave similar results.

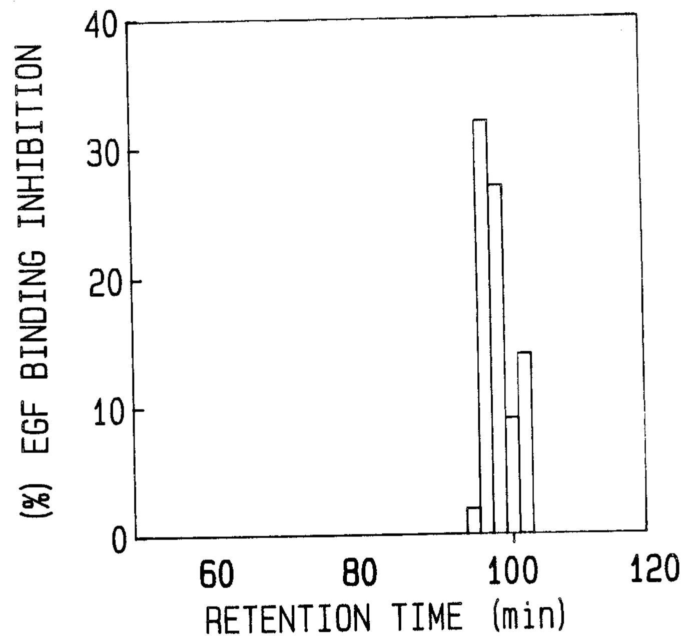

FIG. 4. EGF RRA on size exclusion HPLC fractions of TSP. Fractions shown in Fig. 3 were also analyzed for the ability to inhibit binding of [^{125}I]iodo-EGF in an EGF RRA and expressed as percent [^{125}I]iodo-EGF binding inhibition. Similar results were obtained in three separate experiments.

displace [^{125}I]iodo-EGF from its receptor in the RRA (Fig. 4). The representative profiles shown in Fig. 3 and 4 were obtained under physiological salt conditions, as outlined in *Materials and Methods*. When 1 M ammonium acetate, pH 4.5, was used as the column elution buffer, results similar to those presented in Figs. 3 and 4 were obtained. In 50% of the profiles it was found that the peak tube of EGF growth activity was off set by one

tube, but was not separated from the peak tube of radioreceptor activity. The reason for this shift in the peak tubes with the different assays is currently unknown, but may be due to the presence of the growth inhibitor. However, with no exception, any peak tube containing EGF growth-promoting activity also contained EGF receptor-binding activity. Murine EGF was used to determine the retention time of native EGF on the size exclusion column. The murine EGF was detected with the growth assay and found to elute at 125 min. [^{125}I]Iodo-EGF was also used and found to elute at the same retention time (*i.e.* 125 min). Therefore, native EGF and the EGF-like substance in TSP appear to have different apparent mol wt. [^{125}I]Iodo-EGF was applied and eluted from the column in the presence and absence of TSP to determine whether components in TSP would give a larger apparent mol wt. No difference was observed in the elution profile of [^{125}I]iodo-EGF when TSP was present. The results indicate that the EGF-like substance in TSP has an apparent molecular mass of 35K by size exclusion chromatography.

The peak containing EGF activity was pooled and termed EGF activity gel filtration pool. The amount of EGF activity present in this sample was quantitated with the RRA and growth assay (Table 1). The specific activity of the EGF activity (nanograms of EGF per μg protein) was increased approximately 5-fold with the RRA and 530-fold with the growth assay. A 2-fold increase in the amount of protein assayed in the RRA gave an approximately 2-fold increase in nanograms of EGF detected. Therefore, with the RRA, a linear relationship exists in the amount of TSP assayed and EGF receptor-binding activity. Due to the sensitivity of the growth assay, lower amounts of protein were assayed than with the RRA. A similar specific activity was observed for the growth assay and the RRA when 1.6 μg protein were used (Table 1). However, a 2-fold decrease in protein in the growth assay resulted in an 8-fold decrease in specific activity (nanograms of EGF per μg protein). Therefore, a nonlinear relationship exists when the growth assay is used to assay EGF growth activity in the TSP gel filtration fraction. The reason for the nonlinearity in the growth assay is unknown at present and may be due to the presence of the growth inhibitor.

Reverse phase chromatography was also used to fractionate TSP. Elution of TSP from a C4 reverse phase column was accomplished with an acetonitrile gradient. Fractions were collected and subsequently analyzed for EGF-like activity. TSP contained a peak of EGF growth activity which eluted with a 52-min retention time (Fig. 5). Because TSP contained an EGF growth inhibitory substance, as shown in Fig. 1, the fractions from the C4 column were also analyzed in the EGF growth assay in the presence of 3 ng EGF (Fig. 6). Although the baseline

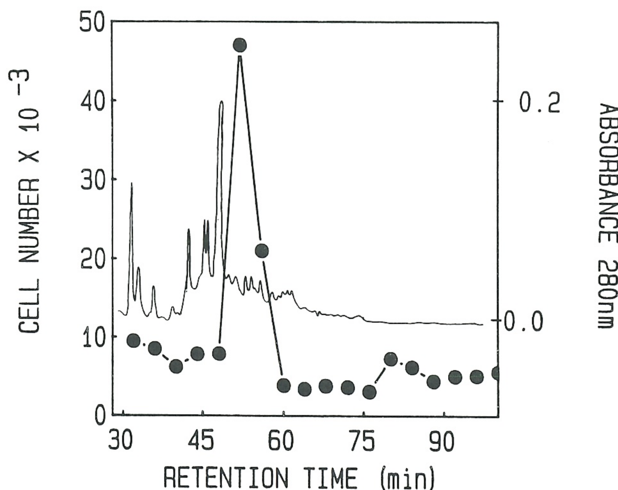

FIG. 5. Reverse phase HPLC of TSP. Collected fractions were analyzed for the ability to stimulate MK cell growth (●–●), represented as cell number × 10^{-3}, and protein elution was monitored at 280 nm. Similar results were obtained in three separate experiments.

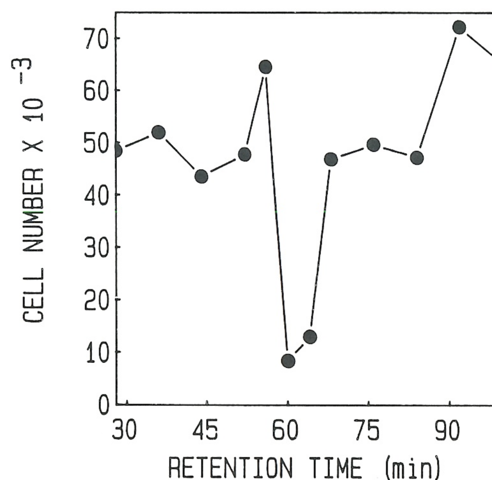

FIG. 6. EGF growth assay on reverse phase HPLC fractions of TSP. Fractions shown in Fig. 5 were analyzed for the ability to inhibit MK cell growth in the presence of 3 ng EGF, represented as cell number × 10^{-3}. Similar results were obtained in three separate experiments.

of EGF-stimulated growth was variable, several fractions significantly inhibited the growth-promoting effects of EGF. In the presence of EGF a peak containing the inhibitory protein was observed with a 60-min retention time (Fig. 6). Therefore, the reverse phase chromatographic procedure used separated the EGF-like substance in TSP from the EGF growth inhibitory activity. This observation implies that the EGF-like substance and the EGF growth inhibitory activity are distinct activities. Fractions from the C4 were also analyzed in the EGF RRA. The same fractions that contained growth-promoting activity were detected in the EGF RRA (Fig. 7). The first peak tube of the EGF growth inhibitory activity also was reactive in the RRA. Therefore, some overlap of the EGF-like substance peak and EGF growth inhib-

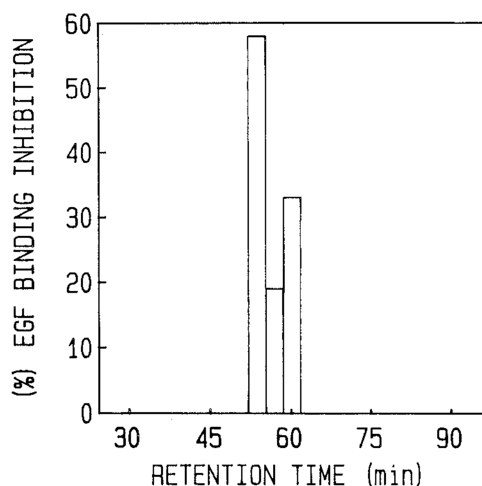

FIG. 7. EGF RRA on reverse phase HPLC fractions of TSP. Fractions shown in Fig. 5 were also analyzed for the ability to inhibit binding of [^{125}I]iodo-EGF in an EGF RRA and expressed as percent [^{125}I]iodo-EGF binding inhibition. Similar results were obtained in three separate experiments.

itor peak appears to occur. Unlabeled murine EGF and [^{125}I]iodo-EGF eluted with a 44-min retention time on the reverse phase column. This observation indicates that the hydrophobicity and elution profile of native murine EGF are different from those of the EGF-like substance in thecal/interstitial cell secreted protein.

Antisera were obtained to murine EGF (generously provided by Dr. S. Cohen, Vanderbilt University) and human EGF (Calbiochem, La Jolla, CA) in an attempt to immunoprecipitate the EGF-like substance in TSP. Using several established techniques with either second antibody or protein-A procedures (21, 22), no detectable immunoprecipitate was detected from thecal cell secreted proteins radiolabeled with [^{35}S]cysteine, [^{35}S]methionine, and [^{3}H]glycine. These results indicate that the EGF-like substance in TSP is not immunologically similar to murine or human EGF with the procedures and reagents used.

Discussion

One hypothesis tested in the current study is that the cells outside of the basal lamina of the follicle secrete proteins that can act as paracrine factors to influence cells on the inside of the basal lamina. Of particular interest are the secretion products of thecal cells that may regulate granulosa cell proliferation and differentiation. This hypothesis has been initially tested in the rat model because of the extensive use of the rat system. An examination of ovary sections from diethylstilbestrol-treated rats under the light microscope shows that granulosa cells are the major cell type present, while thecal and interstitial cells contribute equally to the rest of the ovarian cell population. The granulosa cell preparation used has previously been shown to have a low percentage of contaminating cell types (23). Unfortunately, the immature rat ovary has limitations due to its small size, and it is not feasible to dissect out sufficient quantities of thecal cell layers to generate the amount of conditioned medium required for the current study. Because of this limitation we have obtained a mixed cell population from ovaries that have been depleted of granulosa cells. The major cell types in this preparation include thecal cells and stromal interstitial cells. Therefore, the cell preparation used is referred to as thecal/interstitial cells. Initial characterization of this thecal/interstitial cell culture indicated undetectable levels of aromatase activity, which implies the absence of granulosa cell contamination. However, these cultures did produce androgens in response to gonadotropin treatment, which confirms the presence of thecal cells. In the current study, thecal/interstitial cell cultures are used to examine the possible local ovarian synthesis of an EGF-like substance. Determination of the site of synthesis of an EGF-like substance will require the isolation and culture of individual cell types, including thecal and stromal interstitial cells. The observations presented demonstrate local production of an EGF-like substance by a thecal/interstitial cell preparation, but do not indicate the specific site(s) of synthesis.

An EGF RRA and growth assay were used to detect the presence of an EGF-like substance. GSP had undetectable levels of EGF activity with either of the EGF assays. This observation indicates that either granulosa cells do not produce EGF or a more sensitive assay will be required. In contrast, TSP did contain an EGF-like substance, as detected with both the RRA and growth assay. Therefore, thecal/interstitial cells appear to produce an EGF-like substance that can bind to the EGF receptor and promote the growth of an EGF-dependent cell. Initial characterization of the EGF-like substance produced by thecal/interstitial cells indicates an apparent molecular mass of 35K by size exclusion chromatography. The elution profile of murine EGF isolated from the salivary gland on the HPLC size exclusion column was different from that of the EGF activity in TSP. The aggregation of the EGF-like substance with other proteins in TSP or association with an EGF-binding protein, which would result in a larger apparent mol wt for the EGF activity, appeared unlikely and was supported by the observation that high salt acidic buffers did not alter the apparent mol wt. Therefore, results indicate that the apparent mol wt of murine EGF and the EGF-like substance in TSP are different. Reverse phase chromatography also demonstrated that murine EGF and the EGF-like substance in TSP have different profiles. Results indicate that the EGF activity in TSP is more hydrophobic than murine EGF. The EGF-like substance in TSP

also appeared to be immunologically distinct from both mouse and human EGF. Therefore, the EGF-like substance produced by thecal/interstitial cells has biochemical properties unique compared to those of murine EGF. These observations imply that the EGF activity in TSP is possibly a precursor, a unique form of EGF, or a different protein. EGF-like substances with unique biochemical properties have been isolated from a number of tissues and physiological fluids (24). Native EGF is a 6K polypeptide derived from a 128K precursor molecule (24, 25). This precursor molecule appears to be cleaved at different sites in different tissues. For example, a 46K species has been detected in the male mouse submaxillary gland in addition to the 6K EGF, whereas in the mouse kidney a 130K protein is generated with little or no 6K EGF (26). It is possible that the 35K EGF-like substance in TSP may be an active species synthesized by ovarian cells from an EGF precursor molecule. It is also possible that the 35K protein is not EGF, but, rather, a different protein. An example of such a protein is transforming growth factor-α (TGFα), which can bind to the EGF receptor and promote the growth of EGF-sensitive cells (27, 28). TGFα has been shown to be a different gene product than EGF (29). Purification and biochemical characterization of the EGF-like substance produced by thecal/interstitial cells will be required to identify and establish the relationship of the EGF activity in TSP to authentic EGF.

In addition to the EGF-like substance produced by thecal/interstitial cells, an EGF inhibitory activity was detected. This inhibitory activity reduced the ability of EGF to stimulate cell growth. Reverse phase chromatography indicated that the EGF-like substance and the EGF inhibitory activity are different proteins. Therefore, thecal/interstitial cells also produce a substance that can inhibit the ability of EGF to stimulate cell proliferation. Within the ovary there are several developmental stages in which the inhibition of cell growth is required, including the primordial and atretic follicles. Locally produced growth inhibitors could play a role in regulating cell growth at these stages of development. An example of a protein that could have this type of inhibitory activity is TGFβ (30, 31). TGFβ has been shown to inhibit the growth-promoting effects of EGF on many EGF-sensitive cell types (31, 32). Further investigation of the EGF inhibitory activity will require purification and characterization of the protein.

Several growth factors are known to influence the growth and differentiation of granulosa cells; however, with the exception of IGF, the identification of the source of these proteins (*i.e.* derived from the circulation or by local production) and their physiological significance is uncertain. As previously discussed, EGF has the ability to regulate granulosa cell function, growth, and differentiation. Levels of high affinity, low capacity EGF receptors on granulosa cells are regulated through the actions of hormones, such as gonadotropins (15), and growth factors, such as TGFβ (33). Hormone receptor levels for FSH and LH can, in turn, be regulated by the actions of EGF (34). Due to the diverse and often dramatic effects of EGF on granulosa cell growth and differentiation, it has been postulated that EGF may have an *in vivo* role in regulating ovarian function (6, 23). The observations presented in the current study indicate that thecal/interstitial cells are capable of producing an EGF-like substance that could act as a paracrine factor to regulate granulosa cell growth and differentiation. This EGF-like substance may also act as an autocrine factor within the ovary due to the previous observations that EGF can regulate steroidogenesis in thecal or interstitial cells (35). The physiological importance of the actions of EGF on ovarian cell function, growth, and differentiation is supported by the observation of local ovarian synthesis of an EGF activity. Although the cell type in the thecal/interstitial cell preparation that is responsible for the production of the EGF-like substance is not known, the thecal cells are a likely candidate. Thecal cells are in close proximity to granulosa cells, they develop in concert with granulosa cells during the antral stages, and interactions between thecal and granulosa cells are essential for maintenance of the dominant follicle. In support of this is the observation that extracts of thecal cells obtained from porcine follicles contain a growth factor for granulosa cells (36). Investigation of the site of synthesis, site of action, and biochemical characterization of the EGF-like substance produced by thecal/interstitial cells will be required to develop a better understanding of the physiological significance of an EGF-like substance in the ovary.

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