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Fibronectin as a Marker of Granulosa Cell Cytodifferentiation

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ABSTRACT. The hormonal regulation of fibronectin secretion by rat granulosa cells in culture was investigated: fibronectin was measured by a competitive enzyme-linked immunoadsorbant assay. Granulosa cells isolated from 25-day-old diethylstilbestrol-primed rats and cultured under defined conditions in the absence of hormones secreted low levels of fibronectin during the first 24 h of culture, after which there was a rapid increase in secretion until 72 h. In contrast, cultures treated with a combination of NIH-FSH-15 (200 ng/ml) and insulin (5 μg/ml) secreted low levels of fibronectin throughout the culture period. Subsequently, it was found that both FSH and insulin could independently suppress the increase in fibronectin secretion found in control cultures. Combined treatment with FSH and insulin resulted in a level of fibronectin which was the same as either FSH or insulin alone. The actions of FSH and insulin were dose dependent; 10 ng FSH/ml and 2.5 μg insulin/ml were required to produce a maximum suppression. The ability of $(Bu)_2$cAMP (1.0 mM) to suppress fibronectin secretion suggested that the action of FSH on this parameter was mediated via the production of cAMP. Testosterone and estrogen alone did not influence secretion and did not modulate the actions of FSH and insulin. At the time at which FSH induces the cytodifferentiation of granulosa cells in culture, assessed by the increase in aromatase activity, fibronectin secretion is suppressed. The inverse relationship between fibronectin secretion and the induction of those granulosa cell functions essential for the development of the preovulatory follicle indicates that fibronectin may provide a useful marker for the stage of cytodifferentiation and follicular maturation. (*Endocrinology* **117:** 886–892, 1985)

THE PRIMARY follicle formed during fetal life or shortly after birth remains in this embryonic form until it receives the stimulus to grow. Once growth is initiated, there is a slow phase of growth during which the granulosa cells proliferate and the surrounding stromal cells restructure to form the thecal layers around the periphery of the follicle. The majority of follicles, however, do not reach maturity but become atretic. Those follicles selected to ovulate enter a rapid phase of growth before ovulation which is characterized by increased mitotic activity, concomitant cytodifferentiation, and morphological maturation of the granulosa cells (1–3). The selection, as well as the subsequent development of the preovulatory follicle, depends upon the presence of LH and FSH. LH acts on thecal cells to stimulate the production of androgens which are utilized by granulosa cells for the synthesis of estrogens and also augments various actions of FSH (4, 5). FSH acts exclusively on granulosa cells to increase the activities of aromatase, cholesterol side chain cleavage, 3β-hydroxysteroid dehydrogenase, and 20α-hydroxysteroid dehydrogenase, to promote the acquisition of LH and PRL receptors, and stimulate the secretion of proteoglycans and plasminogen activator (1, 2).

Preovulatory follicles continue to grow in an environment in which other follicles become atretic. The eminence of the dominant follicles may be achieved by establishing autocrine and paracrine mechanisms of regulation. To further our understanding of the environment in which follicles develop, we have recently examined the radiolabeled proteins secreted by rat granulosa cells in culture (6). A component of extracellular matrix, fibronectin, was found to be the major radiolabeled secreted protein in unstimulated cells. As the cytodifferentiation of granulosa cells was induced by hormones, the secretion of fibronectin was inhibited (6). In the present paper we have quantitated the levels of fibronectin secreted and examined the effects of hormones on the production of this component.

Materials and Methods

Immature Wistar Crl: (W1) BR rats were obtained from Charles River Canada (Montreal, Quebec, 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

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by sc injection. DES acts as a granulosa cell mitogen to increase the yield of cells. Ovine FSH (NIADDK-oFSH-15; 20× NIH-FSH-S1 by the human CG (hCG) augmentation bioassay of Steelman-Pohley) was provided by the Pituitary Hormone Distribution Program, NIADDK. Bovine insulin, human fibronectin, Tween-20, activated charcoal, and $(Bu)_2$cAMP were purchased from Sigma Chemical Company (St. Louis, MO) and rat fibronectin and specific antiserum from Calbiochem (La Jolla, CA). [1,2-^{3}H]Testosterone (40 Ci/mmol) was obtained from New England Nuclear (Boston, MA), and [1β-^{3}H]testosterone was prepared from this as described previously (7).

Preparation of cell cultures

Animals 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 (8). Cells (~100 μg protein) were plated into 24-multiwell Falcon tissue culture plates (Falcon Plastics, Los Angeles, CA) as 1.0-ml aliquots 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 glycine. 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. The cells were allowed to attach to the surface of the plate for 3 h before the addition of hormones to the existing culture medium. After 24 and 48 h in culture, all of the medium was routinely replaced and the treatments were repeated.

Assay for fibronectin

Fibronectin was measured by a competitive enzyme-linked immunoadsorbant assay (ELISA) using the following procedure. Immulon 2 microtiter plates (Dynatech Labs, Alexandria, VA) were coated overnight at 4 C with 200 ng human fibronectin/well in 100 μl 20 mM Na_2CO_3 buffer, pH 9.6. The standard curve for human fibronectin (0–1000 ng) was set up in triplicate in 100 μl 0.05% (vol/vol) Tween-20 in 10 mM NaH_2PO_4 containing 0.85% NaCl, pH 7.4 (Tween-PBS), and incubated overnight at 4 C in plastic tubes with 100 μl Tween-PBS containing the goat antiserum raised against rat fibronectin. The final dilution of the antiserum was 1:20,000. Duplicate samples of the medium from the granulosa cell cultures were preincubated as described for the standard fibronectin samples. The remainder of the assay was carried out at room temperature. The coating antigen solution was removed from the plate, and the wells were washed once with PBS and then three times with Tween-PBS. The last wash was left on for 5 min. The coated plates were incubated with 200 μl preincubated antigen antibody mixture/well for 30 min, then washed three times with Tween-PBS. Alkaline phosphatase conjugated to antigoat immunoglobulin G raised in rabbits was freshly diluted to 1:500 with Tween-PBS and 100 μl added to each well. This was incubated for 45 min, and the plates were washed three times with Tween-PBS. Plates were then washed once with diethanolamine buffer (5 mM $MgCl_2$, 10% diethanolamine in water, pH 9.8, stored in the dark). Each well was incubated with 120 μl freshly prepared 4-nitrophenyl disodium orthophosphate (British Drug Houses, Toronto, Ontario, Canada), 1 mg/ml diethanolamine buffer. Incubation time averaged 1 h but varied according to the ambient temperature of the laboratory. Plates were read on a microELISA densitometer (Titertek, Multiskan, Flow Laboratories, Inc., Canada) using a 405-nm filter.

Assay for aromatase activity

After the cells were cultured for 48 h, the medium was removed and replaced with 0.5 ml fresh culture medium. Aromatase activity in the cells was determined by adding [1β-^{3}H] testosterone (0.3 μCi; 0.25 μM) to each culture well and incubating for 2 h at 37 C. The aromatization of [1β-^{3}H]testosterone was assessed from the stereospecific release of tritium to produce ^3H_2O, according to a previously validated assay method (7). Blank values were established from identical incubations in the absence of cells. In the experiments described here, the nonspecific release of ^3H_2O was less than 100 cpm. The aromatization reaction was stopped by transferring the incubation medium to tubes, washing the culture wells with 0.2 ml PBS, and adding activated charcoal (25 mg in 0.1 ml water) to the combined medium and PBS. After incubation for 2 h at 4 C, the charcoal was centrifuged down at 3000 × g for 30 min. A 0.5-ml aliquot of the supernatant containing ^3H_2O, was counted in 5 ml Aquasol (New England Nuclear Corp.) in a liquid scintillation counter.

Assay for protein content

At the end of the culture period in each experiment, the protein content of the attached cells was determined by the method of Lowry *et al.* (9) after solubilization in 0.5 ml 1% sodium dodecyl sulfate containing 1 mM EDTA. All the data shown in Figs. 2–6 are expressed as the amount of fibronectin secreted, or the ^3H_2O released, per milligram of cellular protein present in the monolayer culture.

The amount of total protein secreted into the culture medium was measured using the method of Bradford (10).

Statistical analysis

All the results are presented as the mean ± SE of determinations of cells from three replicate cultures within each treatment group and are derived from a single representative experiment. Observations were confirmed in two or more independent experiments. Mean values were compared using Student's t test.

Results

ELISA assay for the measurement of fibronectin

The goat antiserum used in the ELISA assay was raised against rat serum fibronectin. The displacement of human fibronectin from the antifibronectin antibodies by rat serum fibronectin, human serum fibronectin, and fibronectin secreted by rat granulosa cells is shown in

Fig. 1. The parallel displacement curves for fibronectin from rat and human serum indicate that the more readily available fibronectin from human plasma can be used as a valid standard for the measurement of rat fibronectin. Furthermore, the parallel displacement curves for rat serum fibronectin and rat granulosa cell fibronectin imply that the fibronectins are antigenically similar. The sensitivity of the ELISA assay was found to be 1–5 ng fibronectin. The ELISA assay was linear from 5–100 ng with an interassay coefficient of variation of 5% and an intraassay variation of 10%. Components in culture medium and the hormones utilized in the study did not cross-react with the fibronectin antibody. It was demonstrated previously that only one protein, fibronectin, secreted by rat granulosa cells could be immunoprecipitated with rat serum fibronectin antibody (6).

Effects of FSH and $(Bu)_2$cAMP on fibronectin secretion

Granulosa cells from DES-primed immature rats were cultured in the absence or presence of FSH, and all the medium was removed and replaced with fresh medium at 24, 48, and 72 h. The amount of fibronectin was quantitated in each sample of medium by the ELISA assay. The results were expressed as the amounts of fibronectin which had accumulated over each time period. The amount of protein in the granulosa cell monolayer was determined after 72 h of culture, and this value was used to normalize the fibronectin secreted throughout the culture period. In previous experiments we found no significant changes in the amount of cellular protein present after 24, 48, and 72 h of culture. In unstimulated control cultures, fibronectin secretion was low during the first 24 h, after which there was a rapid increase in secretion until 72 h. In contrast, treatment with FSH from the time of plating inhibited the increase in fibronectin secretion observed in control cultures, resulting in low amounts being secreted throughout the culture period (Fig. 2). The amount of total protein secreted by unstimulated granulosa cells into the medium during the collection period for 48–72 h of culture was 90 μg/mg cellular protein whereas FSH-treated cells secreted 130 μg/mg cellular protein. When this was correlated with the amount of fibronectin secreted during the same time period, fibronectin constituted 10% of the total secreted proteins by unstimulated cells. The results obtained using the ELISA assay agree with previous observations which showed that fibronectin became a major radiolabeled protein secreted with time in culture of unstimulated granulosa cells. The increase in the intensity of radiolabeled proteins on the fluorograms of control cultures did not occur when the cultures were stimulated with FSH alone or with FSH, insulin, and testosterone (6). When the ELISA assay was used, FSH was found to

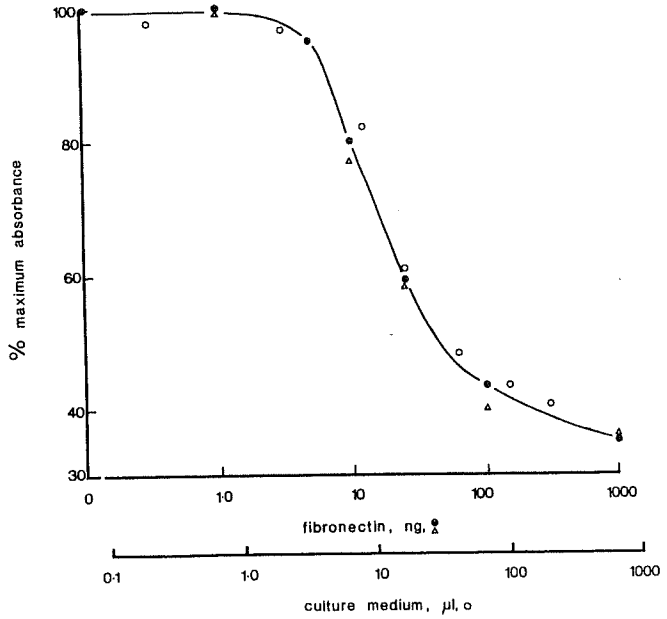

FIG. 1. ELISA displacement curves with human fibronectin (●), rat fibronectin (△), and granulosa cell-conditioned medium (○). *Points* represent the mean of triplicate determinations with a coefficient of variation less than 10%.

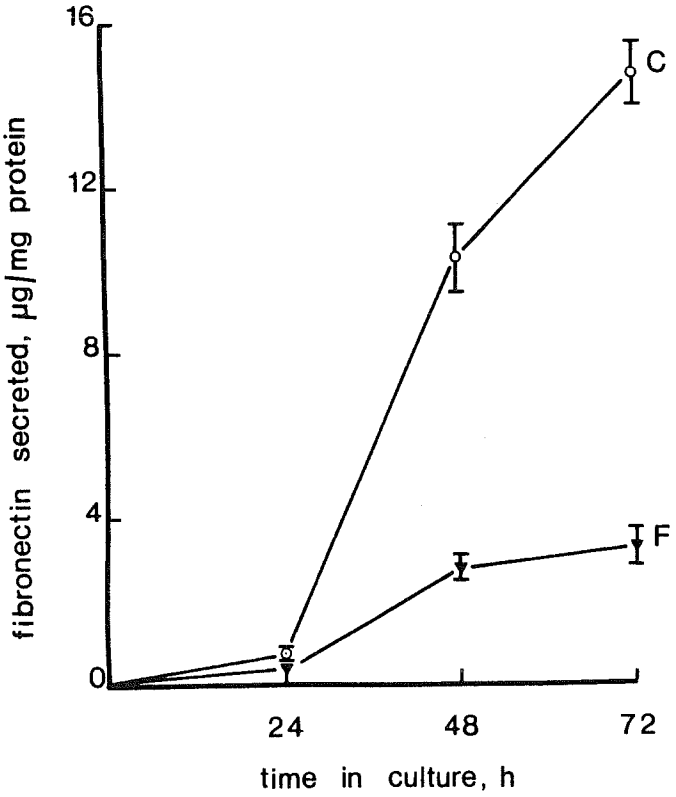

FIG. 2. Cumulative secretion of fibronectin by granulosa cells over a 72-h culture period under control conditions (C) and when treated with 200 ng NIH-FSH-15/ml (F). Results are the mean of triplicate cultures ± SE. The inhibitory effect of FSH was significant ($P < 0.001$) after 48 and 72 h of culture.

suppress the secretion of fibronectin in a dose-dependent manner; 0.5 ng FSH/ml was the minimal effective concentration, and 10 ng/ml produced a maximal response (Fig. 3).

Since $(Bu)_2$cAMP is able to exert similar effects to FSH on other differentiated functions of granulosa cells, we examined its ability to influence fibronectin secretion. Cells were cultured for 48 h under control conditions or with either 200 ng FSH/ml or 1.0 mM $(Bu)_2$cAMP. In the presence of $(Bu)_2$cAMP, fibronectin secretion was suppressed to an extent that was comparable to that elicited by FSH (Table 1).

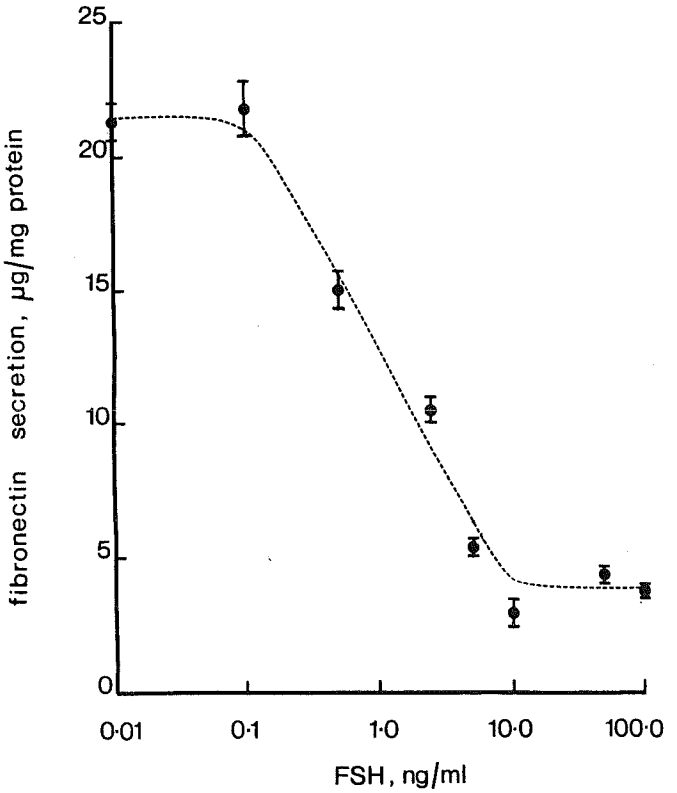

FIG. 3. Logarithmic dose-response curve for the effect of NIH-FSH-15 on the amount of fibronectin secreted by granulosa cells over a 48-h culture period. Each value is a mean of three replicate cultures ± SE.

TABLE 1. The ability of $(Bu)_2$cAMP to suppress the secretion of fibronectin by rat granulosa cells

Treatment	Fibronectin secretion (µg/mg cell protein)
None	18.9 ± 1.1
FSH	1.7 ± 0.1
$(Bu)_2$cAMP	1.4 ± 0.2

Granulosa cells isolated from 25-day-old DES-primed rats were cultured for 72 h with FSH (200 ng NIH-FSH-15/ml) or 1.0 mM $(Bu)_2$cAMP. The fibronectin was assayed in the culture medium collected at 24-h intervals, and the data were expressed as the total fibronectin secreted by 1 mg cell protein in the monolayer over the 72-h culture period. Values are the mean ± SE (n = 3).

Effects of insulin on fibronectin secretion

Our previous survey of radiolabeled proteins secreted by granulosa cells also revealed that insulin and testosterone were effective in suppressing the increase in fibronectin production (6). These experiments, however, did not indicate whether either or both of the hormones was active. Consequently, we have used the ELISA assay to measure fibronectin secretion in cultures treated with insulin and/or testosterone and compared this effect with that produced by FSH. As shown in Fig. 4, insulin independently suppressed the secretion of fibronectin to an extent comparable to that of FSH. Combined treatment with FSH and insulin resulted in a level of fibronectin which was the same as either FSH or insulin alone. Testosterone alone did not influence secretion and did not modulate the actions of FSH or insulin. Since estrogen augments FSH actions on aromatase activity, the effect of this steroid was also tested and found to have no effect alone or in combination with FSH or insulin (Fig. 4).

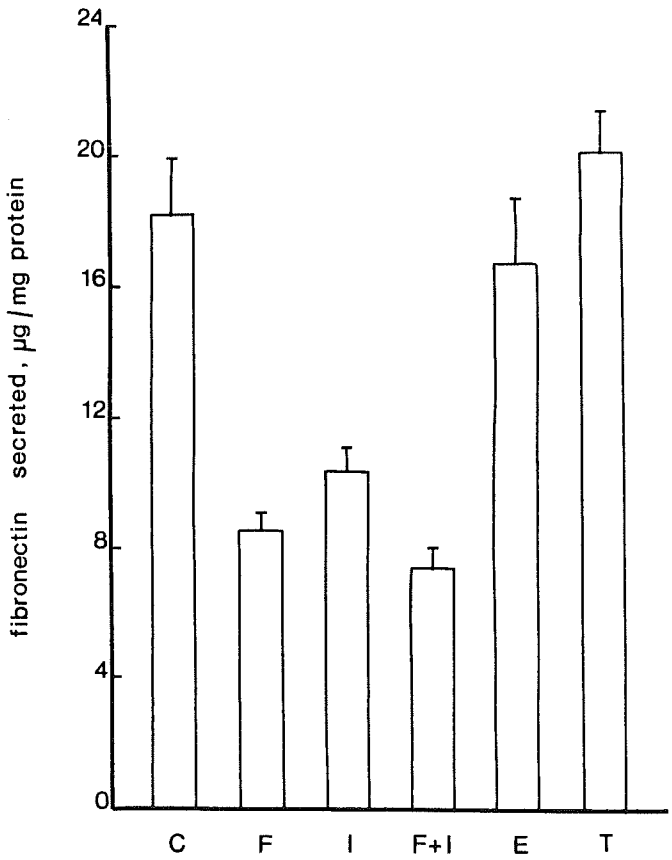

FIG. 4. Effect of 200 ng NIH-FSH-15/ml (F), 5 µg insulin/ml (I), 5×10^{-7} M testosterone (T), and 5×10^{-7} M estrogen (E) on the secretion of fibronectin by cultured granulosa cells over a 48-h culture period. Results are the mean of triplicate cultures ± SE. The amounts of fibronectin secreted by F, I, and F plus I-treated cells are significantly different from controls ($P < 0.001$). The values after either E or T treatment are not significantly different from controls.

The ability of insulin to maintain a lower level of fibronectin secretion compared to control cultures was dose dependent; 50 ng insulin/ml caused a significant reduction whereas 2.5 μg/ml were required to produce a maximal suppression (Fig. 5).

Comparison of fibronectin secretion and aromatase activity after FSH treatment

Since FSH is required for the normal cytodifferentiation of granulosa cells, the ability of FSH to modulate aromatase activity, which is a useful index of the stage of differentiation, was compared with its effects on fibronectin secretion within the same cultures. As shown in Fig. 6, FSH induced aromatase activity at a time at which it suppressed the secretion of fibronectin.

Discussion

The results presented provide the first indication that hormones known to influence granulosa cell cytodifferentiation can also influence the secretion of a component of extracellular matrix, namely fibronectin. These observations give additional insight into the changes which occur in granulosa cell functions during the development of the follicle.

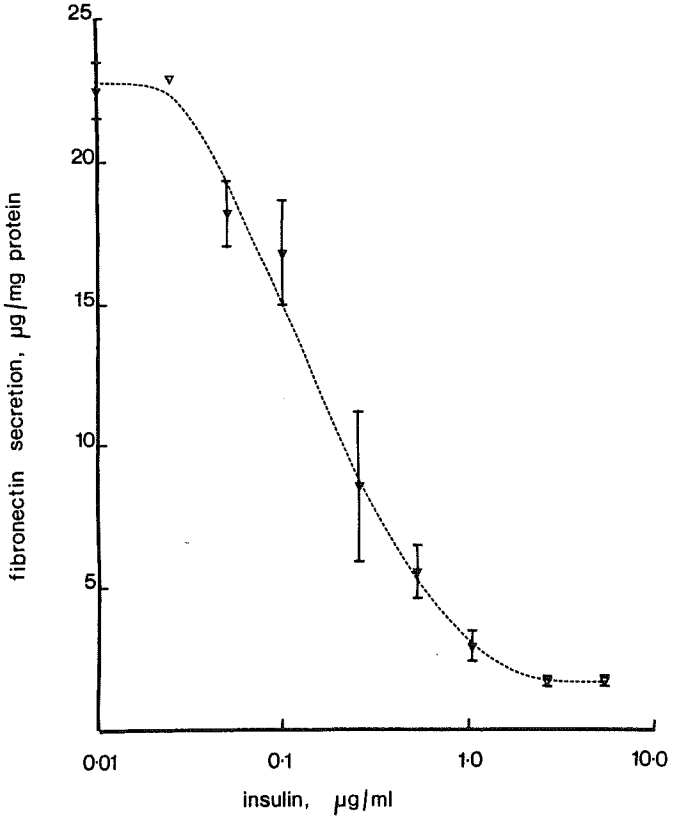

FIG. 5. Logarithmic dose-response curve for the effect of insulin on the amount of fibronectin secreted by granulosa cells over a 48-h culture period. Each value is a mean of three or four replicate cultures ± SE.

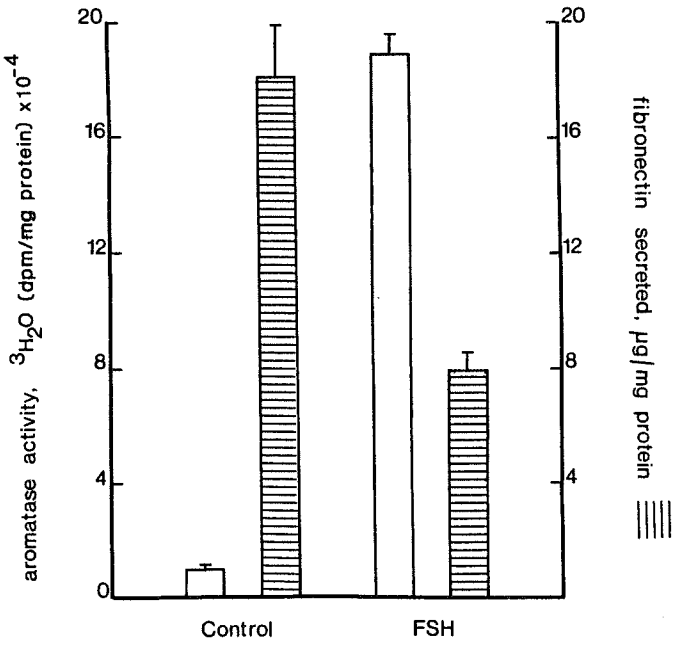

FIG. 6. Comparison between the effects of 200 ng FSH/ml on aromatase activity (*open bars*) and the amount of fibronectin secreted (*hatched bars*), in the same granulosa cell preparation. After 48 h in culture, aromatase activity was assessed by incubating cells for 2 h with 0.3 μCi [1β-^{3}H]testosterone (0.25 μM) and measuring the amount of ^3H_2O released. The amount of fibronectin secreted under control conditions and after treatment with FSH for 48 h was measured by the ELISA assay. Each value is mean of three replicate cultures ± SE. The differences between the control and the FSH-treated groups are significant in each set of experiments ($P < 0.001$).

Granulosa cells isolated from immature follicles are not steroidogenic and do not possess significant numbers of LH and PRL receptors (11, 12). When these cells are placed in culture under control conditions, the above properties are not acquired but rather the cells secrete fibronectin. Fibronectin became a major secreted protein from 48–72 h of culture, constituting 10% of the total secreted protein. The conclusion that a component of extracellular matrix is a major secretory product of unstimulated immature granulosa cells supports our previous report in which a 220,000-dalton protein, identified as fibronectin by immunoprecipitation, comprised a major portion of the total [^{35}S]methionine-radiolabeled proteins (6).

When immature granulosa cells are treated with FSH in culture, they undergo cytodifferentiation characterized by the appearance of steroidogenic enzymes, specific cell surface, and secreted proteins (1, 2). As these end responses are induced by FSH, fibronectin secretion is maintained at a reduced level. Our previous analysis of cellular radiolabeled proteins showed that fibronectin was a minor component which did not change with time under control culture conditions and decreased slightly after hormonal stimulation. The hormonal inhibition of

secretion of fibronectin is due, therefore, to a decrease in synthesis and not to an increase in deposition (6). Fibronectin is the only granulosa cell product whose synthesis is known to be suppressed by FSH and therefore may provide a useful marker for the stage of granulosa cell differentiation.

Under serum-free conditions, insulin promotes the proliferation of rat ovarian cell lines (13) and primary cultures of bovine and porcine granulosa cells (14, 15). In addition to the effects on growth, insulin has been implicated in the maintenance of differentiated properties of primary cultures of porcine granulosa cells (15). May *et al.* (16) found that insulin was required for FSH-induced production of LH/hCG receptors in porcine granulosa cells. Furthermore, insulin alone slightly increased progesterone production but significantly enhanced the actions of FSH and hCG (15). In human granulosa cells, insulin alone stimulated the synthesis of estradiol-17β from exogenous testosterone and augmented the action of FSH on aromatase activity (17). In addition to these effects on various differentiated functions of granulosa cells, FSH and insulin can promote morphological maturation. Immature porcine granulosa cells respond to insulin with changes in morphology which are characteristic of more highly differentiated cells (16). Rat granulosa cells undergo morphological maturation in response to FSH (3). The present study shows that insulin can suppress the synthesis of fibronectin to an extent similar to that of FSH and adds support to the concept that as granulosa cells mature and acquire differentiated functions the production of fibronectin is suppressed.

The concentrations of insulin required to influence fibronectin secretion were much higher than physiological concentrations; 50 ng insulin/ml caused a significant reduction whereas 2.5 μg/μl was required to produce a maximum suppression. If these effects are important physiologically, then they indicate that other insulin-like factors may be involved. Insulin interacts with a low affinity with the somatomedin C receptors, which raises the possibility that somatomedin C or a similar factor may play a physiological role. In this regard it is interesting to note that somatomedin C can augment the FSH-induced stimulation of progesterone production by rat granulosa cells and is secreted by porcine granulosa cells (18, 19).

Even though both FSH and insulin influence granulosa cell differentiation, they have different mechanisms of action. FSH acts by stimulating adenylate cyclase to increase intracellular levels of cAMP (20). The role of cAMP as a mediator of FSH action is supported by the ability of cAMP analogs, $(Bu)_2$cAMP and 8-bromo-cAMP to mimic the various effects of FSH, including the action on fibronectin as shown here (21). The mechanism of insulin action is not as well defined, but it is known not to act through cAMP as an intracellular mediator (22). The present study demonstrates that both FSH and insulin alone can inhibit fibronectin secretion but, when added together to the cultures, the effects are not additive but similar to either alone. These observations imply that the mechanism by which fibronectin secretion is inhibited is complex and not simply related to cAMP levels. Furthermore, this study suggests that agents which promote granulosa cell differentiation, independent of mechanism, may in turn influence the secretion of fibronectin, supporting the concept that fibronectin could be used as an additional marker of the stage of differentiation.

Fibronectin has been shown to be secreted by most endothelial and mesenchymal cells. In general, epithelial cells do not synthesize fibronectin, although there are some exceptions (23). It is known that fibronectin plays an important role in cell adhesion, shape, and migration (24).

The function of fibronectin secretion by granulosa cells can be speculated from its apparent inverse relationship with the stage of cytodifferentiation of the granulosa cell. Since fibronectin promotes cell adhesion and cell proliferation (24), its secretion by granulosa cells may provide an essential component of the substratum on which thecal cells become organized into the outer wall of the follicle. Another possibility is that the secreted fibronectin may be a constituent of the environment in which the oocyte develops and matures before the time at which it is bathed in follicular fluid which contains fibronectin derived from the serum. In support of these speculations on the physiological role of fibronectin are the previous observations that fibronectin influences the differentiated state of several other systems. For example, fibronectin inhibits myoblast fusion and glycosaminoglycan synthesis by chondrocytes (25, 26). Fibronectin also regulates adiopocyte differentiation by inhibiting the normal gene expression of lipogenic proteins (27). Further studies on the expression of fibronectin by granulosa cells throughout the development of the follicle will provide more insight into its functional role.

The results of the present study demonstrate that fibronectin secretion may be a useful marker of granulosa cell differentiation. Further examination of the substances which influence fibronectin secretion by granulosa cells will provide insight into the factors which control follicular development and ovarian function.

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