Hormonal regulation of cytodifferentiation and intercellular communication in cultured granulosa cells

(gonadotropin receptors/gonadotropin-releasing hormone/gap junctions/cyclic AMP/progesterone)

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ABSTRACT Granulosa cells from immature hypophysectomized diethylstilbestrol-treated rats displayed pronounced intracellular and intercellular changes after 48 hr of exposure to follicle-stimulating hormone (FSH) in vitro. As determined by light and electron microscopy, most of the FSH-treated cells became highly aggregated and grew in multilayered clusters. Numerous gap junctions were seen between cells, indicating the presence of significant intercellular communication. Microvilli densely covered the surface of the hormone-stimulated cells, which contained enlarged mitochondria with convoluted cristae, characteristic of steroidogenic cells. Luteinizing hormone receptors, identified by autoradiography with 125I-labeled human chorionic gonadotropin, were mainly associated with aggregated cells, whereas single cells were usually free of the labeled hormone. Addition of a gonadotropin-releasing hormone agonist prevented the appearance of luteinizing hormone receptors and markedly impaired cyclic AMP and progesterone production, as well as the morphological changes induced by FSH. The majority of the granulosa cells grown in the absence of either hormone assumed a flattened, smooth shape and grew primarily in monolayers. The maintenance of cellular aggregation and intercellular communication by FSH, and its inhibition by gonadotropin-releasing hormone, may play an important role in the cytodifferentiation of ovarian granulosa cells.

Differentiation of granulosa cells of the ovarian follicle in vitro is accompanied by the sequential development of plasma membrane receptors for follicle-stimulating hormone (FSH; follitropin) and luteinizing hormone (LH; lutropin) (1, 2). The induction of LH and progesterin receptors in follicular cells is believed to be promoted by the dual effect of estrogen and FSH (3). Other morphological and functional changes that occur during granulosa cell differentiation include follicular antrum formation (3, 4) and development of intercellular junctions (5), as well as progesterone secretion (3, 4). It was recently shown that ovarian tissue contains receptors to gonadotropin-releasing hormone (GnRH; gonadoliberin) (6) and that GnRH agonists block gonadotropin-induced formation of LH receptors and progesterone production in both granulosa cells (7, 8) and luteal cells (6, 9, 10). These findings indicate that hypothalamic peptide hormones can exert a direct effect on ovarian function, though the mechanisms of LH receptor induction and differentiation of granulosa cells by FSH and its inhibition by GnRH remain to be defined.

Recently, induction of LH receptors by FSH has been described in cultured granulosa cells obtained from hypophysectomized diethylstilbestrol (DES)-treated immature rats (11). We have employed this system to analyze the morphological and biochemical changes associated with the process of granulosa cell differentiation in vitro. This study has revealed that FSH-treated granulosa cells undergo pronounced changes in vitro that are strikingly similar to the in vivo process of differentiation. These include the development of LH receptors and the capacity for progesterone and cyclic AMP formation, as well as cell association and intercellular communication. This work has also demonstrated that GnRH agonists inhibit the specific morphological and biochemical processes that are associated with FSH-induced granulosa cell differentiation.

MATERIALS AND METHODS

Cell Culture. Female hypophysectomized rats 25 days old were obtained from Hormone Assay Laboratories (Chicago). Silastic 10-mm capsules containing DES were implanted subcutaneously at the time of hypophysectomy in order to stimulate granulosa cell proliferation (12). Five days after surgery and DES treatment, ovaries were removed and granulosa cells were isolated by modification of previously described techniques (12, 13). Ovaries were incubated in McCoy’s 5A medium (modified, without serum) supplemented with 10 mM Hepes at pH 7.4, 4 mM l-glutamine, penicillin at 100 units/ml, streptomycin sulfate at 100 μg/ml, 6.8 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), and 0.5 M sucrose, added to disrupt intercellular gap junctions (13, 14). Granulosa cells were released by puncturing the ovaries with a 27-gauge needle and expressing the remaining cells through a 40-mesh stainless steel grid. Cell viability was determined by trypan blue exclusion and was normally between 60% and 70%. Approximately 1 × 10⁶ viable granulosa cells were added to 35-mm plastic tissue culture dishes in a total volume of 1.0 ml of the above medium without EGTA or sucrose. Ovine FSH (NIH-FSH-S13, 15 units/mg with LH contamination of 0.05 NIH-LH-S1 units/mg), 200 ng/well, and the GnRH agonist [d-Ala²-des-Gly⁸-GnRH N-ethylamide (GnRHα) (Peninsula Laboratories, San Carlos, CA) were added in 10-μl volumes at the beginning of culture. Cells were cultured in serum-free medium (7) at 37°C in a humidified 95% air/5% CO₂ environment. After 48 hr of culture, medium was removed and analyzed for extracellular cyclic AMP and progesterone by radioimmunoassay (9). The content of LH receptors during cell culture was measured after binding of 125I-labeled human chorionic gonadotropin (125I-hCG) as described (9).

Light Microscopy. For examination of cultured cells, sterile glass coverslips (9 × 9 mm) were introduced into 35-mm culture dishes.

Abbreviations: DES, diethylstilbestrol; FSH, follicle-stimulating hormone (folitropin); LH, luteinizing hormone (lutropin); hCG, human chorionic gonadotropin; GnRH, gonadotropin-releasing hormone (gonadoliberin); GnRHα, [d-Ala²-des-Gly⁸-GnRH N-ethylamide; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid. * Permanent address: Department of Hormone Research, The Weizmann Institute of Science, Rehovot, Israel.

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wells just before plating of the cells. Cell cultures and cell suspensions (before culturing) were fixed with 3% (wt/vol) glutaraldehyde in Millonig’s phosphate buffer, pH 7.4 (15), and were visualized by phase-contrast optics (Leitz Ortholux light microscope) at ×400 magnification.

**Autoradiography.** Cell cultures after various treatments (see below) were washed once with fresh medium and then incubated for 1 hr at 37°C in the presence of 6 × 10⁶ cpm/ml of ¹²⁵I-hCG (approximately 10⁵ cpm/ng). Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled hormone (100 international units). At the end of the incubation period with radiolabeled hormone, cells were washed three times with fresh media supplemented with 0.1% bovine serum albumin, fixed with 3% glutaraldehyde in Millonig’s buffer, scraped from the plastic wells with a rubber policeman, and centrifuged at 400 × g for 5 min. Cells were resuspended in fresh fixative solution, and the bound radioactivity was determined in a gamma spectrometer. Samples were pelleted in a Beckman Microfuge (model 152), postfixed in 1% osmium tetroxide, and embedded in Epon as described (16). One-micrometer sections were processed for light microscopy autoradiography (16), with exposure time to the photographic emulsion (Ilford L4) of 10–20 days. Sections were stained with 1% toluidine blue and photographed at ×630 magnification.

**Electron Microscopy.** Cells were fixed, scraped, and centrifuged as described above. After osmium fixation they were stained en bloc with 2% aqueous uranyl acetate. Sections of the cell pellets embedded in Epon were stained with uranyl acetate and lead citrate (17) and photographed in a Philips 300 electron microscope at original magnifications of ×3300 and ×33,000.

**RESULTS**

**Effects of FSH and GnRH on LH Receptors, Cyclic AMP, and Progestrone.** When granulosa cell suspensions were grown in culture for 48 hr in the presence of FSH, there was a 10-fold increase in binding of ¹²⁵I-hCG, accompanied by marked parallel increases of both cyclic AMP and progesterone formation (Table 1). Addition of the GnRH agonist together with FSH almost abolished the formation of LH receptors, and this addition reduced the synthesis of cyclic AMP by 82% and that of progesterone by 86%. In freshly cultured cells, cyclic AMP and progesterone levels were lower than in the unstimulated cells after 48 hr in culture (data not shown).

**Morphological Observations at the Light Microscope Level.** Freshly prepared cells from ovaries of DES-treated immature rats consisted mainly of single rounded cells, and occasionally there were small clusters of aggregated rounded cells (Fig. 1A). The number of cells in such aggregates did not usually exceed five to seven. When cells were grown for 48 hr in culture with FSH, much larger aggregates were formed (Fig. 1B), consisting of up to several dozen cells. The cells in the aggregated form acquired a shape like that of epithelial cells and were tightly packed. The bottom layer of cells in the aggregates attached to the plastic dish were flattened. Often, neighboring aggregates of cells were interconnected to form a chain-like structure. When cells were grown in the presence of FSH plus GnRHα, most of the intact cells grew in a monolayer of flattened cells (Fig. 1C). Cell debris and aggregates of damaged cells were occasionally seen over the cells that attached to the plastic dish. When cells were grown for 48 hr in the absence of FSH and GnRHα, a monolayer of flat cells was observed (Fig. 1D). Cells grown with GnRHα alone, or with GnRHα plus FSH, formed a monolayer that was less flattened than the untreated cells.

**Light Microscopic Autoradiography.** In order to visualize the LH receptors in granulosa cells, cultures or cell suspensions were incubated with a saturating concentration of ¹²⁵I-hCG. Under such conditions, the labeling of cells cultured in the presence of FSH was 6 × 10⁶ cpm/10⁸ cells. A 100-fold excess of unlabeled hormone (100 international units) reduced the binding by 97%. The radioactivity bound by cells at zero time of culture and at 48 hr of culture without FSH was only 6% and 5%, respectively, of that bound by the FSH-stimulated culture. As can be seen from Fig. 1E, most of the silver grains (which represent the bound hormone) were confined to highly aggregated cells, whereas single cells usually showed only a very low density of silver grains. Occasionally, some single cells also showed a high density of labeling. However, in serial sections it was observed that most of these cells were associated with neighboring cells that could be seen at different levels of sectioning (not shown). The label was mostly associated with cells in the aggregate that were tightly packed and epithelial in shape, while more rounded cells showed less dense labeling.

**Electron Microscopy.** Granulosa cells freshly obtained from DES-treated animals were rounded and essentially devoid of microvilli (Fig. 2A). The cell membrane was ruffled in some areas. Occasionally, clustered cells contained remnants of gap junctions, which could be seen between cells as well as within cells (not shown). When cells were grown in the presence of FSH for 48 hr, dramatic changes occurred in their morphological appearance. Most of the cells became highly aggregated and irregular in shape, with numerous long microvilli covering their surface (Fig. 2B). Frequently, large gap junctions could clearly be identified between these cells, with a typical pentalaminar or heptalaminar structure and total thickness of 180–200 Å (Fig. 2C). The total length of the individual junctional elements was often more than 1 μm. The mitochondria of FSH-treated cells were enlarged (compare with Fig. 2A), while the endoplasmic reticulum and Golgi complex were well developed. In contrast, cultures treated with GnRHα plus FSH displayed pronounced morphological differences. Most of the intact cells were flattened and did not show gap junctions or microvilli, and their mitochondria were not enlarged. Although cell aggregates containing elements of gap junctions and microvilli could occasionally be seen, such aggregated cells usually showed regressive changes (not shown). Cells grown in the absence of hormones showed ultrastructural features similar to those of cells incubated with FSH and GnRHα, with an even more flattened appearance.

**DISCUSSION**

The present studies have demonstrated that FSH is able to induce not only the formation of LH receptors and biochemical responses in cultured granulosa cells but also striking morphological changes in the organization of these cells. These FSH-induced changes in vitro in many respects resemble the differentiation of follicular tissue in vivo (18, 19). Thus, formation of gap junctions between cells, the appearance of microvilli on cell surfaces, and the development of large mitochondria, smooth
endoplasmic reticulum, and Golgi apparatus were seen in the hormone-treated cells. Moreover, we have shown by autoradiography that receptors for LH appear mainly in cell aggregates that morphologically resemble the differentiated follicular tissue in vivo (18, 19).

One of the most striking observations was that the presence of FSH caused granulosa cells to grow in multilayered aggregates rather than in monolayers. Because the initial cell suspensions consisted mainly of single cells and small aggregates, and cultures without FSH form monolayers, it is evident that FSH plays an important role in this aggregation phenomenon.

Although we have observed that some degree of aggregation may occur after culture for 24 hr without the hormone, this progressively disappears during the second day of culture. Therefore, FSH may play a more critical function in promoting and maintaining cellular aggregation rather than in inducing cell clustering.

These data suggest that FSH has an important effect on the regulation of cellular communication between the differentiating granulosa cells. Although remnants of gap junctions can still be detected after the combined treatment with EGTA and sucrose solutions used for tissue dissociation, it is evident that the
FIG. 2. Electron microscopy of cultured granulosa cells. Thin sections of granulosa cells after tissue dissociation (A) and after 48 hr in culture in the presence of FSH (B and C), and in the presence of FSH plus GnRHa (D). (A) This single cell shows a rounded profile and the plasma membrane is essentially devoid of microvilli. Small mitochondria (m) are seen in a neighboring cell. (B) A typical aggregate of cells, formed during culture with FSH. Cells are covered with numerous long microvilli (v) and are irregular in shape. Gap junctions (arrows and circle) are seen between cells. The mitochondria (m) are enlarged and the Golgi complex is well developed. Several endocytic vesicles (e) are seen within cells and contain fragments of membranes and electron-dense granulated material. (C) An enlarged image of the gap junction circled in B. It has a typical pentalaminar structure with an overall thickness of 180–200 Å (opposing arrows). (D) Cultures in the presence of FSH plus GnRHa show flattened cells devoid of microvilli. The mitochondria are small (compared to B) and many endocytic vesicles (e) can be seen within the cells. (A, B, and D: ×6900; bar represents 2 μm. C: ×71,000; bar represents 0.2 μm.)
number and size of these junctional elements increase dramatically in culture. This reflects in vitro observations that gap junctions in the ovary are mainly associated with mature granulosa cells of antral follicles (5, 20). Because follicular gap junctions can be found in immature DES-treated animals (21), the possibility that both estrogens and FSH are important for the maintenance of ovarian cell communication in vitro cannot be excluded. Likewise, because gap junctions permit transport of ions and small molecules such as cyclic AMP (22), chemical and electrical coupling between cells may also affect the maturation of granulosa cells both in vitro and in vivo.

The mechanism by which FSH facilitates the induction of LH receptor formation in granulosa cells is not yet clear. Our finding that the receptor appeared primarily in aggregated cells suggests that contact between cells and intercellular communication may play an important role in LH receptor formation. Indeed, we have previously observed that the LH receptor population in the preovulatory follicle, mainly confined to the outer layers of granulosa cells (23) and that these layers are much more tightly packed and contain more junctional elements than the inner layers of granulosa cells.

The appearance of microvilli in steroidogenic cells has been previously observed both in vivo (18) and in vitro (24). We have demonstrated that FSH induced the formation of both microvilli and LH receptors in cultured granulosa cells, suggesting that these two phenomena may be linked. LH receptors are localized mainly on microvilli in the corpus luteum of the ovary (25, 26), as well as in dispersed Leydig cells of the testis (27). Preliminary observations with high-resolution autoradiography in granulosa cells indicate that most of the 125I-hCG binding sites in these cultures are likewise associated with microvillous processes.

We have also demonstrated in this work and elsewhere (28) that the level of cyclic AMP is markedly increased during the 48 hr of culture in the presence of FSH. Moreover, we have now obtained evidence that both β-h bromo-cyclic AMP and cholera toxin mimic the effect of the FSH-induced appearance of LH receptors in dispersed cells into multilayered aggregates (unpublished observations). The notion that high levels of cyclic AMP are essential for granulosa cell differentiation is in line with several recent observations that this nucleotide promotes cellular and tissue differentiation in a wide variety of systems (29, 30), including formation of insulin receptors in fibroblasts and lymphocytes (31). Conversely, much evidence has indicated that GnRH agonists have a direct inhibitory action in the ovary on LH receptor formation and steroidogenesis (6–10, 32). We have clearly demonstrated in this work that GnRHα interferes with both accumulation of cyclic AMP and induction of morphological changes by FSH in granulosa cells. Thus, the possibility that some of the effects of GnRH on granulosa cell differentiation are mediated by the modulation of cyclic AMP levels in FSH-stimulated cells should be considered.

It was already reported that FSH induced pronounced morphological changes in granulosa cells obtained from preovulatory ovarian follicles (33). This included rounding of the cells, disappearance of microvilli, and rearrangement of microfilament bundles in the cells. However, these effects were reversible after several hours despite the continuous presence of the hormone. In contrast, we have observed that FSH has a prolonged action on several aspects of granulosa cell differentiation, including LH receptor formation, steroidogenesis, and cyclic nucleotide accumulation. This evidence suggests that FSH may exert different effects depending upon the stage of granulosa cell development. The organization and maintenance of cellular communication is affected by the action of this hormone and suggests that many of the effects of FSH in vivo can likewise be induced in vitro.

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