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Biochemistry

Human pituitary and placental hormones control human insulin-like growth factor II secretion in human granulosa cells

(progesterone/gonadotropins/human growth hormone/human chorionic somatomammotropin/prolactin)

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ABSTRACT **Human granulosa cells cultured with calf serum actively proliferated for 18–20 generations and secreted progesterone into the medium; progesterone levels appeared to decline with increase in generation number. Cells cultured under serum-free conditions secreted significant amounts of progesterone and insulin-like growth factor II (IGF-II). The progesterone secretion was enhanced by the addition of human follitropin, lutropin, and chorionic gonadotropin but not by growth hormone. These cells, when challenged to varying concentrations of human growth hormone, human chorionic somatomammotropin, human prolactin, chorionic gonadotropin, follitropin, and lutropin, secreted IGF-II into the medium as measured by specific IGF-II RIA. Among these human hormones, chorionic gonadotropin, follitropin, and lutropin were most effective in inducing IGF-II secretion from these cells. When synthetic lutropin-releasing hormone and α-inhibin-92 were tested, only lutropin-releasing hormone was effective in releasing IGF-II. The results described suggest that cultured human granulosa cells can proliferate and actively secrete progesterone and IGF-II into the medium. IGF-II production in human granulosa cells was influenced by a multi-hormonal complex including human growth hormone, human chorionic somatomammotropin, and prolactin.**

The insulin-like growth factors (IGF-I and IGF-II) are a family of low M_r single-chain peptides with considerable structural and functional similarities to proinsulin (1–3). These growth factors appear to follow a divergent pattern of regulatory mechanisms. The IGF-I levels in plasma are low at birth and gradually increase with age, thus closely following the actions of growth hormone (4–6). Conversely, IGF-II is considered a fetal hormone and appears partially dependent on growth hormone (7, 8). Although the liver is considered the primary site of synthesis of these factors, recent studies have shown that several other tissues including the testes and the ovary (9–11) are also capable of producing IGFs.

The exact role of IGFs in gonadal function is not clear. Attempts to demonstrate the production of IGF-I by cultured granulosa and Sertoli cell (12, 13) have been inconclusive. We have recently identified significant amounts of IGF-II in human follicular and seminal fluids (14). We report the production of IGF-II by human granulosa cells under serum-free conditions and present evidence for multihormonal regulation of IGF-II release by these cells.

MATERIALS AND METHODS

IGF-II was synthesized by the solid-phase method as described (15). Human growth hormone (hGH) (16), and human chorionic somatomammotropin (hCS) (17) were isolated and characterized in our laboratory. Follitropin (FSH), luteinizing hormone (LH), and prolactin (PRL) were obtained from the National Hormone and Pituitary Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. hCG (human chorionic gonadotropin) and fibroblast growth factor were gifts from H. Papkoff and D. Gospodarowicz (both of UCSF) respectively. Epidermal growth factor was purchased from Collaborative Research (Waltham, MA), collagenase and deoxyribonuclease were purchased from Worthington, Dispase, grade II was provided by Boehringer Mannheim, ^{3}H-labeled progesterone was supplied by Amersham, and sheep anti-rabbit gamma globulin was from Antibodies, Inc. Ham's F-12 nutrient media, fetal calf serum, serum substitutes, lipids, and STV (0.01 M PBS, containing 0.02% EDTA and 0.05% trypsin) were obtained from the Tissue Culture Facility of this campus. Human transferrin, penicillin, and streptomycin were purchased from Sigma; tissue culture flasks (75 cm^2) and 24-well culture plates were from Corning Glass Works.

Human Granulosa Cell Cultures. Human follicular fluid was collected from women who were undergoing *in vitro* fertilization procedures at the Department of Obstetrics and Gynecology of this university. The cell culture method was essentially as reported (18, 19), and briefly described as follows: Granulosa cells in the follicular fluid were pooled from different sized follicles and separated by centrifugation at 500 × *g* in a table-top clinical centrifuge for 10 min. The cell suspension was washed three times with 5 ml of sterile phosphate-buffered saline (PBS) and resuspended in 10 ml of fresh PBS containing 10 μg of deoxyribonuclease, 2.5 mg of collagenase, and 5 mg of Dispase, grade II. The cell suspension was incubated at 37°C with gentle shaking for 30 min in a shaking water bath. During this period, cells were resuspended with the help of a Pasteur pipet three or more times. The cell pellet was separated by centrifugation, layered onto a Ficoll-Paque solution (Pharmacia), and placed in a 15-ml tube, and then the centifugation step was repeated. The granulosa cell-enriched fraction was carefully removed, mixed with 25 ml of Ham's F-12 solution containing 10% fetal calf serum, 50 international units of penicillin per ml, and 50 μg of streptomycin per ml (medium A) in a culture flask (75 cm^2) and incubated in a humidified incubator at 37°C in 92% air/8% CO_2. After 24 hr of incubation, the medium was replaced with fresh medium A containing epidermal growth factor (50 ng/ml) and fibroblast growth factor (2 ng/ml) and incubation was continued for 4 days before the cells were subcultured. Granulosa cells were routinely passaged (usually 1:1) as the cells reached confluency. At each passage the medium was separated and stored at −20°C until assayed in

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Abbreviations: IGF, insulin-like growth factor; hGH, human growth hormone; PRL, human prolactin; hCS, human chorionic somatomammotropin; FSH, human follitropin, follicle-stimulating hormone; LH, luteinizing hormone, human lutropin; hCG, human chorionic gonadotropin; LH-RH, luteinizing hormone-releasing hormone; PBS, phosphate-buffered saline.

progesterone RIA, and phase-contrast micrographs of the cultures were taken. Granulosa cells were allowed to grow in a serum-free medium containing 10% serum substitutes supplemented with 2 μg of insulin per ml, 5 μg of human transferrin per ml, and 1 ml of lipid solution (1 mg of oleic acid/2 mg of lecithin/4 mg of cholesterol/1 ml of ethanol). The cells were cultured for 4 days under this condition, and the medium retained for progesterone estimation. IGF-II was identified in the medium using an immunoaffinity column and further analyzed by IGF-II RIA.

Affinity Chromatography. Antibodies generated against synthetic IGF-II were purified using a protein-A Sepharose column (14). Purified antibodies were coupled to CNBr-activated Sepharose 4B at a ratio of 2.5 mg/ml of the gel, and this gel was used to construct a 10-ml affinity column. Forty milliliters of serum-free collected medium was extracted with acid/ethanol (14) and the clear supernatant was lyophilized. The dry powder was dissolved in PBS, pH 7.4, and applied to the column; the column was washed extensively with the powder-containing buffer and with 0.05 M NH_4OAc, pH 4.5, to remove unbound proteins. Bound IGF-II was eluted with 0.2 M NH_4OAc, pH 3.0.

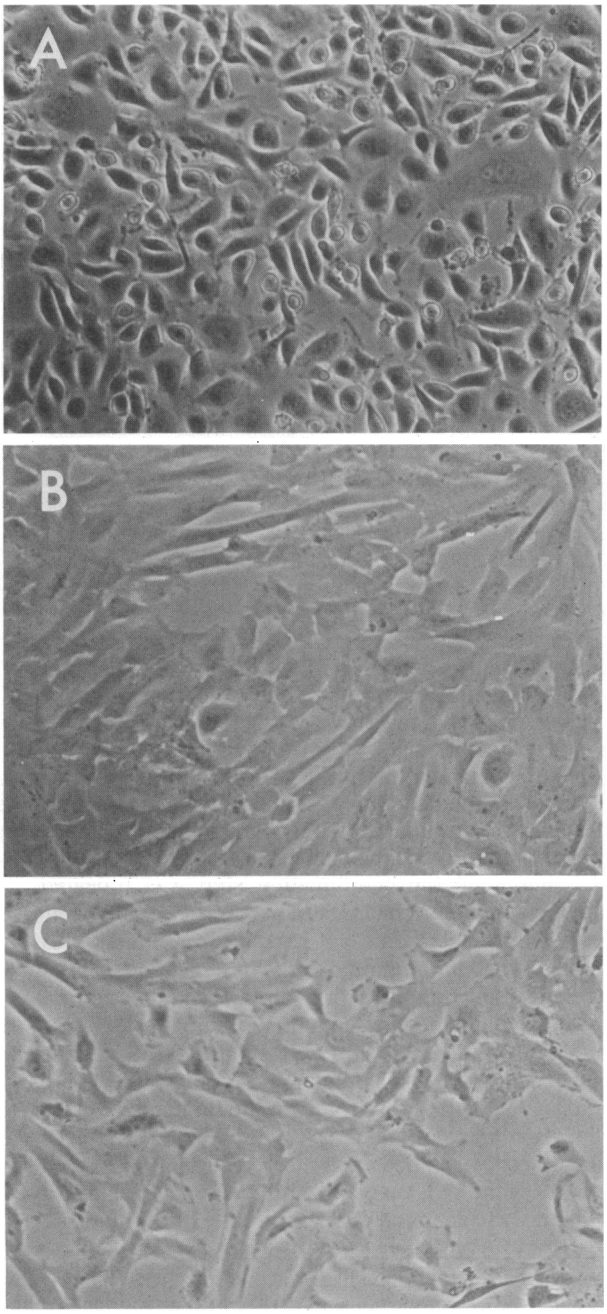

FIG. 1. Human granulosa cell cultures maintained in the presence of epidermal growth factor and fibroblastic growth factor. (*A*) Passage 1; (*B*) passage 4; and (*C*) passage 8. (Magnification ×150.)

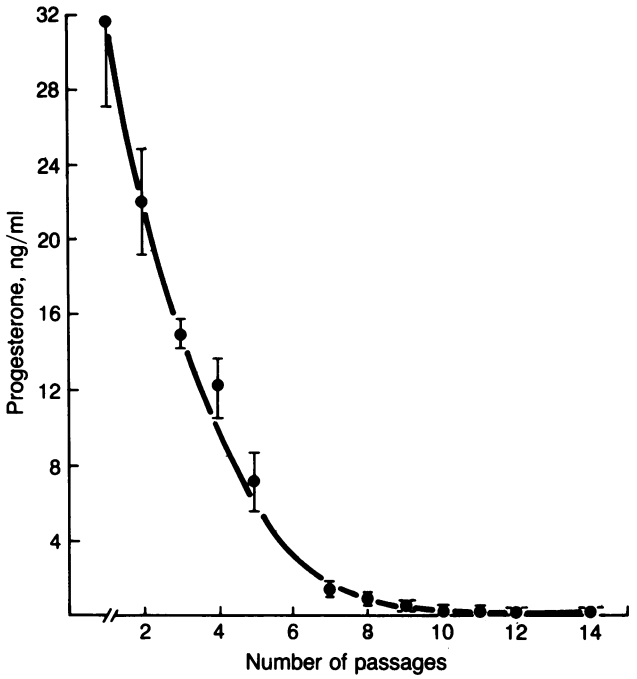

FIG. 2. Progesterone content in cultured granulosa cell media as a function of passages. Cells were grown to confluency, and at each passage the medium was collected and assayed for progesterone by RIA. Data are reported as means ± SD of six different cultures.

Effect of Addition of hGH, PRL, hCS, LH, FSH, and hCG on Granulosa Cell IGF-II Production. The cells grown to confluency were harvested by trypsinization in 5 ml of 0.01 M PBS, pH 7.4, containing 0.02% EDTA and 0.05% trypsin, mixed with 95 ml of fresh medium A, seeded in 24-well plates, and incubated for 24 hr as described above. Cells from one flask were usually distributed to four plates, and each well under these conditions contained 5×10^4 cells per ml. At the time of experimentation the serum-containing medium was replaced with 1 ml of medium containing serum substitute, and various hormones at different concentrations were added in 50-μl volumes. After 48-hr incubation, the medium was separated, centrifuged for 10 min to remove any cell debris, and stored at −20°C. The controls received only medium. Each sample was tested in five or more wells.

Radioimmunoassays. The media recovered from the granulosa cell cultures were directly assayed for IGF-II (200 μl per tube) in the homologus RIA as described (14). The progesterone assay was done as described (20) in duplicate tubes (100 μl per tube), and data were analyzed by the Student's *t* test.

Table 1. IGF-II and progesterone content in cultured human granulosa cell media as estimated by RIA

Batch	IGF-II,* ng/ml	Progesterone,* ng/ml
1	179 ± 27	9 ± 2
2	196 ± 11	11 ± 2
3	202 ± 23	13 ± 2
4	116 ± 11	23 ± 4
5	128 ± 10	18 ± 2

*Mean ± SEM (n = 3).

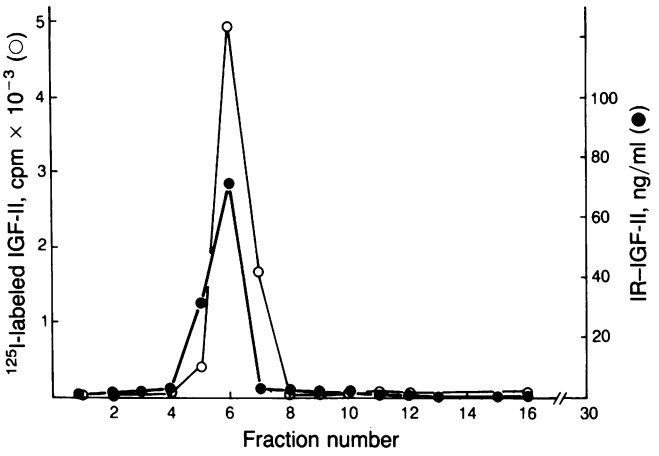

FIG. 3. Elution of IR-IGF-II (●) and ^{125}I-labeled IGF-II (○) from immunoaffinity column (10 ml). The serum-free medium that was extracted with acid/ethanol (40 ml) was dissolved in 10 ml of PBS, and clear mixture was applied onto the column. Unbound fractions (data not shown) were successfully washed with PBS and 0.05 M NH_4OAc, pH 4.5. The bound IGF-II fraction was eluted with 0.2 M NH_4OAc, pH 3.0, and all the samples were lyophilized and then resuspended in 3 ml of PBS and assayed (200 μl per tube) in IGF-II RIA (see ref. 14). ^{125}I-labeled IGF-II was also applied and eluted under similar conditions.

RESULTS

The culture method used for bovine granulosa cells (18, 19) can be used for human granulosa cells. Granulosa cells were pooled irrespective of the size of the follicles and cultured initially for 3–4 days. The life span of these granulosa cells cultured with serum, epidermal growth factor, and fibroblast growth factor ranged from 12 to 15 generations. Cells examined from different batches exhibited similar morphological appearance in the first two generations. These cells were small, rounded, and densely packed (Fig. 1*A*); during later generations the cells gradually became enlarged and appeared angular (Fig. 1*B*), and after four to six generations, a mixture of round and enlarged cells was present. When the cultures were 12–15 generations old, only enlarged cells were evident (Fig. 1*C*). Addition of epidermal growth factor (50 ng/ml) and fibroblast growth factor (2 ng/ml) enhanced the doubling time. Some batches of granulosa cells reached 18–20 generations under these conditions. The medium recovered during each passage was used to monitor the progesterone secretion by these cells (Fig. 2). Progesterone secretion was maximal during the initial passages and gradually declined by five to six generations, becoming undetectable at eight to ten generations.

Granulosa cells maintained under serum-free conditions for 4 days were strikingly similar to those cultured with serum—these cells secreted significant amounts of progesterone and IGF-II (Table 1). The progesterone production was similar to that of cells cultured with serum (see Fig. 2). Amounts of IGF-II and progesterone secreted from these cells varied from batch to batch (see Table 1). The IGF-II in the collected medium displaced the labeled IGF-II bound to the antibody in a dose-related manner (data not shown). Furthermore, the IGF-II present in the acid/ethanol extract of the medium eluted in the same position as that of ^{125}I-labeled IGF-II in an IGF-II affinity column (Fig. 3).

Granulosa cell cultures at three to four generations were suitable for IGF-II and progesterone secretion experiments. Cells were allowed to grow in 24-well plates and were challenged by various hormones. As shown in Fig. 4, these cells responded to LH, FSH, and hCG and enhanced the secretion of progesterone in a dose-dependent manner, but these cells failed to respond to hGH regardless of the concentration used. However, hGH, PRL, hCS, LH, FSH, and hCG effectively induced IGF-II secretion from granulosa cells in a dose-related manner (see Fig. 5). The concentrations of hGH, PRL, and hCS required to release significant amounts of IGF-II were markedly higher (100 ng/ml) than those of FSH, LH, or hCG (10 ng/ml). Among all these hormones tested, hCG, LH, and FSH were the most effective—as little as 2 ng/ml induced an 8- to 10-fold ($P < 0.05$) increase of IGF-II secretion into the medium as compared with controls, whereas synthetic LH-RH (20–100 ng/ml) induced only a minimal amount of IGF-II secretion. The synthetic inhibin-like peptide now designated as α-IB-92 (21) was not effective at any dose level. In fact, these two synthetic peptides served as controls.

DISCUSSION

Ovarian follicular growth and maturation are an orderly process and depend on several coordinating signals. Pituitary gonadotropins and ovarian steroids have predominant roles in granulosa cell physiology (22). Recently, these IGFs have

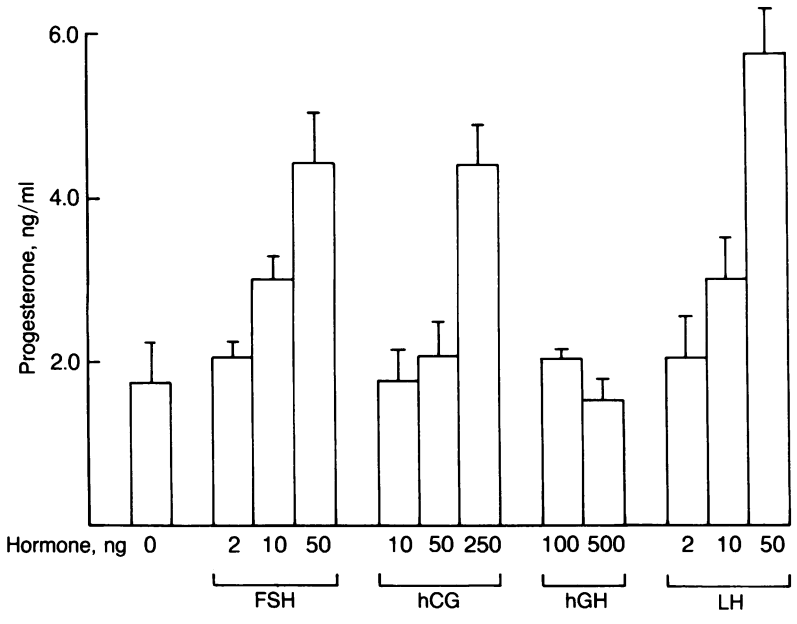

FIG. 4. Effect of various hormones on granulosa cell progesterone secretion into the medium. Cells from passages three were cultured in a serum-free condition in 24-well plates. hGH, LH, FSH, and hCG were added at different concentrations in 50 μl, and the cells were incubated for 48 hr; the medium was subsequently analyzed for progesterone. Mean ± SEM (n = 5 wells).

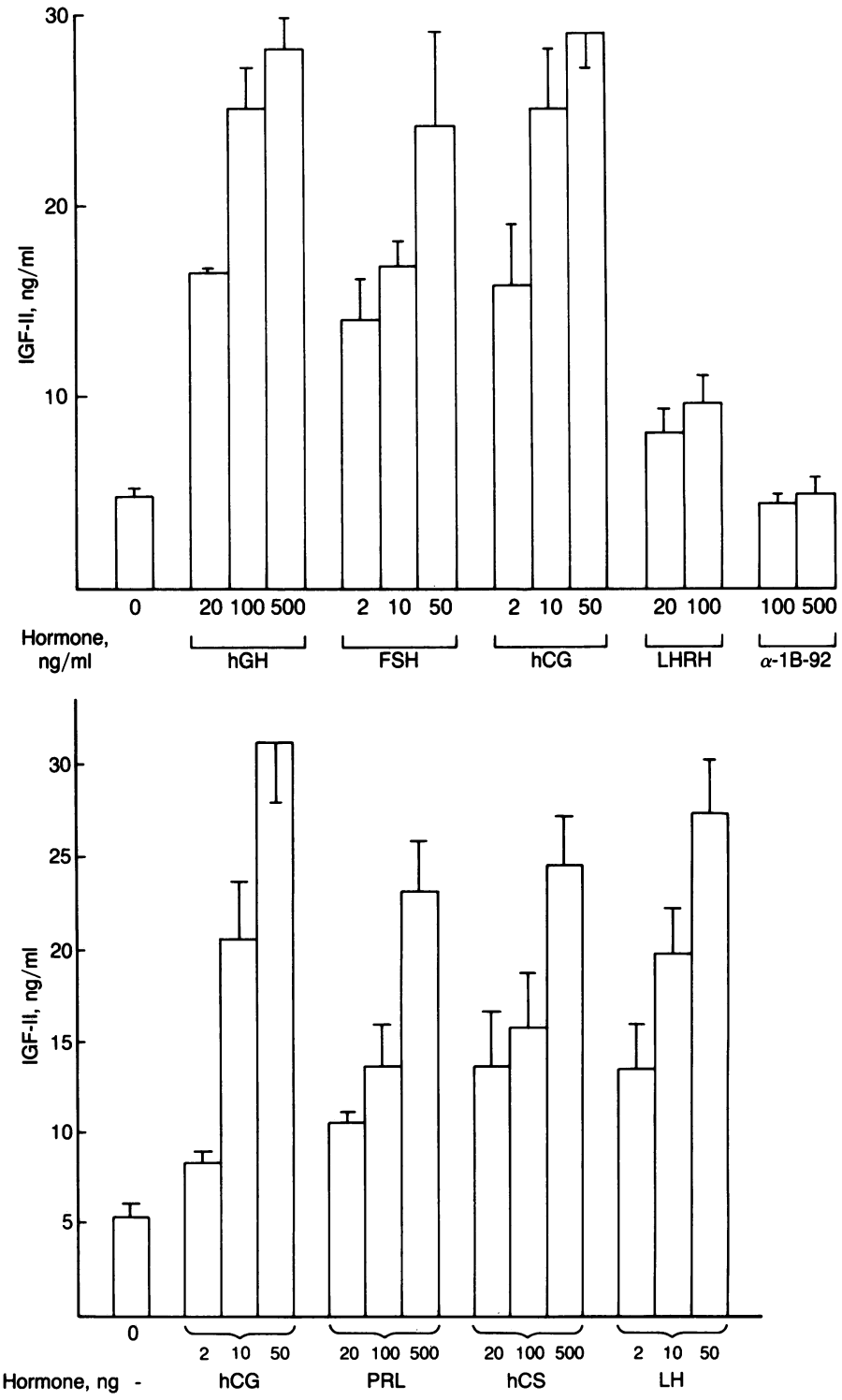

FIG. 5. Effect of various hormones on granulosa cell IGF-II secretion into the medium. Cells were treated with different concentrations of hGH, FSH, and hCG (*Upper*), hCG, PRL, hCS, and LH (*Lower*) for 48 hr, and the medium was analyzed for IGF-II by RIA. LH-RH and α-IB-92 (*Upper*) were used as control peptides. Data are reported as the mean ± SEM (n = 5 wells).

been implicated as intragonadal peptides that regulate local cellular activity (11), and we have identified significant amounts of IGF-II immunoreactivity in human follicular and seminal fluids (14). The IGF-II in these fluids may be assumed to be secreted by the gonadal cells.

Cultured human granulosa cells actively proliferate for at least 12–15 generations. Although, the proliferation is similar to that of the bovine granulosa cells, bovine cells can reach 50–60 generations (18, 19). Some of our batches of human granulosa cells reached 18–20 generations. This difference in cell life span could be due to the pooling of cells from various sized follicles, in addition to species specificity. There was a remarkable change in the morphological appearance of these cells from early to middle and late generations. Progesterone secretion as a function of generations appeared to be maximal during early generations and declined with increasing life span (Fig. 2).

Three to five generations of granulosa cells were used to study IGF-II secretion because these cells retained their ability to secrete significant amounts of IGF-II and proges-

terone even in the absence of any external stimuli (Table 1). In addition, these cells responded to LH, FSH, and hCG in releasing progesterone in a dose-related manner (Fig. 4). hGH, PRL, hCS, LH, FSH, and hCG induced the secretion of IGF-II in a dose-related manner (Fig. 5). However, 10-fold higher concentrations of hGH, PRL, and hCS were required to induce significant amounts of IGF-II into the medium as compared with hCG, LH, and FSH concentrations. These latter three hormones effectively secreted 3- to 5-fold of IGF-II even at a concentration of 2 ng/ml, and this secretion was significantly different from controls ($P < 0.005$).

IGF-II secretion by human granulosa cells appears to be under multihormonal regulation. Whether these hormones have any influence on the induction of IGF-II gene expression or are simply involved in some way in the releasing mechanism remains to be seen.

That IGF-I synergizes with FSH in inducing LH receptors and enhances progesterone production in granulosa cells has been reported (23–25). These actions appear to be mediated by specific receptors on these cells. Indeed, the presence of specific binding sites of IGFs on rat granulosa cells has been suggested (25). Preliminary studies indicate the presence of high-affinity binding sites for IGF-II on human granulosa cells (unpublished observations). Recent evidence indicating the role of EGF in spermatogenesis of the mouse (26) supports the idea that growth factors play an important role as intragonadal regulators of gonadal function.

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