IGF-I and EGF influence on steroid secretion and morphology of human granulosa cells of IVF-cycles and natural cycles in vitro

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Summary: The effect of IGF-I, EGF, PDGF and FGF on human granulosa cells of IVF-cycles, and the effect of IGF-I on granulosa cells of natural cycles (day 7 to 13 of cycle) were evaluated in vitro with and without hCG stimulation. At concentrations of 1 ng and 5 ng per ml culture FGF and PDGF did not alter progesterone and estradiol secretion or the morphology of preovulatory granulosa cells. At 1 ng, 2 ng and 3 ng per ml medium EGF significantly enhanced basal progesterone and estradiol secretion and significantly decreased hCG stimulated estradiol production of preovulatory granulosa cells. While IGF-I treatment with and without hCG stimulation did not alter steroid secretion of preovulatory granulosa cells, the progesterone secretion of granulosa cells of natural cycles was increased by combined treatment with 10 I.U. hCG + 25 ng IGF-I per ml culture medium. Increased steroid secretion was related to reduced cell spreading. Our results provide evidence for the facultative role of IGF-I and EGF as an autocrine/paracrine modulator of ovarian function. IGF-I may play a role in regulation of ovulation induction and luteinisation as IGF-I and hCG act synergistically in increasing progesterone secretion of granulosa cells of natural cycles in vitro and are known to stimulate each other's receptor expression.

Key words: Growth factors; Granulosa cells; Steroid secretion; Morphology.

INTRODUCTION

Recent studies have focussed the potential role of growth factors as paracrine/autocrine modulators of ovarian function. Basic fibroblast growth factor (FGF) was regarded as an intraovarian inducer of oocyte maturation ([1]), and may regulate the stimulating effect of FSH on the ovary ([2]).

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Plateled derived growth factor (PDGF) was found to amplify the epidermal growth factor (EGF), and insulin-like growth factor I (IGF-I) induced granulosa cell proliferation ([3]). Granulosa cells secrete both IGF-I ([4]) and IGF binding proteins ([5]) and have IGF receptors ([6]). IGF-I potentiates the stimulatory action of FSH on LH receptor expression ([6]) and on the steroid secretion of granulosa cells in vitro ([7]), while FSH and LH increase the IGF binding of granulosa cells ([8]). This synergistic relationship suggests that IGF-I may have an important role in luteinisation and ovulation. On the other hand EGF seemed to inhibit the FSH induced development of LH receptors ([9]).

Endo *et al.* ([10]) described an inhibitory effect of EGF on the hCG induced steroid secretion and ovulation in rabbit ovary. In this study, the effects of IGF-I, EGF, FGF and PDGF on human preovulatory granulosa cells of stimulated IVF-cycles and of IGF-I on human granulosa cells of the follicular phase of natural cycles were evaluated in vitro.

MATERIALS AND METHODS

Material

Ham's F10 medium, fetal calf serum (FCS), penicillin, streptomycin, IGF-I, EGF, FGF and PDGF were provided from Boehringer Mannheim (Mannheim, Germany). Phosphate buffered saline (PBS) was obtained from Serva (Heidelberg, Germany), Percoll® from Pharmacia (Uppsala, Sweden), human chorionic gonadotrophin (hCG) from Serono (Freiburg im Breisgau, Germany) and Coat-A-Count® Progesterone and Coat-A-Count® Estradiol radioimmunoassay kits from Diagnostic Product Corporation (Los Angeles, USA).

Granulosa cells

Preovulatory granulosa cells were yielded by follicular aspirates of 27 patients of our IVF-program undergoing oocyte retrieval. The patients obtained ovarian hyperstimulation with hMG. Ovulation induction was performed by injection of 10000 I.U. of hCG. Granulosa cells of the follicular phase of natural cycles were yielded by 13 patients with regular cycles who were undergoing laparoscopy for benign gynaecological diseases on day 7 to 13 of cycle. In two patients the day of the cycle could not be determined due to irregular cycles in one case and a hysterectomy several years before in the other case. The patients gave their informed consent for follicular puncturing.

Granulosa cell culture

The follicular aspirates were centrifuged for 10 minutes at 1000 rpm and the cell pellet was resuspended with cold PBS. The resuspended cells were layered on a 45% Percoll® colum and centrifuged for 20 minutes at 2000 rpm to separate the red blood cells. The granulosa cells were aspirated from the interphase, washed twice with cold PBS and counted in Neubauer cytometers using trypan blue dye exclusion test. 10^5 viable cells per 1 ml Ham's F10 medium containing 10% FCS, 100 I.U. penicillin and 100 µg streptomycin were plated in multiwell culture dishes (Nunc, Denmark). The influence of IGF-I on preovulatory granulosa cells in vitro were tested at 5 ng and 25 ng per ml medium. EGF was used at concentrations of 1 ng, 2 ng and 3 ng per ml medium; FGF and PDGF at concentrations of 1 ng and 5 ng per ml medium. The effect of the growth factors was evaluated with and without hCG stimulation of the cultures (10 I.U./ml medium). In the cultures of granulosa cells obtained from natural cycles IGF-I was used at a concentration of 25 ng with and without 10 I.U. hCG per ml culture medium.

The cultures were maintained for 8 to 14 days at 37° Celsius in a 5% CO_2-gas atmosphere. Cell morphology and growth was checked every day by an Olympus invert phase contrast microscope. The culture media were exchanged daily and stored at −4° Celsius until hormone measurements were made.

Hormonal Assays

Progesterone and estradiol concentrations in the culture media were evaluated in duplicate by radioimmunoassay.

Statistical Evaluation

The influence of the growth factors on the steroidogenesis of the granulosa cell cultures was evaluated for significance by the Mann-Withney-U-Wilcoxon test. For significance evaluation the data of the experiments were pooled. In the figures the data of individual representative experiments were given.

RESULTS

Effect of FGF and PDGF on preovulatory granulosa cells.

At neither tested concentrations of 1 ng and 5 ng per ml culture medium, did FGF and PDGF alter the basal or the hCG stimulated progesterone and estradiol secretion of human preovulatory granulosa cell in vitro. The morphology of the granulosa cells in vitro was not influenced by FGF or PDGF.

Effect of EGF on preovulatory granulosa cells.

Under basal culture conditions, the stellate granulosa cells begin to spread after three to five days in culture. This phenomenon was prevented by stimulation with hCG. The granulosa cells maintained their stellate form and developed

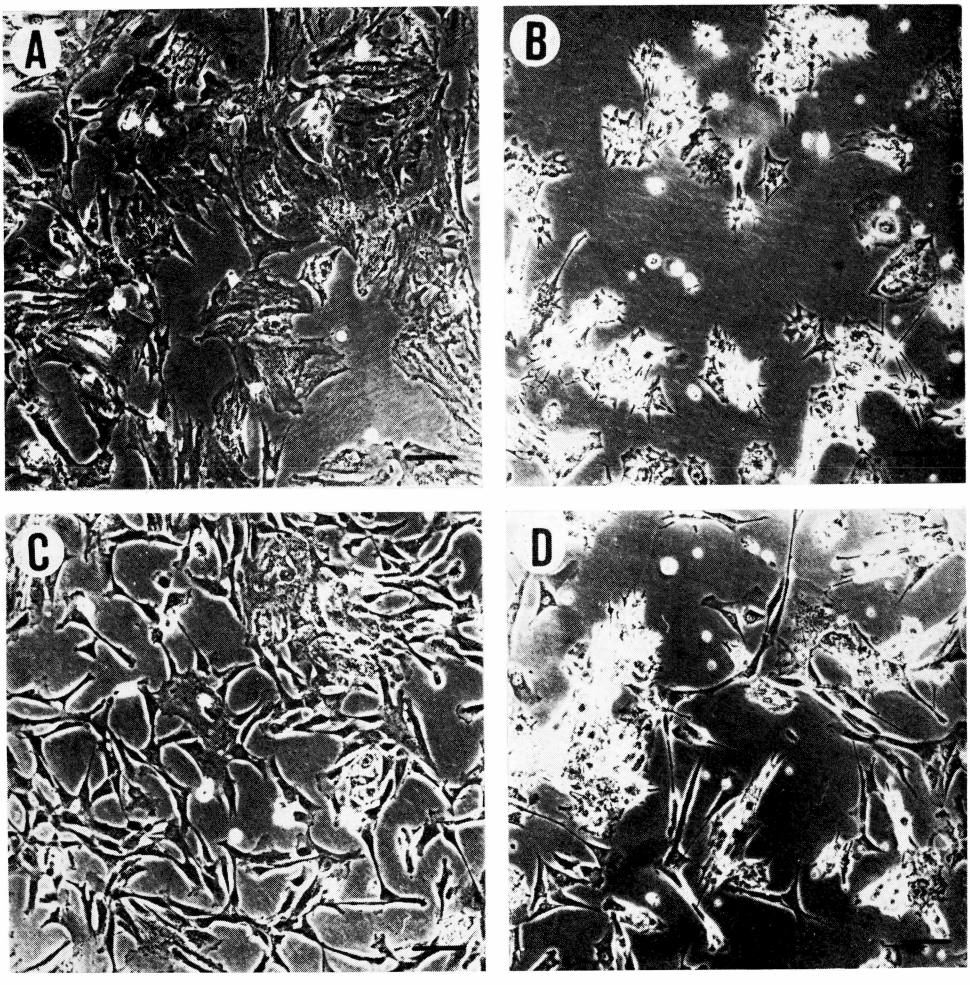

Plate 1. — Effect of EGF on morphology of human preovulatory granulosa cells in vitro of one patient (day 8 of culture): *a*) in medium Ham's F10 the granulosa cells were spread; *b*) the stimulation with hCG (10 I.U./ml) resulted in reduced cell spreading; *c*) granulosa cells influenced by EGF (2 ng/ml) have fibroblastoid cell shape; *d*) the combined influence of hCG and EGF lead to elongation of filopodia compared to pure EGF (—— $\widehat{=}$ 0.1 mm).

longer filopodia. EGF treatment leads to an elongate fibroblastoid cell shape with bipolar filopodia. Additional stimulation of the cells with hCG resulted in elongation of the filopodia (Plate 1). This effect of EGF on the morphology was observed at all tested concentrations.

EGF increased basal progesterone secretion of the granulosa cell cultures [significant at days 6 and 10 ($p < 0.05$), days 7 and 8 ($p < 0.01$)] and, slightly, the basal estradiol secretion [significant at day 8 ($p < 0.01$), day 12 ($p < 0.05$)]. The hCG stimulated progesterone secretion was not affected by EGF, whereas, the hCG stimulated estradiol secretion was decreased by EGF [significant at days 12 and 14 ($p < 0.01$)] (Fig. 1 and 2).

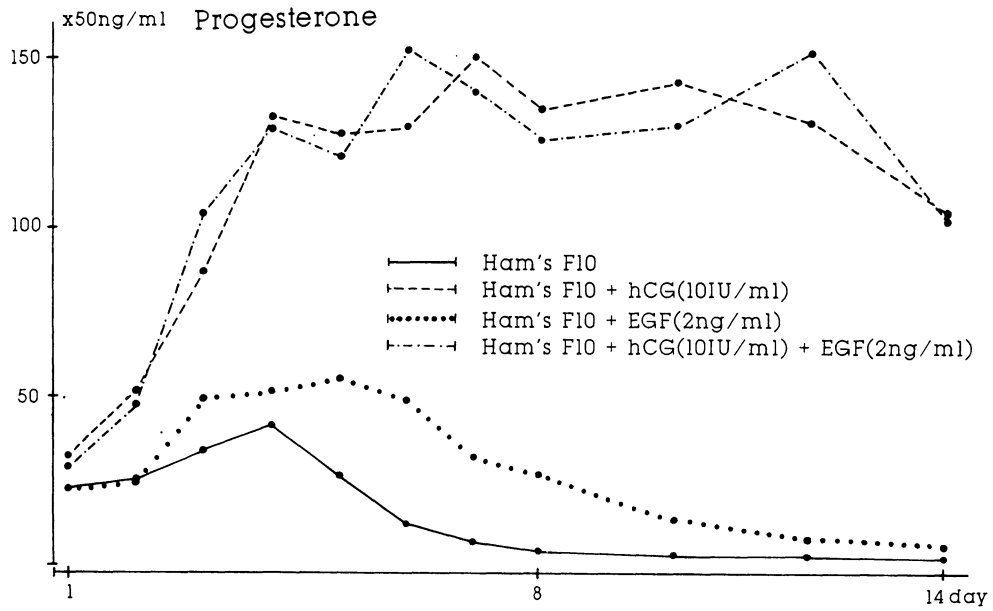

Fig. 1. — Influence of EGF (2 ng/ml) on basal and hCG stimulated progesterone secretion of human preovulatory granulosa cells in vitro (10^5 cells/ml). Each dot represents the mean of 2 wells of the granulosa cell culture of one patient.

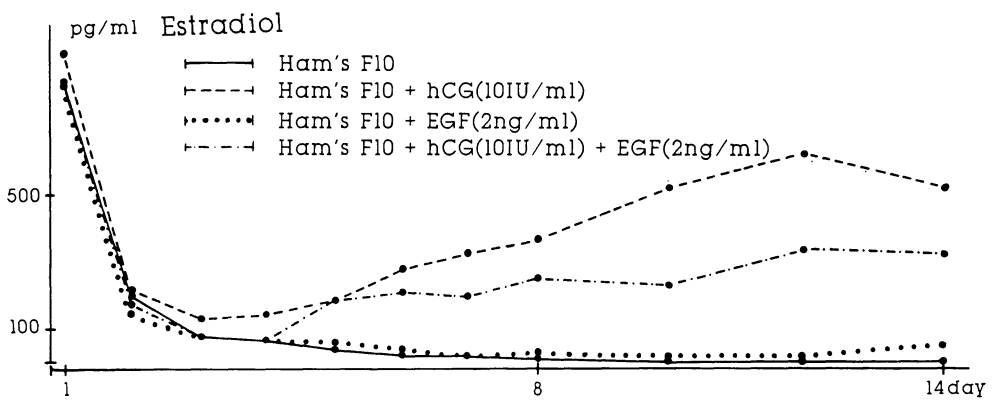

Fig. 2. — Influence of EGF (2 ng/ml) on basal and hCG stimulated estradiol secretion of human preovulatory granulosa cells in vitro (10^5 cells/ml). Each dot represents the mean of 2 wells of the granulosa cell culture of one patient.

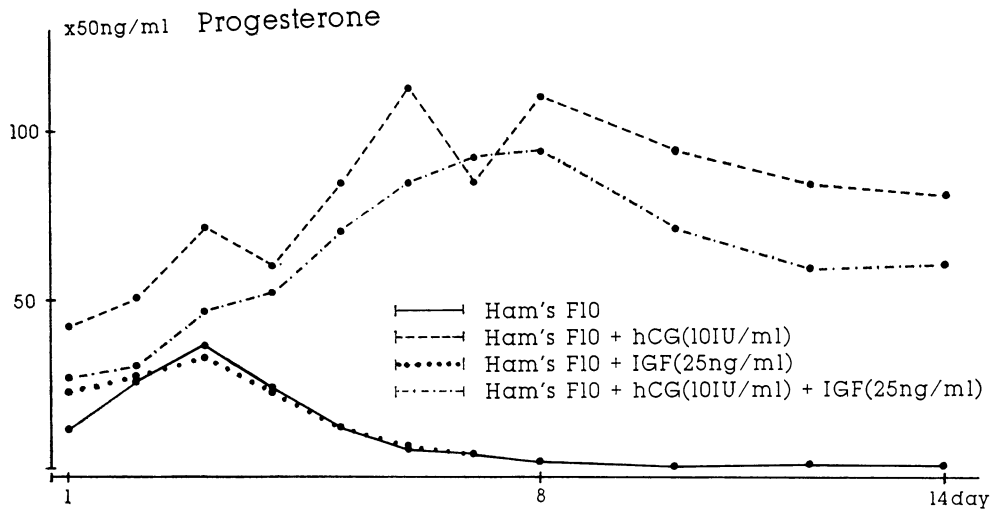

Fig. 3. — Influence of IGF-I (25 ng/ml) on basal and hCG stimulated progesterone secretion of human preovulatory granulosa cells in vitro (10^5 cells/ml). Each dot represents the mean of 2 wells of the granulosa cell culture of one patient.

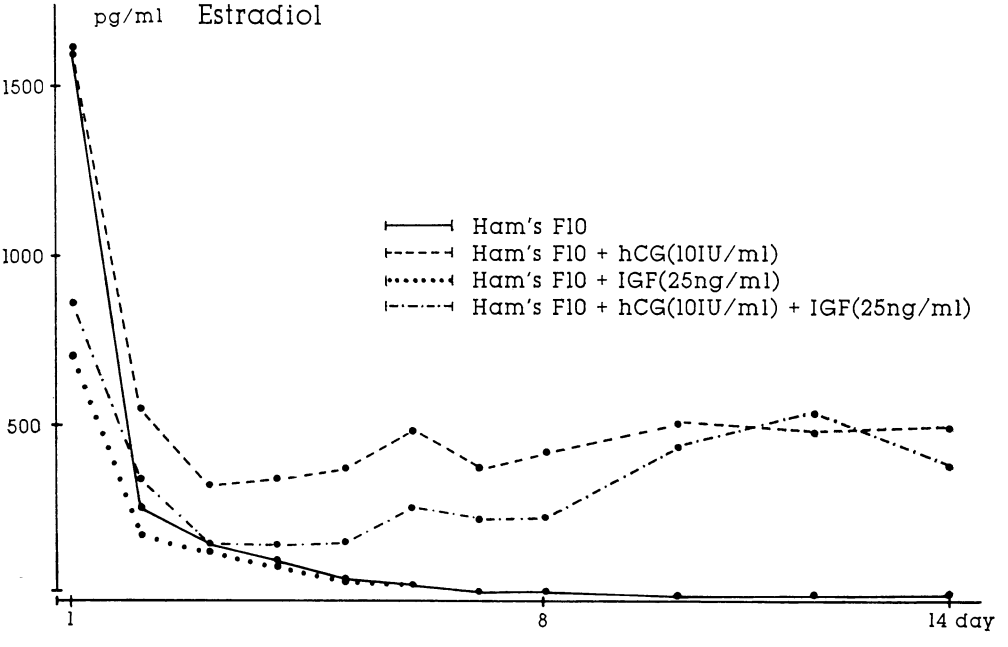

Fig. 4. — Influence of IGF-I (25 ng/ml) on basal and hCG stimulated estradiol secretion of human preovulatory granulosa cells in vitro (10^5 cells/ml). Each dot represents the mean of 2 wells of the granulosa cell culture of one patient.

At tested concentrations the effects of EGF were not dose-dependent.

Effect of IGF-I on preovulatory granulosa cells.

At 5 ng and 25 ng per ml culture medium IGF-I did not alter basal or hCG stimulated steroid secretion of preovulatory granulosa cells in vitro (Fig. 3 and 4). The morphology of preovulatory granulosa cells was not influenced by IGF-I.

Effect of IGF-I on granulosa cells of natural cycles.

The basal progesterone secretion increased during the first 2 to 5 days of culture similarly to the cultures of preovulatory granulosa cells, or declined immediately. The basal estradiol secretion dropped within 48 hours similarly to preovulatory granulosa cell cultures. The mode of steroid secretion did not depend on the age of the cycle. The progesterone secretion, but not the estradiol secretion, was slightly increased by stimulation with 10 I.U. hCG per ml culture medium. The addition of 25 ng IGF-I per ml to the culture medium did not affect the steroid secretion. In contrast, the progesterone secretion increased when 25 ng IGF-I plus 10 I.U. hCG per ml were added to the culture medium (Fig. 5 and 6). These observations were in agreement with our own results of steroid secretion of bovine granulosa cell in vitro (unpublished data). Statistical evaluation was not considered because of the lesser number of cells that could be obtained.

In the first days of culture, the granulosa cells from natural cycles showed a small cell body without filopodia or a stellate cell form similar to preovulatory granulosa cells of stimulated cycles. After three to five days in culture, the cells began to spread. HCG did not alter cell morphology. During the first days of culture, granulosa cells exposed to IGF-I had longer filopodia compared to granulosa cells in medium Ham's F10. IGF-I did not prevent cell spreading, unlike the combined influence of IFG-I and hCG (Plate 2).

DISCUSSION

The present results suggest that IGF-I and EGF influence granulosa cell function. In accordance with Tapanainen *et al.* ([11]) FGF did not alter hormone secretion or morphology of human preovulatory granulosa cells in vitro. Oury and Darbon ([2]) found an inhibitory effect of FGF on FSH induced LH receptor expression, and Channing *et al.* ([12]) found that FGF was more inhibitory to steroid secretion of granulosa cells of small follicles. These observations may explain the lack of effect of FGF on steroidogenesis of preovulatory granulosa cells, in which luteinisation and LH receptor expression have already started.

In contrast, IGF-I and EGF modulate steroid secretion of granulosa cells. The increased steroid secretion in EGF treated preovulatory granulosa cells and in IGF-I + hCG stimulated granulosa cells of natural cycles was accompained with reduced cell spreading. This relationship between cell shape and steroidogenic activity has been described earlier in studies concerning collagen cultures of granulosa cells ([13, 14]).

EGF-binding capacity was related to granulosa cell differentiation ([15]). EGF receptor expression of granulosa cells was decreased by hCG treatment ([16]), whereas EGF treatment inhibit FSH induced LH/hCG receptor expression ([9]) and FSH induced oestrogen production ([17]). In accordance with our findings of reduced hCG estradiol secretion of human preovulatory stimulated granulosa cells in vitro by EGF Endo *et al.* ([10]) reported reduced hCG induced estradiol and progesterone secretion of prefused rabbit ovaries by EGF. As they

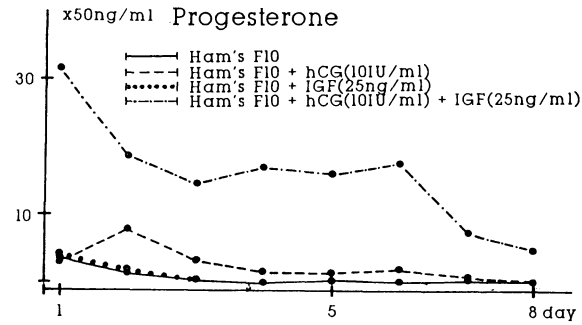

Fig. 5. — Influence of IGF-I (25 ng/ml) on basal and hCG stimulated progesterone secretion of human granulosa cells of natural cycles in vitro. Each dot represents 1 well of the granulosa cell culture of one patient.

were not able to confirm the decreased progesterone secretion in vitro Endo *et al.* ([10]) suggested that EGF inhibits the stimulatory effect of hCG by attenuating cell communications. Our results of decreased hCG stimulated estradiol secretion of granulosa cells in vitro by EGF did not support this concept. It may be speculated that the inhibition of the hCG induced estradiol secretion in our study is caused by inhibition of LH/hCG receptor expression or inhibition of aromatase activity as progesterone secretion was not affected by EGF treatment. Jones *et*

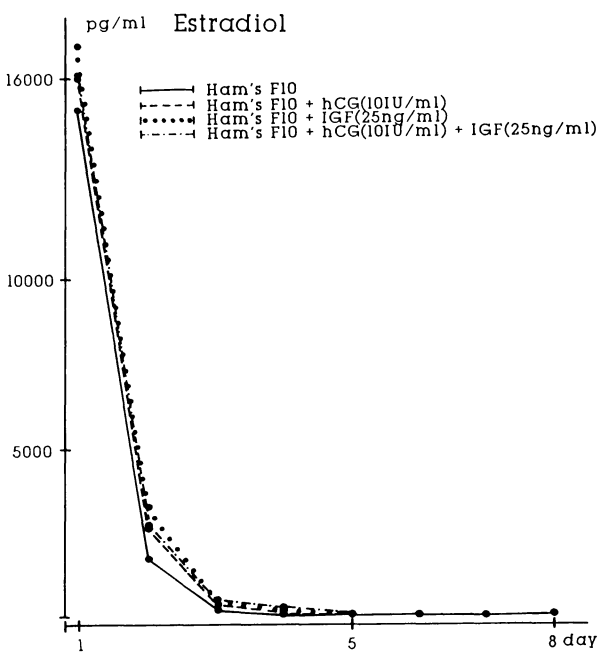

Fig. 6. — Influence of IGF-I (25 ng/ml) on basal and hCG stimulated estradiol secretion of human granulosa cells of natural cycles in vitro. Each dot represents 1 well of the granulosa cell culture of one patient.

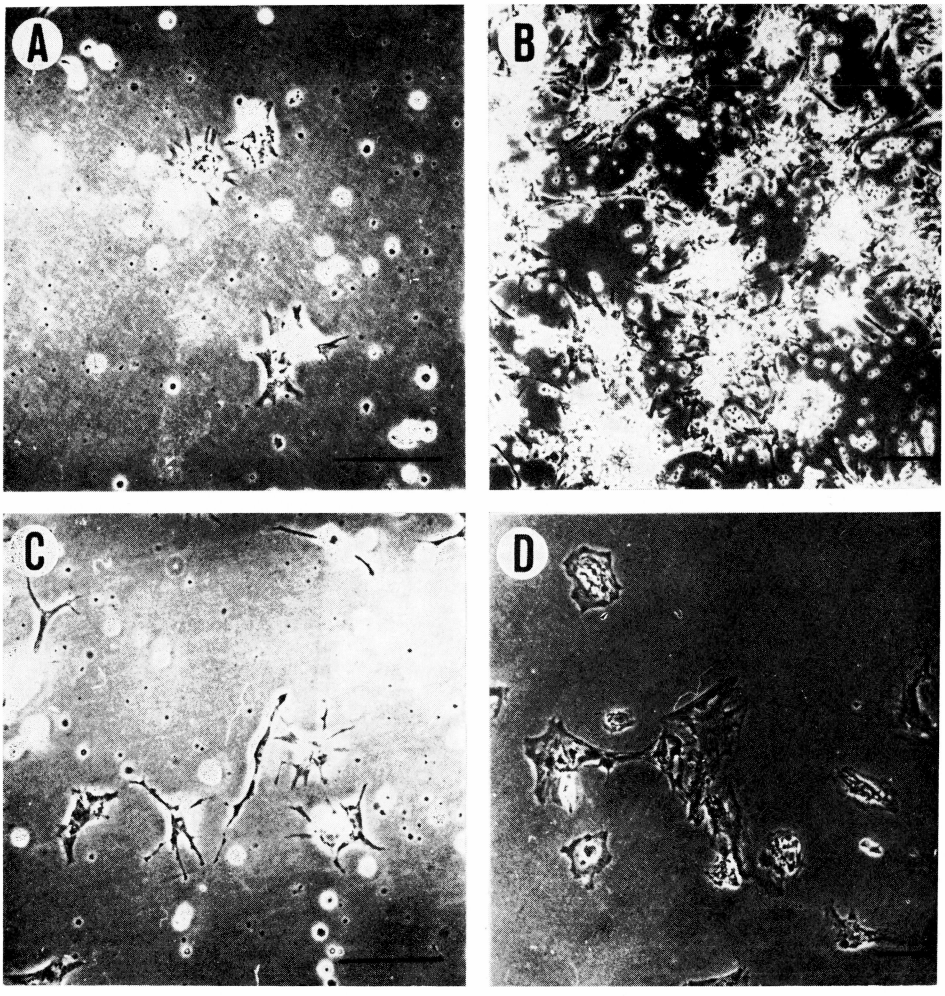

Plate 2. — Morphology of human granulosa cells of natural cycles in vitro: *a*) stellate granulosa cells yielded from a follicle at day 9 of cycle (day 1 of culture); *b*) granulosa cells yielded from a follicle at day 11 of cycle (day 1 of culture); *a, b*) the morphology was similar to the morphology of preovulatory granulosa cells in vitro; *c*) IGF-I treatment (25 ng/ml) resulted in elongation of filopodia (day 1 of culture); *d*) spreaded granulosa cells at day 8 of culture; *a, c, d*) the cells originate from the same patient (—— $\widehat{=}$ 0.1 mm).

al. ([18]) reported dose-dependent enhancement of basal progesterone but not estradiol secretion of granulosa cells by EGF. The wider dose ranges of EGF and the addition of androstenedione to culture medium may explain the differences from our results. The inhibitory effect of EGF on basal progesterone of human granulosa cells ([12]) was not confirmed by our data.

The ability of IGF-I to increase steroid secretion may be different in cultures of preovulatory granulosa cells of stimulated IVF-cycles and of granulosa cells of natural cycles. IGF-I is known to increase

FSH induced LH receptor expression ([6]), whereas FSH and LH enhance IGF-I binding capacity ([8]). Treatment with hCG or IGF-I alone did not influence the steroid secretion of granulosa cell cultures of natural cycles. This may be due to the lack of sufficient binding capacity. A potential synergistic up-regulation of LH and IGF-I receptors may explain the rise of progesterone secretion by combined treatment of cultures with IGF-I and hCG. In the preovulatory preluteinized granulosa cells of IVF-cycles, IGF-I may not be able to increase LH receptor expression further. This may explain the lack of effect of IFG-I on preovulatory granulosa cells. In contrast to our results, Bergh *et al.* ([19]) observed a stimulatory effect of IGF-I at 1 ng to 100 ng per ml on progesterone and estradiol secretion both of granulosa cells of natural cycles and stimulated IVF-cycles. These differences may be explained by the lower FCS (1%) concentrations in their experiments.

The results of our study provide evidence for the facultative role of IGF-I and EGF as an autocrine/paracrine modulator of ovarian function. EGF seemed to inhibit the stimulatory action of hCG on granulosa cells and to enhance basal steroid secretion. Hsu *et al.* ([20]) found the EGF-like activity in follicular fluid inversely correlated with the follicle size. Along with the ability of EGF to stimulate granulosa cell proliferation ([15]), and to inhibit FSH induced LH-receptor expression ([9]) the potential role of EGF in follicular development and prevention of luteinisation may be suggested. IGF-I may be considered as a modulator of ovulation induction and luteinisation as IGF-I and hCG act synergically in increasing progesterone secretion of granulosa cells of natural cycles in vitro and are known to stimulate each others receptor expression.

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