ESTABLISHMENT OF A HUMAN GRANULOSA CELL CULTURE FOR EVALUATION OF THE BIOLOGICAL ACTIVITY OF HUMAN RECOMBINANT GONADOTROPHINS

Summary of Ph.D. Thesis

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INTRODUCTION

The relative contributions of follicle stimulating hormone (FSH), luteinizing hormone (LH) and human chorionic gonadotrophin (hCG) to folliculogenesis and subsequent formation of the corpus luteum have been a subject of interest for many years. The use of human granulosa cells obtained as a by-product of in vitro fertilization (IVF) procedures, is favoured as an *in vitro* model of ovarian function, because they can be easily harvested and cultured from follicle aspirates. These cells, however, are capable of responding with an increased steroid production to all three gonadotrophins. Therefore, the interpretation of the results obtained from *in vivo* or *in vitro* studies was compromised by the impurity of the hormone preparations used in these studies. The recent availability of recombinant FSH (rFSH), recombinant LH (rLH) and recombinant hCG (rhCG) provides an opportunity to investigate the fine regulation of the ovarian function *in vivo* or to examine their *in vitro* effects on granulosa cell steroidogenesis separately.

Recombinant gonadotrophins are produced by recombinant DNA technology involving the use of a Chinese Hamster Ovary cell line, providing gonadotrophins with several advantages as compared with urine-derived preparations, such as fully controlled production process, high purity and specific activity and excellent safety profiles. These compounds have pharmacokinetic characteristics similar to those of pituitary and urine-derived gonadotrophins and have been extensively studied in clinical trials. Recombinant FSH is the most advanced in terms of clinical assessment for the induction of multifollicular development in assisted reproduction techniques (ART). Recombinant FSH, completely free from other gonadotrophin activity, has been reported to induce normal multifollicular development with a

concomitant increase in serum oestradiol concentrations in IVF patients, suggesting that, after pituitary desensitization, the residual levels of LH (0.3-1.0 mIU/ml) synergizing with locally produced IGF-I and inhibin are sufficient to supply androgen substrates for oestradiol biosynthesis. Higher LH levels during the preovulatory period may impair gametogenic events due to an overstimulation of androgen production by the theca cells. On the other hand, the lack of LH activity in hypogonadotropic women results in very low oestradiol levels during follicular stimulation by rFSH. Therefore, rLH appears to be ideal adjunct therapy to rFSH in such patients in order to obtain adequate follicular steroidogenesis, as demonstrated by oestradiol secretion, endometrial growth and luteal phase progesterone secretion. In addition, rhCG was recently used successfully to trigger final follicular maturation prior to ART after rFSH-induced follicular development. The above results provide evidence that, in the course of ovarian hyperstimulation (where the aim is to obtain as many eggs as possible for fertilization *in vitro*), the role of LH during normal follicular development is focused on the production of the androgens necessary for oestrogen biosynthesis. By comparison, in normal ovulating patients, where the aim is to achieve monovulation and subsequent conception *in vivo*, the importance of LH in the selection of a dominant follicle and in the induction of ovulation is not questioned.

Besides clinical trials, the direct effects of recombinant gonadotrophins on human ovarian steroidogenesis are also extensively studied. The influence of rFSH on human granulosa cells from patients with spontaneous and hyperstimulated cycles in short-term cultures has already been demonstrated. Recombinant FSH induced dose-related increases in oestrogen production by the granulosa cells from normal preovulatory

follicles. However, cells from preovulatory follicles after the LH surge or from gonadotrophin-stimulated cycles exhibited no stimulatory responses to rFSH. Additionally, apart from the limited information concerning the stimulatory action of rLH on progesterone production, no exact information is available on the direct effects of rLH and rhCG on granulosa cell steroidogenesis

OBJECTIVES

The aims of the present study were

1. To establish a useful culture of human granulosa cells harvested from follicular aspirates of women undergoing *in vitro* fertilization.

2. To investigate the basal oestrogen and progesterone release from the granulosa cells *in vitro*.

3. To study the granulosa cell steroid production in the presence of appropriate substrates for oestradiol (testosterone and androstenedione) and progesterone (fetal calf serum as a cholesterol source) biosynthesis in order to provide optimum culture conditions for stimulation studies.

4. To investigate the effects of recently developed recombinant human gonadotrophins (rFSH, rLH and rhCG) on ganulosa cell steroidogenesis in long-term cultures. The effects of recombinant gonadotrophins were compared by using the same batch of granulosa cells in order to balance the possible intersubject variations in gonadotrophin sensitivity.

MATERIALS AND METHODS

Human Recombinant Gonadotrophins

Human recombinant gonadotrophins (rFSH, specific activity 15,000 IU/mg; rhCG specific activity 10,000 IU/mg; rLH specific activity 14,900 IU/mg) were generously supplied by Serono.

Protocol of Ovarian Hyperstimulation and Cell Isolation

Luteinized granulosa cells were obtained from women undergoing oocyte retrieval for IVF. Reasons for infertility were tubal occlusion, reduced male factor, polycystic ovarian disease (PCOD) or unexplained infertility. The mean age of these patients was 32.9 years (range: 23 to 45 years). Gonadotrophin therapy was preceded by complete desensitization of the pituitary gland with 0.1 mg per day of triptorelin (Decapeptyl®). Multiple follicular development was achieved with pure FSH (225 IU per day; Fertinorm HP®) until follicular maturity. For ovulation induction, 10,000 IU hCG (Pregnesin®) was injected 36 h prior to ultrasound-guided transvaginal follicle aspiration. Oocytes were immediately removed from the follicular fluid and processed for fertilization. Follicular fluids aspirated from the same patients were pooled and centrifuged for 3 min at 100 g. The resulting pellets were resuspended in PBS containing 1 % FCS and washed with PBS+1% FCS. Final pellets were incubated with 20 U/ml hyaluronidase for 12 min at 37°C in a humidified atmosphere of 95% air + 5% CO_2. Cell suspensions were washed and layered on 50% Percoll and centrifuged for 10 min at 2,000 g. The granulosa cells were aspirated from the interface and subsequently washed with culture medium (M199 supplemented with 100 µg/ml gentamicin, 2 mM L-glutamine and 10% FCS). Finally, the cells were

resuspended in a small volume of culture medium and counted in a hemocytometer. Cell viability was determined by trypan blue exclusion and was found to be consistently higher than 80%. For the correlation study, aliquots were taken from individual granulosa cell preparations to determine the 24-h basal oestrogen production. For the stimulation studies, cells prepared from 2 or 3 patients on the same day were pooled to obtain a sufficient number of granulosa cells.

Cell Cultures

Granulosa cells were plated in multivell plastic dishes at a density of 100,000 viable cells/well and cultured at 37°C in a humidified atmosphere of 95% air+5% CO_2. After a 24-h incubation, the cells were attached to the plates, forming monolayer cultures. For the correlation study, the spent media from the individual cell cultures were removed and stored for oestradiol analysis and the cultures were terminated. For other studies, the media were removed, and fresh media were added to the wells. Incubation was continued uninterrupted for 48 h except for the study of the daily progesterone and oestradiol release from the unstimulated granulosa cells, where the cultures were maintained for up to 5 days with a daily medium change. After 3 days of preincubation, the media were replaced by fresh media containing 1% FCS for oestradiol stimulation studies and 2.5% FCS for progesterone stimulation studies. The cultures were maintained for an additional 6 days in the presence of near-physiological concentrations of rFSH (1-10-100 mIU/ml), rhCG (0.01-0.1-1-10 IU/ml) or rLH (0.001-0.01-0.1-1 IU/ml). The stimulation of oestradiol production was performed in the presence or absence of 10 ng/ml testosterone. For the evaluation of the influence of FCS on the basal and rhCG-stimulated progesterone production, spent media supplemented with 10% FCS were

replaced after the initial 3-day preincubation by fresh media containing different concentrations (0-10% v/v) of FCS, and the cultures were maintained for up to 9 days in the presence or absence of 0.1 IU/ml rhCG. In all cases, the media were changed every 48 h and stored at -20°C for subsequent analysis.

Steroid Assays

Progesterone and oestradiol levels were determined by competitive enzymeimmunoassay and radioimmunoassay, respectively. The specific antibodies displayed no cross-reactivity with the steroids used for the study. The average intra- and interassay coefficients of variation were 6.9 and 9.4, respectively, for progesterone and 5.1 and 7.5, respectively for oestradiol. The assay sensitivity was 2 ng/ml for progesterone and 10 pg/ml for oestradiol.

Statistical Analysis

Experiments were performed in duplicate or triplicate. All experiments were repeated at least three times to ensure reproducibility. Data are presented as means ± SEM of combined data obtained from the same type of experiments. Linear regression analysis was performed to correlate the serum oestrogen levels with the oestrogen levels produced by the granulosa cells. The statistical significance of combined data was determined by Student's t-test for paired samples. All statistical parameters were calculated with Statistical Package for Social Sciences (SPSS) 6.0 software. Values of $P<0.05$ were considered to be significant.

RESULTS AND DISCUSSION

In this study, the *in vitro* biopotency of recombinant gonadotrophins (rFSH, rhCG and rLH) was assessed on the basis of their ability to stimulate the oestradiol and progesterone secretion of luteinized human granulosa cells in long-term cultures. Prior to an evaluation of the basal and the stimulated steroid production, granulosa cells in cultures were identified by specific inhibin immunostaining.

Basal Oestrogen Secretion

During the first day of incubation, the granulosa cells produced a high basal level of oestradiol and exhibited wide variations in oestrogen secretion. The individual differences found in basal granulosa cell oestrogen production were also reflected in the serum oestradiol concentrations measured on the day of hCG administration, since a close correlation ($r=0.84$, $n=21$) was observed between the serum oestradiol concentrations and the *in vitro* basal oestrogen production of granulosa cells obtained from the same patient when assayed within the first day of culture. Thus, granulosa-lutein cells taken from patients with relatively low serum oestradiol levels (low responders to gonadotrophin stimulation) on the day of hCG injection produce less oestrogen *in vitro* as compared with cells from higher gonadotrophin responders. Since granulosa cells from woman at risk of OHHS (very high responders) were reported to secrete more oestradiol in culture than did cells from normal patients, our results support the concept that elevated serum oestrogen levels in women at risk of OHSS appear to be not only a result of the cumulative contribution from an increased number of preovulatory follicles: the individual follicles also possess an increased capacity for oestrogen production.

The basal oestrogen levels decreased to less than 50% of initial values after a 3-day preincubation and were less than 5% at the end of the culture. Thus, the granulosa cells were capable of secreting oestradiol spontaneously throughout the whole culture without androgen and gonadotrophin support. Although the basal oestrogen production gradually decreased during the culture, high aromatase activity was present in the granulosa cells as evidenced by the dose-dependent increases in oestrogen production in the presence of aromatizable androgens. The cells maintained their oestrogen-producing capacity for at least 9 days in the absence of added gonadotrophins.

Basal Progesterone Secretion

Besides the high aromatase activity, granulosa cells were able to secrete high amount of progesterone. The reason, why these cells produce both oestradiol and progesterone during the culture may be the heterogeneity of the cell population present in the follicular fluid at the time of ovum collection. The granulosa cells secrete oestrogens in response to FSH stimulation, whereas the luteal cells are responsible for progesterone biosynthesis. Large luteal cells secrete more progesterone basally than do small luteal cells, and in contrast, small luteal cells produce more progesterone in response to LH/hCG. The baseline progesterone production gradually increased during the first few days of culture, reaching its maximum on the day 3 of culture, followed by a subsequent decline, which could be partly explained by the lower FCS levels present in the culture medium. If we accept that progesterone release is a marker of luteinization, the increasing progesterone/ oestradiol ratio during the preincubation period is direct evidence that the granulosa cells after follicle aspiration tend to luteinize in the culture.

In our experiments, the granulosa cells produced significantly lower levels of progesterone in the absence of serum than in its presence. Furthermore, the stimulation of the granulosa cells by rhCG caused only moderate increases in progesterone production in the absence of serum. The addition of serum to the culture medium resulted in dose-dependent increases in both baseline and rhCG-induced progesterone production. Our results clearly demonstrate that the granulosa-lutein cells require both gonadotrophin induction and serum supplementation (cholesterol substrate) for progesterone synthesis.

Recombinant Gonadotrophin-Stimulated Oestrogen Production

For the stimutation studies, granulosa-lutein cells were preincubated for 3 days in a culture medium containing 10% FCS prior to gonadotrophin induction, to allow them to recover after *in vivo* overstimulation. In the absence of exogenous androgens, rFSH stimulated the oestrogen production of human granulosa cells in a clear, dose-dependent manner. The reduction in baseline oestrogen levels during the culture made the cells more sensitive to rFSH. In the presence of testosterone, the cells produced a considerably higher baseline level of oestradiol, which remained constant up to the end of the culture. However, under these conditions the oestrogen accumulation was not significantly augmented by the presence of rFSH, suggesting that human granulosa-lutein cells in culture for 9 days are still able to convert androgens to oestrogens without the addition of rFSH. The lack of response to rFSH in the presence of testosterone could not be explained by the direct inhibitory effect of androgens, since the non-aromatizable androgen 5α-dihydrotestosterone had no influence on either the basal or the rFSH-induced oestrogen production.

In contrast with rFSH, marked changes were noted in the ability of the granulosa cells to respond to rhCG with respect to oestradiol production during the culture. Recombinant hCG inhibited oestrogen production within days 3 and 5 of culture, being significant at doses higher than 0.01 IU/ml, whereas no effect was found during the next stimulation interval (between days 5 and 7). During the final induction period (between days 7 and 9) rhCG produced an increase in oestradiol level, with a significant stimulation occurring at a dose of 0.1 IU/ml. The rhCG-dependent rise in oestradiol level was more pronounced in the absence of androgens, in accord with previous findings.

Recombinant LH was ineffective in stimulating the oestradiol production of the granulosa cells at any dose tested in either the presence or the absence of exogenous androgens. This is in contrast with the findings of other studies, where purified LH standards were investigated. The stimulatory effect of LH described in these studies may be accounted for by an FSH contamination of the LH preparations.

Recombinant Gonadotrophin-Stimulated Progesterone Production

Until this study, the stimulatory action of FSH on granulosa-lutein cell progesterone secretion was thought to be caused by LH activity present in the FSH preparations. However, our data provide direct evidence that rFSH completely devoid of LH activity is still able to stimulate progesterone production.

In accordance with several previous observations involving the use of purified gonadotrophin preparations, rhCG enhanced the progesterone production by the granulosa cells. Maximum progesterone stimulation

occurred at a dose of 0.1 IU/ml rhCG. The amount of rhCG required to elicit the maximum progesterone responssis the dose found effective in oestradiol stimulation and also the peak serum hCG level measured after 10,000 IU hCG administration for ovulation induction.

Similarly to rFSH and rhCG, rLH augmented progesterone production in a clear, dose-dependent manner. These data confirm results obtained after purified LH stimulation. In a recent paper, the effect of rLH on progesterone production was found to be biphasic, with lower responses at higher doses. The stimulatory effect of rLH in these experiments provided evidence that the ineffectiveness of rLH in the stimulation of the granulosa cell oestrogen biosynthesis could not be explained by the lack of response of the cells to rLH.

Although the results obtained from *in vitro* experiments should not extrapolate directly to *in vivo* mechanisms, our data suggest that in human luteinized granulosa cells, rFSH is capable of stimulating both oestradiol and progesterone production, whereas rLH and rhCG alone appear to be involved only in progesterone biosynthesis.

PUBLICATIONS

I. **Földesi,I.,** Breckwoldt,M., Neulen,J. (1998) Oestradiol production by luteinized human granulosa cells: evidence of the stimulatory action of recombinant human follicle stimulating hormone. Human Reproduction, **13,** 1455-1460.

II. **Földesi,I.,** Breckwoldt,M., Neulen,J. (1999) Granulóza sejtek in vitro sejtkultúrája, mint az ovariális működés modellje: a rekombináns gonadotropinok hatásainak vizsgálata a sejtek szteroid bioszintézisére. Magyar Nőorvosok Lapja, **62,** 373-380,