Expression of Insulin-Like Growth Factor (IGF), IGF Receptor, and IGF-Binding Protein Messenger Ribonucleic Acids in Luteinized Granulosa Cells from Different Size Follicles After Controlled Ovarian Hyperstimulation

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Purpose: This study was conducted to determine the gene expression of insulin-like growth factors (IGFs), their receptors, and binding proteins (IGFBPs) in human luteinized granulosa cells collected during ovum retrieval.

Methods: cDNA probes were used to analyze the gene expression of IGF-I, IGF-II, IGF type 1 receptor (IGFR-1), IGF type 2 receptor (IGFR-2), and IGFBP-1 to -6 in luteinized granulosa cells.

Results: The levels of IGFBP-5 transcripts of the luteinized granulosa cells increased with increasing follicular fluid (FF) volume when follicles were grouped into 3 volume categories of ≤ 1.5 mL; > 1.5 mL but ≤ 4.5 mL; and > 4.5 mL (0.28 ± 0.64 vs. 0.41 ± 0.76 vs. 0.46 ± 0.92 , $p = 0.037$).

Conclusions: The increased levels of IGFBP-5 transcripts in luteinized granulosa cells found with increased follicular fluid volume after COH may favor a shift toward diminished action of IGFs in larger follicles immediately prior to ovulation.

KEY WORDS: Controlled ovarian hyperstimulation; follicle; granulosa cell; IGF; IGFBP; IGF receptor.

INTRODUCTION

Insulin-like growth factors, their receptors, their binding proteins, and binding protein proteases are important in folliculogenesis and steroidogenesis (1,2). IGFs have been shown to augment granulosa cell proliferation and steroidogenesis and inhibit apoptosis in preantral and antral follicles (3,4). The intraovarian IGF system not only may be implicated in follicular growth and atresia but, when disordered, may also be

involved in the pathophysiology of polycystic ovarian syndrome (PCOS) (5).

It has been demonstrated in human follicular fluid (FF) of the physiological menstrual cycle that dominant follicles have a greater concentration of IGF-I in FF than their cohorts (6). Varied stages of follicular development during the physiological menstrual cycle are not directly comparable with the cohort follicles of variable size that have previously undergone COH. COH exposes cohort follicles to high concentrations of follicle stimulating hormone (FSH) and ovulatory dose concentrations of human chorionic gonadotropin (hCG). Our prior study found that levels of IGF-I in FF decrease with increasing volume of FF in desensitized, COH cohort pre-ovulatory follicles (7). This finding suggests immediately prior to ovulation that there may be a shift toward diminished action of IGF-I in larger follicles

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after COH. Nevertheless, concentrations of IGFs and IGFBPs in FF may not necessarily indicate the production rates or activities within the follicles. IGF-I in FF might derive mainly from the circulation and IGFBP-I in FF might be mainly produced locally (7). In addition, IGF receptors play an important role in mediating the activities of IGFs. However, the hormonal milieu within the FF, as well as the expression of messenger ribonucleic acids (mRNA) in the IGF system remains unclear.

Expression of mRNAs encoding the IGF system may depend on the follicle size (8). It has been demonstrated physiologically that IGFBP production in the developing ovarian follicle is dependent on follicle size and is regulated by IGF-I and gonadotropins (9,10). Yet, there is paucity of data comparing the gene expression of IGFs, IGF receptors, and IGFBPs in human luteinized granulosa cells from different size follicles after COH. This study sought to determine the gene expression of IGF system and to correlate the results with IVF outcome.

MATERIALS AND METHODS

Controlled Ovarian Hyperstimulation

Patients underwent COH for ART using the so-called long protocol (11). Briefly, subcutaneous luprolide acetate (Lupron; Abbott Australasia PTY, Kurnell, New South Wales, Australia) was administered from Day 21 of the last menstrual cycle preceding the ovum retrieval. Exogenous follicle stimulating hormone (Metrodin; Serono Laboratories, Rome, Italy) administration was initiated after pituitary desensitization was achieved, as indicated by a serum estradiol (E_2) level ≤ 35 pg/mL (conversion factor to SI unit, 3.67) and the diameter of all follicles ≤ 10 mm. Daily doses of Metrodin were continued on an individual basis depending on the follicular growth. Ten thousand IU of human chorionic gonadotropin (Pregnyl; Organon, West Orange, NJ) was administered when the two largest follicles reached a mean diameter of 16 mm with consistent levels of E_2 . The ovum retrieval was scheduled 35–37 h later. Fertilization was confirmed by the presence of two pronuclei about 18 h after sperm insemination or intracytoplasmic sperm injection (ICSI).

Collection of Luteinized Granulosa Cells

Luteinized granulosa cells and oocytes were collected via transvaginal ultrasound-guidance. To

obtain uncontaminated luteinized granulosa cells, because the puncture needle was not withdrawn before entering the next follicle, only the first, clear aspirate from each ovary was used for study. In addition, because approximately 1.5 mL FF remained within the aspiration tubing system after the collection of luteinized granulosa cells and oocytes, we used the actual collected volume of FF and disregarded the estimated remaining FF.

Isolation of Granulosa Cells and Purification of RNA

Histopaque (histopaque 1077, Sigma, St. Louis, MO, USA) was used to isolate granulosa cells from follicular fluid. The total RNA of the isolated granulosa cells were extracted by the guanethidium isothiocyanate method (Trizol, Gibco BRL, Gaithersburg, MD, USA). The details of the extraction procedures were performed according to the manufacturer's instructions. The extracted RNA was quantified using a spectrophotometer (DU640, Beckman, Fullerton, CA, USA).

Semiquantitative RT-PCR

One-step RT-PCR was performed using a commercial kit (Boehringer Mannheim, Mannheim, Germany). The reaction was carried out in a final volume of 25 μ L containing 1.5 mM of $MgCl_2$, 5 mM NH_4Cl , 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.4 mM dNTPs, 4 μ M digoxigenin-conjugated dUTP, with 1U of AMV reverse transcriptase, 1U *Tfi* DNA polymerase, 200 ng of RNA and 0.4 mM of each primer. The primers used in the reactions were shown in Table I (12–17). In order to compare the PCR product in a semiquantitative way, experiments were performed to determine the optimal number of cycles that would yield lineal phase of amplification. Accompanying the amplifications of the targets, amplification of the β -actin-specific sequence was used as internal control. All of the reactions were performed in the same thermocycler (DNA thermocycler 480, Perkin Elmer, Norwalk, CT, USA). The cDNA was first synthesized at 48°C for 45 min. Then the template was denatured at 94°C for 3 min and amplified as follows: IGF-II (94°C, 1 min; 56°C, 1 min; 72°C, 1 min; 28 cycles); IGFBP-2 (94°C, 1 min; 52°C, 1 min; 72°C, 1 min; 24 cycles); IGFBP-4 (94°C, 1 min; 62°C, 1 min; 72°C, 1 min; 28 cycles); IGFBP-5 (94°C, 1 min; 54°C, 1 min; 72°C,

Table I. Primers and the Length of Product of RT-PCR^a

Primers	Length of product (bp)
IGF-II Forward: AAGTCCGAGAGGGACGTGT Reverse: AACCTGATGGAAACGTCCGT	356
IGFR-1 Forward: AACCACGAGGCTGAGAAGCT Reverse: CAGCATAATCACCAACCCTC	447
IGFR-2 Forward: TGCACGACTTGAAGACACGC Reverse: CCAAGTAGGCACCACTAAGC	339
IGFBP-2 Forward: AGGTTGCAGACAATGGCGAT Reverse: GTAGAAGAGATGACACTCGG	497
IGFBP-4 Forward: GCCACTTGCGCCCTGGGCTT Reverse: GTCAGCCAGCTGGTGGCAGTCCAGCTC	595
IGFBP-5 Forward: GGAATTCGAAGCAGTGAATAAGGACCG Reverse: ACTCAACGTTGCTGCTGTCGAAGGTGT	376
β -actin Forward: CGACGAGGCCAGAGCAAGAGA Reverse: TCCAGGGCGACGTAGCACAGCTT	504

^a Reverse transcriptase polymerase chain reaction.

1 min; 24 cycles); IGFR-1 (94°C, 1 min; 53°C, 1 min; 72°C, 1 min; 28 cycles); IGFR-2 (94°C, 1 min; 56°C, 1 min; 72°C, 1 min; 28 cycles); and β -actin (94°C, 1 min; 60°C, 1 min; 72°C, 1 min; 26 cycles). A final extension was performed for 5 min at 72°C. PCR products were resolved on a 2% agarose gel and blotted to a nylon transfer membrane (Hybond-N⁺, Amersham Life Science, Chalford, Buckinghamshire, England). The image was obtained by chemiluminescence. Briefly, the membrane was incubated in alkaline phosphatase-conjugated antidigo-xigenin antibody solution (Boehringer Mannheim, Mannheim, Germany), CSPD substrate (Boehringer Mannheim, Mannheim, Germany), and exposed to Film Medical X-ray film (Fuji photo film, Tokyo, Japan). The intensity of the PCR product bands were quantified using a densitometer (Model GS-700 Imaging Densitometer, Bio-Rad Laboratories, CA, USA). mRNA expression of IGF-I, IGFBP-1, -3, and -6 were not detectable by RT-PCR in luteinized granulosa cells in preliminary series, and were therefore excluded in later experiments. Relative levels of IGF-2, IGFR-1, IGFR-2, IGFBP-2, IGFBP-4, and IGFBP-5 signals were normalized to that of the β -actin internal control. The length of the PCR products are shown in Table I (12–17).

Statistical Analyses

All statistics were performed using the Statistical Package for the Social Sciences (SPSS-PC, version 7.0). The samples were classified into three groups according to the volume of follicular fluid. Luteinized granulosa cells aspirated from the follicle were classified into three follicular volume groups as follows: ≤ 1.5 mL, $1.5 \text{ mL} < \text{follicle} \leq 4.5 \text{ mL}$, and follicle $> 4.5 \text{ mL}$. Kruskal–Wallis test was used to compare differences in the mRNA expression of IGF-2, IGFR-1, IGFR-2, IGFBP-2, IGFBP-4, and IGFBP-5 among the three groups. Chi-square analysis was used to compare differences in oocyte fertilization rates among the three groups.

RESULTS

During the period from September 1998 to September 1999, luteinized granulosa cells were collected from 79 patients who underwent 84 ovum retrieval procedures for ART. The mean age of the patients was 32.1 ± 4.1 years (range 21–40 years). Indications for ART were endometriosis in 23 cases, male factor in 20 cases, tubal factor in 17 cases, unexplained infertility in 15 cases, and other in four cases.

A total of 144 samples met the criteria for study. Some samples were of inadequate volume for testing of all items of the IGF system. RT-PCR revealed an intense genetic expression of IGF-II, IGFR-1, IGFR-2, IGFBP-2, -4, and -5. Figure 1 shows agarose gel

Fig. 1. Results of semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) in human luteinized granulosa cells after controlled ovarian hyperstimulation. The upper band of each column represents a 504 bp β -actin-specific products, which serves as an internal control. The lower band of each column is the target products, IGF-II, IGFR-1, IGFR-2, IGFBP-2, IGFBP-4, and IGFBP-5, respectively.

Table II. mRNA Expression of IGF-II, IGFR-1, IGFR-2, IGFBP-2, -4, and -5 by RT-PCR^a of Luteinized Granulosa Cells After Controlled Ovarian Hyperstimulation

	Group 1 ^b	Group 2	Group 3	<i>p</i>
IGF-II	1.00 ± 4.00 (33)	1.48 ± 4.31 (45)	1.03 ± 1.89 (28)	0.071
IGFR-1	2.60 ± 12.15 (43)	1.86 ± 5.59 (54)	0.89 ± 1.12 (29)	1.141
IGFR-2	0.50 ± 1.34 (31)	0.82 ± 2.89 (42)	0.66 ± 1.51 (19)	0.209
IGFBP-2	0.18 ± 0.46 (30)	0.15 ± 0.39 (42)	0.20 ± 0.33 (18)	0.426
IGFBP-4	0.18 ± 0.53 (26)	0.27 ± 0.40 (32)	0.16 ± 0.27 (12)	0.486
IGFBP-5	0.28 ± 0.64 (43)	0.41 ± 0.76 (54)	0.46 ± 0.92 (32)	0.037

Note. Values are the mean ± SEM, with sample numbers in parentheses.

^a Reverse transcriptase polymerase chain reaction.

^b Group 1, follicular fluid volume ≤1.5 mL; Group 2, >1.5 mL but ≤4.5 mL; Group 3, >4.5 mL.

containing the RT-PCR products of IGF-II, IGFR-1, IGFR-2, IGFBP-2, -4, and -5. Semiquantitative comparison of the RT-PCR products was performed using β -actin transcripts as the reference. For mRNA encoding IGFBP-5, expression was significantly higher with increasing volume of FF when follicles were categorized into three groups of ≤1.5 mL; >1.5 mL but ≤4.5 mL; and >4.5 mL (0.28 ± 0.64 vs. 0.41 ± 0.76 vs. 0.46 ± 0.92 , $p = 0.037$) (Table II). For mRNA encoding IGF-II, IGFR-1, IGFR-2, IGFBP-2, and -4, no effect of size was found among the three FF volume groups, ≤1.5 mL; >1.5 mL but ≤4.5 mL; and >4.5 mL (Table II). The fertilization rate did not appear to be correlated with the volume of FF (Table III).

DISCUSSION

We investigated the genetic expression of IGFs, IGF receptors, and IGFBPs of luteinized granulosa cells obtained from different size follicles after COH for ART in order to determine whether differences exist in the IGF system among different size follicles under ovarian hyperstimulation, and whether these

differences may impact oocyte quality and embryo quality. Few studies have addressed this issue.

In this study, we could not detect mRNA expression of IGF-I, IGFBP-1, -3, and -6 in follicles which had undergone COH when this detection was attempted immediately preceding ovulation in the preliminary series. Previous studies found that follicular size affected the expression of these items, and that detection of the genetic expression depended on the methodology used (18–20). In situ hybridization could not demonstrate mRNA expression of IGF-I and IGFBP-6 in granulosa cells of unstimulated human ovaries (17,19). IGFBP-1 and -3 have been demonstrated in granulosa cells of dominant follicles by in situ hybridization, but not in granulosa cells of small antral follicles (19). RT-PCR could detect IGF-I expression in granulosa cells from 11-mm follicles, but not in 14-mm ones (16). IGFBP-1 mRNA expression was not detectable by RT-PCR in the granulosa cells of 11- and 14-mm follicles (16). In the present study, approximately 1.5 mL of FF remained within the aspirating tubing system after collection, and this portion of the fluid was disregarded in the statistical analysis. Consequently, follicles less than 11 mm were practically excluded from the present study. RT-PCR analysis has been previously reported to reveal IGFBP-6 mRNA in granulosa cells of unstimulated ovaries, but the follicular sizes were not described (16).

Follicular IGFBPs could play an important role in decreasing the bioavailability of IGFs through sequestration. Although follicular fluid levels of IGFs and IGFBPs were not assayed in the present study, the gene products appeared to colocalize with their corresponding mRNA (19). Studies of the function of IGFBP-5 on human follicular cells are lacking. Care must be executed when applying findings to other species (21), but IGFBP-5 is not necessarily implicated as an atretogenic agent (22). It has been demonstrated that mouse IGFBP-5 transcript levels were elevated in the granulosa cells of healthy primary and secondary follicles but decreased in subsequent follicular stages and in atretic follicles (22).

The increasing IGFBP-5 production in luteinized granulosa cells of larger follicles in the present study may imply a shift toward diminished action of IGFs at the time immediately preceding ovulation. This finding is consistent with and complementary to our previous finding on the concentrations of IGF-I, and IGFBP-1 in FF collected during ovum retrieval (7). In our previous study, we found decreased levels of follicular IGF-I with unaltered levels of IGFBP-1 in larger follicles which had undergone COH for ART (7). This

Table III. Oocyte Fertilization in Relation to Three Follicular Fluid Volume Groups

	Group 1 ^a	Group 2	Group 3
Fertilized	19	21	21
Unfertilized	1	5	3

^a Group 1, follicular fluid volume ≤1.5 mL; Group 2, >1.5 mL but ≤4.5 mL; Group 3, >4.5 mL.

finding, as well as the findings of the present study, also favor a shift toward diminished action of IGFs at the time of immediately prior to ovulation. The results of this study do not necessarily reflect the situation that exists in normal unstimulated follicles (6). Thecal cells, in addition to granulosa cells, also play a modulatory role in folliculogenesis and steroidogenesis. It has been shown that thecal tissue is an essential part of the IGF system in the ovary (16). Although impractical, it would be of interest to investigate mRNA expression of thecal cells and oocytes under the situation of COH.

The fertilizability of oocytes may be interpreted as an indicator of oocyte quality. Despite the different mRNA expression of IGFBP-5 in this study, FF volume was not related to oocyte fertilization when follicles were divided into the three fluid volume groups of ≤ 1.5 mL; > 1.5 mL but ≤ 4.5 mL; and > 4.5 mL. This result is complementary to our previous finding that no relationship could be identified between levels of IGF-I or IGFBP-1 in FF and oocyte fertilizability (7). These findings suggest that there is no discernable difference in the status of the maturational process according to FF volume, given that the FF volume must exceed the 1.5 mL that remained within the aspiration tubing system used in this study.

In conclusion, the increase in the mRNA expression of IGFBP-5 in luteinized granulosa cells with increasing FF volume demonstrated in this study may favor a shift toward diminished action of IGFs at the time immediately prior to ovulation after COH. This finding is consistent with our previous findings on the concentrations of IGF-I, and IGFBP-1 in FF collected during ovum retrieval (7).

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