Follicular Fluid Insulin-like Growth Factor-I and Insulin-like Growth Factor–Binding Protein-1 and -3 Vary as a Function of Ovarian Reserve and Ovarian Stimulation

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Purpose: *Follicular fluid concentrations of insulin-like growth factor (IGF)-I, IGF-II, IGF-binding protein (BP)-1, and IGFBP-3 in 57 women undergoing in vitro fertilization and embryo transfer were examined to determine whether levels reflected differences in patients' exposure to gonadotropin stimulation and a diminished ovarian reserve.*
Methods: *Preovulatory follicular fluid was obtained from both gonadotropin-stimulated and unstimulated cycles. Subjects were grouped according to normal or decreased ovarian reserve and whether or not they received gonadotropin stimulation.*
Results: *The mean follicular fluid concentrations of IGF-I and IGFBP-1 were significantly lower in the "decreased" ovarian reserve group compared with the "normal" ovarian reserve group, with no change in estradiol or IGF-II levels. This resulted in a decreased molar IGF-I: BP ratio and an increased molar IGF-II:IGFBP-1 ratio. In unstimulated cycles, mean follicular fluid concentrations of IGFs did not differ significantly compared with those in stimulated cycles, whereas concentrations of IGFBP-1 and IGFBP-3 were significantly lower, leading to higher molar ratios of the IGFs to the binding proteins.*
Conclusions: *Follicular fluid IGF and binding proteins vary as a function of ovarian reserve and gonadotropin stimulation. This may reflect either differences in oocyte quality or a suboptimal follicular fluid environment.*

KEY WORDS: follicular fluid; insulin-like growth factors; in vitro fertilization; ovarian reserve.

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INTRODUCTION

In human in vitro fertilization (IVF), lower implantation rates are found in perimenopausal patients compared with women demonstrating normal ovarian reserve. This may be due partly to poorer embryo quality and increased aneuploidy seen in older patients (1). Lower implantation rates are also typical of patients utilizing gonadotropin stimulation compared with women undergoing natural ("unstimulated")-cycle IVF (2). Finally, diminished rates may also be secondary to the direct effect of gonadotropin stimulation on either the endometrium or the oocyte (3).

We chose to characterize the preovulatory follicles of "poor responders," analyzing their composition of estradiol, insulin-like growth factors (IGFs), and binding proteins (BPs) to determine whether differences existed between women exhibiting abnormal and normal responses to gonadotropins in hope of explaining the poor outcomes typically seen in these patients. We also chose to compare preovulatory follicular fluid growth factor composition obtained from patients undergoing unstimulated IVF with those undergoing gonadotropin stimulation to determine whether there was an effect of gonadotropin stimulation on the concentration of IGFs and their BPs.

IGF-I and IGF-II are local regulators of follicular function in conjunction with gonadotropins and may be regulators of follicular development and intraovarian sex steroid production (4,5). *In vivo* IGFs are complexed with a high affinity to a family of IGFBPs. IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-4 have been identified in follicular fluid from normally cycling women (6). IGFBP-3 (150 kd) is a principle IGFBP in adult serum, and it circulates as a complex consisting of IGFBP-3, an acid-labile subunit, and IGF peptide (7). IGFBP-3 mRNA is present in granulosa cells of

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estrogen-dominant follicles, however, most of it comes from outside the follicles (8). IGFBP-1 is a growth hormone–independent (28kd) carrier of IGF-I and plays a minor role in the serum but is expressed in the granulosa cells of the dominant follicle following the luteinizing hormone (LH) surge (9). IGF-I and IGF-II are present in follicular fluid at concentrations in the range of that seen in serum. There is a greater concentration of IGF-II in dominant follicles compared with small follicles. This is consistent with immunohistochemical studies that show that IGF-II is produced locally in the preovulatory granulosa cells and may be the principal intraovarian growth factor (10,11). In addition, except for one study (12), follicular fluid IGF-II, not IGF-I levels, correlated significantly with follicular fluid diameter and estradiol levels (13). In humans, it is unclear whether IGF-I is produced locally in the ovary (10,11,14) or whether it plays a role in folliculogenesis. Patients with Laron dwarfism (15) have very low levels of IGF-I but do exhibit ovarian function, and pregnancies have been reported. However, differences in their implantation rates have not been studied. On the other hand, there is evidence that IGF-I is important in oocyte maturation, as concentrations of IGF-I are significantly higher in the follicular fluid of mature oocytes that fertilize and cleave compared with those that do not fertilize (16) or when oocytes are immature (17).

Because IGF-I and -II appear to play a critical role in each of the major steps involved in follicular function leading to ovulation, we chose to compare the local periovulatory follicular fluid environment of dominant follicles in four groups of patients. We hypothesized that the follicular fluid environment would be less optimal in patients with decreased ovarian reserve and in patients receiving gonadotropin stimulation. We also measured IGFBP-1 and -3 levels to assess the levels of free IGFs.

MATERIALS AND METHODS

The study was reviewed and approved by the Institutional Review Board of Columbia Presbyterian Medical Center, New York.

Subjects

The study group comprised women undergoing IVF-embryo transfer (ET) with decreased reproductive ovarian reserve, which we defined as patients 40 years of age or older or younger patients with either day-3 FSH levels of $\geq$15 mIU/ml or maximum estradiol levels of $\leq$500 pg/ml following controlled ovarian hyperstimulation with $\geq$225 IU FSH/day. Therefore, the study group was a mixture of older patients (60%) and patients showing a poor response to gonadotropins (40%). The control group consisted of women aged younger than 40 years, with basal FSH levels less than 15 mIU/ml and maximum estradiol levels greater than 500 pg/ml.

Patients had a history of regular ovulatory menstrual cycles as documented by midluteal progesterone levels greater than 10 ng/ml or in-phase late luteal endometrial biopsy. They also had tests demonstrating normal prolactin levels, thyroid function, and androgen levels and a normal body mass index (18–25 kg/m^2). Patients had either tubal or male-factor infertility and a normal history and physical examination and were prescribed no medication other than fertility drugs.

Protocol

Patients were not randomized. They were divided into four groups according to ovarian reserve and gonadotropin stimulation. Patients deliberately chose unstimulated cycles for either financial reasons, fear of medication, or a previous poor response to gonadotropin stimulation. Patient characteristics in the four groups are presented in Table I. Of note, two-thirds of the patients in group II were 40 years of age or older, while all patients in group IV were younger than 40 years.

Groups I and II received luteal-phase (d21) gonadotropin-releasing hormone agonist leuprolide acetate (LA; TAP Pharmaceuticals, Deerfield, IL), 1.0 mg/day. The dose of LA was decreased to 0.5 mg/day when the serum estradiol level was less than 30 pg/ml and 225 IU of human menopausal gonadotropin was begun. The medication dose was modified upward according to individual response as needed. When a minimum of two follicles greater than 18 mm and a serum estradiol level measuring greater than 500 pg/

Table I. Patient Characteristics of Groups I–IV Expressed as Mean ± Standard Error

Group	Ovarian reserve	Age (yr)	FSH (IU/L)	hMG (amp)
I (n = 24)	Normal	29.1 ± 0.9	7.5 ± 0.9	3.5 ± 0.2
II (n = 15)	Decreased	40 ± 0.7	9.2 ± 1.7	5.1 ± 0.2
III (n = 11)	Normal	31.6 ± 0.6	6.8 ± 0.8	Unstimulated
IV (n = 7)	Decreased	33.6 ± 2	19.3 ± 0.5	Unstimulated

ml was attained, the patient was given 10,000 IU of human chorionic gonadotropin (hCG; Profasi; Serono Laboratories, Inc., Randolph, MA) intramuscularly.

Groups III and IV underwent baseline ultrasound scan examinations during their first 3 days of menstruation. Serial transvaginal ultrasonography was begun on day 7 of the cycle. When follicle maturity was obtained, evidenced by a dominant follicle of greater than 16 mm and serum estradiol levels greater than 200 pg/ml, hCG was administered. Cycles demonstrating a positive urinary LH surge prior to hCG administration were canceled.

Oocyte aspiration was performed transvaginally under ultrasound-directed guidance. In patients undergoing stimulated cycles (Groups I and II) the first follicle of greater than 20 mm was aspirated and analyzed. Because the puncture needle was not withdrawn between aspiration of the follicles, only the first clear aspirate from the ovary was used for the assay to avoid blood contamination. Only clear aspirates containing one mature oocyte were used; those containing more than one egg or no eggs were not used. In patients undergoing unstimulated cycles, the aspirate from the dominant follicle was obtained. Mature oocytes were classified on the basis of morphology and the appearance of the oocyte cumulus corona complex.

Oocytes were cultured in modified Ham's F-10 medium (GIBCO, Grand Island, NY) supplemented with 6% human albumin fraction V (National Hospital Buffer) and then inseminated. Follicular fluid aspirates (1–3 ml) not contaminated with blood were spun at 2500 rpm at 4°C for 10 min using a tabletop centrifuge to remove cellular debris and were stored at −70°C. Subsequently, they were thawed and analyzed in duplicates for IGF-I, IGF-II, insulin, IGFBP-1, IGFBP-3, and estradiol. Follicular fluid was normalized to total protein levels obtained by the Bradford technique rather than follicular fluid volume.

Assays

Follicular samples were assayed in duplicate using two-site ^{125}I immunoradiometric assay (IRMA) or radiometric assay kits (Diagnostic Systems Laboratories, Webster, TX) and all were run in the same assay. Insulin was measured by radioimmunoassay with an intraassay coefficient of variation (CV) of 5.7% and an interassay CV of 7.7%. Total IGF-I was measured using a two-site coated tube IRMA following acid/alcohol extraction to separate the BPs with an intraassay CV of 5.3% and interassay CV of 8.4%. Total IGF-II was measured after separation from the BPs, obtaining an intraassay CV of 6.3% and an interassay CV of 8.9%. Total IGFBP-3 assays had an intraassay CV of 3.9% and an interassay CV of 7.6%. Total IGFBP-1 was measured by an IRMA assay recognizing equally all phosphorylated and dephosphorylated forms. The intraassay CV was 4.0% and the interassay CV was 7.5% for IGFBP-1. The intraassay CV was 5.0% and the interassay CV was 8.6% for estradiol.

Statistics

Differences between the means of the log-transformed data for each growth factor were compared between groups by means of one-way analysis of variance and the multiple-comparison test of Bonferroni. Statistical significance was defined as P less than 0.05. Linear regression analysis (the method of least squares) and the Pearson correlation coefficient was used to define the age association with follicular fluid values.

RESULTS

Follicular Fluid Data

The mean concentrations of IGF-I, IGF-II, IGFBP-1, and IGFBP-3 and estradiol in the follicular fluid of the four groups of patients are shown in Table II. There were no differences in follicular fluid IGF-II levels between groups. IGF-I levels, on the other hand, varied with ovarian reserve, decreasing with less responsiveness. In unstimulated cycles, there were similar

Table II. Follicular Fluid Levels of IGFs, IG-FBP-1, IGFBP-3, and Estradiol Expressed as Mean ± Standard Error for the Four Groups of Patients*

Group	IGF-I (ng/ml)	IGF-II (ng/ml)	IGFBP-1 (ng/ml)	IGFBP-3 (ng/ml)	Estradiol (n*M*)
I	199 ± 16	565 ± 55	176 ± 14	2712 ± 107	1461 ± 11
II	105 ± 12[a]	632 ± 50	97 ± 4[a]	2518 ± 164	1162 ± 15
III	186 ± 18	547 ± 83	102 ± 8[c]	1995 ± 155[c]	1592 ± 15
IV	73 ± 15[b]	630 ± 51	55 ± 10[d]	2223 ± 250	1239 ± 10

* Significantly different at $P < 0.05$: (a) group II vs. I; (b) group IV vs. I and II; (c) group III vs. I; (d) group IV vs. I and II.

levels of IGF-I and IGF-II. However, BP-1 and BP-3 levels were significantly lower, consistent with higher free IGFs. Mean follicular fluid estradiol levels (n*M*) were slightly higher in normal ovarian response patients but were not statistically significant (Table II). Follicular fluid levels of insulin also were not different (range, 7–10 μIU/ml; data not shown).

The molar ratios between IGF-I and IGFBP-1 and IGFBP-3 decreased with lower ovarian reserve and with gonadotropin stimulation (Fig. 1). IGF-II-to-IGFBP-1 and IGFBP-3 molar ratios also decreased with gonadotropin stimulation due to the lower levels of BPs. However, free IGF-II in the follicular fluid of patients with decreased ovarian reserve appears to be increased. Linear regression analysis demonstrated that there was a significant negative correlation between age and IGF-I (stimulated cycles; $r = -0.52$, $P < 0.05$; unstimulated cycles, $r = -0.77$, $P < 0.05$) as well as the IGF-I-to-IGFBP-1 molar ratios (stimulated cyles, $r = -0.56$, $P < 0.05$) and IGF-I-to-IGFBP-3 molar ratios (stimulated cycles, $r = -0.6$, $P < 0.05$; unstimulated cycles, $r = -0.67$, $P < 0.05$), consistent with a more rapid decrease in IGF-I than IGFBP levels.

DISCUSSION

This study uniquely examines follicular fluid growth factors and BP levels in both stimulated and unstimulated cycles. We chose to compare stimulated and unstimulated cycles because gonadal stimulation invariably effects implantation. This effect relates both to the endometrium and the oocyte. We hypothesized that follicular development may be compromised by high dosages of gonadotropins used during in vitro fertilization. We also examined the importance of ovar-

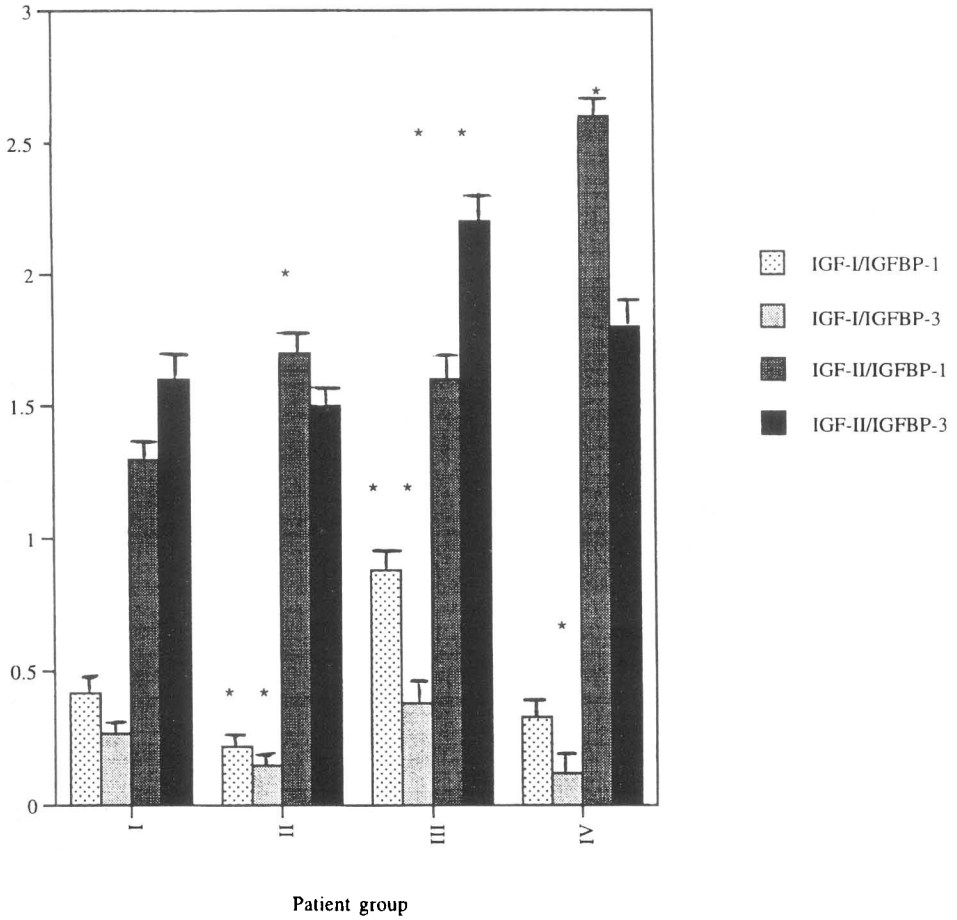

Fig. 1. Follicular fluid IGF-I:IGFBP-1, IGF-I:IGFBP-3, IGF-II:IGFBP-1, and IGF-II:IGFBP-3 molar ratios were calculated to evaluate changes in free IGF-I. * Statistically significant difference compared with the stimulated normal ovarian reserve group (control) ($P < 0.05$).

ian reserve by comparing women with decreased ovarian reproductive function defined by an age of 40 or greater or a poor response to gonadotropins or elevated FSH levels to normal controls.

Our findings are consistent with those of Klein *et al.* (18), who measured serum and preovulatory follicular fluid levels of IGF-I and IGF-II in younger and older patients and found a similar decrease in follicular fluid IGF-I levels in the older group with no change in IGF-II. However, Seifer *et al.* (19) showed follicular fluid concentrations of IGF-I and IGF-II to be significantly lower in the "low" FSH group compared with the "high" FSH group in stimulated cycles. Their high FSH group (>18 IU/L) had a mean age of 32.6 years, so they were not seeing our age-related decreased in IGF-I levels. Both studies did not measure IGFBPs.

Previously it has been shown that IGF-I and IGBP-3 serum levels decline with age (7,20,21). Likewise, we noted that older patients have lower preovulatory follicular fluid levels of IGF-I and IGFBP-3. This was anticipated because serum levels correlate well with follicular fluid levels (11,13) and local follicular fluid IGF-I is derived from serum. In addition, follicular fluid IGFBP-1 levels reflects the diminished local production by the granulosa cells, which also decrease with age and decreased ovarian reserve. Since the decline in circulating IGF-I is more rapid than in IGFBP-1, there is a decrease in the IGF-I to IGFBP-1 ratio seen with age and decreased ovarian reserve. However, younger patients with elevated FSH also have lower IGF-I levels. Although our patient number is low in group IV, there is a suggestion that the decreases we see in the IGF-I levels in patients with decreased ovarian reserve may be due to more than just age alone and may be related to the elevated FSH levels. Therefore, in this select group of patients with IGF-I deficiency in the follicular fluid, there may be a place for augmentation of intrafollicular IGF-I by either the addition of growth hormone to ovulation induction protocols or the addition of IGF-I to the culture medium *in vitro* following oocyte aspiration. Studies using adjunctive growth hormone in poor responders have been mixed in showing a benefit, despite increases in follicular fluid IGF-I after treatment (22–26). None of the studies showed a significant inprovement in pregnancy rates. However, two had a significant improvement in the fertilization rate after growth hormone (23,25) and two showed a decreased gonadotropin requirement (22,23). Therefore, one has to be more selective in candidates for growth hormone treatment by documenting IGF-I or growth hormone deficiency. Another approach may be the addition *in vitro* of IGF-I to the culture medium, as IGF-I has been shown in human granulosa cells to augment gonadotropin-stimulated progesterone production and aromatase activity and increase DNA synthesis (8).

IGF-II most likely is derived by local production, as there is no correlation between the concentration in serum and follicular fluid, and IGF II mRNA and protein have been detected in granulosa cells of large antral follicles (11). Unlike free IGF-I, follicular fluid concentrations of free IGF-II may increase with age because we have found a decrease in the levels of binding proteins without a subsequent decrease in IGF-II levels. The relative importance of IGF-I compared with IGF-II in regulating follicular development is not completely understood. If we assume that IGF-II and estradiol, not IGF-I, are important markers for normal oocyte development, then we conclude that follicular development and granulosa cell function are not deficient in patients with lessened ovarian reserve and may even be better, as reflected by lower levels of binding proteins with similar IGF-II and estradiol levels. This would be consistent with the primary defect being the oocyte.

Our patients with unstimulated cycles with decreased ovarian reserve (group IV) differed from our patients with stimulated decreased ovarian reserve (group II). Group II consisted of a heterogeneous patient population of older women and women requiring larger amounts of gonadotropin stimulation due to elevated FSH levels or poor response to stimulation. Group IV were women who were all younger than 40 with elevated FSH levels and a history of a poor response to gonadotropin stimulation. However, since group II is heterogeneous, there is no way to exclude the possibility that the observed differences are due to an age effect or due to the higher dosages of gonadotropins used in this group. FSH may be directly modulating the IGF and IGFBP levels in follicular fluid.

We have also shown that stimulation correlates with higher follicular fluid IGFBP-1 levels and, to a lesser extent, IGFBP-3 levels without altering follicular fluid IGF-I or II levels. Because the amount of free IGF-I and IGF-II is dependent on the equilibrium between the total IGF and the total IGF binding capacity in serum or follicular fluid, we speculate that the amount of free IGF is increased in follicular fluid of unstimulated cycles. However, at the cellular level both inhibitory and stimulatory effects of IGFBP-3 and IGFBP-1 on IGF-I activity have been described (27–29). To assess this further, we would need to measure directly free IGF levels and measure levels of specific proteases that lower the effects of the IGFBPs from IGFs (30).

Because ovarian follicular development is dependent on the synergistic action of gonadotropins and growth factors (31), stimulation with exogenous gonadotropins may inhibit the IGF system by increasing concentrations of follicular fluid BPs.

We conclude that reproductive aging and exogenous gonadotropin stimulation affect the follicular fluid growth factor milieu. Differences exist in the follicular fluid environment of unstimulated cycles compared with that in stimulated cycles and in cycles with a decreased ovarian reserve. To summarize, there are decreased levels of the BPs that are associated with increased bioavailable levels of IGFs in unstimulated cycles. In cycles of patients with a decreased ovarian reserve, there is a decrease in IGF-I but not IGF-II and a decrease in IGFBP-1 and 3, leading to decreased bioavailable IGF-I but increased IGF-II. Follicular fluid levels of growth factors and binding proteins in group IV distinctly differ, implying that ovarian responsiveness and reproductive function play a role independent of the patient's chronological age. There are two possibilities. The first is that the oocyte is defective, and not the follicular fluid environment, as reflected by similar IGF-II and estradiol levels with lower levels of binding proteins seen in patients with a decreased ovarian reserve. We speculate that a second possibility of poor follicular environments as reflected by lower IGF-I may contribute to differences in implantation rates seen in the patients with a decreased ovarian reserve by adversely affecting oocyte viability; the addition of growth hormone or IGF-I in these deficient patients may be a useful strategy. In addition, high doses of gonadotropins may adversely affect the oocytes by increasing levels of BPs.

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