Insulin-like growth factor binding protein-2, 28 kDa and 24 kDa insulin-like growth factor binding protein levels are decreased in fluid of dominant follicles, obtained from normal and polycystic ovaries


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Summary

In order to investigate potential changes in insulin-like growth factor binding proteins (IGFBPs) during human follicle maturation, we examined the IGFBP profiles in follicular fluid from follicles in different stages of maturation. Samples were obtained from ovaries of women with regular menstrual cycles and of subjects with cycle abnormalities and polycystic ovaries (diagnosed as polycystic ovary syndrome (PCOS)) and analyzed by Western ligand blotting. IGFBPs of 43 kDa, 37 kDa, 31 kDa, a doublet around 28 kDa and a minor band of 24 kDa were detected in follicle fluid of normal non-dominant (size < 10 mm) and atretic (androstenedione/estradiol ratio > 4) follicles of both regularly menstruating women and PCOS patients. The 43 and 37 kDa IGFBPs could be identified as IGFBP-3 and the 31 kDa IGFBP as IGFBP-2, whereas the 28 kDa IGFBP could not be identified as IGFBP-1, all by immunoblotting techniques. A dramatic decrease in IGFBP-2, the 28 kDa and 24 kDa IGFBPs was observed in follicular fluid of dominant follicles (size > 10 mm) of both regular menstruating individuals and one PCOS patient as compared with follicular fluid of normal non-dominant or atretic follicles. These observations indicate that the PCOS follicle may not be different from normal with respect to IGFBP profiles. Furthermore, these results suggest that at least one of these IGFBPs might be involved in human folliculogenesis.
Introduction

The insulin-like growth factors (IGFs) are low molecular weight peptides with both metabolic and mitogenic properties believed to play a role in ovarian function [1,2]. In the human preovulatory granulosa cell, expression of IGF-II mRNA but not IGF-I mRNA has been detected [3]. Human granulosa cells contain transcripts for both the type I and type II IGF receptor [4] and contain the type I IGF receptor on their surface [5]. In human granulosa cells, isolated from polycystic ovaries, IGF-I synergizes with follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG) in increasing 17β-estradiol (E2) production, suggesting a role for IGF-I in enhancing gonadotropin action in human folliculogenesis [6].

In serum and extracellular fluids, the IGFs are present bound to high affinity binding proteins (IGFBPs). These IGFBPs comprise a family of six structurally related proteins [7], which modulate IGF action [8]. In human follicular fluid several of these IGFBPs have been found and one such species, IGFBP-1, was localized in luteinized granulosa cells [9]. Human granulosa cells express mRNA for IGFBP-1 [10]. In addition, IGFBP-1 was reported to inhibit IGF induced proliferation of human granulosa cells, suggesting that IGFBP-1 is one of the endogenous factors regulating growth and differentiation of human granulosa cells [11]. IGFBP-2, -4 and -5 mRNA expression was found mainly in granulosa of atretic follicles and IGFBP-4 and -5 gene expression was located primarily in theca and stromal cells. Gene expression of IGFBP-3 was detected in theca of all stages tested and was selectively expressed in granulosa cells of dominant follicles [12]. IGFBP-3 has been shown to inhibit IGF-I and FSH induced estrogen production by cultured human granulosa cells [13], suggesting that this binding protein may modify FSH actions.

Since there is mounting evidence that the IGF system, complete with ligands, IGF-receptors and IGFBPs, is present in the human ovary, we decided to investigate potential changes in IGFBPs during follicle maturation. Therefore, we examined the IGFBP profiles in fluid from follicles in different stages of maturation obtained from ovaries of women with regular menstrual cycles and of PCOS patients presenting with cycle abnormalities.

Materials and Methods

Follicular fluid samples

16 women volunteered to participate in this study, which was approved by the local Ethics Review Committee. Their mean age was 33 years (range 27–44) and they all were regularly menstruating with a mean cycle length of 28 ± 2 days (mean ± S.D.). Follicular fluid samples were collected through puncture of individual follicles as described previously [14]. In addition, follicle fluid was obtained from five infertile PCOS women. Four patients presented with amenorrhea and one with severe oligomenorrhea (cycle length 72–84 days). All five fulfilled our criteria for transvaginal sonographic diagnosis of polycystic ovaries; (1) ovarian volume above 8.0 ml, (2) > 11 follicles between 2 and 10 mm in size in each ovary, and (3) increased ovarian stroma echogenicity [15]. The mean age of these patients was 28 years, two women were obese (BMI > 25 kg/m²) and hirsute, one woman was hirsute only. Serum hormone concentrations were 10.6 (range 7.9 to 12.1) IU/l for luteinizing hormone (LH), and 6.9 (range 3.9 to 8.3) IU/l for follicle stimulating hormone (FSH) estimated by immunoradiometric assays. Radioimmunoassay serum determinations for serum testosterone were 2.9 (range 1.6 to 7.2) nmol/l. All FF samples were divided into three groups (depending on their androstenedione/estradiol (AD/E₂) ratio as described by McNatty et al. [16]) and pooled per individual. Group A (normal non-dominant follicles): AD/E₂ ratio < 4 and a diameter < 10 mm, group B (atretic follicles): AD/E₂ ratio > 4 and group D (dominant follicles): AD/E₂ ratio < 4 and a size > 10 mm.
Western ligand blotting

Western ligand blots were prepared essentially as described by Hossenlopp et al. [17]. FF samples (3 μl) were applied to a 9% sodium dodecylsulfate (SDS)-polyacrylamide gel, run under non-reducing conditions, and the separated proteins were transferred onto nitrocellulose filters by electroblotting. The filters were washed with 3% NP-40 in 100 mM Tris-HCl, pH 7.5/0.9% NaCl (TBS), 3% BSA in TBS, and then incubated with 500,000 cpm [125I]IGF-II (kindly provided by Dr. S. van Buul-Offers, Wilhelmina Childrens Hospital, Utrecht The Netherlands) for 14 h at 4°C. Subsequently, filters were washed and exposed to Kodak X-Omat AR film with intensifying screen at -70°C for up to 14 days.

Immunoblotting

After autoradiography, filters were immunostained using either a monoclonal antibody against IGFBP-1 [18], a polyclonal antibody against IGFBP-2 (kindly provided by Dr. J. Schwander, Kantonspital Basel, Switzerland) or a polyclonal antibody against IGFBP-3 (kindly provided by Dr. R. Rosenfeld, Stanford University Stanford, USA) using standard procedures. Briefly, filters were incubated with 3% BSA in TBS followed by incubation with an IGFBP specific antibody. Filters were washed in 0.1% NP-40 in TBS and incubated with an alkaline-phosphatase coupled second antibody. Finally, filters were washed and stained with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Boehringer, Mannheim).

Quantification of IGFBP levels was performed by densitometric scanning of autoradiograms using an image analyzer (IBAS 2000 Zeiss Kontron, Oberkochen, Germany) and the Kontron IBAS1 version 4.4 software program supplied. Mean intensities were calculated from measurements of autoradiograms of at least four samples and statistical confidence of differences calculated using Student's t-test.

Results

Follicle fluid (FF) samples obtained from individuals with regular cycles were subjected to SDS-PAGE and Western ligand blotting using iodinated IGF-II. As shown in Fig. 1, two major bands of 43 and 37 kDa, corresponding to the glycosylated forms of IGFBP-3, were detected. In addition a band of 31 kDa, a doublet around 28 kDa and a minor band of approx. 24 kDa (corresponding to the molecular mass of IGFBP-2, IGFBP-1 and IGFBP-4, respectively) were seen (Fig. 1). Using a polyclonal antibody specific for IGFBP-3, we were able to show that the 43 and 37 kDa bands were indeed isoforms of IGFBP-3 (Fig. 2a). The 31 kDa band was identified as IGFBP-2 by immunoblotting (Fig. 2b). However, the 28 kDa IGFBP doublet did not react with a specific antibody against IGFBP-1, whereas the antibody clearly detected the positive control (IGFBP-1 in amniotic fluid), indicating that this 28 kDa IGFBP was not IGFBP-1 (Fig. 2c).

Comparison of the Western ligand blot profile of the IGFBPs in FF of follicles in different stages of development, revealed that in all dominant follicles, IGFBP-2, the 28 kDa and the 24 kDa IGFBP were strongly decreased, as compared to normal follicles.

Fig. 1. Autoradiograph of a representative Western ligand blot of equal volumes (3 μl) of follicular fluid from normal non-dominant follicles (left lane) and atretic follicles (right lane) obtained from one individual. Molecular mass markers (in kDa) are indicated by arrows.
in two samples classified as FF of atretic follicles, a decrease in IGFBP-2 and the 28 kDa and 24 kDa IGFBPs was observed (results not shown).

In FF samples obtained from five PCOS patients IGFBP profiles were also determined. When compared to FF of normal and atretic follicles of regular cycling women, no dramatic difference in IGFBP profiles could be observed (Fig. 4). One of the PCOS patients developed a dominant follicle. In FF of this follicle the same decrease in IGFBP-2, 28 kDa IGFBP and 24 kDa IGFBP was observed as seen in the dominant follicles of regular cycling individuals (Fig. 4), showing no difference in IGFBP profile between FF of follicles obtained from PCOS patients or regularly menstruating women.

In order to quantify differences in IGFBP levels, autoradiograms were analyzed by densitometric scanning. The mean relative IGFBP-2 levels in fluid of normal non-dominant (100 ± 52%) and atretic follicles (50 ± 23%, n = 6) was not significantly different (P > 0.05). Also no significant difference between the relative 24 kDa IGFBP levels could be observed (100 ± 39%, n = 6 for normal non-dominant and 69 ± 21%, n = 6 for atretic follicles). Valid quantifi-
culation of the differences in IGFBP-2, the 28 kDa IGFBP and the 24 kDa IGFBP levels between follicle fluid of normal non-dominant and dominant follicles could not be made due to the dramatic decrease in the intensities of these IGFBPs in fluid of the dominant follicles.

Discussion

Results presented show that follicle fluid of healthy follicles obtained from regularly menstruating women contain IGFBPs with a molecular mass of 43, 37, 31, 28 and 24 kDa. Using standard immunoblotting techniques, we were able to identify the 43 and 37 kDa bands as IGFBP-3 and the 31 kDa protein as IGFBP-2. The 28 kDa doublet, however, could not be identified as IGFBP-1. Recently, Cataldo and Giudice also reported the presence of a 28 and 24 kDa band in follicular fluid. The 28 kDa IGFBP was shown to be glycosylated, and upon deglycosylation this protein most likely comigrated with 24 kDa IGFBP, because no additional band was found. Since indeed a glycosylated variant of IGFBP-4 was reported [19,20], the authors suggested that the 28 kDa and 24 kDa IGFBPs found in follicular fluid were the glycosylated and non-glycosylated forms of IGFBP-4 [21]. Alternatively, the 28 kDa glycosylated IGFBP could be IGFBP-6. This IGFBP has been shown to contain a potential Asn-linked glycosylation site, but is up to now only detected in porcine follicular fluid [22]. Furthermore, no IGFBP-6 mRNA expression could be observed in any cell type of the human ovary [12]. Finally, since the 28 kDa IGFBP is a doublet, the possibility exists that this doublet represents both IGFBP-4 and IGFBP-6.

Comparison of the IGFBP profiles in follicular fluid samples of follicles in different stages of development revealed that their was no dramatic change in IGFBPs in follicular fluid of normal healthy non-dominant follicles as compared to atretic follicles. Only in two out of 12 pools of atretic follicles a decrease in IGFBP-2, the 28 kDa and 24 kDa IGFBP (presumably IGFBP-4), was observed. Furthermore, no differences in IGFBP profiles were observed between follicular fluid samples obtained from PCOS patients and regularly menstruating women. From one PCOS patient follicular fluid was obtained from a dominant follicle, showing the same decrease in IGFBP-2, the 28 kDa and 24 kDa IGFBPs as seen in regularly menstruating women, indicating that with respect to the IGFBPs, follicular fluid of PCOS patients is not different from normal. These observations are in contrast with previous findings. Cataldo and Giudice described 3-, 6- and 19-fold higher amounts of IGFBP-2, 28 kDa and 24 kDa IGFBPs, respectively, in follicular fluid of atretic compared to healthy follicles and these changes in IGFBP levels were also observed in follicular fluid obtained from three PCOS patients [21,23]. Some of this disagreement might be explained by the way atretic and healthy follicles are defined. We have classified the follicles according to AD/E2 ratios as validated previously [16], whereas E2/T was used by Cataldo and Giudice. Secondly, our group of PCOS patients is defined according to rigid clinical (obesity, hirsutism), endocrine (hyperandrogenemia) and sonographic (polycystic ovaries) criteria, as described previously [15]. Finally, the most striking differences between atretic and healthy non-dominant follicles noted [21] might be found only in a subgroup of these follicles (namely those with a E2/T ratio of 136–500).

A dramatic decrease in IGFBP-2, the 28 kDa and 24 kDa IGFBPs was observed in all samples of dominant follicles tested as compared to healthy developing non-dominant follicles. This finding seems of special interest since several studies indicate that the IGFBPs may play a role in regulating the potential of IGF to enhance gonadotropin action and subsequent follicle development [24–26]. Both IGFBP-1 and IGFBP-3 have been shown to inhibit IGF-I and FSH induced estrogen production by cultured human granulosa cells. These IGFBPs were also capable of inhibiting the IGF-I, but not FSH induced progesterone response [13], demonstrating the inhibitory effect of these IGFBPs on the IGF-I-
stimulated granulosa cell steroidogenesis. Furthermore, IGFBP-1 has been shown to inhibit the DNA amplification induced by IGFs in human granulosa-luteal cells [11]. Also, a role for the IGFBPs not directly related to their IGF binding capacity has been proposed. Bicsak et al. showed that, like an IGF-I antiserum, IGFBP-2 and -3 were capable of inhibiting steroid production by granulosa cells, but that IGFBP-3 was 2–3-fold more potent then IGFBP-2. Furthermore, the IGFBPs had no effect on the IGF-I stimulated cAMP production induced by FSH, suggesting that IGFBPs may have alternative ways of action, other than IGF sequestration [27].

In summary, it may be concluded from this study that IGFBP-2, the 28 kDa and 24 kDa IGFBPs are decreased in the dominant follicles as compared to normal healthy non-dominant and atretic follicles. A decrease of the inhibitory IGFBPs may result in an increase in free IGFs resulting in a stimulation of the mitogenic response of granulosa cells and an amplified steroidogenic response to FSH stimulation. This in turn facilitates the follicle to gain dominance. Furthermore, no differences in the IGFBP follicle fluid profile between PCOS and regularly menstruating women could be observed, which may suggest that with respect to intra-ovarian IGFBPs, PCOS patients are not different from normal.

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References


