Modulation of the insulin-like growth factor-binding proteins by follicle size in the human ovary

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Abstract

The IGFs are believed to play an important role in the regulation of steroidogenesis and follicular maturation in the human ovary. The activities of the IGFs are regulated by a family of binding proteins (IGFBPs) which are subject to a number of potential post-translational modifications. The aim of this study was to investigate both the production and modification of the IGFBPs in follicular fluid and in medium conditioned by granulosa cells and theca from individual follicles at different stages of maturation.

In follicular fluid from healthy, dominant follicles there was an increase in the amount of IGFBP-2, -3 and -4 present as lower molecular weight forms (23 kDa, 29 kDa and 16.5 kDa respectively) in comparison to that seen in atretic follicles from the same ovary. Furthermore for IGFBP-4, this fragmentation was confirmed to be attributable to the presence of a specific protease which could be inhibited not only by the addition of metal ion chelators or serine protease inhibitors, but also by the addition of other recombinant unsaturated IGFBPs, particularly IGFBP-3. IGF-I did not modulate the activity of the IGFBP-4 protease in solution but was able to prevent the inhibition seen with IGFBP-3.

Analysis of granulosa cell conditioned medium from the same series of healthy and atretic follicles revealed that IGFBP-2 and -4 were the predominant IGFBPs with no fragments seen on immunoblotting. In contrast, IGFBP-3 in conditioned medium from theca of atretic follicles was always found as an intact doublet, but was found partially fragmented (30 and 32 kDa) in medium conditioned by theca from healthy, dominant follicles with the proportion of IGFBP-3 in this lower molecular weight or fragmented doublet increasing with follicular maturation. A similar situation was also found for IGFBP-4 with the progressive increase in the amount of the 15 and 16.5 kDa fragments. IGFBP-2 was always found to be intact. Finally, IGFBP production from stroma explants was also examined. This revealed a wide variation in IGFBP pattern between different ovaries, although there was a remarkable degree of consistency between different stroma explant cultures from the same ovary. Immunoblotting for IGFBP-3 revealed that, where present, it existed as both an intact and a lower molecular weight doublet and that IGFBP-2 was again always found to be intact.

In conclusion we have demonstrated alterations in the proteolytic modification of the IGFBPs which differ in the various follicular compartments and are closely linked to the stage of follicular development.

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Introduction

The insulin-like growth factors (IGF-I and -II) are pluripotent molecules which are capable of exerting both metabolic (insulin-like) and mitogenic actions. With the use of animal models a number of investigators have demonstrated that IGFs play an important role in ovarian folliculogenesis (Adashi et al. 1985, Hsu & Hammond 1987, Monget et al. 1993). Similar actions have also been found on addition of IGF-I to cultured human granulosa cells (Erickson et al. 1989, Mason et al. 1993a), indicating an equally important role for these growth factors in man. However, analysis of the expression of IGF-I and -II gene transcripts within the ovary has revealed some major variations between species. In the rat, IGF-I expression was confined to the granulosa cells (Oliver et al. 1989) whereas in human granulosa cells IGF-II rather than IGF-I is expressed (Geisthovel et al. 1989). Furthermore in the human ovary, IGF production is not confined to the granulosa cells as we have previously demonstrated the presence of both IGF-I and -II in cultured explants of theca and of stroma (Mason et al. 1996).

Under normal physiological situations, the IGFs are bound to a family of specific binding proteins (IGFBPs), which currently consists of six members, IGFBP-1 to -6. Analysis of the IGFBPs produced by theca and stroma explants by Western ligand blotting revealed that the most intense bands were those denoting IGFBP-2 and a 24 kDa IGFBP, presumably non-glycosylated IGFBP-4.
(Mason et al. 1996). In addition, by the use of sensitive immunoradiometric assays, IGFBP-1 was found to be produced not only by theca and stromal explants cultures, but also by cultures of granulosa cells under basal conditions (Mason et al. 1993b). Other IGFBPs have also been identified in conditioned medium from granulosa cells obtained from unstimulated ovaries (that is from women undergoing natural cycles) and these include IGFBP-2, IGFBP-3 and IGFBP-4 (Cataldo et al. 1993). Using in situ hybridisation El-Roeiy et al. (1994) found that small, androgen dominant follicles expressed mRNA for IGFBP-2, -3, -4 and -5 in either the theca layer only (IGFBP-3) or in both theca and granulosa cells, as found for IGFBP-2, -4 and -5. In contrast, in dominant follicles mRNA for IGFBPs-1 to -5 was found either in the theca layer (IGFBP-2) or in granulosa cells (IGFBP-1) or in both (IGFBP-3, -4 and -5). In other cell culture systems the IGFBPs have been shown to enhance and/or inhibit IGF actions. However, to date, very little is known about their roles in the various compartments of the human ovary with the only published data demonstrating that both IGFBP-3 and -1 can inhibit not only IGF-induced production of oestradiol by granulosa cells but can partially inhibit follicle-stimulating hormone (FSH)-stimulated steroidogenesis (Mason et al. 1992).

In the last 5 years a further level of regulation of IGFBP activity has been described, namely that of proteolytic modification. Although originally described in the circulation of pregnant women (Giudice et al. 1990a, Hossenlopp et al. 1990) subsequent work has shown that modified or cleaved binding proteins are present in most, if not all, extracirculatory fluids (Lalou & Binoux 1993, Dodd et al. 1995, Xu et al. 1995, Matsumoto et al. 1996) of normal healthy adults. Furthermore, IGFBP specific protease activity has been characterised in a number of cell culture models (Conover et al. 1993, Frost et al. 1993, Angelloz-Nicoud & Binoux 1995) indicating the widespread occurrence of proteases and their potential importance as a method of modulating the actions of the IGFBPs. In accordance with this, IGFBP specific protease activity has also been found in both stroma and theca explant conditioned medium (Mason et al. 1996).

Analysis of follicular fluid has identified the presence of a number of IGFBPs (Giudice et al. 1990b, Holly et al. 1990), including IGFBP-3, -2 and -1. In addition, differences have been seen on Western ligand blotting between the IGFBP profiles of attetric and developing oestrogenic follicles from normally cycling women (Cataldo & Giudice 1992), with the former apparently having greater amounts of IGFBP-2 and the 28 and 24 kDa IGFBPs.

In this study we have analysed the IGFBP profile and the presence of modified forms of IGFBP-2, -3 and -4 in follicular fluid from follicles ranging in size from 3 to 21 mm and in conditioned medium from granulosa cells as well as from theca and stroma explants.

**Materials and Methods**

**Patient details**

Ovaries were obtained from 17 women undergoing bilateral oophorectomy for non-ovarian, benign gynaecological disease with tissue being obtained as a result of procedures which the gynaecologist deemed necessary for the clinical management of the patient. None of the patients had received any medication for stimulation or suppression of ovarian function for at least 3 months prior to surgery. Informed consent was obtained from each patient prior to surgery and approval for these studies was granted by the Kensington, Chelsea and Westminster Health Authority ethics committee. All patients were having regular menstrual cycles and cycle stage at time of surgery was random.

A serum pool from 25 healthy adult volunteers was used as normal reference material along with a pool comprising serum taken for routine investigations from 10 women in their final trimester of pregnancy.

**Tissue culture**

After dissecting the intact follicles from the stroma, the follicular fluid was aspirated. Follicular health was assessed according to follicular fluid levels of oestradiol (E2) and androstenedione (A) and by granulosa cell content, where a healthy follicle was regarded as one having an A:E2 of less than four according to the criteria of McNatty & Baird (1978). In total, follicular fluid from 38 individual follicles ranging in size from 3 to 21 mm was examined in this study. Those follicles which contained no blood clots were incised and the granulosa cells were gently removed from the surface. The granulosa cells were then incubated as previously described (Mason et al. 1990) for 48 h, but at a density of $1 \times 10^6$ viable cells per well in 1 ml serum-free Medium 199 (Gibco-BRL, Paisley, Strathclyde, Scotland) with 200 mm glutamine and the antibiotics, penicillin and streptomycin.

The theca layer of each follicle was then removed and cut into small pieces (wet weight varied from 0.4 to 2.4 mg). Pieces of stroma from the centre of each ovary were cut into pieces of between 4 and 5 mg. Each piece of tissue was washed twice before incubating for 48 h in 1 ml serum-free Medium 199. At least three pieces of theca were incubated separately from each follicle. Following the 48 h incubation the wet weight of each thecal or stromal explant was recorded, the medium collected and frozen at $-20^\circ$C until analysed.

Granulosa cells and theca explants from the individual follicles were cultured separately, allowing analysis of the IGFBPs in follicular fluid, theca and granulosa cell conditioned medium from any one follicle.
Materials

All chemicals (except where stated) were purchased from Sigma (Poole, Dorset, UK). Recombinant non-glycosylated IGFBP-3 was a gift from Dr C Maack, Celtrix (Santa Clara, CA, USA), recombinant IGFBP-2 was generously provided by Dr G Heinrich, Sandoz Pharma Ltd (Basel, Switzerland), recombinant IGFBP-1 was a gift from Dr J Cox, Synergen Inc. (CO, USA), recombinant IGFBP-4 was purchased from Austral Biologicals (San Ramon, CA, USA) and recombinant IGF-I and -II were purchased from Kabi Pharmacia (Stockholm, Sweden). IGF-I, -II, IGFBP-1, -3 and -4 were all iodinated using the Chloramine T method. The IGFBP-3 specific antibody (SCH-2/6) was raised in our laboratory against non-glycosylated IGFBP-3, the IGFBP-2 monoclonal antibody was a kind gift from Dr G Heinrich, Sandoz Pharma Ltd and the IGFBP-4 antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA).

Western ligand blotting

The IGFBP profile was visualised using a modification of the technique of Western ligand blotting as previously described by Coulson et al. (1991) following separation using a 12.5% SDS-polyacrylamide gel. 2-5 µl serum or follicular fluid were loaded per lane or the equivalent of 332-5 µl granulosa cell conditioned medium. For theca and stroma explant cultures 124 µl conditioned medium were loaded per lane with the volume added from each replicate being normalised for tissue weight.

Immunoblotting

Following ligand blotting the membranes were immunoblotted using specific antibodies for IGFBP-3 (SCH-2/6 at 1:15 000 dilution) and IGFBP-2 (EN22/386 at 1:5000 dilution) utilising the method previously described (Cwyfan Hughes et al. 1995) or for IGFBP-4 (No. 06-109 at 1:1000 dilution) using the method supplied by Upstate Biotechnology Inc. After incubating with the relevant second antibody conjugated to horseradish peroxidase, the signal was visualised using enhanced chemiluminescence (ECL, Amersham International, Amersham, Bucks, UK).

The membranes were either immunoblotted initially for IGFBP-3 and then subsequently for IGFBP-2, after neutralisation of the residual peroxidase activity by incubating for 2 h in a 3% solution of hydrogen peroxide. Alternatively they were immunoblotted for IGFBP-4 using a buffer of PBS+3% milk instead of Tris-buffered saline with 0.2% Tween 20 and 5% milk. Gels were quantified by densitometry using a Bio–Rad G5–690 Imaging densitometer.

Protease assays

IGFBP protease activity was assessed in the follicular fluid using a method based on that described by Lamson et al. (1991). Briefly, this involved incubating the sample for 24 h at 37 °C with either 30 000 c.p.m. [125I]IGFBP-1, [125I]IGFBP-3 or [125I]IGFBP-4 before separating any radiolabelled fragments on a 12.5% SDS-polyacrylamide gel for IGFBP-3 and -1 or on a 14% SDS-polyacrylamide gel for IGFBP-4.

Results

Follicular fluids

The IGFBP profiles of follicular fluid obtained from four different ovaries are shown in Fig. 1a. As previously described IGFBP-3 was the predominant binding protein in both atretic and healthy follicles. Analysis of the IGFBP-3 by immunoblotting revealed the presence of a 29 kDa band (Fig. 1b), in addition to the two intact bands, both in follicular fluid and in serum. The intensity of the band denoting this potential fragment was found to be consistently increased in the healthy, dominant follicle (53% of the total being fragmented) in comparison to the atretic follicles (32% of total IGFBP-3 fragmented) from the same ovary (Fig. 1b). An alteration in the ratio of intact to fragmented IGFBP-3 is usually associated with the induction of proteolytic activity directed against this binding protein, however incubation of follicular fluid with radiolabelled [125I]IGFBP-3 failed to result in its fragmentation irrespective of whether the follicle was healthy or atretic (Fig. 2a). Variations in the IGFBP profile were seen between healthy and atretic follicles on ligand blotting for the bands denoting IGFBP-2 and non-glycosylated IGFBP-4; with diminished intensity for both in the dominant follicles (Fig. 1a). Analysis of IGFBP-2 by immunoblotting revealed the presence of three bands in follicular fluid at 34, 25.5 and 23 kDa representing intact and two lower molecular weight forms respectively (Fig. 1c). In contrast, only two bands at 34 and 25.5 kDa are found in the serum (Fig. 1c). In the follicular fluid from any one ovary, the intensity of the 25.5 kDa band remained apparently constant with the intensity of the intact band being considerably greater in atretic follicles than in the dominant follicle. Conversely, the 23 kDa band (which to date we have not observed in any other body compartment) was found to be elevated in healthy, dominant follicles consistent with a decrease in the 34 kDa band (Fig. 1c). Immunoblotting for IGFBP-4 demonstrated the presence of three bands presumably representing a glycosylated form of IGFBP-4 at 26 kDa, non-glycosylated IGFBP-4 at 24 kDa and a fragment at 16.5 kDa (Fig. 1d). These three bands were seen both in normal serum and in follicular fluid from atretic follicles. In contrast, only two IGFBP-4 bands were...
seen in the fluid of healthy follicles; with an increase in the intensity of the band denoting fragmented IGFBP-4 to an average of 85% of the total in comparison to atretic follicles from the same ovary where the fragments only represented, on average, 22% of the total IGFBP-4 (Fig. 1d).

The apparent absence of 24 kDa IGFBP-4 in the fluid from healthy follicles was found to be attributable to the presence of proteolytic activity which cleaved \(^{[125]I}\)IGFBP-4 into two bands of equal intensity with molecular weights of 13 and 17-5 kDa (Fig. 2b, lanes 4, 6 and 7). Preliminary characterisation of this proteolytic activity confirmed that the enzyme or enzymes responsible were metallo-/serine proteases with an identical fragmentation pattern and inhibitor profile to that seen in serum from the final trimester of pregnancy (data not shown). Furthermore, the activity in follicular fluid could be greatly reduced in a dose-dependent manner by the addition of recombinant IGFBPs, those assessed being IGFBP-1, -2 and non-glycosylated IGFBP-3 (Fig. 3a, lanes 6, 7, 9 and 10); for example, in the absence of IGFBP-3 the fragmentation was 28% of the total, 22% with 0-1 m IGFBP-3, 14 and 7% with 0-5 m and 1 m IGFBP-3 respectively. In addition this inhibition could be reversed by preincubating the recombinant IGFBP with an equimolar amount of IGF-1 (Fig. 3a, lane 8). IGF-1 alone was found not to modulate the activity of this protease in solution (Fig. 3a, lane 5), neither was the binding protein acting as an alternative substrate for the protease as shown by the failure to fragment IGFBP-1 (Fig. 3b, lane 3) or IGFBP-3 (Fig. 2a). Interestingly, IGFBP-3 was found to be a better inhibitor of this protease...
activity than either of the other two binding proteins. Approximately 10-fold more IGFBP-2 or -1 was required to achieve the same level of inhibition (data not shown).

**Granulosa cells**

Granulosa cell conditioned medium from healthy, dominant follicles and small, atretic follicles were examined from two ovaries (ovary 2 and 3) whose follicular fluids are shown in Fig. 1; the IGFBP profile is shown in Fig. 4a. The predominant binding proteins appeared to be IGFBP-2 in the medium from atretic follicles (Fig. 4, lane 2), whereas in granulosa cell medium from dominant follicles there were two bands denoting IGFBP-2 and a 26 kDa band, presumably glycosylated IGFBP-4 (Fig. 4, lane 3). These findings were confirmed by immunoblotting (Fig. 4b and c) with no demonstrable modifications of either binding protein.

**Theca explants**

The IGFBP profile from theca explant conditioned medium obtained from four follicles (two different ovaries)
is shown in Fig. 5a. The most intense band on Western ligand blotting, irrespective of follicle size, appeared to be non-glycosylated IGFBP-4. Immunoblotting for IGFBP-4 revealed that although the majority was intact there were two bands (possibly fragments) present at 16-5 and 15 kDa whose intensity increased from 2-5 to 38% as the follicle matured (Fig. 5d). Examination of the endogenous IGFBP-3 by immunoblotting also demonstrated maturation changes in that IGFBP-3 present in small, atretic follicles existed predominantly as an intact doublet (41-5 and 44 kDa) whereas in the conditioned medium from theca of dominant follicles a doublet of molecular weights 30 and 32 kDa was seen as well as the intact IGFBP-3 (Fig. 5b). It was of interest to note that, as with IGFBP-4, the proportion of the IGFBP-3 found in these lower molecular weight forms increased from 2 to 78% as the size of the dominant follicle increased (Fig. 5b). Furthermore, the size of the lower molecular weight forms seen on immunoblotting for IGFBP-3 and -4 did not resemble that found in the follicular fluid obtained from the same follicles. This would be consistent with there being differences in the modulation of these IGFBPs in the theca explants in comparison to those found in the follicular fluid.

In contrast, when the endogenous IGFBP-2 was analysed, only the intact 34 kDa binding protein was found both in atretic and healthy follicles (Fig. 5c).

**Stroma explants**

Analysis of stroma conditioned medium revealed a considerable variation of IGFBP profiles between different ovaries (Fig. 6), although the profile obtained from any one ovary appeared to be consistent irrespective of the original location of the explant within that ovary. As with the conditioned medium from the theca explants, only intact IGFBP-2 was found on immunoblotting (Fig. 6) whereas the IGFBP-3 was again found as both an intact doublet and as a lower molecular weight doublet with identical molecular weights to those found for theca (Fig. 6).

![Figure 6](image-url)

**Figure 6 Autoradiograph of the IGFBP pattern of 2.5 µl of normal adult serum (NAS) and medium conditioned by theca explants.**
**Discussion**

In this study we have examined the IGFBP pattern of the various cellular components of individual follicles from human ovaries. Analysis of follicular fluid from healthy, dominant follicles and atretic follicles demonstrated pronounced variations in IGFBP patterns. There was an apparent increase in IGFBP-2 and -4 in the fluid from atretic follicles, as has been previously reported (Calaldeo & Giudice 1992). Closer examination of the endogenous binding proteins in follicular fluid from dominant follicles revealed that the majority of the IGFBP-2 and -4 present was as lower molecular weight or fragmented forms which were not visible by ligand blotting. In follicular fluid the IGFBP-2 present is denoted by three bands; the smallest band at 23 kDa, which has, to date, only been found in the ovary, represents the major form of this binding protein in the fluid of dominant follicles. Analysis of the other components revealed only intact IGFBP-2 in conditioned medium from granulosa cells, theca and stroma explants, while in the circulation both the intact and the 25·5 kDa band were seen. The finding of lower molecular weight forms of endogenous IGFBP-2 only in follicular fluid indicates the presence of a specific mechanism for modulating this binding protein which is not seen in any other compartment of the ovary, nor is it the same as that found in the circulation. The exact modulation undergone by IGFBP-2 to produce these two lower molecular weight bands is not currently known; however it is possible to speculate that they arise due to the activity of a specific protease, as similar activity has been demonstrated by Besnard et al. (1996) in ovine follicular fluid.

In contrast, IGFBP-3 in the follicular fluid exhibited a similar profile on immunoblotting as that seen in the circulation, with an increase in the intensity of the 29 kDa fragment band in fluid from dominant follicles although no IGFBP-3 proteolytic activity could be detected. This led us to hypothesise that the 29 kDa band in follicular fluid was derived from the circulation, the increase seen in dominant follicles reflecting the increase in vascularity of the theca layer at this time. In the theca and stroma explants a completely different modification of the endogenous IGFBP-3 was found with the appearance of a lower molecular weight doublet as well as the intact doublet on immunoblotting. Analysis of follicles at various stages of maturation revealed that this lower molecular weight doublet was only present in theca from dominant follicles with the proportion of the IGFBP-3 appearing in this doublet increasing with the size of the follicles.

On immunoblotting for IGFBP-4, three bands were seen both in follicular fluid and in serum: a 26 kDa presumably glycosylated variant, a 24 kDa non-glycosylated form and a 16·5 kDa fragment. In the circulation and in fluid from atretic follicles, the 24 kDa form predominated whereas in follicular fluid of dominant follicles the majority of the IGFBP-4 present was fragmented, which would be consistent with the presence of specific proteolytic activity directed against this binding protein. It was of interest to note that the 26 kDa glycosylated IGFBP-4 in these samples appeared to be more resistant to proteolysis. Detectable IGFBP-4 protease activity was found in the follicular fluid obtained from the dominant follicles which produced the same molecular weight fragments as those seen following incubation with late pregnancy serum. Preliminary characterisation by Chandrasekher et al. (1995) demonstrated that this enzyme activity was due to a metallo-/serine protease. Not only have we confirmed this but we have described a potential novel regulator of this proteolytic activity. In this paper we have provided the first evidence that other IGFBPs can influence the IGFBP-4 protease activity found in follicular fluid. The addition of unsaturated recombinant IGFBP-1, -2 or -3 is able to dramatically reduce the activity of this protease. IGFBP-3 was a more potent inhibitor than either IGFBP-2 or -1 and this inhibition could be completely reversed by preincubating the IGFBP-3 with an equimolar amount of IGF-I. We have also shown that the IGFBPs were acting as specific inhibitors of this IGFBP-4 protease activity and were not merely acting as alternative substrates for the enzyme.

From these results we can hypothesise that in atretic follicles, where the IGF concentrations are low in the follicular fluid, the majority of the IGFBP-3 is unsaturated and can therefore block the IGFBP-4 protease. This would then result in IGFBP-4 being able to inhibit IGF activity. In contrast, in the healthy, dominant follicle the increased concentration of IGF (including IGF-II produced by the granulosa cells) in the fluid would result in the majority of the IGFBP-3 being saturated with IGF and thus unable to suppress the IGFBP-4 protease activity. Once cleaved the IGFBP-4 has a lower affinity for IGF-I and no longer inhibits IGF activity (Conover et al. 1993), making the latter more available to the cell receptors allowing the IGFs to push the follicle into final maturation.

The source of this protease was investigated by analysing endogenous IGFBP-4 from the theca and granulosa cells from healthy, dominant follicles and atretic follicles. Although granulosa cells have been proposed as the site of production of this protease (Chandrasekher et al. 1995) we found no evidence in granulosa cell conditioned medium of any fragmentation or lower molecular weight forms of IGFBP-4. In contrast, in the theca conditioned medium the majority of the IGFBP-4 was present in the 24 kDa non-glycosylated form along with two possibly fragmented lower molecular weight forms of 16·5 and 15 kDa which appeared in theca from dominant follicles. These results imply that neither the granulosa cells nor the theca cell layer are the site of production of the IGFBP-4 protease found in follicular fluid, but further studies are needed using radiolabelled IGFBP-4 as the substrate to confirm this finding.
In summary we have described a novel regulator of the IGFBP-4 peptidase found in follicular fluid of dominant follicles. Unsaturation binding proteins, particularly IGFBP-3, were found to inhibit this peptidolytic activity and peptidosecretion of an equimolar amount of IGFBP-4 was able to reverse the inhibition. Furthermore, we have shown that under basal conditions in the human ovary there are changes in the IGFBP pattern which appear to be associated with follicle maturation. Such co-ordinated changes within the different compartments of the ovary must indicate that the IGF system plays a sophisticated role in the regulation and integration of follicular development and maturation.

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