Development, validation and application of an ultra-sensitive two-site enzyme immunoassay for human follistatin

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Abstract

Recent studies have found follistatin to be an important regulator of activin bioactivity. Whilst a number of assay formats have been described, all are of limited sensitivity and require the use of isotopes. Many use polyclonal antibodies. Furthermore, a wide range of follistatin preparations have been used as standards, complicating inter-laboratory comparison.

We now describe an ultra-sensitive two-site enzyme immunoassay using a pair of mouse monoclonal antibodies raised against follistatin 288. The presence of sodium deoxycholate and Tween 20 in the diluent gave results for total (free and activin-dissociated) follistatin. The assay had a detection limit of <19 pg/ml and recovery of spiked follistatin 288 from amniotic fluid, serum, seminal plasma, human follicular fluid and granulosa cell conditioned medium averaged 100·7 ± 7·5%, 89·1 ± 5·5%, 98 ± 4·9%, 96 ± 7·2% and 123·9 ± 11% respectively. The intra- and interplate coefficients of variation were <5%. An excess of activin-A (50 ng/ml) prior to assay did not affect follistatin recovery. Inhibin-A, inhibin-B, activin-A, activin-B and activin-AB had minimal cross-reactivity (<0·3%). However, follistatin 315 had a significant cross-reaction (9·9%).

Serially diluted human samples gave dose–response curves parallel to the standard. Pooled human follicular fluid contained high concentrations of follistatin (~242 ng/ml). Follistatin was also found in maternal serum during pregnancy (first trimester ~0·8 ng/ml, third trimester ~2·8 ng/ml), normal male serum (~0·45 ng/ml), amniotic fluid (sixteen week ~3·63 ng/ml, term ~0·89 ng/ml), seminal plasma (2·4–30 ng/ml) and human granulosa cell conditioned media (~0·44 ng/ml). Serial serum samples taken throughout the menstrual cycle of ten women showed fluctuating follistatin concentrations (~0·62 ng/ml) with no apparent relationship to the stage of the cycle. Interestingly, pooled serum from postmenopausal women appeared to have higher follistatin levels than any of the normal women (~1·4 ng/ml).

The possible presence in certain samples of mixtures of follistatin isoforms with different immunoreactivities poses major problems of interpretation in this and all other current follistatin immunoassays. Further work is needed to identify the major immunoreactive forms in different tissues and fluids. Nevertheless, the new assay has a number of advantages over previous assays and should prove a useful tool for various clinical and physiological studies.

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Introduction

Follistatin is a monomeric glycosylated polypeptide chain which was initially identified in and isolated from both bovine and porcine follicular fluids on the basis of its inhibition of pituitary follicle-stimulating hormone (FSH) secretion (Robertson *et al.* 1987, Ueno *et al.* 1987, Ying *et al.* 1987). It is well documented that follistatin exerts its inhibitory effect on FSH secretion by neutralizing activin bioactivity (Nakamura *et al.* 1990, Kogawa *et al.* 1991, Shimonaka *et al.* 1991, de Winter *et al.* 1996). There are two main forms of mature mammalian follistatin (FS) which occur as a result of alternative modes of precursor mRNA splicing, giving core proteins of 315 amino acids and the carboxy-truncated variant of 288 amino acids (FS315 and FS288) (Shimasaki *et al.* 1988, Michel *et al.* 1990, Inouye *et al.* 1991). Further variations in the molecular weight of mature follistatin occur as a result of varying degrees of glycosylation (Inouye *et al.* 1991, Sugino *et al.* 1993).

Previous immunoassays for follistatin have either been radioimmunoassay (RIA) or immunoradiometric assay (IRMA) formats (Table 1). The most sensitive of these assays has a detection limit of 500 pg/ml follistatin. This is two orders of magnitude less sensitive than the inhibin enzyme-linked immunosorbent assays (ELISAs) from our laboratory (Groome *et al.* 1994, 1995, 1996). These previous follistatin assays have other disadvantages, including

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Table 1 Characteristics of previous immunoassays

	Assay format	Sensitivity	Reference	Antibodies used	Standard used
Specific for total or free follistan					
Total	IRMA	0·5 ng/ml	Wakatsuki *et al.* (1996)	Mouse monoclonal, rabbit polyclonal	rh-FS315
Free	IRMA	0·5 ng/ml	Wang *et al.* (1996)	Mouse monoclonal	rh-FS288
Total	RIA	2·5 ng/ml	Gilfillan & Robertson (1994)	Rabbit polyclonal	bFS 35 kDa
Total	RIA	4·0 ng/ml	Khoury *et al.* (1995)	Mouse polyclonal	rh-FS288
Total	RIA	1·6 ng/ml	Klein *et al.* (1993)	Rabbit polyclonal	bFS 35 kDa
Total	RIA	0·92 ng/ml	Sugawara *et al.* (1990)	Rabbit polyclonal	pFS 32, 35, 39 kDa mixture
Total	ELISA	0·019 ng/ml	Present study	Mouse monoclonal	rh-FS288

the use of hazardous short-lived isotopic reagents. All except Wang *et al.* (1996) use polyclonal antisera. By contrast, previous immunoassays developed in this laboratory for inhibin-A, inhibin-B, inhibin Pro-αC, activin-A and activin-AB all use exclusively monoclonal antibodies, a non-isotopic detection system and are highly sensitive (Groome *et al.* 1994, 1995, 1996, Knight *et al.* 1996, Evans *et al.* 1997).

The aim of the present work was to develop a similar ELISA for total follistatin. Use is made of a dissociating solution (Poncelet & Francimont 1994, McFarlane *et al.* 1996) to disrupt activin/follistatin complexes. The validated assay appears to be a suitable tool for physiological and clinical studies. In order that our collaborators using the assay have a reference on the assay validation to cite in their work, we present here these studies.

Materials and Methods

Antibody production

Female Balb/c mice were immunized subcutaneously with 20 μg recombinant human (rh)-FS288 (a gift from the National Hormone and Pituitary Program and the National Institute of Diabetes and Digestive, and Kidney Diseases, Bethesda, MD, USA) in an emulsion with Freund's complete adjuvant. The immunization was repeated on two further occasions at monthly intervals in Freund's incomplete adjuvant, before finally boosting intravenously with rh-FS288 (total 100 μg in saline). The spleen was removed and the splenocytes were fused to Sp2/0 myeloma cells using polyethylene glycol following a standard fusion protocol (Galfre & Milstein 1981). Hybridoma supernatants were screened on a 96-well plate coated with rh-FS288 (0·2 μg/ml in 0·2 M sodium bicarbonate buffer, pH 9·4) following a screening protocol described elsewhere (Groome *et al.* 1995). Positive clones were expanded and recloned in methyl cellulose (McCullough & Spier 1990). The supernatants from these single clones were titrated against rh-FS288 (0·2 μg/ml) under a standard ELISA protocol (Groome *et al.* 1995). On the basis of these experiments two clones (29/9 and 17/2) were selected and isotyped with a commercial kit (Sigma Chemical Co., Poole, Dorset, UK). Both clones were found to secrete immunoglobulin G1 (IgG1) antibodies, and were grown to produce ascitic fluid in pristane-primed BALB/c mice (Harlow & Lane 1988). Purification of IgG was carried out using protein-G affinity chromatography (Prosep-G; Bioprocessing, Consett, Co. Durham, UK).

29/9-Coated 96-well plates

29/9 Monoclonal antibody, diluted in 0·2 M sodium bicarbonate buffer, pH 9·4, was coated by simple adsorption onto 96-well ELISA plates (Life Technologies Ltd, Paisley, Renfrewshire, UK) overnight at room temperature (10 μg/ml). The following day the plates were banged to dryness on paper towelling, then 100 μl dry coat reagent/well (Bionostics, Wyboston, Bedfordshire, UK) were added to the plates. After 1 h incubation at room temperature the plates were banged to dryness and stored in a sealed box.

Sample/standard preparation

All samples used in this study were obtained from patients who had given informed consent for collection approved by the appropriate ethical committee. Menstrual cycle serum samples were taken from normal women who had given informed consent for blood collection approved by the Oxfordshire Health Authority. The standard material used in the assay was rh-FS288. Both standards and samples were diluted in dissociating solution (84 mmol sodium deoxycholate, 3·4% (v/v) Tween 20, 1% (w/v) BSA, 5% (v/v) mouse serum in PBS). Standards were prepared by serially diluting the stock rh-FS288 to give a high standard of 2500 pg/ml and a low standard of 19·53 pg/ml.

ELISA for total follistatin

Duplicate 50 μl amounts of standard/sample were added to wells on the plate, which was then sealed and incubated

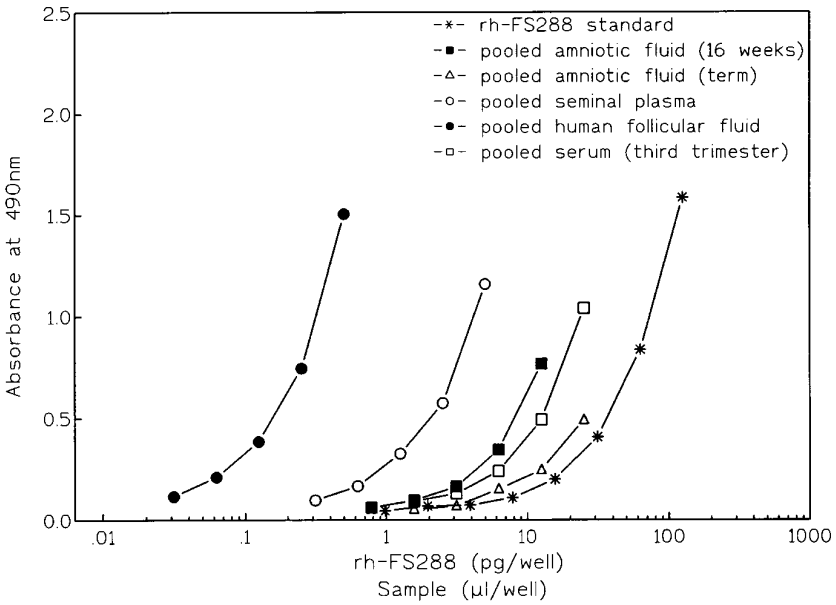

Figure 1 Dose–response curves for various human biological fluid samples containing follistatin using the optimized ELISA procedure.

overnight stationary at room temperature in a sealed moist box. The following day, the plate was washed and to each well was added 50 µl of approximately 1 µg/ml solution of the Fab fragment of clone 17/2, which had previously been coupled to alkaline phosphatase by heterobifunctional chemistry as described previously (Ishikawa *et al.* 1983). This was diluted in Tris conjugate buffer: 1% (w/v) BSA in 25 mM Tris–HCl pH 7·5 containing 0·15 M NaCl and 0·5% (v/v) Tween 20. The optimal concentration of this material differed from batch to batch and was determined empirically. After 2 h incubation in a moist box at room temperature, the plate was washed thoroughly, and banged to dryness on paper towelling. Alkaline phosphatase substrate (50 µl) was added to each well (ELISA Amplification System; Life Technologies Ltd) and the plate incubated for 2 h stationary at room temperature. Amplifier solution (50 µl; from the above kit) was then added to each well and the ensuing chromogenic reaction was stopped by adding 0·4 M HCl (50 µl/well) once colour began to develop in the zero analyte wells and the top standard had an absorbance of approximately 1·8. The well absorbances were read at 490 nm with a reference wavelength set at 620 nm using a microplate reader (EL340; Bio-Tek Instruments, Winsooki, VT, USA). Data were interpreted using Kineticalc EIA software (Bio-Tek Instruments).

Statistical analysis

To determine whether dose–response relationships of serially diluted standard and test samples were identical (parallel), the slope values (± 95% confidence intervals) of log-log transformed data for each response curve were compared by linear regression. The curves were deemed to be parallel if the slopes (± 95% confidence intervals) were found to overlap.

Results

Validation studies

Specificity The cross-reactivities of inhibin-A, inhibin-B, activin-A, activin-B, -activin-AB and FS315 were all

Table 2 Concentrations of follistatin (ng/ml except where stated otherwise) in various human biological fluid samples. Values are means ± S.D.

	Follistatin (ng/ml)
Subject group (*n*)	
First trimester serum (4)	0·847 ± 0·26
Third trimester serum (7)	2·80 ± 0·93
Normal menstrual cycle serum (10)	0·627 ± 0·196
Normal male serum (3)	0·450 ± 0·16
Week 16 amniotic fluid (5)	3·63 ± 1·50
Term amniotic fluid (7)	0·890 ± 0·32
Seminal plasma (4)	12·89 ± 9·9
Human granulosa cell cultured media (13)	0·44 ± 0·33
Pooled postmenopausal serum	1·42
Pooled human follicular fluid	252
Placental homogenates (ng/g) (3)	3·11 ± 0·4

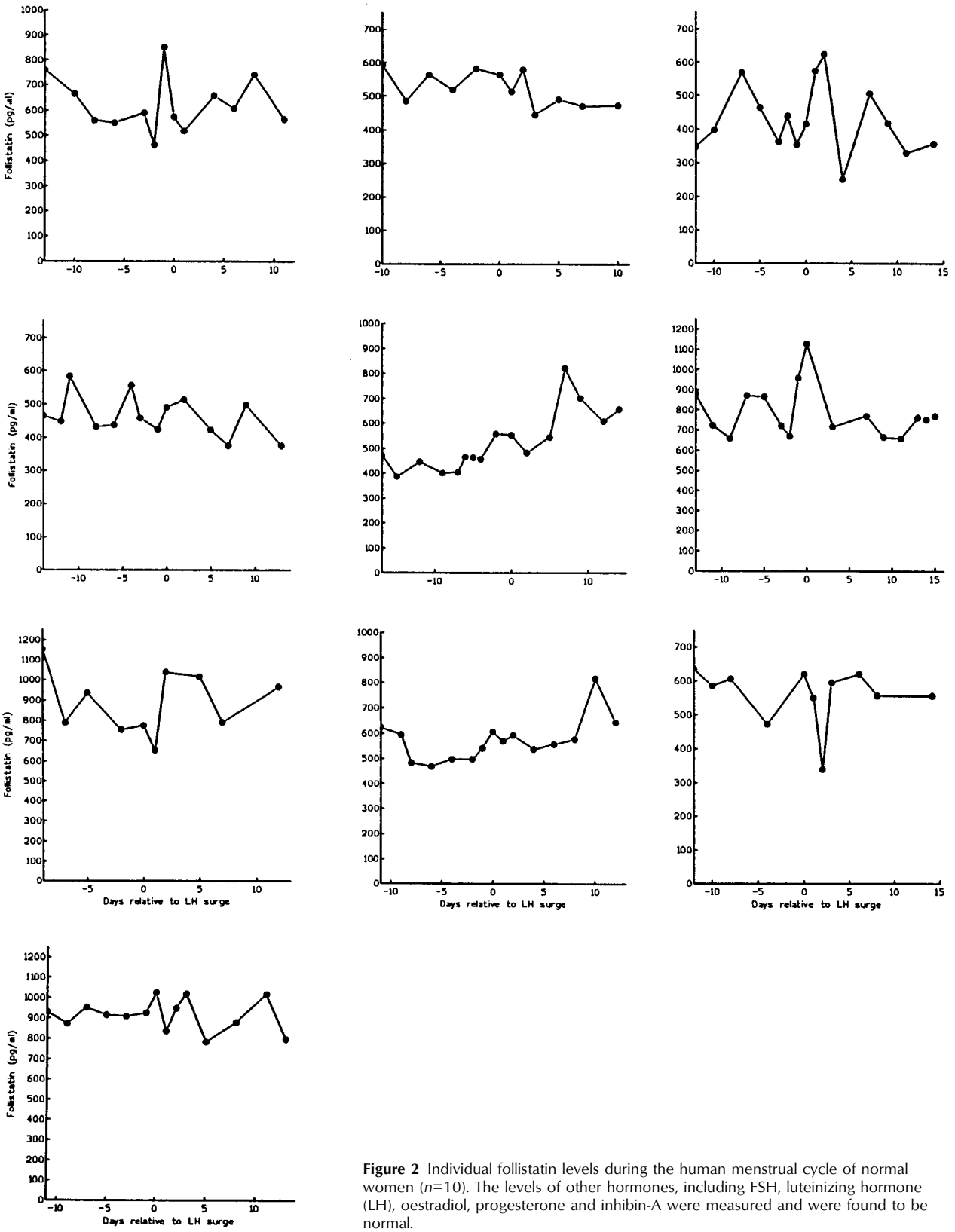

Figure 2 Individual follistatin levels during the human menstrual cycle of normal women (n=10). The levels of other hormones, including FSH, luteinizing hormone (LH), oestradiol, progesterone and inhibin-A were measured and were found to be normal.

tested in the optimized assay. None of the above showed any cross-reaction (<0·3%) with the exception of FS315 (9·9%). Further specificity experiments were conducted to determine whether activin or inhibin interfere with the response signal to follistatin. Preincubation of FS288 standard (0·63 ng/ml) with a large excess of activin-A (50 ng/ml) for 3 h before assay was found to have no effect on follistatin immunoreactivity, indicating that the assay can measure total follistatin. This was also the case when follistatin was preincubated with inhibin-A (50 ng/ml).

Assay range and sensitivity The range of rh-FS288 standards used in the assay was 19–2500 pg/ml. The detection limit of the assay, defined as the dose of follistatin which generated an absorbance value=$x_{\text{true blanks absorbance}}$+ 5 × S.D. zero-dose sample (Porstmann & Kiessig 1992), was <19 pg/ml.

Accuracy This was determined by 'spiking' various human test samples with a known amount of follistatin 288 standard (0·5 ng/ml) prior to assay and assessing the percentage recovery. Mean recovery values of exogenous follistatin from follicular fluid, seminal plasma, amniotic fluid, serum and granulosa cell conditioned medium were 96 ± 7·2, 98 ± 4·9, 100·7 ± 7·5, 89·1 ± 5·5 and 123·9 ± 11% respectively. Parallelism (Fig. 1) between the standard and the various biological fluids tested was confirmed by regression analysis of log-log transformed data. The slopes of the lines (± 95% confidence intervals) were shown to overlap. Thus it can be stated that the slopes of the lines were not significantly different.

Precision Replicates (n=8) of various concentrations of rh-FS288 standard were assayed on separate plates (n=4) to determine assay coefficients of variation. The mean intra- and interplate coefficients of variation were both <5%. The coating antibody used for each plate was from the same batch, as was the detection antibody. Due to both these antibodies being produced by monoclonal cells lines there is no reason for the precision of the assay to vary from batch to batch.

Follistatin concentrations in human samples Table 2 shows the concentrations of follistatin measured in various human biological fluids. Human follicular fluid contained the highest concentration of follistatin (~250 ng/ml). In pregnancy, serum follistatin levels were found to increase from 847 ± 260 pg/ml (n=4) in the first trimester, to 2800 ± 930 pg/ml (n=7) in the third trimester. By contrast, concentrations in human amniotic fluid were found to decrease as gestation progressed from 3630 ± 1500 pg/ml (n=5) at week 16, to 890 ± 320 pg/ml (n=7) at term. Placental homogenates collected at term (n=3) had 3·11 ± 0·4 ng/g follistatin.

Fig. 2 shows the individual follistatin levels during the menstrual cycle of normal women (n=10). There was no consistent trend in the serum follistatin levels. Concentrations ranged from ~250 pg/ml up to ~1120 pg/ml with no relationship to the phases of the cycle. Follistatin in the pooled serum of postmenopausal women (1424 pg/ml) was present at higher concentrations than that of normal menstruating women.

Discussion

The development of immunoassays for follistatin has been complicated by certain characteristics of the molecule. In some ways these are similar to difficulties previously experienced in FSH assay development (Storring 1992). The presence of multiple molecular forms in biological fluids in a variety of species (Robertson *et al.* 1987, Sugino *et al.* 1993, Yokoyama *et al.* 1995), together with varying degrees of glycosylation, combined with the possibility of proteolysis during sample handling all complicate follistatin assay development. Since there is no widely accepted method and no commercially available follistatin assay, the literature now contains a description of seven distinct immunoassays from groups using different labels and polyclonal or monoclonal antibodies (Table 1). Six of these are claimed to measure total follistatin, whereas Wang *et al.* (1996) claim that their assay is specific for free follistatin. Due to the absence of an international follistatin standard, some groups used recombinant FS288 or FS315 as standards. Others used purified individual porcine (p) or bovine (b) isoforms, or even mixtures of these. Naturally, the absolute levels of follistatin reported by the various groups vary widely.

In consequence of the above, it is impossible to reconcile all the data emerging from groups using the various assays. No assay appears to have been widely adopted, indeed the majority of assays are only used in the laboratories where they were developed. Several examples can be given of the contradictory information appearing in the literature. Gilfillan and Robertson (1994) report significantly lower total serum follistatin concentrations in human luteal phase compared with follicular phase. By contrast, Khoury *et al.* (1995) report no statistical variation across the menstrual cycle. The present study supports the latter finding. Serum follistatin does not appear to be primarily an ovarian product.

Several groups agree that, in the serum of pregnant women, total follistatin concentrations rise steadily with increasing length of gestation (Khoury *et al.* 1995, Wakatsuki *et al.* 1996). Our data support these findings (maternal serum follistatin concentrations: first trimester 0·847 ± 0·26 ng/ml, third trimester 2·80 ± 0·93 ng/ml). In marked contrast Gilfillan and Robertson (1994) found no variation throughout human pregnancy, indeed in their study all of the serum samples had follistatin concentrations within a narrow range (3·9–5·2 ng/ml). This included normal male serum, hypogonadal male serum, normal

female serum, serum samples from pregnant women and serum samples from postmenopausal women. Wakatsuki *et al.* (1996) reported higher levels of follistatin in postmenopausal women. Whilst Gilfillan and Robertson (1994) also reported an increase with age, the magnitude was much smaller than in the Wakatsuki study. Khoury *et al.* (1995) found no difference with increasing age. Our data suggest that postmenopausal serum levels are elevated to the upper end of the range found in normal women.

The assay described by Wang *et al.* (1996) uses two monoclonal antibodies and the assay is claimed to be specific for free follistatin. This claim is based on the data shown in Fig. 2 of their paper in which activin appears to be able to inhibit the signal obtained from follistatin. However, close examination of the figure, and in particular its legend, show that approximately 20% of the signal remains even in the presence of a vast excess of activin-A (approx. 150-fold molar excess). Since only a small molar excess of activin is needed to convert all the follistatin into activin complexes we do not believe the data are correctly interpreted i.e. the assay does not specifically measure free follistatin. We would expect almost complete inhibition of signal with a small molar excess of activin-A if the assay were truly measuring free follistatin. It seems to us that a more likely interpretation of the data is that incubation of samples with the immobilized monoclonal antibody allows dissociation of the complexes permitting measurement of initially activin-bound follistatin. Thus, in modest activin excess recovery of follistatin remains quantitative. However, as activin excess increases the available solid phase antibody becomes insufficient to displace all the activin from follistatin. If this interpretation is correct, the concept of being able to assay free follistatin specifically is invalid. Further work would be needed to accept the validity of a free follistatin assay.

A major problem in all follistatin assays is the choice of standard and the ability of assays to measure each of the different follistatin isoforms. For the three main forms so far described, i.e. FS288, FS303, FS315, it would be desirable to have three totally specific assays. No assay so far described achieves this goal. Gilfillan and Robertson (1994) reported that in their assay the potencies of other forms relative to the bFS 35 kDa standard were as follows: 31 kDa, 4·1, 39 kDa, 2·6 and 45 kDa, 5·9. Wakatsuki *et al.* (1996) developed an assay for FS315 but provided no evidence for lack of reactivity with the other forms. Wang *et al.* (1996) developed an assay for FS288 but provided no evidence that FS315 did not cross-react. In our experience most antibodies raised to FS288 would react well with FS315 and *vice versa*.

Very little information is currently available about the relative concentrations of FS288, FS303 and FS315 in different biological fluids and tissues. If some tissues or fluids contain only one of the isoforms then it is of little consequence if the assay used to detect it has a potential to cross-react with other forms. However, problems of interpretation may occur if such an assay is used on other samples which contain a mixture of forms, possibly with different biological functions. In this case, precise quantification is impossible. In addition to the three major isoforms there exist a range of glycosylation variants. Nobody has tested the reactivity of each of these in immunoassays, further adding to the complexity of the situation.

As a result of these considerations all follistatin immunoassays currently available, including that in the present study, pose problems in the interpretation of results. One advantage of the present assay is that it is based on monoclonal antibodies which should give long term reproducibility of results in different laboratories. In contrast to previous studies, we have measured the cross-reaction of the FS315 variant compared with FS288 (9·9%). Recent work suggests that follistatin in human serum may be predominantly FS315, whereas FS288 is the dominant form in follicular fluid (Schneyer *et al.* 1996). If this is the case, then our estimates of follicular fluid concentrations based on FS288 standards are probably reasonably accurate ($\sim$250 ng/ml). However, we would expect our assay to record serum concentrations approximately 10% of their true values. This seems to be the case since Khoury *et al.* (1995) report serum concentrations of 8·09 ng/ml in the normal human menstrual cycle, compared with 0·5–1·0 ng/ml in the present study. Wakatsuki *et al.* (1996) report human female serum levels as 12·5 ng/ml. It is not known which forms of follistatin are present in most tissues and fluids and thus it is possible that mixtures may be found. Although our assay sensitivity for serum forms is reduced, this is compensated for by the very high starting sensitivity. Thus, normal serum levels are still readily measurable. In further studies we hope to determine the extent of cross-reactivity of FS303. We also plan to identify the major forms of follistatin in various human tissues and fluids. Only with this information can meaningful physiological studies be carried out by current immunoassays.

Our goal was to try to develop an optimized total human follistatin assay suitable for a wide range of clinical and physiological studies. The assay described here is similar in format to the inhibin assays from our laboratory. A special dissociating solution (Poncelet & Francimont 1994, McFarlane *et al.* 1996) allows the assay to measure total follistatin by disrupting activin/follistatin complexes. A pair of high affinity monoclonal antibodies used in sandwich amplified ELISA format make the assay ultrasensitive (19 pg/ml), much more sensitive than the best of the previous assays (see Table 1). Use of alkaline phosphatase-labelled detection antibody and dry antibody-coated microplates give the reagents a long shelf life. The assay has been validated for human follicular fluid, human serum, amniotic fluid, seminal plasma and granulosa cell conditioned medium. It seems likely to find a wide range of applications. In due course, similar immunoassays specific for FS315 and FS303 will be needed. In view of

the high degree of homology between human, bovine and porcine follistatins, we were surprised to find that our assay has limited ability to recognize animal follistatins. This is probably because antibodies are produced preferentially to the less conserved regions.

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