Follistatin serum concentrations during full-term labour in women – significant differences between spontaneous and induced labour

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Abstract

Follistatin has been isolated from human placenta and has been identified in human foetal membranes and fluids. Serum follistatin levels in women rise during pregnancy particularly near term. In this study, we examined the effect of induction and stage of labour on maternal plasma concentrations of follistatin. Women who gave birth after a normal pregnancy were retrospectively divided into three groups: those who went in labour spontaneously ($n=33$), needed induction by amniotomy and IV oxytocin ($n=18$) or underwent planned caesarean section ($n=10$). Serum was collected at 38–40 weeks of gestation, periodically through labour with a vaginal examination and once within 36 h postpartum and assayed for oestradiol, progesterone, prolactin and C-reactive protein. Follistatin was measured using a rabbit antiserum (#204) raised against purified 35 kDa bovine follistatin. Human recombinant follistatin was used as both standard and tracer. Concentrations of follistatin at 38–40 weeks of gestation were significantly different between groups. Those who had a spontaneous labour had concentrations higher than those who were induced. Similarly, those who were induced had concentrations higher than those who underwent a caesarean. In the spontaneous group, follistatin rose during labour, peaking at 57.9 ± 5.48 ng/ml at > 3 cm of cervical dilation, and after delivery follistatin decreased to 26.16 ± 3.4 ng/ml at 24 h post-delivery. In induced patients follistatin continued increasing to peak following delivery at 26.9 ± 3.0 ng/ml and decreased at > 3 h post-delivery. Follistatin concentrations in caesarean section patients at 24 h post-surgery (18.53 ± 3.74 ng/ml) were not different from that before the surgery and were comparable with the other two groups. Follistatin is clearly implicated in the onset of labour; however, further studies with a larger cohort of women are necessary to determine the nature of its role.

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Introduction

The monomeric activin-binding protein follistatin, isolated in 1987, on the basis of its role in the suppression of follicle stimulating hormone (Robertson *et al.* 1987, Ueno *et al.* 1987), has now been implicated in a multitude of physiological systems in human females and males, but in the latter it usually remains unchanged. Follistatin is widely recognised for its ability to modulate the actions of activin, a member of the transforming growth factor β superfamily. There have been up to six isoforms isolated, formed from alternate splicing sites and a variety of glycosylation and post-translational proteolytic cleavage, with the splice variants being follistatin-315 (FS315) and follistatin-288 (FS288; Sugino *et al.* 1993). Follistatin preferentially binds activin with high affinity, with some isoforms having the ability to interact with heparan

sulphate proteoglycans on cell surface membranes (Nakamura *et al.* 1991).

Follistatin has been implicated in the female reproductive axis, pregnancy (reviewed by Phillips & de Kretser 1998) and embryonic development (Tashiro *et al.* 1991, Mummery & van den Eijnden-van Raaij 1993, Tuuri *et al.* 1994). The reported concentrations of total follistatin through pregnancy have been somewhat inconsistent between assays and groups. Previous cross-sectional studies of maternal serum in pregnancy show that activin concentrations increase in women in the third trimester, with activin A being the predominant isoform in all pregnant women (Draper *et al.* 1997, Petraglia 1997, Schneider-Kolsky *et al.* 2000, Wang *et al.* 2002).

Woodruff *et al.* (1997) reported that while total follistatin concentrations increased in late pregnancy,

in all women there were no significant increases in the small percentages of free follistatin. Khoury *et al.* (1995) also reported increases in total follistatin across pregnancy but did not clearly define gestational period (Khoury *et al.* 1995). In studies by Evans *et al.* (1998) and O'Connor *et al.* (1999), the results for total follistatin show a similar trend of increasing follistatin with gestational progression. The increased concentration of follistatin observed in the final trimester of gestation has led to interest in follistatin as a marker for the onset of parturition. However, the Schnieder-Kolsky *et al.* (2000) study to determine the involvement of both activin and total follistatin (FS288) across parturition in patients undergoing spontaneous labour, induction of labour and caesarean section delivery showed no significant changes in either protein.

Both follistatin and activin have been implicated in inflammatory pathways involved in wound repair, systemic inflammation and illnesses such as meningitis in both males and females (Klein *et al.* 1996, Jones *et al.* 2000, Keelan *et al.* 2000, Wankell *et al.* 2003). Such inflammatory pathways can initiate the acute phase response, involving the acute phase marker C-reactive protein (CRP) and/or stress response which can stimulate the production of cortisol from the adrenals.

Follistatin's activin regulatory role is well established, and there is evidence suggesting that this partnership interacts with prolactin (PRL) during decidualisation (Jones *et al.* 2002). Early studies on cultured stromal cells indicate that activin promotes decidualisation (Jones *et al.* 2002) with stromal cells producing both activin receptor and activin β_A subunits in high concentrations with onset of decidualisation. One of the earliest markers of decidualisation is production of PRL, by decidualised endometrial stromal cells as well as the pituitary (Thraill *et al.* 1989, Frank *et al.* 1995). Jones *et al.* (2002) established in *in vitro* cultures that decidualisation had occurred through the increased levels of PRL; however, the production of PRL ceased upon administration with follistatin.

Due to the inconsistencies in the current published data (likely to be due to measurement of differing follistatin isoforms), we measured total follistatin (FS288) in blood samples from labouring and non-labouring patients using an antibody and an assay (O'Connor *et al.* 1999) distinct from that used by Schneider-Kolsky *et al.* (2000) study. This research has endeavoured to establish any links between follistatin and the acute phase response, using well-established markers of this pathway CRP and the stress marker cortisol. Additionally, this study reviewed the routine hormones of pregnancy, oestradiol and progesterone, to establish any links with the actions of follistatin and as a serological measure of placental expulsion.

Results

Oestradiol and progesterone

Results from the traditional gestational hormones, oestradiol and progesterone, show the expected pattern of concentration changes across the sample period from antenatal through 24 h post-delivery (data not shown), i.e. concentrations peak in early labour with respect to the antenatal period that fall rapidly by the early postpartum period when the placenta is removed.

Oestradiol and progesterone show that in early labour, spontaneous onset patients have higher concentrations ($P < 0.05$) of both of these hormones when compared with the induced patient group (oestradiol: $91.4 \text{ nmol/l} \pm 15.5$ and $65.1 \text{ nmol/l} \pm 8.78$ respectively; and progesterone: $314.5 \text{ nmol/l} \pm 32.0$ and $224.8 \text{ nmol/l} \pm 32.9$ respectively). As labour progressed, there was a trend to maintaining an increased concentration of both hormones in the spontaneous patients; however, it was not deemed significant from the induced patient group.

Cortisol

In both vaginal delivery groups, cortisol (Fig. 1) rises from the antenatal period across labour to peak at the early postpartum sample (spontaneous Ante: $663.9 \text{ nmol/l} \pm 52.6$, Lab2: $1108.1 \text{ nmol/l} \pm 89.9$, Post1: $1691.6 \text{ nmol/l} \pm 251.2$; induced Ante: $421.1 \text{ nmol/l} \pm 43.6$, Lab2: $987.9 \text{ nmol/l} \pm 194.3$, Post1: $1424.21 \text{ nmol/l} \pm 154.21$). In the spontaneous labour patients, these results showed a significant pattern of increasing concentrations ($P < 0.02$). Whilst induced patients showed a similar pattern, the antenatal samples in this group were significantly less than all subsequent samples ($P < 0.03$).

Figure 1 Profile of cortisol across the antenatal period, Lab1 (0–3 cm vaginal dilation) Lab2 (> 3 cm dilation), Post1 (0–3 h post delivery) and Post2 (3–24 h post delivery). Significant differences within each group (Spon, Induced and LSCS) are indicated with letters. Significant differences between groups are indicated numerically.

In the late postpartum sample period, a decrease in the spontaneous and induced patient groups is seen. Interestingly, in late postpartum, induced patients have the highest concentration ($906.7 \text{ nmol/l} \pm 132.4$) when compared with spontaneous labour and caesarian section (LSCS) deliveries ($708.5 \text{ nmol/l} \pm 96.1$ and $571.7 \text{ nmol/l} \pm 106.4$ respectively).

Prolactin

As shown in Fig. 2, in the spontaneous labour patients, there is a trend to increasing PRL between the antenatal period and the final postpartum sample ($2773.6 \pm 242.3 \text{ } \mu\text{IU/ml}$ to $4823.9 \pm 581.2 \text{ } \mu\text{IU/ml}$). Induced patients show PRL reaching its significant peak ($P < 0.004$) at late labour ($5449.40 \pm 890.71 \text{ } \mu\text{IU/ml}$) and then dropping significantly to reach to early labour levels in the first postpartum period ($3906.89 \pm 806.32 \text{ } \mu\text{IU/ml}$).

Follistatin

Follistatin concentration changes in maternal serum over time are profiled in Fig. 3. In the spontaneous labour patients, follistatin showed significant differences between antenatal, early labour, late labour and early postpartum samples, whilst the induced patients showed significant differences between labouring samples and the early postpartum sample ($P < 0.01$).

Of great interest are the differences seen between these two patient groups within both labouring samples and early postpartum samples. In early labour, spontaneous patients have significantly higher concentrations of follistatin ($P < 0.01$) than induced patients ($23.4 \text{ ng/ml} \pm 1.84$ to

Figure 2 Profile of prolactin across the antenatal period, Lab1 (0–3 cm vaginal dilation) Lab2 (> 3 cm dilation), Post1 (0–3 h post delivery) and Post2 (3–24 h post delivery). Significant differences within each group (Spon, Induced and LSCS) are indicated with letters. Significant differences between groups are indicated numerically.

Figure 3 Profile of follistatin across the antenatal period, Lab1 (0–3 cm vaginal dilation) Lab2 (> 3 cm dilation), Post1 (0–3 h post delivery) and Post2 (3–24 h post delivery). Significant differences within each group (Spon, Induced and LSCS) are indicated with letters. Significant differences between groups are indicated numerically.

$13.9 \text{ ng/ml} \pm 2.54$ respectively), whilst in late labour there is a twofold difference which peaks in the spontaneous group ($P < 0.0004$). When the two groups are compared at late labour, follistatin concentrations are $57.9 \text{ ng/ml} \pm 5.48$ and $20.5 \text{ ng/ml} \pm 1.73$ respectively. Follistatin remains significantly elevated ($P < 0.0003$) in spontaneous labour patients in the early postpartum ($57.6 \text{ ng/ml} \pm 5.1$) when compared with induced patients ($26.9 \text{ ng/ml} \pm 3.0$). The induced patients show follistatin continues to rise from labour, into the early postpartum period and reaches its peak concentration here ($26.9 \text{ ng/ml} \pm 3.0$ to $20.5 \text{ ng/ml} \pm 1.73$ in late labour). In the later postpartum period, we see follistatin concentrations in both patient groups decreasing.

C-reactive protein

As seen in Fig. 4, CRP concentrations in all patient groups remain relatively steady from antenatal period and across labour into the early postpartum period. However, in the late postpartum period, we see a four- to fivefold increase in CRP in all patients. Spontaneous patients increase significantly ($P < 0.0006$) from early postpartum $13.4 \text{ mg/l} \pm 3.96$ to $43.7 \text{ mg/l} \pm 12.0$ in the later postpartum sample. Induced and LSCS patients increase from $11.0 \text{ mg/l} \pm 2.61$ and 4.92 mg/l to $44.1 \text{ mg/l} \pm 9.69$ and $55.2 \text{ mg/l} \pm 12.2$ respectively ($P < 0.0003$).

Discussion

This current study has shown that there are clear indications for the involvement of follistatin in the parturition process. Follistatin concentrations were

Figure 4 Profile of C-reactive protein across the antenatal period, Lab1 (0–3 cm vaginal dilation) Lab2 (>3 cm dilation), Post1 (0–3 h post delivery) and Post2 (3–24 h post delivery). Significant differences within each group (Spon, Induced and LSCS) are indicated with letters. Significant differences between groups are indicated numerically.

significantly higher in labouring samples in the spontaneously labouring patient when compared with the induced patient. Concentrations were higher in spontaneous patients with <3 cm cervical dilation and follistatin concentrations in the spontaneously labouring patient were greater than twofold those measured in induced patients in late labour when dilation >3 cm.

The Schneider-Kolsky *et al.* (2000) assessment of follistatin and activin across spontaneous parturition in 36 women correlated results with cervical dilation, and no changes in follistatin concentrations were shown. In the same study, nine women undergoing inductions had serum collected at 60-min intervals from the time of the amniotomy to delivery and they also showed no significant changes in either follistatin or activin concentrations. A number of women in this study had pre-existing medical conditions such as gestational diabetes and non-proteinuric hypertension (Schneider-Kolsky *et al.* 2000). Previous studies have shown that follistatin concentrations may alter with inflammatory conditions, creating the possibility that concentrations measured by Schneider-Kolsky *et al.* were influenced by pre-existing conditions in the patients (Schneider-Kolsky *et al.* 2000).

The multiplicity of isoforms of follistatin, the high affinity for activin and its 85% homology between species have made the development of accurate assays for follistatin a difficult task (Sugino *et al.* 1993, Evans *et al.* 1998). The Schneider-Kolsky *et al.* (2000) study utilised a follistatin assay that was different from the one used in the present study. The antibody used by Schneider-Kolsky *et al.* (2000) is a monoclonal mouse antibody raised against human recombinant rhFS288, whilst the O'Connor *et al.* (1999) assay, as utilised in this

study, uses a polyclonal rabbit antibody raised against purified 35 kDa bovine follistatin. As indicated by the variability of results in previous studies, the different antibodies are probably measuring differing follistatin isoforms. When considering all follistatin results, including those in the present study, one must consider the multiplicity of follistatin isoforms and the potential diversity of actions of each of these isoforms.

The lack of agreed standards for measuring follistatin and the availability of ten different assays makes the interpretation across studies difficult. At least two groups indicate that their assay determines the levels of unbound follistatin (Wang *et al.* 1996, McConnell *et al.* 1998), whilst the other assays measure total (bound and unbound) follistatin (Sugawara *et al.* 1990, Klein *et al.* 1991, Gilfillan & Robertson 1994, Khoury *et al.* 1995, Wakatsuki *et al.* 1996, Evans *et al.* 1998, McConnell *et al.* 1998, de Kretser *et al.* 1999, O'Connor *et al.* 1999).

When considering the diverse and often contradictory data on follistatin, one must consider the multiple isoforms of follistatin and the potential that they may have different physiological functions. The strongest evidence for differing functions comes from the different distribution of isoforms FS3155 and FS288 between tissues, and the different interactions with cellular membranes (Sidis *et al.* 2006). Recent work by Sidis *et al.* (2006) showed that follistatin isoforms differentially regulated testicular tumour cells, with FS288 suppressing and FS315 enhancing the activin-driven proliferation of these cells, so that it remains possible for follistatin isoforms to have different modes of action in pregnancy and parturition (Sidis *et al.* 2006). Despite evidence in other studies to the contrary, it is clear from this present study that follistatin does have a role to play in the onset and parturition process, even though we are probably measuring a different isoform from other across-labour studies. Data from Schneider-Kolsky *et al.* (2000) showing no changes to follistatin in labour, along with this study's finding, suggest that there are follistatin isoforms that are differentially regulated across the parturition of women.

This study has shown that oestradiol and progesterone concentrations drop below antenatal concentrations following delivery, confirming the placenta as the major source of these hormones. Studies have shown high concentrations of follistatin in the placenta, amnion and choriodecidua, implying that these tissues are potential sources for follistatin in maternal serum (Kaiser *et al.* 1990, Mercado *et al.* 1993, Yokoyama *et al.* 1995, Petraglia 1997, Keelan *et al.* 1999). In this study, follistatin concentrations decreased at the same time as both oestradiol and progesterone, although they failed to reach antenatal levels until the late postpartum period. Kogure *et al.* (1996) indicated that the half-life for follistatin is ~4 min, hence if the placenta was the source of the follistatin in maternal serum levels should

return to antenatal concentrations or below within the first postpartum period (<3 h from delivery; Kogure *et al.* 1996). These current results suggest that follistatin increases seen in labour are from a different source or in addition to the placental follistatin. Phillips & de Kretser (1998) demonstrated that follistatin mRNA and protein are widely distributed throughout the body, so that there are many possible sources for the increases seen in maternal serum, such as the vascular endothelial cells (Phillips & de Kretser 1998).

Work by Sidis *et al.* (2006) indicated that the biological activity of follistatin is due to its ability to interact at the cell surface through heparan sulphate proteoglycans (Sidis *et al.* 2006). Studies on the myometrium have shown that whilst other myometrial proteoglycans decrease, heparan sulphate proteoglycans increase by 46% in active labour (Hjelm *et al.* 2002) which can potentially increase the interactions with follistatin. This period coincided with the highest concentrations of follistatin measured in this study. This study has showed follistatin concentrations to increase through parturition (Fig. 3), peaking with established labour >3 cm dilation. It also shows that follistatin is elevated in women who are in spontaneous labour when compared with those undergoing an induction. Of particular interest, in spontaneous labour follistatin is significantly higher than antenatal levels even at a minimal cervical dilation (0–3 cm), indicating that follistatin is rising whilst the pre-labour stage of cervical ripening occurs. Although the study by Schneider-Kolsky *et al.* (2001) showed neither activin β_A subunits nor any of the three activin receptors were immunolocalised to the myometrial smooth muscle cells in active labour, perhaps the elevations noted in this study are due to the actions of the follistatin/activin complex or those of follistatin individually. The follistatin/activin complex or follistatin individually may be acting in concert with the increasing number of uterine myometrial heparan proteoglycans through labour in uterine remodelling essential for birth.

Studies on stress have linked PRL and cortisol release in the physiological stress response (Armario *et al.* 1996, Richter *et al.* 1996). Whilst the current study did not show these links in the data (Figs 1 and 2), perhaps in a larger study, when samples could be analysed with respect to labour length, we may find such correlations. Knowing that PRL is influenced by activin, stress, cortisol, feeding status, etc., it seems that complete understanding of roles of PRL at parturition is multifactorial, and therefore a much larger patient cohort and rigorous sampling protocols are necessary to tease out its influence, if any, on parturition.

When reviewing follistatin results along with the stress marker, cortisol, and the acute phase protein, CRP (Figs 3 and 4), it can be observed that follistatin concentration increases in early labour in the spontaneous onset patients, well before either of these

proteins. This suggests that follistatin is increasing due to stimuli other than that of stress or the acute phase response. While follistatin has been implicated in the acute phase response, this study clearly shows that the acute phase response triggered postpartum is distinct from that of follistatin increases seen during spontaneous labour. Perhaps the increases in follistatin that this study documents in early labour are the trigger for the adrenal glands to release glucocorticoids (Vanttinen *et al.* 2002) that then initiate or propagate the release of the acute phase response proteins from the hepatocytes in the liver to assist in the postpartum 'mop up' and uterine remodelling. The results showed follistatin concentrations remain elevated above antenatal concentrations in the postpartum period, suggesting that the placenta is not the major source of the follistatin seen in maternal serum.

Whilst this study has shown that follistatin concentrations increase significantly across labour, suggesting that it is involved with the spontaneous onset of parturition, there are still many aspects that need to be considered for further research. More frequent samples late in gestation would help establish these initial findings that follistatin is involved in the cervical ripening stage of parturition prior to active labour as well as a longer period of sampling *post partum*. We have previously reported that follistatin in the sheep rises and reaches maximal levels in the 36–48 h following parturition; therefore, a longer sampling period would be valuable to assess this in women (Rae *et al.* 2003).

Overall, this study has shown follistatin is clearly implicated in the spontaneous onset of labour in women. Further research is required to determine the exact role it plays during parturition.

Materials and Methods

Experimental subjects

Obstetric patients of the Tamworth Base Hospital in the New England Area Health Service were recruited into the study. Patients who suffered any pre-existing conditions, e.g. gestational diabetes, were excluded from the study. Any patients who had pre-labour membrane rupture or those who needed emergency or surgical intervention in labour were also excluded. Patients who suffered any adverse birth outcome were also excluded from the study. All women recruited to the study were in good health at the time of recruitment.

The mean age of women used in the study was 27.8 years (range of 18.8–42.7 years). The gestation of the women was calculated from a definite last menstrual period date or by paediatric judgment post-delivery using the well-established Dubowitz scoring system (Dubowitz & Dubowitz 1977, Bhagwat *et al.* 1990, Eggarth *et al.* 2002). The average gestation of women in the study at term is 39.1 weeks with a range of 36–44 weeks. Using a patient cohort of 61 women who delivered by the end of the study, patients were separated according to whether delivery was a planned caesarean section or from spontaneous or induced onset of labour. Women who

failed to go into spontaneous labour by 40 weeks plus 10 days were induced. Inductions were performed using amniotomy and IV oxytocin (syntocin) administered until the delivery. The characteristics of each of these groups are shown in Table 1.

This study was approved by the New England Area Health Service Clinical Research Group and Ethics Committee, and the University of New England Ethics Committee.

Sample collection

Venous blood samples were collected into 5 ml vacuette Z serum separator clot activator tubes (Greiner, St Gallen, Switzerland). These samples were centrifuged at 2683 g (4000 r.p.m.) for 10 min and immediately frozen at -15°C and stored until assayed. Blood samples collected from patients have been grouped according to: antenatal (Ante); early labour, <3 cm vaginal dilation (Lab1); late labour >3 cm vaginal dilation (Lab2); early postpartum, 0–3 h post-delivery (Post1) and late postpartum, >3 h and <36 h post-delivery (Post2).

Assays

Samples were analysed for oestradiol, progesterone, cortisol and PRL using the automated clinical analyzer Access2 (Beckman-Coulter Australia Pty Ltd, Gladesville, NSW, Australia). CRP was analysed using the automated clinical analyzer Synchron (Beckman-Coulter Australia Pty Ltd).

Total follistatin was determined in plasma using a RIA as previously described by O'Connor *et al.* (1999). Briefly, the assay used a rabbit antiserum (#204) raised against purified 35 kDa bovine follistatin. A human recombinant follistatin (FS288) was used as both standard and tracer. The dissociation reagents were modified to 10% Triton X-100, 3% sodium deoxycholate and 0.5% SDS. Cross-reactivity with inhibin and activin was <0.5%. The detection limit was 0.25 ng/ml and the intra- and inter-assay coefficients of variation were 6 and 12% respectively.

Data analysis

The raw hormone concentration data were log transformed to normalise the data and equalise the variance. The data were then analysed by two-way ANOVA using the Student–Newman–Keuls post test to compare individual means. All the calculations were carried out using the general linear model procedure and repeated statement of the SAS computer package (SAS Institute Inc., Cary, NC, USA). The values were presented as mean $\pm$ s.e.m. and were considered significant at $P < 0.05$.

Table 1 Characteristics of patients using type of onset as criteria for inclusion.

Characteristics	Spontaneous onset	Induced onset	Caesarean section
No. of deliveries	33	18	10
Gestation (weeks; median)	39.3	38.6	39.7
Age (years; median)	27.6	26.7	30.1
Length of labour (h; median)	6.5	4.5	0.0

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