Follistatin concentrations in maternal and fetal fluids during the oestrous cycle, gestation and parturition in Merino sheep

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The aim of this study was to investigate the changes in follistatin, an activin binding protein, during the oestrous cycle, gestation and parturition in ewes using a radio-immunoassay for total follistatin, which uses dissociating reagents to remove the interference of activin. Follistatin concentrations remained unchanged (2.7 ± 0.2 ng ml⁻¹) during the oestrous cycle and decreased as pregnancy progressed. Follistatin concentrations in allantoic fluid also decreased during gestation, whereas in amniotic fluid follistatin concentrations reached a peak at day 75 of gestation (9.8 ng ml⁻¹) and had decreased to 4.4 ng ml⁻¹ at day 140. Follistatin concentrations in fetal blood (7.0 ± 0.5 ng ml⁻¹) did not change from day 50 to day 140 of gestation but were significantly higher than in matched maternal samples (3.1 ± 0.3 ng ml⁻¹). Circulating follistatin

in ewes was significantly increased on the day of parturition (5.6 ± 0.6 ng ml⁻¹) compared with the days before parturition (2.7 ± 0.4 ng ml⁻¹), but had decreased by day 2 after birth. Blood samples from newborn lambs showed that plasma follistatin concentration (13.4 ± 2.3 ng ml⁻¹) was significantly higher than that of the mothers and remained high for at least 7 days after birth. These data support previous studies of the human menstrual cycle indicating that follistatin is not an endocrine signal from the ovary; however, in contrast to human pregnancies, follistatin concentrations in sheep decreased and become high only after or during parturition. This difference observed between species may reflect different physiological effects of follistatin or may be the result of measurement of different isoforms.

Introduction

Activins are homo- and heterodimers of the inhibin β_A and β_B subunits, which form activin A, activin B and activin AB. The activins were initially identified by their capacity to stimulate the secretion of FSH by pituitary cells in culture (Ling *et al.*, 1986; Vale *et al.*, 1986). More recent studies have shown that activins have a wide range of actions in a variety of other tissues (Mather *et al.*, 1997). Activins were first implicated in pregnancy when the mRNAs for the β_A and β_B subunits were identified in the placenta (Meunier *et al.*, 1988) and fetal membranes (Petraglia *et al.*, 1993). The dimeric proteins were subsequently identified in the placenta (de Kretser *et al.*, 1994a; Yokoyama *et al.*, 1995). High concentrations of activin A have been recorded in ovine amniotic fluid (Wongprasartsuk *et al.*, 1994; de Kretser *et al.*, 1994b) and allantoic fluid (Foulds *et al.*, 1998). A number of studies have shown that activin A concentrations are high in the plasma of pregnant women (Muttukrishna *et al.*, 1996; Woodruff *et al.*, 1997; Fowler *et al.*, 1998) and, more recently, O'Connor *et al.* (1999) showed that activin A concentration increases throughout human pregnancy, particularly in the third trimester. The

inhibin/activin subunits are expressed by granulosa cells in developing antral follicles and are probably important in regulating proliferation and function of granulosa cells (Findlay, 1993), and, hence, follicular development (Schneyer *et al.*, 2000). Activin A has also been shown to vary biphasically during the menstrual cycle in women, with peaks occurring mid-cycle and during the late luteal early follicular phase (Muttukrishna *et al.*, 1996).

The role of activin in many biological systems is thought to be modulated by follistatin, a high affinity activin binding protein, which can neutralize most of the biological actions of activin (Nakamura *et al.*, 1990; Kogawa *et al.*, 1991; Mather *et al.*, 1993). Follistatin was first identified in follicular fluid as an inhibitor of FSH secretion by the pituitary, although its potency was only 10–30% of that of inhibin (Robertson *et al.*, 1987; Ueno *et al.*, 1987). Subsequently, follistatin was shown to be a binding protein of both activin and inhibin, and, more recently, several bone morphogenic proteins (BMPs) (Iemura *et al.*, 1998). Follistatin exists as a variety of isoforms, which are the product of at least two alternatively spliced forms; there are at least six forms of this protein with molecular masses ranging from 31 kDa to 45 kDa after proteolytic cleavage and variable glycosylation (Robertson *et al.*, 1987; Ueno *et al.*, 1987; Sugino *et al.*, 1993). Recent data using surface

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plasmon resonance technology demonstrate that some isoforms have a higher affinity for activin than do others (Hashimoto *et al.*, 2000); hence, these isoforms may have different physiological functions.

Follistatin has been isolated from human placenta and has been identified in human and ovine fetal membranes and fluids (de Kretser *et al.*, 1994a,b; Petraglia *et al.*, 1994a,b). Serum follistatin concentrations increase during pregnancy in women, particularly near term (Woodruff *et al.*, 1997; O'Connor *et al.*, 1999). Follistatin mRNA and protein have been localized to the granulosa cells in the ovary (Nakamura *et al.*, 1991). Although expression of follistatin mRNA is at a maximum in the rat ovary at oestrus, corresponding protein concentrations in the ovary and serum do not change significantly (Mercado *et al.*, 1993). These data are supported by studies of women showing little change in follistatin concentrations during the menstrual cycle (Khoury *et al.*, 1995).

The aim of the present study was to study comprehensively the concentrations of follistatin in maternal plasma during the ovine oestrous cycle and pregnancy, and to measure follistatin concentrations in fetal plasma and fluids. The secondary aim was to assess the usefulness of sheep as a model for these kinds of studies, thus allowing more intensive investigation than is possible with human subjects.

Materials and Methods

Ethical approval of animal experiments

These experiments were approved by the University of New England Animal Ethics Committee and conform to the NHMRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes.

Animal treatments

A group of five Merino ewes was kept with two castrated male Merino sheep (wethers) that had received injections of 50 mg mixed testosterone esters (Durateston; Intervet, Castle Hill) over the previous 2 months. The wethers were harnessed with marking crayons so that the day of oestrus could be determined. Blood samples were taken once a day until the ewes were marked again, indicating the next oestrus. A flock of 50 ewes was kept with intact rams harnessed with marking crayons to determine the conception date. Blood samples were taken once a week and groups of five ewes were killed at days 50, 75, 100, 125 and 140 of gestation. Cord blood, amniotic and allantoic fluid samples were also collected. A further group of five ewes was allowed to give birth and blood samples were collected once a day for the last 10 days before parturition and on days 1, 2 and 7 after parturition. Blood samples were collected from the lambs of these ewes, together with a further group of seven lambs and ewes, on days 1, 2 and 7 after parturition.

Blood samples were collected by venepuncture from the jugular vein and added to heparin-coated tubes. The use of heparin as an anticoagulant does not interfere with the

assay. The blood was centrifuged at approximately 1400 g for 30 min and the plasma was removed and stored at -20°C until assayed.

Hormone assays

Follistatin was measured using a radioimmunoassay as described by Phillips *et al.* (1996). In brief, this assay uses a rabbit antiserum (No. 202) raised against bovine 39 kDa follistatin purified from follicular fluid. A heterologous bovine follistatin pool (35, 39 and 45 kDa), which was isolated from bovine follicular fluid as described by Klein *et al.* (1991), was used as standard and tracer. The follistatin pool (2.5 μg) was iodinated (0.5 mCi) using Iodogen (500 ng; Pierce, Rockford, IL). Separation of bound from free follistatin was achieved by goat anti-rabbit second antibody precipitation in the presence of 0.9% (w/v) saline. Bovine inhibin, human recombinant inhibin and human recombinant activin show $<0.5\%$ crossreactivity in the assay. Dissociating reagents were used to eliminate the interference of activin, as described by McFarlane *et al.* (1996). The sensitivity of the assay was 1.0 ng ml^{-1} and the inter- and intra-assay coefficients of variation were 13.0 and 8.0%, respectively.

Samples were assayed for progesterone using a similar procedure to that described by McFarlane *et al.* (1990). The antiserum was prepared using progesterone-11 α -HS-BSA (No. 230; Bioquest Limited, North Ryde). Each sample was extracted in duplicate with a ten times volume of diethyl ether. The sensitivity of the assay was 25 pg ml^{-1} . The inter-assay coefficient of variation was 11% and the intra-assay coefficient of variation was 4%.

Data analysis

Parallel-line statistics (Finney, 1978), regression analysis and one-way ANOVA followed by a Student–Newman–Keuls multiple range test were performed using the SAS computer software package (SAS Institute Inc., Cary, NC). The data were log-transformed before analysis to normalize the data and to equalize the variance.

Results

Blood samples were collected once a day from a group of five ewes from the day they showed oestrus until they were marked again, indicating the start of the next ovarian cycle. The concentrations of follistatin measured in these daily samples are shown (Fig. 1). Follistatin concentrations did not change significantly during the oestrous cycle (Fig. 1), with an overall mean concentration of $2.7 \pm 0.2\text{ ng ml}^{-1}$.

Follistatin concentrations in plasma samples taken at weekly intervals from pregnant and non-pregnant ewes from the day of oestrus until day 140 of gestation are shown (Fig. 2). In the non-pregnant ewes, follistatin concentrations did not change significantly and although the concentrations appeared to increase in the last half of gestation this increase was not significant. In the pregnant ewes, follistatin

Fig. 1. Concentration of follistatin in plasma samples from cyclic ewes, with day 0 as the day of oestrus. Data are mean $\pm$ SE ($n = 5$).

concentrations decreased significantly overall ($P < 0.02$) from 4.2 ± 0.1 ng ml $^{-1}$ to 2.8 ± 0.1 ng ml $^{-1}$ at day 140 of gestation. Plasma follistatin concentrations were significantly lower in pregnant ewes than in non-pregnant ewes from day 100 to day 140 of gestation. This period coincided with the increase in plasma progesterone concentration, which occurred at approximately day 90 of gestation (Fig. 2).

Allantoic fluid was collected from groups of ewes killed at approximately days 50, 75, 100, 125 and 140 of gestation. Regression analysis demonstrated that follistatin concentrations, although variable, decreased significantly ($P < 0.001$) from day 50 to day 140 of gestation (Fig. 3). The follistatin profile in amniotic fluid was different from that observed in allantoic fluid, and the concentrations of follistatin in amniotic fluid were significantly ($P < 0.05$) lower than those recorded in allantoic fluid. The peak concentrations were on days 75 and 100, which were significantly ($P < 0.01$) higher than on days 50, 125 and 140 (Fig. 4).

Follistatin concentrations in fetal plasma were significantly higher ($P < 0.01$) than in maternal plasma from day 75 to day 140 of gestation. Although the fetal plasma concentrations of follistatin showed a decrease with gestational age, this was not significant. Matched maternal samples taken just before the ewes were killed did not change significantly from day 75 to day 140 of gestation (Fig. 5).

Blood samples were collected once a day from day 10 before the expected day of parturition, on the day of birth and on days 1, 2 and 7 after birth to monitor the changes in follistatin concentration at about the time of parturition more closely. A significant ($P < 0.05$) increase in follistatin

Fig. 2. Weekly plasma concentrations of follistatin in (a) non-pregnant control ewes ($n = 5$) and concentrations of (b) follistatin and (c) progesterone in pregnant ewes ($n = 10$ –35) from the day of oestrus (day 0) until day 140 of gestation. Data are mean $\pm$ SE.

concentration in ewes was observed on the day of parturition and on day 1 after parturition (Fig. 6). These concentrations decreased on day 2 and by day 7 after parturition concentrations were not significantly different from those before parturition.

Follistatin was measured in blood samples taken from lambs and their mothers on the day of parturition and on days 1, 2 and 7 after parturition (Fig. 7). Follistatin concentrations in the plasma of the lambs were significantly higher ($P < 0.001$) than plasma concentrations in the

Fig. 3. Follistatin concentrations in sheep allantoic fluid collected at days 50, 75, 100, 125 and 140 of gestation. A linear regression line ($R = -0.7208$) described by the equation $y = -0.0081x + 2.4141$ is shown fitted to the data. Note the logarithmic scale.

Fig. 4. Follistatin concentrations in sheep amniotic fluid ($n = 3-13$) collected at days 50, 75, 100, 125 and 140 of gestation. Data are mean $\pm$ SE. ^{ab}Concentrations with different letters are significantly different ($P < 0.05$).

Fig. 5. Follistatin concentrations in plasma taken from sheep fetuses (■; $n = 4-10$) at days 75, 100, 125 and 140 of gestation compared with follistatin concentrations in matched maternal plasma (○) at the same times. Data are mean $\pm$ SE.

Fig. 6. Daily follistatin concentrations in ewes ($n = 10$) from day 10 before parturition to day 7 after lambing. Day 0 is the day of parturition. Data are mean $\pm$ SE. *Concentrations with asterisks are significantly different from other values ($P < 0.05$).

Fig. 7. Follistatin concentrations in the plasma of lambs (■, $n = 10$) and ewes (□, $n = 10$) on the day of birth and on days 1, 2 and 7 after birth.

mothers. Although the concentrations appeared to decrease over the week, this decrease was not significant. The follistatin concentrations observed in the lambs were also significantly higher ($P < 0.01$) than those measured in fetuses during late gestation.

Discussion

In the present study we have described the profiles of circulating follistatin in ewes during the oestrous cycle and during pregnancy using an assay that is not interfered with by activin. There were no significant differences in plasma follistatin concentrations in ewes during the oestrous cycle. Although follistatin mRNA in rats (Nakatani *et al.*, 1991) and sheep (Tisdall *et al.*, 1994), and follistatin protein as determined by immunocytochemistry in cattle (Singh and Adams 1998), increase with follicular development this is not reflected in plasma follistatin concentrations. This finding is in contrast to that reported previously by Klein *et al.* (1993), but is similar to that reported in humans (Khoury *et al.*, 1995). The previous study by Klein *et al.* (1993) used a radioimmunoassay for follistatin that was subsequently found to be interfered with by activin (de Kretser *et al.*, 1994a). It is likely that the increased follistatin concentration reported by Klein *et al.* (1993) in the luteal phase of the oestrous cycle may be a result of interference with activin or other growth factors, which bind to follistatin. The data from this study and previous studies using a variety of assays for follistatin (Kettel *et al.*, 1996; Evans *et al.*, 1998; McConnell *et al.*, 1998) support the concept proposed by Khoury *et al.* (1995) that follistatin, or at least the isoforms detected in the assays, does not have an endocrine role during the oestrous or menstrual cycle.

Follistatin concentrations measured in human maternal plasma using a variety of assays increase as pregnancy progresses, particularly in the last trimester (Wakatsuki *et al.*, 1996; Woodruff *et al.*, 1997; Fowler *et al.*, 1998; O'Connor *et al.*, 1999; Schneider-Kolsky *et al.*, 2000). In contrast, the plasma concentrations of follistatin in sheep did not increase during pregnancy but instead decreased slightly. The decrease in follistatin concentrations during gestation in sheep was unexpected but may indicate that there are species differences in the physiology of follistatin during pregnancy. The secretion of inhibin A does not increase during the follicular phase of the oestrous cycle in ewes (Knight *et al.*, 1998), in contrast to the increase observed during the human menstrual cycle (Groome *et al.*, 1994) and the rat oestrous cycle (Fahy *et al.*, 1996). During pregnancy in ewes, plasma inhibin A concentrations decreased at day 60 and remained low until late in gestation (Findlay *et al.*, 1991; Knight *et al.*, 1998), whereas in humans circulating inhibin A concentrations increase, particularly in the third trimester (Muttukrishna *et al.*, 1995).

Follistatin content in the allantoic fluid compartment was highest early in gestation and decreased significantly ($P < 0.01$), as determined by log-linear regression analysis, as pregnancy progressed. In contrast, the concentrations of follistatin in amniotic fluid were highest at mid-gestation and then decreased until term. These findings are similar to data from human pregnancies showing that the concentrations were higher near mid-gestation and significantly lower near term (Evans *et al.*, 1998). The source of follistatin in these fluids is likely to be the fetal membranes (Keelan *et al.*, 1999) or the placenta (de Kretser *et al.*, 1994a; Petraglia *et al.*, 1994b; Yokoyama *et al.*, 1995) or both, which have been shown to produce follistatin. The difference in patterns of concentrations between the allantoic and the amniotic fluids implies that the source of the follistatin in the compartments is not the same and is differentially regulated.

Follistatin concentrations in plasma from sheep fetuses were up to three times higher than matched samples of maternal plasma. Follistatin is expressed by a number of tissues in the fetus (Roberts and Barth, 1994) and expression may be increased in these tissues to modulate the effects of activins and other transforming growth factor β (TGF- β) family members during development. Evidence from studies of liver after partial hepatectomy show that follistatin mRNA is upregulated and that exogenous intravenous follistatin promotes more rapid regrowth of the liver than in untreated animals (Kogure *et al.*, 1995, 1996).

Follistatin concentrations increased significantly on the day of parturition and remained high for at least 24 h before decreasing on day 3 after parturition. This profile is very similar to that observed after surgery (Klein *et al.*, 1993) or after injection of interleukin 1 β (Phillips *et al.*, 1996). Inflammatory cytokine concentrations increase during labour in humans (Opsjn *et al.*, 1993) and stimulate activin A secretion by the placenta (Mohan *et al.*, 2001). However, the observation that follistatin concentration was increased in maternal plasma after the expulsion of the fetal mem-

branes indicates that the source of follistatin is probably maternal rather than fetal. This increase in follistatin after parturition may be due to the tissue damage and cytokine cascade resulting from the birth and it is possible that follistatin is not directly implicated in parturition.

After birth, plasma concentrations of follistatin in the lambs were increased compared with concentrations a few days before parturition. These high concentrations were significantly greater than those observed in matched maternal plasma and remained high for at least 7 days after birth. Although similar data are not available from other species, a study by Kettel *et al.* (1996) indicates that follistatin is higher in adults and postmenopausal women than in girls in early adolescence. Although these results are not directly comparable, they do indicate that circulating follistatin may be dynamically regulated during development and that sexual maturation may occur in response to the changing concentrations of activin.

A number of other follistatin-like molecules have been reported (Zwijssen *et al.*, 1994; Hayette *et al.*, 1998; Tsuchida *et al.*, 2000) that may crossreact in the follistatin assays used in the present study. Of these, the follistatin-like protein (FLRG) is most closely related (Hayette *et al.*, 1998); it has two follistatin domains that are able to neutralize the bioactivity of activin (Tsuchida *et al.*, 2000). It is also highly expressed in a range of tissues including the placenta (Tsuchida *et al.*, 2000); thus, if it crossreacts in a follistatin assay it will contribute to the overall concentrations reported. Until these proteins have been tested for crossreactivity in the various assays used to measure follistatin and until our knowledge of their physiology improves some caution must be taken when interpreting the current follistatin assay data.

In conclusion, the results of the present study indicate that follistatin has an important role during development and early neonatal life, and that the follistatin dynamics during pregnancy in sheep are not similar to those in humans. Hence, in this respect, the sheep is perhaps not a good model of human pregnancy. However, undertaking these comparative studies is likely to enhance our understanding of the roles of follistatin and activin during pregnancy and parturition in general.

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