Amniotic fluid concentrations of dimeric inhibins, activin A and follistatin in pregnancy

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Abstract

Objective: The feto-placental unit is the major source of circulating concentrations of inhibin A and activin A in human pregnancy. The aim of this study was to measure the amniotic fluid concentrations of inhibin A, inhibin B, activin A and follistatin in pregnancies bearing male and female fetuses.

Design and Method: Amniotic fluid samples collected by amniocentesis were stored at -20°C . Dimeric inhibins, 'total' activin A and 'total' follistatin were measured using specific two-site enzyme immunoassays. Samples were assayed blindly and the information on fetal sex was obtained from the cytogenetics laboratory.

Results: Data show that amniotic fluid concentrations of inhibin A, inhibin B and activin A gradually increase with gestation whilst concentrations of follistatin are similar between weeks 15 and 20 of pregnancy. Mean amniotic fluid levels of inhibin A and inhibin B at 16 and 17 weeks gestation and mean activin A levels at 15 and 16 weeks gestation are considerably lower in pregnancies with male ($n = 24$) compared with female ($n = 28$) fetuses. Levels of follistatin are not different in the male and female fetal pregnancies at any studied gestation.

Conclusions: The results indicate that amniotic fluid contains high concentrations of inhibins (A and B), activin A and follistatin in early pregnancy suggesting that these hormones are produced by the fetal membranes and may be involved in the development of the fetus.

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Introduction

Inhibins are glycoprotein hormones consisting of two dissimilar subunits. Inhibin A is an α - β_A dimer and inhibin B is an α - β_B dimer. Activins are homodimeric glycoprotein hormones. Activin A is a β_A - β_A dimer, activin AB is a β_A - β_B dimer and activin B is a β_B - β_B dimer. Follistatin is a structurally distinct single chain glycoprotein hormone with functional similarity to inhibin. Within the last decade follistatin has also been identified as a high affinity activin binding protein (1).

Classically, inhibins and activins were isolated and purified based on their effect on pituitary follicle-stimulating hormone (FSH) secretion. Inhibins inhibited pituitary FSH secretion and activins stimulated FSH secretion. Inhibins, activins and follistatins were initially purified and characterised from ovarian follicular fluid in different species. Extragonadal sources have also been identified for these proteins (2). Development of specific assays for inhibin A (3, 4), inhibin B (5), activin A (6), activin AB (7) and follistatin (8) has enabled us to measure the specific molecular form of dimeric protein.

Using these new specific assays, high concentrations of inhibin A (9) and activin A (10) have been measured throughout pregnancy. However, inhibin B concentrations in maternal circulation during pregnancy have been reported to be very low, with levels close to or at the detection limit of the assay (11, 12). Studies in early pregnancy support a feto-placental origin for inhibin A and activin A (13–15). Messenger RNA for inhibin α subunit has been identified in the syncytiotrophoblast and inhibin/activin β_A and β_B mRNAs have been identified in both cytotrophoblast and syncytiotrophoblast in the placenta (2). Immunocytochemical staining has also shown the presence of the α subunit, β_A subunit and the β_B subunit protein in syncytiotrophoblast (16, 17) and the decidua (18).

Recently two studies have reported amniotic fluid levels of inhibin A and inhibin B in normal (19) and abnormal pregnancies affected with Down's syndrome (20). However, levels of activin A and follistatin have not been studied in the amniotic fluid in the second trimester. The aim of this study was to investigate the concentrations of inhibin A, inhibin B, activin A and follistatin in amniotic fluid of pregnancies bearing male and female fetuses in the second trimester.

Materials and methods

Amniotic fluids

Amniotic fluid samples ($n=146$) were collected at amniocentesis performed for clinical diagnostic reasons. Amniotic fluid was centrifuged to remove any fetal cells and the supernatant was frozen at -20°C until assayed for inhibin A, inhibin B, activin A and follistatin. All samples analysed were from genetically normal fetal pregnancies. Samples were analysed blind and the information on fetal sex was subsequently obtained from the cytogenetics laboratory. The results of assays from female fetus bearing pregnancies and male fetus bearing pregnancies were analysed to study any difference in levels of inhibins, activin A and follistatin. For statistical analysis, samples were separated into male ($n=13$, $n=28$, $n=15$, $n=7$, $n=5$) and female ($n=12$, $n=24$, $n=24$, $n=10$ and $n=8$) fetus bearing pregnancies at 15, 16, 17, 18 and 20 weeks respectively. Gestational age was determined by ultrasound and the date of the last menstrual period.

Hormone assays

Inhibin A Amniotic fluid concentrations of dimeric inhibin A were measured in duplicate $10\ \mu\text{l}$ aliquots as described elsewhere (4). The mean intra- and interassay coefficients of variation were 4.3% and 5.1% respectively. The minimum detection limit of the assay for human recombinant inhibin A (NIBSC, South Mimms, UK) was 2 pg/ml.

Inhibin B Amniotic fluid concentrations of dimeric inhibin B were measured in $10\ \mu\text{l}$ duplicate aliquots using an enzyme immunoassay (EIA) as described in detail elsewhere (5). An in-house standard preparation (partially purified human follicular fluid) was standardised against human recombinant inhibin B (Genentech, San Francisco, CA, USA) and was used as the assay standard. The minimum detection limit of the assay for human recombinant inhibin B was 10 pg/ml. The mean intra- and interassay coefficients of variation were 6.2% and 7.2% respectively.

Follistatin Amniotic fluid concentrations of 'total' follistatin (follistatin 288) were measured in duplicate as previously described (8). The minimum detection limit of the assay for human recombinant follistatin standard was 20 pg/ml. The mean intra- and interassay coefficients of variation were <10%. The assay cross reacts 9.9% with follistatin 315.

Activin A Amniotic fluid concentrations of 'total' activin A were measured using an EIA specific for 'total' activin A as described in detail elsewhere (6). The mean intra- and interassay coefficients of variation were 6.5% and 7.7% respectively. The minimum detection

limit of the assay for human recombinant activin A (Genentech) was 50 pg/ml.

Statistical analysis

Unpaired Student's *t*-tests were carried out to investigate the difference in amniotic fluid concentrations of the hormones between male and female fetus bearing pregnancies at a particular gestation. One-way analysis of variance with Bonferroni Dunn *posthoc* test was carried out to investigate the changes in levels across the gestations. Correlation analysis (Pearson correlation) was used to study the relationship between the hormones with increasing gestation time.

Results

Amniotic fluid levels of inhibin A increased with gestation from 15 to 18 weeks and then remained similar up to 20 weeks (Fig. 1a). Levels of inhibin A tended to be higher in female (0.68 ± 0.08 ng/ml; 1.19 ± 0.6 ng/ml respectively) than in male (0.55 ± 0.05 ng/ml; 0.75 ± 0.12 ng/ml respectively) bearing pregnancies at 16 and 17 weeks gestation, but this difference did not reach statistical significance (Fig. 1b and Table 1).

Inhibin B concentrations in the amniotic fluid increased progressively from 15 to 20 weeks ($P < 0.001$; ANOVA; Fig. 2a). Although levels of inhibin B also appeared higher in the amniotic fluid from female fetus bearing pregnancies (0.26 ± 0.04 ng/ml, 0.42 ± 0.06 ng/ml, 0.66 ± 0.24 ng/ml and 0.6 ± 0.17 ng/ml

Figure 1 (a) Scatter plot of individual concentrations of amniotic fluid inhibin A at different gestations. (b) Mean $\pm$ S.E.M. concentrations of inhibin A in male (open bars) and female (solid bars) fetus bearing pregnancies.

Table 1 Concentrations of inhibin A, inhibin B, activin A and follistatin in male and female bearing pregnancies from 15 to 20 weeks gestation. Results are means $\pm$ S.E.M.

	Weeks gestation				
	15	16	17	18	20
Inhibin A (ng/ml)					
Female	0.32 $\pm$ 0.06	0.68 $\pm$ 0.08	1.19 $\pm$ 0.6	0.68 $\pm$ 0.08	0.71 $\pm$ 0.12
Male	0.36 $\pm$ 0.06	0.55 $\pm$ 0.05	0.75 $\pm$ 0.12	0.55 $\pm$ 0.05	0.56 $\pm$ 0.3
Inhibin B (ng/ml)					
Female	0.09 $\pm$ 0.02	0.26 $\pm$ 0.04	0.42 $\pm$ 0.06	0.66 $\pm$ 0.24	0.6 $\pm$ 0.17
Male	0.15 $\pm$ 0.04	0.2 $\pm$ 0.04	0.26 $\pm$ 0.07	0.32 $\pm$ 0.09	0.41 $\pm$ 0.19
Activin A (ng/ml)					
Female	5.17 $\pm$ 0.62	5.8 $\pm$ 0.8	4.5 $\pm$ 0.48	5.8 $\pm$ 0.8	8.9 $\pm$ 1.7
Male	3.9 $\pm$ 0.06	4.84 $\pm$ 0.39	4.89 $\pm$ 0.62	7.39 $\pm$ 0.6	5.21 $\pm$ 2.4
Follistatin (ng/ml)					
Female	1.8 $\pm$ 0.12	1.57 $\pm$ 0.12	1.48 $\pm$ 0.1	1.49 $\pm$ 0.19	1.4 $\pm$ 0.15
Male	1.63 $\pm$ 0.16	1.93 $\pm$ 0.15	1.62 $\pm$ 0.18	1.57 $\pm$ 0.6	1.36 $\pm$ 0.52

respectively) than from male fetus bearing pregnancies (0.2 $\pm$ 0.04 ng/ml, 0.26 $\pm$ 0.07 ng/ml, 0.32 $\pm$ 0.09 ng/ml and 0.41 $\pm$ 0.19 ng/ml respectively) from 16 to 20 weeks, the difference was not statistically significant (Fig. 2b and Table 1).

Total activin A levels also rose slightly from 15 to 20 weeks gestation (Fig. 3a) but with no statistical significance. Activin A levels also appeared to be higher in female fetus bearing pregnancies (5.17 $\pm$ 0.62 ng/ml, 5.81 $\pm$ 0.81 ng/ml respectively) than in male fetus bearing

pregnancies (3.9 $\pm$ 0.06 ng/ml, 4.84 $\pm$ 0.39 ng/ml) at 15 and 16 weeks gestation (Fig. 3b and Table 1) but the difference was not statistically significant.

Follistatin levels did not alter between 15 and 20 weeks gestation (Fig. 4a). Levels of follistatin were also similar in male and female fetus bearing pregnancies (Fig. 4b and Table 1) at the studied gestations.

Correlation analysis showed that amniotic fluid concentrations of inhibin A and follistatin were not significantly correlated with any other parameters

Figure 2 (a) Scatter plot of individual concentrations of amniotic fluid inhibin B at different gestations. (b) Mean $\pm$ S.E.M. concentrations of inhibin B in male (open bars) and female (solid bars) fetus bearing pregnancies. $P < 0.001$ (ANOVA) – significant increase with gestation. **Figure 3** (a) Scatter plot of individual concentrations of amniotic fluid activin A at different gestations. (b) Mean $\pm$ S.E.M. concentrations of activin A in male (open bars) and female (solid bars) fetus bearing pregnancies.

Figure 4 (a) Scatter plot of individual concentrations of amniotic fluid follistatin at different gestations. (b) Mean $\pm$ S.E.M. concentrations of follistatin in male (open bars) and female (solid bars) fetus bearing pregnancies.

measured. Inhibin B levels were significantly correlated with activin A levels ($r = 0.2$, $P < 0.02$).

Discussion

We have investigated the amniotic fluid concentrations of inhibin A, inhibin B, activin A and follistatin from 15 to 20 weeks gestation and in male and female fetus bearing pregnancies. This is the first study to report activin A and follistatin levels in the amniotic fluid in the second trimester of male and female bearing pregnancies. Maternal serum concentrations of inhibin A and activin A have been reported to be high in the circulation throughout pregnancy (9, 10). However, inhibin B levels are low and near the detection limit of the assay (11, 12). In pregnancy, the major source of inhibin A and activin A is the fetoplacental unit (13–15). However, the fetoplacental source of inhibins and activin A in maternal serum could be different from the source of amniotic fluid inhibins and activin A because inhibin B is not detectable in maternal circulation but is present in high quantities in the amniotic fluid.

Studies have been carried out on the cellular localisation of inhibin/activin subunits using specific antibodies to the α , β_A and β_B subunits in the placenta. In the first and second trimester placenta, positive staining for α and β_A subunits was clearly observed in the syncytial layer, with very faint staining for β_B subunits (16). The epithelial layer of the amnion

showed an intense fluorescent staining for β_B subunit and positive signals were also observed for the α and β_A subunits (21, 22). Maternal decidual cells stained with both inhibin α and β_B antisera showed a similar localisation. Cells stained with β_A subunit were sparse and followed a distribution pattern different from that of cells stained with α or β_B antisera (18).

This study's data are consistent with other reports showing that high concentrations of inhibins (19, 20) and 'total' activin A (23) are present in the amniotic fluid. In our study, we have investigated the levels of inhibins (A and B), activin A and follistatin in the amniotic fluid of male and female bearing pregnancies.

The follistatin assay used in this study essentially detects follistatin 288 and cross reacts considerably with follistatin 315 (9.9%). The amount of the different molecular forms of follistatin in the amniotic fluid is not known although follistatin 315 is reported to be the major follistatin form in human circulation (24). However, high concentrations (1–2 ng/ml) of follistatin 288 are present in amniotic fluid. This study shows that follistatin levels tend to decrease in amniotic fluid between 15 and 20 weeks gestation, in contrast to the pattern seen for inhibins and activin A. However, there is no significant correlation between follistatin and activin A or inhibins.

Measurements in peripheral serum collected from adult men have identified mainly inhibin B (25), activin A (6) and follistatin (8) with inhibin A levels close to the detection limit of the assay. In contrast, amniotic fluid contains high concentrations of inhibin A, inhibin B, activin A and follistatin whether the fetus is male or female. However, it is very interesting that inhibin B levels are high in the amniotic fluid and hardly detectable in the maternal circulation indicating that the source of these proteins in the maternal circulation is different from the source in the amniotic fluid. It will be interesting to investigate the levels of these proteins in the uterine vein and compare it with maternal circulating levels.

High concentrations of inhibins, activin A and follistatin in the amniotic fluid suggest a biological function for these proteins in this fetal compartment in pregnancy. Little is known about the physiological role of these proteins in pregnancy although the maternal circulating levels and amniotic fluid levels are higher in pregnancy compared with the menstrual cycle.

Activin-A is reported to play a role in early embryo development and mesoderm induction in other species. However, there are no reported studies on the effect of activin/inhibin on human embryo development (26).

In summary, amniotic fluid is a source of inhibin A, inhibin B, activin A and follistatin 288 and 315 in second trimester pregnancy. Levels of inhibin B progressively rise between 15 and 20 weeks gestation whereas inhibin A, activin A and follistatin levels do not change significantly. Levels of all proteins do not vary significantly between pregnancies bearing male and female fetuses.

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