Second trimester levels of maternal serum total activin A and placental inhibin/ activin α and βA subunit messenger ribonucleic acids in Down syndrome pregnancy

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Abstract

Objectives: Previous data have shown that inhibin A (α/βA) is increased about twofold in maternal serum samples from Down syndrome pregnancy. Our objectives were to determine whether activin A (βA/βA) was similarly increased in maternal serum from pregnancies affected with fetal Down syndrome, and to investigate whether increased expression of each inhibin/activin subunit occurred in placental tissue from cases of fetal Down syndrome.

Design and methods: Maternal serum total activin A levels were measured in 20 cases of fetal Down syndrome and 100 unaffected pregnancy samples. In addition, analysis of inhibin/activin α and βA subunit mRNA levels was performed in placental tissue extracts from six cases of fetal Down syndrome and six tissues with a normal karyotype.

Results: The median total activin A level in the Down syndrome cases was 0·82 MoM (multiples of the median); values did not differ significantly (P = 0.36, Mann–Whitney U analysis) from those in unaffected pregnancies. The inhibin α subunit/GAPDH mRNA ratio, but not that of βA subunit/ GAPDH mRNA, was significantly greater (P < 0.01, ANOVA) in placental tissue from Down syndrome than in control placental tissue.

Conclusions: Unlike inhibin A, activin A is not significantly increased in Down syndrome relative to unaffected pregnancy. Furthermore, increased amounts of maternal serum inhibin A in Down syndrome pregnancy probably result from increased placental expression of inhibin α, but not βA, subunit.

Introduction

Down syndrome, trisomy 21, is the leading cause of severe mental retardation in industrialized countries. During the second trimester of pregnancy, fetal Down syndrome is associated with increased maternal serum levels of a variety of secretory products of the placenta, including progesterone, human placental lactogen, schwangerschaftsprotein 1, human chorionic gonadotropin (hCG), and the free α and β subunits of hCG (1). hCG and free β-hCG are now commonly used in screening serum for fetal Down syndrome and their levels in Down syndrome are, on average, about double those in unaffected pregnancies (2). Recently, the amounts of another placental protein, inhibin A were also shown to be about twice as great in maternal serum from Down syndrome than in serum from unaffected pregnancies (3–8). This increase in serum inhibin A is important clinically: inhibin A increases the prenatal detection of Down syndrome by 7–22% when used as a serum marker in conjunction with hCG and alpha fetoprotein, with or without unconjugated estriol (uE3).

Inhibin A is a dimeric protein consisting of an α and βA subunit. In addition to the inhibin A heterodimer, free α subunits (8, 9) and a βA subunit homodimer (10–12), called activin A, circulate in maternal serum. As activin A is another placental protein (12) with a putative role in fetal development, an objective of the present study was to determine whether activin A was also increased in maternal serum of pregnancies affected with fetal Down syndrome.

The mechanisms leading to increased inhibin A in Down syndrome are unknown, but could include increased gene expression of one or both subunits, increased protein translation or a lower rate of protein degradation. Each inhibin subunit is translated from a separate gene (13), but as is true of other placental

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proteins showing increased serum levels during Down syndrome pregnancy, neither is located on chromosome 21. As both inhibin $\alpha$ and $\beta A$ mRNA have been demonstrated in the placenta (14–16), a second objective of the present study was to explore whether increased expression of each inhibin subunit occurred in placental tissue from cases of fetal Down syndrome.

Material and methods

Serum samples
Twenty cases of fetal Down syndrome, from which there was available sufficient serum in freezer storage, were identified among samples from women who had second-trimester triple-marker screening at Women and Infants Hospital between April 1992 and January 1994. Each case sample was matched to five samples from unaffected singleton pregnancies for duration of freezer storage (about 1 week), and all samples were collected during 15–20 completed weeks of gestation. Levels of inhibin A were previously found to be increased (1.95 MoM) in this set of Down syndrome maternal serum samples (8).

Total activin A assay
All serum samples were coded and sent from Women and Infants Hospital to the University of Pisa for analysis, so that the assay could be performed without knowledge of which samples were cases and which were controls. A two-site ELISA (10) was used to measure activin A in both free and follistatin-bound complexes (total activin A). The colorimetric signal was developed using a commercially available ELISA amplification system (ImmuNo Select ELISA Amplification System, Dako, Milan, Italy) according to the manufacturer’s instructions. The activin A plates were read at 490 nm on an automated EIA plate reader (Basic Radim Immunoassay Operator (BRIO), Radim Spa, Pomezia, Italy). The assay limit of detection was <100 pg/ml. The intra- and interassay coefficients of variation were <7%.

Tissue preparation
Placental tissues (trophoblast and amnion/chorion membranes) were collected at 18–20 weeks of gestation from six women carrying fetuses affected with Down syndrome as previously assessed by amniocentesis, and from six pregnant women with normal tissue karyotype as controls. Permission to obtain the tissues was granted by the Human Investigation Committee of Pisa.

Amnion/chorion membranes were peeled from the decidua and then decidua was carefully dissected from the chorion. Trophoblast and amnion/chorion membranes were macroscopically identified and a histological evaluation was carried out in parallel. The pieces of trophoblast and membranes were immediately treated for extraction of total RNA according to the method of Chomczynski & Sacchi (17). Total RNA extracted from trophoblast, amnion and chorion membranes was quantified by UV absorption at 260 nm.

Reverse transcriptase-polymerase chain reaction
The presence of inhibin/activin $\beta A$, inhibin $\alpha$ subunits and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs was demonstrated by amplifying respective target sequences by PCR according to the instructions provided with the GeneAmp amplification reaction kit (Perkin Elmer, Milan, Italy). One microgram of total RNA was reverse transcribed to prepare cDNA. PCR was performed on the entire cDNA product with Taq (Thermus aquaticus) DNA polymerase and the manufacturer’s recommended buffers.

Reaction conditions for reverse transcription were as follows: 1 mmol/l deoxyxynitrophenyl triphosphate, 1 unit RNAsin, 100 pmol random hexamers and 200 units reverse transcriptase. The reaction was run at 42 °C for 1 h. The mixture was then heated at 99 °C for 5 min and quick-chilled on ice.

Specific oligonucleotide primers designed to amplify sequences of inhibin/activin $\beta A$, inhibin $\alpha$ subunit (18) and GAPDH (19) cDNAs are shown in Table 1. Computer analysis performed to study the possible secondary structure of the different cDNAs and to compare the synthesized oligomers with the human sequences in the MicroGenie (Beckman, Palo Alto, CA, USA) gene database bank revealed no more than 74% homology in the former and 72% in the latter among all other genes. Sequence homology among the different oligomers used in the present study was also avoided, excluding possible cross-reactions. In each

| Table 1 Sequence of GAPDH, inhibin/activin $\beta A$ and inhibin $\alpha$ subunits sense and antisense primers. |
|-----------------------------|----------------------------------|
| GAPDH, sense               | 5’-TGATGACATCAAGAAGGTTGTGAAG-3’ |
| GAPDH, antisense           | 5’-TCCTTGGAGGCCATGTGGGCCAT-3’  |
| $\beta A$, sense           | 5’-GTTTGCGCATCGAGAAGACG-3’     |
| $\beta A$, antisense       | 5’-GAGGTTGGCAAGGGCTATGGGCCGCAT-3’ |
| $\alpha$, sense            | 5’-CAGCCACAGATGCCAGCTGT-3’     |
| $\alpha$, antisense        | 5’-CTCCGGAGGCCCTCTGACAGGCCCAG-3’ |
amplification reaction. 15 pmol [γ³²P]ATP end-labeled correspondent primers were used. Amplification products were visualized on a 4% agarose gel. The gel was stained with ethidium bromide and viewed on an ultraviolet light box. To estimate differences in mRNA expression, the cycle number for each cDNA was determined in preliminary experiments in order to achieve the exponential phase and to avoid the plateau effect. In all the amplification procedures, the negative control was a blank prepared using all reagents and substituting 2 µl water for RNA.

For the βA subunit, cDNA amplification was performed with 24 thermal cycle steps (94°C, 1 min; 60°C, 1 min; 72°C, 3 min). For the α subunit, cDNA amplification was performed with 24 thermal step cycles (94°C, 1 min; 60°C, 1 min; 72°C, 1 min). For GAPDH, nine thermal cycles (94°C, 20 s; 59°C, 5 s; 75°C, 70 s) were followed by 20 cycles (94°C, 20 s; 58°C, 10 s; 75°C, 70 s). An aliquot of the PCR mixture was run on an 8% acrylamide gel and stained with ethidium bromide. Bands were cut out and dissolved in 0.025 mol/l periodic acid at 50°C for 60 min to dissolve the gel completely before counting in a β-scintillation counter. Recovery of radioactivity from the gel was 95%. Data were expressed as the ratio of inhibin subunit cDNAs to their correspondent GAPDH cDNA. mRNAs were then estimated using the ratio of βA and α subunit to GAPDH. The experiments were repeated at least three times and qualitatively similar results were always obtained.

Results

Total activin A serum levels

Levels of maternal serum total activin A were compared in 100 unaffected pregnancies and in 20 Down syndrome pregnancies between 15 and 20 weeks of gestation. Activin A levels did not differ significantly (P = 0.36, Mann–Whitney U analysis) between unaffected (median = 1.45 ng/ml, range = 0.3–7.0 ng/ml) and Down syndrome (median = 1.20 ng/ml, range = 0.4–3.0 ng/ml) pregnancies. Total activin A levels were not significantly correlated with gestational age in the time period studied (r = 0.10, P = 0.35), and therefore a single median value was determined for the unaffected pregnancies. The MoM (multiples of the median) values in the Down syndrome cases and controls are shown in Fig. 1. The median total activin A value in the Down syndrome cases was 0.82 MoM.

Expression of inhibin α and inhibin/activin βA mRNAs

The inhibin α subunit/GAPDH mRNA ratio was significantly greater (P = <0.01, ANOVA) in placental tissue from Down syndrome (0.11 ± 0.02) than in control tissue (0.04 ± 0.01; Fig. 2, left panel). In contrast, there was no significant difference in the βA subunit/GAPDH mRNA ratio in Down syndrome (0.52 ± 0.27) and control (0.69 ± 0.09) placental tissue (Fig. 2, right panel). A representative photograph of the RT-PCR results is shown in Fig. 3.

Discussion

The measurement of inhibin A (α/βA) is a useful addition to second-trimester screening of maternal serum for fetal Down syndrome, showing approximately twofold increases in affected pregnancies (3–8). The present results demonstrate increased amounts of α subunit mRNA in placental tissue from Down syndrome pregnancies, suggesting that increased α subunit expression is part of the mechanism leading to increased levels of inhibin A in serum. Furthermore, previous data have shown that circulating levels of precursor forms of the inhibin α subunit are not significantly different in Down syndrome and unaffected pregnancies (8). These data, taken together, suggest that when α subunit expression is upregulated, it is preferentially post-translationally processed from the precursor form to dimeric inhibin A in Down syndrome pregnancy.

In contrast to α subunit mRNA levels, no differences were seen in the amounts of βA subunit mRNA between Down syndrome and unaffected placental tissue. Similarly, there were no significant differences observed in second-trimester maternal serum levels of the βA homodimer, activin A, in Down syndrome and unaffected pregnancies. These results are supported by a previous preliminary study of total activin A in a separate, smaller sample set in which minimal differences were observed between maternal levels of serum activin in Down syndrome (1.25 MoM) and unaffected pregnancies (20). In addition, using a less specific activin assay that does not measure activin–follistatin...
complexes, levels of follistatin-free activin were not significantly different in Down syndrome and unaffected maternal serum samples (8). There is no assay available currently to measure $\beta_A$ monomer specifically, and no data showing that the free $\beta_A$ subunit of inhibin/activin circulates as a monomer in maternal serum.

Overall, these data suggest that increased $\alpha$, but not $\beta_A$, subunit expression in placenta may contribute to the twofold increase in maternal serum inhibin A seen in Down syndrome pregnancy. However, not all mRNAs are translated into protein. Although the secretion of inhibin and activin from placental tissues in culture was not directly studied, the results in serum suggest that the production of inhibin and activin protein does parallel the levels of mRNA found in second-trimester placental tissues. Co-localization of inhibin $\alpha$ and $\beta_A$ subunit mRNAs in placental cells (21, 22) may facilitate inhibin A secretion during pregnancy. In addition, levels of translation or the relative stability of the $\alpha$ and $\beta_A$ subunit mRNAs may influence the ratio of inhibin A to activin A production. In fact, recombinant cells expressing $\alpha$ subunit in excess of $\beta_A$ subunit preferentially produced inhibin A over activin A (13), a finding consistent with the mRNA and serum results observed in Down syndrome pregnancy.

The mechanisms driving inhibin A production in Down syndrome pregnancy are unknown. Some biologically active molecules with the potential to regulate inhibin release from human placental cells have been identified using in vitro models. For example, hCG, cAMP, prostaglandin, epidermal growth factor, transforming growth factor-$\alpha$, dexamethasone, gonadotropin-releasing hormone, epinephrine and phorbol ester have been shown to stimulate the release of inhibin from cultured placental cells (reviewed in (23)). Any one or a combination of these factors may be involved in regulating inhibin A production or secretion during pregnancy.

In summary, total activin A levels were not significantly different in Down syndrome and unaffected pregnancy. $\beta_A$ subunit mRNA levels also were not different, but those of inhibin $\alpha$ subunit mRNA were significantly increased in Down syndrome placental tissues compared with those of a normal karyotype. Taken together with previous studies, these data suggest that levels of inhibin A, but not of activin A, are a useful serum marker for detection of fetal Down syndrome, and that increased serum inhibin A probably results from increased expression of inhibin $\alpha$ subunit mRNA in the placenta.

Figure 2 Inhibin and activin subunit mRNA levels in Down syndrome (hatched bars) and unaffected, control (open bars) placental tissues. Left panel: inhibin $\alpha$ mRNA levels relative to GAPDH mRNA. Right panel: inhibin/activin $\beta_A$ mRNA levels relative to GAPDH mRNA. *$P<0.01$.

Figure 3 Representative example of the expression of inhibin $\alpha$ and activin $\beta_A$ subunit mRNAs by RT-PCR in trophoblasts of a normal karyotype (lane 1) and a Down syndrome tissue (lane 2). Lanes 3 and 4 are positive (term pregnancy, 40 weeks gestation) and negative (run without template) controls respectively. The size marker (M) is BioMarker LOW from Bio Ventures Inc (Irvine, CA, USA).
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