Inhibin and activin production and subunit expression in human placental cells cultured in vitro

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Introduction

Inhibins and activins are dimeric proteins of the transforming growth factor (TGF) β superfamily (Massague, 1990; Roberts et al., 1990). They are a combination of three related protein subunits (α, βA or βB). The mRNA levels of these subunits were studied quantitatively during in-vitro differentiation of human cytotrophoblast cells into syncytium, using Northern blot analysis and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. The corresponding protein concentrations were determined by specific enzyme-linked immunosorbent assays for inhibin A, B, pro αC and activin A in cellular protein extracts and culture medium (n = 5). Immunofluorescence studies showed syncytium formation after 48 h. The α subunit was present before plating and increased at 48 h (P < 0.001) while the βA subunit was weak before plating and increased at 24 h. The βB subunit was not detected. With respect to corresponding protein synthesis, inhibin A (α + βA) had risen after 48 h in cellular protein extract and after 72 h in culture medium, while activin A (βA + βB) was detected after 24 h, with no significant variations in culture medium. There was a good correlation between inhibin A and α subunit expression (r = 0.736, P < 0.001), as well as between activin A and βA subunit expression (r = 0.755, P < 0.001). This study showed that mRNA expression parallels protein synthesis of inhibin and activin in trophoblast cells. Inhibin A synthesis appears to be dependent on α subunit mRNA expression, rather than on the βA subunit which controls activin A synthesis. This study has also shown that isolated cytotrophoblast cells do not produce dimeric inhibin. However, during the transformation of cytotrophoblast cells into syncytium, βA subunit mRNA expression may be an indicator of cell fusion.

Key words: activin/inhibin/mRNA/placenta/trophoblast

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remains unknown whether the production of these proteins parallels the level of their mRNA.

The in-vitro transformation of isolated cytotrophoblast cells into an endocrine active syncytiotrophoblast has been well-described (Kliman et al., 1986) and is widely used to study hormone synthesis and secretion from trophoblast cells. The aims of this study on human placental cells cultured in vitro were (i) to determine the relation between mRNA expression and inhibin and activin synthesis in vitro; (ii) to determine which cell population (cytotrophoblast or syncytiotrophoblast) produces these proteins; and (iii) to explore a possible relationship between syncytiotransformation and the mRNA expression level of inhibin and activin subunits.

Materials and methods

Cell culture

Term placenta (37–40 weeks pregnant) were collected immediately after elective Caesarean section, from five uncomplicated pregnancies. Cytotrophoblast cells were isolated using a modification of Kliman’s method (Kliman et al., 1986). Villous tissue was dissected free of membranes and main vessels, washed with ice-cold 9% NaCl and minced into small pieces. The tissue (~60 g) was then digested in 250 ml of 0.25% dispase II (Boehringer Mannheim, Mannheim, Germany) solution in Ca^2+-, Mg^2+-free Hank’s balanced salt solution (HBSS-CMF, GibcoBRL Life Technologies, Paisley, UK) for 45 min at 37°C in an oscillating water bath; 10 mg deoxyribonuclease I (Boehringer Mannheim) were then added and digestion allowed to proceed for a further 15 min. The tissue fragments were allowed to settle down for 1 min. The supernatant containing dispersed cells was decanted, filtered through a 100 μm nylon filter and centrifuged for 10 min at 300 g in four 50 ml tubes. The pelleted cells were resuspended in 8 ml of Iscove’s modified Dulbecco’s medium (IMDM; Gibco BRL Life Technologies) containing 2 mmol/l L-glutamine, 25 mmol/l HEPES (pH 7.4), 50 IU/ml penicillin and 50 μg/ml streptomycin, pooled, filtered through a 40 μm nylon filter and resuspended in 6 ml of the same medium. The isolated cells were then subjected to a further purification by density centrifugation with a discontinuous 5–70% Percoll (Pharmacia Biotech, Buckinghamshire, UK) gradient, made of 5% steps of 3 ml each by dilution of 90% Percoll in HBSS–CMF, in a 50 ml centrifuge tube. The digested cells were layered over this pre-formed Percoll gradient and centrifuged at 1200 g at room temperature for 20 min. The purified cytotrophoblasts were collected in the region corresponding to a density of 1.048–1.062 g/ml (estimated by the position of density marker beads run in a parallel Percoll gradient tube), washed with supplemented IMDM, and resuspended in 10 ml of 37°C trypsin solution (0.5 g/l porcine trypsin and 0.5 mmol/l EDTA. 4Na of HBSS; Sigma, St. Louis, MO, USA) for 1 min. The reaction was stopped with 5 ml of fetal calf serum (FCS; GibcoBRL Life Technologies) and placed on ice; another 5 ml FCS was layered in the bottom of the tube before centrifuging for 5 min at 1000 g at room temperature. The pelleted cells were washed and filtered through a 40 μm nylon filter. Finally they were resuspended in supplemented IMDM enriched with 15% FCS and antibiotics and counted by an haemocytometer. Cell viability was assessed by Trypan Blue exclusion.

The purified cytotrophoblasts were plated on 60 mm Primaria Petri dishes (Becton Dickinson, Bedford, MA, USA) at a density of 0.4×10^6 viable cells/cm^2 in 4 ml supplemented IMDM enriched with 15% FCS and cultured at 37°C in a humid atmosphere of 5% CO₂. The medium was changed daily and cells cultured for 5 days after plating. On each day, culture medium was recovered and kept frozen at −20°C until the assays were performed. Cells were lysed with TriReagent (Molecular Research Center, Cincinnati, Ohio, USA) and kept frozen at −80°C to further extract total RNA and protein. For immunofluorescence study, the cells were plated in Sonic Seal Slide wells (Nunc A/S, Roskilde, Denmark) at the same density in 1 ml supplemented IMDM. All experiments were repeated five times, each time on independently isolated trophoblast cell cultures.

Immunofluorescence

Trophoblast cultures were analysed at 24, 48 or 72 h after plating. Cells were rinsed with phosphate buffered saline (PBS) at 4°C and fixed for 10 min at 4°C in 100% methanol, rinsed again with PBS and stored at 4°C in PBS for no longer than 4 days. Preparations were preincubated in PBS containing 0.5% bovine serum albumin (BSA), 2.5% normal goat serum and 0.1% saponin for 15 min at 4°C, then incubated with primary monoclonal antibody diluted in the same solution overnight at 4°C. The following mouse primary antibodies (all from Sigma) were used: monoclonal anti-vimentin (1:200), monoclonal anti-desmosomal protein (1:400) and monoclonal anti-alkaline phosphatase (1:4000). The primary antibody was omitted in control slides. Cells were rinsed for 5 min six times in PBS containing 0.1% saponin, followed by a 1 h incubation at room temperature with fluorescein-conjugated goat anti-mouse immunoglobulin G F(ab')2 fragments (Boehringer Mannheim, 1:500 in the same solution). Cell nuclei were counterstained with 5 μg/ml propidium iodide in washing solution for 5 min at room temperature, followed by six rinses. Slides were mounted using VectaShield mounting medium (Vector Laboratories, Burlingame, CA, USA) and fluorescence was examined using confocal microscopy.

RNA and protein extractions

Total RNA was extracted from frozen samples using the acid guanidine thiocyanate–phenol–chloroform method (Chomczynski et al., 1987), quantified by absorbance at 260 nm, and divided into aliquots as ethanol/sodium acetate precipitate. Proteins were isolated by the same method, resuspended in PBS 1% sodium dodecyl sulphate (SDS), and quantified with the Bradford method using BSA as standard (BioRad Protein Assay; Biorad, Hercules, CA, USA).

Reverse transcriptase–polymerase chain reaction

We used the Superscript One-step reverse transcription–polymerase chain reaction (RT–PCR) system (GibcoBRL) to amplify target sequences of inhibin/activin βA and βB, inhibin α subunits and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference mRNA (Dveksler et al., 1992; Licht et al., 1993). Both cDNA synthesis and PCR were performed consecutively in a single tube using gene-specific primers from 1 μg total RNA in the presence of recombinant ribonuclease inhibitor (RNase Out; GibcoBRL). The 5’ sense and 3’ antisense oligonucleotides used as primers are listed in Table 1 (Voutilainen et al., 1991; Dveksler et al., 1992). RT–PCR was carried out using a DNA Thermal Cycler 9700 (Perkin-Elmer, Forester City, CA, USA) to obtain a single band after agarose electrophoresis. Specificity of the PCR products was checked by digestion with restriction enzymes. The cycle number necessary for an exponential phase of the PCR reaction was determined for each cDNA. For the α, βA subunits and GAPDH, reverse transcription was performed at 50°C for 30 min and cDNA amplification at respectively 25, 18, and 19 thermal cycle steps (94°C for 15 s; 56°C for 30 s; 72°C for 90 s). For the βB subunit, reverse transcription was performed at 54°C for 30 min and cDNA amplification was achieved with 30 thermal cycle steps (94°C for 15 s; 66°C for 30 s; 72°C for 90 s).
PCR products were quantified by ion-exchange high performance liquid chromatography (HPLC). Each PCR product was diluted in 25 mmol/l Tris–Cl, pH 8 and 25 mmol/l Tris–Cl, 1 mol/l NaCl, pH 8 was used as elution gradient. Chromatograms showed a single peak for each PCR product and the product level was estimated by peak surface measurement. Each inhibin subunit peak surface value was expressed as a ratio to their corresponding GAPDH peak surface value.

**Northern blot analysis**

For each sample, 15 μg of total RNA were size-fractionated by electrophoresis on 1% agarose/2.2 mol/l formaldehyde gel and transferred to Hybond-N nylon membranes (Amersham, Buckinghamshire, UK). The same 3′-antisense oligonucleotides used as primers in PCR reactions were used as probes. Those oligonucleotides were 3′ end-labelled with [α-32P]dATP (6000 Ci/mmol; Amersham) using terminal deoxynucleotidyl transferase and purified with Sephadex G50 columns (PharmaciaBiotech). Blots were prehybridized in 10% dextran sulphate, 1% SDS, 1 mol/l NaCl, and 25 μg/ml Torula yeast RNA (Voutilainen et al., 1991), 1 ml/10 cm2 membrane, at 60°C for 4 h. Hybridization was performed in the same solution after addition of the probe at 106 cpm/ml for 18 h. Membranes were washed four times for 20 min in 1× sodium chloride/sodium citrate (SSC), 0.1% SDS. Kodak BioMax Transcreen intensifying screen and Kodak BioMax MS film were used for autoradiography at −70°C for 10 days for inhibin subunits and 3 days for GAPDH.

 Autoradiograms were scanned and hybridization signals were estimated by densitometric analysis (Scan Analysis, Biosoft, UK) after background subtraction. Each inhibin subunit mRNA was expressed as the ratio of the corresponding GAPDH density in the same sample.

**Inhibin A, B, pro αC and activin A assays**

Activin A, inhibin A, B, and pro αC were tested with a two-monoclonal antibody solid-phase sandwich microtitre plate enzyme-linked immunosorbent assay (Serotec, Oxford, UK) as described (Fowler et al., 1998). The assay sensitivity was 100 pg/ml for activin A, 2 pg/ml for inhibin A, 15 pg/ml for inhibin B and 2 pg/ml for inhibin pro αC. Inter-plate and intra-plate coefficients of variation were all <10%. Each value was normalized to the protein content of the corresponding cell lysate.

**HCG, oestradiol, oestriol and progesterone assays**

HCG was tested using a chemiluminescent immunometric assay (DPC, Belgium) according to the manufacturer’s protocol. The assay has a sensitivity of 1.1 mIU/ml. Intra- and inter-assay variation coefficients were <10%. Oestradiol (BioMériel, France), oestriol and progesterone (Amerlex, Amersham, UK) were tested using radioimmunoassay, according to the manufacturer’s protocol. The sensitivities were 7 pg/ml, 0.08 ng/ml and 0.1 nmole/l respectively. The intra- and inter-assay coefficients of variation were: 6.3 and 8.8% for oestradiol, 2.7 and 6.7% for oestriol, 3.6 and 5.4% for progesterone. Each value was normalized to the protein content of the corresponding cell lysate.

**Statistical analysis**

Results are presented as mean ± SEM. Comparisons between days of culture were made using a one-way analysis of variance (Statview, Abacus concepts). In case of heterogeneous distributions (P < 0.05; analysis of variance), post-hoc tests (Fisher’s Protected Least Significant Difference) were used to compare subgroups. Correlation studies were performed with a correlation coefficient (Z-score).

**Results**

Isolation and purification of cytotrophoblast cells from term placenta yielded 1–2×10⁶ cells/g wet weight of tissue. This represented ~1–3% (by DNA content) of the placental tissue. The viability of these cells was >90% when assessed by Trypan Blue exclusion. By phase contrast microscopy, the cells appeared round (mean diameter 10–20 μm) with a large nucleus and a poorly developed cytoplasm. The protein content of the culture remained constant throughout the 5 day culture period (711 ± 19 μg per culture dish after 24 h, and 806 ± 55 μg per culture dish after 120 h, F = 2.157, P = 0.12).

Immunofluorescence study (Figure 1) showed that only 5.6 ± 0.6% of cells were labelled for vimentin (specific for cells of mesenchymal origin; Osborn et al., 1983), at 24 h. Most cells were positive for the desmosomal protein, but remained mononucleated at 24 h, showing a pavement-like pattern. At 48 h, immunofluorescence staining of desmosomal protein disappeared between some cell nuclei, as the cells became multinucleated. There was no difference between 48 and 72 h in syncytium formation. Although most cells were multinucleated, a limited number of mononucleated cells connected to syncytial cells by desmosomes were still present. Immunoreactivity of placental alkaline phosphatase antigen (specific for syncytiotrophoblast cells; Bulmer et al., 1985), increased with days in culture but not all syncytial cells expressed this antigen.

The mRNA expression level of the α, βA and βB subunits were first studied in isolated cytotrophoblast cells before plating and over the 5 days of cell culture. Northern blot analysis of the α subunit showed a major 1.54 kb mRNA and two minor mRNAs (3.15 and 5.51 kb). The reference GAPDH gene probe revealed a major 1.60 kb mRNA and a minor 3.24 kb mRNA. Significant variations of the mRNA expression level of the α subunit (F = 77.135, P < 0.001) were observed during the in-vitro transformation of cytotrophoblast cells into syncytium (Figure 2A and B). The expression was present before plating, with a slight increase after 24 h (P < 0.05). A marked increase was observed after 48 h (P < 0.001) with a maximum level after 72 h (P < 0.01) and a decrease was only observed at 120 h (P < 0.001). RT–PCR analysis showed similar results (F = 5.374, P = 0.003).
Northern blot analysis with the βA subunit probe showed a major 2.83 kb mRNA and five minor mRNAs (1.71, 3.6, 6.3, 7.2 and 11.6 kb). Significant variations of their expression over the culture period were also observed ($F = 20.263$, $P < 0.001$) (Figure 2A and B). βA subunit expression was almost undetectable before plating, but showed a marked increase after only 24 h ($P < 0.001$). Another slight increase could also be observed after 72 h ($P = 0.016$), followed by a decrease after 120 h ($P < 0.05$). RT–PCR analysis showed the same variations (data not shown). No βB subunit expression could be found either by Northern blotting, or by RT–PCR (data not shown).

The secretory function of cultured cells was then evaluated by HCG, oestradiol, oestriol and progesterone release in the medium (Figure 3). Different secretion profiles were observed. HCG (Figure 3A) showed large variations with days of cell culture ($F = 13.252$, $P < 0.001$): the secretion had started to rise after 48 h (not significant), increased after 72 h ($P < 0.001$), then declined ($P = 0.05$). Variations of oestradiol secretion (Figure 3B) were also highly significant ($F = 50.096$, $P < 0.001$) but the secretion increased after only 48 h ($P < 0.05$) with a maximum concentration after 72 h ($P < 0.001$) followed by a plateau. Oestriol was never detected in culture media. There were no significant variations in progesterone secretion (Figure 3C).

The capability of cytotrophoblast cells to produce and secrete inhibin A, B, pro αC and activin A during their syncytial transformation in vitro were then studied by measurement in culture media and cell protein extracts (Figure 3). Significant variations with days of culture were observed for inhibin A (Figure 3D; $F = 17.571$, $P < 0.001$) and inhibin pro αC (Figure 3E; $F = 10.524$, $P < 0.001$) in culture medium. Inhibin A was almost undetected after 24 h but had risen significantly after 72 h ($P < 0.001$) followed by a decrease observed after 120 h ($P < 0.05$). A similar profile was observed in cell protein extracts, with a significant rise after 48 h ($P < 0.001$) and a maximum concentration after 72 h ($P < 0.001$). Inhibin pro αC was detected from the first day of cell culture. After a small decrease after 48 h in cell culture medium (not significant), inhibin pro αC showed a significant peak after 72 h in both culture medium ($P = 0.005$) and cell protein extract ($P < 0.001$). Activin A was detected from the first culture day, showed significant variations over days of culture only in cell protein extracts ($F = 4.932$, $P < 0.005$) with a peak after 72 h ($P < 0.001$) and a decrease at the end of the culture ($P < 0.05$). No inhibin B was detected in either culture medium and cell protein extract over the 5 day culture period.

A relationship between gene expression and protein secretion was investigated using correlation analysis. With respect to inhibin A, there was a better correlation with the α subunit mRNA ($r = 0.736$, $P < 0.001$) compared to the βA subunit mRNA ($r = 0.420$, $P < 0.05$). Similarly, activin A showed a better correlation with the βA subunit mRNA ($r = 0.755$, $P < 0.001$) compared with the α subunit mRNA ($r = 0.494$,
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**Figure 2.** (A) Northern blot analysis. The main mRNA revealed with oligonucleotide probes for α, βA and GAPDH before plating (day 0) and over the 5 day culture period (days 1–5) are presented. (B) Expression levels of inhibin and activin subunits mRNAs before plating and over the 5 day culture period. Results are presented as density ratio to GAPDH mRNA hybridization after scan densitometry of Northern blot analysis (solid bars for α subunit mRNA and open bars for βA subunit mRNA). Values are presented as mean ± SEM, n = 5. Statistically significant differences compared with the mean value on the previous day, *P < 0.05 and **P < 0.001 respectively.

P < 0.05). Inhibin pro αC was correlated with the α subunit mRNA (r = 0.475, P < 0.05) but not with the βA mRNA.

Comparison between hormones tested in culture medium over the 5 days culture period showed highly significant correlation (P < 0.001) between HCG and oestradiol (r = 0.761), inhibin A and HCG (r = 0.879), inhibin A and oestradiol (r = 0.779), inhibin A and inhibin pro αC (r = 0.903) and activin A and progesterone (r = 0.796).

**Discussion**

To the best of our knowledge, this is the first quantitative report of inhibins and activin A expression in human trophoblast cells cultured *in vitro*, at both mRNA and protein levels. The method used to isolate and purify cytotrophoblast cells from term placenta derives from a well-established method (Kliman et al., 1986). We used dispase II rather than trypsin for tissue dissociation because it is less harmful to cell membranes (Green et al., 1979; Keelan et al., 1994). Membrane integrity is known to be essential for cell attachment and subsequent cell fusion, and this was confirmed in the present study.

Cell clumping was avoided by minimizing exposure time in trypsin (Karl et al., 1992), combined with filtration. As reported by others (Richards et al., 1994; Henson et al., 1996), isolated cells were also plated at high density to favour syncytium formation (Ringler and Strauss, 1990). The protein content in our cell culture conditions is comparable with other reports (Henson et al., 1996).

By vimentin immunolabelling, only 5% of cells in these cultures are of mesenchymal origin (fibroblasts, endothelial cells, macrophages, lymphoid cells; see Osborn and Weber, 1983). By 24 h after plating, most of the cells contain desmosomal protein, demonstrating epithelial origin (this antibody does not stain hemi-desmosomes). Syncytial transformation, suggested by the disappearance of desmosomal protein staining between adjacent cells, had occurred after 48 h of cell culture (Douglas and King, 1990). The increasing number of trophoblast cells expressing alkaline phosphatase antigen, specific for syncytiotrophoblast microvillous membrane (Bulmer and Johnson, 1985), parallels syncytium formation.

However, the 72 h delay before significant increase in HCG or oestradiol suggests step-wise differentiation of trophoblast cells *in vitro*: aggregation, syncytium formation and functional maturation, in agreement with other reports (Bax et al., 1989; Ringler and Strauss, 1990).

No expression of the βB subunit mRNA was found in placental cells cultured *in vitro*. This confirms that inhibin B during pregnancy is mainly produced by decidual cells (Petraglia et al., 1990) or by fetal membranes (Wallace et al., 1997; Riley et al., 2000). Previous studies yielded conflicting results about the cellular localization of inhibin products in placental tissue: syncytiotrophoblast for some (Minami et al., 1992; McCluggage et al., 1998), cytotrophoblast for others (Petraglia et al., 1987, 1991). According to the mRNA expression of inhibin subunits in isolated cytotrophoblast cells before plating, only a significant α subunit expression with almost no βA subunit mRNA expression can be detected. Cytotrophoblast...
cells are thus able to produce monomeric inhibin pro αC protein, but not dimeric inhibin A. The use of an antibody to α subunit explains the results shown in other studies.

Correlation studies between gene expression and protein secretion lead us to suspect that inhibin A synthesis is mainly dependent on the expression of the α subunit mRNA and not on the expression of the βA subunit mRNA, which determines activin A synthesis. There is a parallelism between protein production and mRNA expression during transformation in vitro of cytotrophoblast cells into syncytium.

In this model, the rise in mRNA expression of inhibin α subunit observed at 48 h coincides with syncytium formation and the rise of inhibin A in cell extracts. Inhibin A released in culture medium showed a significant increase only after 72 h. Therefore, the in-vitro differentiation of cytotrophoblast cells into syncytium shows multiple steps: cell aggregation, fusion into syncytium and production of a syncytial specific protein, followed by secretion of this protein in culture medium. We propose that α subunit mRNA expression is an early indicator of syncytium formation in vitro.

Activin A secretion and βA subunit mRNA expression show only a slight increase with syncytium formation. Activin A mRNA could therefore be an indicator of cytotrophoblast cell aggregation. This process requires cell movement and the presence of serum in the culture medium is thought to provide motility factors (Bloxam et al., 1997), although differentiation also occurs in serum-free medium when cells are cultured on extracellular matrix-coated surfaces (Ringler and Straws, 1990). The presence of activin receptors on cytotrophoblast cells have been demonstrated (Peng et al., 1993) and Caniggia et al. have shown that activin A stimulates the outgrowth of cytotrophoblast cells (Caniggia et al., 1997). Moreover, activin A activity is not counterbalanced by inhibin A at this time of the culture. Therefore, activin A could also be implicated in the syncytium formation process, particularly in the aggregation step.

The strong correlations between inhibin A and HCG or oestradiol, and between activin A and progesterone, suggest possible mutual interactions in their synthesis and secretion, as previously suggested for HCG and immunoreactive inhibin (Petraglia et al., 1987). Another possible explanation is the simultaneous synthesis of these hormones as the cells transform into syncytium.

In conclusion, this study shows that the mRNA expression

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**Figure 3.** Hormone secretion by trophoblast cells in culture. (A) HCG, (B) oestradiol, (C) progesterone, (D) inhibin A, (E) inhibin pro αC and (F) activin A in culture medium (open squares) and in cell protein extract (filled circles) were followed over the 5 day culture period (medium changed daily). Values are normalized to the protein content of cell lysates, and are means ± SEM, n = 5. Statistically significant differences compared with mean values on the previous day, *P < 0.05 and **P < 0.001 respectively.
of the α and βA subunits parallels inhibin and activin production in trophoblast cells. Inhibin A synthesis seems to be dependent on α subunit mRNA expression and not on βA subunit mRNA expression, the latter controlling activin A synthesis. Isolated cytotrophoblast cells from human term placenta are not able to secrete dimeric forms of inhibin or activin. During transformation of cytotrophoblast cells into syncytium, βA subunit mRNA expression may be an indicator of cell aggregation, while α subunit mRNA expression may be an indicator of cell fusion.

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