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N-glycans of human amniotic fluid transferrin stimulate progesterone production in human first trimester trophoblast cells *in vitro*

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Abstract

Aims: During pregnancy, the placenta produces a variety of steroid hormones and proteins. Several of these substances have been shown to exert immunomodulatory effects. Progesterone is thought to mediate some of these effects by regulating uterine responsiveness. The aim of this study was to clarify the effect of amniotic fluid transferrin and its N-glycans on the release of progesterone by first trimester trophoblast cells *in vitro*.
Methods: Cytotrophoblast cells were prepared from human first trimester placentae by trypsin-DNAse dispersion of villous tissue followed by a percoll gradient centrifugation and depletion of CD45 positive cells by magnetic cell sorting. Trophoblasts were incubated with varying concentrations (50–300 μg/ml) of transferrin from human amniotic fluid and serum as well as with N-glycans obtained from amniotic fluid transferrin. Culture supernatants were assayed for progesterone by enzyme-immunometric methods.
Results: The release of progesterone increased in amniotic fluid transferrin- and N-glycan-treated trophoblast cell cultures compared to untreated trophoblast cells. There was no stimulating effect of serum transferrin on the progesterone production of trophoblast cells.
Conclusions: The results suggest that amnion-transferrin and especially its N-glycans modulate the endocrine function of trophoblasts in culture by up regulating progesterone secretion.

Keywords: N-glycans; progesterone production; transferrin; trophoblast cells.

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Introduction

During pregnancy the immune response of the mother against certain antigens is reduced [5, 8]. One molecule out of a variety that regulate uterine immune responsiveness is progesterone [1, 7]. Siiteri et al. [18] found that progesterone inhibits the inflammatory response. The suppression of mixed lymphocyte culture responses by progesterone has been also described [2]. Due to its lipid solubility, progesterone incorporates into the lymphocyte plasma membrane interfering with membrane dynamics. This promotes a locally suppressed activity at the maternal-fetal frontier.

In most mammals, progesterone production is initially located in the corpus luteum of the ovary, shifting to the trophoblast as pregnancy proceeds. Progesterone biosynthesis in the human placenta is regulated by a variety of hormones. Progesterone synthesis is also regulated by estradiol-17-beta and auto regulated by progesterone [7] itself. Feinberg et al. [6] showed that progesterone production was significantly stimulated by interleukin-1 alpha (IL-1 α), interleukin-1 beta (IL-1 β) and tumor-necrosis-factor-alpha (TNF-α) in chorion carcinoma cells. In addition, progesterone plays also a role in maintenance of pregnancy. New results of Meis et al. [15] and da Fonseca et al. [4] show that administration of progesterone is able to prevent preterm delivery.

During gestation, the fetus requires large amounts of iron. The major iron source is maternal holo-serum transferrin [19, 22]. Transferrin-dependent iron transfer is mediated by transferrin receptors, which are present on the maternal-facing plasma membrane and on the fetal-facing basal plasma membrane of placental syncytiotrophoblasts [11, 22].

Transferrin is present in amniotic fluid in relatively high concentrations, showing a different glycosylation compared with serum transferrin [20]. The biological function of human amniotic fluid transferrin (hAFT) is still unknown. In addition, trophoblast cells also synthesize

transferrin [24], which shows a special glycosylation pattern [21]. We found identical carbohydrate structures of hAFT and trophoblast transferrin [20, 21]. In this study, we investigated the influence of hAFT and its N-glycans on the release of progesterone of first trimester trophoblast cells *in vitro*.

Material and methods

Purification of transferrin from amniotic fluid

Human amniotic fluid was dialyzed against 50 mmol/l NH_4HCO_3 buffer, pH 8.3 and fractionated on a DEAE-Sepharose CL-6B column (5×15 cm; Pharmacia, Uppsala, Sweden). The material was further purified on a Superdex 75 column (1.6×60 cm; Pharmacia). Buffer exchange was performed on a Sephadex G 25 column equilibrated with 50 mmol/l Tris-HCl, pH 7.2. Subsequently, transferrin-containing material was applied to a protein A column (2×1 ml, Pharmacia), equilibrated with 20 mmol/l NaH_2PO_4, pH 7.0 to remove remaining IgG. The elution was performed isocratically at a flow rate of 1 ml/min. The main fraction containing transferrin was pooled and used for the final purification. Monoclonal antibodies directed against human chorionic gonadotropin (1 mg, IgG1, clone 816, Dianova, Hamburg, Germany) were coupled to CNBr-Sepharose 4B (1 ml, Pharmacia). After blocking the remaining active groups with glycine, the material was loaded on a column and equilibrated with 20 mmol/l NaH_2PO_4, pH 7.0. The transferrin containing material was then applied to the column and eluted at a flow rate of 1 ml/min. The main fraction containing pure amniotic fluid transferrin was concentrated by ultra filtration (PM 10 membrane, Amicon). The purity was checked by SDS-PAGE and N-terminal amino acid analysis. Quantification of the isolated transferrin was performed on a VITROS 250 analyzer (Ortho-Clinical Diagnostics GmbH, Neckargemünd, Germany).

Preparation of hAFT-N-glycan-Asn

Purified and lyophilized human amniotic fluid transferrin (111 mg) was dissolved in 1.5 ml of 15 mmol/l $CaCl_2$ buffer containing 1.5 μmol of NaN_3. To the solution were added 2.2 mg of protease type XIV from *Streptomyces griseus* (Sigma), pH was adjusted to 8.5 and the mixture was incubated at room temperature for three days. After removal of the precipitate by centrifugation, the supernatant was lyophilized and purified by gelfiltration on a Hi Load Superdex 30 column (1.6×600 cm, Pharmacia), equilibrated and eluted with 100 mmol/l NH_4HCO_3 buffer at a flow rate of 750 μl/min (detection: 214 and 254 nm). Carbohydrate-containing fractions were analyzed by TLC (1 mol/l ammonium acetate: isopropanol = 1:2) and detected with a 1:1 mixture of 1 mol/l H_2SO_4 and 0.2% resorcine monomethylether in ethanol. The pooled fractions were lyophilized and the remainder (11.7 mg) was dissolved in 150 μl of 15 mmol/l $CaCl_2$ buffer containing 1.5 μmol of NaN_3. To the solution were added 0.22 mg of protease type XIV from *Streptomyces griseus* (Sigma), the pH was adjusted to 8.5 and the mixture was incubated at room temperature for three days. The reaction mixture was purified on a Hi Load Superdex 30 column (1.6×600 cm, Pharmacia), equilibrated and eluted with 100 mmol/l NH_4HCO_3 buffer at a flow rate of 750 μl/min (detection: 214 and 254 nm). The fractions (4 ml) eluting between 77 and 95 min were lyophilized, analyzed by 500 MHz-^{1}H-NMR and pooled. Yield: 5.14 mg (4.6%); Rf = 0.08–0.13 (1 mol/l ammonium acetate: isopropanol = 1:2).

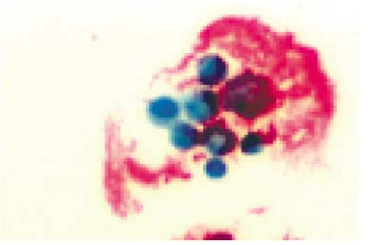

Figure 1 Isolated trophoblast cells express cytokeratin 7 as a specific marker for trophoblast tissue detected with monoclonal mouse antibodies of the IgG subtype and staining with fast red; 40×lens.

Culture of first trimester trophoblast cells

Trophoblast cells were isolated from four different human first trimester placentas obtained after legal abortion. Tissue dissected from the placenta was minced and transferred to HBSS-HEPES-buffer containing trypsin (10000–13000 U/mg) and DNAse I (0.1 mg/ml). The cell suspensions obtained by three digestion steps were separated from tissue fragments. The first step of throphoblast cell purification was performed on a Percoll (Pharmacia) gradient [9, 12]. Trophoblast cells collected at the interphase were removed, washed, and incubated with an anti-CD45 monoclonal antibody (Dianova, Hamburg, Germany) followed by rat anti-mouse magnetic MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). For the depletion of leukocytes, cells were applied to a MiniMACS column (Miltenyi Biotec), and purified trophoblast cells were obtained [10]. Trophoblast identity of the isolated cells was determined morphologically by light microscopy, by their ability to produce specific hormones (hCG, progesterone, hPL), and by immunostaining with a monoclonal mouse anti-cytokeratin 7 antibody (Progen Biotechnik, Heidelberg, Germany) (Figure 1) and a polyclonal rabbit anti hCG antibody (Dako, Hamburg, Germany) (Figure 2). Isolated cells were CD45 negative (Figure 3) whereas depleted leucocytes showed a strong CD45 staining (Figure 4). Trophoblast cells were cultured in DMEM medium with 10% inactivated fetal calf serum and with antibiotics and antimycotics.

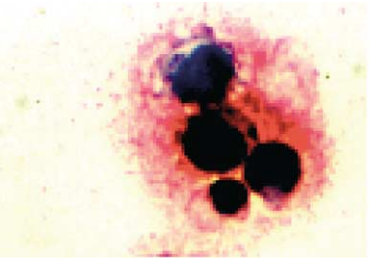

Figure 2 Isolated trophoblast cells also produce hCG detected in the cytoplasma of trophoblasts with polyclonal rabbit antibodies and staining with the fast red reagent; 40×lens.

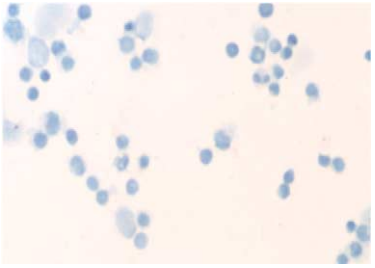

Figure 3 Isolated trophoblast cells after purification with magnetic cell sorting are negative for CD45 (incubation with a monoclonal mouse anti CD 45 antibody (IgG) and staining with fast red); 10×lens.

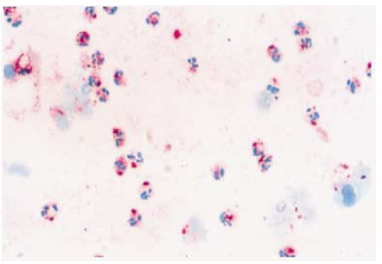

Figure 4 Expression of CD45 is shown in depleted placental immune cells after magnetic cell sorting and incubation with a monoclonal mouse anti CD 45 antibody (IgG) and staining with fast red; 10×lens.

Immunocytochemistry

Isolated trophoblast cells were grown on 3-well multitest slides (Roth, Karlsruhe, Germany) to subconfluency, dried, wrapped and stored at −80°C. After thawing, cells were briefly fixed with formalin (Merck, Darmstadt, Germany, 5% in PBS, 5 min) and permeabilized with digitonin (Ysat, Wernigerode, Germany, 6 μg/ml in PBS, 15 min). Both treatments do not interfere with binding even in cases where epitopes are known to be sensitive to formalin [9]. Antibodies were diluted with cell culture medium and incubated overnight at 4°C. Antibodies used for the experiments are listed in Table 1. After washing, slides were stained with the Vectastain® Elite ABC-Kit (Vector Laboratories, Borlingame, CA). Visualization was performed with the Fast-Red reagent.

Treatment of trophoblast cultures with hAFT, serum transferrin and N-glycan-Asn of hAFT

Purified first trimester trophoblasts were adjusted to a cell concentration of 1×10^6 cells/ml. Cell suspensions were incubated in the presence of amnion-transferrin (100–200 μg/ml), human apo-serum transferrin (200 μg/ml, Sigma-Aldrich Chemie GmbH, Germany) or varying concentrations (100–300 μg/ml) of N-glycan-Asn of hAFT. Untreated cell cultures of each placenta were used as controls. The effect of transferrin on the secretion of progesterone for syncytiotrophoblasts was studied by simultaneously comparing the kinetics of progesterone release of untreated (controls) and transferrin-stimulated trophoblast cell cultures. At designated times, aliquots of the culture media were removed, frozen at −20°C and replaced with fresh medium. Cells were cultured for 8 and up to 72 hours.

Identification of secreted hormones

The secretion of progesterone was determined by means of the analyzer SR1 from BioChemImmunoSystems GmbH. Progesterone was analyzed by competitive enzyme immuno assay, respectively. Specific antibodies were labeled with fluorescein. After reaction with anti-fluorescein antibody and binding to magnetic particles the separation was performed. Color intensity was measured at 3 different wave lengths. Samples were diluted 1:3 with hormone-free diluent because of the intensively colored culture medium. Sensitivity and the intra-assay coefficient of variation of progesterone EIA (<6.4 nmol/l and 6.8% at a medium value of 11.5 nmol/l; 5.4% at 65.6 nmol/l, respectively) demonstrate the accuracy of the applied methods.

Statistical analysis

Observations for the stimulation experiments at each sampling time were compared using Wilcoxon's signed rank tests. The size of increase in progesterone levels after addition of the transferrins was compared using this test. Statistical computerization of the analysis utilized the SPSS software package. $P<0.05$ was considered statistically significant.

Results

Identification of hAFT and isolation of hAFT N-glycans

hAFT was isolated from human amniotic fluid in a four-step procedure using anion exchange chromatography followed by gel filtration and immunoadsorption. The reddish-colored protein migrated similar to human serum transferrin as a single band at 78 kDa in SDS-PAGE under reducing conditions (Figure 5). Sequence analysis of the first 6 N-terminal amino acids of amnion transferrin revealed Val-Pro-Asp-Lys-Thr-Val, identical with the N-terminal amino acid sequence of human serum transferrin. The purified human amniotic fluid transferrin was digested twice with a protease cocktail from *Streptomyces griseus*. This resulted in complete digestion of transferrin to glycopeptides to respective N-glycan-

Table 1 Antibodies used in this study.

Antigen	Antibody	Isotype	Concentration/Dilution	Source
CD 45 (LCA)	CBL 124	Mouse IgG	1:100	Dianova
CK 7	Ks 7.18	Mouse IgG	1:50	Progen Biotechnik
hCG	A 0231	Rabbit IgG	Ready to use	Dako

CK=cytokeratin, LCA=leucocyte common antigen.

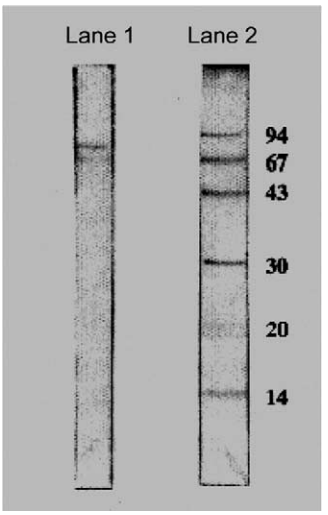

Figure 5 SDS-polyacrylamide gel analysis of purified human amnion fluid transferrin, reduced with 2-mercaptoethanol (Lane 1). Lane 2, molecular weight markers (numbers in kD). The gel was stained with Coomassie blue.

asparagines (N-glycan-Asn) as determined by 500 MHz-^{1}H-NMR. Only those fractions of N-glycans carrying asparagine as the single amino acid were pooled and will be referred to as N-glycan-Asn from hAFT.

Stimulation of progesterone production by human amniotic-fluid-transferrin

To test the effect of hAFT on progesterone secretion of trophoblast cells *in vitro*, we stimulated these cells with increasing concentrations of amniotic fluid transferrin and its N-glycans. In our experiments, trophoblast cells were isolated from four different human first trimester placentae. Analysis of progesterone in stimulated and unstimulated cell cultures revealed that amnion transferrin-stimulated trophoblast cells and cells, which were stimulated with N-glycans of hAFT, show a higher secretion of progesterone compared to unstimulated cells over a cultivation period of 72 h (Figure 6). There was a significant difference between the progesterone production of stimulated and unstimulated cell cultures ($p < 0.002$ for 100 μg hAFT, $p < 0.002$ for 200 μg hAFT and $p < 0.046$ for 200 μg hAFT-N-glycan-Asn, $p < 0.028$ for 300 μg/ml hAFT N-glycan-Asn). The stimulation effect of hAFT and its N-glycans is dose-dependent. Cells stimulated with 200 μg/ml hAFT showed the highest stimulation effects compared to 100 μg/ml hAFT stimulated cells. Cells stimulated with N-glycan-Asn from hAFT (300 μg/ml) showed the highest stimulation rates compared to 200 μg/ml and 100 μg/ml N-glycan-Asn. Trophoblast cells stimulated with serum-transferrin did not show an increase in progesterone production as compared to unstimulated controls.

Discussion

Our investigations show that especially the N-glycans of human amniotic fluid transferrin (hAFT) stimulate the secretion of progesterone in human first trimester trophoblast cells in a time- and dose-dependent manner. In a recent study [21] we found differences in the N-glycan structures between hAFT and serum transferrin. Previously unknown (α1–3)-fucosylated N-glycans were found, containing sialyl Le^x motifs. Furthermore compared to serum transferrin, a higher degree of (α1–6)-fucosylation and an increase in branching from di- to triantennary compounds has been detected. For the first time the presence of O-glycans was described in transferrin. Recently Verrijt et al. [23] demonstrated that human trophoblast cells isolated from term placentae synthesize transferrin. This transferrin isolated from cytotrophoblast and syncytiotrophoblast cells was shown to be different from both, maternal and fetal serum transferrin. The iso-electric points were found at lower pH

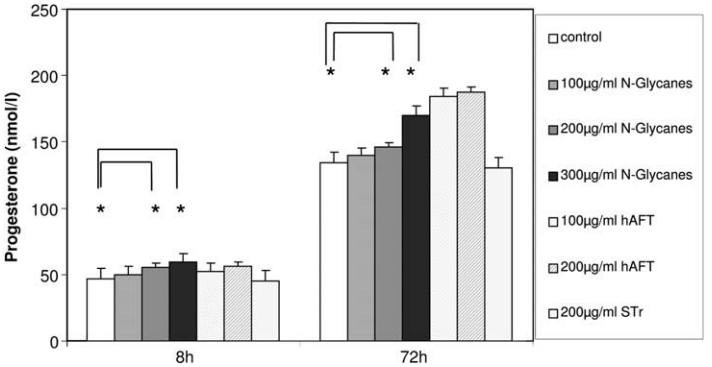

Figure 6 Stimulation of progesterone production of isolated first trimester trophoblast cells induced by N-glycans of hAFT and for comparison hAFT and serum transferrin. The cells (1×10^6/ml supplemented cell culture medium) were cultured for 8 and 72 hours without (control), with 100 μg/ml, 200 μg/ml or with 300 μg/ml hAFT N-glycans or 100 and 200 μg/ml hAFT or 200 μg/ml serum transferrin. Data represent the means ± SD of four cultures, Asterisks represent significant differences between N-glycan stimulated cultures and controls.

compared to the iso-electric points of maternal and fetal serum transferrin. A higher degree of sialylation and an increase in branching was found in cytotrophoblast transferrin. This is in agreement with our investigations of the carbohydrate structures in hAFT, suggesting the trophoblast origin of hAFT. Because the trophoblast is able to produce transferrin with a special glycosylation, we speculate that progesterone production in trophoblast cells can be stimulated by trophoblast produced transferrin or in detail by its glycans in an auto- or paracrine mechanism.

The human placenta produces approximately 300 mg of progesterone and about 100 mg of estrogens per day. Progesterone facilitates the maintenance through a multitude of mechanisms, e.g. by acting on the myometrium and the uterine cervix. There is evidence that progesterone has an immunosuppressive effect and can regulate the cytokine network in the uterus [3]. Progesterone may play an important role in the adaption of the maternal cardiovascular system to pregnancy.

At the beginning of gestation, progesterone is synthesized by the corpus luteum graviditatis and the trophoblast. Already in the 5th week of gestation, the trophoblast becomes the main source for progesterone synthesis. Biosynthesis of progesterone in the human placenta is regulated by a number of substances. Feinberg et al. [6] described that IL-1 α, IL-1 β and TNF α significantly stimulated progesterone production of the trophoblast tumor cell line JEG-3. Seki et al. [16] found that in primary placental cells, IL-1 beta had no effect on the progesterone production. Studies of Shanker et al. [17] with first trimester or term human placental minces showed that addition of estradiol-17-beta increased the progesterone production. Addition of the aromatase inhibitor CGS 16949 A and the estrogen receptor antagonist ICI 182780 inhibited progesterone production. RU 486 and ZK 98299, which are progesterone receptor antagonists, significantly modulated progesterone synthesis. Shanker et al. concluded that progesterone synthesis in human placenta is regulated by estradiol-17-beta and autoregulated by progesterone itself. Osteogenic protein-1 (OP-1/BMP-7), a member of the transforming growth factor (TGF-β) superfamily of proteins, reduced the secretion of progesterone in cytotrophoblast cultures of early and full term placentas [14]. Results obtained in this study show that progesterone synthesis during pregnancy is up regulated by trophoblast produced transferrin. In granulosa cells on the other hand, transferrin inhibits progesterone synthesis [11]. This result suggests that transferrin is able to regulate progesterone synthesis in a variety of tissues. During pregnancy however special glycosylated transferrin up-regulates progesterone. This effect could be important for the maintenance of pregnancy. As stated by the studies of de Fonseca et al. and Meis et al. [4, 15], administration of progesterone can prevent preterm labor. On the basis of our results we may speculate that also administration of special glycosylated transferrin could be useful for prevention of preterm birth. This has to be evaluated in larger studies.

In summary, this study provides evidence that N-glycan-Asn of hAFT, which differ from serum transferrin glycans, are able to stimulate the progesterone secretion of trophoblast cells *in vitro*. Because hAFT is produced by trophoblast cells itself, an auto- or paracrine mechanism of progesterone stimulation can be discussed.

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