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Human Amniotic Fluid Glycoproteins Expressing Sialyl Lewis Carbohydrate Antigens Stimulate Progesterone Production in Human Trophoblasts in vitro

Udo Jeschke[a] Ioannis Mylonas[a] Dagmar-Ulrike Richter[b] Annett Streu[b]
Heiner Müller[b] Volker Briese[b] Klaus Friese[a]

[a]1st Department of Obstetrics and Gynecology, Ludwig Maximilian University of Munich, Munich, and
[b]Department of Obstetrics and Gynecology, University of Rostock, Rostock, Germany

Key Words
Amniotic fluid glycoproteins · Progesterone production · Trophoblast cells

Abstract
Background: Progesterone is thought to mediate immune modulator effects by regulating uterine responsiveness. The aim of the study was to clarify the effect of transferrin and glycodelin A (former name PP14) as sialyl Lewis X-expressing glycoproteins on the release of progesterone by trophoblast cells in vitro. ***Methods:*** Cytotrophoblast cells were prepared from human term placentas by standard dispersion of villous tissue followed by a Percoll gradient centrifugation step. Trophoblasts were incubated with varying concentrations (50–300 μg/ml) of human amniotic fluid- and serum-transferrin as well as with glycodelin A. Culture supernatants were assayed for progesterone, human chorionic gonadotropin (hCG) and cortisol by enzyme immunometric methods. ***Results:*** The release of progesterone is increased in amniotic fluid transferrin- and glycodelin A-treated trophoblast cell cultures compared to untreated trophoblast cells. There is no relation between transferrin and the hCG or cortisol production of trophoblast cells. ***Conclusion:*** The results suggest that sialyl Lewis carbohydrate antigen-expressing amniotic fluid glycoproteins modulate the endocrine function of trophoblasts in culture by upregulating progesterone production.

Introduction

During pregnancy the immune response of the mother against certain antigens is reduced [1, 2]. Progesterone is one of a variety of molecules that regulate uterine immune responsiveness [3, 4] by inhibiting the inflammatory response [5]. The suppression of mixed lymphocyte culture responses by progesterone has also been described [6]. Due to its lipid solubility, progesterone incorporates into the lymphocyte plasma membrane interfering with membrane dynamics [7]. This promotes a locally suppressed activity at the maternal-fetal frontier [8].

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

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Dr. Udo Jeschke
1st Department of Obstetrics and Gynecology, Ludwig Maximilian University of Munich
Maistrasse 11
DE–80337 Munich (Germany)
Tel. +49 89 5160 4266, Fax +49 89 5160 4916, E-Mail udo.jeschke@med.uni-muenchen.de

hormones. Progesterone synthesis is also regulated by estradiol-17β and autoregulated by progesterone itself [4]. Feinberg et al. [8] showed that progesterone production was significantly stimulated by interleukin (IL)-1α, IL-1β and tumor necrosis factor-α (TNF-α) in chorion carcinoma cells. In addition, progesterone also plays a role in the maintenance of pregnancy, and administration of progesterone is able to prevent preterm delivery [9, 10].

During gestation, the fetus requires large amounts of iron. The major iron source is maternal serum transferrin [11, 12]. 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 [13, 14].

Glycodelin, previously known as placental protein 14 (PP14), is an approximately 28-kD glycoprotein with a unique carbohydrate configuration, consistent with sialylated LacdiNAc structures that are very unusual for mammals [15]. Glycodelin is a major reproductive glycoprotein with several functions in cell recognition and differentiation [16]. Under physiological conditions glycodelin is mainly synthesized in secretory endometrial glands [17, 18] and gestational decidua [19]. Although the precise function of glycodelin A has still not been elucidated, it can inhibit the activity of natural killer (NK) cells [20] and therefore play a major role during implantation and the early development of the fetal-placental unit. Interestingly, glycodelin A levels rise rapidly in early pregnancy, with the highest concentrations being found in the decidua between the 6th and 12th week of gestation [21], suggesting important function in early pregnancy.

In the present study, we investigated the influence of the human amniotic fluid glycoprotein transferrin, human holo-serum transferrin and apo-serum transferrin and glycodelin A on progesterone release of trophoblast cells in vitro.

Materials and Methods

Purification of Transferrin from Amniotic Fluid (hAFT)

Human amniotic fluid transferrin (hAFT) was purified as previously published [22]. Briefly, amniotic fluid was dialyzed and fractionated on a DEAE-Sepharose (Amersham Biosciences, Uppsala, Sweden). Transferrin-containing fractions were further purified on Superdex 75 (Amersham Biosciences). Subsequently, transferrin-containing material was applied to a protein A column (Amersham Biosciences) to remove the remaining IgG. Monoclonal antibodies directed against human chorionic gonadotropin (hCG; Dianova, Hamburg, Germany) were coupled to CNBr-Sepharose 4B (Amersham Biosciences) to remove traces of hCG. Transferrin-containing material was applied to the column; the main fraction containing pure amniotic fluid transferrin was concentrated by ultrafiltration. 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).

Purification of Glycodelin A from Amniotic Fluid

Glycodelin A was purified as recently published [23]. Briefly human amniotic fluid was dialyzed and fractionated on DEAE-Sepharose (Amersham Biosciences). Glycodelin A was further purified on octyl-Sepharose Cl-4B (Amersham Biosciences). Final purification was achieved by hydrophobic interaction chromatography on Resource-Phe (Amersham Biosciences). Glycodelin A-containing fractions were concentrated by ultrafiltration.

Culture of Trophoblasts

Cytotrophoblast cells were isolated from term human placentas following spontaneous delivery or planned cesarean section and were processed according to Kliman et al. [24] with slight modifications [23]. Briefly, villous tissue was minced and transferred to a trypsin/DNAse I mixture (142.4 mg trypsin and 5 mg DNAse I; Sigma-Aldrich, Taufkirchen, Germany). The remaining tissue fragments were digested twice in trypsin/DNAse I solution (71.2 mg trypsin and 5 mg DNAse I; Sigma-Aldrich). The cell suspensions obtained by the three digestion steps were pooled and loaded on a preformed Percoll (Amersham Biosciences) gradient. The density zone containing trophoblast cells was removed. The trophoblast identity of the isolated cells was confirmed by their ability to produce specific hormones (hCG, hPL and progesterone) and by immunostaining with an anti-cytokeratin antibody (anti-cytokeratin AE1/AE3; Roche, Mannheim, Germany). Viability of trophoblast cells was tested using a trypan blue exclusion assay (Sigma-Aldrich, Munich, Germany).

Treatment of Trophoblast Cultures with hAFT, Serum Transferrin and Glycodelin A

Trophoblasts were adjusted to a cell concentration of 1×10^6 cells/ml in RPMI 1640 culture medium supplemented with 10% fetal calf serum and gentamycin (200 μg/ml) and incubated in the presence of varying concentrations (50–250 μg/ml) of amnion transferrin, varying concentrations (100–300 μg/ml) of human holo- and apo-serum transferrin (Sigma-Aldrich) and varying concentrations (50–250 μg/ml) of glycodelin A. The effect of the glycoproteins on the secretion of progesterone was studied by simultaneously comparing the kinetics of progesterone release of untreated (controls) and glycoprotein-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 up to 64 h.

Identification of Secreted Hormones

The analyzer SR1 from BioChemImmunoSystems GmbH determined the secretion of progesterone. Samples were diluted 1:3 with hormone-free diluents because of the intensively colored culture medium. The 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.

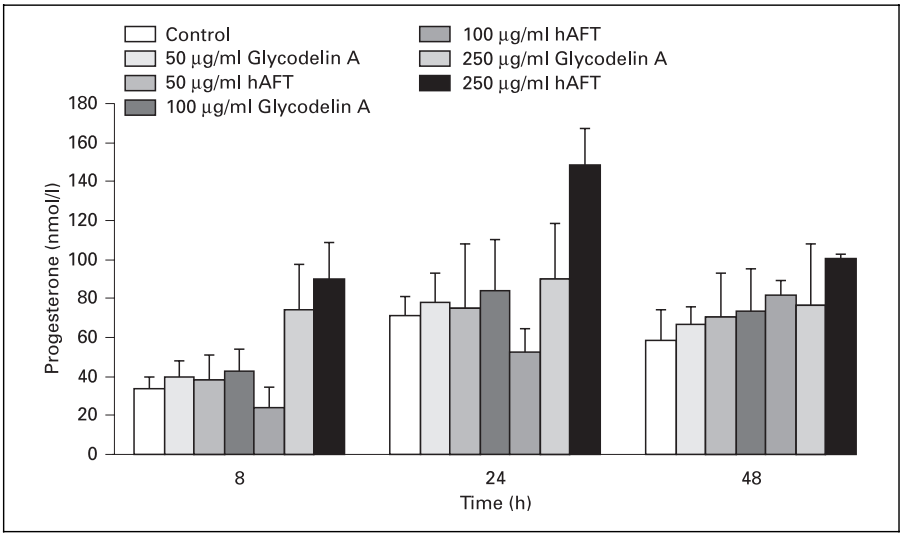

Fig. 1. Effect of human amnion fluid transferrin (hAFT) and glycodelin A on the progesterone production of trophoblast cells in vitro. Data (mean ± SD) represent progesterone concentration in aliquots of the culture media at designated times, n = 4 for each group. Differences between the controls and stimulated cultures are significant for cultures stimulated with 50, 100 and 250 µg/ml hAFT ($p < 0.002$, 0.002 and 0.004) and cultures stimulated with 100 and 250 µg/ml glycodelin A ($p < 0.001$, 0.001).

Statistical Analysis

The Wilcoxon's signed rank tests for paired samples was used to compare the means. Statistical computerization of the analysis utilized the SPSS software package (SPSS, Chicago, Ill., USA). $p < 0.05$ was considered statistically significant.

Results

Stimulation of Progesterone Production by hAFT and Glycodelin A

Analysis of progesterone in stimulated and unstimulated cell cultures revealed that hAFT- and glycodelin A-stimulated trophoblast cells showed a higher secretion of progesterone compared to unstimulated cells over a cultivation period of 48 h (fig. 1). There was a significant difference between the progesterone production of stimulated and unstimulated cell cultures ($p < 0.002$ for 50 µg hAFT, $p < 0.002$ for 100 µg hAFT and $p < 0.004$ for 250 µg hAFT). The stimulation effect of hAFT is dose-dependent. Cells stimulated with 250 µg/ml hAFT showed the highest stimulation effects compared to 100 and 50 µg/ml hAFT-stimulated cells. The relative stimulation with 50 µg/ml hAFT was 112.4%, with 100 µg/ml hAFT 119.4% and with 250 µg/ml hAFT 122% compared to unstimulated controls. The relative progesterone production of trophoblast cells stimulated by 50 µg/ml glycodelin A was not significantly different from unstimulated controls ($p > 0.4$). In trophoblast cells stimulated with 100 and 250 µg/ml glycodelin A, the progesterone production was elevated to 185.5 and 240.8%, respectively. These differences between stimulated cells and controls were significant ($p < 0.001$ and $p < 0.001$).

No Stimulation of Progesterone Production by Holo- and Apo-Serum Transferrin

Over the cultivation period of 48 h, trophoblast cells from two human term placentas stimulated with 100 and 300 µg/ml of apo- and holo-serum transferrin did not show an increase in progesterone production as compared to unstimulated controls ($p > 0.7$; fig. 2). In addition, we found that neither of the tested transferrins had an effect on the hCG or cortisol release of trophoblast cells (data not shown).

Discussion

At term, the human placenta produces approximately 300 mg of progesterone and about 100 mg of estrogens per day [25]. Progesterone facilitates the maintenance of pregnancy through a multitude of mechanisms, e.g. by acting on the myometrium and the uterine cervix. There is evidence that progesterone has immunosuppressive effects and regulates the cytokine network in the uterus [26]. Interestingly, IL-1α, IL-1β and TNF-α can significantly stimulate the progesterone production of the trophoblast tumor cell line JEG-3 [8]. However, in primary placental cells IL-1β had no effect on progesterone production [27]. Studies by Shanker and Rao [28] showed that incubation of cultured trophoblast cells with estradiol-17β increased progesterone production. The 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

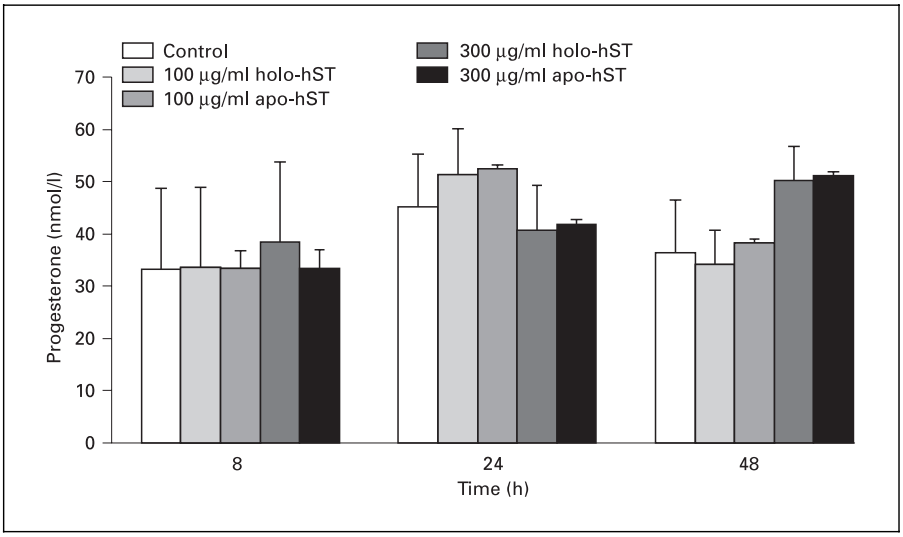

Fig. 2. Effect of human holo- and apo-serum transferrin on progesterone production in trophoblast cells. Trophoblast cells stimulated with 100 and 300 μg/ml of holo- or apo-serum transferrin showed no significantly different progesterone secretion compared to unstimulated controls ($p > 0.7$).

antagonists, also significantly modulated progesterone synthesis. Therefore, progesterone synthesis in the human placenta is regulated by estradiol-17β and autoregulated by progesterone itself. [28]. 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 first-trimester and term placentas [29].

We demonstrate that hAFT and glycodelin A stimulate secretion of progesterone in human trophoblast cells in a dose-dependent manner. In a recent study we showed that both glycoproteins express sialyl Lewis carbohydrate structures and are able to block E-selectin [30].

Recently, it was demonstrated that human trophoblast cells isolated from term placentas synthesize transferrin [31]. This transferrin isolated from cytotrophoblast and syncytiotrophoblast cells was demonstrated to be different from both maternal and fetal serum transferrin. A higher degree of sialylation and an increase in branching was described in cytotrophoblast transferrin. This is in agreement with our findings of the carbohydrate structures in hAFT, suggesting that hAFT was produced by the trophoblast and secreted into the amniotic fluid and not into maternal circulation. In addition, human serum transferrin, isolated from pregnant women, does not contain (α1–3)-fucosylated N-glycans [30]. Because hAFT contains (α1–3)-fucosylated N-glycans, representing sialyl Le^X elements, it is able to bind to selectins as shown by our previous study [30]. Human trophoblast cells express L- and E-selectin [32, 33]. Therefore transferrin produced by trophoblast cells may act in a paracrine or autocrine fashion on trophoblasts and contribute to the amniotic fluid protein pool. Serum transferrin even if it is isolated from pregnant women does not contain (α1–3)-fucosylated N-glycans and therefore does not stimulate progesterone production in trophoblasts. Glycodelin A also contains (α1–3)-fucosylated N-glycans, representing sialyl Le^X elements. In addition, we were able to show that moderate and high concentrations of glycodelin A stimulate the progesterone production of trophoblast cells. Stimulation with hAFT elevated progesterone secretion. Surprisingly, also low concentrations of hAFT increased progesterone values significantly. The hAFT concentrations used in the experiments correspond with the transferrin concentrations in amnion fluid in vivo [34] and are lower than in human serum (factor 33.74). The low progesterone stimulatory effect of hAFT compared to glycodelin A correlates with our measurements on inhibition power of both glycoproteins in E-selectin-mediated cell adhesion [30]. Glycodelin A binds to E-selectin more efficiently compared to hAFT, suggesting a glycosylation in glycodelin that is more suitable to bind to selectins. Because hAFT and serum transferrin are only different in their glycosylation [22] and hAFT in contrast to serum transferrin contains (α1–3)-fucosylated N-glycans, we assume that glycoproteins with these structures are able to stimulate progesterone production in trophoblast cells.

In summary, in this study we could show that glycoproteins expressing sialyl Lewis carbohydrate structures stimulate the progesterone production of trophoblast cells. In combination with previous data [31] that demonstrate the synthesis of differentially glycosylated transferrin by tro-

phoblast cells, a paracrine or autocrine mechanism is suspected. In addition, our results indicate that glycodelin A may have paracrine effects in reproductive tissues and at the maternal–trophoblast interface. The precise mechanism of glycodelin A–trophoblast interaction is under investigation.

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