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Stimulation of hCG protein and mRNA in first trimester villous cytotrophoblast cells in vitro by glycodelin A

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Abstract

Aim: Human chorionic gonadotropin (hCG) is produced by fetal trophoblast cells and secreted into maternal circulation mainly in the first trimester of pregnancy. Another glycoprotein, glycodelin A, is one of the main products of the maternal decidua during this period. The purpose of this study was to investigate the effect of glycodelin A on hCG release by isolated cytotrophoblast cells in vitro.
Methods: Cytotrophoblast cells were prepared from human first trimester placenta and incubated with varying concentrations of glycodelin A. Supernatants were assayed for hCG protein concentrations, and quantification of beta hCG mRNA was carried out by RT-PCR. Expression of hCG was analysed in stimulated trophoblast cells and in unstimulated controls by immunocytochemistry.
Results: Glycodelin A induces a dose-dependent increase of hCG production. An increase of hCG expression was measured at 100 and 200 μg/mL glycodelin-A treatment in trophoblast cell culture by TaqMan assay on mRNA level. We found a moderate staining of hCG in control trophoblast cells, whereas a strong hCG staining was seen in glycodelin A-treated trophoblast cells.
Conclusions: HCG is a marker for the differentiation process of trophoblast cells. Our results suggest that glycodelin A secreted by the decidualized endometrium is involved in the regulation of hormones produced by the trophoblast.

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Introduction

Glycodelin, previously known as placental protein 14 (PP14), is expressed in glandular epithelium of the endometrium [20] and decidua [19]. Glycodelin suppresses the cytolytic capacity of NK-cells (Natural Killer Cells) *in vitro* [32] and it stimulates the Th2-type cytokine production in monocytes [28], shown by an increased production of IL-6 and IL-10 following glycodelin exposure [25, 26]. Glycodelin directly inhibits T cell activity [39] in an early phase of activation by diminishing T cell responses in the contact site at the time of T cell receptor triggering and inducing T cell apoptosis [38]. Our own investigations [48] suggest a relationship between serum levels of PP14 and threatened abortion, whereby serum PP14 levels were significantly lower in patients with threatened abortion at 10 to 20 weeks of gestation than in normal pregnancies. Glycodelin was shown to have a molecular weight of 28 kDa with two identical subunits held together by non-covalent bonds and a carbohydrate content of 17.5% with a unique carbohydrate configuration. Glycodelin, isolated from amniotic fluid (glycodelin A, GdA), contains fucosylated LacdiNAc structures that are very unusual for mammals [8]. Julkunen and co-workers confirmed the homology between GdA and β-lactoglobulins by deducing its complete amino acid sequence [22]. A similar glycoprotein, glycodelin S, is found in seminal plasma, but it is differentially glycosylated from GdA [23].

It is assumed that GdA plays an important role during implantation and the early survival of the developing feto-placental unit. GdA serum levels increase on the 14th day after conception but do not differ from levels in the late secretory phase [21]. A more significant rise of glycodelin A has been found on the 21st and 28th day of pregnancy. The highest serum glycodelin A concentrations have been detected between six and 12 weeks of gestation, followed by a decrease and plateau at 24 weeks. Julkunen et al. [21] measured the content of GdA in amniotic fluid and found the highest values between the 12th and 20th week of gestation with a highest level of 232 μg/mL. This is nearly one thousand fold higher than the serum glycodelin A for equivalent gestational age. Even early radioimmunoassays established that the GdA secretory pattern resembles that of human chorionic

gonadotropin (hCG) [42]. Because these two proteins are different with regards to their tissue of origin (GdA is produced by maternal decidua but hCG is produced by fetal trophoblast), no functional relationship between hCG and GdA is suggested. It is generally accepted that hCG is produced by the syncytiotrophoblast [45], but Hoshina et al. [12] and Boime [3] speculated that hCG could also be produced by cytotrophoblast cells, although the regulation of placental hCG-β expression is still not well understood [27]. In a preliminary study we investigated the expression of βhCG mRNA in panels of different human normal tissues and isolated term trophoblast cells [40]. We found a βhCG stimulation by glycodelin A on mRNA level. The effect of glycodelin A on βhCG regulation is time-dependent. We observed a short increase of βhCG mRNA copy numbers 60 min after glycodelin A treatment. In another study, normal preparations of human term trophoblast cells *in vitro* were used to investigate the effect of glycodelin A on endocrine trophoblast function by measuring the release of hCG in culture medium [17]. Because production of both GdA and hCG are highest in the first trimester of pregnancy, we undertook the present study to investigate the influence and relationship of glycodelin A on βhCG-mRNA and protein expression in isolated first trimester trophoblast cells.

Materials and methods

Placental tissue was obtained from terminations of normal pregnancies between the seventh and 12th week of gestation. A total of 14 placentas was obtained after curettage treatment. All women were between 22 and 36 years, had a singleton pregnancy and showed no abnormalties until the moment of termination. The study was approved by the ethical committee of the University of Rostock, and informed consent to use the tissue was obtained from each patient.

Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, antibiotic and antimycotic solutions, Ultroser G and trypsin-EDTA were obtained from GibcoBRL Life Technologies (Paisley, UK). Trypsin type 3 and DNAse I were obtained from Sigma (Taufkirchen, Germany) and HEPES buffer (1M) and inactivated fetal calf serum (FCS) came from Biochrom (Berlin, Germany).

Preparation and culture of villous cytotrophoblast cells

CTBs were prepared according to a published protocol [15]. Briefly, first trimester placental tissue was minced and digested three times with trypsin and DNAse I in HBSS-HEPES. First purification was performed on a percoll gradient. After centrifugation, CTBs were aspirated and the villous cytotrophoblast cells were immunopurified using antibodies against CD45 and CD9 (Dianova, Hamburg, Germany) and a magnetic cell sorting column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to eliminate contaminating leukocytes (CD45), extravillous trophoblast cells, fibroblasts and other mesenchymal components (CD9). The identity of the isolated villous cytotrophoblast cells was determined morphologically by light microscopy and by immunostaining with a monoclonal mouse anti-cytokeratin 7 antibody (Progen Biotechnik, Heidelberg, Germany). In addition, cells were checked for possible expression of the Thomsen-Friedenreich epitope, which is a marker for syncytiotrophoblast [15]. Isolated cells contained less than 1% digested fragments of the syncytiotrophoblast. Cells were cultured in DMEM medium with 10% inactivated FCS and with antibiotics and antimycotics.

Purification of glycodelin A from amniotic fluid

Glycodelin A was purified from amniotic fluid obtained from the pooled samples obtained from women with normal singleton pregnancies that for diagnostic purposes by two anion exchange chromatography steps, gel filtration and two hydrophobic interaction chromatography steps as described elsewhere [17]. Briefly, amniotic fluid was loaded onto a DEAE-Sepharose column and fractionated on a 50–500 mM NH_4HCO_3-gradient followed by gel filtration and a second anion exchange chromatography step on Resource Q. Final purification was obtained by hydrophobic interaction chromatography on Octyl-Sepharose and Resource-Phe (all columns by Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Identification of glycodelin A by Western blot analysis

Western blot analysis of purified glycodelin A was carried out according to the procedure published elsewhere [2]. Briefly, glycodelin A was detected with a polyclonal rabbit anti glycodelin A antibody (10 μg/mL, Bioscience, Berlin, Germany) followed by anti-rabbit IgG conjugated to HRP (1:1000, Dianova, Hamburg, Germany) and stained with 3-amino-9-ethyl carbazol (Sigma).

Treatment of trophoblast cultures with glycodelin A

Freshly isolated trophoblast cells were incubated at concentrations of 1×10^6 cells/mL in 24-well plates (Greiner Labortechnik, Solingen, Germany) in humidified 5% CO_2-95% air at 37°C in the presence of 50 μg/mL, 100 μg/mL and 200 μg/mL glycodelin A. Untreated cell cultures of the same placentas were used as controls. In addition, samples of the transferrin containing fraction of amniotic fluid were also used as controls to verify the effect of glycodelin A. The effect of glycodelin A on the secretion of markers for syncytiotrophoblasts was studied by comparing the kinetics of hCG mRNA or protein production of untreated (controls) and glycodelin A-stimulated trophoblast cell cultures simultaneously. All experiments were carried out at least in triplicates.

Identification of hCGβ-mRNA transcripts (real-time RT-PCR)

Total RNA was prepared using the acid guanidium thiocyanate-phenol-chloroform protocol [6]. Quality of the RNA samples was determined by electrophoresis through denaturing agarose gels and staining with ethidium bromide. The RNA was quantified and evaluated for purity by UV spectrophotometry. To further assess the quality of RNA, all specimens were tested by analysis of housekeeping gene expression using conventional RT-PCR.

HCG-β primers and probe were designed using the Primer Express™ 1.0 program (PE Applied Biosystems, Foster City, CA).

Oligonucleotide hybridisation probe and primers with the following sequences were synthesized as follows: TaqMan™ probe 5′-CTG CTG AGC ATG GGC GGG ACA T (exon 2), forward primer 5′-CCA AGG ATG GAG ATG TTC CAG, and reverse primer 5′-GCA CGC GGG TCA TGG T. The reverse primer was designed to span an exon/intron junction to avoid amplification of DNA sequences, whereas the forward primer was complementary to the 3′-end of exon 1. Primers and probe were obtained from Applied Biosystems GmbH (Weiterstadt, Germany). The primers yielded a RT-PCR product of 199 nucleotides [40].

The preparation of RNA standard and quantification of βhCG mRNA expression by ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) have been published in detail previously [40, 41]. The TaqMan® EZ RT-PCR Kit (PE, Applied Biosystems) was used for reverse transcription and amplification of both targets and standards. All RT-PCR reactions were performed in triplicate with a final volume of 25 μL. RT-PCR conditions were optimised for primers (300 nM), probe (100 nM), template (100 ng), and manganese acetate concentrations (3 mM). The reaction conditions were 2 min at 50°C, 30 min at 60°C, 2 min at 95°C, 40 cycles with 20 sec at 94°C and 1 min at 60°C [41].

Analysis of hCG protein production

At designated times (24 h and 48 h) aliquots of the culture media were taken, frozen at −40°C and replaced with fresh medium. Cells were cultured for up to 48 h. Secretion of hCG was analysed by the automated hormone analyser "Immulite" (DPC Biermann GmbH, Bad Nauheim, Germany). This assay detects, besides normal hCG, the five irregular forms of βhCG including hyperglycosylated hCG. The results of this assay agree with those of the RIA, and it avoids false-positive values in the samples tested [7]. The sensitivity of the used assay is 1.1 mIU/mL, and the detection limit is 5000 mIU/mL.

Immunocytochemistry

Isolated trophoblast cells were incubated with varying concentrations of glycodelin (0, 100 μg/mL and 200 μg/mL) and grown on three-well multitest slides (Roth, Karlsruhe, Germany) for up to 72 h. At designated times, medium was withdrawn, the slides were air-dried, wrapped and stored at −80°C. After thawing, the 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). The antibody against hCG was diluted to 5 μg/mL with PBS and incubated with the slides overnight at 4°C. As isotype controls of the anti-hCG antibody normal mouse serum as well as a monoclonal antibody against Glycodelin A (A87-B/D2, IgG) were incubated with different slides overnight at 4°C. After washing, Cy3-labelled goat anti-mouse IgG, diluted 1:200, were used as second antibodies for staining of hCG. Slides were examined with a Zeiss (Jena, Germany) Axiophot photomicroscope. Digital images were obtained with a digital camera system (Axiocam; Zeiss) and saved on computer.

Statistical analysis

The SPSS/PC software package, version 6.01 (SPSS GmbH, Munich, Germany), was used for collection, processing, and statistical analysis of all data. Statistical analysis was performed using the non parametrical Wilcoxon's signed rank tests for comparison of the means. $P < 0.05$ was considered statistically significant.

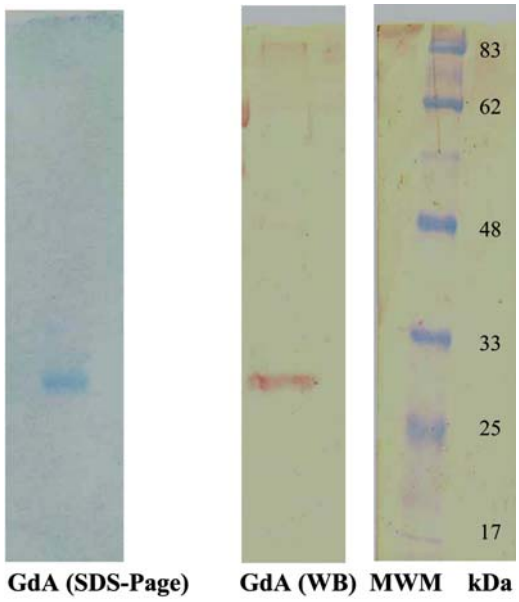

Figure 1 SDS-PAGE analysis of glycodelin A (lane 1, stained with Coomassie Brillant Blue G250, Amersham Biosciences), lane 2; glycodelin A, Western blot analysis detected with a polyclonal glycodelin antibody (Bioscience), lane 3; protein marker (New England Biolabs)

Results

Purification of glycodelin A from amniotic fluid

SDS-PAGE of the purified protein showed one band at 28 kD (Figure 1, Lane 1). In addition, the purified protein was checked for impurities of hCG by the automated hormone analyser "Immulite" (DPC Biermann GmbH, Bad Nauheim, Germany) and did not contain any hCG traces. Identity of purified glycodelin A was checked by Western blot analysis (Figure 1, Lane 2).

Stimulation of hCGβ-mRNA production by glycodelin A

An increase of hCGβ-mRNA expression was measured at 100 μg/mL and 200 μg/mL glycodelin A-treatment in trophoblast cell cultures by the TaqMan™ assay. No significant change of βhCG mRNA expression was seen in the untreated control (Figure 2). Time- and dose-dependency studies showed that the highest number of copies was measured at the higher dose (200 μg/mL) after as little as 30 min, whereas with the lower dose (100 μg/mL) 60 min were required. In both cases the increase was

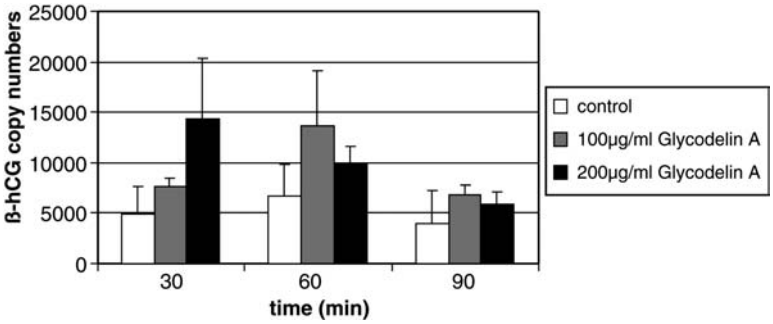

Figure 2 Time-dependent quantification of βhCG mRNA copy numbers (mean values ± SD, n = 3 for every group) per 200 ng total mRNA in glycodelin A (100 μg/mL, 200 μg/mL) – treated or untreated trophoblast cell cultures. Quantification was performed using the external βhCG RNA standard run in parallel.

statistically significant (P = 0.046 and 0.028, respectively). After 90 min, mRNA levels were almost indistinguishable from the controls.

Stimulation of hCG protein production by glycodelin A

The concentrations of hCG released by trophoblast cells, incubated for 24 or 48 h with different concentrations of glycodelin A (0, 50, 100, 200 μg/mL) are shown in Figure 3. The data indicate that glycodelin A stimulates hCG protein production in trophoblast cells in a time and dose-dependent manner. When trophoblast cells were incubated with glycodelin A (50 μg), no significant rise (P = 0.624) of hCG production was observed. A significant increase in hCG production was found for higher concentrations of glycodelin A (100 μg/mL P = 0.036; 200 μg/mL P = 0.028) after prolonged incubation (48 h). Addition of 100 μg/mL glycodelin A led to a rise of the hCG level by the factor 2.2, whereas addition of 200 μg/mL led to a rise of the hCG levels by the factor 3.7. The transferrin containing fractions of amniotic fluid had no effect on the hCG production of trophoblast cells and showed the same values of hCG production as controls.

Expression of hCG on CTB as detected by immunofluorescence

Isolated trophoblast cells from the first trimester of pregnancy showed moderate expression of hCG after 72 h of cultivation and subsequent staining with the anti-hCG antibody MS-1039-P, respectively. Results are documented in Figure 4 A. Glycodelin A-treated trophoblast cells (100 mg/mL, 200 μg/mL) showed a stronger expression of hCG. The same set of antibodies was used as for investigations of controls. Results are documented in Figure 4 B,C. Slides incubated with normal mouse serum or monoclonal anti-GdA antibody A87-B/D2 showed no staining of cytotrophoblast cells (data not shown) .

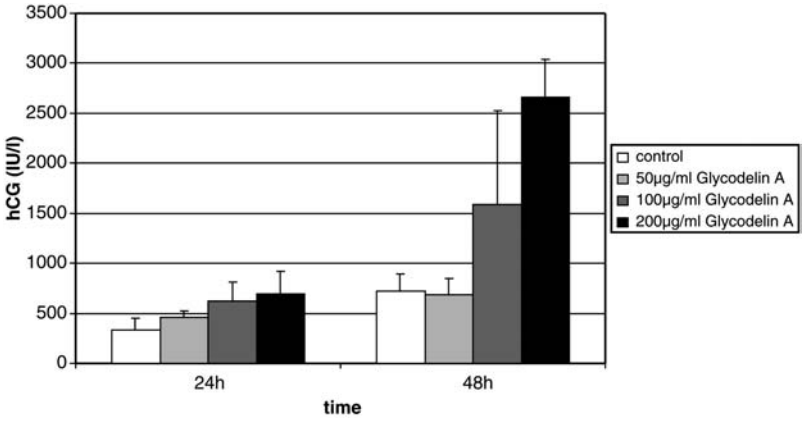

Figure 3 Effect of glycodelin A on the hCG production of cytotrophoblast cells *in vitro*. Data (mean ± SD) represent hCG concentration in aliquots of the culture media at designated times (n = 4 for every group). P = 0.036 (100 μg/mL glycodelin A) and P = 0.028 (200 μg/mL glycodelin A) indicates the significance levels between controls and stimulated cultures. There was no significant difference observed between the hCG production of controls and cells stimulated with 50 μg/mL glycodelin A (P > 0.05).

Comment

The physiological role of hCG during pregnancy is not completely understood. It is generally assumed that hCG production by the trophoblast stimulates progesterone secretion, thus contributing to the maintenance of the corpus luteum of pregnancy. Expression of hCG is detectable as early as at the eight cell stage of the developing embryo [4]. An array of paracrine modulators of hCG synthesis has been identified [14, 18, 29, 33], but less is known about the regulatory elements of the hCGβ gene because highly differentiated cells are required for their regulation [1].

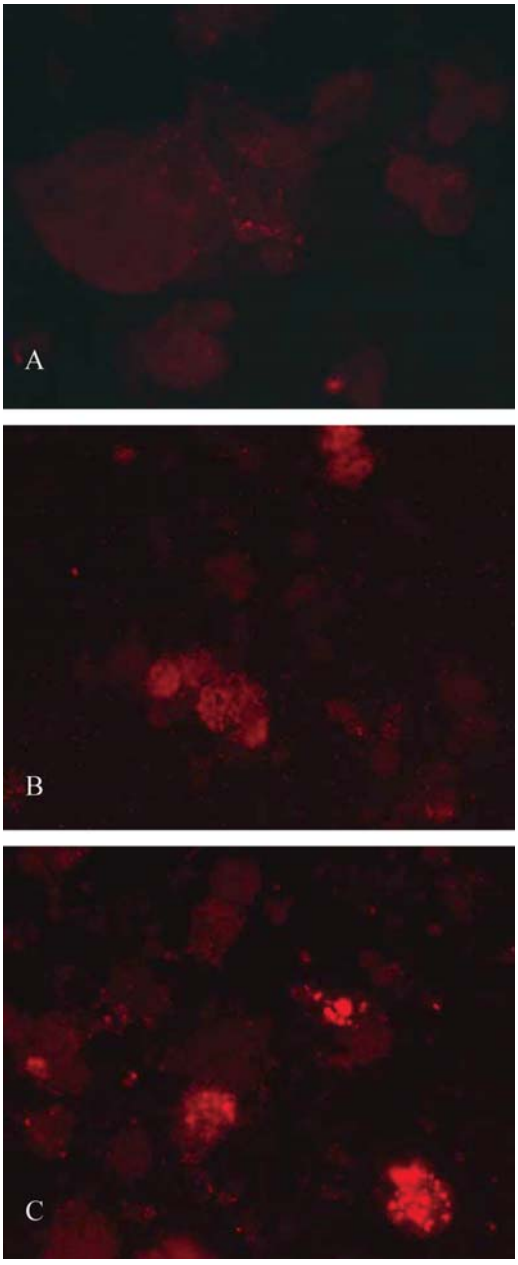

Figure 4 Expression of hCG on cytotrophoblast cells cultured without (A) or with 100 μg/mL glycodelin A (B) and 200 μg/mL glycodelin A (C) after 72 h of incubation. Slides were stained for hCG (mab MS-1039-P). Second antibodies were Cy3- labelled, respectively. 63 × lens.

This study shows that glycodelin A stimulates hCG in isolated trophoblast cells *in vitro* at mRNA level. In addition, glycodelin A caused an increase in hCG release by cultivated cytotrophoblast cells into the medium. This increase was dose-dependent. Addition of 50 μg/mL glycodelin A did not lead to a significant increase of hCG secretion. Addition of higher concentrations (100 μg/mL and 200 μg/mL) of glycodelin A induced a significant increase in secretion of hCG compared to unstimulated controls in trophoblast cells. The range of concentrations of glycodelin used for the experiments is similar to the physiological range of this glycoprotein as found in amniotic fluid during the first trimester of pregnancy [44]. The glycodelin concentration rises during the first eight to nine weeks of gestation, and is maintained until 14–15 weeks of gestation [21]. During this time of pregnancy, fetus-derived trophoblast cells invade deeply into the maternal decidua, and the contact between fetal and maternal cells is most intimate [9]. Glycodelin A is the main product of the decidua in the first trimester of pregnancy [19, 43], whereas also IGF-BP1 and prolactin are synthesized and secreted in high levels by decidual cells [5, 10, 11, 13, 24, 31, 34, 47, 46]. Our results suggest a stimulating effect of glycodelin A on hCG secretion of trophoblasts. To investigate a possible induction of hCG *synthesis* in trophoblasts by glycodelin A, real time RT-PCR was used. The results showed a dose dependent stimulating effect of glycodelin A of trophoblasts on the βhCG mRNA level. There was no significant rise of hCGβ mRNA in unstimulated controls. The addition of high amounts of glycodelin A (200 μg/mL) led to an immediate increase of βhCG mRNA copy numbers by the factor 3 after only 30 min of incubation compared to unstimulated controls. Addition of 100 mg/mL glycodelin A led to an immediate increase of βhCG mRNA copy numbers by the factor 2 after 60 min of incubation. This immediate stimulation effect was also observed in former studies, where glycodelin A stimulated βhCG mRNA synthesis in term trophoblast cells [40] as well as in Jeg 3 trophoblast tumor cells [2]. A delayed stimulation of hCG protein production by glycodelin A was measured in both cell types, which could be explained by a feedback inhibition of the βhCG mRNA synthesis by hCG itself. Feedback inhibition of gene expression is a widespread phenomenon in which the expression of a gene is downregulated by its protein product. Feedback in eukaryotic cells involves time delays resulting from transcription, transcript splicing and processing, and protein synthesis [30].

Thus, our results show that glycodelin A induces both βhCG mRNA synthesis and secretion of hCG in trophoblast cells, although the exact mechanism of action of

Table 1 Antibodies used in the study.

Antigen	Clone	Species and isotype	Concentration/dilution	Source
CD 45	CBL 124	Mouse IgG	1:100	Dianova
CD 9	CBL 560	Mouse IgG	1:100	Dianova
CK 7	Ks 7.18	Mouse IgG	1:50	Progen Biotechnik
Glycodelin	–	Rabbit IgG	10 μg/mL	Bioscience
Glycodelin A	A87-B/D2	Mouse IgG	5 μg/mL	Nemod
hCG	MS-1039-P	Mouse IgG	5 μg/mL	Dianova
TF	A78 G/A7	Mouse IgM	1:4	Serotec

glycodelin A on trophoblast cells is not known. It is already known that glycodelin A regulates uterine immune responsiveness. It has been shown that decidual extracts containing glycodelin A suppress thymidine uptake in normal and mitogen-stimulated human mixed lymphocyte cultures [37]. In addition, glycodelin A decreased the synthesis of IL1 and IL2 and the expression of the IL2-receptor by mitogen-stimulated cells [35, 36]. Here we showed that glycodelin A is involved in stimulation of first trimester trophoblast derived glycoprotein hormone hCG. In a recently published study we could show that glycodelin A induced a reduced expression of hPL in first trimester trophoblast cells. Expression of MUC1 was not affected by glycodelin A. Freshly isolated trophoblast cells showed no Thomsen-Friedenreich (TF) expression, a marker for the syncytiotrophoblast and extravillous trophoblast cells [15] but became positive for this antigen after 96 h of cultivation. Glycodelin A stimulated trophoblast cells inhibit TF-expression after 96 h of cultivation and glycodelin A plasmids induced a significantly higher hCG production in transfected cells compared to cells transfected with the empty plasmid [16]. Results obtained in both studies suggest that glycodelin A is involved in differentiation of trophoblast cells. This is due to the fact that treatment of glycodelin A and its plasmid transfected trophoblast cells stimulated hCG production in isolated first trimester trophoblast cells and inhibited hPL and TF expression.

In conclusion, we have shown that glycodelin A induces synthesis of βhCG mRNA and secretion of hCG in trophoblast cells. These results indicate a function of glycodelin A in trophoblast hCG production. But more information about the exact mechanism of hCG regulation by glycodelin A is required.

Acknowledgements

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