Stimulation of Progesterone, Estradiol and Cortisol in Trophoblast Tumor BeWo Cells by Glycodelin A N-glycans

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Abstract. Background: The immunosuppressive protein glycodelin A (GdA) is secreted by a large number of gynaecological tumors. GdA has a unique glycosylation, including fucosylated LacdiNAc structures. Trophoblast tumor cells produce a variety of steroid hormones and human chorionic gonadotropin (hCG). The purpose of this study was to investigate the effect of GdA N-glycans on hCG, cortisol, estradiol and progesterone release by the chorion carcinoma cell line BeWo in vitro. Materials and Methods: Chorionic carcinoma cells of the cell line BeWo (1x10⁶ cells/ml) were cultivated in the presence of 25 mmol/ml glycodelin A and 25 mmol/ml glycodelin A N-glycans RH-3, RH-4 and RH-5 for up to 72 h. Unstimulated BeWo cells were used as controls. After 24 h, 48 h and 72 h, 1 ml cell culture supernatant was removed, stored at –25°C and replaced by fresh medium. All experiments were carried out at least in triplicates. Collected cell culture supernatants were analysed for hCG, progesterone, estradiol (E2) and cortisol concentration. Results: The production of estradiol, cortisol and progesterone were increased in GdA N-glycan-treated cell cultures as compared to untreated BeWo cell cultures. GdA N-glycans did not stimulate hCG production in BeWo cells. A significant increase in E2 secretion was observed in BeWo cells incubated with glycodelin A and glycodelin A N-glycans (RH-3: 2.6% E2 increase compared to control, p=0.012; RH-4: 4% increase, p=0.017; glycodelin: 7.7% increase, p=0.017). Although an increase in progesterone secretion was observed in BeWo cells incubated with glycodelin A (9.0%) and glycodelin A N-glycans (RH-3: 16.2%, RH-4: 28%, RH-5: 8.7%) compared to controls, these differences were not statistically significant. Conclusion: Estradiol, cortisol and progesterone are hormone markers for BeWo trophoblast tumor cells. The results suggest that GdA with its distinct glycosylation modulates the hormone production of trophoblast tumours.

Glycodelin, formerly named placental protein 14 (PP14), is a 28 kDa glycoprotein with a unique carbohydrate configuration, containing sialylated LacdiNAc structures that are very unusual for mammals (1). Glycodelin, isolated from amniotic fluid (glycodelin A, GdA) is composed of two identical subunits closely connected by non-covalent bonds and a carbohydrate content of 17.5% (2). A similar glycoprotein, glycodelin S (GdS), was found in seminal plasma, but with a different glycosylation compared to GdA (3).

Glycodelin is a major reproductive glycoprotein with several functions in cell recognition and differentiation (4). Under physiological conditions glycodelin is mainly synthesized in secretory endometrial glands (5, 6), gestational decidua (7), seminal vesicles (8), the ovary (9) and in megakaryocytic/erythroid precursors of the bone marrow (10). Furthermore, glycodelin is also expressed in a variety of carcinomas including endometrial, cervical, mammary and ovarian tumors, although the precise role of this glycoprotein in cancer remains unknown (4, 11-16).

There is substantial evidence that GdA may be a mediator for immunomodulatory and immunosuppressive effects in several human tissues. GdA suppresses the release of interleukin-2 (IL-2) and interleukin-2 receptor (IL-2R) from stimulated lymphocytes (17-19). It also inhibits the activity of NK-cells (natural killer cells) (20) and suppresses both the allogenic mixed lymphocyte reaction and lymphocyte responsiveness to phytohemagglutinin (17, 18). The cytotoxic activity of NK-cells is inhibited by GdA in the concentration range of 1 to 50 μg/ml (21). A relationship between low serum levels of GdA and threatened abortion has been also suggested (22). This immunosuppressive effect of glycodelin...
might be mediated by the blocking of E-selectin-mediated cell adhesion (1). The fucosylated LacdiNAc structures are able to bind E-selectin more effectively than sialylated Lewis X antigens (23). Recently, it was demonstrated that both GdA and serum glycodelin, are very efficient inhibitors of the E-selectin-mediated cell adhesion in vitro, suggesting an important role in the metastatic process of cancer cells (24).

The patterns of rise and fall of circulating GdA and human chorionic gonadotropin (hCG) concentrations are similar (25). Both levels rise from implantation until the 10th week of pregnancy and decrease thereafter (26). Therefore, high levels of GdA could play an important role during implantation and early survival of the developing feto-placental unit. These two proteins are different regarding their tissue of origin (glycodelin A is expressed by the decidua and hCG by the fetal syncytiotrophoblast) but are also expressed by a number of gynaecological tumors. For trophoblast tumors, hCG plays the role of a tumor marker protein.

A cell line which expresses high doses of protein hormones, like hCG, and steroid hormones, like estrogens, progesterone and cortisol, is the BeWo cell line. Therefore the culture of trophoblast tumor cells is a good model for tests of the functional relationship between hCG, steroid hormones and glycodelin A, and especially the N-glycans of glycodelin A.

Therefore the purpose of this study was: a) to determine the effect of glycodelin A N-glycans on hCG-secretion of BeWo trophoblast tumor cells and b) evaluation of the effect of glycodelin A N-glycans on secretion of steroid hormones by BeWo cells.

Materials and Methods

Cell culture. The chorionic carcinoma BeWo cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (GibcoBRL Life Technologies, Paisley, Scotland) with 10% inactivated foetal calf serum (FCS) (Sigma, Taufkirchen, Germany) without antibiotics or antymycotics.

Purification of glycodelin A. Glycodelin A was purified from amniotic fluid obtained from pooled samples of women with normal singleton pregnancy which were obtained for diagnostic purposes by two anion exchange chromatography steps, gel filtration and two hydrophobic interaction chromatography steps as described elsewhere (27). Briefly, amniotic fluid was loaded onto a DEAE-Sepharose column and fractionated on a 50-500 mM NH₄HCO₃-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: Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Glycodelin A N-glycans. Glycodelin A N-glycans (RH-3 to RH-5, Figure 1) were obtained from Prof. Dr. van den Eijnden, Department of Medical Chemistry, Vrije Universiteit, Amsterdam, The Netherlands and synthesized according to the publication from Hokke and van den Eijnden (28).

Cultivation of BeWo cells with glycodelin A N-Glycans. Chorionic carcinoma cells of the BeWo cell line (1x10⁶ cells/ml) were cultivated in the presence of 25 mmol/ml glycodelin A and 25 mmol/ml glycodelin A N-glycans RH-3, RH-4 and RH-5 for up to 72 h. Unstimulated BeWo cells were used as controls. After 24 h, 48 h and 72 h, 1 ml cell culture supernatant was removed, stored at –25°C and replaced by fresh medium. Replacement of media and incubation

<table>
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<tr>
<th>RH-3</th>
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<tr>
<td>NeuAc2-6GalNAc1-4GlcNAc1-2Man1-6, Man1-4GlcNAc1-4GlcNAc-Asn</td>
<td>Fuc(1-3), GalNAc1-4GlcNAc1-2Man1-6, Man1-4GlcNAc1-4GlcNAc-Asn</td>
<td>NeuAc2-6GalNAc1-3GlcNAc1-2Man1-3, GalNAc1-3GlcNAc1-2Man1-3</td>
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**Figure 1.** Glycodelin A N-glycans used for the study.
times for hormone measurements were carried out accordingly to recent studies (29). All experiments were carried out at least in triplicate. Collected cell culture supernatants were analysed for hCG, progesterone, estradiol (E2) and cortisol concentration.

Measurement of hCG protein expression. The concentration of hCG in the culture medium was determined by an automated SR1 hormone analyzer (BioChemImmunoSystems GmbH, Freiburg, Germany). HCG (triplicates of each sample) was analyzed using competitive enzyme-immunoassay (EIA) (30). Antibodies used for this EIA are specific for the β-subunit of hCG (BioChem ImmunoSystems GmbH). Samples for hCG were used undiluted. Sensitivity and the intra-assay coefficient of variation were: 1 IU/l, related to 1 IRP 75/537, and 6.4% at 11.1 IU/l, 4.2% at 104 IU/l.

Measurement of progesterone, estradiol and cortisol. The SR1 analyzer from BioChemImmunoSystems GmbH determined the secretion of progesterone and estrogen (31, 32). Samples were diluted 1:3 with hormone-free diluents 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) and estradiol EIA (<15 pg/ml and 4.9% at a medium value of 1800 pg/ml; 4.3% at 461 pg/ml) demonstrated the accuracy of the applied methods.

The concentration of cortisol was also determined with the SR1 analyzer using an immunoenzymometric assay (33). Specific antibodies were labeled with fluorescein. The separation was performed by reaction with anti-fluorescein antibody and binding at magnetic particles. Colour intensity was measured at three different wavelengths. Samples were used undiluted. Sensitivity and intra-assay coefficient of variation of cortisol EIA (<2 ng/ml, and 11.1% at 37.0 ng/ml, 8.2% at 211.0 ng/ml) demonstrated the reliability of the method for these investigations.

Statistical analysis. Statistical analysis was performed using the Wilcoxon’s signed rank tests for comparison of the means over the whole cultivation period. A p<0.05 value was considered statistically significant.

Results

No stimulation of hCG production in BeWo cells. Native glycodelin A isolated from amniotic fluid and glycodelin A N-glycans RH-3 to RH-5 were used to determine the effect of glycodelin A on hCG secretion of trophoblast tumour cells BeWo. No increase in hCG secretion in BeWo cells incubated with glycodelin A and glycodelin A N-glycans (Figure 2) compared to the control cells was found.

Stimulation of progesterone production in BeWo cells. Native glycodelin A, isolated from amniotic fluid and glycodelin A N-glycans RH-3 to RH-5 were used to determine the effect of glycodelin A on progesterone production of trophoblast tumor cells BeWo. Although an increase in progesterone secretion was observed in BeWo cells incubated with glycodelin A (9.0%) and glycodelin A N-glycans (RH-3 16.2%, RH-4 2.8%, RH-5 8.7%, Figure 3) compared to controls, these differences were not statistically significant (RH-3 vs. control p=0.051, RH-4 vs. control p=0.4, RH-5 vs. control p=0.139, glycodelin A vs. control p=0.237).

Stimulation of estradiol production in BeWo cells. Native glycodelin A isolated from amniotic fluid and glycodelin A N-glycans RH-3 to RH-5 were used to determine the effect of glycodelin A on estradiol (E2) secretion of BeWo trophoblast tumour cells. A significant increase in E2 secretion in BeWo cells incubated with glycodelin A and glycodelin A N-glycans was observed (RH-3 2.6% E2 increase compared to control, p=0.401; RH-4 8.9% increase, p=0.012; RH-5 4% increase, p=0.017; glycodelin 7.7% increase, p=0.017, Figure 4).

Stimulation of cortisol production in BeWo cells. Native glycodelin A isolated from amniotic fluid and glycodelin A N-glycans RH-3 to RH-5 were used to determine the effect of glycodelin A on cortisol secretion of BeWo trophoblast tumor cells. An increase in cortisol secretion in BeWo cells incubated with glycodelin A and glycodelin A N-glycans (RH-3 13% cortisol increase compared to control, p=0.036; RH-4 1.4% increase, p=0.735; RH-5 10.3% increase, p=0.069; glycodelin 5.2% increase, p=0.123, Figure 5).

Discussion

In this study we showed that the production of estradiol, cortisol and progesterone were increased in GdA N-glycan-treated cell cultures as compared to untreated BeWo cell cultures. GdA N-glycans did not stimulate hCG production in BeWo cells.

Steroid hormones of placental and fetal adrenal origin have important roles in regulating key physiological events essential to the maintenance of pregnancy and development of the fetus for extrauterine life. The steroidogenic capabilities of choriocarcinoma cells in culture are similar to those of the in vivo placenta and support their use as an experimental model of placental steroidogenesis (34).

Progesterone has suppressive actions on lymphocyte proliferation and activity and on the immune system to prevent rejection of the developing fetus and placenta (35). It also suppresses the calcium-calmodulin-MLCK system and thus activity of uterine smooth muscle, thereby promoting myometrial quiescence to ensure the maintenance of pregnancy (36). In addition, galectin-1 (gal-1), a member of the mammalian β-galactoside-binding proteins, preferentially recognizes Galβ1-4GlcNAc sequences of oligosaccharides associated with several cell surface glycoconjugates. As demonstrated histochemically, the lectin recognizes appropriate glycopeptides on the syncytiotrophoblast and on chorionic carcinoma cells (BeWo) (37). The lectin decreased cellular progesterone production (38). Gal-1-mediated
inhibition of cellular progesterone production was reduced in the presence of a Thomsen-Friedenreich (TF)-polyacrylamide (39) conjugate (40). N-glycans of glycodelin A are also able to bind gal-1 and therefore could inhibit action of gal-1 on progesterone production (32).

Recent findings showed that estrogens have functional influences on the placenta. In particular, the experiments described by Bukovsky et al. (41) showed that an increase in estrogen production in the placenta via co-stimulation of the estrogen receptor-alpha can stimulate the differentiation of...
cytotrophoblast cells. Our own investigations showed that glycodelin A also stimulated estrogen production in trophoblast cells (32). An additional study showed that estradiol (0.05-0.45 μM) stimulated BeWo cell proliferation and increased the percentage of S-phase cells. The BeWo cell ER binds to an ERE consensus sequence and the ER-ERE complex is supershifted by antibodies directed against the ER. Jiang et al. concluded that BeWo cells express a functional ER that is important for the control of BeWo cell proliferation, suggesting a potential role for estrogens in mediating placental trophoblast growth and development (42).

Cortisol, a 64-C21 steroid, belongs to the glucocorticoid hormone group (43). Lacking enzyme P 450c17, the placenta is not capable of synthesizing cortisol from cholesterol, a 65-C27 steroid and pregnenolone, an inactive 65-C21 steroid. However, the placenta expresses P450scc (side-chain cleaving enzyme), 3β hydroxysteroid dehydrogenase and 11β hydroxysteroid dehydrogenase.
(11βHSD) activity (44) and therefore is able to metabolize C19 steroids of maternal and fetal origin for estrogen and cortisol biosynthesis (45). In particular, 11βHSD (46) allows the conversion of cortisone to biologically active cortisol within trophoblasts (44).

The physiological role of hCG during pregnancy is not completely understood. It is generally assumed that hCG production by human trophoblast cells stimulates progesterone secretion, thus contributing to the maintenance of the corpus luteum of pregnancy. Expression of hCG is detectable as early as at the 8 cell stage of the developing embryo (47). An array of paracrine modulators of hCG synthesis has been identified (48-51). Although new insights into the type of hCG-glycosylation and cell that produce hCG have recently emerged (52, 53), the regulation of placental hCG-β expression is still not well understood (54). Analysis on the promoter region of hCG-β showed that this region is quite complex (55) and not the result of a single discrete regulatory element (56). Experiments with cytokines have pointed towards a role for interleukin-1β in hCG regulation (57). We demonstrated that GdA is involved in the stimulation of hCG in first trimester trophoblast cells (29). Production of hCG by isolated first trimester trophoblast cells was described previously (53, 58, 59). In addition, trophoblast cells are the site of production of a hyperglycosylated form of hCG characteristic of early pregnancy (53, 58). Recently it was demonstrated that the carboxyl-terminal sequence (CTP) of hCG generates a specific signal for the apical release of this hormone into the maternal circulation (60). It is also known that O-linked oligosaccharides in the CTP contribute to the apical routing, and that the hyperglycosylation includes all four O-serine linked carbohydrates (61). Recent findings demonstrate that members of the retinoid receptor family such as RXR are expressed in the placenta, and that Pan-RXR and peroxisome proliferator-activated receptor gamma (PPARγ) ligands have stimulatory effects on hCG and in addition on the synthesis of other trophoblast hormones such as leptin (62, 63). GdA, which exhibits significant sequence similarity to β-lactoglobulins, binds retinoids (26, 64). In our earlier studies we showed that glycoladin A, and especially its N-glycans, stimulate hCG primarily in the first trimester (29), in term trophoblast cells (27, 32, 65) and in Jeg3 trophoblast tumor cells (66). In the present study, we showed that glycoladin A and its N-glycans did not stimulate hCG in BeWo cells. In contrast to hCG, production of progesterone, estradiol and cortisol was elevated by glycoladin A and its N-glycans in BeWo trophoblast tumor cells. These findings are in agreement with our other studies on primary trophoblast cells (31, 32, 67-69). Estrogens stimulated BeWo cell proliferation and increased the percentage of S-phase cells (42).

Conclusion

Estradiol, cortisol and progesterone are hormone markers for BeWo trophoblast tumor cells. The results suggest that GdA, with its distinct glycosylation, modulates the hormone production and in addition the proliferation of trophoblast tumors.

Acknowledgements

We thank D. Eckerle and W. Ertl for excellent technical help and Prof. Dr. van den Eijnden and Dr. Hokke, Department of Medical Chemistry, Vrije Universiteit, Amsterdam, The Netherlands for their generous gift of glycoladin A N-glycans. The project was supported by the “Kultusministerium M/V” and the “Deutsche Forschungsgemeinschaft (JE181/4-1)’.

References


Received December 13, 2006
Revised March 2, 2007
Accepted March 5, 2007