Sera of patients with recurrent miscarriages containing anti-trophoblast antibodies (ATAB) reduce hCG and progesterone production in trophoblast cells in vitro

Viktoria von Schönfeldt, Nina Rogenhofer, Katharina Ruf, Christian J. Thaler, Udo Jeschke

Division of Gynecological Endocrinology and Reproductive Medicine, Department of Gynecology and Obstetrics, LMU Munich, Munich, Germany

Department of Gynecology and Obstetrics, LMU Munich, Munich, Germany

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Abstract

Problem: Reproductive failure including recurrent miscarriages (RM) has been suggested to correlate with antibodies that cross react with HLA-negative syncytiotrophoblasts and we have reported that 17% of women with 2 or more miscarriages and 34% of women with 3 or more miscarriages express anti-trophoblast antibodies (ATAB). Until now, the mechanism, how ATAB interfere with pregnancy success is not known. hCG and progesterone both play fundamental roles in supporting human pregnancy. Therefore we investigated the effects of sera of RM patients containing ATAB on the hCG and progesterone production of cells of the choriocarcinoma cell line JEG-3.

Method of study: In vitro study to investigate effects of patient sera with and without ATAB on hCG and progesterone secretion of JEG-3 cells. The presence of ATAB was detected as described earlier. Effects of sera from ATAB positive and ATAB negative RM patients on hCG and progesterone secretion by JEG-3 cells were analysed 12 and 24 h after plating. Sera of women without pregnancy pathologies served as controls.

Results: Sera of ATAB-positive RM patients significantly inhibit hCG secretion of JEG-3 cells for 12 h after plating compared to sera of healthy controls (p = 0.019) and significantly reduce progesterone production for 12 h (p = 0.046) and 24 h (p = 0.027) of co-culture. Sera of ATAB-negative RM patient show no significant effect on progesterone secretion.

Conclusions: Inhibition of hCG and progesterone production might point to a mechanism, how ATAB interfere with early pregnancies.

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1. Introduction

Reproductive failure including recurrent miscarriages (RM) has been associated with antibodies that cross react with HLA-negative syncytiotrophoblasts (Balasch et al., 1981; McIntyre and Faulk, 1983; Johnson et al., 1984), (McIntyre et al., 1986; Kishore et al., 1996). These antibodies seem to appear early in pregnancies and can be identified by absorbing their activity with HLA-negative trophoblasts (McIntyre and Faulk, 1979; Power et al., 1983). Non-HLA anti-paternal specific antibodies may reflect a pathological humoral immune response against trophoblast cells, predisposing for RM (Kajino et al., 1988; Harris and Pierangeli, 1998). By binding to syncytiotrophiand/orendovascular trophoblast harmful immune responses such as complement- or cell mediated cytotoxic reactions might be triggered (Kajino et al., 1988; Nielsen et al., 2010). The precise antigenic epitopes targeted by anti-trophoblast antibodies remain unknown to date.

We recently demonstrated that 17% of women with 2 or more miscarriages and 34% of women with 3 or more miscarriages express anti-trophoblast antibodies (ATAB) (Rogenhofer et al., 2012). For that evaluation we chose the choriocarcinoma cell line JEG-3 because these cells retain many characteristics of normal pregnancy-derived trophoblasts and express a wide range of trophoblast-specific antigens that are candidate targets by maternal antibodies (Ringler and Strauss, 1990; Burt et al., 1991; Vanderpuye et al., 1991; Gobin et al., 1997; Yie et al., 2006). In detail,
Table 1

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Biographic and historic data of patients and controls.</th>
</tr>
</thead>
<tbody>
<tr>
<td>patients (n = 194)</td>
<td>controls (n = 80)</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>33 (21–40)</td>
</tr>
<tr>
<td>Mean pregnancies (range)</td>
<td>4 (2–15)</td>
</tr>
<tr>
<td>Mean deliveries (range)</td>
<td>9 (0–2)</td>
</tr>
<tr>
<td>Mean miscarriages (range)</td>
<td>4 (2–15)</td>
</tr>
<tr>
<td>Primary RM</td>
<td>150 (77.3%)</td>
</tr>
<tr>
<td>Secondary RM</td>
<td>9–20</td>
</tr>
<tr>
<td>Number of miscarriages</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>68 (35.1%)</td>
</tr>
<tr>
<td>4</td>
<td>31 (15.9%)</td>
</tr>
<tr>
<td>5–8</td>
<td>20 (10.3%)</td>
</tr>
<tr>
<td>9–20</td>
<td>5 (2.6%)</td>
</tr>
</tbody>
</table>

Note: NS: nonsignificant; n: number; p-value: significance level; n.s.: non significant; \( % \): percentage; RM: recurrent miscarriages.

they are negative for conventional MHC class I and II antigens but do express monomorphic HLA-G (Kovats et al., 1990; Olivas et al., 2002; Menier et al., 2003). Therefore, there should be no reactivity of antibodies against paternally-inherited HLA-antigens towards JEG-3 cells. This pattern of antigen expression resembles that of extravillous trophoblast cells that are exposed to maternal tissue.

Furthermore, similar to trophoblast cells, JEG-3 cells produce hCG and progesterone.

Several studies conclude that a single hCG measurement in early pregnancy can reliably predict pregnancy outcome (Ali-Sebai et al., 1996; Letterie and Hibbert, 2000; Condous et al., 2005; Ochsenkuhn et al., 2009).

In addition, previous investigations have suggested that the relative power of a combined progesterone and hCG determination in the prediction of pregnancy outcome is even higher. Therefore, the aim of this study was to investigate the differential effects of sera from ATAB-positive and ATAB-negative RM patients on the hCG and progesterone secretion by JEG-3 cells.

2. Materials and methods

2.1. Patients and controls

194 patients with a history of two or more consecutive miscarriages before the 20th week of gestation were evaluated and included in the RM group. The criteria for strengthening the significance of observational studies in epidemiology were accounted for where applicable. All patients underwent an extensive diagnostic work up and were excluded from analysis upon identification of known causes for RM including infectious diseases, uterine anomalies, endocrinologic dysfunctions (polycystic ovary syndrome according to the Rotterdam criteria (Rotterdam ESHRE/ASRM PCOS Consensus Workshop Group, 2004)), hyperprolactinemia, hyperandrogenemia, thyroidal dysfunctions such as hypothyroidism and thyroid autoantibodies, autoimmune disorders (autoimmune antibodies >1:240, antinuclear antibodies IgG and IgM, anti-Ro/SSA, -Jo-1, -SSB/La, -RNP, -Sm, -dsDNA antibody, -AMA), deficiencies in coagulation factors (protein C, protein S, factor XII, antithrombin III) and fetal and parental chromosomal disorders (numerical aberrations). Also excluded were patients with inherited thrombophilias (factor V-Leiden mutation (FVL), the prothrombin (PT) 20210G>A substitution) as well as the homozygous 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C>T exchange and antiphospholipid syndrome according to the international consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (Miyakis et al., 2006).

Biographic and historic data of patients and controls are illustrated in Table 1: 73.2% (n = 142) suffered from primary RM no infant born either dead or alive after the 20th completed week of gestation and/or weighing more than 500 g before the series of miscarriages (Reza, 2010) and 26.8% (n = 52) underwent secondary RM at least one infant born either dead or alive after the 20th completed week of gestation or weighing more than 500 g before the series of abortions miscarriages (Reza, 2010). Healthy control individuals with normal pregnancies, >1 normal term deliveries of healthy, normal weight singletons and without gestational pathology were recruited from our outpatients department from 2004 to 2010.

Blood sampling in all cases was carried out at least 8 weeks after the last pregnancy had ended in order to rule out any immediate interference with gestation. Serum aliquots (100 μL) were frozen and stored at −20 °C for further analyses. Neither RM patients nor controls had ever received transfusions of blood or blood products.

Signed informed consent was obtained from all participants allowing analysis of all clinical and laboratory data mentioned in this paper. The Human Investigation Review Board of the Ludwig-Maximilians-University Munich approved the study (number 238-06).

2.2. JEG-3 cells

JEG-3 cell culture was set up as previously described (Kohler and Bridson, 1971; Paul et al., 2000). In brief, the cells were cultured in complete culture medium RPMI 1640 (Sigma, Deisenhofen, Germany) supplemented with FCS (heat-inactivated, 10% v/v, Dako, Glostrup, Denmark) and Penicillin/Streptomycin (100 IE/ml Penicillin, 50 μg/ml Streptomycin) (Invitrogen, Darmstadt, Germany) at 37 °C in a humidified atmosphere (95% CO2) until 80% sub-confluence was reached.

2.3. Flow cytometry analysis

JEG-3 cells were prepared for flow cytometry as described earlier (Rogenhofer et al., 2012). Cells were detached from the flask by addition of 10 mL ready-to-use Accutase solution containing proteolytic and collagenolytic enzymes in Dulbecco’s Phosphate buffered saline (PBS), pH 7.4 (Sigma, Deisenhofen, Germany, Cat.Nr.A6964) per 75 cm2 surface area and returned to the incubator for 10 min. Cell concentration was determined using a Coulter Z1 counter (Coulter Electronics, Krefeld, Germany). A suspension of 105 cells was transferred into a 5 mL polystyrene round-bottom tube (BD Bioscience, Heidelberg, Germany) and washed twice in RPMI supplemented with 20 mg/mL FCS and 0.2 mM Colchicin (Sigma, Deisenhofen, Germany). Subsequently, cells were re-suspended and incubated with 20 μL of patient, control or standard sample sera for 60 min at 4 °C.

The expression of conventional HLA-A/B antigens by JEG-3 cells was analysed by incubating the cells with a monoclonal mouse anti-HLA-A/B antibody (TP25.99, Sangstat, Nantes, France diluted 1:20 in 20 μL PBS). HLA-G expression was analysed utilizing a monoclonal mouse anti-HLA-G-1 antibody (MEM-G-9, Abcam, Cambridge, UK; diluted 1:20 in 20 μL PBS) (38–40, 46). Purified mouse IgG (Cedarlane Diagnostics, Burlington, Ontario, Canada) at identical IgG concentrations served as control.

Following incubation with human sera or primary monoclonal antibodies, the cell suspension was washed twice in RPMI buffer and incubated with FITC-conjugated secondary antibodies diluted 1:10 in 10 μL PBS for 60 min at 4 °C in the dark. Secondary antibodies were polyclonal goat anti-human IgG/FITC F(ab)2 (Dako, Hamburg, Germany), or FITC-conjugated rabbit anti-mouse IgG (Dako, Hamburg, Germany) respectively.

The cell suspensions were analysed on a Beckton Dickinson flow cytometer (FACScan, Heidelberg, Germany) equipped with a 2.4 mW argon ion laser at an excitation wavelength of 488 nm. The
green signals of FITC on a log scale of each analysed cell fraction were collected using a 590 nm band pass filter. A marker was set in the FITC histogram as the cut-off between the background signals and positive staining which was determined by comparison with negative control samples.

Anti-JEG-3 reactivity of individual test sera was calculated by using the formula:

\[
\text{ACTIVITY OF TEST SERUM (MCS)} - \text{ACTIVITY OF LOW REACTING STANDARD (MCS)} \times 100 = \text{[\%]}
\]

Activity of high reacting standard (MCS) – activity of low reacting standard (MCS)

2.4. Cell viability

Cell viability was investigated by flow cytometry with PI-staining: An aliquot of cells from each experiment was washed twice and incubated with propidium iodide (1 mg/mL, Sigma, Deisenhofen, Germany) for 10 min. The red signals of propidium iodide were quantified by flow cytometry by using a 650 nm band pass filter; the propidium iodide positive cells were considered dead and propidium iodide negative cells were considered alive. Only samples with at least 90% viability were included for the analysis.

2.5. hCG and progesterone production of JEG-3 cells

JEG-3 cells were plated in 24-well-plates to reach a total number of 50,000 cells per well and cultured with 1 mL RPMI 1640+ Glutamax medium per well for 24 h at 37 °C and 5% (v/v) CO₂. Attachment of cells was checked and cell numbers were established. Culture media were refreshed and supplemented with 10% (v/v) patient sera of ATAB-positive or ATAB-negative RM patients or healthy controls. All wells were prepared as duplicates and three independent experiments were set up. Supernatants were analysed for hCG and progesterone production after 12 and after 24 h of culture on an automated in vitro diagnostic system (IMMULITE, Siemens, Munich, Germany) as described recently (Jeschke et al., 2005, 2007).

2.6. Statistical Analyses

Statistical analyses were performed with the Statistical Package for Social Sciences (SPSS for Windows 19.0, SPSS Inc., Chicago IL, USA). Comparisons of pregnancy histories and biographic data of JEG-3 positive and JEG-3 negative patients as well comparison of RM patients and control groups were performed by standard Chi-square test and Mann-Whitney U test. Positive reactivity with JEG-3 cells was defined to be above the 95% confidence-range of the control group. This was calculated according to the formula above. P-values < 0.05 were regarded as statistically significant.

3. Results

3.1. Definition of ATAB-positive sera of patients with recurrent miscarriages by flow cytometry

A minimum of 15,000 cells was analysed in each run. Reactivity was measured in mean channel shifts (MCS), and anti-JEG-3 reactivity was expressed as percentage of the difference between the reactivity of two in house standard samples that were included in each assay. The standard sample with high reactivity consisted of a pool of 10 patient sera, identified as highly reactive in preliminary experiments. These patients had experienced a mean number of 4 (minimal-maximal: 3–8) unexplained miscarriages. While meeting the criteria of RM patients, we did not include them in our study group. Identical serum aliquots of each of these individuals were pooled in order to obtain a positive standard sample and this respective anti-JEG-3 reactivity was arbitrarily defined as 100%. The standard sample with low reactivity consisted of a blood group AB standard sample provided by our local blood bank (registration number 505110) and this anti-JEG-3 reactivity was arbitrarily defined as 0%.
3.2. Cytotoxicity of ATAB positive sera tested with a cell viability assay

Only samples with at least 90% viability were included for the analysis. The intra-assay coefficient of variation (intra-assay CV) was calculated from 5 independent measurements of a given serum within one measurement and calculated to be 8.9%. The inter-assay CV was calculated from 5 different measurements of a given serum at 5 different time points and calculated to be 13.3%. The intra-assay coefficient of variation (intra-assay CV) was calculated from 5 independent measurements of a given serum within one measurement and calculated to be 8.9%.

3.3. Sera of ATAB-positive RM patients reduced hCG expression of JEG-3 cells

Sera of ATAB-positive RM patients significantly inhibit hCG secretion of JEG-3 cells for 12 h compared to the sera of healthy controls [7.19 ± 2.91 µIU/mL vs. 13.9 ± 0.14 µIU/mL, controls; p = 0.019]. Sera of ATAB-negative RM patients leave the hCG secretion of JEG-3 unaltered (Fig. 1a). At 24 h after addition of patient sera, no significant difference of hCG secretion is detected between the ATAB and control group.

3.4. Sera of ATAB-positive RM patients inhibits progesterone expression of JEG-3 cells

Sera of ATAB-positive RM patients also decrease progesterone secretion of JEG-3 cells for 12 h and 24 h (Fig. 1b) compared to the sera of healthy controls [12 h: 0.72 ± 0.13 ng/mL vs. 1.68 ± 0.11 ng/mL, controls (p = 0.046); and 24 h: 2.39 ± 0.16 ng/mL vs. 2.81 ± 0.23 ng/mL, controls (p = 0.027)]. In addition, sera of ATAB-negative RM show no significant effect compared to controls.

4. Discussion

In this study, we demonstrate a significant inhibitory effect of sera from anti-trophoblast-antibody-positive (ATAB) patients with recurrent miscarriages (RM) on hCG and progesterone production of JEG-3 choriocarcinoma cells in vitro. Inhibition was not related to negative effects on cell viability and only samples with more than 90% of viable cells were used for analysis. Inhibitory effects on hCG secretion were found at early time points after plating (12 h) and inhibitory effects on progesterone secretion persisted up to 24 h after plating. Surprisingly, the serum of ATAB negative RM patients had also some effects on progesterone and hCG expression compared to the control serum. Although these differences were not as significant as with the ATAB positive sera, we have to acknowledge that sera of RM patients in general contain substances that may influence the endocrine output of trophoblast cells. It is known that serum of RM patients contains a variety of cytokines and other factors that bind to G-protein coupled receptors like IL-6 (Arslan et al., 2004). In addition, RM is accompanied with an increase in IL-15 expression (Toth et al., 2010). By binding G-protein coupled receptors (GPCRs) these substances may influence steroid hormone synthesis, as steroid hormone synthesis is influenced mainly by a GPCR driven pathway in the trophoblast (Zosmer et al., 1997).

The steroid hormone progesterone and the protein hormone hCG both play a fundamental role in supporting human pregnancy. 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 stereospecific abilities of choriocarcinoma cells in culture are similar to those of the in vivo placenta and support their use as an experimental model of placenta steroidsogenesis (Bahn et al., 1981).

Progesterone has suppressive actions on lymphocyte proliferation and activity and on the immune system to prevent rejection of the developing fetus and placenta (Stites and Siiteri, 1983). 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 (Pepe and Albrecht, 1995). A down regulation and even a plateau of hCG in ongoing pregnancy are accompanied with a negative pregnancy outcome and an onset of abortion. For that reason, the measurement of hCG in the first trimester is the main prognosticator for a successful pregnancy.

The physiological role of hCG during pregnancy is not completely understood. It is generally assumed that hCG production by human trophoblast supports maintenance and function of the corpus luteum, its production of progesterone, thus ensuring a normal course of pregnancy. Expression of hCG is detectable as early as at the 8 cell stage of the developing embryo (Bonduelle et al., 1988). An array of paracrine modulators of hCG synthesis has been identified (Jameson and Hollenberg, 1993; Pestell et al., 1994; Johnson et al., 1997; Mochizuki et al., 1998). Although new insights about the type of hCG-glycosylation and the cell types that produce hCG have recently emerged (Ulloa-Aguirre et al., 2001; Kovalevskaya et al., 2002), the regulation of placental hCG-β expression is still not well understood (Maston and Ruvolo, 2002). Analysis on the promoter region of hCG showed that this region is quite complex (Albanese et al., 1991) and not a result of a single discrete regulatory element (Hollenberg et al., 1994). Experiments with cytokines have pointed towards a role for interleukin-1β in hCG regulation (Seki et al., 1997). We demonstrated that Gα1 is involved in the stimulation of hCG in first trimester trophoblast cells (Jescke et al., 2005).

On the other hand, the JEG-3 trophoblast tumor cell culture system was already proven as a useful cell culture system for both, the identification of ATAB and the regulation of hCG and progesterone in vitro.

The cell line JEG-3 contains trophoblastic tumour cells at various stages of differentiation. JEG-3 cells compose of invasive and cytotrophoblast cells (Bergemann et al., 2003). The differentiation of cytotrophoblast cells is associated with activation of –α and hCGB subunit genes. These intermediates are transient, they differentiate to either extravillous or syncytiotrophoblasts (Hoshina et al., 1984). Furthermore chorion carcinoma cells consisting of clusters of cytotrophoblast like and large multinucleated cells express –α and hCGB mRNA (Matsuo et al., 1985). In all cases, these cells produce large amounts of progesterone.

4.1. In summary

Anti-trophoblast antibodies (ATAB) were characterised recently by binding to choriocarcinoma cell line JEG-3. These cells retain many characteristics of normal pregnancy-derived trophoblasts and express a wide range of trophoblast-specific antigens that are candidate targets by maternal antibodies. Progesterone and hCG release was found to be decreased in ATAB-treated JEG-3 trophoblast cells compared to untreated cells. Inhibition of both or any of these hormones might point to a mechanism, how ATAB interfere with early pregnancies.

References


Kohler, R., immune trophoblast tnf-alpha, (alpha, trophoblast A.
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