Progesterone enhances HLA-G gene expression in JEG-3 choriocarcinoma cells and human cytotrophoblasts in vitro

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BACKGROUND: Evidence suggests that HLA-G plays a critical role in maternal immune tolerance to the fetus. However, regulation of HLA-G gene expression is not well understood. Many studies have suggested that progesterone may also be important in suppressing maternal immune response to the fetus. Therefore, we hypothesized that this steroid hormone may play a role in regulating HLA-G gene expression. The objective of the study was to explore potential effects of progesterone on HLA-G gene expression in vitro. METHODS: Cultured first trimester trophoblasts and JEG-3 choriocarcinoma cells were treated with progesterone and its antagonist RU486. HLA-G gene transcription was determined by real-time PCR while HLA-G translation was investigated by a specific enzyme-linked immunosorbent assay for HLA-G and western blot analysis. RESULTS: HLA-G mRNA and protein expression in trophoblasts and JEG-3 cells were elevated by progesterone in dose- and time-dependent manners. The effect of progesterone can be completely inhibited by co-incubation with RU486 at the same concentrations. CONCLUSION: Progesterone has an up-regulatory effect on HLA-G gene expression in first trimester trophoblasts and JEG-3 cells in vitro.

Key words: HLA-G/JEG-3/progesterone/RU486/trophoblast

Introduction

How the fetal–placental unit, a semi-allograft, escapes maternal immune rejection during pregnancy is a fundamental question in reproductive immunology. Placental trophoblast cells must play an essential role, since they are the only cells of this unit which come into direct contact with maternal tissue at the interface (Tony et al., 1997). Multinucleated syncytiotrophoblast cells cover the ‘floating’ chorionic villi, which are bathed in maternal blood. These express neither HLA class I nor class II antigens. Extravillous cytotrophoblast cells invade the uterine decidua layer and those that reach maternal vessels replace their endothelial cells to line these vessels. These cells mainly express the non-classical class I antigen, HLA-G, which is minimally polymorphic and its expression is limited to a few other adult or fetal tissues including oocytes (Jurisicova et al., 1996), early embryos (Jurisicova et al., 1996), thymus (Crisa et al., 1997), and activated monocytes (Moreau et al., 1999). Some expression of HLA-C has also been reported in extravillous cytotrophoblast (King et al., 2000). Through alternative splicing of mRNA, a secreted or soluble form of HLA-G also exists (Fujii et al., 1994).

These unique characteristics of HLA-G have led to the hypothesis that HLA-G may play a vital role in maternal immune tolerance to the fetus (Kovats et al., 1990). Experimental evidence from our group and others has suggested that HLA-G in both membrane and soluble forms may protect the fetal–placental unit from natural killer cell lysis (Kovats et al., 1991; Chumble et al., 1994; McMaster et al., 1995) and cytotoxic T-cell activity (Deniz et al., 1994; Kapasi et al., 2000).

An understanding of the regulation of HLA-G gene expression is essential in order to fully appreciate the functional aspects of this protein. HLA-G gene expression is known to be developmentally regulated (i.e. decreases during human gestation), but the exact mechanisms of how HLA-G gene expression is regulated are still poorly understood.

Progesterone is an essential steroid for maintenance of pregnancy. Functions attributed to progesterone in pregnancy include: stimulation of growth and differentiation of the endometrium to allow for implantation, inhibition of myometrial contractions, and induction of immune tolerance to the fetus (Szekeres-Bartho and Szekeres, 1992; Parronchi et al., 1995). The exact mechanisms by which progesterone induces immune tolerance are not well characterized. For these studies, we hypothesized that one mechanism is through modulation of placental HLA-G gene expression. To evaluate this hypothesis, we studied the effects of progesterone on HLA-G expression in both cultured isolated first trimester cytotrophoblasts and the JEG-3 choriocarcinoma cell line.
Materials and methods

Cell culture
The JEG-3 choriocarcinoma cell line shares several features with primary cytotrophoblast cells, including expression of HLA-G (Ringer and Strauss, 1990). Like the trophoblast HLA-G, the JEG-3 cell HLA-G heavy chain is recognized by both of the monoclonal antibodies (mAb) (2C/C8 and 3C/G4) used in our enzyme-linked immunosorbent assay (ELISA) (see below). Using a cell line had the advantage of avoiding contamination by other cell types that may also have progesterone receptors. JEG-3 human choriocarcinoma cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown in Roswell Park Memorial Institute 1640 medium containing 10% fetal calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. For HLA-G protein expression studies, cells were cultured in a 24-multiwell plate, and 6-multiwell plates (Nunc, Rochester, NY, USA) were used for HLA-G mRNA expression studies.

Ethics approval for collection of first trimester placental tissue from therapeutic abortion specimens for these studies was obtained from the human ethics committee of the Sunnybrook and Women’s College Health Sciences Centre, Toronto, Canada. Purified human cytotrophoblast cells were isolated as previously described (Librach et al., 1994). For HLA-G protein and mRNA expression studies, these cells were cultured in 24- and 6-multiwell plates (Nunc) respectively.

Induction experiments
Progesterone and the progesterone antagonist RU486 (Sigma, St Louis, MO, USA) were used to determine whether progesterone has an effect on HLA-G expression in JEG-3 cells and trophoblasts. For these experiments, the cells were incubated with either serum-free medium (control) or serum-free media containing various doses of progesterone and/or RU486. For the dose-dependent studies, cells were collected after exposure for 24 h. For time-course studies, cells were harvested after different exposure times. Since the maximal progesterone effect was observed after 3–4 h of treatment, for experiments using western blot and real-time PCR techniques, cells were collected after exposure for 4 h. For HLA-G protein expression analysis, the cells were lysed in 0.05 mol/l Tris–HCl buffer containing 0.5% NonidetP-40, 2.5 mmol/l EDTA and 200 mmol/l phenylmethyl-sulphonyl fluoride at 4°C and the cell lysate supernatant was obtained by centrifugation at 16 000 g for 5 min at 4°C.

ELISA for HLA-G protein in cell lysate and conditioned culture medium:
Purification of HLA-G protein standard has been described in detail previously (Kapasi et al., 2000). Briefly, 1 g of placental tissue was homogenized and lysed and the lysate was passed through sequential 4H84 mAb (against HLA-G) followed by bMM1 mAb (against β2-microglobulin) affinity columns. Isolated HLA-G protein was analysed by both sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) stained with Coomassie blue and western blot with the 4H84 mAb. The purified protein contained both the surface (38–39 kDa) and secreted forms (34–35 kDa) of HLA-G and no classical class I molecules were detected in the preparation (Kapasi et al., 2000). The ELISA used in the experiments was similar to that described previously, except that our mAb, 2C/C8 was substituted for 4H84 (Yie et al., 2004, 2005a,b). By competition assay, 2C/C8 [an immunoglobulin (Ig) G3 mAb] has substantially the same binding site on the HLA-G heavy chain as 4H84 (an IgG1 mAb) (see US Patent No. 6,613,538 for a full description and validation of this assay). As previously described, our mAb, 3C/G4 (an IgA mAb), binds to a distinct site on the HLA-G heavy chain. Briefly, each well of a 96-well immunoplate (Corning, NY, USA) was filled with 0.1 ml of 2C/C8 mAb (10 μg/ml) and kept at 4°C overnight. Each well was washed three times with a 0.01 mol/l phosphate-buffered saline (PBS) washing solution containing 0.05% Tween-20 and blocked with 5% milk for 4 h at room temperature. Duplicate conditioned cultured medium samples and cell lysate samples were added to a final volume of 0.1 ml in each well and incubated at 4°C overnight. Wells were washed three times with the washing solution and to each well 0.1 ml of biotinylated 3C/G4 (0.4 mg/ml) was added. The plate was incubated for 2 h at room temperature. All the wells were washed four times before adding 0.1 ml/well of a 1:2000 dilution of streptavidin–horseradish peroxidase (HRP) (Sigma) in 0.01 mol/l PBS containing 1% bovine serum albumin, and then incubated for 1 h at room temperature. Wells were then washed four times before adding 100 μl of tetramethylbenzidine substrate solution (Sigma). After 10–15 min incubation at room temperature the reactions were stopped by the addition of 50 μl of 1 mol/l HCl to each well and the plate was read at 450 nm on an automated ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). Curve fitting and dose interpolation were performed using software associated with the reader.

Our ELISA has a lower limit of detection of 10 ng/ml. The variation coefficients within and between batches were 4.4 and 9.3% respectively. Accuracy of the assay was 94.6%. Serially diluted cell lysate and conditioned medium samples gave dose responses parallel to that of the native purified HLA-G standard.

The total protein concentrations in each cell lysate sample were determined by using a Bio-Rad protein kit (Bio-Red, Hercules, CA, USA). HLA-G concentrations in each cell lysate sample were then normalized using the total protein concentrations in each sample. Normalized HLA-G values in samples treated with progesterone and/or RU486 were compared with controls.

Western blot analysis
The same amounts of total proteins (5 mg) in each cell lysate sample were separated by 12% SDS–PAGE and electroblotted to PVDF membranes (Invitrogen, Carlsbad, CA, USA), according to published methods (Towbin et al., 1979). Membranes were blocked in 0.1% PBS containing 0.1% Tween-20 (Sigma) and 5% Block solution (Roche, Montreal, Quebec, Canada) for 1 h at room temperature, and then incubated with mAb 3C/G4 diluted 1:500 in the PBS at 4°C overnight. On the next day, membranes were washed with 0.01% PBS/0.1% Tween-20 solution (3×10 min) and incubated with HRP-conjugated goat anti-mouse IgG (Sigma) diluted 1/1000 for 1 h at room temperature. Membranes were then developed with 3,3′-diaminobenzidine (Sigma) after another three washes.

Real-time PCR analysis
Total RNA was extracted using Trizol® reagent (Gibco/BRL, Burlington, ON, Canada) according to the manufacturer’s manual. cDNA were prepared from the total RNA using oligo (dT) primers and Moloney murine leukaemia virus reverse transcriptase (Gibco/BRL). The RT reaction was performed at 42°C for 50 min.

Real-time PCR was performed with the SYBR Green Mix (ABI, Forster City, CA, USA) on an ABI Prime 7900 Sequence detector. Specific primers for HLA-G were 5′-AGCTGTTGTTGTCCTTC-3′ for forward; and 5′-GGCCAGAAAACTGCTT-3′ for reverse; primers for β-actin were 5′-CCACCCGGGAAGAGATA-3′ for forward; and 5′-CCAGAGGCTACAGGGATG-3′ for reverse. The HLA-G and β-actin primers flanked 106 and 97 bp regions respectively. For each PCR product the melting curve was determined. The Comparative threshold cycle number (2–ΔΔCt) method was used after a validation experiment demonstrated that efficiencies of target (HLA-G) and reference (β-actin) were approximately equal. Ct values define the
threshold cycle of PCR, at which amplified products were detected. ΔCt was the difference in the threshold cycles for HLA-G and β-actin (internal control). Our results were represented as fold changes in expression of HLA-G after treatment with progesterone/RU486 relative to controls (−ΔΔCt) that were calculated from the arithmetic formula 2−ΔΔCt (Livak and Schmittgen, 2001).

**Statistical analysis**

All experiments were performed in triplicate and all experiments were repeated at least three times on different occasions. All data were analysed for the comparison of group means using one-way analysis of variance. P < 0.05 was considered statistically significant.

**Results**

**Effect of progesterone on HLA-G protein expression**

To observe any effect of progesterone on steady state of HLA-G protein expression in vitro, JEG-3 cells and trophoblasts were incubated in serum-free medium containing progesterone from 0 (control) to 1000 ng/ml for 24 h. The quantitative data of JEG-3 and trophoblasts in HLA-G proteins before and after exposing to progesterone, detected by the ELISA, were listed in Table I. Figure 1A–D shows that exogenous progesterone increases HLA-G protein levels in both cultured JEG-3 and cytotrophoblasts in a dose-dependent manner as compared to control. The increases were statistically significant when cells were treated with a dose of 100 ng/ml of progesterone or higher (F = 8.02, P = 0.0012 for JEG-3-conditioned media, F = 5.08, P = 0.0012 for JEG-3 cell lysates, F = 4.83, P = 0.0017 for cytotrophoblast-conditioned media and F = 18.3, P < 0.0001 for cytotrophoblast cell lysates). In western blot experiments, two distinct bands were detected with the Ab. The lower mol. wt band presumably represents the soluble form of the molecule. The band density increased in a dose-dependent manner (Figure 1E), paralleling our results obtained by ELISA.

In order to explore the time-course of this effect, JEG-3 cells were treated with or without 100 ng/ml of progesterone in serum-free medium from 0 to 48 h. It took 4–5 h to achieve maximal effect on HLA-G protein expression (Figure 2A).

In western blot experiments, JEG-3 cells were treated with or without 1000 ng/ml of progesterone in serum-free medium from 0 to 4 h. The strongest Ab reaction was found at 3 h after treatment with 1000 ng/ml of progesterone, whereas there was no change in untreated samples during the 4 h incubation (Figure 2B).

**Table 1.** Quantitative data of HLA-G proteins, detected by the enzyme-linked immunosorbent assay, after exposing the cells to 0 to 1000 ng/ml of progesterone for 24 h in both JEG-3 and trophoblast cells.

<table>
<thead>
<tr>
<th>Progesterone (ng/ml)</th>
<th>JEG-3</th>
<th>Trophoblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell lyse (mg/mg protein)</td>
<td>Medium (mg/ml)</td>
</tr>
<tr>
<td>0</td>
<td>3.83 ± 0.32</td>
<td>71.7 ± 2.7</td>
</tr>
<tr>
<td>1</td>
<td>4.14 ± 0.38</td>
<td>96.2 ± 7.2</td>
</tr>
<tr>
<td>10</td>
<td>4.57 ± 0.65</td>
<td>109.2 ± 2.6</td>
</tr>
<tr>
<td>100</td>
<td>5.39 ± 0.75</td>
<td>144.2 ± 2.2</td>
</tr>
<tr>
<td>1000</td>
<td>5.57 ± 0.60</td>
<td>177.3 ± 3.0</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

**Effect of progesterone on HLA-G mRNA expression**

In order to determine whether progesterone also had an effect on the steady state of HLA-G mRNA expression in vitro, a real-time PCR assay was carried out. Progesterone at 10 ng, 100 ng and 1000 ng/ml enhanced HLA-G mRNA expression...
Progesterone enhances HLA-G gene expression by 2.36-, 10.53- and 17.58-fold respectively, as compared to controls (F = 4.54, P = 0.0387) (Figure 3A).

The time-course of progesterone induction of HLA-G mRNA was also investigated. HLA-G mRNA was increased 17.16-fold after 1 h with 1000 ng/ml progesterone (F = 52.4, P < 0.0001), whereas there was no significant change in control cells during the 4 h incubation (F = 2.61, P = 0.0778) (Figure 3B).

**Effect of progesterone on HLA-G expression: RU486 experiments**

To determine whether the effect of progesterone on HLA-G expression required progesterone receptor binding, cultured JEG-3 cells in wells were all exposed to 100 ng/ml of exogenous progesterone and varying doses of the progesterone antagonist RU486 for 24 h. As measured by ELISA, addition of RU486 from 1 to 1000 ng/ml significantly inhibited the up-regulatory effect of progesterone in a dose-dependent manner with maximal inhibition at 100 ng/ml (Figure 4A and B) (F = 2.57, P = 0.0332 for cell lysate and F = 4.2, P = 0.0153 for conditioned media).

Similarly, RU486 blocked the up-regulatory effect of progesterone observed by both western blot analyses (Figure 4C) and real-time PCR techniques (F = 5.60, P = 0.0230) (Figure 4D) in a dose-dependent manner.

**Discussion**

The present study demonstrates for the first time that progesterone has an up-regulatory effect on HLA-G gene expression in cytotrophoblast and JEG-3 choriocarcinoma cells *in vitro*. The effect is dose dependent with maximal enhancement of
both steady state mRNA and protein expression with a progesterone concentration of ~100 ng/ml or higher. Our time-course study revealed maximal HLA-G production after 4 h, suggesting that at least part of the progesterone effect is likely through regulation of transcription. During pregnancy, circulating progesterone concentrations rise to 200 ng/ml and at placental maternal–fetal interface progesterone concentrations appear to reach levels as high as 3 μg/g tissue (Szekeres-Bartho and Szekeres, 1992). Our in vitro findings of a clear regulatory effect of progesterone on HLA-G expression suggest that progesterone may play a physiological role in the regulation of HLA-G gene expression in vivo.

It is now clear that a successful pregnancy requires a number of immune modulation factors from both mother and fetus. Progesterone has been suggested to be an important immune modulator during pregnancy for many years (Stites and Siiteri, 1983). However, the precise underlying mechanisms for how progesterone modulates the immune system are still under investigation. Growing evidence indicates that progesterone has many effects on the immune system during pregnancy such as triggering suppressor T-cell generation (Brierley and Clark, 1987), blocking cytotoxic T-cell activity (Mannel et al., 1990), reducing natural killer cell activity (Hansen et al., 1992), inducing lymphocyte blocking proteins (Barakonyi et al., 1999) and modifying the cytokine response (Parronchi et al., 1995; Piccinni et al., 1995; Choi et al., 2000). Whether these effects are through the progesterone receptor remains controversial (Szekeres-Bartho et al., 1989; Mansour et al., 1994; Schust et al., 1996).

Sex steroid hormones are known to act through intracellular receptors. Both progesterone receptor (PR) mRNA and protein have been identified in trophoblasts of human placenta from first trimester to term by various techniques (Rossmanith et al., 1997). The present study shows that the stimulatory effect of progesterone can be completely blocked by co-incubation with a corresponding concentration of RU486 (Figure 4). The mechanism of action of RU486 involves strong binding affinity to PR followed by transconformation changes in the ligand-binding domain (Cadepond et al., 1997). Direct activation of gene expression after steroid hormone receptor binding usually occurs within 1–4 h (Landers and Spelsberg, 1992). The time-course of progesterone effect that we observed further suppG gene expression via its intracellular receptor (Figures 2 and 3B).

Activated PR will bind a specific DNA sequence, the progesterone response element (PRE), and will initiate the transcription of target genes (Giangrande and McDonnell, 1994).
1999). However, HLA-G gene promoter has no classical PRE sequence. Therefore, molecular mechanisms implicated in progesterone enhancement of HLA-G gene expression remain to be further elucidated.

In summary, the present study demonstrates that HLA-G gene expression is up-regulated by progesterone. Future studies in our laboratory will address whether this effect is mediated by a novel PRE in the HLA-G gene promoter region or through some other mechanism.

References

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