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Effect of Glucocorticoids on Transcriptional Status of HLA-G in Human Trophoblast Cells from Full Term Placenta

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ABSTRACT There are evidences which suggest that HLA-G molecule plays an important role in immune tolerance, protecting the potentially allogenic fetus from maternal immune attack. Regulation of HLA-G expression is not well characterized; however, studies suggest a possible role of glucocorticoids in modulation of HLA-G gene expression. Therefore, we tested this hypothesis by inducing the HLA-G expression levels in full term placenta using two glucocorticoids -Dexamethasone and Hydrocortisone, JEG-3 and JAR cell lines were used as a positive and negative controls. Cultured trophoblast cells were treated with Dexamethasone and hydrocortisone. HLA-G transcription was determined by semi-quantitative RT-PCR. Choriocarcinoma JEG-3 (HLA-G $^{+ve}$) and JAR (HLA-G $^{-ve}$) cell lines were obtained from American Type Culture Collection (ATCC). The level of HLA-G mRNA transcripts in trophoblast cells were elevated by Dexamethasone and hydrocortisone in dose and time dependent manners. Glucocorticoids have an up-regulatory effect on HLA-G transcripts in trophoblast cells.

INTRODUCTION

Human leukocyte antigen (HLA)-G, gene was first identified by Geraghty et al. (1987) and was found to be located at 6p21.3. HLA-G is a major histocompatibility complex–I (MHC-I) molecule and characterized by a limited polymorphism, expressed mainly at the feto-maternal interface and play an essential role in maternal immune tolerance to the fetus (Kovats et al. 1990). Extensive studies have been carried out on its functions in fetal–maternal immune maintenance. These studies suggested that HLA-G serves as a protection factor for the fetus from maternal allorecognition by the ability of HLA-G to modulate both the functions of immune component cells, such as natural killer (NK) cells, T lymphocytes, antigen presenting cells and cytokine balance during pregnancy (Poehlmann et al. 2006; Yan et al. 2007). The mechanisms that underlie the functions of HLA-G involve its interaction with various receptors such as immunoglobulin-like transcripts 2, 4 (ILT2, ILT4), and killer immunoglobulin receptor 2DL4 (KIR2DL4) expressed on these immune cells (Rajagopalan et al. 1999; Yan et al. 2005). The expression of this molecule is limited to a few other adults or fetal tissues including oocytes, thymus and activated monocytes (Moreau et al. 1999).

In contrast to classical HLA class-I genes, the primary transcript of the nonclassical HLA-G gene gives rise to four membrane bound isoforms (HLA-G1, HLA-G2, HLA-G3, HLA-G4 and three soluble isoforms HLA-G5, HLA-G6 and HLA-G7 through alternative splicing mechanisms (Paul et al. 2000). In case of pregnancy all the isoforms were seen in first trimester and second trimester placenta and the expression reduced during gestation but in recurrent spontaneous abortion an altered expression of HLA-G was seen (Aldrich et al. 2001; Ober et al. 2006).

In present study, glucocorticoids are used to induce HLA-G expression in trophoblast cells obtained from full term pregnancy. Glucocorticoids (Dexamethasone and Hydrocortisone) have potent anti-inflammatory and immunosuppressive properties. Glucocorticoids (GC) is a key regulator of placental gene expression and are used to improve the outcome of pregnancy in women undergoing assisted conception by in vitro fertilization-embryo transfer (IVF-ET)

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(Boomsma et al. 2007), and in women with a history of recurrent miscarriages (Quenby et al. 2005). These hormones are expressed by the placental tissues widely in addition to their appropriate receptors. They act as positive or negative regulators of transcription of genes such as collagen, fibronectin, integrin, prostaglandin dehydrogenase (Patel et al. 1999), human chorionic gonadotropin (HCG) (Ringler et al. 1989) plasminogen activator inhibitor-1 (Ma et al. 2002), and glucose transporters (Hahn et al. 1999). Through semiquantitative determination of mRNA levels, Moreau and co-workers (Moreau et al. 2001) had demonstrated for the first time that HLA-G mRNA expression in choriocarcinoma cells (JEG-3) as well as in cultured trophoblast cells obtained from 1st trimester terminated pregnancy explants can be upregulated by glucocorticoids such as dexamethasone and hydrocortisone. Expression of HLA-G protein has also been shown to be upregulated by the effect of progesterone (Yie et al. 2006) which is another important steroid hormone, as well as by reversal of DNA methylation (Moreau et al. 2002) emphasizing the role of epigenetic mechanisms involved in the regulation of HLA-G expression. In order to examine the effects of glucocorticoides on the expression of HLA-G mRNA during full term pregnancy. We conducted this study using dexamethasone and hydrocortisone as candidate glucocorticoids and induced HLA-G mRNA expression in cultured trophoblast cells obtained from term placenta. We have used JEG-3 (HLA-G^{+ve}) and JAR (HLA-G^{-ve}) cell lines as positive and negative controls. These cell lines are established from human choriocarcinoma cells derived from first trimester trophoblast (Kitano et al. 1988). They are potentially suitable "in vitro" models for the regulation of the expression of HLA-G in placenta.

MATERIALS AND METHODS

Purification of Villous Cytotrophoblasts from Term Placenta

Term placental tissues (N=20) obtained immediately after uncomplicated cesarean sections. These samples were collected from "Queen Mary's Hospital, Lucknow", Uttar Pradesh, India. Cytotrophoblast cell isolation and primary culture were performed as described (Kliman et al. 1986; and Alsat et al. 1991) with some modification. Briefly, chorionic villi were minced into small pieces and incubated for 30 minutes at 37ºC in 0.25% trypsin, 0.02% EDTA. The resultant cell suspension was filtered through muslin cloth, washed, and layered over a discontinuous (5-70% in 5% steps) percoll gradient (Sigma-Aldrich). After centrifugation at 800g for 25min, the cells at the interface was removed, washed, and re-suspended in RPMI-1640 media with 2mM L-glutamine, and antibiotics at a concentration of $1x10^6$ cells/ml. The cell suspension was allowed to settle on 35mm plastic culture dishes for 30 min. at room temperature so that contaminating macrophages got adhered to the plates. The non-adherent cells were then plated on to 35mm culture dishes, which were pre-coated with laminin (20µg/ml, sigma Chemicals Co, St. Louis, MO, Cat No. L2020) for 45min at room temperature. The histopathology of all the cells was carried out to confirm that at least 90% of the cells were trophoblast cells.

The human gestational choriocarcinoma cell lines 'hcc', JEG-3 (HTB-36) and JAR (HTB-144) were obtained from the American type culture collection (ATCC, Rockville, MD, USA). These cells were cultured in RPMI-1640 (Sigma-Aldrich), containing 10% fetal bovine serum (Life technology) and 100 IU/ml streptomycin-penicillin solution (Sigma-Aldrich) and other supplements.

Cell Treatments

Harvested trophoblast cells were incubated in serum free medium (control) or in serum free medium containing various doses of dexamethasone and hydrocortisone (0-1000ng/ml)**.** In order to evaluate time dependent responsiveness, cells were harvested after different exposure times (0hr- 72hrs).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells using the Tri-reagent (Invitrogen USA). cDNA was prepared from 5ug of total RNA using oligo (dT) primers and moloney murine leukemia virus reverse transcriptase (Stratagene). The RT reaction was performed at 42ºC for 50min. RT-PCR amplification were carried out using two HLA-G specific primers, G.257 (Ex-2 specific; 5'-GGAAGAGGAGACACGGAACA-3') and G.1225 (3'-UTR specific; 5'-TGAGACAGAGACGGAGA

CAT-3') .In addition, RT-PCR amplification of â-actin (endogenous control) forward: 5'-GAAGCATTTGCGGTGGACGAT-3' reverse: 5'-TCCTGTGGCATCCACGAAACT-3' was carried out to estimate the amount of RNA in all samples. PCR products were analyzed by electrophoresis in 1.5% agarose gel pre-stained with ethidium bromide and gel densitometry analysis was performed with normalized â-actin values as reference.

Statistical Analysis

Differences in normalized HLA-G expression levels between the treated and untreated cells were tested for statistical significance using Fisher's exact test with Bonferroni correction. The magnitude of the effect was estimated by odds ratios and their 95% confidence intervals (Windows 11.0.0.2001; SPSS Inc.). *P* values less than or equal to 0.05 were considered significant.

RESULTS

Expression Pattern of HLA-G Transcripts in Trophoblast Cells

To determine the expression patterns of HLA-G transcripts in trophoblast cells originating from the different gestation periods, a semi-quantitative RT-PCR was carried out. In 1st and 2nd trimester of pregnancy all the HLA-G transcripts were seen and this was decreased during gestation, in full term pregnancy only G1/G5 and G3 were seen. We used JEG-3 cell line as +ve control which revealed all the isoforms while none was observed in JAR cell line which is negative Control (Fig. 1). Both these cell lines were taken by us as +ve and –ve controls.

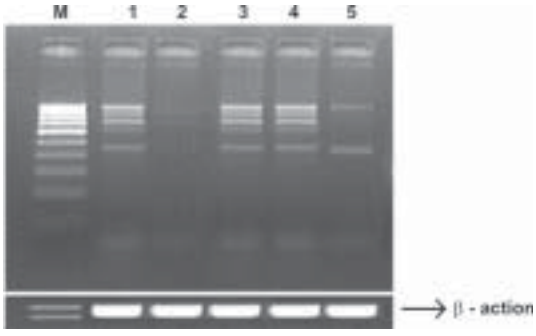

Fig. 1. RT-PCR results shows from first trimester terminated, 2nd trimester and full term pregnancy samples. M= 100bp DNA Ladder, Lane -1 represents JEG-3 as a positive control and Lane-2 represents JAR as a negative control . Lane-3and4 shows 1st trimester and 2nd trimester placental samples. Lane-5 shows only G1 and G3 transcripts of HLA-G in full term pregnancy. Beta-actin used as an endogenous control

Glucocorticoid Elevates HLA-G Transcripts in Cultured Trophoblast Cells

HLA-G induction experiments using glucocorticoids drugs-dexamethasone and hydrocortisone were performed in cultured trophoblast cells from full term placental tissues. For these experiments the cells were incubated with either serum free or serum free medium containing various doses (0-1000ng/ml) of the drugs. For the dose-dependent studies, cells were collected after exposure for 72 hrs. In order to determine the time responsiveness of the drugs, cells were incubated with 1000ng/ml of both dexamethasone and hydrocortisone from 0-72 hrs. The effect of drugs was observed after 24hrs of treatment and a significant increase in HLA-G transcripts were observed after 72 hrs of incubation (Fig. 2).

In order to study the effect of glucocorticoids on steady state expression of HLA-G mRNA, a semi-quantitative RT-PCR was carried out. When trophoblast cells were incubated for 72hrs in a serum free medium containing increasing doses of dexamethasone and hydrocortisone, dose dependent increase in HLA-G transcript was seen at drug concentrations ~500ng/ml and higher

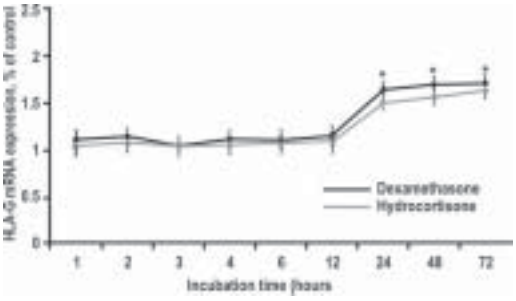

Fig. 2. For determination of the time responsiveness of the drugs, the harvested cultured cells were treated with 1000ng/ml of both dexamethasone and hydrocortisone from 0-72 hrs incubation times. The maximal effect of drugs was observed after 72hrs of treatment

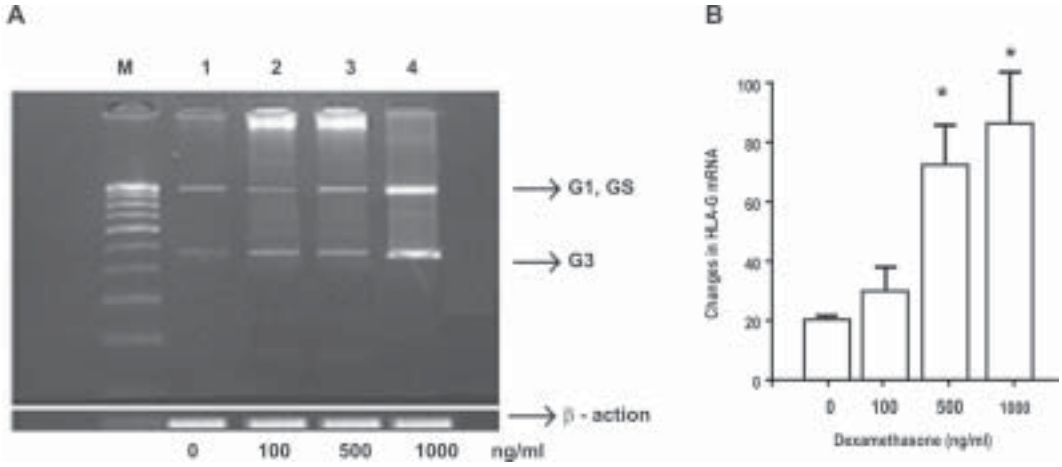

Fig. 3 (A) Effect of increasing doses of Dexamethasone on steady state HLA-G mRNA expression by cultured trophoblast cells. Cells were cultured for 72hrs in serum free medium containing increasing drug conc. ranging from 0-1000ng/ml. (B) Densitometric analysis shows changes in HLA-G transcripts with or without drugs. Deaxamethsone showed 4fold increase relative to control. (*P < 0.05 compared to control culture).

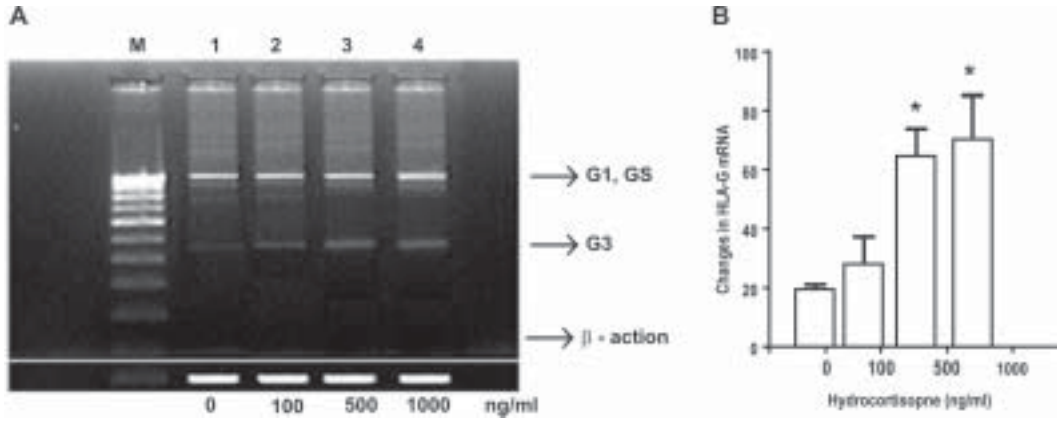

Fig. 4 (A) Effect of increasing doses of Hydrocortisone on steady state HLA-G mRNA expression by cultured trophoblast cells. Cells were cultured for 72hrs in serum free medium containing increasing drug conc. ranging from 0-1000ng/ml. (B) Densitometric analysis shows changes in HLA-G transcripts with or without drugs. Hydrocortisone showed 3.5 fold increase relative to control. (*P < 0.05 compared to control culture)

(Fig. 3A and 4A). We obtained marginally higher expression of HLA-G transcript with dexamethasone as compared to hydrocortisone (Fig. 5). Upon densitometric analysis, HLA-G1, G3 and G5 showed dose dependent increase in mRNA expression (Fig. 3B and 4B) out of which G1 and G5 were found to be predominantly expressed.

DISCUSSION

In the present study we have investigated the effects of two glucocorticoids–dexamethasone and hydrocortisone, on HLA-G mRNA expression in cultured trophoblast cells and demonstrated that the HLA-G transcripts were

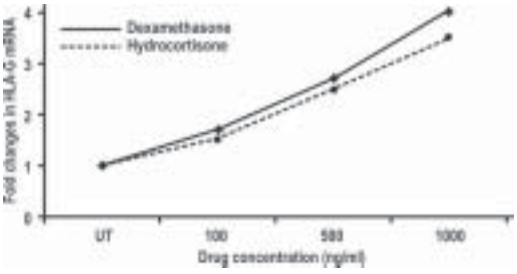

Fig. 5. Comparative representation of increase in HLA-G mRNA in cultured trophoblast cells after treatment with or without both the drugs. Dexamethasone showed 4 fold increase whereas Hydrocortisone showed 3.5 fold

up regulated by both the drugs, albeit higher by dexamethasone as compared to hydrocortisone. The importance of HLA-G in immune tolerance has, in the past few years, been widely demonstrated in fetal tolerance by the mother, allograft tolerance (Rouas-Freiss et al. 1997; Kovats et al. 1990) and immune escape of the tumors (Amiot et al. 2003). Altered protein sequence as well as reduced expression of HLA-G has been variously implicated to have an association with recurrent miscarriages as suggested by various polymorphic (Aldrich et al. 2001) as well as functional (Ober et al. 2006) studies.

We have investigated the effects of dexamethasone and hydrocortisone on HLA-G mRNA expression in trophoblast cells and observed a dose-dependent increase in the levels of HLA-G mRNA isoforms. JEG-3 (HLA-G^{+ve}) and JAR (HLA-G^{-ve}) cells were used as positive and negative controls.

Glucocorticoids are an essential component of normal pregnancy with pluripotent effect on decidualization and implantation (Arcuri et al. 1996, 1997), placental development (Malassine Cronier 2002), fetal brain development, and lung maturation and parturition (Challis et al. 2001; Whittle et al. 2001). The concentration of glucocorticoids markedly increases in maternal plasma and amniotic fluid near parturition whether occurring before or at term. Glucocorticoids support a TH_2 cytokine-secreting profile which is requisite for successful pregnancy outcome (Almawi et al. 1999). Trophoblast, a unique lineage without counterpart in adult tissues, is in direct contact with maternal blood and tissue. The major graft rejection-promoting molecules, human leukocyte antigens (HLAs), are tightly regulated in these cells, with none of HLA-A, HLA-B, or HLA class II antigens are expressed. The HLA class Ib antigens, HLA-E, HLA-F, and HLA-G, are detectable on some subpopulations. Our study has focused on the regulation of expression of HLA-G, which circulate in maternal blood and are present at high levels in the pregnant uterus, by the intervention of therapeutic glucocorticoids. HLA-G was the first trophoblast HLA class I antigen to be identified and remains an antigen of great interest and focus of experimental evaluation. Trophoblast cells produce general soluble immunosuppressant, such as progesterone and prostaglandins, and specific suppressor molecules. These molecules include HLA-G and HLA-E, which are present at high levels at the maternal-fetal interface, and tolerogenic molecules, such as transforming growth factor-â1 (TGF-â1) and interleukin-10 (IL-10).

The importance of different HLA-G isoforms is a matter of continuing debate. However, the full-length HLA-G1 isoform, which is structurally like classical HLA class- I (Lee et al. 2005), has been widely investigated over the past few years, and considered to be functional isoform. Also it has been well established that both the full length membrane bound and soluble isoforms, HLA-G1 and HLA-G5 respectively are stable and functionally active. Moreover, only HLA-G1 and HLA-G5 are predominant isoforms in human preimplantation embryos (Blaschitz et al. 2005). The classical HLA class I á1 domain, residues 77–80 have been described as an important NK cell receptor recognition site (Ulbrecht et al. 2004). In this region, Met^{76} and Glu^{79} are unique to HLA-G (in all alleles described to date) (Ven et al. 1998), suggesting that expression of even one HLA-G isoform on HLA class I-positive targets constitutes an additional protective mechanism against NK over activation through the interaction with inhibitory KIR, in this case, at the feto-maternal interface. In the present study we have observed differential expression of HLA-G3 isoform across different drug concentrations (Fig. 4), which may have contributed to the overall observation of HLA-G transcript up regulation. However, our data confirms to the up regulatory effects of dexamethasone and hrdrocortisone on HLA-G transcription, as various isoforms of HLA-G are an outcome of alternative transcription of the primary transcript.

Previously it has been shown that glucocor-

ticoids on the upregulation of HLA-G expression has been demonstrated in cultured trophoblasts (Moreau et al. 2001), and Yei et al. (2006) had demonstrated the up regulatory effect of progesterone on HLA-G expression in JEG-3 cells. The effect of epigenetic regulation of HLA-G expression has also been shown through reversal of HLA-G repression by demethylation of the DNA (Moreau et al. 2002).

In the present report, we have demonstrated the effect of two glucocorticoid drugs–dexamethasone and hydrocortisone on non-classical HLA-G in cultured trophoblast cells obtained from full term placenta. As we have seen that both the drugs up regulate the HLA-G expression in time dependent manner demonstrating that the potential mechanism of HLA-G expression could be altered by various therapeutic drugs and that can be exploited as a possible therapeutic modality for pregnancy disorders of immunological etiology including recurrent miscarriages. In these women there is no expression of any of the HLA- G isoforms. Further our study illustrates that presence of HLA –G is required for the maintenance of pregnancy in the early gestational period.

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