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The human leukocyte antigen G promotes trophoblast fusion and β-hCG production through the Erk1/2 pathway in human choriocarcinoma cell lines

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ABSTRACT

The human leukocyte antigen G (HLA-G) is expressed on the fetal–maternal interface and plays a role in protecting fetal-derived trophoblasts from the maternal immune response, allowing trophoblasts to invade the uterus. However, HLA-G also possesses immune suppressing-independent functions. We found that HLA-G expressing BeWo choriocarcinoma cells increased cell–cell fusion compared to control BeWo cells under forskolin treatment. Regardless of forskolin treatment, the expression of fusogenic gene mRNAs, including syncytin-1, the transcription factor glial cell missing 1 (Gcm1), and beta human chorionic gonadotropin (β-hCG) were elevated. HLA-G up-regulates β-hCG production in human choriocarcinoma cells because HLA-G knockdown in JEG-3 cells induces a dramatic decrease in β-hCG compared with control cells. The defect in β-hCG production in HLA-G knocked-down cells could not be completely overcome by stimulating hCG production through increasing intracellular cAMP levels. HLA-G expressing cells have increased phosphorylation levels for extracellular signal-regulated kinase1/2 (Erk1/2) in BeWo cells. The Erk1/2 pathway is inactivated after the inhibition of HLA-G expression in JEG-3 cells. Finally, Erk1/2 inhibition was able to suppress the increased hCG production induced by HLA-G expression. Together, these data suggest novel roles for HLA-G in regulating β-hCG production via the modulation of the Erk1/2 pathway and by inducing trophoblast cell fusion.

1. Introduction

The placenta is a critical organ in implantation and fetal development [1]. Trophoblast fusion and the consequential formation of the multinucleated syncytiotrophoblast layer are essential determinants of placentation [2]. During implantation, intercellular fusion of trophoblast cells surrounding the inner cell mass leads to the first generation of syncytiotrophoblast, which is capable of penetrating the uterine epithelium [3]. Without the formation of this multinucleated layer, invasion and implantation of blastocyst would not occur [4]. Syncytiotrophoblasts secrete reproductive hormones, including human placental lactogen (hPL) and human chorionic gonadotrophin (hCG) [3]. hCG, a hormone highly associated with human reproduction, plays important roles in placental, uterine and fetal development [5]. hCG is composed of an α subunit and a unique β subunit. The concentration of β-hCG is commonly used to determine levels of total hCG. The embryo first produces β-hCG at the eight-cell stage [6]. In the first week of pregnancy, hCG is an autocrine factor, acting on promoting the implantation of pregnancy [7]. Abnormally low levels of β-hCG clearly mark failing pregnancies, including early pregnancy loss, spontaneous abortion [5].The cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway was known to stimulate β-hCG biosynthesis in choriocarcinoma cell lines [8]. Two mitogen-activated protein kinase (MAPK) family members, Erk1/2 and p38, are involved in cAMP/PKA-induced β-hCG secretion [9,10].

The human leukocyte antigen G (HLA-G) belongs to the HLA class Ib of molecules, which are specifically expressed at the maternal–fetal interface [11]. The mRNA and protein of HLA-G is expressed in human embryos and especially on the trophectoderm, which differentiates into cytotrophoblasts and syncytiotrophoblasts [12]. HLA-G in soluble form is also expressed in the cytotrophoblasts which undergo the syncytial fusion with the syncytiotrophoblast [13]. HLA-G has been characterized as an immunosuppressor [14] and is associated with several common pregnancy complications, including unexplained miscarriage and pre-eclampsia [13,15]. Several studies have investigated the expression of soluble HLA-G (sHLA-G), which exists in early embryonic culture supernatants, and its correlation with pregnancy outcome [16,17]. However, the non-immune function that

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HLA-G plays in trophoblast differentiation along with the fusion pathway and secretion of β-hCG has not been well addressed.

Human choriocarcinoma cell lines have served as useful models for investigating the factors that regulate trophoblast fusion and the synthesis and secretion of β-hCG. Among those cell lines, HLA-G is expressed on JEG-3 but not on BeWo cells [11]. BeWo cells exhibit a low spontaneous fusion efficiency, which can be significantly increased by up-regulating the intracellular cAMP concentration [8].

The aim of this study was to investigate the potential role of HLA-G in the processes of trophoblast cell–cell fusion and synthesis of β-hCG. The role of HLA-G in modulating β-hCG production was investigated by loss- and gain-of-function studies on two human choriocarcinoma cell lines. Finally, we showed that β-hCG upregulation was attenuated by an inhibitor to the Erk1/2 MAPK pathway, which was activated on HLA-G expressing cells.

2. Materials and methods

2.1. Reagents

Reagents were purchased from the following sources: monoclonal antibodies to HLA-G (4H84, Abcam), actin (C4, Abcam), E-cadherin (H-108, Santa Cruz Biotechnology); polyclonal antibody to β-hCG (ab9376, Abcam), MEK1/2 inhibitor U0126 (9903), MEK1 inhibitor PD98059 (9900), antibodies to phospho-MAPK family pathway (9910, Cell Signaling Technology), p38 inhibitor SB202190 (559388, Calbiochem), hygromycin B (H7772) and forskolin (F6886, Sigma), DAPI and secondary antibodies (Invitrogen). The following siRNA oligonucleotides were obtained from Ambion: HLA-G siRNA (siHLA-G), GGUAUGAACAGUAUGCCUATT; control siRNA (siControl), CCCGUAUACGACACCGAGUAGUCUU.

2.2. Cell lines and transfection

The human choriocarcinoma cell line JEG-3 (ATCC) was cultured in DMEM: Ham's F12 (1:1, Gibco) supplemented with 10% FBS (Gibco) and 20 mM HEPES (Gibco). BeWo cells (ATCC) were cultured in Ham's F12K medium (Gibco) supplemented with 10% FBS. For the inhibitor treatments, the cells were starved for 24 h and pretreated with inhibitors or DMSO (Sigma) as a vehicle control for 1 h. Thereafter, 10% FBS was added into the indicated medium. For the siRNA transient transfection, the cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

2.3. Lentiviruses

In our culture system, BeWo cells were resistant to plasmid transfection using the Lipofectamine 2000 reagent. Consequently, a lentiviral system was employed to generate stable control and HLA-G expressing cells. Lentiviruses were generated by co-transfecting the packaging plasmid (CMVΔ8.92 and pMDG) and iDuet-101a or iDuet-101a-HLA-G-FLAG (NCBI Reference Sequence: NM_002127.5) constructs into the HEK293FT cell line. The BeWo cells were infected with the control and HLA-G expressing lentivirus. Hygromycin B (100 μg/ml; Sigma) was used to select for infected cells at 48 h post-infection, and the resistant cells were pooled and expanded for following studies.

2.4. RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen). Five micrograms of total RNA was reverse transcribed to cDNA using Superscript II reverse transcriptase regents (Invitrogen). The sequencing real-time PCR reaction contained 2 μL cDNA template, each specific primer at a concentration of 0.4 μM, and 10 μL of two times SYBR® Premix Ex Taq™ II (TaKaRa). The sequences of the forward and reverse primers were 5′-TGGAACAACTTCAGCACAGA-3′ and 5′-GCCATTCAAACAACGATAGG-3′ for syncytin-1, 5′-CTGACA AGGCTTTTTTCTTCACA-3′ and 5′-CCAGACGGGACAGGTTT-3′ for Gcm1, 5′-GCTACTGCCCCACCATGACC-3′ and 5′-ATGGACTCGAAGCG CACATC-3′ for β-hCG, 5′-GCCATCAATGACCCCTTCATT-3′ and 5′-TTGACGGTGCCATGGAATTT-3′ for GAPDH. GAPDH was employed as the internal control. Each reaction was performed in triplicate.

2.5. Immunofluorescence and cell fusion analysis

After the appropriate treatment, cells cultured on glass coverslips were fixed with 3.7% paraformaldehyde in PBS. Following a 30-min incubation period with 10% donkey serum to block the nonspecific binding of the antibodies, the cells were incubated with primary antibodies overnight (4 °C). The secondary antibody incubation was performed at room temperature for 1 h. Confocal microscopy was performed using a Zeiss LSM 780 microscope (Carl Zeiss) with a 20× objective. BeWo cells were fixed and immunostained. Intercellular boundaries were stained with an E-cadherin antibody, and the nuclei were stained with DAPI. Ten random fields were photographed for a cell fusion analysis. The total number of nuclei, the number of nuclei within multinucleated (more than two nuclei) syncytia and the number of syncytia were counted. Ratios of the fused cell number to the total number of nuclei were calculated as the cell fusion index to quantify the cell fusion ratio as described previously [18].

2.6. Western blotting

To detect non-phosphorylated protein, cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Roche). The BCA assay (Pierce) was employed to determine protein concentrations. For phosphorylated protein detection, cells were lysed in two times sample buffer. Membranes were incubated with primary antibodies overnight (4 °C) and secondary antibodies (Pierce) for 1 h at room temperature. ECL kits (Pierce) were used for chemiluminescence detection.

2.7. β-hCG quantification in the conditional medium

The culture medium was collected and centrifuged at 12,000 rpm for 5 min to remove cell debris. Solid phase sandwich ELISA was performed to estimate the secreted β-hCG level using a β-hCG ELISA kit following the manufacturer's instructions (SUNBIO).

2.8. Statistical analysis

For a comparison of two treatments, statistical analyzes were performed using SPSS 17.0 (SPSS) with Student's *t* test. At least three replicates were performed for each experiment. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. HLA-G promotes BeWo cell fusion and fusogenic gene expression

The BeWo cells are characterized as an *in vitro* model for mimicking trophoblast differentiation from cytotrophoblasts to the syncytiotrophoblasts phenotype [8]. To estimate the role of HLA-G in trophoblast fusion, BeWo cells stably expressing HLA-G and control BeWo cells generated using HLA-G expressing and control lentiviruses, were treated with 50 μM DMSO or forskolin for 48 h,