INTRODUCTION

Maternal immunological tolerance of the fetal placenta involves the recognition of foreign antigens, especially, the paternally-inherited fetal antigens in such a way that does not induce an anti-fetal maternal immune response.1 Trophoblast cells located between the fetus and mother, can bias the maternal immune system toward immunological acceptance of fetal antigens. The mechanisms by which lymphocyte tolerance toward trophoblast cells have not been fully elucidated. However, different evidences have suggested that immune costimulator B7 family plays an important role in this process.

B7s providing second signals to immunological cells are key regulators of the adaptive immune response. Individual members of the B7 family can be both positive and negative regulators of the immune response depending on their specific counterreceptors.2 B7-H1(PD-L1) is a negative regulator of the immune response in this family, which is important for formation and maintenance of maternal immunological tolerance of the fetal placenta.2 It is a surface-bound ligand and it binds to the lymphocyte-expressed receptor, PD-1. This interaction inhibits antigen-specific activation.

ABSTRACT

Objective: This paper is aimed at to evaluate B7-H1 expression as induced by human cytomegalovirus (HCMV) in extravillous cytotrophoblast cell line HPT-8 and possible underlying mechanism.

Method: Real time PCR and flow cytometry were used to determine B7-H1 mRNA and protein before and after HCMV infection in HPT-8 cells. Western blot analysis was used to determine the level of MAPK phosphorylation in HPT-8 cell lines infected with HCMV.

Results: 100TCID50 was found to be the most effective dose, capable of stimulating B7-H1 mRNA and protein expression in HPT-8 cells. When empty control group was considered to have a B7-H1 mRNA value of 1, B7-H1 mRNA was 4.32 in 100TCID50 group. In flow cytometry study, mean fluorescence intensity (MFI) of 100TCID50 group was 16.14, while empty control group was 1.34. Both mRNA and protein expression were found to be significantly increased (P < 0.05) in 100TCID50 group compared to empty control group. The result of Western blot analysis showed increase in B7-H1 expression caused by the extracellular signaling that was related to ERK activation and the ERK inhibitor U0126 was found to reverse this increase.

Conclusion: HCMV upregulates B7-H1 expression in human extravillous cytotrophoblast cell line HPT-8, which is related to MAPK activation. Our result would be helpful in finding better therapies against intrauterine HCMV infection.

KEY WORDS: Cytomegalovirus, Cytotrophoblast, B7-H1, MAPK.
and cytokine production by T cells. In the human placenta, B7-H1 proteins are expressed selectively on trophoblast cells, including villous and extravillous cytotrophoblast cells, and syncytiotrophoblast cells. Blockage of maternally-derived B7-H1 has been found to result in rejection of allogeneic fetuses in mice. It has also been shown that persistent expression of PD-1 on T cells contributes to chronic viral infections due to its inhibition of immune response. In summary, these studies have offered strong evidence that this pathway plays a role in tolerance to both self and foreign antigens.

HCMV is an important reason for intrauterine infection which is one of the most common causes of microcephaly, sensorineural deafness, and retardation of cognitive abilities and intrauterine growth. In this paper, we report the influence of HCMV infection on the expression of immune co-suppressive molecule B7-H1. We also checked signaling protein’s phosphorylation after HCMV infection. Our results suggested HCMV was a strong inducer of B7-H1 expression in extravillous cytotrophoblast cell line HPT-8, this might explain why HCMV was a common factor for intrauterine infection and might provide clues for future anti-HCMV intrauterine infection strategy.

METHODS

Reagents: Extravillous cytotrophoblast cell line HPT-8, Human embryonic lung fibroblasts (HEL) and HCMV AD169 Standard strain were purchased from American Type Culture Collection (ATCC; Manassas, VA, U.S.). Cell culture reagents were all obtained from Gibco (Carlsbad, CA, U.S.). TRIzol Reagents, OPTI-MEM and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA, U.S.). Anti-human B7-H1 antibody (clone MIH1) were from eBioscience (San Diego, CA, U.S.). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit IgG secondary antibody were obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, U.S.). All other antibodies were obtained from Cell Signaling TechnologyO (Beverly, MA, U.S.). JNK inhibitor-SP600125, ERK inhibitor-U0126, P38 inhibitor-SB203580 were all from Calbiochem (U.S.). Reverse transcriptase was obtained from Fermentas. SYBR Green PCR master mix for real time PCR was from Applied Biosystems. 18S (NCBI: NR_046235.1) were 5′- GGACACGGACAGGATTGACA -3′ and 5′- ACCCACGGAATCGAGAAAGA -3′. The primers used for B7-H1 (GeneBank: BC113734.1) were 5′- ACAGAGGGCCGGGCTGTGA -3′ and 5′- AGCGGTACACCCCTGCATCCT -3′. The reaction were conducted by 40 cycles of 95°C 15 s and 60°C 1 min. PCR products for both B7-H1 and 18S were confirmed by sequencing. We used the DDCT method for calculating: mean relative quantification=2\(^{-\text{meanDDCT}}\) (DCTtarget-DCTendogenous control). B7-H1 levels in the control group were designated as 1.

Flow cytometry: As after transcription, some other reasons, for example post transcriptional, translational mechanisms could also influence gene expression. We used flow cytometry to determine HCMV protein. For this, 6×10⁵ HPT-80 cells were cultured in 6-well plates. Cells were treated and cultured in media with different HCMV titers for 24 hours. Cells were harvested using trypsin and cell density was adjusted to 1×10⁵/ml with PBS. Two hundred microliters of cell suspension was incubated in the dark with PE-labeled mouse antihuman B7-H1 monoclonal antibody for 30 min. FACS Calibur (Becton Dickinson) flow cytometry was used for this experiment. Each experiment was repeated at least 3 times.

For checking the role of different MAPKs, we determined B7-H1 expression by inhibiting different MAPKs. In these studies, stock solutions of MAPK inhibitors were prepared in DMSO. The final concentration of SP600125 (10 μM), U0126 (10 μM), and SB203580 (20 μM) were added to each group and DMSO final concentration never exceeded
0.05%. Different titers of HCMV with medium were added to each group after one hour. The control group was treated with the same amount of DMSO but no MAPK inhibitors. Cells were cultured conventionally for 24 h and the expression of B7-H1 protein was detected by flow cytometry.

**Protein preparation and immunoblotting:** First, 8×10^5 HPT-8 cells were cultured in a 100 mm dish and treated as above. The cells were lysed using RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF, and protease inhibitors (Complete Mini; Roche, Switzerland)]. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferring to a nitrocellulose membrane, the membrane was immunoblotted with anti-human phosphorylated ERK1/2 (P-ERK1/2), P38 or JNK/JNK respectively. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody, and visualized by enhanced chemiluminescence reaction using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, U.S.). The membranes were stripped and reprobed with antibodies recognizing the corresponding non-phospho proteins.

**Statistical analysis:** The statistical analysis software package SPSS11.5 was used in this study. Values were means ± SEM. One-way ANOVA was used to analyze data from every group, and the LSD t-test was used to analyze multi-comparison of every group at the same time. *P*-values of less than 0.05 were indicative of statistical significance. All experiments had been repeated for at least 3 times in triplicate.

**RESULTS**

**Virulence:** TCID50 of HCMV AD169 standard strain was measured in HEL cells. According to Reed-Muench calculation, the TCID50 of HCMV AD169 standard strain in this cell came to 10^{-3.29}.

**Influence of HCMV on B7-H1 mRNA:** Eight hours after HCMV infection, HCMV significantly induced B7-H1 mRNA expression in HPT-8 cells as determined by real time PCR (Fig.1A). There were statistically significant differences between the control and the treated groups with viral titers greater than 5 TCID50 (*P*<0.01).

**Influence of HCMV on B7-H1 protein:** Level of B7-H1 protein was measured using flow cytometry (Fig.1B). Changes in B7-H1 expression were assessed by comparison of mean fluorescence intensity (MFI). In all groups, the MFI values were significantly different from the empty control group (*P*<0.05).

**Influence of HCMV infection on the phosphorylation of MAPK in HPT-8 cells:** In order to determine possible mechanisms of HCMV induced B7-H1 expression in HPT-8 cells, we did Western-Blot to check the phosphorylation of MAPKs that could involved in the expression of B7-H1 mRNA. In experiments before, the ability of HCMV to stimulate B7-H1 protein was more obvious when viral titer was 100 TCID50. Therefore, an HCMV titer of 100 TCID50 was used in all following experiments. Total cellular protein was extracted at different time points after addition of viral solution. ERK, P38/P-P38, and JNK/JNK levels did not differ before and after viral infection. However, P-ERK was changed when viral solution was added to the cell. The P-ERK level increased after 5 min and reached its peak at 15 min and gradually returned to its original level at 120 min (Fig.2).
Influence of MAPK inhibitors on B7-H1 protein expression caused by HCMV infection: Media containing HCMV with different inhibitors of MAPK (JNK inhibitor- SP600125, ERK inhibitor-U0126, P38 inhibitor-SB203580) were added to HPT-8 cells. The B7-H1 protein level was measured using flow cytometry. MFI was used to represent the actual level. No statistically significant differences were observed among the DMSO group, SP600125, and SB203580 (Fig.3). When ERK inhibitor (U0126) was used, however, the concentration of B7-H1 protein decreased significantly ($P < 0.05$).

**DISCUSSION**

Our results suggested that HCMV infection could cause upregulation B7-H1 mRNA and protein. We also found activation of Erk1/2, one of MAPK pathways, to be involved in this process.

In the human placenta, B7-H1 protein is expressed selectively on trophoblast cells. This suggests that B7-H1 is important to maternal immunological tolerance of the fetal placenta. Many studies have clearly demonstrated that B7-H1 has dual roles in immunological tolerance: induction and maintenance of peripheral tolerance. The precise mechanism underlying B7-H1 expression in trophoblast cells is unclear.

Several signal transduction pathways have been reported to participate in the regulation of B7-H1 expression. Crane showed that B7-H1 expression and function in breast and prostate cancer was mediated by the PI3K pathway. B7-H1 expression was shown to be dependent on the ERK and p38 mitogen-activated protein kinase (MAPK) signaling pathways in colon cancer, in anaplastic large cell lymphoma and Hodgkin lymphoma. Our study suggests that the ERK 1/2 pathway and resultant increase in B7-H1 transcription are involved in HCMV-induced B7-H1 regulation in trophoblast cells.

ERK is one important mammalian MAPK pathway. The JNK and p38 MAPK pathways are also important. HCMV infection has previously been shown to induce activation of ERK1/2 and to be related to viral survival in infected cells. Using his own studies and those of others, Melnick found that upregulation of ERK phosphorylation is necessary to initial CMV-induced pathogenesis.

HCMV is an important reason for intrauterine infection in perinatal period. In this paper, we
Fig. 3: MAPK inhibitors on B7-H1 protein expression caused by HCMV infection, as measured by Flow cytometry. Different inhibitors were added to each group 1 hour before HCMV treatment. The control group was treated with the same amount of DMSO but no MAPK inhibitors. Each experiment was repeated at least 3 times.

have reported the influence of HCMV infection on the expression of B7-H1 and also determined the possible mechanisms underlying. Our result suggested HCMV is a strong inducer of B7-H1 expression in extravillous cytotrophoblast cell line HPT-8, this might explain why HCMV was an important reason for intrauterine infection and might provide useful clues for future anti-HCMV therapies.

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Authors Contribution:
WRG and JHZ: Designed the study protocol, conducted most of the study and prepared the primary manuscript. ZC, LL and HX: Conducted cell culture, virus titer determination and infection, contributed in the statistical analysis and editing of manuscript. LHL, XHZ and QST: Conducted inhibitory study and western-blot. SHC: Revised the study design, final revision of manuscript for publication and submitting the paper.

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