ERK MAP-Kinase is Involved in the B7-H1 Expression Induced by Etoposide In Retinoblastoma Cells

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Abstract: The effect of etoposide on B7-H1 expression was determined by the Reverse-Transcription Polymerase Chain Re-action (RT-PCR), real-time PCR and flow cytometry analysis in Y79 cells. Then, the involvement of Mitogen-Activated Protein Kinases (MAPKs) signal pathways were tested by Western blotting and signal transduction inhibitor assays. Furthmore, specific small interfering RNA (siRNA) targeting B7-H1 was transfected into Y79 retinoblastoma cells using liposome. Silencing of B7-H1 expression was measured by RT-PCR and Western blotting assays. Etoposide increased B7-H1 mRNA and protein levels in Y79 cells. The effect of etoposide on B7-H1 expression peaked at the concentration of 5 μg mL^{-1} as confirmed by RT-PCR, real-time PCR and flow cytometry assays (p<0.01). The phosphorylation status of Extracellular Signal-Regulated Kinase (ERK), c-Jun N-terminal Kinase (JNK) were constitutively activated by etoposide and MEK inhibitor simultaneously reduced the expression of B7-H1 induced by etoposide (p<0.01). B7-H1 siRNA significantly silenced B7-H1 expression in Y79 cells as confirmed by RT-PCR and Western blotting assays (p<0.01).

INTRODUCTION

Suppression of immune responses is the critical mechanism of the evasion of tumor immunity. Even the presence of activited tumor-specific T cells in the tumor sites is unable to inhibit tumor growth (Jantzer and Schendel, 1998; Lee *et al.*, 1999). Adoptive immunotherapy using *in vitro* activated T cells has not been satisfactory in tumor treatment (Abken *et al.*, 2002; Cohen *et al.*, 2001). As if there is a microenvironment of immune privilege in the protection of tumor from immune destruction (Hanson *et al.*, 2000). Some soluble factors and molecules have been found to be upregulated in the tumor sites which can inhibit the immune responses. These include transforming growth factor-β IL-10, Fas, Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) and RACS1 (Dunn *et al.*, 2002; Khong and Restifo, 2002; Smyth *et al.*, 2001).

B7-H1 is a newly discovered membrane molecule which plays an important role in tumor immune evasion (Dong and Chen, 2003). The expression of human B7-H1 mRNA is detected in many tissues (Dong *et al.*, 1999). In contrast to mRNA expression, there is little B7-H1 protein expression in the tissues rich in mRNA expression

(Dong *et al.*, 2002). B7-H1 is expressed widely in diverse human tumor tissues including ovarian cancer, lung cancer, esophageal cancer, gastric cancer, rectal cancer, pancreatic cancer, bladder cancer and prostate cancer (Dong and Chen, 2006). Although, this molecule is highly expressed in tumor cells *in vivo*, it is low or absent *in vitro*. It may be that *in vivo* microenvironment increases B7-H1 expression (Thompson *et al.*, 2009). Studies have shown that local tumor expression of B7-H1 has a co-stimulatory effect on T lymphocytes and inhibits immune reaction by activation of memory T lymphocytes (Chen, 2004).

Mitogen-Activated Protein Kinase (MAPK) family is an important signaling system in mediating cellular responses. According to sequence homology and function, MAPK family is divided into four categories, namely Extracellular Signal-Regulated Kinase (ERK), c-Jun N-terminal Kinase (JNK)/Stress-Activated Protein Kinase (SAPK), p38 and ERK5/BMK1(Chang and Karin, 2001). Nuclear transcription factors, cytoskeletal proteins and enzymes phosphorylated by activated MAPK play critical roles in cells proliferation, differentiation, transformation and apoptosis regulation. They are also closely related to inflammation, cancer and many other diseases.

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Retinoblastoma is a genetically determined tumor which represents the most common intraocular cancer of childhood (Aerts *et al.*, 2006). It may occur at any age but most often in younger children, usually before the age of three years. Left untreated, retinoblastoma is always fatal and the patients die of intracranial extension and disseminated disease within 2 years. Enucleation is still often necessary in the treatment of retinoblastoma (Chintagumpala *et al.*, 2007). Repeated cycles of chemotherapy with carboplatin, vincristine and etoposide combined with cryotherapy and laser photocoagulation have improved the ocular salvage rate for children (Antoneli *et al.*, 2006) and treatment in the early stages of disease holds a promising prognosis for survival and salvage of visual function.

In this study, researchers investigated the effect of etoposide on B7-H1 expression in retinoblastoma cells and the role of MAPK signaling pathway in this process. Furthermore, to explore the potential of immunotherapy for retinoblastoma, researchers studied the outcome of using small molecules RNA interference technology.

MATERIALS AND METHODS

Cell culture: Human retinoblastoma cell, Y79 cell line was purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 (Gibco, USA) with 10% Fetal Bovine Serum (FBS, Gibco, USA). The medium was changed every 2-3 days and cultures were maintained in an incubator at 37°C in 5% CO_2/95% air atmosphere.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Cells were first cultured in RPMI1640 with 0.1% FBS for 24 h. Then, the medium was removed and cells were washed with PBS, cultured again in RPMI1640 without serum. For testing the effects of etoposide, 5×10^5 of Y79 cells were incubated in 6 well plates and etoposide (Sigma) dissolved in Dimethyl Sulfoxide (DMSO, Sigma) was administered. The final concentrations of etoposide in the wells were 2.5, 5, 10, 20 and 40 μg mL^{-1}, respectively.

TRIzol-Reagents (Invitrogen) were used to extract total cellular RNA at 24 h according to the manufacturer's instruction. Reverse Transcription (RT) was performed using 1.5 μg of total RNA and 0.2 μg of random hexamer primers (Fermentas) following the manufacturer's protocol for synthesis of first strand cDNA. Reagent boxes (Fermentas) were used in PCR. Ribosomal RNA (rRNA) 18S was used as the internal reference. The primers used for B7-H1 were: forward 5'-ACAGAGGG CCCGGCT GTTGA-3', reverse 5'-AGCGGTACACCCCTGCATCCT-3'

(95 bp PCR product). The primers used for 18S were: forward 5'-CAG CCACCCGAGATTGAGCA-3', reverse 5'-TAGTAGCGACGGGCGGTGTG-3' (254 bp PCR product). PCR was carried out at 94°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec for a total of 40 cycles and then 72°C for 10 min. The PCR products were detected by 1.5% agarose gel electrophoresis and confirmed by sequencing.

Real-time PCR: For real-time PCR analysis of ILK mRNA, total RNAs were extracted using TRIzol reagent and cDNA (from 200 ng RNA) was synthesized. The real-time PCR was conducted in triplicates using the SYBR Green PCR master mix (Applied Biosystems) on an Applied Biosystems 7500 real-time cycler. The primers for B7-H1 were the same as those in RT-PCR. The primers used for 18S were: forward 5'-GGACACGGACAGGATTGACA-3', reverse 5'-ACCCACGGAATCGAGAAAGA-3'. The Threshold Cycle (Ct) values were analyzed using the comparative Ct ($\Delta\Delta$Ct) Method. The amount of target was determined by normalizing to the endogenous reference (18S) and relative to the control group.

Flow cytometry analysis: After 24 h of culture, the Y79 cells were harvested and suspended in PBS and cell density was adjusted to 1×10^6 mL^{-1}. The cells were cultured with 5% goat serum for 10 min at room temperature. Then, the phycoerythrin conjugated anti-B7-H1 antibody (diluted 1:100, eBioscience, USA) was added and the cells were incubated for 30 min at 4°C followed by washing with cold PBS. The cells were analyzed on a FACScan (Becton Dickinson, USA). Data were collected and analyzed with CellQuest Software. Changes in B7-H1 expression after stimulation of the cells were assessed by a comparison of the staining intensity ratios which were calculated by dividing the Mean Fluorescence Intensity (MFI) of cells stained with the B7-H1 antibody by the MFI of the cells stained with the corresponding isotype control antibody.

Western blot analysis: The Y79 cells were inoculated 6 well plate and administrated with 5 μg mL^{-1} of etoposide. Cells were collected at 0, 15, 30, 60 and 120 min by centrifugation and washed twice with PBS then lysed in ice-cold RIPA buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.25% deoxycholate, 1.5 mM MgCl$_2$, 1 mM EGTA, 1 mM Phenylmethylsulfonylfluoride (PMSF), 10 mM zNaF, 10 mM pervanadate, 10 μg mL^{-1} leupeptin and 10 μg mL^{-1} aprotinin] on ice, using 100 μL of lysis buffer per well. After 10 min on ice, lysates were transferred into microcentrifuge tubes and centrifuged at 12,000×g for 5 min at 4°C. After measuring protein concentration, 35 μg of total protein was subjected to

sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with mouse anti-human Phosphorylated ERK1/2 (P-ERK1/2) monoclonal antibody, mouse anti-human ERK1/2 monoclonal antibody, mouse anti-human phosphorylated P38 monoclonal antibody, rabbit polyclonal anti-human P38 antibody (1:1000 dilution,Cell Signaling Technology), rabbit anti-human phosphorylated JNK/JNK polyclonal antibody (1:1000 dilution, Bioworld) at 4°C overnight, rabbit anti-human p-JNK/JNK antibody (1:1000 dilution, Bioworld) or rabbit anti-human p-Jun/Jun antibodies (1:1000, Bioworld). Horseradish Peroxidase (HRP)-conjugated goat anti-mouse or rabbit IgG secondary antibody (1:5000, Jackson Immunoresearch Laboratories Inc. West Grove, PA) was used. Enhanced Chemiluminescence substrate (ECL, Themo scientific) was applied to detect the secondary antibody according to the manufacturer's instructions and the membranes were placed against autoradiographic films (Koda, USA). Monoclonal antibody against β-actin (1:2000, Beyotime Company, China) was used as an internal standard for verifying protein loading.

MAPK signal transduction inhibitors assay: For this study, stock solutions of MAPK inhibitors (JNK inhibitor-SP600125, ERK inhibitor-U0126, P38 inhibitor-SB203580, Calbiochem, USA) were prepared in DMSO and diluted to the final concentration in the culture medium. The final concentration of DMSO never exceeded 0.05%. SP600125 (10 μM), U0126 (10 μM) and SB203580 (20 μM) were added to each groups, respectively. After 1 h, 5 μg mL^{-1} of etoposide was added. The control group was treated with the same amount of DMSO used in treatment groups. Cells were cultured conventionally 24 h and expression of B7-H1 was detected by flow cytometry.

Plasmid vector transfection of siRNA targeted B7-H1: The pGCsilencer^{TM} U6/Neo/GFP/RNAi plasmid was purchased from Shanghai Jiao Company. The target sequence was as follows: CTGAGAAT CAACAC AACAA. SiRNA targets human B7-H1 (Genbank accession number AF177937) at nucleotides 640-658 bp. Y79 cells were inoculated at the density of 5×10⁵ mL^{-1} in RPMI-1640 medium without calf serum and antibiotics at 1.5 mL cell suspension each well in 6 well plates. Transfection was carried out using Lipofectamine 2000 reagent (Invitrogen). About 5 μL liposomes and 750 ng plasmid DNA were diluted in 250 μL serum-free Opti-MEM I (Invitrogen), respectively. Diluted liposomes were mixed with diluted plasmid DNA

and the mixture was incubated for 20 min at room temperature for complex formation. The entire mixture was added to the cells in the well. Negative Control siRNA (CSI) and transfection reagent (NC) were set up as controls. At 24 h after transfection, 5 μg mL^{-1} etoposide was administrated. After another 24 h, the cells were harvested for RT-PCR and flow cytometry analyses.

Statistical analysis: All experiments were repeated three times. Statistical analysis Software SPSS11.5 was used. Values are expressed as means±SEM. One-way ANOVA was used to analyze the overall data of every group and the Least Significant Difference (LSD) t-test was used to analyze multicomparison of every group at the same time. The level of confidence for statistical significance was determined to be p<0.05.

RESULTS

Etoposide increased B7-H1 mRNA and protein expression in cultured Y79 cells: Cultured Y79 cells were treated with epotoside and B7-H1 mRNA expression was determined by RT-PCR and real-time PCR. After 24 h, Increased expression of B7-H1 mRNA was detected (Fig. 1a and b). The positive effect of epotoside on B7-H1
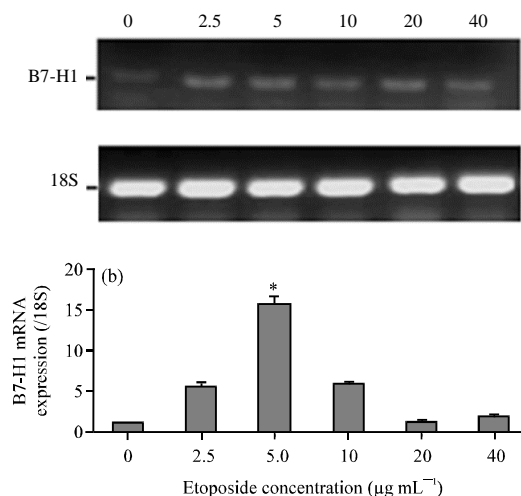

Fig. 1: Effect of etoposide on expression of B7-H1 mRNA. a) RT-PCR of B7-H1. 18S mRNA was co-amplificated as internal control; b) real-time PCR of B7-H1. The amount of B7-H1 mRNA was obtained by normalizing to the endogenous reference (18S) and relative to 0 μg mL^{-1} group. After 24 h, increased B7-H1 mRNA was observed with increasing etoposide concentration and the effect peaks at 5 μg mL^{-1} etoposide concentration (*p<0.01 vs. other groups)

mRNA expression peaked at 5 μg mL^{-1} and then weakened with higher concentrations. Significant difference was detected between the 5 μg mL^{-1} group and the other groups (p<0.01). Consistent with the expression of B7-H1 mRNA described above, the B7-H1 protein level in Y79 cells was also significiantly enhanced by epotoside treatment for 24 h as evidenced by flow cytometry (Fig. 2a and b). The strongest fluorescence intensity of B7-H1 was observed at 5 μg mL^{-1} group, compared with the other groups, the differences were statistically significiant (p<0.01).

The ERK signaling pathway is involved in B7-H1 expression induced by etoposide in Y79 cells: Researchers next aimed to clarify the mechanisms of B7-H1 expression induced by etoposide in Y79 cells. As discussed earlier, MAPK signaling pathways play an important role in cancer biology. Researchers hypothesized that epotoside regulates B7-H1 expression by some of the MAPK signaling pathways in Y79 cells. Based on the above results, researchers selected the 5 μg mL^{-1} etoposide group as the study group. Western
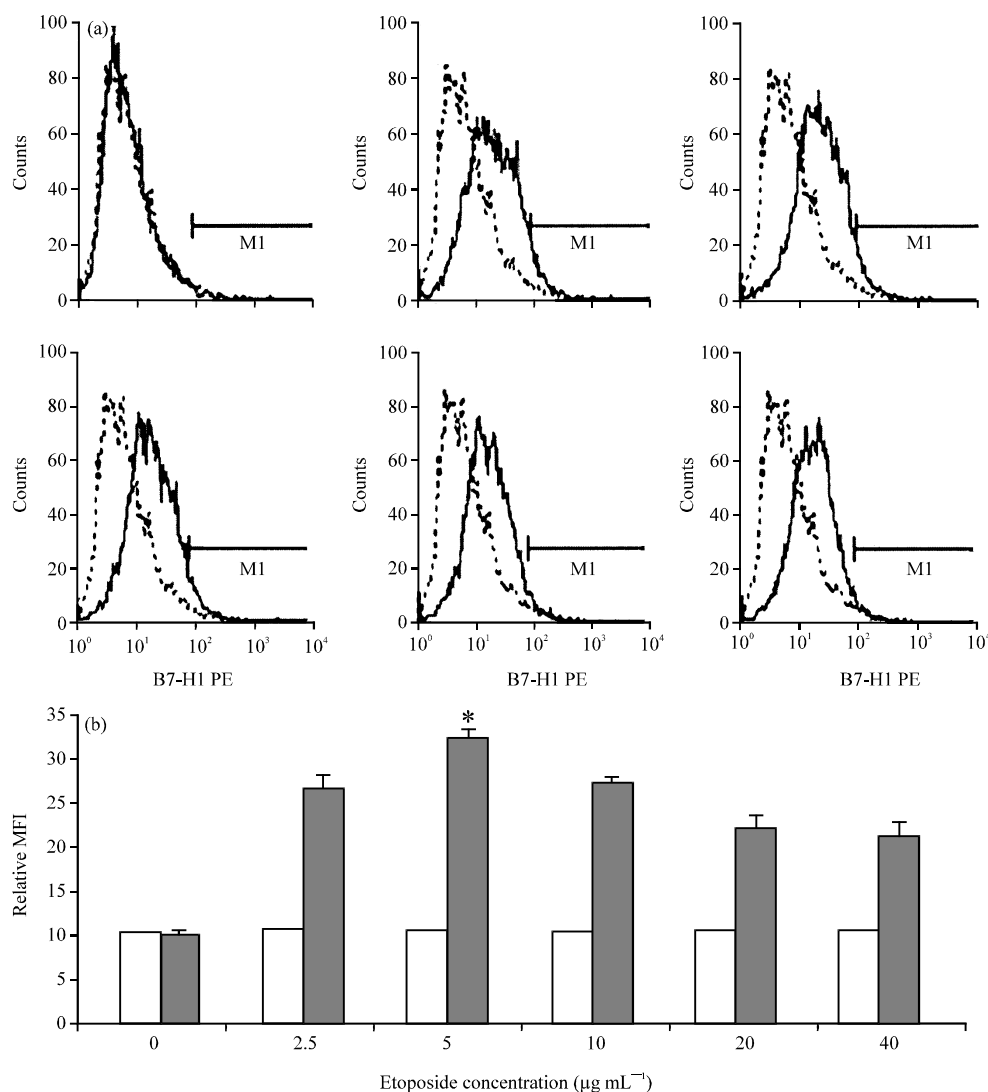

Fig. 2: B7-H1 protein expression induced by etoposide; a) Flow cytometeric analysis of B7-H1 expression on Y79 cells after 24 h incubation with etoposide. Representative histograms are demonstrated. Solid traces: staining with B7-H1 antibody; dotted traces: staining with an isotype antibody as the negative control; b) The experiment was repeated three times. The black histograms represent B7-H1 expression and the open histograms show the negative control. MFI = Mean Fluorescence Intensity. The level of B7-H1 expression was the highest at 5 μg mL^{-1} of etoposide (*p<0.01 vs. other groups)
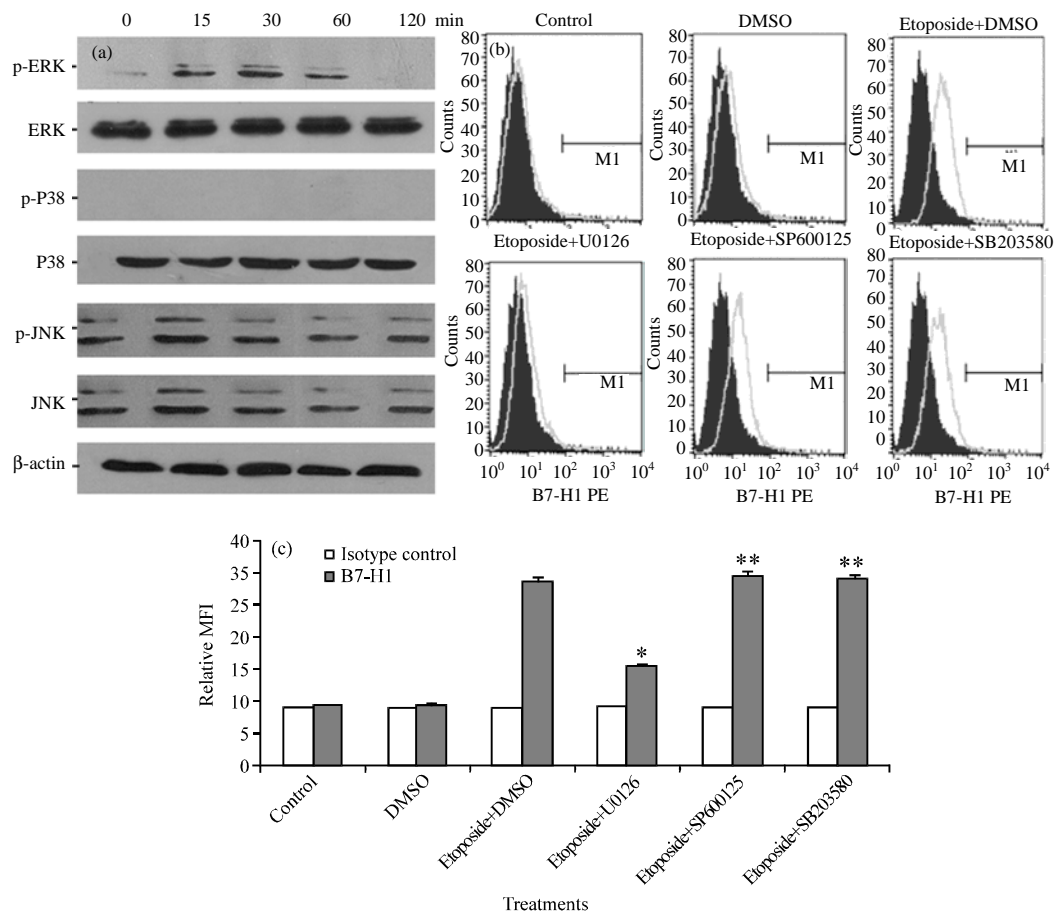
Fig. 3: The etoposide-induced B7-H1 expression was suppressed by the blockade of Extracellular Signal-Regulated Kinase (ERK) signaling pathway in Y79 cells; a) Western blot analysis of ERK, JNK, P38 phosphorylation in y79 cells. The cells were subjected to analysis after treatment with etoposide (5 μg mL^{-1}). There are activation of ERK and JNK but not P38 signaling pathways; b) Flow cytometric analysis of B7-H1 expression on Y79 cells after 24 h incubation etoposide (5 μg mL^{-1}) with or without 1 h pretreatment with signal transduction inhibitors, i.e., 10 μM UO126 (MEK1/2), 10 μM SP600125 (JNK) or 20 μM SB203580 (p38). The histogram data represent one typical result out of three independent experiments. The grey histograms represent B7-H1 expression and the open histograms show the negative control; c) Data in graphs show the mean of the MFI of three experiments. U0126 reduced the etoposide induced B7-H1 expression (*p<0.01 vs. Etoposide+DMSO group). The effect of etoposide was not influenced by SP600125 and SB203580 (**p>0.05 vs. Etoposide+DMSO group)

blotting verified the constitutive activation of ERK and JNK but not P38 signaling pathways (Fig. 3a). To determine the signaling pathways involved in B7-H1 expression induced by epotoside, researchers applied different signal transduction inhibitors to Y79 cells. Pretreatment with the MEK inhibitor U0126 strongly reduced the etoposide induced B7-H1 expression (Fig. 3b and c; p<0.01). The effect of etoposide was not blunted by JNK inhibitor SP600125 and p38 inhibitor SB203580 (Fig. 3b and c, p>0.05).

B7-H1 siRNA knock down the expression of B7-H1 induced by etoposide in Y79 cells: The pGCsilencer™ U6/Neo/GFP/RNAi plasmid was successful to transfect Y79 cells. The transfected cells showed green fluorescence under fluorescence microscope (Fig. 4a). The B7-H1 siRNA effectively reduced the mRNA expression (Fig. 4b). Consistent with the mRNA expression, flow cytometry analysis showed that B7-H1 protein level in Y79 cells induced by etoposide was also significantly decreased by B7-H1 siRNA transfection (Fig. 4c, p<0.01).
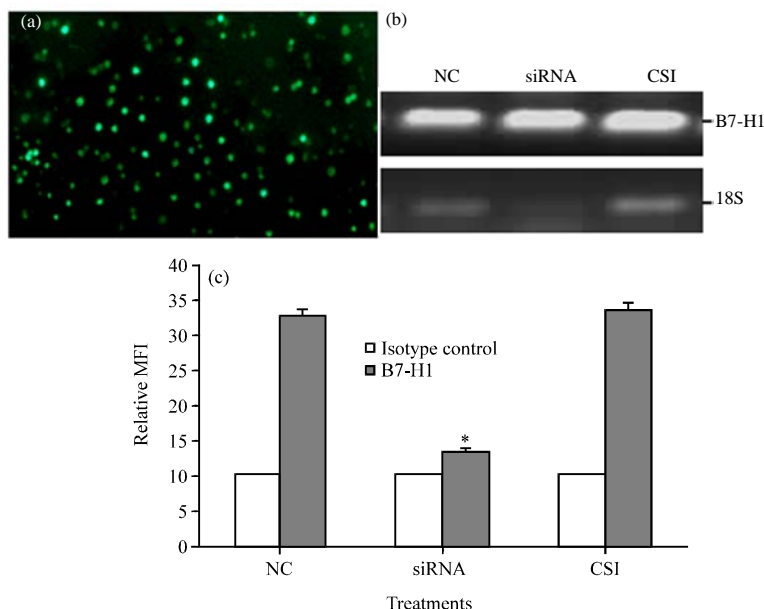
Fig. 4: Effect of B7-H1 siRNA on etoposide-induced expression of B7-H1 in Y79 cells; a) The B7-H1 siRNA plasmid was successful to transfect Y79 cells (green); b) RT-PCR 18S mRNA was co-amplificated as internal control. Decreased B7-H1 mRNA could be observed in B7-H1 siRNA group; c) The results of flow cytometric analysis of B7-H1 show that B7-H1 siRNA has knocked down the expression of B7-H1 protein induced by etoposide ($*p<0.01$ vs. NC and CSI groups)

No significant difference in B7-H1 protein expression was detected between the NC and the CSI groups ($p>0.05$).

DISCUSSION

B7-H1 is a newly discovered costimulatory molecule in immune supression. B7-H1 is a ligand of PD-1 that expressed by activated T cells following TCR (T cell receptor) stimulation with cognate antigen peptides that are presented in the complex of MHC class I or II. B7-H1 expressed by tumor cells induces the apoptosis of tumor-reactive CD8 T cells (Muhlbauer *et al.*, 2006) on the other hand, B7-H1 expressed by tumor-associated DCs induces IL-10 an immunosuppressive cytokine, in tumor-specific T cells (Curiel *et al.*, 2003). These findings indicate that B7-H1 can lead to immune escape of tumor cells. The expression of B7-H1 in the tumor surface can act as receptor in anti-apoptotic signal transmission and escape from the attack by cell toxic T lymphocytes (Azuma *et al.*, 2008). B7-H1 mRNA is abundant in many tissues. But the protein is detected only in tumor tissues and immune privileged organs (such as eye and placenta). B7-H1 protein doesn't exsist in cultured tumor cell lines *in vitro*. Several cytokines can induce expression of B7-H1 protein *in vitro* such as γ-Interferon (IFN-γ)

(Rameshwar, 2008). The expression of B7-H1 can be detected in a variety of tumor cells but the regulatory mechanisms are different.

Chemotherapy is a common means for treating cancer. But drug resistance is an important factor in decreasing efficacy. Researchers studied the effect on expression of B7-H1 in retinoblastoma cells by etoposide. The results of RT-PCR and fluorescent quantitation PCR showed that etoposide induced the expression of B7-H1 mRNA. Flow cytometry also showed that it stimulated expression of B7-H1 protein significantly. The expression of B7-H1 was associated with concentration of etoposide. It peaked at 5 µg mL^{-1} and then did not increase as the dose increased. In Yoshihiko's research (Usui *et al.*, 2006) it was found that Y79 cells expressed B7-H1 induced by IFN-γ. When the Y79 cells and T lymphocytes were in mixed culture, B7-H1 binded with PD-1 in T cells surface. T lymphocyte proliferation was inhibited and escape from tumor immunity. Based on this study, it suggests that expression of B7-H1 induced by etoposide may be an important reason of resistance in retinoblastoma chemotherapy.

Earlier studies show that there are many signaling pathways involved in regulating the expression of B7-H1 in tumor cells: JAK/STAT signaling pathway is involved in the expression of B7-H1 in lung and liver cancer stimulated by IFN (Lee *et al.*, 2006) whereas PI3K pathway

in glioma cells (Parsa *et al.*, 2007). The expression of the molecule stimulated by IFN-γ and TOLL-like receptor ligands in multiple myeloma cells is mediated by MEK (Liu *et al.*, 2007). MEK signaling pathway is also involved in the expression of B7-H1 in anaplastic large cell lymphoma and Hodgkin lymphoma cells (Yamamoto *et al.*, 2009). The results demonstrate that etoposide activated ERK1/2 phosphorylation strongly in Y79 cells and that this signaling pathway may be involved in the expression of B7-H1. The effect of MEK inhibitor-U0126 indicates that etoposide stimulates the expression of B7-H1 by phosphorylation of ERK1/2.

Researchers further investigated the role of small RNA molecule interference in this process. RNA interference (RNAi) is the homologous mRNA degradation induced by double-stranded RNA (dsRNA) and the corresponding gene silencing. Exogenous or endogenous dsRNA is imported into cells and it activates Dicer enzyme complex (helicase and endonuclease) in cells which can identify unusual double-stranded RNA to cut short or siRNA (Lopez-Fraga *et al.*, 2009). Homologous siRNA was synthesized *in vitro* in this study. B7-H1 siRNA was successfully transfected into retinoblastoma cells with cationic liposome transfection reagent carried by plasmid vector. B7-H1 siRNA can inhibit the expression of B7-H1 induced by etoposide at both mRNA and protein levels.

CONCLUSION

The results demonstrated that etoposide promoted B7-H1 expression via activating phosphorylations of the ERK signal pathway in human retinoblastoma cells. These findings might open up the prospective of immunotherapy as an important treatment for retinoblastoma.

LIMITATIONS

The current study is that it is only an *in vitro* experiment. The results do indicate the need for further animal experiments and clinical studies to determine the molecular mechanism (s) of etoposide on human retinoblastoma cells. The findings from the investigation open up the prospective for immunotherapy as an important adjunct treatment to reduce drug resistance and enhance the effect of chemotherapy.

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