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CLINICAL STUDY

In vitro expression of HPV16 E7 linked to HMGB1 immunoadjuvant in mammalian cells

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ABSTRACT

BACKGROUND: E7 is the major transforming protein of human papillomavirus (HPV) that plays important role in maintaining the proliferative state in HPV-infected cells. Furthermore, high mobility group 1 protein (HMGB1) is a highly conserved component of chromatin that can be secreted by macrophages and activated monocytes and thus functions as an inflammation mediator.

METHODS: In the current study, cloning of HMGB1 gene and also HPV16E7-HMGB1 was performed in pEGFP-N1 eukaryotic expression vector in order to evaluate their expression in mammalian cells. For this purpose, the HEK-293T cells were transfected by pEGFP-E7, pEGFP-HMGB1 and pEGFP-E7-HMGB1 using TurboFect delivery system. The levels of protein expression were assessed by flow cytometry and fluorescent microscopy at 48 hr after transfection, as well as by western blot analysis using anti-GFP polyclonal antibody.

RESULTS: Our data showed a clear band of ~ 684 bp and ~ 981 bp related to HMGB1 and E7-HMGB1 genes in agarose gel, respectively. The expression of HMGB1-GFP and E7-HMGB1-GFP proteins was confirmed for the bands of ~ 53 kDa and ~ 64 kDa in the transfected cells using western blot analysis, respectively. The linkage of HMGB1 gene to E7 could likely neutralize the negative charges of E7, thus a clear band of 64 kDa was detected instead of 76 kDa in western blot analysis. Moreover, the percentage of expression for E7-GFP, HMGB1-GFP and E7-HMGB1-GFP was 76 %, 55 %, and 52 %, in comparison with pEGFP-N1 (~82 %) as a positive control. Indeed, HMGB1 linked to HPV16 E7 gene decreased transfection efficiency of E7 DNA in HEK-293T cells.

CONCLUSION: Generally, the electrophoretic mobility of HPV16 E7 was changed due to the linkage of HMGB1 gene. Furthermore, the fusion protein could be efficiently expressed in mammalian cells for the next use in immunotherapy *(Fig. 3, Ref. 51).* Text in PDF *www.elis.sk.*

KEY WORDS: high mobility group box 1, HEK-293T, pEGFP-N1, cell transfection.

Introduction

Human papillomaviruses (HPVs) are responsible for malignant tumor formation in genital regions (*e.g.*, high-risk or oncogenic HPVs), as well as benign anogenital warts (*e.g.,* low risk or non-oncogenic HPVs) (1-3). Among the main genotypes of high-risk HPVs, HPV-16 is the most common genotype and responsible for ~ 60% of cervical cancer cases in the world (3-7). The studies showed that E6 and E7 oncoproteins expressed in the early stages of HPV infection maintain the proliferative state in HPV-infected cells (8-10). HPV16 E7 is a small protein with a natively unfolded domain that is constitutively expressed in all layers of the infected epithelium. According to the reports, the E7-specific T-cell responses could provide protection against the growth of a transformed tumor cell line. Thus, E7 protein is considered as a tumor antigen for development of immunotherapy (11-19). Among different therapeutic vaccines, DNA vaccines have been widely used to develop vaccines against various infectious diseases and cancer. Recently, several strategies have been applied to increase the potency of DNA vaccine such as the use of adjuvants, and delivery systems (20). High mobility group protein B1 (HMGB1) is a highly conserved nuclear protein that was proposed as a novel effective adjuvant in DNA vaccination against a lethal mucosal influenza A/PR/8/34 challenge. Indeed, HMGB1 adjuvant is able to enhance adaptive effector and memory immune responses (21). HMGB1 acts as a multi-functional protein and an extracellular signaling molecule during recombination, transcriptional regulation, inflammation, cell differentiation, cell migration, and tumor metastasis (22-26). Due to its role in inflammation, HMGB1 was used for treatment of rheumatoid arthritis and also sepsis (27, 28). HMGB1 can facilitate the binding of p53 to DNA using the DNA bending (29-34). In the current study, we constructed the DNA encoding HMGB1 gene as well as the E7-HMGB1 fusion and evaluated their expression in a human cell line using fluorescent microscopy, flow cytometry, and western blotting.

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Materials and Methods

Construction of pEGFP-HMGB1 and pEGFP-E7-HMGB1

The full length of HMGB1 gene (Access No: NM_010439) was obtained from the pBluescript II SK+ vector containing the coding sequence of HMGB1 protein (Synthesized by BioMatik co., Canada). For preparation of pEGFP-HMGB1 and also pEGFP-E7-HMGB1 constructs, the HMGB1 gene (~ 684 bp) was cloned into the *Kpn*I /*Age* I sites of pEGFP-N1 (Clontech, Palo Alto, CA, USA) and pEGFP-E7 (previously provided by Bolhassani *et al*., 35), respectively. The forward and reverse primers for amplifying the HMGB1 (HMG-F & HMG-R) were designed as follows (the enzyme restriction sites are shown in bold).

HMG-F: 5′ AGG TAC CGA GGG ATC CAT GGG CAA A 3′

HMG-R: 5′ AAC CGG TAA TTC TTC TTC ATC TTC GTC TTC C 3′

PCR analysis was performed by PCR thermal cycler under standard conditions (94°C for 1 min; 55°C for 45 s and 72°C for 1 min; 30 cycles) and the product was separated on a 0.8 percent agarose gel. The bands corresponding to the expected PCR products size were gel purified (QIAquick gel extraction kit protocol, QIAGEN), digested with *Kpn*I and *Age*I, and ligated into a similarly digested expression vectors (pEGFP-N1 & pEGFP-E7) using T4 DNA ligase (Fermentas, Germany). The ligation mixtures were used to transform *E. coli* DH5α strains. The plasmid DNAs were purified from recombinant clones by an alkaline lysis method. The presence of the inserted HMGB1 fragment was confirmed by PCR and restriction enzyme digestion as detected on gel electrophoresis and finally sequenced using the dideoxy chain termination method on an automated sequencer. DNA constructs containing HMGB1 (pEGFP-HMGB1 and pEGFP-E7-HMGB1) were purified in large-scale using Midi-kit (Qiagen). DNA concentrations were determined by the absorbance measured at 260 nm.

In vitro expression of HMGB1, and E7-HMGB1 proteins in HEK-293 T cells

Human HEK-293 T cells (provided from the cell bank of Pasteur Institute of Iran) were maintained in complete RPMI (Sigma) medium supplemented with 10% fetal calf serum (FCS, Gibco) at 37°C and 5% CO_2 atmosphere. Then, the cells were seeded into a 12-well plate and transfected using TurboFect as *in vitro* delivery reagent. For DNA transfection with TurboFect (cationic polymer, Fermentas), the recombinant pEGFP-HMGB1 and pEGFP-E7-HMGB1 vectors (~ 1–2 µg) were pre-incubated with 4 µl of reagent in a final volume of 25 µl and incubated at room temperature for 20 min to form the DNA/TurboFect complexes. The complexes were then added to each well containing cells and medium. Cells were harvested 48 h post-transfection, washed, and re-suspended in PBS, to determine the proportion of fluorescent cells expressing HMGB1 or E7-HMGB1 proteins using flow cytometry. The quality of protein expression was also detected by fluorescent microscopy and western blotting. The pEGFP-N1 was used as a positive control.

Western blot analysis

HEK-293 T cells were washed with PBS and lysed in whole-cell lysis buffer (10% glycerol, 0.5 mM EDTA, 1 mM DTT, 2 mM natrium fluoride, 0.2% Triton X-100 in PBS pH=7.4) supplemented with protease inhibitor (Sigma). Proteins were separated on 12.5% (w/v) polyacrylamide gel and transferred to nitrocellulose membrane (Millipore). The anti-GFP polyclonal antibody (Acris, USA, 1:10000 v/v) was used to confirm HMGB1-GFP, E7-HMGB1-GFP protein expression under standard procedures. The immunoreactive protein bands were visualized using peroxidase substrate named 3, 3‘-diaminobenzidine (DAB, Sigma).

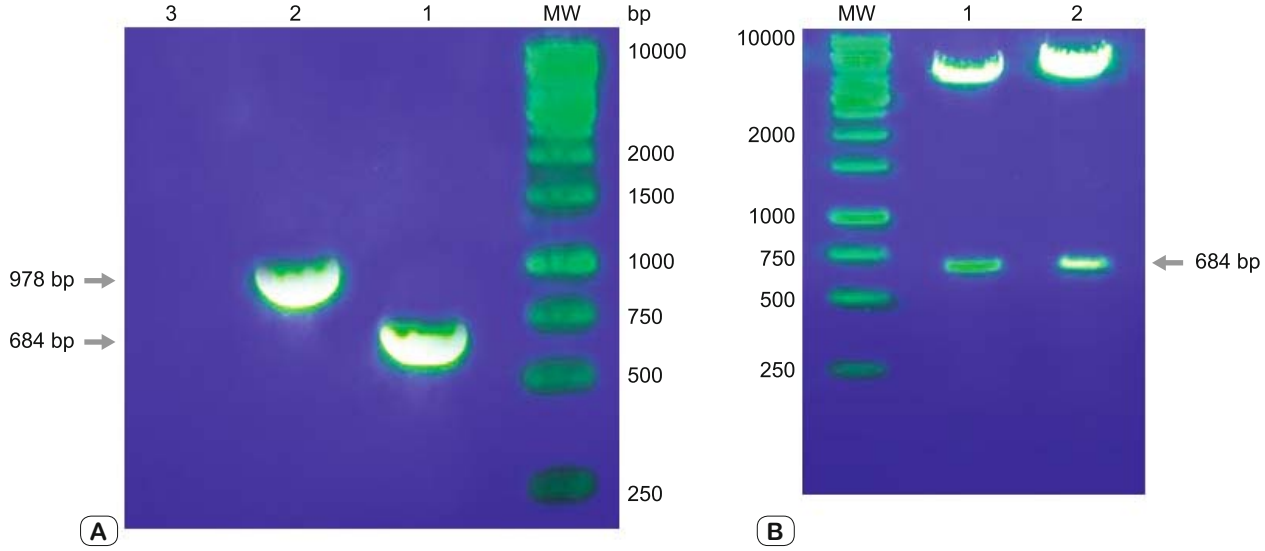

Fig. 1. Cloning of HMGB1 into the multiple cloning sites (MCS) of pEGFP and pEGFP-E7 vectors: A) PCR analysis of HMGB1 (lane 1, ~ 684 bp) and E7-HMGB1 (lane 2, ~ 978 bp) from pEGFP-HMGB1 and pEGFP-E7-HMGB1 vectors, respectively. B) The confirmation of *HMGB1* gene (~ 684 bp) by digestion from pEGFP-HMGB1 vector (lane 1), and pEGFP-E7-HMGB1 vectors (lane 2); MW is molecular weight marker (Fermentas, Germany).

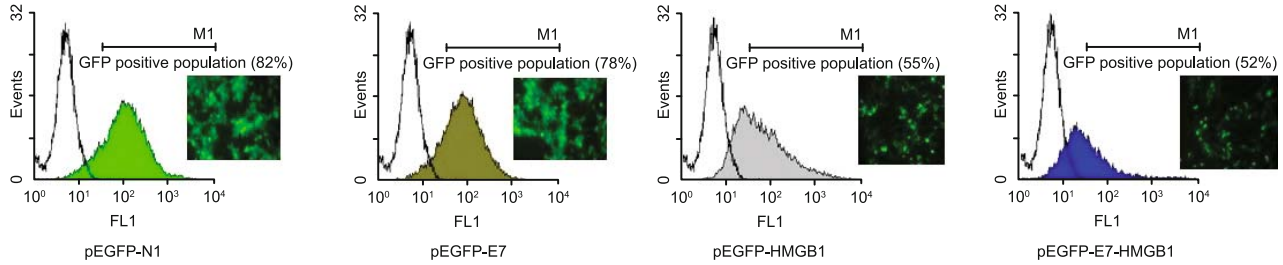

Fig. 2. Expression analysis of GFP, E7-GFP, HMGB1-GFP and E7-HMGB1-GFP proteins in HEK-293T cells transfected by pEGFP-N1 (A), pEGFP-E7 (B), pEGFP-HMGB1 (C), and pEGFP-E7-HMGB1 (D) using flow cytometry and fluorescent microscopy.

Statistical analysis

Statistical analysis (Student's t-test) was performed by Prism 5.0 software (GraphPad, San Diego, California, USA) to analyze the percentage of HMGB1-GFP, and E7-HMGB1-GFP expression using flow cytometry. The value of $p < 0.05$ was considered statistically significant. Similar results were obtained in two independent experiments.

Results

Generation of HMGB1 and E7-HMGB1 DNA constructs

At first, the HMGB1 gene was inserted into the N-terminal of pEGFP-N1 and pEGFP-E7 expression vectors without stop codon for construction of pEGFP-HMGB1 and pEGFP-E7-HMGB1. The recombinant plasmids as well as pEGFP-N1 were prepared in large scale and confirmed by PCR and digestion. The results showed that HMGB1 and E7-HMGB1 migrated as the clear bands of ~ 684, and 978 bp in agarose gel, respectively (Figure 1).

HMGB1 linked to HPV16 E7 gene decreased transfection efficiency of E7 DNA in HEK-293T cells

In vitro DNA delivery of HMGB1 and E7-HMGB1 was performed by TurboFect as the transfection reagent. GFP expression was evaluated by fluorescence microscopy and flow cytometry at 48 h after transfecting HEK-293T cells. GFP fluorescence was observed in the cells that received 1-2 µg of pEGFP-N1, pEGFP-E7, pEGFP-HMGB1, and pEGFP-E7-HMGB1 vectors. The levels of protein expression were 82% for pEGFP-N1; 78% for pEGFP-E7; 55% for pEGFP-HMGB1, and 52% for pEGFP-E7-HMGB1 using TurboFect reagent. The level of GFP expression detected by pEGFP-E7 delivery was significantly higher than that by pEGFP-HMGB1 and pEGFP-E7-HMGB1 transfection ($p < 0.05$, **Figure 2**). The efficiencies of HMGB1 and E7-HMGB1 gene expression were similar at 48 h after cell transfection.

Identification of HMGB1 and E7-HMGB1 gene expression using western blot analysis

Western blot analysis was performed using anti-GFP polyclonal antibody to ensure the proper expression of HMGB1 and E7-HMGB1 proteins. The specific bands with expected size of 53 kDa, and 64 kDa were detected for HMGB1-GFP, and E7-HMGB1-GFP expressed from pEGFP-HMGB1, and pEGFP-E7-HMGB1 vectors in the transfected cells, respectively as shown in **Figure 3**. Indeed, HMGB1 or E7-HMGB1 expression was detectable in transfected cells as compared to untransfected cell extracts by western blotting. The results showed that the linkage of HMGB1 gene to E7 could likely neutralize the negative charges of E7, thus a clear band of 64 kDa was detected instead of 76 kDa in western blot analysis.

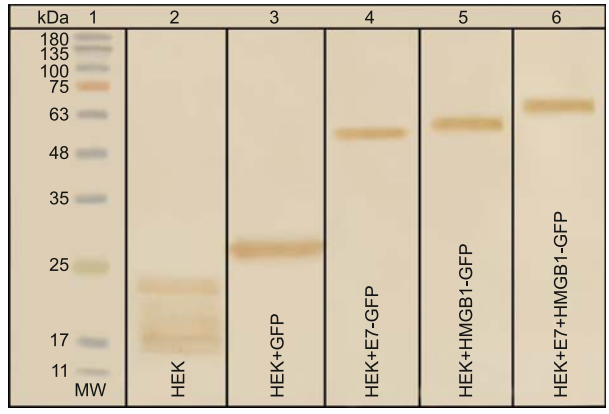

Fig. 3. Western blot analysis using an anti-GFP antibody: The results showed the expression of the full-length GFP (lane 3, ~ 27 kDa), E7-GFP (lane 4, ~ 50 kDa), HMGB1-GFP (lane 5, ~ 53 kDa), and E7-HMGB1-GFP (lane 6, ~ 64 kDa) proteins. A detectable band was not observed in untransfected cells (lane 2) as a negative control. MW is molecular weight marker (lane 1, Fermentas).

Discussion

HPV 16 is the most important HPV type which can be detected in about half of all invasive cervical cancers (36). Great efforts are recently undertaken to develop therapeutic vaccines against HPV infections. HPV E7 protein is expressed in all tumor tissues and used as an effective antigen in vaccine design (37, 38). In the current study, the expression of E7, HMGB1 and E7-HMGB1 in human cell line was evaluated and compared by both western blotting and flow cytometry. In order to achieve these goals, the HMGB1 (~ 684 bp) and E7-HMGB1 (~ 978 bp) genes were cloned into eukaryotic expression vector (pEGFP-N1). The correct cloning was confirmed by digestion and PCR analysis. Then, HEK-293T cells were transfected by the purified plasmid DNAs using TurboFect reagent. As known, transfection is a powerful analytical tool that delivers for-

eign nucleic acids into cells to generate genetically modified cells. The transfection methods are classified into three groups such as biological, chemical, and physical delivery systems (39). Herein, we used a cationic polymer (TurboFect) for DNA delivery. Western blotting is a widely used technique for the detection and analysis of proteins based on their ability to bind to specific antibodies (40-42). In this study, anti-GFP antibody was used to bind GFP fused to proteins of interest. Western blot analysis showed the presence of the prominent protein bands related to HMGB1 and E7-HMGB1 that were not detectable in un-transfected cell lysates. In the presence of sodium dodecyl sulphate (SDS) and 2-mercaptoethanol (2-ME), the E7-GFP protein migrated as a 50 kDa protein (23kDa E7 + 27kDa GFP) during poly acrylamide gel electrophoresis. The theoretical molecular mass of E7 protein is nearly 11 kDa. The previous data indicated that the substantial net negative charge of the wild type E7 protein is responsible for its anomalous electrophoretic behavior. This electrophoretic behaviour of the HPV16E7 protein was associated with the amino terminal half of the protein (43, 44). Our findings indicated that the linkage of HMGB1 to E7 decreased protein size from ~ 76 kDa (23kDa E7+ 25 kDa HMGB1+ 27 kDa GFP) to ~ 64 kDa (11kDa E7+ 25 kDa HMGB1+ 27 kDa GFP) in western blot analysis. Indeed, the HMGB1 likely led to charge neutralization and protein migration in normal size. In addition, the cell expression of HMGB1 and E7-HMGB1 was similar and significantly lower than E7 protein. This result showed lower uptake of HMGB1 and also E7-HMGB1 as compared to E7 protein in HEK-293T cells. Fluorescence microscopy as an efficient and unique tool also showed DNAs transport and their expression to living cells because of its specificity, and high sensitivity. The studies indicated that fluorescence microscope can detect the fluorescence emitted from labeled molecules in biological samples as images or photometric data from which intensities and emission spectra can be deduced (45, 46). Generally, the expression systems utilizing mammalian cells are able to generate recombinant proteins with proper folding, post-translational modifications which are important for their biological activity. Up to now, a number of mammalian cell lines have been used for protein expression including HEK-293 (Human embryonic kidney) and CHO (Chinese hamster ovary). HEK-293 cells exhibited the highest level of polyethyleneimine (PEI)-mediated transfection for GFP expression (~ 50-80% of cells), and were widely used for production of recombinant proteins both by transient transfection as well as by the formation of stable cell lines (47). Our data also represented high efficiency of HEK-293 T for GFP expression using Turbofect (~ 82%) as well as high expression of the E7 (~ 76%), HMGB1 (~ 55%), and E7-HMGB1 (~ 52%) proteins using flow cytometry analysis. Flow cytometry technique provides qualitative and quantitative analysis of single cells using fluorescent markers such as GFP (48-50). GFP reporter gene is a useful approach for evaluation of gene expression (51) as observed in our study.

Conclusion

In summary, the recombinant HMGB1 and E7-HMGB1 plasmids could be delivered into HEK-293T cells using TurboFect cationic polymer and efficiently expressed *in vitro*. Regarding the suitable expression of DNA constructs, they will be used for *in vivo* studies as a therapeutic DNA vaccine in future.

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