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EXPERIMENTAL STUDY

Delivery of HIV-1 Nef linked to heat shock protein 27 using a cationic polymer is more effective than cationic lipid in mammalian cells

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ABSTRACT

BACKGROUND: Different adjuvants and delivery systems have been used to enhance the potency of DNA vaccines against viral diseases. Among them, heat shock proteins (HSPs) are stress proteins that have multiple roles such as chaperon activity and anti-apoptotic and adjuvant properties. The goal of this study was to compare the expression of HIV-1 Nef, Hsp27 and Hsp27-Nef genes transfected in HEK-293T mammalian cells by TurboFect and Lipofectamine as a cationic polymer and lipid, respectively.

METHODS: At first, the pEGFP eukaryotic vectors encoding HIV-1 Nef, Hsp27 and Hsp27-Nef genes were generated and transfected in HEK-293T using TurboFect and Lipofectamine delivery systems. Then, the expression of proteins was evaluated and compared using fluorescent microscopy, flow cytometry and western blotting 48 hr after transfection.

RESULTS: The accuracy of the DNA constructs was confirmed on agarose gel electrophoresis to be ~ 720 bp, ~ 648 bp, and ~ 1368 bp bands for Hsp27, Nef, and Hsp-Nef, respectively. The expression analysis in the transfected cells showed that the delivery of genes using TurboFect was significantly higher than that using Lipofectamine. Furthermore, transfection of Hsp27 gene was more effective than that of Nef gene using both delivery systems. Hsp27 linked to Nef could also increase its delivery and expression in HEK-293T cells.

CONCLUSION: Generally, Hsp27 can be used as a suitable carrier in DNA vaccine design against HIV-1 infections *(Fig. 5, Ref. 28)*. Text in PDF *www.elis.sk.*

KEY WORDS: small heat shock protein, HIV-1 Nef, Hsp27, gene delivery.

Introduction

Human immunodeficiency virus (HIV) weakens the immune system causing acquired immune deficiency syndrome (AIDS). The major cells targeted by HIV include macrophages, T-cells, and dendritic cells (DCs). Different receptors are required in this way, such as CD4 and CCR5 or CXCR4 on T-cells, galactocerebroside (Gal-C) on macrophage, and DC-SIG on DCs for viral spreading (1, 2). The HIV genome contains three major genes encoding fifteen viral proteins essential for the viral life cycle: a) *gag, pol, env* genes expressing enzymes, structural and envelope proteins, respectively; b) *tat, rev* genes encoding regulatory proteins, and c) *vpr, vip, vpu*, and *nef* encoding accessory proteins (3). Therefore, researchers designed a way of stimulating the immune system by using viral genes as antigens. Nef protein (MW: 27 kDa) is expressed early in viral life cycle and has important roles including inhibition of CD4, MHC I, MHC II activity, stimulation of replication and viral infection, and induction of apoptosis in both uninfected and infected cells. Generally, the studies showed that the lack of Nef reduces the infectivity of the virus (4–9). Indeed, expression of Nef protein in the first phase of the infection, its existence during the period of infection, and having epitopes for T-cells are the advantages of Nef as a good antigen candidate for HIV vaccine development. However, recent findings indicated low permeability of the DNA constructs and their poor immunogenicity. Thus, such vaccines should be improved by delivery systems and adjuvants (10). There are three major tools for gene delivery including viral, physical and chemical methods. Due to some drawbacks of both viral and physical methods, a variety of chemical transfection systems were developed, such as lipids, cationic polymers and calcium phosphates. The cationic polymers (*e.g.*, TurboFect) and lipids (*e.g.*, lipofectamine) could generate compact and stable complexes with negatively charged nucleic acid molecules, protect DNA from degradation, and facilitate efficient DNA delivery into the cells (11, 12). On the other hand, an adjuvant enhances the immunity to an antigen in conjugate and complex forms. Heat shock proteins (HSPs) are highly conserved proteins that play main roles in maintaining protein homeostasis during cellular stress. They were classified based on their molecular weights including HSP90, HSP70, HSP60 and small HSPs. Many

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HSPs exhibited the regulatory and stimulatory roles on immune responses (13–15). Small Hsps are the members of HSP family with molecular weights from 12 to 43 kDa. These proteins have a similar structure such as N-terminal domain, α-crystalline, and C-terminal domain (16). Treatment of macrophage with the recombinant Hsp27 (rHsp27) led to an increase in the expression of both pro-inflammatory cytokines (*e.g.*, IL-1β , TNF-α), and anti-inflammatory cytokines (*e.g.*, IL-10, GM-CSF) (17–19). Herein, we prepared DNA constructs of Hsp27, Nef, and Hsp27-Nef for evaluation of their expression in mammalian cell line. Then, these constructs were transfected to the HEK-293T cell line using TurboFect and Lipofectamine transfection regents and their expression were assessed by fluorescent microscopy, flow cytometry and western blotting. The data showed that both systems were suitable for delivery of DNA constructs in a certain dose, but the transfection efficiency of TurboFect system was higher than that of Lipofectamine.

Materials and methods

Construction of the recombinant plasmids

The full length of Hsp27 gene was prepared in a prokaryotic expression vector (pQE30, Biomatik Co., Canada). To generate pEGFP-Hsp27, Hsp27 fragment was subcloned into the pEGFP-N3 eukaryotic expression vector in *Nhe*I/ *Sal*I sites (Fermentas). HIV-1 Nef sequence was subcloned from pUC-19 into the pEGFP-N1 in *Nhe*I/*Pst*I sites. To make pEGFP-Hsp27-Nef, at first, pUC-Nef and pQE30-Hsp27 were digested by *Bam*HI/*Pst*I and *Bgl*II/*Pst*I, respectively. Then, Nef fragment was ligated to Hsp27 in pQE30-Hsp27 (*Bam*HI and *Bgl*II are compatible enzymes) using T4 DNA Ligase (Fermentas). Finally, Hsp27-Nef fusion was cut from pQE-Hsp27-Nef, and subcloned into pEGFP-N1 using *Nhe*I/*Pst*I restriction endonucleases. The *E. coli* DH5α competent cells were transformed by all plasmids by heat shock for 90 second at 42 °C. The single clones were cultured overnight in Luria-Bertani (LB) broth at 37 °C. Finally, the recombinant plasmids were purified with plasmid DNA extraction mini kit (Yekta Tajhiz Azma, Iran) and confirmed by digestion and sequencing.

Cell Culture

Human Embryonic Kidney 293T cells (HEK-293T, Pasteur Institute of Iran) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), supplemented with 10% fetal bovine serum (FBS, Gibco), pen/strep (100U/ml penicillin and 0.1mg/ml Streptomycin, Gibco). The cell cultures were incubated at 37 °C and 5% CO_2 conditions. One day before transfection, 5×10^5 cells were seeded into the 6-well plates to achieve exponential growth phase.

Transfection

HEK-293T cells at 70–80 % confluency were transfected with pEGFP-Hsp27, pEGFP-Nef, pEGFP-Hsp27-Nef, and pEGFP-N1 as a positive control using TurboFect (Thermo scientific) and Lipofectamine™2000 (Invitrogen) reagents. To generate TurboFect-plasmid DNA complex, 7 µl of TurboFect and 4 µg of plasmid were mixed and incubated for 15 minutes at room temperature. Finally, the complexes were added dropwise to each well in serum-free medium. Six hours after the cell transfection, the medium was replaced with the pre-warmed complete medium.

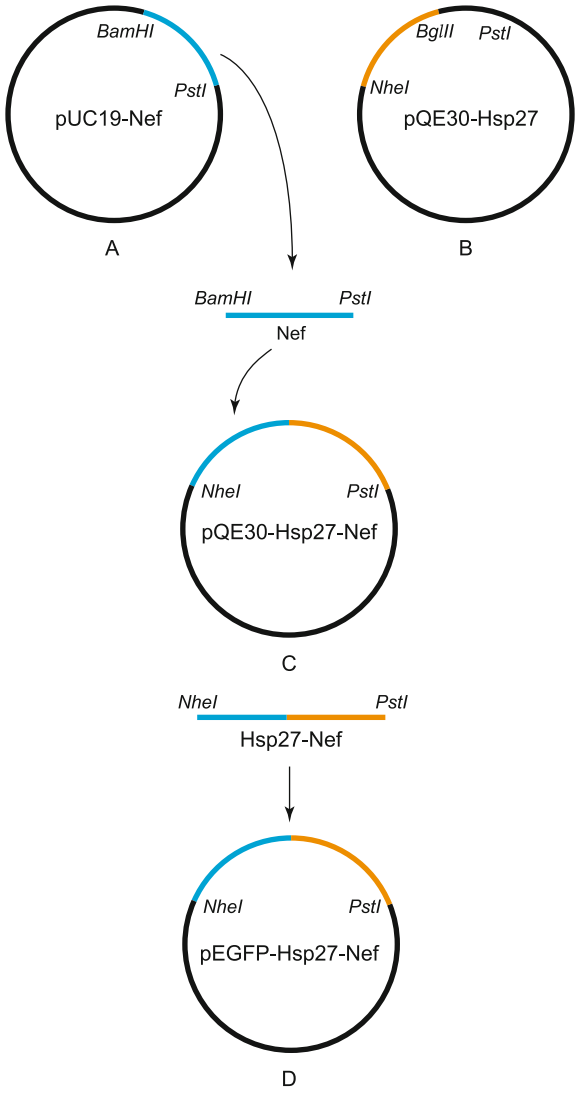

Fig. 1. The detailed process of pEGFP-Hsp27-Nef construct: A) Nef fragment was digested from pUC-19 with *Bam*HI/*Pst*I, B) pQE30-Hsp27 was digested with *Bgl*II/*Pst*I, C) Nef fragment was ligated into the linearized pQE-Hsp27 with T4 DNA ligase, D) Hsp27-Nef fusion was cut from pQE30 and subcloned into pEGFP-N1 with *Nhe*I/*Pst*I restriction enzymes.

To produce lipofectamine-plasmid DNA complex, 150 µl of serum-free medium was mixed with 10 µl of lipofectamine and incubated for 5 minutes at room temperature. Then, 150 µl incomplete DMEM was mixed with 4 µg of plasmids, added to lipofectamine solution, mixed gently, and incubated for 30 minutes at room temperature to form the DNA-lipofectamine complexes. After that, transfection complexes were added to each well and the medium was replaced after 6 hours of incubation at 37 °C with pre-warmed DMEM, 10% FBS and 1/100 penicillin/streptomycin. After 48 hours, transfection efficiency using TurboFect as well as

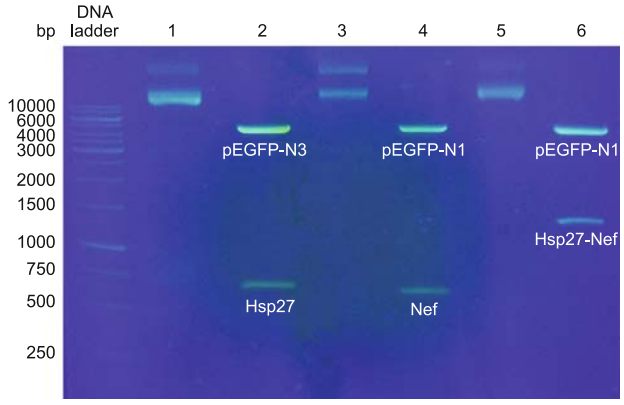

Fig. 2. Confirmation of the recombinant plasmids by double-digestion on gel electrophoresis: Lanes 1, 3, 5 represent the purified plasmids of pEGFP-Hsp27, pEGFP-Nef, pEGFP-Hsp-Nef on 1% agarose gel, respectively; Lanes 2, 4, 6 denote the double digested products using *Nhe*I/*Sal*I for pEGFP-Hsp27 (~ 720 bp), *Nhe*I/*Pst*I for pEGFP-Nef (~ 648 bp), and pEGFP-Hsp27-Nef (~ 1368 bp), respectively.

lipofectamine was evaluated by fluorescent microscopy, flow cytometry, and western blotting.

Transfection assay

Fluorescent microscopy and Flow cytometry

Fluorescent microscopy and Flow cytometry were used to determine transfection efficiency. The levels of Nef-GFP, Hsp27-GFP, Hsp27-Nef-GFP, and GFP protein expression was estimated after 48-h transfection using fluorescent microscopy (Envert Fluorescent Ceti, Korea) and quantified by a fluorescence-activated cell sorting (FACS) caliber flow cytometer (Partec, Germany). For flow cytometry analysis, the cells were harvested by trypsin and the cell pellets were resuspended in 1ml PBS (pH = 7.4). The expression of fluorescent genes was measured in the FL1 channel using an excitation filter (485 nm) and emission filter (535 nm). The untransfected HEK-293T cells and transfected cells with pEGFP-N1 were used as negative and positive controls, respectively. 10,000 cells were counted in each analysis.

Western blotting

For western blotting, the cells were harvested by trypsin, and the cell pellets were resuspended in PBS. Total cellular proteins were solved in 6X sample buffer containing Tris-HCl (0.5 M), glycerol, SDS, and 2-Mercaptoethanol (2 %). The samples were separated on 12.5 % acrylamide gel and transferred to nitrocellulose membrane. The membrane was incubated in blocking buffer (TBS 10X, 0.1 % Tween20, BSA, Merck) and washed with TBS10X and 0.1 % Tween20. Then, anti-GFP polyclonal antibody conjugated with horseradish peroxidase (1: 10000 v/v) was used to detect the proteins of interest in the presence of DAB substrate (Roche Diagnostics-Germany).

Results

Confirmation of the DNA constructs

The Hsp27 was correctly cloned in pEGFP-N3 as a 720 bp fragment on 1 % gel electrophoresis after digestion with *Nhe*I/*Sal*I enzymes. Furthermore, the clear bands of 648 bp for Nef gene and 1368 bp for Hsp27-Nef fusion were appeared on gel electrophoresis after digestion of pEGFP-Nef and pEGFP-Hsp-Nef with *Nhe*I/*Pst*I enzymes as shown in Figures 1 and 2.

Transfection assay using fluorescent microscopy and flow cytometry

The DNA transfection efficiency of Turbofect and Lipofectamine was demonstrated by fluorescent microscopy and flow

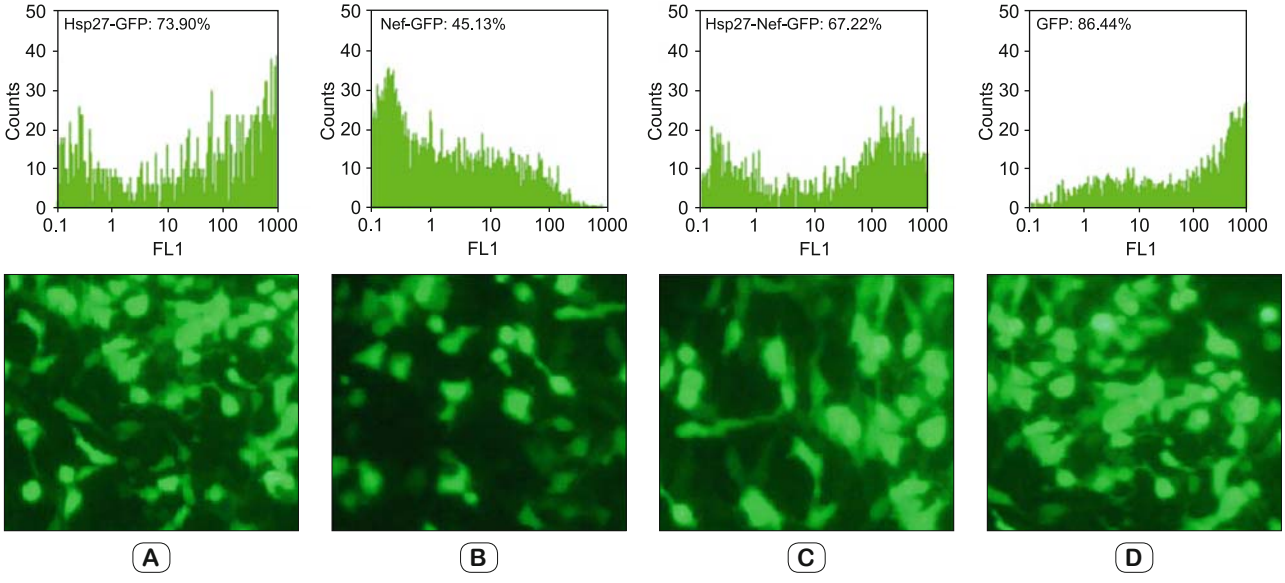

Fig. 3. Analysis of Hsp27-GFP (A), Nef-GFP (B), Hsp27-Nef-GFP (C), and GFP (D) expression in HEK-293T cells by Turbofect transfection reagent using Fluorescent microscopy and flow cytometry. The pEGFP-N1 (D) was used as a positive control.

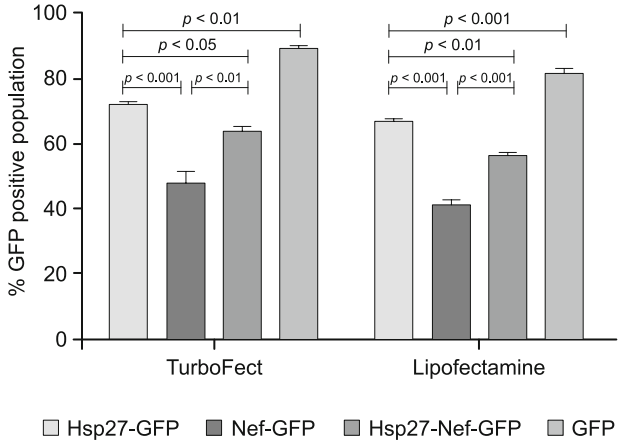

Fig. 4. Comparison of TurboFect and Lipofectamine delivery: The transfection efficiency of TurboFect was significantly higher than that of Lipofectamine.

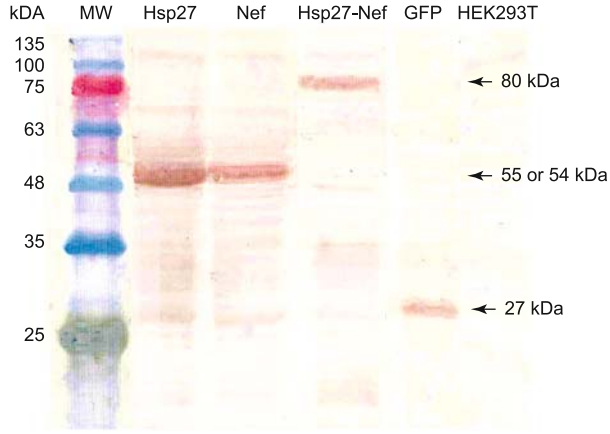

Fig. 5. Identification of protein expression in HEK-293T cells 48 h after transfection using western blot analysis. The expression of Hsp27-GFP (~ 54 kDa), Nef-GFP (~ 53 kDa), and Hsp27-Nef-GFP (~ 80 kDa) proteins was detected by an anti-GFP antibody as compared to the un-transfected cells. The GFP expression (~ 27 kDa) was applied as a positive control. MW is the molecular weight marker.

cytometry as shown in Figure 3. Flow cytometry analysis showed that the permeability of pEGFP-Hsp27, pEGFP-Nef, and pEGFP-Hsp27-Nef using Turbofect transfection system was greater than that achieved with Lipofectamine ($p < 0.05$). These results were determined by the percentage of protein expression using GFP reporter marker. The transfection efficiency of Turbofect and Lipofectamine reagents was summarized in Figure 4. The percentage of Hsp27-GFP, Nef-GFP, Hsp27-Nef-GFP, GFP expression were 72.19 ± 0.60, 47.66 ± 3.58, 63.76 ± 1.49, 89.13 ± 0.83 for TurboFect system, and 66.72 ± 0.80, 41.36 ± 1.38, 56.20 ± 0.65, 81.39 ± 1.45 for lipofectamine, respectively. Regarding these data, Hsp27 could increase Nef DNA delivery in the cells more significantly than Nef DNA alone using both TurboFect and lipofectamine transfection reagents ($p < 0.05$).

Western blotting

Western blot analysis showed a successful expression of Hsp27-GFP, Nef-GFP, Hsp27-Nef-GFP, GFP proteins using anti-GFP antibody (Fig. 5). In The data indicated clear bands of ~ 54 kDa, ~ 53 kDa, ~ 80 kDa, and ~ 27 kDa for Hsp27-GFP, Nef-GFP, Hsp27-Nef-GFP, and GFP, respectively in western blotting.

Discussion

The main goal of the current study was to prepare the fusion of Hsp27 and Nef for the first time and evaluate its expression levels after transfection in mammalian cells using TurboFect and Lipofectamine delivery systems. *In vitro* transfection of different cell lines is an important challenge for evaluating the gene expression. To achieve the highest transfection efficiency, it is needed to assess various gene delivery systems. The ideal gene delivery systems should have high transfection efficiency and low toxicity in the cells (12). HIV-1 Nef is considered a key factor in HIV pathogenesis (20, 21). The studies showed that transfection of Nef gene in HEK- 293 was performed by a liposomal transfection agent named Effectene, exhibiting the ability of this system to transport Nef into the cell (22). Another study showed that HIV-1 Nef gene was transfected into human coronary arterial endothelial cells (HCAEC) and Jurkat cells with lipofectamine LTX reagent, and the transfection efficiency was 70 % and 20 %, respectively (23). Among other lipid-based transfection reagents, flow cytometry analysis showed that GFP expression in different myeloma cell lines with Lipofectamine 2000 has higher efficiency as compared to LyoVec lipolex. Efficiency rates of pEGFP-N1 transfection in HEK293-FT, NS0 and Ag8 were efficiently higher than those of SP2/0, NS1 and P3U1, which were hardly transfected by these reagents (24). Our results demonstrated that cationic TurboFect polymer was more effective than lipofectamine, a cationic lipid, for transfection and expression of Hsp27, Nef, and Hsp27-Nef genes into HEK-293T cells. Furthermore, the analysis of HIV-1 Nef and Hsp27-Nef delivery using TurboFect and lipofectamine showed that Hsp27 could increase Nef expression in HEK-293T cells. Indeed, transfection of Hsp27 gene was also higher than that of Nef gene using both delivery systems. A study indicated that the efficiency of HIV-1 MPER-V3 gene expression in HEK-293T using Lipofectamine and Turbofect was 55 % and 65 %, respectively, indicating higher efficiency of TurboFect reagent (25). Physical methods such as electroporation were also compared with chemical methods of DNA transfer. For example, lipofectamine showed greater efficiency in transfection (~42.4 %) than in electroporation (~37.3 %) in an adherent human lung cancer cell line (Mehr-80 cells). In addition, both lipofectamine and electroporation reagents indicated higher transfection efficiency than other techniques such as SuperFect and DEAE-dextran reagents (26). Similar to our study, Hauser and colleagues demonstrated that the transfection of Hsp70-HPVE7 fusion into HEK-293A cells by Lipofectamine Plus was successful in comparison with each gene, alone suggesting the use of Hsps for increasing the potency of DNA vaccine *in vivo* (27). Another study also represented that transfection of Hsp27 with lipofectamine2000 in RGC-5 cells

was significantly elevated (~25-fold) under stress conditions as compared to untransfected cells (28).

Conclusion

Although different systems were used to transfer genes with various efficiency, in this study we showed that the efficiency of TurboFect transfection reagent of genes is higher than that of lipofectamine, which makes the former a more suitable tool for *in vitro* delivery in the future. Our observation showed the efficiency of Hsp27 to increase Nef delivery in DNA vaccines against HIV-1 infections. However, further study is required to investigate the Hsp27 mechanisms for enhancing gene delivery.

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