Original Article

Construction of pLLO vector encoding truncated form of Listeriolysin O as molecular adjuvant for DNA vaccine studies

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Abstract

Background: The major problem of DNA vaccine is less immunogenicity of them verses other killed or live whole organism vaccines therefore adjuvants for use in this kind vaccines is very necessary. Genetic adjuvants with bacterial sources are an appropriate approach to modulate immune responses to DNA vaccines. Listeria Monocytogenes proteins such as Listeriolysin O (LLO) with CD4 and CD8 epitopes can be as an adjuvant to initiate both innate and adaptive immune responses if the protein cytotoxicity can be eliminated. Herein we constructed a truncated LLO plasmid as genetic adjuvant and tested it in combination with a DNA construct as a model vaccine.

Materials and Methods: About 1340bp of the 5' end of whole LLO gene was amplified by PCR on DNA purified from Listeria Monocytogenes. Sequential sub cloning of truncated LLO into the Xho I/EcoRV sites of pcDNA3.1 plasmid, downstream of CMV promoter was done. pLLO plasmid was transfected to HEK293T cell line by lipofection method. LLO protein expression from transiently transfected 293T cell lysates was confirmed by western blotting. Then the adjuvant activity of LLO in BALB/c mice model was analyzed using proliferation test.

Results: Double digestion of pLLO plasmid with the enzymes that were applied for cloning led to the isolation of two fragments with expected sizes. The final plasmid was also confirmed following sequencing reactions. Moreover, expression of LLO was evidenced in transfected 293T cells, compared to non-transfected controls. In vivo study was shown, high significant proliferative responses in LLO co-immunization pattern.

Conclusion: In the DNA vaccine study, LLO co-administration plasmid could be a suitable genetic adjuvant to enhance cellular immune response of vaccine.

Keywords: DNA vaccine, molecular adjuvant, Listeria Monocytogenes, listeriolysin O

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Introduction

The general goal of vaccination is the production of a robust immune response to the administered antigen able to give constant long term protection against infection (1). To attain this goal with DNA vaccine often requires the genetic adjuvants to improve the efficacy of vaccine (2). Whole alive or killed microorganisms are too harmful and toxic to be applied as adjuvants.

Therefore, the bacteria-derived materials are using as a main potential source of adjuvants (1). This adjuvant activity is mediated through activation of Toll-like receptors that mediates the danger signals activating the host immune defense system (1).

Listeria Monocytogenes is a gram-positive, facultative intracellular bacterium that expresses a pore-forming toxin known as listeriolysin O (LLO) that is essential for its pathogenesis and allows L.

monocytogenes to escape from endocytic vesicle into the host cell cytosol, where the bacteria are able to multiply proficiently (3). LLO is a member of a large family of cholesterol-dependent cytotoxins (CDCs) which its activity is dependent on cholesterol linkage (4). LLO is of signaling molecules that promotes a variety of cellular responses involving production of cytokines, modifications of immunosuppression, alterations epigenetic and influx of calcium signaling (5). This multifaceted feature of LLO is because of its ability to act as pathogen-associated molecular patterns (PAMPs) and recognition by Toll-like receptor 4 (TLR4) which is a pathogen recognition receptor (PRRs)(6). LLO improves antigen presentation in the context of MHC class I molecules and enhances T cell-mediated immune responses when mixed or conjugated to antigens (7, 8). These activities appeared to be linked to different LLO domains, however, full length of LLO has toxicity effect which associated with its pore-forming. To overcome this obstacle, a detoxified, no hemolytic form of LLO (dtLLO), with deletion of c terminal of protein sequence that are crucial for its binding to cholesterol was constructed (9), This study has demonstrated that truncated LLO plasmid could be cloned and expressed in mammalian cells without cytotoxicity to be used as a potent adjuvant in Vaccine design .

Methods

Plasmid construction. In this study, Listeria Monocytogenes (serotype 2) were purchased from Persian Type Culture Collection and bacterial genomic DNA was extracted according to the manual of high pure PCR template preparation kit (Roche, Germany). To amplify the LLO truncated form (5 to 415 aa of LLO full length protein), we amplified LLO using FLLO XhoI and R1LLO primers for fist PCR then FLLO XhoI and R2LLOECoRV primers for nested PCR (table 1). The two PCR reaction (25 μl, total) included 1ug of extracted genome in first PCR and 1ul of prior PCR product in the second PCR, 10 pmol of each primers ,2.5mM Mgcl2, 0.2mM dNTP and 1 U of Pfu DNA polymerase, and after the initial denaturation at 95 °C for 5 min, thermal program of 95 °C for 15 sec, 55 °C for 60 sec and 72 °C for 120 sec was applied for 40 cycles

and final extension at 72°C for 10 min then the second PCR product was visualized under a UV trans illuminator in 1.5% agarose gel containing safe stain DNA, and purified with PCR product purification Kit (Roche, Germany). The purified PCR product and pcDNA3.1(Invitrogen, CA, United States) were digested with XhoI and EcoRV restriction enzyme and then the PCR product was subcloned into the XhoI/ EcoRV sites of pcDNA3.1 under cytomegalovirus (CMV) promoter(figure 1) and transformed into DH5α Ecoli strain competent cell. The pcDNA3.1-LLO (pLLO) construct was confirmed by restriction enzyme study and bidirectional sequencing.

Expression of recombinant truncated LLO fragment. Expression of LLO fragment was analyzed in transfected 293T cells with the pLLO by the turbofect kit (Thermo Scientific, CA). One day after transfection, the medium was changed with complete medium to grow for 48 hours. Finally, the transfected cells were collected to detect the LLO truncated protein. The un-transfected cells served as the negative control. The collected cells were washed two times in PBS and lysed with lysis buffer (1% Nonidet P-40, 10 mg/L- phenyl methyl sulfonyl fluoride 50 mM-tris Cl, pH 8.0) for 0.5 hr on ice and then the lysates were centrifuged at 800 g for 15 minutes. The supernatants were collected to be evaluated by the western-blot method.

Western Blotting. The recombinant LLO protein, were resolved by 15% gel. The protein bands were transferred to nitrocellulose membranes (Amersham, United Kingdom). Subsequently, blocking buffer (3% non-fat skimmed plus 0.05% tween 20) was utilized for 1.5 hr at room temperature to block membrane. The membrane was incubated with 1:2000 diluted of Anti-Listeriolysin (LLO) antibody (ab43018 Abcam USA) in blocking buffer as the primary antibody for two hr at room temperature, then washed three times for five minutes with TBST (tris buffered saline containing 0.1% Tween 20). After washing, the membrane was incubated with HRP-conjugated anti rabbit against IgG (Invitrogen, Germany), diluted at 1:10000 in TBST for one hr at room temperature and then, washing was performed as mentioned before. Chemiluminescent HRP substrate (Amersham, UK) was added to the membrane for seven minutes at room temperature. In the following,

the produced light was recorded on a film.

Protocol of Immunization. Female BALB/c (H-2d) mice , 6 week old were immunized i.d in three times with two weeks interval using by 100 µg of purified plasmids pLLO, pc-NS3 (HCV Non-structural protein 3 plasmid as immunogenic plasmid control) (10) or pLLO plus pc-NS3 (co-administration) . All experiments were approved by accordance with the national experimental guidelines and ethics council (ethics number: D52/639).

Cell Proliferation Assays. Two weeks post-immunization, five mice of each group were sacrificed according animal ethics protocols and spleens were harvested aseptically. Proliferation assays were performed by using the BrdU(5-bromo-2 deoxyuridine, Roche, Germany) ELISA Roche cell proliferation kit. Briefly, The RBC lysed splenocytes were cultured as triplicates (1×10^6 cells/well) in a 24-well culture plate stimulated with 3 µg/mL of recombinant NS3 protein(rNS3). Con A (concanavalin A, 5 µg/mL; Sigma, USA) was added in positive wells to ensure the cells were healthy and un-stimulated wells were used as negative controls. After 60 hours incubation, 20 µL/well BrdU solution was added and re-incubated for 12 hours at 37°C, and finally the proliferation analysis was performed according to the roche BrdU protocol (Roche, Germany). Stimulation index was calculated by dividing the OD rNS3 stimulated wells to OD un-stimulated blank for each group.

Results

PCR amplification and construction of recombinant plasmid. The LLO PCR products were analyzed on 1% agarose gel in parallel with 1kb DNA ladder marker. The expected 1355 bp PCR product fragment was observed. The XhoI andEcoRV digested pcDNA3.1 plasmid and LLO PCR product were purified by gel extraction method (figure 2A). The LLO PCR product was ligated to pcDNA3.1 plasmid then the ligated plasmid (figure 1) was transformed into E.coli DH5α competent cell. The recombinant plasmid was confirmed by restriction enzyme analysis (figure 2B) and sequencing analysis.

Expression of the LLO in mammalian cells. During 48 hours after transfection the cells were monitored for cell cytotoxicity. No remarkable cytotoxic effect was seen comparing to control. The expression of LLO truncated gene was evaluated in transfected HEK293T cells by western blotting using Anti-Listeriolysin (LLO) antibody (Abcam USA). Mock-transfected HEK293T cell was served as the negative control. In western blot result was shown The expression of LLO fragment in transfected HEK293T lysate and no expression in the negative control HEK293T cells (figure 3).

Cell Proliferation Assay. The mice groups were immunized i.d. with 100 µg pLLO alone, 100 µg pc-NS3 alone, or 100 µg pLLO plus 100 µg pc-NS3. The immunization was performed in three times with two weeks interval. The specific proliferation was measured 2weeks following last immunization. To analyze proliferative responses, splenocytes of immunized mice were stimulated with NS3 protein while incorporation of BrdU into the stimulated splenocytes was detected by ELISA. As shown in Figure 4, NS3-specific proliferations were significantly augmented in immunized concurrently with pc-NS3 and pLLO group compared with pLLO alone or pc-NS3 alone ($P < 0.05$).

Discussion

The relatively low efficacy of DNA vaccines in inducing immune responses, especially in large animal species has impaired their practical use. Therefore considerable effort expended on improving DNA vaccine, using different adjuvants(2, 11, 12). The construction of genetic adjuvants of infectious agents are another alternative approach when used as co-delivered vector in order to modulate immune responses to DNA vaccines. Listeria Monocytogenes proteins such as Listeriolysin O (LLO)which has some CD4 and CD8 epitopes can be act as an adjuvant to initiate both CD4 + and CD8 + immune responses(13), trigger signals to activate deferent aspects of the immune systems for antitumor activity(14), reduce tolerance of "self-/tumor-associated antigens" and expand the effects of "non-self-foreign" antigens as do classical adjuvants. Additionally, purified LLO when mixed with foreign proteins and administered to mammalian cells, can transfer protein in cytosol and has been exploited for delivery to host cells both in vitro and in vivo. In general, LLO protein contains all

Table 1: Primers sequence, PCR product size and temperature melting of primers

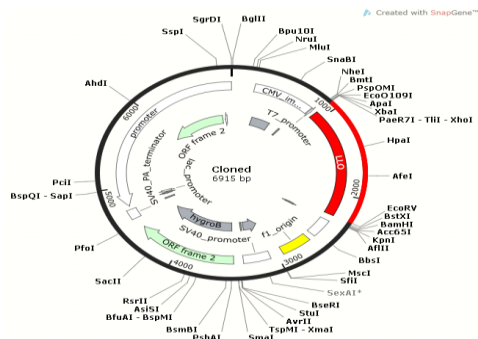| Primers			5'→3'	PCR Product size (bp)	Tm (°c)
1st	Forward	FLLOXhoI	CGC CTC GAG ACC ATG CTA GTT TTT ATT ACA C	1333	60
	Reverse 1	R1LLO	GTG GTG GTG GTG ATC ATA ATT TAC TTC ATC C		
2nd	Forward	FLLOXhoI	CGC CTC GAG ACC ATG CTA GTT TTT ATT ACA C	1355	60
	Reverse 2	R2LLOECoRV	ATA G A TA T C ATTA ATG ATG GTG GTG GTG GTG ATC		

Figure 1. Schematic presentation of recombinant pLLO.

Figure 3. Analysis of truncated LLO protein Expression by Western Blotting With LLO Antibody in 293 Cells

key factors of an ideal adjuvant but full length of LLO protein has a strong apoptogenic aspect that induces death in activated T cells and inhibit their responses (15). Moreover, this protein causes cell death and other marked negative effects in dendritic cells that inhibits antigen presentation (16, 17). Previous study was shown that high innate and adaptive immunogenic effects of LLO protein is induced by a separate portion of its cytotoxic activity domain(13). Currently, the crystal structure study of LLO has been shown which rod-like whole LLO protein has four distinguished domains, referred to as D1 to D4 detail(18). Among of all domains, D4 with
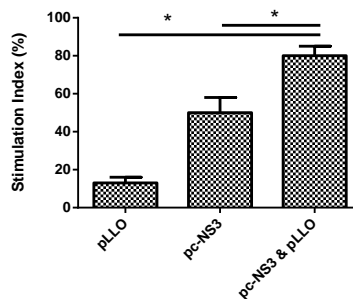

Figure 4. Specific cell proliferation assay: Two weeks after last immunization, The Splenocytes of three immunized mice groups were resuspended in RPMI 1640 containing 10% FBS and the rNS3-specific proliferation following stimulation with 3 μg/ml recombinant NS3 was determined by cell proliferation ELISA, BrdU colorimetric kit. The mean stimulation index values are shown for each group. (* indicates statistical significance, P value < 0.05).
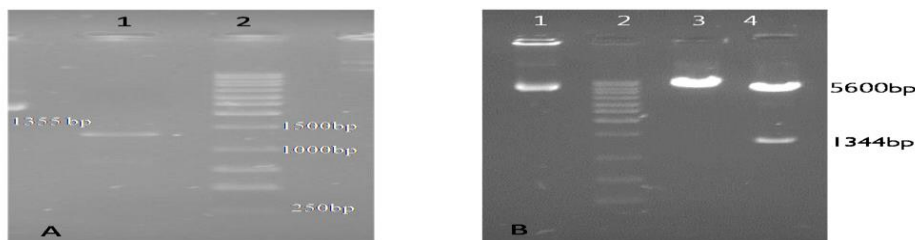

Figure 2. Purified PCR amplification of LLO and restriction analysis for confirmation of pLLO. A) Lane 1: Purified PCR Product of LLO truncated fragment(5 to 415 aa of LLO full length protein) which is 1355bp in size, Lane 2: 1kb DNA ladder marker.B) Lane 1: pcDNA3.1 plasmid, Lane 2: 1kb DNA ladder marker, Lane 3: pcDNA3.1-LLO(pLLO) digested by EcoRV (linear pLLO plasmid), Lane 4: pLLO digested by XhoI and EcoRV restriction enzymes.

conserved structural motif in the C terminal region, is responsible to membrane binding and cytotoxic activity(19). Previous study was shown that truncated form of LLO (including domains 1-3), lacking domain 4, is the main region for the induction of proinflammatory cytokines and innate immune response(20). Carrero et al. studies have shown that conversion of tryptophan to alanine at residues 491 and 492 led to a reduction in cytolytic and hemolytic effects to 95% without a significant decrease in the binding, processing and presentation of immunodominant peptides to T cells. Thus it can be concluded that LLO immunogenicity is independent of cytotoxic activities (13).

Conclusion

Our result indicated that pLLO gene fragment encoding only domains 1-3 of whole LLO gene was constructed successfully and cloning accuracy was confirmed by restriction enzyme study and sequencing. Then the ability of this plasmid to express on mammalian cell line was confirmed by western blotting assay. The NS3 specific proliferation results, following mice immunization shown in figure 4 demonstrates the significant high proliferation response in pc-NS3/pLLO group compared to pc-NS3 group. According to this finding, pLLO as co-administration plasmid could be a potent genetic adjuvant to enhance immune response of DNA vaccine.

Conflicts of Interest

There is no conflict of interest.

Acknowledgment

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