Original Article

Targeting MCF-7 Cell Line by Listeriolysin O Pore Forming Toxin Fusion with AHNP Targeted Peptide

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Abstract

Background: Tumor-targeting peptides are attracting subjects in cancer therapy. These peptides, which are widely studied, deliver therapeutic agents to the specific sites of tumors. In this study, we produced a new form of recombinant listeriolysin O (LLO) with genetically fused Anti-HER2/neu peptide (AHNP) sequence adding to its C-terminal end. The aim of the study was to engineer this pore-forming toxin to make it much more specific to tumor cells. **Materials and Method and Results:** Two forms of the toxin (with and without peptide) were subcloned into a bacterial expression plasmid. Subcloning was performed using a polymerase chain reaction (PCR) product as a megaprimer in a quick-change PCR to introduce the whole insert gene into the expression plasmid. After expression of two recombinant forms of LLO in BL21 DE3 cells, purification was performed using Ni-NTA affinity column. MDA-MB-231 and MCF-7 cell lines (as negative and positive controls, respectively) were treated with both LLO toxins to evaluate their cytotoxicity and specificity. The IC_{50} of LLO on MDA-MB-231 and MCF-7 cells was 21 and 5 ng/ml, respectively. In addition, IC_{50} for the fusion AHNP-LLO toxin was 140 and 60 ng/ml, respectively. It was found that the cytotoxicity of the new engineered AHNP-LLO toxin has decreased by about 9x compared to the wild-type toxin and the specificity of the AHNP-LLO toxin has been also reduced. **Conclusions:** Results show that the C-terminal of the LLO should not be modified and it seems that N-terminal of the toxin should be preferred for engineering and adding peptide modules.

Keywords: *Breast cancer, listeriolysin O, quick-change polymerase chain reaction, tumor-targeting peptide*

Introduction

Finding a new treatment for cancer is one of the priorities of many research laboratories. There are many treatments for cancer, such as chemotherapy and radiation therapy. Cells with higher growth rate are better targets for treatment in chemotherapy, and this is the Achilles' heel of this therapeutic approach and low selectivity in targeting malignant cells. For this reason, therapeutic approaches need to be determined with higher selectivity and specificity. Other traditional chemotherapy problems are the resistance of tumor cells, partly due to the high doses of chemical drugs.[1,2] Biological treatments based on the monoclonal antibodies, enzymes, and toxins have created new ways of dealing with the specificity issue in cancer therapy. Especially, combination therapy using chemotherapeutics and biological molecules could be selective to make the therapeutic approaches more and more specific.[3,4] The need to transfer more doses of drug to the site of tumors and reduce the side effects of the therapy introduced antibodies as the first candidates for tumor-selective targeting. However, the large size of antibody, having a costly and productive production process, low tissue penetration, and liver and reticuloendothelial system uptake are among the drawbacks of antibodies to be used as specificity making element in fusion protein therapeutics. The success and the problems of using antibodies in therapeutic protein conjugates and also engineering strategies to overcome their shortcomings have been reviewed elsewhere.[5-7] However, several therapeutic antibodies and other chimeric molecules based on the antibodies have been approved so far, and many more exist in preclinical and clinical trials. Using targeting peptides is another tool to make specificity in a toxic protein molecule, which should be a tumor therapeutic. Small size, low cost

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of production, and high tissue penetration of peptides have led to extensive research in discovery and utilizing peptides in making specificity in killing moieties. Tumor-targeting peptides and their efficiency evaluation are also reviewed elsewhere.[8-11] Several researchers have attempted to make specific therapeutics or diagnostics with tumor-targeting peptides.[12-15] Toxins are attached to a variety of cells by their catalytic domain and cause them to die. For the specific targeting of the catalytic domain of toxins, some engineering should be done on toxic molecules. Listeriolysin O (LLO) is an essential virulence factor for *listeria monocytogenes* that promotes bacterial escape from phagosomal space into the cytoplasm. LLO with molecular weight of 58 kDa belongs to the family of cholesterol-dependent cytolysins (CDCs). LLO molecules insert into the eukaryotic cell membrane when attached to cholesterol-binding receptor, creating pores of differing sizes. This ability of LLO to form pores in eukaryotic membrane is the basis of its cytolytic activity.[16-18] Although the potential of pore-forming toxins to deliver a broad range of molecules to the target cells has been studied by many groups, due to the lack of specificity in binding to the target membranes, they are not still a real candidate for cancer therapy. The only engineering strategy for a specific targeting of tumor cells with LLO molecule has been the fusion of an antibody fragment against the tumor-antigen Lewis Y to the toxin molecule.[19] This study presents a new approach for the specificization of the LLO molecule for a particular type of cancer cell. Our goal was to determine whether there is a possibility to have more specific pore-forming toxin using targeting peptides which target some kinds of cells with more selectivity. This has been performed by fusing a targeting peptide sequence to the C-terminal of LLO molecule.

Materials and Methods

Escherichia coli DH5α and BL21 (DE3) strains were prepared from Pasteur Institute, Iran. In addition, DNA gel recovery kits, T4 DNA ligase, plasmid extraction kit and DpnI enzyme were provided from Sinaclon Co. (Iran) and Thermo Fisher Scientific Co., respectively. Other chemicals were prepared from molecular biology grade providers (Sigma and Merck Co., Germany).

Recombinant listeriolysin O expression and purification

pPSG-IBA35-LLO expression construct was a gift from Dr. R. Stachowiak. *E. coli* DH5α cells were cultured and chemically competented by $CaCl_2$ conventional method. Competent cells were transformed with 30 ng of expression construct, and one of the transformants was used for the amplification of the construct. The colony was cultured in a 5-ml tube containing 50 μg/mL of ampicillin, at 37°C overnight. Recombinant plasmid has been extracted by column method (QIAprep Spin Miniprep Kit, Qiagen™). In another transformation experiment, the same amount of expression construct was used to transform BL21 DE3 competent cells.

A number of colonies of BL21 DE3 transformants were cultured in Lysogeny broth (LB) medium containing 50 μg/mL of ampicillin, at 37°C, induced by addition of 0.1-mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) when $OD^{600\ nm}$ reached approximately 0.7. Expression of the recombinant LLO protein has been checked in colonies by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Fresh aliquots of recombinant LLO protein-expressing clones were used for inoculation of another 100-mL LB medium. Clones were cultured until the $OD^{600\ nm}$ of approximately 0.7, and expression was induced by adding 0.1-mM IPTG. After overnight induction, the bacteria have been harvested by centrifugation at 6000×g for 5 min. Bacteria pellet was bead beated for 20 s and 15 times with a benchtop vortex with maximum shaking speed (2000 rpm) with 30-s intervals incubating on ice. Concentration of ingredients lysis buffer contained Tris, 100 mM, pH = 8.0; NaCl, 300 mM; 2 ME, 5 mM; glycerol, 10%. The soluble fraction was centrifuged and the supernatant was loaded onto a column filled with Ni-NTA agarose resin (Qiagen Inc.) to purify the recombinant His-tagged LLO protein. Equilibration and washing buffers of the chromatography were the same as lysis buffer. Elution buffer was also the same as lysis buffer except for the 300-mM imidazole. Eluted fractions were analyzed by 10% SDS-PAGE for the presence of recombinant LLO protein, and fractions containing recombinant protein were dialyzed against the same buffer as lysis buffer with 50-mM NaCl.

Epitope tagging

A set of primers was ordered to amplify the LLO gene containing the 33 extra nucleotides encoding AHNP peptide targeting Her2 antigen on the breast cancer cells. Forward primer was set to bind to + 100-nucleotide upstream of the LLO-coding sequence in the expression vector pPSG-IBA35. Reverse primer was composed of three regions; a 5' region complementary to the pPSG-IBA35 vector backbone after the cloning site, a middle part of primer encoding AHNP targeting peptide, and a 25 bp region at 3'end complementary to the last nucleotides of LLO gene. Polymerase chain reaction (PCR) of the LLO gene using these primers has led to the production of chimeric LLO gene [Figure 1]. This PCR product was gel purified and used in a quick-change PCR step to synthesize the remaining parts of the pPSG-IBA35 vector. Product of the quick-change step was cleaned up, digested with DpnI, and transformed into *E. coli* DH5α competent cells. The next steps were the same as native LLO protein expression and purification.

Hemolysis assay

LLO hemolytic activity was expressed as hemolytic unit (HU) per milliliter of solution or per milligram of total protein. The HU is the smallest amount of toxin that

liberates half the hemoglobin (50% lysis) from a suspension of erythrocytes.

The hemolytic activity of LLO and LLO-AHNP was checked out with red blood cell (RBC) hemolysis assay. RBC was washed three times with phosphate-buffered saline (PBS) and resuspended in PBS with 2-mM DTT. In $OD^{600\ nm}$ should be absorbed between 1 and 1.5. Serial 1/2 dilutions of toxins were prepared in PBS. A 20-µl aliquot of each toxin was added to a total volume of 400-µl RBC suspension, and after 10 min of incubation at 37°C, absorbance of the sample was measured at 600 nm. The percentage of hemolysis was calculated as the following formula: Hemolysis (%) = $(A_{max}\text{-}A_{test})/(A_{max}\text{-}A_{min}) \times 100$.

Cell culture and cytotoxicity assay

The MCF7 (breast carcinoma) and MDA-MB-231 (breast carcinoma) cell lines were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS). For all treatments, cells with over than 95% viability as assessed by trypan blue dye exclusion assay were used. The MCF7 and MDA-MB-231 cells at 10^6 cell/ml dilution were prepared after three steps of washing with PBS to remove any FBS in the cell suspension. Serial 1/2 dilutions of toxins were prepared in PBS. A 50 µl of each LLO and LLO-AHNP toxins was added to 50-µl aliquots of the two cell lines, and the viability of the cells was analyzed after 5-min incubation at 37°C.[20] Each experiment was performed in triplicate. IC_{50} of the effects of each toxin on the two cell lines was calculated by sigmoid regression of viability data against the toxin concentrations.

Results

Inserted AHNP peptide-coding sequence was verified by DNA sequencing (data not shown). Expression constructs were transformed into BL21 DE3 cells. Recombinant LLO and LLO-AHNP proteins were obtained from induced *E. coli* cells (molecular weight: 58 and 60 kDa, respectively) as single polypeptide bands on the SDS-PAGE at the end of the purification procedure [Figure 2]. Protein concentration was measured by Bradford procedure. Purified wild-type LLO and LLO-AHNP protein concentrations were 425 and 163 µg/ml, respectively.

Hemolytic activity

Hemolysis assay was performed to show pore-forming activity of wild-type and AHNP-fused LLO toxins [Figure 3]. The result demonstrates that LLO and LLO-AHNP conserved the hemolytic activity approximately at the same extent. IC_{50} values for the hemolytic activity of wild-type LLO and AHNP-LLO were calculated as 258 ± 97 ng/ml and 147 ± 11 ng/ml, $P = 0.25$, showing that there is no significant difference between two forms of toxin.

Cytotoxicity of listeriolysin O-AHNP toxin

Cytotoxic effect of the wild-type LLO and AHNP-peptide-fused LLO toxins were examined on two cell lines including MCF-7 and MDA-MB-231 using serial dilutions of toxins in a trypan blue dye exclusion assay as described in material and methods section, and data analysis was carried out with GraphPad Prism 6 Demo. IC_{50} of each toxin was calculated using sigmoidal regression on the dose–response curve of the toxin. As seen in Table 1, adding a AHNP-peptide to the C-terminal of LLO toxin has attenuated the cytotoxic effect of the toxin by about 10-fold. Since MCF-7 cell line expresses Her2 antigen on its, it has been selected. Both types of the toxin have shown to be more potent on MCF-7 cells compared with MDA-MB-231 cell (IC_{50} values of 3–5 times less) [Figure 4].

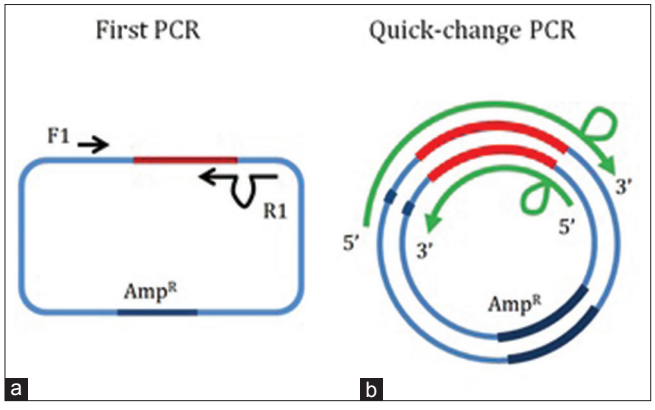

Figure 1: Design of epitope-tagging protocol. For introducing the AHNP peptide-coding sequence into pPSG-IBA35-listeriolysin O expression vector, a reverse primer was designed containing two arms complementary to the 3'-end of the listeriolysin O gene and the vector backbone downstream of the gene and an intervening part coding the AHNP peptide. A simple polymerase chain reaction with this primer and a forward primer on the upstream of the gene has led to the polymerase chain reaction product (a), which was used in the second step as the megaprimer (1.65 kbp) in quick-change polymerase chain reaction (b)

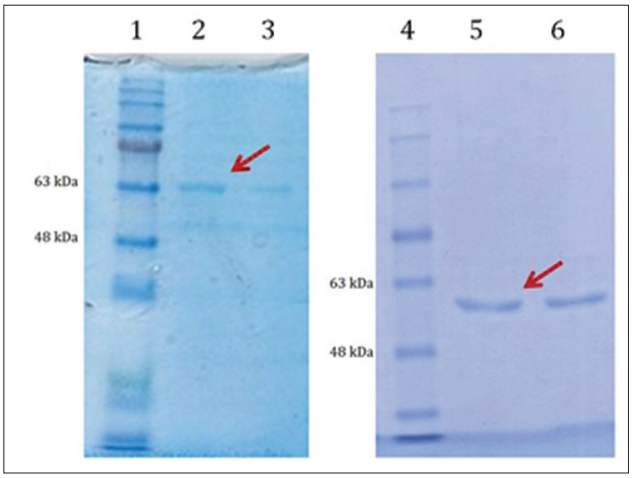

Figure 2: Purification of the two forms of listeriolysin O. Two forms of the toxin (wild-type and fusion with AHNP targeting peptide) were purified using Ni-NTA resin. As shown with arrow in lanes 2 and 5, both toxins were purified as single bands

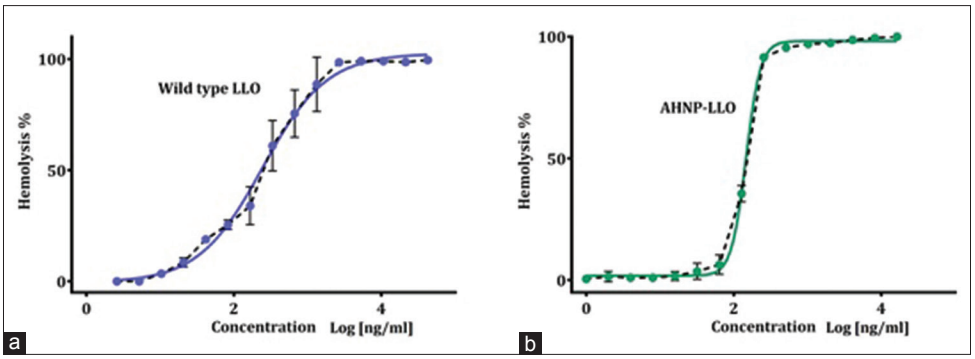

Figure 3: Hemolysis activity of wild-type (a) and engineered (b) listeriolysin O. Serial dilutions of the toxins were used to lyse human red blood cells. Sigmoidal fit was performed to find the $I_{cs}0$ of each toxin. All analysis was carried out in GraphPad Prism 6

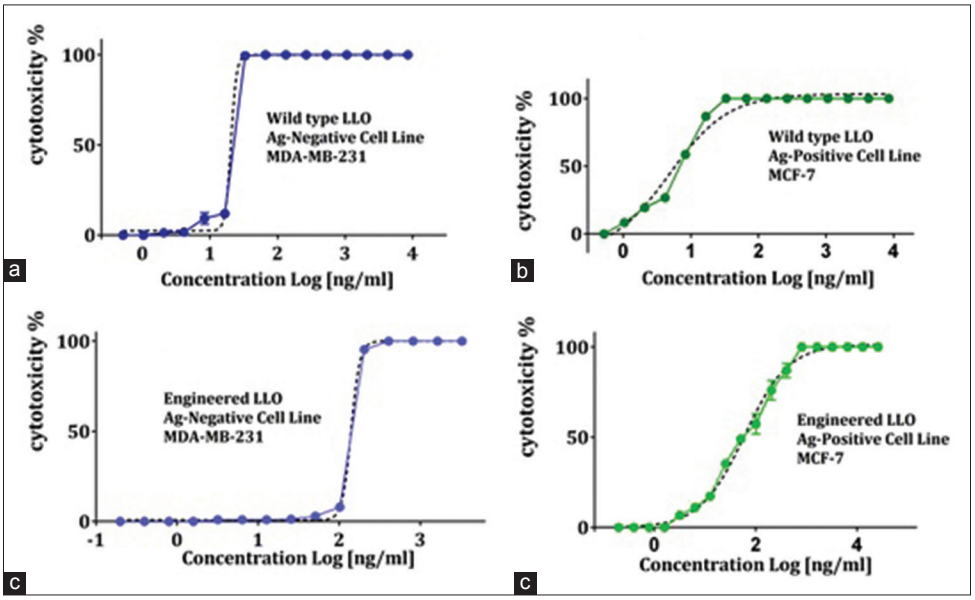

Figure 4: Cytotoxicity of toxins on antigen-negative and antigen-positive cell lines. Antigen-negative (a, c) and antigen-positive (b, d) cell lines were cultured and exposed to serial dilutions of the two forms of listeriolysin O (wild-type and engineered). Trypan blue staining was performed to distinguish between live cells and dead ones in each dilution. The sigmoidal fit was performed to find the $I_{cs}0$ of each toxin. All analysis was carried out in GraphPad Prism 6

Table 1: IC_{50} values of toxins cytotoxic effects on two cell lines

Cell line type	MDA-MB-231 (Ag^- cell line)	MCF-7 (Ag^+ cell line)
Wild-type LLO	20.49	4.85
AHNP-LLO	140.02	59.84

Discussion

Targeting peptides in many studies have been used to enhance the specificity in biological macromolecules such as toxins and enzymes.[10] Targeted drug delivery shows unique characteristics of tumor cells for the aims of enhanced specificity of treatment and reduces their side effects.[21] Bacterial toxins can efficiently kill the cells, and thus, many toxins have been studied as potential anticancer agents.[22]

The recent discoveries of molecular biology provide essential information on the enzymes and carrier proteins. The LLO toxin gene was cloned into pUC57 vector and send to us. Instead of cutting the gene with restrictive enzymes and entering into the pET system, we subjected the gene to the pPsg expression vector using a quick-change PCR. By eliminating restrictive enzymes, we decreased the costs and fixed the enzyme-related problems including preserving them as well as reducing their activity over a long period of time. The results of this cloning method were in accordance with the study of Glomski *et al*., in 2002, who entered the LLO toxin gene into the pET29b expression vector by means of PCR and megaprimer production using the QuikChange II site-directed mutagenesis kit. After production and purification of the recombinant LLO protein on SDS-PAGE, a single band was found in the range of 58 kDa.[23] In a study by Camilla *et al*., LLO toxin gene was introduced into *E. coli* bacterium by the PET-3a expression plasmid and recombinant toxin was produced and purified.[24] In the present study, the purification steps are approximately the same as the methods taken by

Camila.[24] In our study, the final amount of the purified protein for LLO and LLO-AHNP was 425 μg/L and 163 μg/L, respectively. The evaluation results of the hemolytic activity of LLO recombinant toxin correspond to the observations gained by Geoffroy *et al*. The $I_{C5}0$ value, the concentration in which 50% of the erythrocytes lyse, was reported, respectively, 24 ng/ml for LLO and 36 ng/ml for LLO-AHNP in the current study. Since the more hemolysis strength of LLO over LLO-AHNP observed, these toxins are likely to have the binding site in their C-terminal sides, as it is suggested in Robin's work.[25] Furthermore, the presence of the AHNP-targeting peptide in the C-terminal toxin interferes with the binding of toxin to the membrane of target cells. The results obtained from the study by Camilla *et al*. showed that the size of the toxin on the SDS-PAGE gel, observed as a single band, was 56 kDa. In a study by Robin *et al*. in 2005, three different expression structures for the production of LLO toxin were compared. In the first structure, using the intein fusion system, they could not do the purification by column. In the second structure, by putting His-Tag in the N-terminal of the toxin gene, 350 μg/L of protein was purified, and in the third structure, 250 μg/L protein was purified by placing His-Tag in the C-terminal of the toxin gene. In this study, it was indicated that setting His-Tag in the C-terminal of the toxin leads to the reduction of the protein expression, which its cause is still unknown.[17] In this work, we removed the signal sequence of the protein and then sent it to synthesis.

In this study, we examined the effects of LLO recombinant toxin and LLO-AHNP toxin on MCF-7 and MDA-MB-231 cell lines of breast cancer, and it resulted in cytotoxic activity of recombinant toxin against MCF-7 and MDA-MB-231 cell lines, which is dependent on protein concentration. According to the results, the cytotoxicity effects of LLO-AHNP is less than LLO toxin and as it was explained, that is probably due to the placement of the AHNP peptide in the C-terminal toxin and intervention in the bonding domain of toxin. To determine IC_{50} value and cell viability rate, we used the trypan blue test. Having determined the average IC_{50} obtained from the effects of both natural and engineered toxins on the MDA-MB-231 and MCF-7 cell lines, the results were as follows:

1. The lethal effect of natural toxin on MDA-MB-231 cell line as a negative control was four times higher than MCF-7 cell line as a positive control, indicating a negative receptor effect on the toxin lethal properties
2. The effect of engineered toxin on the MCF-7 cell line is about two times lower than the MDA-MB-231 cell line, which shows that the AHNP receptor has no effect on the C-terminal side of LLO toxin
3. Comparing the activities of both natural and engineered toxins in MDA-MB-231 cell as a negative control indicates that the engineered toxin (LLO-AHNP) has more than 9 times activity loss in an AHNP receptor-free cell. In other words, the addition of the AHNP peptide has resulted in the toxin activity loss of more than seven times in the negative AHNP-R cell.

Conclusions

Our results indicated that the C-terminal of the LLO should not be modified and it seems that N-terminal of the toxin should be preferred for engineering and the addition of peptide modules. At the end, this study is fundamental and further studies should be done on this subject.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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