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Towards prostate cancer gene therapy: Development of a chlorotoxin-targeted nanovector for toxic (melittin) gene delivery

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ABSTRACT

Prostate cancer is the second leading cause of death due to cancer in men. Owing to shortcomings in the current treatments, other therapies are being considered. Toxic gene delivery is one of the most effective methods for cancer therapy. Cationic polymers are able to form stable nanoparticles via interaction with nucleic acids electrostatically. Branched polyethylenimine that contains amine groups has notable buffering capacity and the ability to escape from endosome through the proton sponge effect. However, the cytotoxicity of this polymer is high, and modification is one of the applicable strategies to overcome this problem. In this study, PEI was targeted with chlorotoxin (CTX) via *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) cross-linker. CTX can bind specifically to matrix metalloproteinase-2 that is overexpressed in certain cancers. Melittin as the major component of bee venom has been reported to have anti-cancer activity. This was thus selected to deliver to PC3 cell line. Flow cytometry analysis revealed that transfection efficiency of targeted nanoparticles is significantly higher compared to non-targeted nanoparticles. Targeted nanoparticles carrying the melittin gene also decreased cell viability of PC3 cells significantly while no toxic effects were observed on NIH3T3 cell line. Therefore, CTX-targeted nanoparticles carrying the melittin gene could serve as an appropriate gene delivery system for prostate and other MMP-2 positive cancer cells.

Keywords:
Prostate cancer
Polyethylenimine
Chlorotoxin
SPDP
Melittin
PC3 cell line

1. Introduction

Prostate cancer is the second leading cause of cancer death in men. About one in 39 die of prostate cancer and about one man in seven is diagnosed with it (American Cancer Society, 2016). Depending on the situation, there are different treatment options for men with prostate cancer such as surgery, radiation therapy, hormone therapy, chemotherapy, and cryotherapy (Park et al., 2011). However, an ongoing endeavour to improve therapy and reduce the death rate by different research groups brings new hopes. Gene therapy is a potential approach to delivering effective genes to target cells (Soltani et al., 2013). For cancer gene therapy, exogenous genes are transferred to malignant human cells in order to cause cell death (Harrington et al., 2001). Since naked genes are not able to cross cell membranes and other barriers, an appropriate vector should be designed to transfer the genes efficiently and protect it from degradation in the blood stream (Li et al., 2015). Non-viral vectors are appropriate vehicles for gene delivery because they have low cytotoxicity and good biocompatibility compared to viral vectors (Sieradzki et al., 2014). However, low transferring efficiency is one of the most important disadvantages of these vectors (Pezzoli and Candiani, 2013). Cationic polymers are able to interact electrostatically with negatively charged nucleic acids in order to form stable nanoparticles. Thus, these polymers have been extensively used for gene delivery (Grigsby and Leong, 2010). Polyethylenimine is one of the first cationic polymers that has been utilised for gene delivery since 1995. Because of high DNA binding efficiency and the ability to escape from endosome through the proton sponge effect, PEI has been considered a gold standard for polymer-based gene vectors (He et al., 2010). Many reports have demonstrated that linear and branched PEIs with a molecular weight of 25 kDa have high transfection efficiency (Huh et al., 2007).

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Branched polyethylenimine contains primary, secondary, and tertiary amines and notable buffering capacity. So transfection efficiency and also cytotoxicity of this polymer increases compared to the linear PEI (Pathak et al., 2009). The modification of the polymer is the most attractive strategy to overcome the cytotoxicity effect. The amino groups of PEI can be modified by targeting agents (He et al., 2010). Molecules that have specific receptors on target cells have been used as targeting agents (Wong and Jameson, 2012). Chlorotoxin is a 36-amino acid peptide with four disulfide bonds. It was originally isolated from *Leiurus quinquestriatus* (scorpion) venom (Ojeda et al., 2015). It was shown that Chlorotoxin (CTX) can bind specifically to matrix metalloproteinase-2 (MMP-2) containing complex and inhibit the enzymatic activity of it in vitro. MMP-2 is overexpressed in certain cancers and has a key role in the tumour metastasis process (Deshane et al., 2003). When CTX interacts with MMP-2 containing complex receptors, cellular internalization of nanoparticles occurs (Veiseh et al., 2011, 2009). CTX has been shown to specifically recognize different tumours, including the brain, prostate, skin, sarcoma, intestinal, and colorectal cancers. So, it can be used as a cancer cell targeting ligand (Fu et al., 2012).

In addition to designing an appropriate vector, the selection of the proper gene to target tumour cells and to kill them is the key (Shalev et al., 2001). Melittin is the main component of bee venom contains 26 amino acid residues. Previous studies have reported that melittin has an anti-cancer activity against leukaemia, renal, lung, liver, prostate, bladder, and mammary cancer cells (Heinen and Gorini da Veiga, 2011). Park et al. reported that in prostate cancer, apoptosis was induced via down-regulation of NF-κB by melittin, so cancer cell growth can be inhibited by this peptide. Melittin can also block chemotherapeutic resistance in the advanced prostate cancer (Park et al., 2011). Because cellular lytic activity of melittin is nonspecific, nanoparticles carrying the melittin gene can be used as anti-tumour agents (Gajski and Garaj-vrhovac, 2013).

In this study, a 25 kDa branched PEI polymer modified with chlorotoxin targeting peptide by SPDP cross-linker is introduced as an efficiently targeted nanocarrier. This can bind to prostate cancer cells specifically and deliver the gene encoding cytotoxic peptide melittin to them.

2. Materials and method

2.1. Materials

Escherichia coli strains were obtained from Invitrogen. Vectors used were pIRES2-EGFP (clontech, USA) and pET28a (Novagen, USA). Restriction enzymes, T4 DNA ligase and DNAseI, Plasmid extraction kit and *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), isopropyl β-D-1-thiogalactopyranoside (IPTG (were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ni-NTA was purchased from Qiagen (Hilden, Germany). Branched PEI (25 kDa), 2,4,6-trinitrobenzenesulfonic acid (TNBS), phenylmethylsulfonyl fluoride (PMSF), Fluorescein isothiocyanate (FITC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Munich, Germany). Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI) and fetal bovine serum (FBS) were purchased from GIBCO (Gaithersburg, MD), and *N*-(2-hydroxythyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) and Ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Darmstadt, Germany).

2.2. Cell lines

PC3 (PC-3) human prostate cancer cell line (metalloproteinase 2 positive) (ATCC CRL-1435) and NIH3T3 fibroblast cell line (metalloproteinase 2 negative) (ATCC CRL-1658) were obtained from Pasteur Institute of Iran and cultivated in RPMI 1640 and DMEM respectively supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Methods

2.3.1. Cloning

2.3.1.1. Cloning of melittin gene. The gene encoding Melittin containing Kozak consensus sequence on 5′-end, was synthesized by TAG Copenhagen A/S (Denmark) and double digested with *Nhe*I and *Bam*HI and ligated into digested/dephosphorylated pIRES2-EGFP expression vector. The cloning was confirmed using DNA sequencing by Macrogen (South Korea).

2.3.1.2. Cloning of chlorotoxin (CTX) gene. The gene encoding His_6 tag-CTX was designed and synthesized by TAG Copenhagen A/S (Denmark) with N-terminal *Nco*I and C-terminal *Hin*dIII restriction sites. Then gene of interest was double digested with *Nco*I/*Hin*dIII and ligated into digested/dephosphorylated pET28a expression vector. The sub-cloning was confirmed using DNA sequencing by Macrogen (South Korea).

2.3.2. Expression and purification of chlorotoxin

To optimize expression level and obtain a higher level of soluble peptide, the expression plasmid containing CTX was transformed into two *E. coli* strains BL21and Rosetta2 (DE3). A single colony of BL21 and Rosetta2 carrying the desired construct was inoculated into 5 ml Terrific broth (TB) medium (12 g/l tryptone, 24 g/l yeast extract, 0.4% (v/v) glycerol and 0.17 M KH_2PO_4, 0.72 M K_2HPO_4) containing 50 μg/ml kanamycin and grown overnight at 37 °C. Then 50 μl of culture was used to inoculate 10 ml of fresh TB medium containing 50 μg/ml kanamycin and the cells were incubated at 37 °C with vigorous shaking until the OD_{600} reached to 0.6 and 0.9. The peptide expression was induced by IPTG with a final concentration of 0.1 and 1 mM at 37, 22 and 18 °C for 4, 12 and 18 h, respectively. Next, the cells were harvested by centrifugation (5000*g*, 4 °C for 20 min) and the cell pellet was resuspended in lysis buffer (50 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 8). Then the cells were lysed by sonication on ice. The cell lysate was centrifuged at 12000*g* at 4 °C for 20 min. The samples of both insoluble (precipitation) and soluble fractions (supernatant) were placed on 17.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels, and protein bands were visualized by Coomassie Brilliant Blue staining after SDS-PAGE separation.

Finally, the insoluble fraction was solubilized in 6 different solubilization buffers including: (S_1) 50 mM Tris–HCl, 5 mM EDTA, 1 mM PMSF, 6 M β-mercaptoethanol, and 8 M urea, pH 8.5, (S_2) 50 mM Tris–HCl, 5 mM EDTA, 1 mM PMSF, and 6 M GdnHCl, pH 8.5, (S_3) 50 mM Tris–HCl, 5 mM EDTA, 1 mM PMSF, and 2 M urea, pH 12, (S_4) 50 mM Tris–HCl, 5% glycerol, 0.1 mM EDTA, 50 mM NaCl, and 0.4% sarkosyl, pH 7.9, (S_5) 50 mM Tris–HCl, 5 mM EDTA, 1 mM PMSF, 6 M *n*-propanol, and 2 M urea, pH 8.5, (S_6) 50 mM Tris–HCl, 5 mM EDTA, 1 mM PMSF, 6 M β-mercaptoethanol and 2 M urea, pH 8.5 (Singh et al., 2015). To determine the optimal conditions for the solubilization of inclusion bodies, soluble fractions of samples (supernatant) were placed on 17.5% SDS-PAGE gels, and protein bands were visualized by Coomassie Brilliant Blue staining. According to the results, the best solubilization buffer was selected for purification of the CTX peptide.

For purification of CTX, the culture and induction was done in large volume (500 ml). Following centrifugation, the pellet was resuspended in lysis buffer (50 mM Tris–HCl, 5 mM EDTA, and 1 mM PMSF, pH 8.5) and lysed using sonication. After further centrifugation (20,000*g*, at 4 °C for 20 min) the pellet was solubilized in the selected buffer. Afterward, the solubilized peptide was loaded onto a Ni-NTA column. Refolding of the bound peptide is performed using a linear 6–0 M urea gradient. Finally, the peptide was eluted with elution buffer (50 mM Tris, 500 mM NaCl, and 250 mM imidazole, pH 8) and the eluted fractions were analysis by SDS-PAGE and western blot.