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Full Length Research Paper

Using the DNA-chitosan complex as vaccine nanoparticle in cancer

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Abstract

Development of effective cancer vaccine targeting HER2 can improve HER2-specific immunity in cancer cells with HER2 overexpression. In recent years, nanocarriers for gene delivery become a potential strategy in cancer treatment. Cationic polymers have been used as gene carriers. Chitosan has been used with some advances such as biocompatibility, low immunogenicity, minimal cytotoxicity and interacts with negatively charged DNA resulting in nanoparticles of various sizes. The aim of this study was to develop DNA vaccine nanoparticle and to investigate the immune response in rabbit. Human epidermal growth factor type 2 receptor extracellular domain (HER2 EDC) was cloned into the cytomegalovirus promoter-based constitutive expression vector pcDNA.31, resulting in a DNA vaccine vector named pcDNA3.1-EDC. This vector was used to formulate plasmid DNA-chitosan nanoparticle (PDCN) complex using coacervation method. Characterization of these nanoparticles as the morphology, size and zeta potential was observed with sizes of 209 nm, unform size distribution, and spherical shape, and encapsulation efficiency about 57%. This study also investigated the efficiency of plasmid DNA-chitosan nanoparticle complex, showing a higher level of HER2 protein in rabbit serum when PDCN were injected intramuscularly as compared with control and naked DNA. Besides that, Immunoglobulin G (IgG) results showed that, nanochitosan solution is nontoxic and can be used as a good gene delivery system for cancer therapy.

Keywords: HER2, plasmid DNA vaccine, chitosan nanoparticle, immune respond

INTRODUCTION

Human epidermal growth factor receptor 2 (HER2) is a 185- kDa transmembrane protein encoded by HER2 or the c-erbB-2 proto-oncogene on chromosome 17q21. It is a member of the HER family of transmembrane receptors that are vital factors in regulating of many different cellular processes such as proliferation, differentiation, migration, and survival (1). Amplification or overexpression of HER2 protein have been indicated a popular case in the development of many tumors including 20-30% of invasive breast cancers, 54-100% of colorectal cancers, 25% of ovarian cancers, 17-82% of pancreatic cancers and 34% of prostate cancers (2-7). The HER2 protein has three domains: a 105-kDa extracellular domain (EDC), a short transmembrane region, and an intracellular tyrosine kinase domain. The ECD of HER2 can be cleaved from the surface of cancer cells and released into the serum. This ECD can be

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measured with enzyme-linked immunosorbent assays (ELISAs) without any significant cross-reactivity with other members of the HER receptor family (8).

Over two past decades, monoclonal antibodies (mAbs) targeting HER2 have been developed. Trastuzumab is a humanized mAb against the extracellular domain of HER2 has been approved by the FDA in 1998 for metastatic HER2 overexpressing breast cancer (9). However, a major limit of immunotherapy with trastuzumab is the development of drug resistance when it was used in treating the metastatic setting (10, 11). The presence of cellular and/or humoral immune responses against HER2 in patients with HER2 overexpressing tumors have shown in previous studies (12-14). Such immune responses can be associated with slower tumor development at the early stages of the disease (15). Therefore, the development of various anti-HER2 vaccine strategies has been motivated. Moreover, the field of DNA vaccine has attracted the interest of scientists in early 1990s with low effect to human immune responses (16). The previous pre-clinical studies used DNA vaccines alone injected intramuscularly (17,18). Effective delivery systems are required to deliver DNA to antigen-presenting cells and to specific organs. In recent years, various nano-sized drug or gene carriers such as micelles, nanoparticles (NPs), polymer–drug conjugates and stealth liposomes have been investigated in order to minimize side effects of anticancer drugs and enhance the antitumoral drug efficacy in cancer therapy (19,20). Chitosan (CS) is a natural biodegradable polysaccharide derived from chitin and it has a positive charge and is mucoadhesive (21). Moreover, chitosan is biocompatible with living tissues and it does not cause allergic reactions and rejection. It also breaks down slowly to products which are completely absorbed by the human body. Therefore, it is used extensively in drug and gene delivery applications (22-26). The aim of study was to develope DNA vaccine delivery using chitosan to demonstrate the immune respond in rabbit via evaluation of serum HER2 EDC protein and IgG levels between the plasmid DNA with nanochitosan-encapsulated plasmid DNA.

MATERIALS AND METHODS

Materials

High molecular weight chitosan (400 kDa) was purchased from Sigma-Aldrich (St. Louis, Missouri). Escherichia coli DH5α (E. coli DH5α) was used as the host for plasmid cloning and was cultured in Luria broth supplemented with50-µg/ml kanamycin. Restriction enzymes HindHI and XhoI were purchased from Invitrogen (USA). The QIAGEN gel extraction kit (QIAGEN, Valencia, California). IgG Elisa Quantitation Set (Bethyl, USA). For immunohistochemistry analysis the HER2/neu Elisa Kit was purchased from Siemens (Tarrrytown, USA).

Animals.

Female rabbits aged 6 to 8 weeks were purchased from Hanoi Medical University (Dong Anh's breeding factory, Hanoi, Vietnam) with characteristics such as weight of 2.8 – 3.5 kg, Vietnam rabbit (Holland spices), gray color, vaccination of some bacterial and fungi disease, collecting the strong rabbits with some parameters like shine hair, clear eyes, eat well.

DNA Vaccine Plasmid Preparation

The gene encoding the human HER2-EDC antigenic region was obtained from pENTR223.1 vector (Havard, USA) containing the HER2 gene by using forward (5'-CACCCCTGTAAGCTTATGTGTAAGGGC and reverse (5'-GGCCCTCACCTCGAGCCGTGCT) primers containing two HindHI and XhoI restriction enzyme sites, respectively, a fragment of 500 nucleotides was generated by PCR. The PCR fragment was purified using the QIAGEN gel extraction kit and was ligated into the pcDNA3.1 (+) vector using T4 DNA ligase. pcDNA3.1 (+) plasmids containing EDC gene (pcDNA3.1-EDC) were transfected in the Escherichia coli DH5α (E. coli DH5α) cells. The recombinant clones were analyzed by restriction enzyme analysis and DNA sequencing. For the production and purification, the E. coli DH5α cells transformed with pcDNA3.1-EDC were grown in LB medium. These plasmids were purified by using the PureYield™ Plasmid Maxiprep System (Promega) and used for transfection, encapsulation, and immunization studies.

Synthesis of Chitosan Nanoparticle

Plasmid DNA-chitosan nanoparticles (PDCN) were formulated by the complex coacervation method as described earlier with modifications (27). Chitosan solution (4 mg/mL in 5 mM sodium acetate buffer, pH 5.5) and plasmid DNA solution (0.1 mg/mL in 50 mM sodium sulfate) were preheated to 55°C and promptly vortexed for 30-40 seconds. Nanochitosan (NC) solution without plasmid DNA will be prepared to do negative control.

Measurement of Characteristics of Plasmid DNA-chitosan Nanoparticle

The Average Size of Nanoparticles

The mean size of plasmid DNA-chitosan nanoparticle solution were analyzed by Zetasizer 3000HS (Malvern instruments Ltd., United Kingdom).

Surface Morphology

The shape and surface morphology of plasmid DNA-chitosan nanoparticles were determined by scanning electron microscopy (SEM) system (Hitachi S-4800 FE-SEM, USA).

DNA Encapsulation Efficiency

Plasmid DNA-chitosan nanoparticles were centrifuged at 20.000 rpm for 30 min. The supernatants containing free plasmid DNA were determided by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). The DNA encapsulation efficiency was calculated by the amount of DNA encapsulated comparing with the total amount of DNA used in formulation, multiplied by 100.

Rabbit Immunization

Female rabbits used for the experiments were housed at an approved animal facility under a standard day and night cycle and provides clear food. Three different experiment groups were used for immunization consist of nanochitosan solution, naked DNA (pcDNA3.1-EDC) and PDCN. Rabbits were injected intramuscularly with 50 µg of plasmid DNA in different groups. Preperations were repeatly injected in 3 times for 3 weeks (1 nasal/week). Then, the serum samples of rabbits were collected before injection and after the third injection with 15 day to check the expression levels of IgG and HER2 EDC protein.

RESULTS

Cloning and expression of HER2 EDC gene

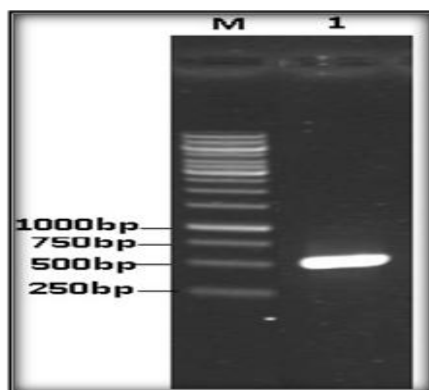


Figure 1: Agarose gel electrophoresis of PCR products of HER2 EDC gene. Lane 1, DNA marker of 1kb (Fermentas) ; lane 2, PCR products of HER2 EDC gene with size of 500 nucleotides.

HER2 EDC gene amplified by PCR with the specific primers was showed in method. This DNA fragment is about 500 bp in length (Figure 1). After cloning in pcDNA3.1 vector generating pcDNA3.1-EDC vector and these vectors were transfected into E. coli cells. The expression of pcDNA3.1-EDC vectors in E. coli cells was shown in Figure 2.

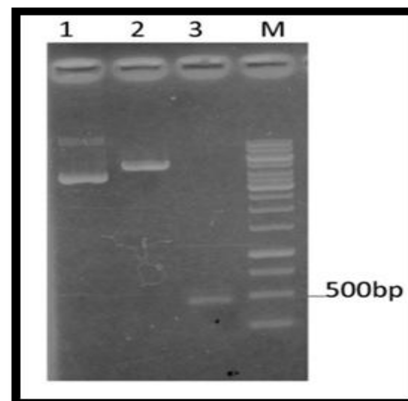


Figure 2: The expression of pcDNA3.1 vector containing HER2 EDC gene in E. coli cells. Lane 1, plasmid pcDNA3.1; lane 2, plasmid pcDNA3.1-EDC; lane 3, HER2 EDC gene; lane M, DNA marker of 1kb.

Characterization of Plasmid DNA-chitosan Nanoparticles

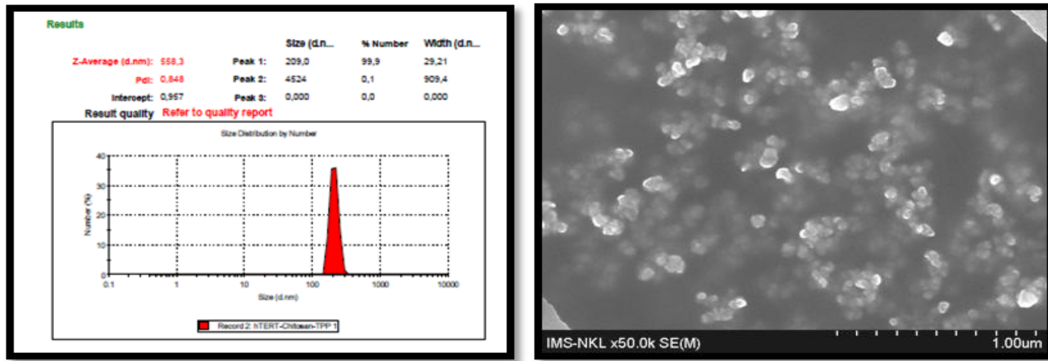
Plasmid pcDNA3.1-EDC encapsulated in chitosan have been formulated using the complex coacervation with modification. Important parameters of PDCN such as mean size, zeta potential and morphology have been investigated by the Malvern Zetasizer. The mean size of nanoparticles was optimized to 209 nm with a narrow distribution (Figure 3A). The shape of PDCN was spherical (Figure 3B).

Quantitation of Immunoglobulin G

Groups of rabbit were intramuscularly immunized with four doses of normal saline, nanochitosan (NC) solution, PDCN solution and naked pcDNA3.1-EDC for 3 nasals at 1-week intervals. Serum samples of different rabbit groups were collected before injection and after 3rd injection. IgG levels in serum were determined by indirect ELISA. The results in figure 4 showed that total IgG levels in serum of the rabbits injected with saline and naked pcDNA3.1-EDC were higher after injecting 3rd as compared to before injecting. However, total IgG levels of CN and PDCN groups were lower after injecting than before injecting.

Measurement of HER2 EDC Protein

To investigate the serum HER2 ECD protein, serum samples were measured by Elisa assay. HER2 EDC protein levels of CN, PDCN and pcDNA3.1-EDC groups were about 280, 474 and 408 pg/mg, respectively. The results showed that the secretion of HER2 EDC protein was low in CN group. However, HER2 EDC levels of rabbit immunized with PDCN were considerably increased compared to those of rabbit immunized with CN or naked pcDNA3.1-EDC (Figure 5).



3A 3B

Figure 3: The mean size of plasmid DNA-chitosan nanoparticles was analyzed by dynamic light scattering (Figure 3A) and surface morphology of PDCN was determined by SEM (Figure 3B).

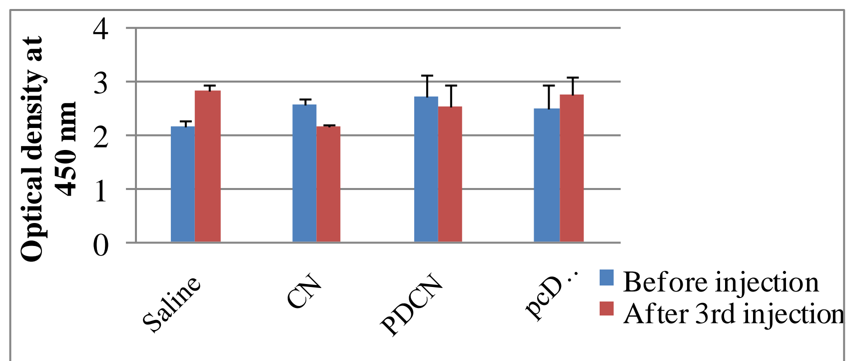


Figure 4: Elisa analysis of total IgG levels in serum of rabbits. IgG levels in rabbit serum were determined in four groups of normal saline, nanochitosan (NC), PDCN and naked pcDNA3.1-EDC before injection and after 3rd injection.

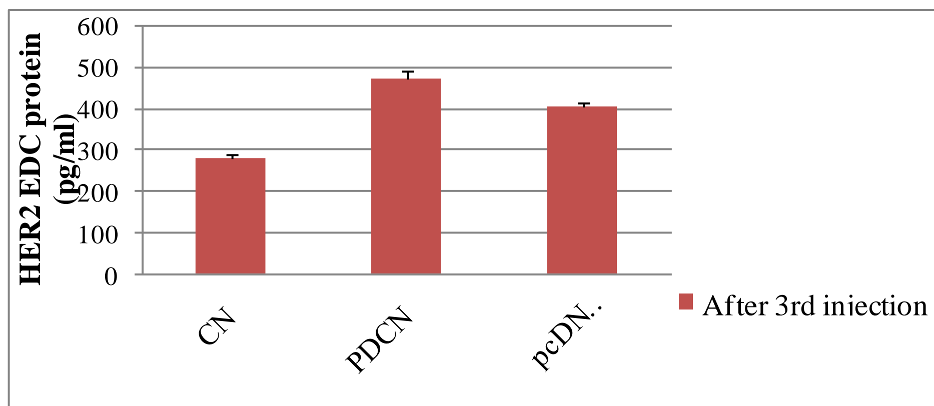


Figure 5: Elisa analysis of total HER2 EDC protein levels in serum of rabbit groups. Rabbits were intramuscularly immunized with three doses of NC, PDCN and naked pcDNA3.1-EDC after 3rd injection.

DISCUSSION

Chitosan has been using as the drug or gene delivery system because of the small particle size from 10nm to 1000nm, which is easy to pass through the cell and chitosan is a non-toxic biodegradable polycationic polymer (28). Nanoparticles are used as non-viral vectors for gene delivery, or as delivery carriers for protein molecules (29). Encapsulation of plasmid DNA in chitosan nanoparticles is dependent on various parameters such as the ratios of moles of the amine

groups of cationic polymers to those of the phosphate ones of DNA, molecular weight of chitosan, degree of deacetylation, polymer charge density, polymer structure, and pH value (30, 31). In this study, PDCN solution was produced through the complex coacervation method with the chitosan/DNA ratio of 1/2 and important parameters are suitable for an efficient gene delivery. Therefore PDCN solution has high stability to inject into rabbit.

The hosts which are the mammals suitable for experiment of immunization are rabbit, mouse, goats, etc. The rabbit was the suitable host for immune research because the immune response system of rabbit are high specificity and affinity than other spices with the diversity and the antigen recognition of rabbit are wider than that in others (32). Moreover, major components in the immune system are antibodies. IgG is the main antibody isotype in blood and extracelluar fluid and its effect is to control infection of body tissues and protect the body from infection (33). Therefore, results of total IgG levels after injecting 3rd were decreased in rabbits immunized with CN and PDCN than in those in saline and naked DNA as compared to before injecting and this result suggested that chitosan controlled the infection of tissues and protect the rabbit from infection.

In addition, in breast cancer patients, high serum HER2 ECD levels have been documented in many studies and measurement of serum HER2 ECD levels is in good concordance with primary breast tumour HER2 status in most cases (34-36). A stable and strong response of immunity by cancer vaccines is expected to lead to establishment of immune memory, resulting in a prevention of tumor recurrence. However, an immunological tolerance against HER2 anti-gen produces a barrier to effective vaccination against this oncoprotein (37). Therefore, the current challenge for designing vaccines is to find the best conditions to break this immunological tolerance. In our study, the antigen-specific HER2 response was higher in rabbits immunized with naked pcDNA3.1-EDC and PDCN indicating that intramuscular vaccination and chitosan could be convenient choices for the delivery of DNA vaccines against HER2.

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