·Gastric Cancer Column·

Preparation and antitumor effects of nanovaccines with MAGE-3 peptides in transplanted gastric cancer in mice

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[Abstract] Background and Objective: As a prospective vaccine carrier, nanoparticles can protect antigens from degradation and enhance immune response. This study prepared nanovaccines with MAGE-3-derived $CD4^+$-$CD8^+$T cell epitope peptides, and investigated its character and antitumor effects on transplanted gastric cancer in mice. **Methods:** We adopted the self-assembly method to prepare peptide/chitosan conjugated with deoxycholic acid (chitosan-deoxycholic acid) nanoparticles. We observed the appearance of the chitosan-deoxycholic acidnanoparticles through a transmission electron microscope (TEM) and analyzed the peptide content and its release pattern by fluorescence spectrophotometry. We observed tumor-suppression efficacy in vivo through animal experiments. **Results:** We successfully prepared nanoparticles with MAGE-3 peptide antigen, and its encapsulation efficiency and loading level were about 37% and 17.0%, respectively. These nanoparticles presented a delayed release pattern in phosphate buffered saline (PBS) at pH 7.4, and the full release time was about 48 h. In 2 mg/mL lysozyme, the nanoparticles showed a sudden release, and the full release time was about 24 h. ELISPOT and cytotoxic experiments showed that the MAGE-3 peptide loaded nanoparticles could stimulate immune response in vivo and could generate MAGE-3-targeted cytotoxic T lymphocytes (CTLs), and kill MAGE-3-specific tumor cells. Tumor suppression experiments showed that the regression ratio of the peptide-loaded nanoparticles group was 37.81%. **Conclusions:** MAGE-3 peptide/chitosan-deoxycholic acidvaccine-loaded nanoparticles can stimulate antitumor immune response in vivo and can regress the growth of mouse forestomach carcinoma cell line MFC.

Key words: Peptide vaccine, nanoparticles, self-assembly, gastric carcinoma, antitumor effect

In recent years, peptide vaccines have become a major focus of tumor vaccine research due to such advantages as specificity, fewer side effects, and ease of synethesis. However, because of its low immunogenicity, peptide vaccines are susceptible to enzymolysis in vivo, its bioavailability is low, and its biologic stability is poor. Therefore, a suitable carrier is required to overcome these shortcomings. As carriers for multipeptide vaccines, studies on nanomaterials have made considerable progress. They can protect antigens and enhance their immunogenicity. At the same time, they can be directly drained to regional lymph nodes because of their particle sizes, bearing a certain property of 'lymphatic targeting'. However, the biocompatibility of some traditional nanomaterials is poor and the process of preparation is complex, involving some chemical mediators in the synthesis, resulting in their being easy-to-damage molecules. Molecular self-assembly is a new concept proposed in recent years, using noncovalent interactions between molecules to construct nanomicelles loaded drugs to protect the bioactive molecules from destruction [1]. In this study, we constructed nanopeptide vaccines through molecular self-assembly to study in-vitro immune mechanisms and the antitumor effects in tumor-bearing mice.

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Materials and methods

Materials and equipment

Male 615 mice of clean grade, aged 6–8 weeks (MHC Background: $H\text{-}2K^K$) were purchased from Tianjin Institute of Hematology, with production license No. SCXK (Tianjin) 2004-0001. The mouse forestomach carcinoma cell line MFC was purchased from Shanghai Cell Bank of the Chinese Academy of Sciences. Enzyme-linked immunosorbent spot

(ELISPOT) assay kits, lymphocyte separation media, and Lympho-Spot™ serum-free media were purchased from Dakewe Biotech Co., Ltd. RPMI-1640 medium was purchased from GIBCO. Fetal calf serum was purchased from Hangzhou Sijiqing Biological Co., Ltd. The mouse recombinant cytokines recombinant murine granulocyte macrophage-colony stimulating factor (rmGM-CSF) and recombinant murine interleukin-4 (rmIL-4) were purchased from Guangzhou, Huida Biotechnology Co., Ltd; MAGE-3 peptides (sequence: FITC-YEEYYPLIFLDNDQETMETSEEEEYEEYYPLIF, No. ZSH-1, from N terminal to C terminal, more than 90% purity) were purchased from Shanghai Huiyuan Biosciences Co., Ltd.

The FACSAsia flow cytometer was from Becton Dickinson, the JEM-100CX II-type field emission scanning electron microscopy was from Japan Electronics Co., Ltd., the superconducting nuclear magnetic resonance (NMR) was an INOVA300 by Varian, the refrigerated centrifuge was an Avanti J-25 Elutriation Centrifuge from Beckman Coulter, Brookhaven Instruments Corporation, and the RF-5301PC fluorescence spectrophotometer was from Shimadzu, Japan.

Preparation of chitosan-deoxycholic acid nanomicelle and characterizations

Preparation of chitosan-deoxycholic acid nanomicelle and NMR characterizations A total of 0.2 g of chitosan was placed into round-bottom flasks and 20 mL of 1% acetic acid solution was added, then 2–3 mL of anhydrous ethanol was added after mixing at room temperature for 30 min. A defined amount of deoxycholic acid and 1- (3-dimethylamino-propyl)-3-ethyl carbodiimide hydrochloride (EDC) was weighed to be dissolved in 7 mL of anhydrous ethanol, which was slowly added to the reaction system mentioned above drop by drop, sustaining the reaction for 24 h at room temperature. After the reaction, ethanol/ammonia solution (7/3, *V*/*V*) was used to adjust the pH value to weak alkaline (7–8) and precipitations obtained after centrifugation were washed in ethanol 2–3 times. Followed by another centrifugation, ethanol was removed and precipitations were dissolved in a small amount of water and freeze-dried for 48 h to get the chitosan-deoxycholic acid products.

Chitosan-deoxycholic acid samples, with D_2O as the solvent and acetone as the internal standard, were measured at room temperature and processed through pressurized water peak technology.

Observation of chitosan-deoxycholic acid nanomicelles with transmission electron microscopy (TEM) Chitosan-deoxycholic acid micelle droplets were placed on copper mesh. We used phosphotungstic acid negative staining to take TEM photographs and observe its morphology.

Preparation of nanovaccines loaded MAGE-3 peptides

The chitosan-deoxycholic acid conjugate was weighed and dissolved in 5 mL of phosphate buffered saline (PBS) with pH 5.8. After magnetic stirring for 4 h, 5 mg/mL of the peptide solution was added drop by drop. Followed by gentle mixing, the mix was placed in a refrigerator at 4℃ for 3 h. Afterward, the mix was centrifuged at 20 000 × *g* for 30 min and placed at 4℃ to be tested.

Study of nanovaccine-loaded MAGE-3 peptides and drug-release properties We used an fluorescence spectrophotometer over a wavelength range of 480–600 nm to scan the peptide solutions with different concentrations (with pH 5.8 and pH 7.4 PBS as a solvent, respectively) and made a standard curve.

We calculated the encapsulation efficiency (EE) of drug-loaded micelles and the loading level (LL) (using the pH 5.8 PBS standard curve to calculate), and determined the cumulative release rate (CRR) of peptides at 37℃ (using the pH 7.4 PBS standard curve to calculate) with the following formula:

$EE = (W_0–WF)/W_{ch}$, $LL = (W_0–W_F)/W_0$, $CRR = W_t/W$

W_0 is the total weight of the multipeptide vaccine. W_F is the weight of free peptides. W_{ch} is the total weight of the amphiphilic chitosan. W_t is the amount of free peptides in solution at the moment t. W is the amount of the total peptide load.

Dendritic cells (DC) phagocytosis experiments of nanovaccine loaded MAGE-3 peptides

Culture of peptide-pulsed dendritic cells Under sterile conditions, the hind limbs of the mice were stripped and the RPMI-1640 medium was used to wash the medullar canal to make a single-cell suspension. Cells were seeded into sterile dishes with 5 × 10^6/mL. RPMI-1640 (containing 10% FBS, 40 ng/mL rmGM-CSF, and 40 ng/mL rmIL-4) were added and cells were incubated in 5% CO_2 at 37℃ overnight to induce maturation of dendritic cells. The culture medium was changed at day 3 and day 6. Cells collected between days 6–8 were mature DCs. DCs in the RPM-1640 medium (containing 10% FBS and 40 ng/mL rmGM-CSF) were incubated overnight, and then 50 μg/mL peptides were added and incubated in 5% CO_2 at 37℃ for another 4 h (with a concentration of 1 × 10^6/mL).

Detection of the intake capacity of mouse bone marrow DCs A total of 106 DCs were seeded into each well of 24-well plates. Free FITC-MAGE-3 peptides with different concentrations of 10 μg, 30 μg, 50 μg, and 70 μg, and nanovaccine loaded MAGE-3 peptides with different concentrations of 10 μg, 30 μg, 50 μg, and 70 μg were added. We used PBS as the negative control group. Incubated for 30 min in 5% CO_2 at 37℃, cells were washed in PBS three times. Then fixing solutions were added and phagocytic rates were detected by flow cytometry.

Immunization of animals and separation of mouse spleen lymphocytes

A total of 30 mice 615 were randomly divided into 5 groups, with 6 mice for each group. Drugs were administrated subcutaneously at the base of the tail on the day when groups were divided (d0) and 7 days afterwards (d7). Administrations were as follows: in the PBS group, 0.2 mL per mouse each time; in the blank nanomicelle group, nanomicelle solutions without loads were injected with 0.2 mL per mouse each time (with a concentration of 2.565 mg/mL); in the peptide group: 300 μg of free peptides were injected with 0.2 mL per mouse each time (with a concentration of 1.5 mg/mL); in the peptide + incomplete Freund adjuvant (IFA) group, 300 μg of the peptides were injected and the peptides were mixed with an equal volume of IFA with 0.2 mL per mouse each time (0.1 mL of 3.0 mg/mL peptide + 0.1 mL IFA); in the nanopeptide group, a nanomicelle

solution containing 300 μg of peptides were injected with 0.2 mL per mouse each time (with a concentration of 1.5 mg/ mL). On the 7th day after the last immunization, mouse spleens were taken under sterile conditions and lymphocytes were separated with a lymphocyte separation medium. Then Lympho-Spot™ was added for resuspension and the cells were counted.

ELISPOT test

According to the ELISPOT reference manual, spleen lymphocytes from mice immunized with different drugs were the effector cells and the MAGE-3-positive MFC cells were the target cells. A negative control group without target cells and a PHA-simulated positive control group were set up in each group. Each experiment was carried out three times and an average was obtained. Effector cells and target cells with the ratio of (E:T = 30:1) were seeded into 96-well plates. Cells were incubated at 37℃ to react for 20 h and then cells were washed followed by adding the upper antibody and horseradish peroxidase (HRP). After a color reaction in the dark for 20 min, the cells were washed with distilled water to terminate the reactions. We used a 1200-dpi scanner to read the number of spots and subtracted the number of spots of the negative control to get the average for each group.

Cytotoxicity test

We adjusted the concentration of effector cells of the splenic T lymphocytes to 1×10^6/mL. After double dilution, the effector cells and the target cells (E:T) with ratios of 2:1, 5:1, and 10 :1 were seeded into 96-well plates as the experimental group. They were incubated in 5% CO_2 at 37℃ for 4 h. At the same time, control groups of the experimental system were set up, which were medium background holes, target cells that released spontaneous holes (without CTL effector cells), and target cells with the maximum released holes (0.1 mL of target cells were added before the end of the culture). Three parallel holes were set up for each group. We used a standard ^{51}Cr release test to detect kill rates.

Antitumor effects of nanovaccines loaded MAGE-3 peptide on tumor-bearing mice

There were 30 mice 615. A total of 1×10^8/mL of exponential growing MFC cells were mixed fully and planted in the left anterior axillary at 0.2 mL per mouse. We used vernier callipers to measure the longest diameter (a) and shortest diameter (b) of the subcutaneous tumor and calculated tumor volume according to the Steel formula $V=0.5 \times a \times b^2$. We used RTV = V_t/V_0 (V_0 is TV that is measured when mice are grouped and treated; V_t is TV that is measured each time) to calculate the relative tumor volume. We used T/C(%) = (TRTV/CRTV) × 100 (TRTV is the relative tumor volume of the treatment group; CRTV is the relative tumor volume of the negative control group) to calculate the relative tumor proliferation rate. Until the tumor was palpable, mice were randomly divided to be treated. The delivery approach was the same with the method mentioned above. The survival of the mice in each group was recorded.

Statistical analysis

All data were analyzed using SPSS version 13.0. The experimental results we re measured by mean ± standard deviation. Differences among the different groups were analyzed by one-way ANOVA. Statistical significance was assumed when $P < 0.05$.

Results

Magnetic characterization of chitosan-deoxycholic acid

The peak between 0.6–2.5 in Figure 1 shows the magnetic resonance peak of deoxycholic acid and the peak between 3–5 shows the magnetic resonance peak of the chitosan main chain, confirming coupling between deoxycholic acid and chitosan.

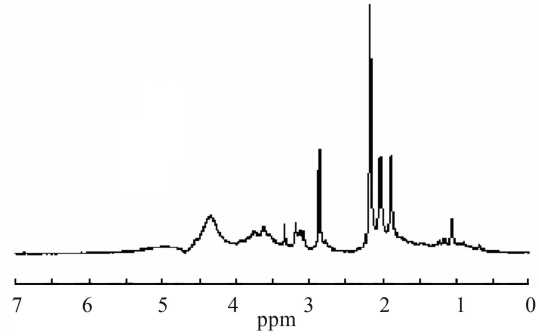

Figure 1 The ^1H NMR spectra of Chitosan-deoxycholic acid

There is a DC peak at 0.6–2.5, indicating that chitosan–deoxycholic acid was synthesized.

Morphology and size of chitosan-deoxycholic acid

Chitosan-deoxycholic acid micelles observed by TEM were particles of 30–50 nm, round or oval in less uniform size, which are shown in Figure 2.

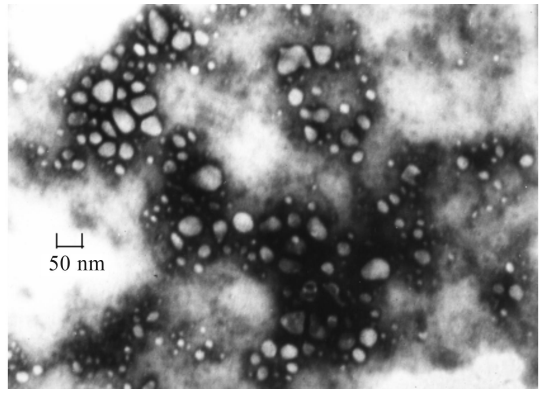

Figure 2 The TEM image of chitosan-deoxycholic acid micelles

The nanomicelles are elliptical and uniform in shape, and 30–50 nm in diameter.

Drug-loading properties of nanovaccine loaded MAGE-3 peptides

Fluorescence spectrophotometer analysis was used to make the standard curves. The result was as follows: I = -3.10 + 74.20C (R = 0.998). According to the working curve, the load factor was 37.3% and the drug loading was 17.0%.

In-vitro drug release behaviors of nanovaccine loaded MAGE-3 peptides

Results obtained from the working curve made by fluorescence spectrophotometer analysis were as follows: I = -0.86 + 43.45C (R = 0.998). Release curves were made according to the concentration of peptides obtained from the standard curve. As shown in Figure 3A, peptides of the drug-loaded nanoparticles released slowly. About 48 h later, the release was completed. While in the lysozyme solution, as shown in Figure 3B, the release of the peptides reached a plateau after about 24 h, suggesting that lysozymes had a certain role in promoting the drug release of chitosan drug-loaded nanomicelles while still maintaining a degree of slow-releasing effects.

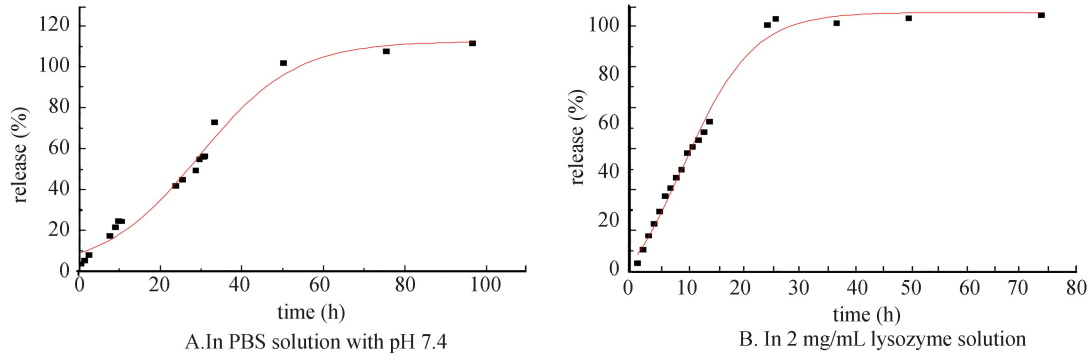

Figure 3 The release pattern of peptide loaded nanoparticles

A, the peptide is completely released from nanoparticles within 48 h; B, the peptide is released from nanoparticles within 24 h in a lysozyme solution, indicating that lysozyme has a promoting effect on peptide-loaded nanoparticles.

Dendritic cell phagocytosis experiments of nanovaccine loaded MAGE-3 peptides

Floating and semi-adherent cells were collected and fixed. An electron microscope was used to observe surface protrusions. The cells were found to display typical DC morphologies. Detection by flow cytometry found that the uptake rate of the nanovaccine loaded MAGE-3 peptides was higher than that of the free FITC-MAGE-3 peptide vaccines by DC ($P < 0.05$) in a dose-dependent manner (the 10-μg group: 50.2% vs. 11.2%; the 30-μg group: 62.4% vs. 23.6% ; the 50-μg group: 66.8% vs. 34.0% ; the 70-μg group: 72.4% vs. 40.4% ; and the PBS negative control group: 2.4%).

ELISPOT test

The number of specific T lymphocytes that can secrete IFN-γ in peptides in the nanovaccine group was higher than that of blank nanomicelle group and PBS group ($P < 0.05$). Among them, the number of mouse spleen T lymphocytes in the peptide group was significantly different from in peptide adjuvant group (Figure 4).

Cytotoxicity test

Spleen lymphocytes from mice immunized by the free MAGE-3 peptides, MAGE-3 peptides + IFA, and MAGE-3 peptides vaccines could kill MFC cells that expressed MAGE-3 specifically. The specific cytotoxicity of mouse spleen lymphocytes in the peptide nanovaccine group with various ratios of effector target cells was higher than that in the peptide group, the blank nanomicelle group, or the PBS group ($P < 0.05$). The cytotoxicity of spleen lymphocytes from mice immunized by the blank nanomicelle and PBS to MAGE-3 positive MFC cells was weak (Figure 5).

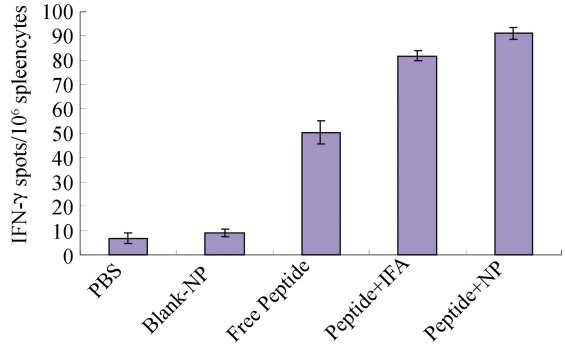

Figure 4 The quantity of IFN-γ secreted by mouse splenic lymphocytes in different groups (by ELISPOT)

The PBS group was given doses of PBS; the blank-NP group, the free peptide group, the peptide + IFA group, and the peptide + NP group were given doses of blank nanoparticles, peptides, peptides mixed with IFA, and peptide-loaded nanoparticles, respectively.

The mouse splenic lymphocytes (effectors) are incubated with MFC (targets) at a set effector/target ratio. The results shown represent the averages and standard deviations of triplicate cultures.

Antitumor effects of nanovaccine loaded MAGE-3 peptides on tumor-bearing mice

There were no significant differences in tumor volume between the different groups before they were divided ($P > 0.05$). Tumor volumes of different groups with different periods of time are shown in Figure 6. With the PBS group as the negative control group, and the nanopeptide, multi-peptide + IFA, and peptide groups as the treatment groups, the tumor inhibition rate was calculated. The tumor inhibition rate of the nanopeptide group was 37.81%, which was significantly different from that of the PBS negative control group ($P = 0.0125$), suggesting that

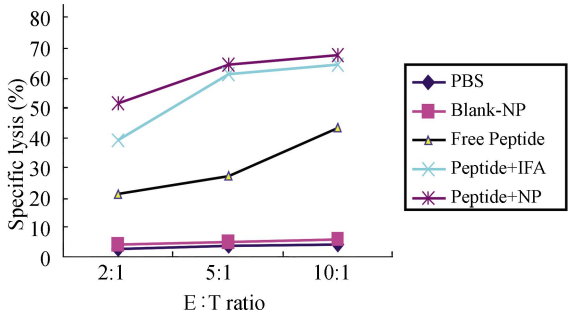

Figure 5 The specific lysis rate of mouse splenic lymphocyte to MFC in different groups.

The PBS group was given dose of PBS; the blank-NP group, the free peptide group, the peptide + IFA group, the peptide + NP group were given doses of blank nanoparticles, peptides, peptides mixed with IFA, and peptide-loaded nanoparticles, respectively.

The MFC cells (targets) are ^{51}Cr-labeld, and incubated with mouse splenic lymphocytes (effectors) at the indicated ratios. Chromium release was measured after 4 h.

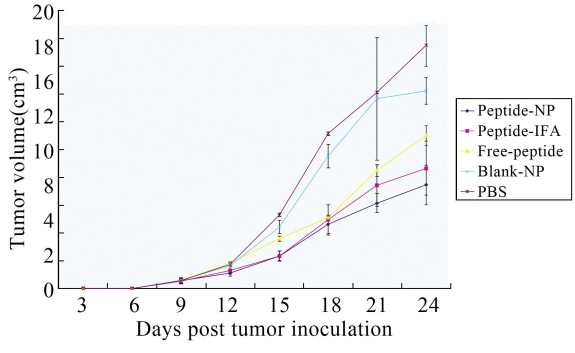

Figure 6. The tumor volume curve in different groups

The PBS group was given dose of PBS; the blank-NP group, the free peptide group, the peptide + IFA group, and the peptide +NP group were given doses of blank nanoparticles, peptides, peptides mixed with IFA, peptide-loaded nanoparticles, respectively.

The tumor volume was calculated according to formula ($V = 0.5 \times a \times b^2$). The results were presented in the curve of the relevant group.

nanopeptides could inhibit tumor growth in mice.

Comparisons of survival time

The analysis of survival time showed that the overall mean period of survival was 27 days and there was no significant difference in survival time among the groups (log-rank x^2 = 5.683, $P > 0.05$), suggesting that the inhibition of tumor growth did not affect of the period of survival, with limited efficacy in extending survival time (Figure 7).

Discussion

The discovery of tumor antigen peptide-based vaccines, the proposal of biologic response modifiers and the application of adoptive cell therapy, and the identification of gene therapy programs were considered to be three major milestones of biological tumor therapy. Compared with traditional vaccines, tumor peptide vaccines have a number of advantages, such as

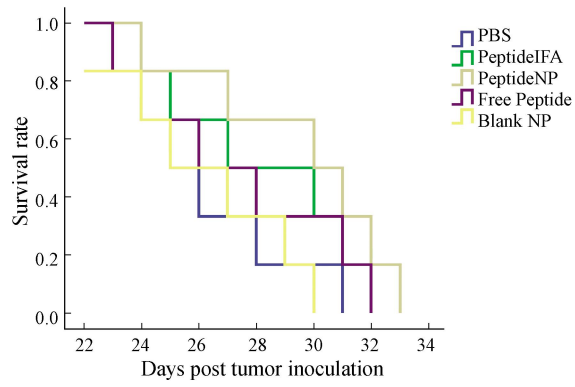

Figure 7 The survival curve in different treatment groups of mice

The PBS group was given dose of PBS; the blank-NP group, the free peptide group, the peptide + IFA group, and the peptide + NP group were given doses of blank nanoparticles, peptides, peptides mixed with IFA, peptide-loaded nanoparticles, respectively. Tumor bearing mice (6 mice in each group) were immunized with PBS, blank-NP, free-peptide, peptide-IFA, and peptide-NP, separately on days 0 and 7. The survival of the mice was monitored.

having a good safety profile, being easy to design and prepare, and being convenient to be synthesized, which can combine with adjuvant treatment and induce a strong CD8$^+$ T cell response[2]. In recent years, along with the development of nanotechnology as well as the development and application of new nanomaterials, nanovaccine strategies of peptide immunotherapy combined with nanotechnology have presented some attractive prospects, whose main advantages are as follows: (1) the protective effects on antigens could prevent their early decomposition and extend the residence time of the antigen in the body to enhance the immune response; (2) as immune adjuvants, nanocarriers themselves can help to expand the nonspecific immune response; (3) besides their small size, nanoparticles have the unique quality of 'lymphatic targeting'.

Most traditional multipeptide vaccines mainly take a single CTL epitope, that is, MHC-I restricted epitope peptides [3]. Zwaveling *et al.*[4] found that co-Th epitopes, that is, MHC-II restricted restricted epitope peptides, could enhance immune response. That may be related to supportive immune function of Th cells, inhibition of immune tolerance by dual-epitopes, and extensions of the duration of antigen presentation[5,6]. MAGE-3 is one of the antigens that is widely expressed in many tumors. Clinical phase I/II trials have confirmed that MAGE-3 combined adjuvant therapy can stimulate specific humoral and cellular immunity[7]. The largest Phase III clinical trial so far (MAGRIT) are still being conducted.

In recent years, protein carrier materials based on amphiphilic graft copolymers have been studied extensively. Akagi *et al.*[8] used γ -PGA and L-PAE to construct amphiphilic nanocarriers by self-assembly, finding that they had a certain superiority as a protein carrier due to their good biocompatibility, biosafety and biostability. Nanopeptide vaccines have also been reported to enhance immune effects and inhibit tumor cell growth [9]. Park *et al.*[10] used chitosan as basic raw materials to construct nanomicelles, which can parcel peptide vaccine well. For this

purpose, on the basis of the preparations of nanomicelles, we used chitosan and deoxycholic acid as our raw materials and took advantage of the electrostatic attractions between peptides and nanomicelles, Van der Waals forces, and so on, to construct drug-loaded nanomicelle units by self-assembly in this study. Experiments of immune activity in vitro and studies on animals found that peptide vaccines with nanomaterials as load units can stimulate more effective immune responses than simple peptides, having some advantages when compared with immunologic adjuvants. Because traditional immune adjuvants have certain toxic side effects, the safety of vaccines based on nanomaterials is superior to that of immunization approaches assisted by traditional adjuvants. However, in animal experiments, the vaccine coadjuvant group had no obvious advantages in extending the survival time of mice, which may be related to factors such as the choice of peptide and adjuvant, the vaccine dose, or immune methods. Whether can we improve immune efficacy through the dual paths of combining humeral immunity with cellular immunity, adjusting the doses of the vaccine, or the frequency of stimulus intervals is one of the research hypotheses we will study in the future. Peptide vaccines with nanomaterials as the carriers have broad prospective applications, requiring us to continue research in this area.

References

[1] Zhou JJ, Chen RF, Tang QB, et al. Preparation of 5-fluorouracil encapsulated in amphiphilic polysaccharide Nano-micelles and its killing effect on hepatocarcinoma cell line HepG2. [J]. Chin J Cancer, 2006,25(12):1459-1463. [in Chinese]

[2] Peng F, Wei YQ. New advance in Clinic trial of cancer vaccines.[J]. Chin J Cancer, 2006,25(8):1059-1062. [in Chinese]

[3] Eggert AO, Andersen MH, Voigt H, et al. Characterization of mouse MAGE-derived H-2Kb-restricted CTL epitopes [J]. Eur J Immunol, 2004 , 34(11):3285-3290.

[4] Zwaveling S, Ferreira MS, Nouta J, et al. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides[J]. J Immunol,2002,169(1):350-358.

[5] Bijker MS, Van Den Eeden SJ, Franken KL, et al. Superior induction of anti-tumor CTL immunity by extended peptide vaccines involves prolonged, DC-focused antigen presentation [J]. Eur J Immunol,2008,38(4):1033-1042.

[6] Melief CJ, Van Der Burg SH. Immunotherapy of established (pre) malignant disease by synthetic long peptide vaccines[J].Nat Rev Cancer, 2008,8(5):351-360.

[7] Vantomme V, Dantinne C, Amrani N, et al. Immunologic analysis of a phase I/II study of vaccination with MAGE-3 protein combined with the AS02B adjuvant in patients with MAGE-3-positive tumors. [J].J Immunother,2004,27(2):124-135.

[8] Akagi T, Wang X, Uto T, et al. Protein direct delivery to dendritic cells using nanoparticles based on amphiphilic poly(amino acid) derivatives[J]. Biomaterials. 2007, 28(23): 3427-3436.

[9] Fifis T, Mottram P, Bogdanoska V, et al. Short peptide sequences containing MHC class I and/or class II epitopes linked to nano-beads induce strong immunity and inhibition of growth of antigen-specific tumour challenge in mice. Vaccine,2004,23(2):258-266.

[10] Park JH, Kwon S, Nam JO, et al. Self-assembled nanoparticles based on glycol chitosan bearing 5beta-cholanic acid for RGD peptide delivery[J].J Control Release,2004,95(3):579-588.