CpG oligodeoxynucleotides augment antitumor efficacy of folate receptor α based DNA vaccine

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Abstract. Folate receptor α (FRα) is overexpressed in a variety of solid tumors and has become an attractive target antigen for immunotherapy purposes. A DNA vaccine was generated by ligation of FRα cDNA into the eukaryotic vector pcDNA3.1. Expression of FRα was confirmed in transiently transfected B16 cells. B16 cell lines that stably express FRα were set up by G418 selection. A total of 100 μg purified plasmid DNA alone or in combination with CpG oligodeoxynucleotides (CpG ODN) was injected intramuscularly in C57BL/6 mice four times at one week intervals. ELISA analysis confirmed that high titers of antibodies against FRα existed in the sera of the experimental animals. Specific cytotoxic T lymphocyte activity against FRα-expressing B16 cells was found and FRα specific lymphocyte proliferation was detected. Coinjection of CpG ODN increased both humoral and cellular immune responses. In the protective model, in which C57BL/6 mice were immunized with the FRα DNA vaccine four weeks before tumor cell inoculation, the growth of tumor was significantly inhibited, and the presence of CpG ODN further increased the inhibitory effect. FRα DNA vaccine alone did not show a significant inhibitory effect in the therapeutic model, in which the DNA vaccine was immediately injected after tumor inoculation. However, FRα DNA vaccine plus CpG ODN showed a significant inhibitory effect in tumor growth. Survival curves for both animal experiments confirmed that mice immunized with pcDNA3.1/FRα plus CpG ODN had a significantly prolonged survival period than that of the pcDNA3.1 control group, the CpG ODN group or the pcDNA3.1/FRα group. The above showed that human FRα based DNA vaccination with CpG ODN as an adjuvant was effective in growth inhibition of a FRα expressing tumor in mice and deserves further evaluation as a possible immunotherapy.

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Abbreviations: FRα, folate receptor α; CpG ODN, CpG oligodeoxynucleotides; CTL, cytotoxic T lymphocyte; TLR9, toll-like receptor 9; GPI, glycosylphosphatidylinositol; 5-MTHF, 5-methyltetrahydrofolate; i.m., intramuscular; LDH, lactate dehydrogenase; E:T ratio, effector cells : target cells ratio

Key words: immunotherapy, FRα, DNA vaccine, CpG ODN, cancer

Introduction

Conventional cancer treatments include chemotherapy, surgery and radiation. They are not very effective in controlling cancers and bring huge suffering to the patients. Novel therapies need to be developed for cancer treatment (1,2).

Most tumor cells express specific antigens that are not found on normal cells. Those so-called tumor-associated antigens allow tumor cells to be recognized and destroyed by the immune system (3). Triggering antitumor immunity by specific vaccination is a safe and effective way to control tumor growth. Comparing with conventional vaccinations such as whole tumor cells, proteins or derived peptides, DNA vaccination is a relatively new method (4). DNA vaccination can generate both humoral and cellular immune responses. Cytotoxic T lymphocyte (CTL) response is regarded critical for tumor cell killing. Furthermore, plasmid DNA is relatively easy to be manipulated to encode desired tumor associated antigens and can be manufactured in large scale without stringent condition requirements compared with protein vaccines, which provides a more practical approach for vaccine development (5).

Although plasmid DNA vaccines are safe and easy to prepare, they are poorly immunogenic molecules. Thus, in order to augment immune responses, a variety of adjuvants have been utilized (6). CpG oligodeoxynucleotides (CpG ODN) are small DNA molecules mimicking the unmethylated CpG motifs which frequently present in bacterial DNA. In mammals, these specific DNA motifs bind and activate toll-like receptor 9 (TLR9), leading to activation, maturation, and proliferation of immune cells. TLR9 is localized in endoplasmatic reticulum, late endosomal and lysosomal compartments of the intracellular milieu. Thus, internalization of pathogen-derived DNA is required for TLR9 triggering, an outcome

that results from either intracellular infection or uptake of bacterial/viral particles by immune cells (7). Once stimulated, TLR9 initiates a response biased towards proinflammatory/Th1 immunity (8). Extensive animal experiments showed that CpG ODN could support the induction of Ag-specific immunity against co-administered peptides and vaccines (9). The early phase I trials showed that CpG ODN was safe and could improve the immunogenicity of co-administered vaccines (10). To increase their DNase resistance, CpG-ODN can be synthesized with a phosphorothioate backbone (11-13).

Folate receptor α (FRα) is a 38 kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein. It binds folic acid and 5-methyltetrahydrofolate (5-MTHF) with high affinity (14). FRα expression in normal tissues is highly restricted and inaccessible to the normal circulation. High expressions of FRα have been described in some cancers, such as non-mucinous ovarian, endometrial, non-small cell lung carcinomas and to a lesser extent in clear cell renal, colorectal and breast cancers. Moreover, FRα expression has been observed in nearly 90% of non-mucinous ovarian cancer and correlated with tumor grade, stage, and aggressiveness. Furthermore, FRα expression remains unchanged in epithelial ovarian and endometrial cancer after chemotherapy. Based on its highly tumor restricted expression profile, FRα represents an attractive candidate for cancer diagnostics and therapeutics (15-19). Several FR-targeted agents are currently in development, representing a promising approach for relevant cancer treatments (20-22).

In this study, we assembled a cytomegalovirus promoter expression vector containing human FRα cDNA, and we evaluated its ability to induce an immune response in mice. We detected both FRα-specific antibodies and cytotoxic T lymphocyte responses, which significantly reduced the '*in vivo*' growth of FRα expressing tumor cells. In addition, the adjuvant effect of CpG ODN was confirmed.

Materials and methods

Reagents, cell lines and animals. CpG ODN was custom-synthesized by Sangon Biotech (Shanghai, China). The sequence of stimulatory phosphorothioate CpG ODN was: 5'-TCCATGACGTTCCTGACGTT-3'. Recombinant human folate receptor α protein, rabbit polyclonal anti-FRα antibody (antigen affinity purified), HRP conjugated goat anti-mouse IgG secondary antibody and HRP conjugated goat anti-rabbit IgG secondary antibody were purchased from Sino Biological Inc. (Beijing, China). G418 sulfate and plasmid purification kits were from Sangon Biotech and Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Lactate dehydrogenase (LDH) kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China).

The ovarian cancer cell line SKOV3 and metastatic melanoma B16 cell line were from Shanghai Cell Biology Institutes (Academia Sinica, Shanghai, China) and were maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum and antibiotics.

Female C57BL/6 (6 weeks, 18-20 g) were purchased from the Yangzhou University Animal Center and used under the experimental animal production license 2121922. All animals were housed in a controlled environment (25˚C; 12 h light-dark cycle), with water and food provided freely. The authors confirm that experiments involving animals adhered to the ethical standards of China Pharmaceutical University and the care of animals was in accordance with the licensing guidelines of China Pharmaceutical University.

DNA vaccine construction. Total RNA was isolated from human ovarian cancer SKOV3 cells. The DNA fragment encoding FRα was amplified using RT-PCR. Reverse transcription was performed at 42˚C using oligo $d(T)_{15}$ as a primer and PCR amplification was carried out for 30 cycles (1 min at 94˚C, 1 min at 55˚C, 1 min at 72˚C) using the following primers specific for FRα gene amplification: CAGTAAGCTTGC CATGGCTCAGCGGATGA (*Hin*dIII); CCGGAATTCTCA GCTGAGCAGCCACAGC (*Eco*RI). The gene was cloned into the eukaryotic vector pcDNA3.1 and the constructed recombinant plasmid was identified by restriction endonuclease digestion and DNA sequencing.

Expression of recombinant plasmid encoding FRα. B16 cells were transfected with a recombinant plasmid pcDNA3.1/FRα or a control plasmid pcDNA3.1 using Lipofectamine 2000 according to the manufacturer's instructions. After incubation for 72 h, the cells were harvested and tested for FRα expression by RT-PCR, western blotting and immunofluorescence. For RT-PCR, the total RNA was isolated and reverse transcribed into cDNA. FRα gene was amplified using previously described primers and analyzed by electrophoresis. For western blotting, collected cell lysates were resolved by polyacrylamide gel electrophoresis and the protein bands were transferred onto a membrane. The membrane was blocked with 5% nonfat dry milk and FRα was detected with rabbit polyclonal anti-FRα antibodies (1:5000) followed by HRP conjugated second antibodies (1:5000). The protein band was visualized with an enhanced ECL chemiluminescent reagent using a Bio-Rad detection system. For cell immunofluorescence staining, the cells were fixed with 4% polyoxymethylene for 20 min. After washing with PBS, the cells were treated with Triton X-100 for 10 min and blocked with 5% BSA for 1 h. Then the cells were incubated with rabbit polyclonal anti-FRα antibodies (1:100) at 4˚C overnight. After being washed with PBS, the cells were incubated with FITC-conjugated goat anti-rabbit IgG secondary antibodies (1:100) for 2 h and visualized with a fluorescent microscope (Olympus) and photographed.

Plasmid DNA preparation. Plasmid DNA was propagated in *E. coli* and was isolated using endonuclease-free plasmid purification kits according to the supplier's protocol. The purified plasmids were dissolved in sterile PBS and used for injection or stored at -80˚C until use.

Preparation of FRα-expressing tumor cell lines. B16 cells were transfected with pcDNA3.1/FRα using Lipofectamine 2000 as described by the manufacturer. After incubation with DNA-lipid complex for 24 h, cells were cultured in fresh growth medium (RPMI-1640 containing 10% fetal bovine serum) with 1000 μg/ml antibiotics G418 for 2 weeks. A G418 dose-response curve was established prior to the selection of the cells. The resistant cells were obtained and serially diluted. Single cells were picked and cultured in presence of G418 for another two weeks to obtain cells that stably express FRα.

Immunization. Wild-type female C57BL/6 mice (4-6 weeks old) were randomly divided into 4 groups with 6 mice in each group. Preliminary experiments were performed to compare the stimulating effect of CpG ODN at different dosages and it was found that 10 μg CpG ODN was a proper dosage (data not shown). In the following immunization, we used 10 μg CpG ODN per mice. Mice receiving a blank vector or 10 μg CpG ODN served as the control groups. In the third group, mice were administered with 100 μg recombinant plasmid pcDNA3.1/FR. In the fourth group mice were injected with 100 μg pcDNA3.1/FR Plus 10 μg CpG ODN. All the reagents were injected in the rectus femoris muscle of both hind legs. Four identical injections were given at one week intervals.

Antibody detection. One week after the fourth immunization, blood samples were collected through the canthus and were kept at 4˚C for 30 min. Then the blood samples were centrifuged at 1500 x g for 10 min, and the supernatants were taken and stored at -80˚C until detection. Microtiter plates (96-well) were coated with 100 μl of 1 μg/ml recombinant human FRα in 0.05 M sodium bicarbonate (pH 9.6) and the plate was kept overnight at 4˚C. After washing three times, the plate was blocked with 0.1 M PBS (pH 7.4) containing 10% (V/V) skim milk at 37˚C for 1.5 h. Then, serial dilutions of mouse sera (diluted in PBS/0.1% BSA/0.05% Tween-20) were added and incubated for 2 h at 37˚C. After washing three times, 100 μl of HRP-conjugated sheep anti-mouse IgG (1:5000) was added and incubated for 1 h at 37˚C. After washing, tetramethylbenzidine (TMB) substrate (100 μl/well) was added and incubated for 15 min. The reactions were stopped with 2M sulfuric acid (50 μl/well). The absorbance of each well at 450 nm was detected with an automated ELISA reader.

Cytotoxic T-lymphocyte (CTL) assays. One week after the last immunization, spleens were isolated from three sacrificed mice of each immunized group. The spleens were ground and passed through a 100 μm filter under sterile conditions. Erythrocytes were lysed using Tris-NH_4Cl (pH 7.2). Splenocytes were washed by PBS and resuspended in RMPI-1640 containing 10% FBS. Then splenocytes of each group were cultured in the presence of 10 μg/ml recombinant human FRα for five days and used as effector cells. The FRα expressing tumor cells were used as target cells. Cytotoxic activity was determined using a lactate dehydrogenase kits. Effector cells were mixed with target cells ($5x10^4$ cells) in triplicate with E:T (effector cells : target cells) ratios of 50:1, 25:1 and 12.5:1. The mixture cells were co-cultured for 4 h at 37˚C in an atmosphere containing 5% CO_2. LDH release under each condition was evaluated according to the instructions of the manufacturer. Cytotoxicity was calculated using the following equation: cytotoxicity (%) = $[(OD_{experiment} - OD_{effector\ spontaneous} - OD_{target\ spontaneous}) / (OD_{target\ maximum} - OD_{target\ spontaneous})]$ x100%.

Lymphocyte proliferation assay. One week after the last immunization, the splenocytes were isolated from each immunized group as described above. Splenocytes ($1x10^5$) were cultured in 100 μl culture medium as blank, or co-cultured with different stimulants including BSA (100 μg/ml) as non-relevant peptide control, recombinant human FRα (100 μg/ml) or 100 μg/ml ConA. Cells were cultured in triplicates in 96-well, flat-bottom plates at 37˚C for 72 h in a 5% CO_2 incubator. MTT dissolved in PBS was added to the cultures at a final concentration of 0.5 mg/ml and incubated at 37˚C for 4 h to form formazan crystals, which were later dissolved in DMSO. The optical density was measured at 540 nm on a Multimode plate Reader. The results were analyzed as the stimulate index (SI) defined as $OD_{experiment} / OD_{blank / pcDNA3.1}$.

Evaluation of the protective effect in C57BL/6 mice. Female C57BL/6 mice (4-6 weeks) were used to evaluate tumor growth inhibition. Immunization procedure was as described above. One week after the final immunization (week 5), the mice were challenged intradermally in the right flank with $2x10^5$ FRα expressing B16 cells. Tumor width and length were measured with a caliper periodically and tumor volume was calculated as $V = (length \times width^2) / 2$. In the survival experiment, the animals were kept for 50 days or until death after tumor challenge.

Evaluation of the therapeutic effect in C57BL/6 mice. Female C57BL/6 mice (4-6 weeks) were challenged with FRα expressing B16 cells on day 0. Four times immunizations with one week intervals were followed as described. Tumor growth was monitored and tumor volume was calculated. In the survival experiment, the animals were kept for 50 days or until death after tumor challenge.

Analysis of FRα protein expression in tumor tissues. Tumor tissues from experimental mice were collected, ground and lysed in RAPI buffer. The proteins were extracted and resolved by SDS-PAGE. Then western blotting was used to detect FRα expression in tumor tissues.

Statistical analysis. Data were expressed as mean ± SD. A two-tailed Student's t-test was used to analyze significance among the groups. A value of $P<0.05$ was considered statistically significant; $P<0.01$ was considered highly statistically significant.

Results

DNA vaccine construction. Human FRα gene was assembled into the pcDNA3.1 expression plasmid under the transcriptional control of a cytomegalovirus promoter. The resulting plasmid (pcDNA3.1/FRα) was assessed for its ability to drive protein synthesis by transient transfection of B16 cells. FRα expression on mRNA level was confirmed with RT-PCR (Fig. 1A) and its expression on a protein level was detected with western blot analysis (Fig. 1B) and immunofluorescence staining (Fig. 1C). These data indicated that the plasmid was functional and capable of inducing expression of the encoded antigen.

Establishment of FRα expressing tumor cell lines. Cells were transfected with pcDNA3.1/FRα. FRα expressing cells were selected with G418 for two weeks. Only the cells with the foreign gene integrated into their genomes could proliferate in the presence of G418. By serial dilution, two FRα expressing B16 cell lines (B62 and C411) were established. Immunofluorescence staining showed that all the cells present in the picture expressed FRα (Fig. 2).

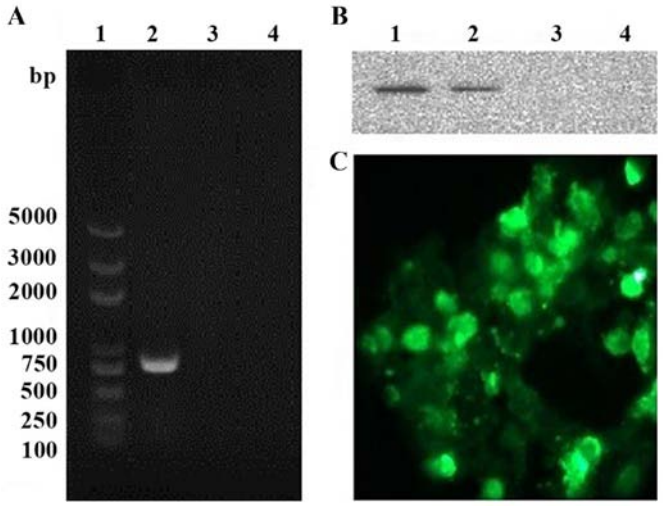

Figure 1. Expression of FRα in pcDNA3.1/FRα transfected B16 melanoma cells. (A) FRα mRNA was detected with RT-PCR. Lane 1, DNA marker; lane 2, B16 cells transfected with pcDNA3.1/FRα; lane 3, B16 cells transfected with pcDNA3.1; lane 4, B16 cells without transfection. (B) FRα protein was detected with western blotting. Lane 1, FRα recombinant protein as a control; lane 2, lysate of B16 cells that were transfected with pcDNA3.1/FRα; lane 3, lysate of B16 cells transfected with pcDNA3.1; lane 4, lysate of B16 cells without transfection. (C) Detection of FRα protein expression on the surface of pcDNA3.1/FRα transfected B16 cells detected by immunofluorescence staining (magnification, x400).

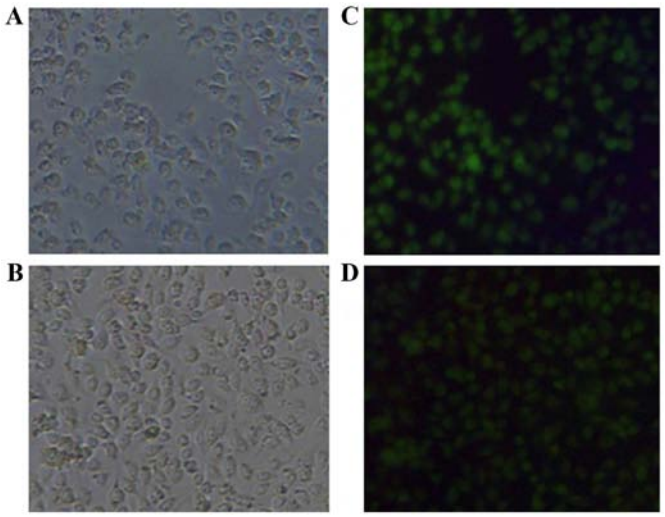

Figure 2. Immunofluorescence staining of two G418 resistant FRα-expressing B16 cell lines C411 (A and B) and B62 (C and D) (magnification, x400).

Humoral immunity induced by the DNA vaccine. To investigate humoral immune response in the mice vaccinated with pcDNA3.1/FRα, mice were immunized four times at one week intervals by intramuscular injections. Serum samples were collected one week after the last immunization and tested by ELISA for their reactivity with recombinant FRα. As shown in Fig. 3, pcDNA3.1/FRα vaccine elicited antibodies against FRα, displaying a very significant difference compared with the pcDNA3.1 group (P=0.00756). The group injected with pcDNA3.1/FRα in combination with CpG ODN showed a very significant difference compared with the pcDNA3.1 group (P=0.00726) and with the CpG ODN group (P=0.00651)

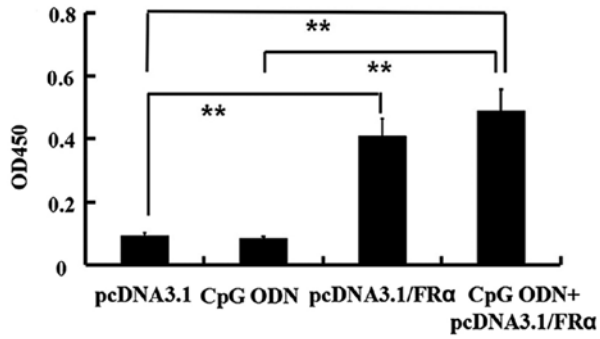

Figure 3. Generation of anti-FRα antibodies in immunized mice. Mice were injected with pcDNA3.1, CpG ODN, pcDNA3.1/FRα alone or in combination with CpG ODN once a week. After four weeks of treatment, blood samples were collected and antibody levels were tested by ELISA. **P<0.01.

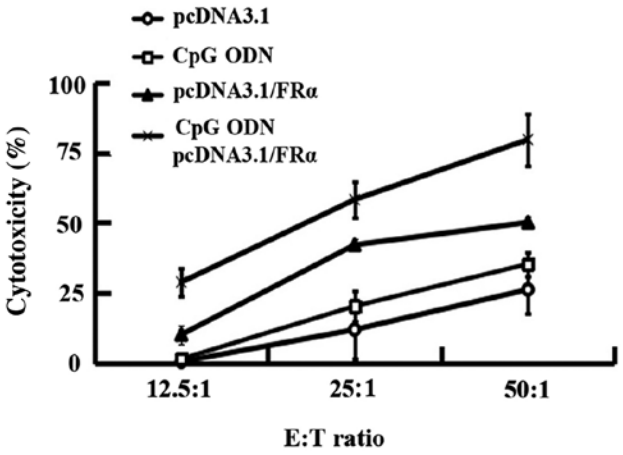

Figure 4. FRα specific CTL activities in immunized mice. Splenocytes isolated from immunized mice of each group were cultured with FRα recombinant protein for five days and used as effector cells. Cytotoxic activity was determined by LDH assay using FRα expressing B16 cells as target cells.

whereas there was not a significant difference compared with the pcDNA3.1/FRα group (P=0.7119).

Cytotoxic T-lymphocyte (CTL) assays. The FRα specific CTL activities of splenocytes from immunized C57BL/6 mice were assessed with FRα expressing B16 cells as target cells using a LDH release method. The effect of pcDNA3.1/FRα plus CpG ODN was tested in comparison with the groups treated with pcDNA3.1, CpG ODN or pcDNA3.1/FRα (Fig. 4). Four injections of pcDNA3.1/FRα resulted in a mean specific killing rate of 10% (E:T ratio of 12.5:1), 42% (E:T ratio of 25:1) or 50% (E:T ratio of 50:1). These killing rates were significantly higher than those of pcDNA3.1 treatment group with P-values of 0.013, 0.012 and 0.016 for the E:T ratio of 12.5:1, 25:1 and 50:1. The killing rates of combined immunization group with pcDNA3.1/FRα and CpG ODN were 28% (E:T ratio of 12.5:1), 58% (E:T ratio of 25:1) or 79% (E:T ratio of 50:1), which were much higher than those of the pcDNA3.1 group (P<0.001) and the CpG ODN group (P<0.001) at the corresponding E:T ratio. Furthermore, the killing rates of combined immunization group (pcDNA3.1/FRα and CpG ODN) were higher than the pcDNA3.1/FRα immunized group with P-values of 0.011, 0.024 and 0.010 at the E:T ratio of 12.5:1, 25:1 and 50:1.

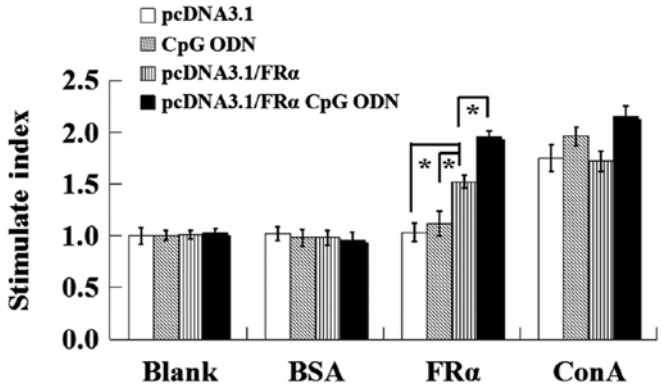

Figure 5. FRα specific lymphocyte proliferation. Mice were injected with pcDNA3.1, pcDNA3.1/FRα, pcDNA3.1/FRα in combination with 10 μg CpG ODN once a week. One week after the fourth immunization, the splenocytes were isolated and cultured without or with different stimulants (BSA, recombinant human FRα or ConA) for 72 h. MTT method was used to test the cell proliferation. The results were analyzed with the Stimulate Index (SI) defined as $OD_{experiment}/OD_{pcDNA3.1\ group\ in\ the\ blank\ group}$. $^{*}P<0.05$, $^{**}P<0.01$.

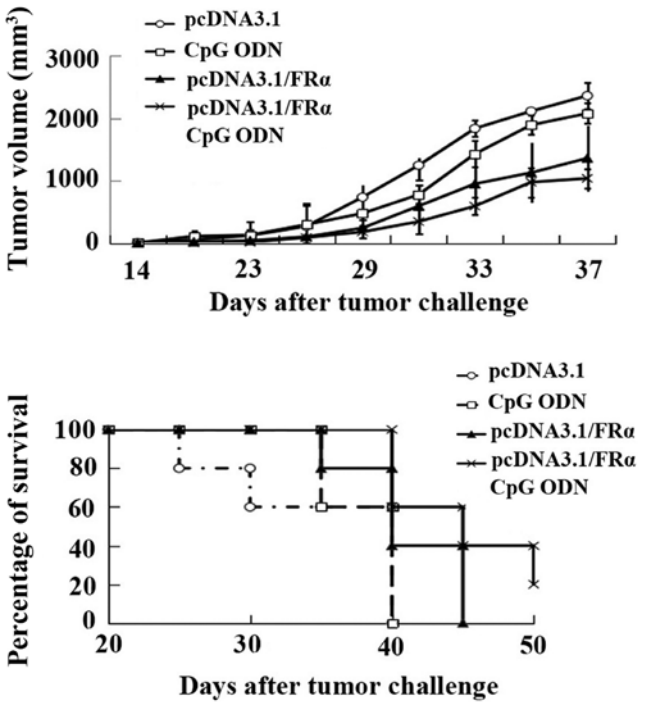

Figure 6. Immunization of mice with pcDNA3.1/FRα alone or in combination with CpG ODNs inhibited the growth of FRα expressing tumors and prolonged their survival. Female C57BL/6 mice were vaccinated at a one week interval with pcDNA3.1, CpG ODNs, pcDNA3.1/FRα and pcDNA3.1/FRα plus CpG ODNs. After four times vaccination, mice were subcutaneously inoculated with FRα expressing B16 cells. Tumor volume was measured for 37 days after tumor challenge (upper panel). Mouse survival rates were monitored for 50 days (lower panel).

Lymphocyte proliferation assays. By treating the isolated lymphocytes with recombinant human FRα, the antigen specific lymphocyte proliferation of immunized C57BL/6 mice was measured using a MTT method and compared with the non-relevant peptide group and the mitogen ConA group. As shown in Fig. 5, there was no proliferation in the blank group or the non-relevant peptide group. With FRα as a stimulant, the stimulate index of mice immunized

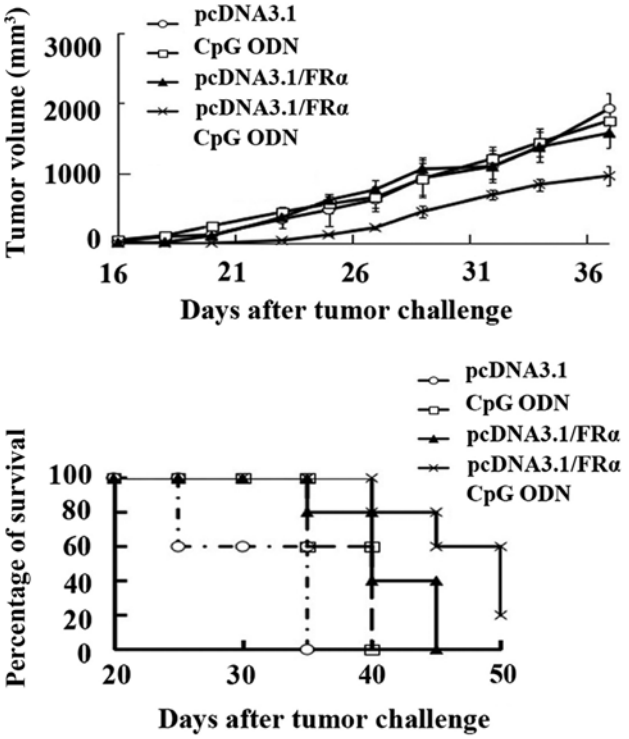

Figure 7. Therapeutic effect of mice immunized with pcDNA3.1/FRα alone or in combination with CpG ODNs. Female C57BL/6 mice were subcutaneously inoculated with FRα transfected B16 cells and then were vaccinated with pcDNA3.1, CpG ODNs, pcDNA3.1/FRα and pcDNA3.1/FRα plus CpG ODNs. The vaccinations were at one-week interval and were given four times. The tumor volume was measured for 37 days after tumor challenge (upper panel). Mouse survival rates were monitored for 50 days (lower panel).

with pcDNA3.1/FRα was significantly higher than that of pcDNA3.1 group (P=0.013) and CpG ODN group (P=0.035). While CpG ODN can further increase this antigen specific lymphocyte proliferation comparing with pcDNA3.1/FRα group (P=0.037). As a mitogen for T cells, ConA stimulated non-specific T cell proliferation and CpG ODN enhanced the stimulating effect of ConA.

Protective effect of DNA vaccination in C57BL/6 mice. One week after the final immunization (week 5), the female C57BL/6 mice (4-6 weeks, 10 mice per group) were challenged subcutaneously with $2x10^5$ FRα expressing B16 cells. Tumor growth was monitored after tumor challenge. As shown in Fig. 6, compared with the empty vector pcDNA3.1 control group, the mice immunized with CpG ODN did not show statistically reduced tumor growth whereas the pcDNA3.1/FRα immunized mice showed significantly reduced tumor growth (P=0.017). The mice injected with pcDNA3.1/FRα plus CpG ODN had reduced tumor growth with a very significant difference compared with the pcDNA3.1 control group (P=0.000281), the CpG ODN group (P=0.001) and the pcDNA3.1/FRα group (P=0.00579).

Furthermore the mice in pcDNA3.1 control group and CpG ODN group all died before day 38, whereas the mice immunized with pcDNA3.1/FRα died before day 45, showing a significant protective effect (P=0.0344). Mice immunized with pcDNA3.1/FRα plus CpG ODN (20%) still survived at day 50, showing a very significant difference compared with

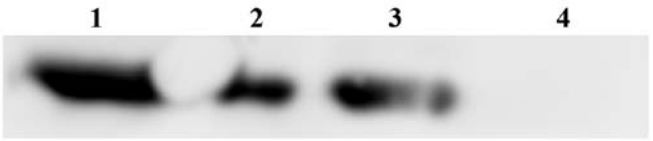

Figure 8. Analysis of FRα protein expression in tumors. Tumor tissues were collected and extracted proteins were resolved by SDS-PAGE. FRα expression was detected by western blotting. Lane 1, tumors of the pcDNA3.1/FRα plus CpG ODNs group; lane 2, tumors of the pcDNA3.1/FRα group; lane 3, tumors of the pcDNA3.1 group; lane 4, tumor cells of untransfected B16 cells.

pcDNA3.1 control group (P=0.00569) and CpG ODN group (P=0.00453), and showing a significant difference compared with pcDNA3.1/FRα group (P=0.046).

Therapeutic effect of DNA vaccination in C57BL/6 mice. To evaluate the therapeutic effect of this DNA vaccine on an existing tumor, mice (10 per group) were inoculated with FRα expressing B16 cells on day 0, and the mice were immunized with different reagents four times at one week intervals. Tumor growth was monitored daily after tumor inoculation. Fig. 7 shows that mice immunized with CpG ODN did not show reduced tumor growth compared with the empty vector pcDNA3.1 control group. The mice receiving pcDNA3.1/FRα did not show significantly reduced tumor growth (P=0.314) compared with the control group. The group injected with pcDNA3.1/FRα in combination with CpG ODN showed reduced tumor growth with a very significant difference compared with the pcDNA3.1 control group (P=0.000337) with the CpG ODN group (P=0.00579), and a significant difference compared with the pcDNA3.1/FRα group (P=0.0251).

The therapeutic experiment was followed up with survival as the end point. The mice in the pcDNA3.1 control group all died before day 34 and all the mice in the CpG ODN group died before day 38. The mice immunized with pcDNA3.1/FRα died before day 42, without a statistically significant prolonged survival compared with pcDNA3.1 control group (P=0.13). For the mice immunized with pcDNA3.1/FRα plus CpG ODN, 20% of the mice survived until day 50, showing a significant difference compared with the pcDNA3.1 control group (P=0.028), the CpG ODN group (P=0.031), and the pcDNA3.1/FRα group (P=0.0265).

Analysis of FRα protein expression in tumor tissues. After DNA vaccine treatment, tumors of mice from different treatment groups were obtained and western blotting was used to detect FRα expression in tumors. Fig. 8 shows that FRα expression was maintained in all the tested tumors.

Discussion

FRα is a tumor associated antigen. Because of its high expression in tumor cells and very limited expression in normal tissues, it is regarded as a promising target for cancer therapy (6).

Though DNA vaccination is an easy method based on its preparation, storage and safety compared with protein and peptide vaccines, its immunogenicity is usually low (11). Adjuvant is often needed to enhance its efficacy. Herein, we chose CpG ODN. It is known that CpG oligonucleotides are excellent adjuvants in murine models. When used in combination with peptide vaccines, it was as potent as the complete Freund's adjuvant regarding the induction of B cell and T cell responses. Furthermore, it is less toxic and it induces a T helper 1 (Th1) response (23). Mineral oil used with Freund's adjuvant kept a sustained release of antigen and at the same time made a local antigen depot (by entrapment of antigen in the mineral oil emulsion) where primed $CD8^+$ T cells may accumulate instead of tumor targeting (24). Alum, the adjuvant that is used routinely in human vaccination, induces the less favorable Th2 response (25).

In this study, we constructed a recombinant plasmid encoding FRα as a DNA vaccine and detected its protective and therapeutic effect in mice models when it was used alone or in combination with CpG ODN. The DNA vaccine by itself or coinjected with CpG ODN both elicited humoral and cellular immune responses. As we expected, CpG ODN as an adjuvant enhanced both humoral and cellular immune reactivity. From the data of the humoral reactivity, although there was not a statistically significant increase in antibody titer after CpG ODN inclusion, we observed that serum from mice injected with pcDNA3.1/FRα plus CpG ODN always had a higher ELISA value compared with the pcDNA3.1/FRα group (data not shown). The pcDNA3.1/FRα and pcDNA3.1/FRα plus CpG ODN vaccines both elicited FRα specific CTL response (Fig. 4) and lymphocyte proliferation (Fig. 5). CTL is a typical $CD8^+$ T cell reaction and antigen specific lymphocyte proliferation is a hallmark of $CD4^+$ cell immunity together with antigen presenting cells. Normally, $CD4^+$ and $CD8^+$ T cells perform their immune functions not in a parallel manner, but together with B cells and other immune cells, they form an immunological network. After activation by DC cells, FRα specific $CD4^+$ T cells helped FRα specific B cells to activate and become plasma cells to produce FRα specific antibodies. The activated $CD4^+$ T cells also helped FRα specific $CD8^+$ T cells to be activated to elicit their CTL function. Furthermore, the $CD4^+$ T cells might also secret cytokines to activate macrophages or other immune cells. All the above may contribute to the antitumor effect of the DNA vaccine.

In the mouse protective model, the DNA vaccine (pcDNA3.1/FRα) showed a significant protecting effect against FRα expressing tumor in tumor growth and animal survival (Fig. 6). Whereas pcDNA3.1/FRα plus CpG ODN displayed a more potent effect than DNA vaccine alone, demonstrating the stimulating effect of CpG ODN on the immune system.

In the therapeutic model (Fig. 7), although vaccination by pcDNA3.1/FRα alone did not show significant effects on tumor growth and animal survival, pcDNA3.1/FRα with CpG ODN did show a significant therapeutic effect. This demonstrated a slow immune reactivity that DNA vaccine can elicit. It took some time for the FRα specific immunity to set up in mice. CpG ODN is an excellent adjuvant in mice and stimulation via TLR9 results in the rapid activation of the innate immune system that in turn supports the induction of an adaptive immune response. CpG ODN accelerated the induction of protective antibodies and generated higher and more persistent antibody titers with protein vaccines (26). Peptide based vaccines by themselves generally failed to elicit strong immune responses (27-30). In an early phase I trial that focused

on CpG ODN as an adjuvant, 10-fold more antigen specific T cells were generated by patients with malignant melanoma immunized with the vaccine containing CpG versus the same vaccine lacking CpG (31). It was reported that recipients of the CpG ODN adjuvant vaccine developed Ag-specific CD8 T cells earlier and with significant higher frequency than the non-CpG group and the antitumor immunity arose more rapidly in patients vaccinated with CpG ODN (32). This is in line with our result. Actually, in immunotherapy of tumors, the situation of therapeutic group is closer to clinical practice. Our result confirmed that combined treatment of DNA vaccine and CpG ODN had potential in growth inhibition of FR-expressing tumors.

The B16 cell clones selected were stably transfected with pcDNA3.1/FRα. It was reported that FRα-transduced C26 cells gradually lost FRα expression and the remaining tumor cells without FRα expression were not attacked by FRα specific immune reactivity (33). Therefore, the tumor growth showed similar growth kinetics with the control group at a later stage. In the present protective and therapeutic animal experiments, mice treated with pcDNA3.1/FRα plus CpG ODN had a tumor which grew slower than that of the pcDNA3.1 or CpG ODN treatment group all through the experiment (37 days). Their growth rate neither speeded up nor showed a similar kinetics with the control group (Figs. 6 and 7). This indicated that the FRα expressing tumor cells did not lose their expression of FRα (Fig. 8), which is important in testing the effect of antitumor agents targeting FRα. Our results also correlate with the clinical research that FRα expression remains unchanged in different cancer after chemotherapy (19).

These work confirmed that CpG ODN was an excellent adjuvant even when administered in solution together with the DNA vaccine. It also confirmed that FRα represents an attractive candidate for cancer immunotherapy.

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