Antigen epitope-expressing cytokines for DNA immunization

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Abstract

Strategies to enhance the efficacy of DNA vaccination against malignancy remain to be established. In this study, a plasmid expressing a tumor antigen incorporated into the signal peptide of human IL-2 was tested as a DNA vaccine in a murine model system. Results showed that antigen-specific CTL responses were elicited by intramuscular injection of these plasmids. Importantly, compared with a minigene vector expressing the same epitope, the OVA epitope-incorporated, IL-2 expression plasmid vaccination was more effective in protecting mice from OVA-expressing tumor challenge. The improved efficacy appears to result from enhanced antigen presentation as well as the immunostimulatory activity of IL-2. This approach may provide new perspectives in designing cytokine-adjuvant DNA vaccines for clinical applications.

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1. Introduction

Nonviral genetic immunization is a promising approach to prevent and treat disease. Plasmid DNA encoding different antigens of viral, bacterial and tumor origin has been demonstrated to provoke immune responses in various species [1,2]. Both humoral and cellular responses can be generated through DNA immunization [3]. Plasmid DNA can be administered either by epidermal delivery (e.g., using gene gun) or by intramuscular inoculation (e.g., through direct injection) [4]. Plasmid DNA is safer and easier to administer than viral vaccines, and its large-scale production and storage is cheaper and easier than protein-based vaccines [5]. Therefore, DNA-based genetic immunization has been intensively investigated for the control of both infectious diseases and malignancy. However, optimal strategies to enhance the efficacy of DNA immunization remain to be established.

Cytotoxic T-lymphocytes (CTLs) play an important role in the clearance of tumor cells in an active immune response during cancer immunotherapy [6,7]. CTL recognize minimal peptides of eight to ten residues, which are presented at the cell surface by MHC class I molecules. Peptides presented by the major histocompatibility complex (MHC) class I are derived from endogenously synthesized proteins. Proteasomes degrade these endogenous proteins to generate presentable peptides [8]. Although, there might be multiple epitopes within an antigen, naturally occurring immune responses focus on relatively few epitopes rather than all possible epitopes [9]. Minigenes encoding minimal MHC class I-restricted peptides have been reported to successfully elicit epitope-specific CTL responses [10].

Cytokines, such as GM-CSF and IL-2, have been used as an adjuvant to enhance the efficacy of DNA immunization [11,12]. In a previous study, we demonstrated that it was feasible to express an antigenic epitope within a cytokine signal peptide, and that the epitope could be processed properly and presented on the cell surface [13]. In
the present study, we assessed the possibility that antigen epitope-incorporated, cytokine-expression vectors could be used as a DNA vaccine. Our data demonstrated that vaccination with antigen epitope-incorporated, cytokine expression plasmid DNA could induce antigen-specific immunity and prevent antigen-harboring tumor development. Importantly, the antigen epitope-incorporated cytokine expression plasmid vaccine was more effective than that of a minigene alone.

2. Materials and methods

2.1. Mice and cell lines

C57BL/6J mice (age 6–12 weeks) were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were maintained under specific pathogen-free conditions in the animal facility at the University of Arizona. B16, a mouse melanoma; EL-4, a mouse thymoma cell line; and E.G7, a clone of EL4 stably transfected with chicken ovalbumin cDNA, were obtained from the American type culture collection (Manassas, VA). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Irvine Scientific), 2 mM glutamine, 1 mM pyruvate, 50 μM 2-mercaptoethanol, penicillin (200 units/ml), and streptomycin (200 μg/ml) at 37 °C in a 5% CO2/95% air atmosphere. For gene-modified cells, Geneticin (600 μg/ml) (Invitrogen, Carlsbad, CA) was added to the medium.

2.2. Peptides

The H-2Kb-restricted peptide SIINFEKL, derived from chicken ovalbumin (OVA), amino acids 257–264, and SVYDFFVWL, derived from the melanoma associated antigen tyrosinase-related protein 2 (TRP-2), amino acids 180–188, were synthesized and purified commercially by Sigma-Genosys (Woodlands, TX).

2.3. Genetic constructs

The plasmid pCMV-IL-2, which expresses human IL-2 directed by the CMV promoter, and the plasmid pCOC-IL-2, which expresses human IL-2 containing the OVA epitope-modified IL-2 signal peptide, were described previously [13]. The IL-2 expression levels were determined and compared by transfection of these constructs into tumor cells. In murine B16 cells, pCOC-IL-2 expressed slightly higher amount of IL-2 compared to pCMV-IL-2 (12554 pg/ml versus 8466 pg/ml) [13]. The plasmid pCMV-MO, which expresses the OVA epitope encoded by a minigene, was constructed as follows. The schematic diagram of the expression cassettes for these plasmids is shown in Fig. 1. To construct pCMV-MO, two primers were synthesized for polymerase chain reaction (PCR) cloning. The up-stream primer 5′-ggggaattcctgcagtcacc-3′ incorporated an Ecol RI site at the 5′ end. The downstream primer 5′-tctcgagctatcagctttcaauggtgtattuc/ctatggggaaacagctgg-3′ incorporated the OVA epitope minigene sequence and an Xho site. The plasmid pCMV-IL-2 was used as a template for PCR and the PCR product was then cloned into the Eco RI and Xho digested pCMV-IL-2 vector. The resulting construct had the OVA minigene replacing the IL-2 gene. All the constructs were verified by DNA sequence analysis.

2.4. OVA epitope presentation assay

Presentation of the OVA epitope was determined using a lacZ/X-gal assay and the OVA-specific T cell hybridoma, B3Z [14]. B16 cells were transfected with plasmids pCMV-IL-2, pCMV-MO, and pCOC-IL-2 by GenePORTER 2 (Gene Therapy Systems, San Diego, CA). At 24 h after transfection, cells were treated with 100 U/ml IFN-γ to upregulate MHC class I expression [15]. At 48 h after transfection, cells were harvested and co-cultured with lacZ-transfected B3Z T cells. OVA peptide-pulsed, IFN-γ-treated B16 cells were used as a positive control. Typically, individual cultures containing 0.5 × 10⁶ transfected cells and 0.5 × 10⁶ B3Z cells, in a total volume of 0.5 ml, were set up in a 24-well plate. Cultures were then incubated for 8 h, fixed, and stained for lacZ expression using X-Gal. The cells were examined for the presence of blue (lacZ expressing) T cells microscopically and photographs were taken.

2.5. DNA vaccination

Plasmid preparations were obtained using the Qiagen Endo-free mega kit (Hilden, Germany). Purified DNA was resuspended in PBS at a final concentration of 0.5 μg/μl. Mice tibialis anterior muscle was pretreated by injection of 100 μl of 10 μM cardiotoxin (Sigma, MO). Five days later, mice received two intramuscular injections of 50 μg DNA at 2 weeks interval.
2.6. IFN-γ production assay

Spleens were harvested from DNA immunized mice 7 days after the last immunization. Splenocytes were cultured at 3 × 10^6 cells/ml in RPMI 1640 medium with mitomycin-C treated EG.7 cells at a 5:1 ratio in 24-well plates. Four days later, live cells were isolated on a lymphocyte-M gradient (Cedarlane, Ontario, Canada) and put back into culture in RPMI medium supplied with 20 U/ml IL-2 for an additional 2 days. T cells were then co-cultured with 5 × 10^3 EL-4, EG.7, OVA peptide-pulsed EL-4 or TRP-2 peptide-pulsed EL-4 cells in 200 μl medium in each well of U-bottom 96-well plates at different ratios. Culture supernatants were collected after 24 h. To determine IFN-γ production, ELISA assays were carried out using an OptEIA Mouse IFN-γ Set (PharMingen, San Diego, CA). The detection limit was 31 pg/ml.

2.7. Cytotoxicity assay

Spleens were harvested from DNA immunized mice 7 days after the last immunization. Splenocytes were cultured at 3 × 10^6 cells/ml in RPMI 1640 medium with mitomycin-C treated EG.7 cells at a 5:1 ratio in 24-well plates. Four days later, live cells were isolated on a lymphocyte-M gradient (Cedarlane, Ontario, Canada) and put back into culture in RPMI medium supplied with 20 U/ml IL-2 for an additional 2 days, and then used in the cytotoxicity assays. Cytotoxicity assays were performed using a CytoTox96® non-radioactive Cytotoxicity Assay kit (Promega, Madison, WI) as previously described. EL-4, EG.7, OVA peptide-pulsed EL-4 and TRP-2 peptide-pulsed EL-4 cells were used as target cells. Briefly, a constant number of target cells and varying numbers of effector cells were co-cultured in 100 μl medium in round-bottom 96-well plates for 6 h. 50 μl of supernatant were harvested and LDH in the supernatant were measured with a 30 min coupled enzymatic assay, which resulted in conversion of a tetrazolium salt into a red formazan product. Absorbance data at 490 nm were collected using a microplate reader.

2.8. Tumor challenge

One week after the last plasmid injection, mice were challenged in the left flank with 1 × 10^6 EL-4 or EG.7 tumor cells. Tumors were measured every other day with calipers once the tumors became palpable. The tumor volume was calculated using the formula: length × width^2 × π/6.

2.9. Statistical analysis

In vitro immune response data were collected from two individual experiments and analyzed by two-tailed Student’s t-test. Tumor protection studies were analyzed by unpaired Student’s t-test.

3. Results

3.1. The OVA epitope expressed by both the minigene and the modified signal peptide can be presented by the tumor cells

Expression and presentation of the OVA epitope encoded by the minigene plasmid (pCMV-MO) and by the OVA epitope-containing IL-2 expression plasmid (pCOC-IL-2) was confirmed by activation of an OVA/H-2Kb-specific T cell hybridoma, B16. B16 tumor cells used to stimulate B3Z T cells were first treated with IFN-γ to increase MHC class I expression. Activated B3Z T cells express lacZ and turn blue when stained with X-gal. OVA peptide pulsed B16 cells were used as a positive control to stimulate B3Z T cells. As shown in Fig. 2, B3Z T cells became activated when stimulated by tumor cells transfected with the OVA epitope-expressing plasmids pCMV-MO and pCOC-IL-2, indicating that presentation of the OVA epitope had occurred. B3Z T cells were activated when stimulated by tumor cells transfected with the OVA epitope-expressing plasmids pCMV-MO and pCOC-IL-2, indicating that presentation of the OVA epitope had occurred.
Spleen cells harvested from DNA immunized mice were stimulated in vitro with EG.7 tumor cells. T cells were then co-cultured with 5 × 10^3 EL-4, EG.7, OVA peptide-pulsed EL-4 or TRP-2 control peptide-pulsed EL-4 cells. Culture supernatants were collected 24 h after stimulation. IFN-γ in the culture supernatants was measured by ELISA assay. Data are representative of two experiments. *P < 0.01, compared with the pCMV-IL-2 immunized group, respectively.

3.2. In vitro IFN-γ production by spleen cells from immunized mice

Spleen cells from plasmid DNA immunized C57BL/6 mice were isolated, stimulated in vitro with mitomycin C-treated EG.7 tumors, and then restimulated with either EL-4 (negative control), EG.7 (positive control), OVA peptide-pulsed EL-4 or TRP-2 peptide-pulsed EL-4 cells (specificity control). IFN-γ secreted into the supernatants was then analyzed by ELISA. As shown in Fig. 3, spleen cells obtained from OVA epitope-expressing pCMV-MO and pCOC-IL-2 plasmid immunized mice secreted significant amounts of IFN-γ when stimulated with EG.7 and OVA peptide-pulsed EL-4 cells, whereas spleen cells generated from the IL-2 alone expressing pCMV-IL-2 plasmid immunized mice did not. As a control, stimulation with EL-4 and TRP-2 peptide-pulsed EL-4 cells did not produce significant IFN-γ in any of the immunized groups. These results indicated that OVA epitope-specific T cells were induced in the pCMV-MO and pCOC-IL-2 immunized mice, but not in the pCMV-IL-2 immunized mice.

3.3. Epitope-specific CTL induction by intramuscular immunization with an cytokine-expressing plasmid DNA

DNA immunization with minigenes has been shown to induce CTL responses [16]. Herein we investigated whether CTL responses could be induced by immunization with plasmid DNA encoding a cytokine gene where the signal peptide was modified with an antigen epitope. Mice immunized with the pCOC-IL-2 DNA plasmid by intramuscular injection were assayed for CTL induction against the OVA epitope. As shown in Fig. 4B, antigen-specific CTL responses were observed as indicated by increased lysis of OVA peptide-pulsed EL-4 tumor cells and OVA-expressing EG.7 cells, as compared to untreated EL-4 and TRP-2 control peptide-pulsed EL-4 cells. As a control, splenocytes obtained from mice immunized with the IL-2 alone expressing plasmid (pCMV-IL-2) lysed each target similarly, indicating that no antigen-specific CTL had been induced (Fig. 4A).

3.4. Decreased tumor development in mice immunized with antigen epitope-incorporated cytokine expression plasmid DNA

Vaccinated mice were challenged with 1 × 10^6 EL-4 or EG.7 cells to determine the ability of the plasmids to induce an antigen-specific protective immune response against the OVA-expressing EG.7 tumor. Approximately 50% of the animals vaccinated with the OVA epitope-incorporated, IL-2
Fig. 5. Protection against EG.7 tumors by DNA immunization. C57BL/6 mice were immunized twice with plasmids pCMV-IL-2, pCOC-IL-2, or pCMV-MO in 2 weeks interval. One week after the second vaccination, mice (6 mice per group) were challenged with $1 \times 10^6$ EG.7 tumor cells. Tumor development was monitored for 45 days. Controls consisted of unimmunized mice challenged with EG.7 tumor cells.

expressing plasmid (pCOC-IL-2) rejected the EG.7 tumor as compared to only 17% of the OVA minigene plasmid (pCMV-MO) vaccinated animals. No protection to EG.7 challenge was observed in animals vaccinated with IL-2 alone expression plasmid (pCMV-IL-2) with all animals developing tumors by day 7 after challenge (Fig. 5). As a control, when vaccinated animals were challenged with the EL-4 tumor, which is the parental tumor of EG.7 but does not express OVA, no protection was seen for any of the plasmid vaccines (Fig. 6A). In addition, the latency and the tumor growth rate in pCOC-IL-2 vaccinated animals were significantly prolonged when compared with that of pCMV-MO (minigene) and pCMV-IL-2 (IL-2 alone) vaccinated animals (Fig. 6B). These results demonstrated that vaccination with plasmid DNA encoding a cytokine gene in which the signal peptide was modified with an antigen epitope could induce protective immune responses to the antigen epitope. Moreover, the immune stimulatory cytokine (IL-2) expressed by pCOC-IL-2 enhanced the efficacy of the DNA immunization.

4. Discussion

DNA-based cancer vaccines are an attractive approach for cancer immunotherapy. After vaccination, plasmid DNA is taken up by host tissue and the encoded antigen is expressed. Active immune responses to the target antigen can be elicited. However, in order to control the tumor, the antitumor immune response needs to be sufficient to overcome any antigen-specific tolerance induced by the tumor. To reach this goal, several strategies designed to enhance the efficacy of DNA immunization have been explored, including co-expression of cytokines as adjuvants, coating DNA with cationic microparticles, and boosting the immunization with viral vectors [17,18]. In this study, we tested the possibility and efficacy of using tumor antigen epitope-incorporated, IL-2 expression vectors as DNA vaccines against cancer.

Immunization with minigene DNA has been reported to exclusively elicit epitope-specific CTL responses [19]. After immunization, the intracellularly expressed antigen epitope binds to MHC class I molecules in the endoplasmic reticulum (ER) and is presented on the cell surface. Numerous studies of T cell activation have revealed that T cells exhibit different levels and types of functional responses in response to different doses of antigen [20]. Therefore, the amount of antigen presented by host cells after immunization is critical in the induction of the immune response. Targeting CTL epitopes to the ER through the addition of an ER leader sequence to the minigenes was found to enhance CTL responses generated by the minigenes [16]. Signal peptides serve as a sorting signal that targets nascent secretory proteins to sites of translocation on the ER membrane, where it is subsequently proteolytically removed from the mature chain by signal peptidase [21,22].

Fig. 6. Tumor development in C57BL/6 mice after DNA vaccination. C57BL/6 mice were immunized twice with plasmids pCMV-IL-2, pCOC-IL-2, or pCMV-MO in 2 weeks interval. One week after the second vaccination, mice (6 mice per group) were challenged with $1 \times 10^6$ EL-4 (A) or EG.7 (B) tumor cells. Tumors were measured every other day once the tumors became palpable. Controls consisted of unimmunized mice challenged with tumors. $P < 0.05$, as compared to unimmunized and pCMC-IL-2 immunized groups.
Because its natural destination is the ER, signal peptides may enhance antigen presentation by providing a pathway for MHC assembly. Khanna et al. reported that ER signal sequences facilitated the transport of peptide epitopes and restored the immunogenicity of a defective antigen processing tumor cell line [23]. Sherritt et al. demonstrated that immunization with tumor-associated epitopes fused to an ER translocation signal sequence was an extremely efficient method of inducing strong immune responses which afforded protection against tumors with down-regulated expression of MHC and peptide transporters [24]. In our previous study, we demonstrated that it was feasible to express an antigenic epitope within a cytokine signal peptide, and that the epitope could be processed properly and presented on the cell surface [13]. In the current study our data demonstrated that antigen-specific immune responses were induced after animals were immunized with an antigen epitope-incorporated cytokine expression plasmid.

Cytokines have been used as adjuvants to enhance the efficacy of DNA immunization. Irvine and his group tested a panel of cytokines given by systemic administration and found that rhIL-2, rmIL-6, rhIL-7 and rmIL-12 were able to enhance the therapeutic responses of DNA vaccination [25]. Co-expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) with antigen in a DNA vaccine has been reported to result in improved immunization [26,11]. Since minigenes do not contain T-helper cell epitopes, an appropriate cytokine environment might be helpful and even necessary to foster activation of antigen presenting cell (APC) and/or CTL for minigene-based DNA immunization. In the present study, we have developed a new strategy to improve the efficacy of DNA vaccination. Through modifying the cytokine signal peptide with an antigen epitope, the antigen epitope and cytokine expression were coupled together in the same plasmid. Our results showed that the antigen epitope expressed within the modified cytokine signal peptide was presented by transfected cells. Importantly, an enhanced vaccination effect was achieved with the antigen epitope-incorporated cytokine expression plasmid as compared to the minigene epitope expression plasmid. The improved antigen presentation might result from the ER-targeting function of the signal peptide, synergizing with the immunostimulatory function of the cytokine simultaneously expressed with the antigen epitope. Moreover, the immunostimulatory activities of the cytokine simultaneously expressed with the antigen epitope can potentiate the immunization effect.

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References


