Engineering Enhancement of Immune Responses to DNA-based Vaccines in a Prostate Cancer Model in Rhesus Macaques through the Use of Cytokine Gene Adjuvants

J. Joseph Kim, Joo-Sung Yang, Kesen Dang, Kelley H. Manson, and David B. Weiner


Abstract

DNA immunization is an important vaccination technique that is being explored as an immunotherapeutic strategy against a variety of infectious diseases as well as cancer. We have been investigating the utility of DNA-based vaccine strategy against prostate cancer. We have developed a DNA vaccine construct that encodes for the human prostate specific antigen (PSA) gene. PSA expression is limited to prostatic tissue, and the level of PSA expression is substantially increased in prostate cancer cells. This tissue specificity makes PSA a potential target for the development of immunotherapies against prostate cancer. A DNA-based PSA vaccine was used to elicit PSA-specific host immune responses in rodent and nonhuman primate models. In an effort to enhance the clinical utility of the DNA-based PSA vaccine, we also examined the use of cytokine gene adjuvants to modulate vaccine-induced immune responses in animal models. We observed that pCPSA vaccine-induced humoral and cellular immune responses can be modulated through the coimmunization with cytokine genes in mice, and these enhancement effects on the PSA-specific cellular responses were extended in macaques. More specifically, coimmunization with interleukin (IL)-2 cDNA construct resulted in a significant enhancement of PSA-specific antibody responses in both mice and macaque models. In contrast, coinjection of IL-12 resulted in reduction of antibody responses in both models. In mice, the groups coimmunized with IL-2, IL-12, or IL-18 showed a dramatic increase in T helper cell proliferation over the results with pCPSA alone. These results support that further evaluation of this vaccination strategy to treat prostate cancer is warranted.

Introduction

Prostate cancer is the second leading cause of cancer-related death in American men (1). In the United States alone, ~184,500 men were diagnosed with prostate cancer, and 39,200 men died of the disease in 1998 (2). Current treatment options for prostate cancer include radical prostatectomy, radiation therapy, or hormonal therapy. Although traditional surgical androgen deprivation has been largely replaced by hormonal therapy, no systemic therapy has clearly improved hormone-refractory disease. Even with early treatment through surgery or radiation therapy, complete eradication of the tumor is not always achieved and can lead to unwanted side effects, such as impotence and urinary incontinence (3-5).

More recently, immune-based therapeutic strategies have been examined as alternatives to current cancer treatments. The prostate is a very specific-function organ that does not produce life-sustaining compounds. Accordingly, antigens that are expressed specifically within the prostate represent tissue-specific antigens. In the case of prostate cancer, these antigens would represent potential targets for immunological intervention. One of these antigens is PSA. PSA is a 240-amino acid member of the glandular kallikrein gene family (6, 7). PSA is a serine protease secreted by both normal and transformed epithelial cells of the prostate gland (6). PSA can be detected in the sera of healthy males at low levels, even without clinical evidence of prostate cancer. However, PSA level is secreted more substantially by cancer cells (6). Because PSA expression appears to be limited to prostate cells, it now is the most widely used marker for prostate cancer (8, 9). Furthermore, the tissue specificity of PSA makes it a potential target for the development of immunotherapies against prostate cancer (10, 11).

We have reported previously on the construction of DNA vaccine that encodes for the expression of human PSA (12). Immunization of this vaccine construct in mice resulted in induction of strong and persistent humoral and cell-mediated immune responses specific to PSA (12). More recently, the DNA-based PSA vaccine was examined in rhesus macaques to study the induction of immune responses and safety profiles after immunization.

DNA vaccination is an important candidate for potential immunotherapy against cancer. DNA immunization strategy delivers DNA constructs encoding for a specific immunogen.

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2 To whom requests for reprints should be addressed, at Department of Pathology and Laboratory Medicine, University of Pennsylvania, 505 Stellar-Chance, 422 Curie Boulevard, Philadelphia, PA 19104. Phone: (215) 349-8365; Fax: (215) 573-9436; E-mail: dbweiner@mail.med.upenn.edu.
4 The abbreviations used are: PSA, prostate-specific antigen; SI, stimulation index; IL, interleukin; LPA, lymphoproliferative assay.
Fig. 1 Induction of antigen-specific humoral and cellular immune responses after DNA immunization. Th1, T helper 1; Th2, T helper 2.

We and others have been investigating the use of molecular adjuvants as a method of enhancing and modulating immune responses induced by DNA immunogens (14–17). Codelivery of these molecular adjuvants consisting of expression plasmid encoding for immunologically relevant molecules, including costimulatory molecules, adhesion molecules, chemokines, and cytokines with DNA vaccine constructs, led to modulation of the magnitude and direction (humoral or cellular) of the immune responses induced in mice (14–17). More recently, the ability of this strategy to significantly modulate DNA vaccine-induced humoral responses in primates has been reported (18). In this report, we examined the modulatory effects of cytokine gene adjuvants to PSA DNA-based vaccines in mice and in rhesus macaques. We observed that antigen-specific humoral and cellular immune responses can be modulated in both rodent and macaque models using the cytokine molecular adjuvant approach.

Materials and Methods

Construction and Expression of pCPSA DNA Constructs

DNA vaccine cassettes expressing PSA were constructed by cloning the complete coding sequence of PSA into clinical
expression vector under the control of cytomegalovirus promoter (12).

**DNA Inoculation of Animals**

**Mice.** The quadriceps muscles of BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN), 6–8 weeks of age, were injected with 50 μg of pCPSA DNA construct formulated in PBS and 0.25% bupivacaine-HCl (Sigma Chemical Co., St. Louis, MO; Ref. 14). The mice were housed in a temperature-controlled, light-cycled room. Their care was under the guidelines of the NIH and the University of Pennsylvania. The control mice were immunized with 50 μg of pCDNA3 vector. Each set of studies was performed three times, and a representative set of results is presented.

**Rhesus Macaques.** Rhesus macaques (Macaca mulatta) were individually housed at the Primedica Mason Laboratories (Worcester, MA). All animal care and use procedures conformed to the revised Public Health Service Policy on Humane Care and Use of Laboratory Animals. Animals were anesthetized with ketamine HCl for all technical procedures. Macaques were immunized i.m. in the quadriceps with DNA preparations formulated in PBS and 0.25% bupivacaine-HCl on multiple occasions.

**ELISA**

Serum antibody reactivity purified PSA protein was analyzed by ELISA, as described previously (18). Briefly, recombinant PSA protein (Fitzgerald Industries) was resuspended in PBS to a concentration of 0.5 μg/ml. Fifty μl (25 ng) of each protein preparation were incubated in each of the ELISA wells overnight at 4°C. Plates were then rinsed with washing buffer (0.45% NaCl in deionized water containing 0.05% Tween 20) and blocked with blocking buffer (5% nonfat dry milk in PBS with 1% BSA and 0.05% Tween 20) for 2 h at 37°C. Serum samples were then diluted in dilution buffer (5% nonfat dry milk in PBS with 0.05% Tween 20) at the appropriate dilutions and incubated in duplicate or triplicate in recombinant protein-coated wells for 1 h at 37°C, washed, and then incubated for 1 h at 37°C with a goat antihuman immunoglobulin-hors eradish peroxidase conjugate (Sigma) diluted in dilution buffer at the concentration suggested by the manufacturer. After extensive washing, the plates were developed with 3,3',5,5'-tetramethylbenzidine dihydrochloride substrate (100 μg/ml), the reaction was stopped with 2 N H2SO4, and color development was quantitated at 450 nm. BSA-coated wells were used as negative binding control wells in these assays. Specific binding (absorbance at 450 nm) was calculated by subtracting A450 values from sera samples bound to BSA (i.e., control) from A450 values from sera samples bound to PSA, i.e., experimental wells (A450experimental – A450control).

**Isolation of Lymphocytes in Mice**

Lymphocytes were harvested from spleens and prepared as the effector cells by removing the erythrocytes and by washing several times with fresh medium as described for macaques. Peripheral blood lymphocytes were prepared as described previously. The isolated cell suspensions were resuspended to a concentration of 5 × 10^6 cells/ml in a medium consisting of RPMI 1640 (Life Technologies, Inc.) with 10% FCS (Life Technologies, Inc.).

**Lymphoproliferative Assay**

A 100-μl aliquot containing 5 × 10^5 cells was immediately added to each well of a 96-well microtiter round-bottomed plate. Recombinant PSA protein (Fitzgerald Industries) at the final concentrations of 5 and 1 μg/ml were added to wells in triplicate. The cells were incubated at 37°C in 5% CO2 for 3 days. One μCi of tritiated thymidine was added to each well, and the cells were incubated for 12–18 h at 37°C. The plates were harvested, and the amount of incorporated tritiated thymidine was measured in a Beta Plate reader (Wallac, Turku, Finland). The SI was determined from the formula: SI = (experimental count/spontaneous count). Spontaneous count wells (media only) included 10% FCS. To assure that cells were healthy, ConA (Sigma) was used as a polyclonal stimulator positive control. The data were analyzed statistically using a paired Student’s t test.

**Results**

**Induction of PSA-specific Immune Responses in Mice.** DNA vaccine constructs expressing human PSA (pCPSA) were cloned into an expression vector under control of a cytomegalovirus promoter as described previously (12). We immunized mice i.m. with this DNA-based PSA vaccine (pCPSA) and investigated the in vivo immune responses generated. Mice received two DNA immunizations i.m. (50 μg each) at 0 and 2 weeks with either the pCPSA or the control pCDNA3 vectors. Sera from immunized mice were collected at 0, 2, 4, and 8 weeks after immunization and were analyzed for specific antibody responses against PSA protein by ELISA at 1:100 dilution (Fig. 2A). The preinjection sera collected at week 0 as well as the control animals immunized with the control vector did not show any PSA-specific antibody response. However, at 1:100 dilution, the PSA-specific antibody response was detected after immunization.

At 1 week after the boost injection, the mice were euthanized, and their spleens were harvested. We isolated lymphocytes from the spleen and tested for T-cell proliferation as described. Recombinant PSA protein (5 and 1 μg/ml) was plated in each well for specific stimulation of T-cell proliferation. The proliferation assay results for the mice immunized with pCPSA vaccine are shown in Fig. 2B. A background level of proliferation was observed in the control group. However, a significant level of T-cell proliferation (SI >2.0) was observed at both 5- and 1-μg/ml concentrations with SIs of 4.0 and 2.3, respectively.

**Induction of PSA-specific Immune Responses in Rhesus Macaques.** We investigated whether the induction of PSA-specific immune responses observed in mice through DNA immunization could also be achieved in rhesus macaques. Four rhesus macaques were immunized i.m. with 500 μg of pCPSA DNA vaccine construct at weeks 0, 4, and 10. The sera samples were collected at various time points after immunization and were assayed for the induction of PSA-specific IgG antibodies. As shown in Fig. 3A, the two macaques immunized with the control pCDNA3 vector did not show any PSA-specific anti-
Body response. In contrast, the PSA-specific humoral response was detected in the immunized macaques as early as 6 weeks after immunization. Overall, the level of PSA-specific antibody response in macaques appeared to be lower than those in mice observed above.

Induction of PSA-specific LPA response was examined in rhesus macaques. As shown in Fig. 3B, a significant level of PSA-specific LPA response (SI >2.0) was observed in PSA-immunized macaques at 14 weeks after immunization. As with the level of the humoral response observed above, the level of PSA-specific LPA response seemed to be lower than the level observed in mice.

**Modulation of PSA-specific Immune Responses in Mice.** Several groups including ours have been investigating the use of molecular adjuvants as a method of enhancing and modulating immune responses induced by DNA immunogens (16, 17). We investigated the immunostimulatory effects this strategy has on the pCPSA vaccine-induced immune responses in mice. IL-2, IL-12, and IL-18 (50 μg of each cytokine gene along with pCPSA) were injected into BALB/c mice i.m. at weeks 0 and 2. The first immune parameter examined was the antigen-specific humoral response. Fig. 4A shows the PSA-specific antibody responses observed over 8 weeks. In this experiment, we observed that coinjection of cytokine genes can differentially modulate the vaccine-induced antibody responses. For instance, coinjection of IL-2 resulted in an enhancement of antibody responses, whereas coinjection of IL-12 resulted in reduction of antibody response. These results are
consistent with the previous observations using DNA vaccine constructs for HIV-1 and herpes simplex virus (15, 17). We also observed that the effects of IL-18 coimmunization were moderate compared with the group immunized with pCPSA alone.

The effects of cytokine coadministration on the PSA-specific T helper cell lymphoproliferative responses were also examined in mice. Activation and proliferation of T helper lymphocytes play an important role in inducing both a humoral and cellular immune responses. At 1 week after the second coinjection with pCPSA and cytokine adjuvants, the mice were sacrificed, the spleens were harvested, and the lymphocytes were isolated and tested for T helper cell proliferation (14). As shown in Fig. 4B, a low background level of proliferation was observed in the control group. A significant level of proliferative responses was observed in the group immunized with pCPSA alone. Moreover, the groups coimmunized with IL-2, IL-12, or IL-18 showed a dramatic increase in T helper cell proliferation over the results with pCPSA alone.

**Modulation of PSA-specific Immune Responses in Rhesus Macaques.** Important for the ultimate use of this vaccine technology in humans is that the results originally observed in mouse systems translate to primate models. Previously, it has been reported that primates may have a limited ability to produce DNA vaccine-encoded proteins through direct genetic inoculation into muscle (19). More specifically, it has been suggested that DNA immunizations alone in primates are not sufficient to generate high levels of antigen-specific antibody responses.

The enhancement effects of cytokine gene coadministration on the PSA-specific immune responses were further examined in macaques. Five groups of two rhesus macaques were immunized with pCDNA3, pCPSA, pCPSA + IL-2, pCPSA + IL-12, and pCPSA + IL-18 at 0 and 4 weeks with 500 μg of each DNA. Similar to what was observed in mice, we observed that coimmunization IL-2 resulted in an enhancement of antibody responses, whereas coinjection of IL-12 seemed to miti-
Fig. 5  A, modulation of PSA-specific antibody responses with cytokine expression cassettes in rhesus macaques. Five groups of two rhesus macaques were immunized with pCDNA3, pCPSA, pCPSA + IL-2, pCPSA + IL-12, and pCPSA + IL-18 at 0 and 4 weeks with 500 μg of each DNA. The sera samples were collected and were assayed for the induction of PSA-specific antibodies by ELISA. B, modulation of PSA-specific lymphoproliferative responses in rhesus macaques. Induction of lymphoproliferative responses against PSA proteins was examined in immunized animals. Each value represents the average of two animals in each group. Data are results from the immunized animals (P < 0.01); bars, SD.

Fig. 5 A. modulation of PSA-specific antibody responses with cytokine expression cassettes in rhesus macaques. Five groups of two rhesus macaques were immunized with pCDNA3, pCPSA, pCPSA + IL-2, pCPSA + IL-12, and pCPSA + IL-18 at 0 and 4 weeks with 500 μg of each DNA. The sera samples were collected and were assayed for the induction of PSA-specific antibodies by ELISA. B, modulation of PSA-specific lymphoproliferative responses in rhesus macaques. Induction of lymphoproliferative responses against PSA proteins was examined in immunized animals. Each value represents the average of two animals in each group. Data are results from the immunized animals (P < 0.01); bars, SD.

As shown in Fig. 5B, induction of lymphoproliferative responses against PSA proteins were examined in immunized animals. We again observed that immunization of macaques with pCPSA vaccine regimen resulted in an induction of PSA-specific lymphoproliferative responses. Coimmunization with IL-2, IL-12, and IL-18 constructs all enhanced the level of PSA-specific lymphoproliferative responses in macaques. In contrast to what was observed in mice, IL-2 coimmunization resulted in the highest level of enhancement, whereas the coimmunizations with IL-12 and IL-18 resulted in more moderate levels of enhancement.

Discussion
Prostate cancer is the most common malignancy in American men and is the second leading cause of cancer-related death in the male population (1). The present treatment for prostate cancer includes radical prostatectomy, radiation therapy, or hormonal therapy. Even the most advanced treatment through surgery or radiation therapy may not achieve complete eradication of the tumor and can lead to unwanted side effects, such as impotence and urinary incontinence (3-5).

One promising novel approach to treat cancer is an immune-based therapeutic strategy. Several immunotherapeutics in different cancer models have been investigated (20). Examples include the injection of live, irradiated, and autologous tumor cells transfected with cytokines (21, 22). Other approaches include recombinant vaccinia virus engineered to express tumor-specific antigens, and more recently, autologous dendritic cells loaded with peptide sequences of cancer antigens have been investigated as possible therapeutic vaccines (11, 23-25). Additionally, a promising approach includes the fusion of cancer cells with dendritic cells as putative immune therapeutic vaccine candidates. This approach is attractive because it appears established that the most effective antigen-presenting cells are mature activated dendritic cells. However, a significant drawback of these approaches is that they require customization for each patient.
DNA vaccination is an important candidate for immunotherapy against cancer. A major focus of developing DNA vaccines against cancer has been the use of tumor-associated antigens. These are proteins produced by tumor cells that can be presented on the cell surface in the context of MHCs (26). The prostate represents a good target for DNA vaccine-based therapy. The prostate is a very specific-function organ that does not produce life-sustaining compounds. PSA is a 240-amino acid serine protease produced by both normal and transformed epithelial cells of the prostate gland, but the cancer cells secrete much higher levels of the antigen (27). Furthermore, the tissue specificity of PSA makes it a potential target for the development of immunotherapies against prostate cancer (10, 11). We have reported previously on the construction of DNA vaccine that encodes for human PSA (12). Immunization of this vaccine construct in mice resulted in induction of strong and persistent humoral and cell-mediated immune responses (12).

Induction of immune responses and safety profiles in rhesus macaques immunized with DNA-based PSA vaccine was examined in a recent study. Because of the high degree of similarity between the rhesus and human prostate gland and PSA (94%), this animal model was well suited to accurately assess the effects of PSA vaccine. In this study, induction of PSA-specific humoral response as well as positive PSA-specific LPA response in the vaccinated macaques was observed. In addition, the stimulated T cells from the PSA-immunized rhesus macaques produced higher levels of T helper 1 type cytokine IFN-γ than the control vector-immunized animals, indicating that T cells were activated by PSA immunization. These results demonstrate the potential utility of targeting PSA as a testable immunotherapy against prostate cancer.

Although the pCPSA immunizations induced humoral and cellular immune responses, the injections did not result in any adverse effects in the immunized macaques, as indicated by complete blood counts, leukocyte differentials, and hepatic and renal chemistries. The macaques appeared healthy, without any physical signs of toxicity, throughout the observation period. These safety observations are consistent with the results observed in humans receiving vaccination with DNA constructs encoding for HIV-1 antigens (28). Overall, the vaccine recipients received the vaccines in a well-tolerated manner, with no significant clinical or laboratory adverse effects measured in all dosage groups.

These early clinical studies have established the ability of the DNA vaccines to elicit immune responses in a safe and well-tolerated manner (29). However, significant efforts have also focused on improving the immune potency of this technology because it is unclear whether, in its present form, its potency will be clinically useful. Improving vaccine potency is a central mission of the field of DNA vaccines. One strategy to enhance immune responses for DNA-based vaccines is the use of molecular adjuvants (13). These molecular adjuvant constructs could be coadministered, along with immunogen constructs, to modulate the magnitude and direction (humoral or cellular) of the immune responses induced by the vaccine cassettes themselves. Such use of molecular adjuvant constructs results in concurrent kinetics of in vivo expression for both the adjuvant and antigen proteins.

In this report, we examined the modulatory effects of cytokine gene adjuvants to PSA DNA-based vaccines in mice and in rhesus macaques. We observed that pCPSPA vaccine-induced humoral and cellular immune responses can be modulated through the coinmunization with cytokine genes in mice, and these enhancement effects on the PSA-specific cellular responses were extended in macaques. For instance, coinmunization of IL-2 resulted in a significant enhancement of PSA-specific antibody responses in both mice and macaque models. These results are consistent with the results obtained by coinmunizing IL-2 cDNA constructs with DNA vaccines for HIV-1 (18). On the other hand, coinjection of IL-12 genes resulted in reduction of antibody responses in both models. In mice, the groups coimmunized with IL-2, IL-12, or IL-18 genes showed a dramatic increase in T helper cell proliferation over the results with pCPSPA alone. These enhancement effects on the PSA-specific cellular responses were extended in macaques, although their effects were less dramatic. To our knowledge, this is the first report of the enhancement of T cell-mediated immune responses using cytokine molecular adjuvants in rhesus macaques.

Overall, the immunomodulatory effects of cytokine gene adjuvants were more moderate compared with those observed in mice. There could be several hypothesis for this observation. One possible cause is that primates may have a limited ability to produce DNA-encoded proteins through direct genetic inoculation into muscle (19). Another potential cause is that human cytokine cDNA constructs were used to immunize macaques, whereas mice cytokine cDNA constructs were used to immunize mice. Although human IL-2, IL-12, and IL-18 are cross-reactive in macaques, there may be some reduction in potency. Using macaque cytokine cDNA constructs to immunize macaques could further enhance their immunomodulatory effects to the levels observed in mice.

References

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