Identification of peptide mimotopes of gp96 using single-chain antibody library

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Abstract Heat shock proteins such as gp96 are immunogenic and are widely used as vaccines in immunotherapy of cancers. The present study focuses on the use of peptide mimotopes as immunotherapeutic vaccines for prostate cancer. To this end, we developed a 15-mer gp96 peptide mimotope specifically reactive to MAT-LyLu gp96–peptide complex using combinatorial single-chain antibody and peptide phage display library. The immunogenicity of the synthesized gp96 mimotope was analyzed initially in normal BALB/c mice in combination with various adjuvants such as complete Freund’s adjuvant (CFA), aluminum salts (ALUM), granulocyte-macrophage colony-stimulating factor (GM-CSF), and liposome, of which CFA served as a positive control. The antibody response was determined and found that the gp96 mimotope with ALUM showed a significant increase in antibody titer, followed by GM-CSF and liposomes. Further, the T cell (CD4⁺ and CD8⁺) populations from splenocytes, as well as IgG isotypes, interleukin-4, and interleukin-5 of gp96 mimotope with ALUM-immunized animals, were analyzed. The results suggest that the gp96 mimotope may elicit a potent and effective antitumor antibody response. Further, the study identifies ALUM and GM-CSF as adjuvant options to drive an appropriate protective immune response as these adjuvants have prior use in humans.

Keywords Heat shock protein · gp96 · Mimotope · Phage display · Prostate cancer

Introduction

Earlier approaches for the search of cancer antigens from wide varieties of cancers including both chemical and spontaneous origins have led to the recognition of heat shock proteins (HSPs) as one of the most effective cancer rejection molecules. This rejection property has been directly attributed to its peptide chaperoning capability (Srivastava 2002). HSPs are a group of ubiquitous intracellular multifunctional proteins; they are of different types with different molecular weights and different intracellular localizations such as HSP60, HSP70, HSP90, and gp96. HSPs are induced under stress conditions and assist vital cellular functions including protein translocation, folding, unfolding, and assembly (Fink 1999; Hartl and Hayer-Hartl 2002). They also play crucial roles in antigen presentation, activation of lymphocytes, macrophages activation, and maturation of dendritic cells (Li et al. 2002; Wallin et al. 2002; Tsan and Gao 2004; Calderwood et al. 2007; Pockley et al. 2008). Consequently, these immunological properties of HSPs pave a novel stalk for the development of tumor immunotherapy. Earlier studies in our laboratory and others reported that immunizing mice with tumor-derived gp96 elicited an antitumor immune response thereby leading to tumor rejection and repression of metastatic tumor progression (Yedavelli et al. 1999; Srivastava et al. 1986). The anticancer immune response of HSPs is attributed to the cross-presentation of chaperone peptide antigens in antigen-presenting cells (APCs) and induces a stronger cytotoxic T-lymphocyte (CTL) response against antigenic peptides (Li et al. 2002; Bendz et al. 2007). Further, studies have demonstrated that tumor immunity of HSP–peptide complex results from the peptides associated with HSPs and not from HSPs or other contaminating proteins (Udono et al. 1994; Binder et al. 2007).

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In addition, the immunogenicity of the HSP-peptide complex also result from a peptide-independent immunomodulatory ability to induce an innate immune response via stimulating the production of pro-inflammatory cytokines including TNF- α , IL-1, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), and the release of NO and C-C chemokines by macrophages and dendritic cells (Janeway and Medzhitov 2002). HSPs also stimulate the cytolytic activity of NK cells thereby contributing to tumor immunity (Multhoff et al. 1999; Gastpar et al. 2004). Thus, the diverse range of immune responses elicited by HSPs allows them to act as potent multivalent vaccines. Further, they elicit immunity to the entire range of epitopes of the cancers thereby avoiding the difficulties in the identification of epitopes from individual cancers and are reported to efficiently abrogate primary and metastatic diseases.

Several murine studies have demonstrated that tumor-derived HSPs, in particular HSP70 and gp96, are potent inducers of immunity stimulating tumor-specific CTL responses. Further, they have been deciphered into ongoing clinical trials, where currently gp96-based vaccines have been used in phase III clinical trials for melanoma and renal cell carcinoma and found clinically advantageous in a subsets of patients, displaying least toxicity and better efficacy (Testori et al. 2008; Assikis et al. 2003; Janetzki et al. 2000; Belli et al. 2002; Mazzaferro et al. 2003). Though gp96-based vaccines efficiently bind the full repertoire of tumor-associated antigens and undergoes T cell priming leading to tumor rejection *in vivo*, the constraints in the availability of sufficient amount of tumor-derived HSPs (Li 1997; Oki and Younes 2004) limits the use of these vaccines plausibly in clinical trials in curing various cancers. An alternative approach to overcome this problem is the use of mimotopes which mimic natural protein epitopes and activate the immune system against the original antigen and provoke specific immune response, for the development of vaccines against tumors (Sharav et al. 2007).

In the present study, we utilized the phage display technique to develop a therapeutic gp96 mimotope that complemented the screening of gp96 isolated from the MAT-LyLu prostate cancer cell line. Further, in order to induce a potent and persistent immune response using peptide-based vaccines, adjuvant support is critical and is considered to be a serious component of a rational peptide vaccine design. Adjuvants improve the performance of vaccines by targeting the antigen to APCs, stimulating cytokines that direct T_H or CTLs responses, promoting cell-mediated immunity and reducing the number of immunizations or the amount of antigen required for protective immunization (Guy 2007). Further use of safe and effective adjuvants also plays a vital role in driving appropriate protective immune response, as adjuvants can cause toxic

side effects. Thus, in the present study, immune responses of gp96 mimotope-based vaccine were studied in BALB/c mice using four types of adjuvants including complete Freund's adjuvant (CFA), aluminum salts (ALUM), GM-CSF, and liposome.

Materials and methods

Growth of cell lines *in vivo* and *in vitro*

Dunning G and MAT-LyLu were grown in culture using RPMI-1640 media supplemented with 10% fetal bovine serum (Gemini, Calabasas, CA, USA), 50 IU/ml of penicillin (Mediatech, Herndon, VA, USA), 50 μ g/ml streptomycin (Mediatech, Herndon, VA, USA), and 2 mM of L-glutamine (Mediatech, Herndon, VA, USA). Tumors used to purify gp96 were obtained by xenografting human prostate cancer cell lines in nude mice, whereas MAT-LyLu and Dunning G tumors were grown by injecting the cell lines into syngenic Copenhagen rats. Upon tumor growth, the animals were euthanized and the excised tumors were snap-frozen in liquid nitrogen and subsequently stored at -80°C and used for gp96 purification.

Gp96 purification

Gp96 was purified from prostate tumor tissues as described earlier by Suto and Srivastava (1995) and Suriano et al. (2005). Tumor tissues (7 g) were homogenized with a Brinkmann homogenizer by incubation on ice in a solution of 30 mM sodium bicarbonate, pH 7.0. Tissue samples were then centrifuged at 7,000 rpm (Beckman Ti-45 rotor) for 10 min after which the supernatant was centrifuged at 27,000 rpm (Beckman Ti-45 rotor) for 90 min. The supernatants were then subjected to successive 50% and 80% ammonium sulfate precipitations, after which the supernatant was applied to concanavalin A-Sepharose column (Pharmacia, Uppsala, Sweden). Glycoproteins were eluted out with 10% methyl- α -D-mannose pyranoside (Sigma Chemical Company, St. Louis, MO, USA). This high concentration of mannose (10%) used completely elutes out bound gp96. The collected fractions were then applied to PD-10 columns (Pharmacia, Uppsala, Sweden) equilibrated with 10 mM sodium phosphate buffer, pH 7.0 containing 300 mM NaCl. After the PD-10 column, samples were loaded on to a DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column equilibrated with 10 mM sodium phosphate buffer, pH 7.0 containing 300 mM NaCl, and bound gp96 was eluted in seven separate fractions with 10 mM sodium phosphate buffer, pH 7.0, containing 700 mM NaCl. The eluted proteins are determined on an SDS-PAGE.

Selection of gp96-specific single-chain antibodies

Gp96 was panned against single-chain antibody (scFv) library by Nissim et al. (1994). The library was subjected to three rounds of panning. Specific scFv phage was affinity selected by using proteins adsorbed to Maxisorp immunotubes (Nunc). For selection of specific gp96, immunotubes were coated with gp96 complex overnight at room temperature (RT) by using 10 µg/ml in phosphate-buffered saline (PBS) for the first round, 5 µg/ml for the second round, and 2.5 µg/ml for the third and fourth rounds of panning. The immunotubes were blocked with Blotto (4% skimmed milk in PBS) for 1 h at RT and then 10^{13} TU scFv phages were added into the immunotube in 2% skimmed milk/2% bovine serum albumin (BSA) in PBS. After 2 h of incubation with rocking at RT, unbound and nonspecifically bound scFv phages were eluted by using ten washes with PBS/0.1% Tween-20 and ten washes with PBS. The specifically bound scFv phage was eluted with 1 ml elution buffer (100 mM HCl, adjusted to pH 2.2 with solid glycine and containing 0.1% BSA) for 10 min at RT. The eluate was neutralized with 60 µl of 2 M Tris base and was used to infect freshly prepared *Escherichia coli* cells. The scFv phages specific for MAT-LyLu gp96 were rescued and used for further rounds of selections, and phage clones specific for MAT-LyLu gp96 were screened using enzyme-linked immunosorbent assay (ELISA).

Panning with X-15 phage peptide library

X-15 phage display peptide library (linear 15-mer) was gifted from George P. Smith, University of Missouri, Columbia, MO, USA. The library was amplified and 1×10^{12} phages were used for panning against E6 scFv clone (specific for gp96-peptide complex) to select affinity peptides to E6 as described by Bonnycastle et al. (1996). Five micrograms of E6 single-chain antibody was coated overnight at 4°C. The wells were washed and blocked with 300 µl Blotto (5% milk, 10 mM EDTA) at RT for 2 h. Subsequently, 50 µl Blotto and 100 µl TBS containing 10^{12} virions were added to each well and incubated at 4°C in a humidified box for 4 h. The wells were washed and bound phages eluted by adding 35 µl of elution buffer (0.1 M HCl, pH 2.2) and incubating at RT for 10 min. Eluted phages were neutralized with 6.6 µl of 1 M Tris, pH 9.1 and then infected into *E. coli* K91 strain and amplified for further rounds of panning. Three rounds of panning were performed to select, enrich, and amplify specific phages. The specificity of phages was tested by immunoscreening and ELISA. Purified DNA from phage clones was sequenced using the sequencing primer GCCAATAGTAGCACCAACGA (Molecular Genetics Instrumentation Facility, University of Georgia, Athens, GA, USA) and peptide sequences obtained.

Peptide used for immunization was synthesized commercially (Synpep, Dublin, CA, USA).

ELISA to detect gp96-specific phage clones

The affinity selected phages were screened for specificity by ELISA (Ashok et al. 2003). Gp96 was coated at a concentration of 1 µg/well in sodium carbonate buffer, pH 9.6 in 96-well microtiter plates and incubated overnight at RT. The nonspecific proteins were washed and wells were blocked with 5% milk-PBST (phosphate-buffered saline 0.05% Tween-20) for 2 h at RT. Followed by addition of 50 µl phage supernatant and incubated for 2 h at RT, 50 µl/well of biotinylated anti-M13 antibodies (1:800 dilution) was added and incubated for 1 h at RT. The plates were washed extensively and developed by adding 50 µl substrate *O*-phenylene diamine (OPD)-hydrogen peroxide. The reaction was stopped by adding 50 µl of 4 N H₂SO₄, and plates were read at 490 nm and referenced at 405 nm.

X-15 vaccination studies in BALB/c mice

Four- to 5-week-old BALB/c mice were purchased from Charles River Laboratories, were placed in groups of three per cage, and were provided with food and drinking water ad libitum and were placed in 12:12 h light/night cycle. Synthetic peptides were conjugated to keyhole limpet hemocyanin (KLH) using 0.25% glutaraldehyde (Gullick 1994). BALB/c mice were administered subcutaneously 100 µg of peptide/mouse with various adjuvants such as CFA, ALUM, GM-CSF, and liposome. Two weeks later (day 14), mice were boosted with 50 µg peptide/animal with same adjuvants above except the CFA which was substituted with incomplete Freund's adjuvant. One week later (day 21), mice were bled through the retro-orbital sinus and screened for antibody production by ELISA. Mice were administered a second booster (day 21) of 50 µg peptide/animal in combination with the same adjuvants. Mice were bled (day 28) through the retro-orbital sinus and screened for antibody production by ELISA essentially as described above, except that the peptides (5 µg/well in 0.25% glutaraldehyde-PBS) were coated onto the plates and the mice sera were used at varying dilutions. Also CD4⁺ and CD8⁺ T cell members were analyzed by flow cytometric analysis. Upon euthanization on day 28, the spleens were collected and used as follows: 2/3 of the spleen was OCT-embedded for immunohistochemistry and 1/3 of the spleen was used for CD4⁺ and CD8⁺ T cell cytometric analysis.

ELISA to detect antibody titer

Antibody production by different adjuvants with X-15 peptide library was screened for specificity by ELISA. X-15 peptide was coated 5 µg/well in 0.25% glutaraldehyde-PBS in 96-

Fig. 1 Identification of specific scFvs reactive to MAT-LyLu gp96. Reactivity of phages from scFv antibody library panned over MAT-LyLu gp96 complex. Different MAT-LyLu gp96 reactive clones such as E6, F3, E10, B9, and H6 are screened by ELISA. All these clones are highly reactive to MAT-LyLu gp96 when compared to gp96 from other sources such as Dunning G, liver, and normal prostate

well microtiter plates and incubated overnight at 4°C. The plates were washed with PBS-T (phosphate-buffered saline with 0.05% Tween-20) three times and then wells blocked with 5% milk-PBS for 2 h at RT. The plates were washed with PBS-T three times. Then, 100 µl of mice sera were added at varying dilutions in respective wells for 2 h at RT. The plates were washed with PBS-T four times and 100 µl of secondary IgG antibody (horseradish peroxidase (HRP)-labeled) solution for 2 h at RT. The plates were washed extensively and developed by adding 100 µl substrate OPD–hydrogen peroxide. The reaction was stopped by adding 50 µl of 4 N H₂SO₄ and plates read at 490 nm and referenced at 405 nm.

Analysis of IgG isotype subpopulation

IgG isotype-specific antibodies were obtained from Biorad. Various IgG isotype antibodies were coated in 96-well plate at 1:100 dilution in a total volume of 100 µl and incubated overnight at 4°C. Wells were then washed with 0.1% TBST then blocked with 5% milk followed by incubation with pre- and post-vaccinated serum at the indicated dilutions for 2 h at 37°C in triplicate. After washing, HRP-labeled secondary antibody was added and incubated for 2 h at 37°C. Following the washing, the reaction was developed with OPD substrate (Sigma). The reaction was stopped with 4 N H₂SO₄, and the results were read at 450 nm with a plate reader.

Determination of interleukin (IL-4 and IL-5) profile

The concentrations of IL-4 and IL-5 in the serum were determined by ELISA. Briefly, a 96-well flat bottom Nunc

Maxisorp™ (Nalge Nunc Intl., USA) was coated with capture antibody (IL-4/IL-5) of interest at a concentration of 0.1 µg/well. The plate was washed and blocked before 100 µl of pre-/post-serum (1:200 dilution) was added to the respective wells. Following a series of stringent washing, the captured cytokine was detected using the specific HRP-conjugated detection antibody. The substrate reagent was added into each well, and following color development, the plate was read at 630 nm wavelength using an ELISA plate reader.

X-15 tumor protection

Four- to 5-week-old Copenhagen rats purchased from Harlan Sprague–Dawley (Indianapolis, MN, USA) were placed in groups of three per cage and were provided with food and drinking water ad libitum and placed in 12:12 h light/night cycle as described earlier (Suriano et al. 2005). The animals were allowed to acclimatize for 1 week before experimentation. Immunogenicity of peptides (X-15 and LX-8b) was tested in syngeneic Copenhagen rats by immunization subcutaneously on days 0, 14, and 21. Here, we used LX-8b, a 12-mer peptide as a positive control of MAT-LyLu gp96–peptide complex mimic. Peptides were conjugated with KLH and then mixed with CFA, followed by injection (100 µg/rat) for the first immunization. Subsequently, boosters were in incomplete Freund's adjuvant (50 µg/rat). On day 14, rats were challenged with 10,000 MAT-LyLu cells/rat intradermally. The rats were checked twice a week for development/growth of tumors, and tumor incidence was calculated at the experimental end point.

Fig. 2 Outline of single-chain and peptide library panning. Experimental scheme of the screening of scFv library and peptide library

Fig. 3 Identification of E6 reactive peptides. Reactivity of phage clones obtained from X-15 peptide library that was panned over E6 clone obtained from scFv phage library. Low reactive B11 clone got from scFv phage library showed comparatively less reactive than E6

Fig. 4 Antibody response of X-15 peptide immunized with different adjuvants (CFA, ALUM, liposome, and GM-CSF) in mice. X-15 peptide showed high IgG titer, a marker of antibody response with CFA immunized mice followed by ALUM, GM-CSF, and liposome. Peptide with CFA immunized group that serves as positive control

showed eight times greater antibody response than normal groups (a). Peptide with ALUM, GM-CSF, and liposome (b–d) vaccinated animals showed six, four, and five times, respectively, higher IgG titer than their normal groups

Fig. 5 Flow cytometry analysis of T cell population in splenocytes. CD4⁺ and CD8⁺ T cell population of vaccinated mice are shown. Both CD4⁺ and CD8⁺ show significant difference compared to pre-vaccinated animals. Asterisk represents significant difference ($p < 0.05$) when compared between naïve and ALUM-treated groups. Each bar represents mean of five mice $\pm$ SD

Statistical analysis

The statistical significance of differential findings between experimental groups and controls was determined by Student *t* test and considered significant if two-tailed $P < 0.05$.

Results

Specificity of MAT-LyLu gp96 reactive scFv

The purified MAT-LyLu gp96–peptide complex was panned against scFv antibody library to identify clones specific for the complex. After three rounds of panning, we obtained several affinity scFv clones (E6, F3, E10,

B9, H6) displaying high affinity which were specific for purified MAT-LyLu gp96–peptide complex and not reactive to either Dunning G, liver, or normal prostate-derived gp96 thus authenticating its specificity (Fig. 1). Gp96 purified from MAT-LyLu, Dunning G, liver, and normal prostate differ only in the peptides or antigens associated with it and not within the structure of gp96 itself as it is a conserved protein and has been shown to possess no sequence variability between various normal tissues and cancers (Srivastava et al. 1987). The E6 clone specific for MAT-LyLu gp96–peptide complex was then panned against the X-15 peptide library, and after three rounds of sequential panning, several clones were selected by immunoscreening. A complete experimental flow chart is presented in Fig. 2. Fifteen peptide clones specific for gp96 (Fig. 3) were isolated and sequenced after which it was determined that all 15 clones shared the same amino acid sequence (GQWQSGDRYWMETST). The resulting peptide was named X-15 and can be considered as a mimotope of MAT-LyLu gp96–peptide complex.

Peptide-specific antibody response in vivo

The use of peptides as vaccines requires help from adjuvants mainly to act as a catalyst in initiating the antigen-specific immune response. To test the immunogenicity of X-15, we utilized three adjuvants which are approved for use in humans: ALUM, liposomes, and GM-CSF. As a positive control, CFA was used. All the adjuvants used displayed comparable IgG titers at both days 14 and 28 as determined by ELISA (Fig. 4a–d). The only difference observed in titers between adjuvants was a robust IgG response with CFA at day 14 (Fig. 4a).

Fig. 6 Analysis of IgG subtypes in X-15 vaccinated mice. Post-serum from X-15 vaccinated mice was analyzed for the various antibody isotypes shown above. The results indicated that X-15-specific antibodies generated in mice were predominantly IgG1, IgG21, IgG2b, Ig λ , and Ig κ . All post-samples were compared to the pre-serum of animals. The experiment shown is mean representative of three separate experiments $\pm$ SD

Cytometric analysis of CD4⁺ and CD8⁺ T cell populations

To further characterize the immune response generated by X-15 in BALB/c mice, we analyzed the percent increase in CD4⁺ and CD8⁺ T cell population, obtained from the splenocyte population, between vaccinated and naïve (non-vaccinated). Significant increases in numbers were observed for both CD4⁺ and CD8⁺ T cells in vaccinated mice compared to non-vaccinated mice (Fig. 5). As expected, based on the high antibody titer, the CD4⁺ T cell numbers were higher than CD8⁺ T cell numbers. Represented in this figure is the cytometric analysis for the X-15-ALUM vaccinated group, but it is important to note that all the groups displayed similar increases in T cell numbers regardless of the adjuvants suggesting that X-15 can be used with various adjuvants in a vaccine setting to elicit a robust immune response.

IgG isotypes and interleukins

Figure 6 shows the distribution of specific IgG and its subtypes present in X-15 vaccinated mice. Among all IgG isotypes (IgG1, IgG2a, IgG2b) were the prominent subtypes, and the light chain were both of κ and λ . IgG1, IgG2a, IgG2b, and Ig κ showed significant differences compared to pre-vaccinated serum samples. The mixture of the isotypes profile may be significant in several different epitopes seen by native B cells.

In X-15 vaccinated rats, a significant increase in IL-4 (Fig. 7) was observed in day 28 compared to pre-serum (day 0) and day 14. No differences in interleukin 5 were observed. Finally, our data suggest that activation of tumor-specific T cells, not only by IgG isotypes but also IL-4, might be a useful indicator for the immunological follow-up of cancer treatment with active immunotherapy.

Fig. 7 Interleukins 4 and 5 profile in pre- and post-vaccinated mice. The above figure represents the interleukin-4 and interleukin-5 analysis in pre- and post-vaccinated mice. IL-4 shows significant difference when compared to control animals. Asterisk represents significant difference ($p < 0.05$) when compared between day 0 and day 28

Fig. 8 Tumor protection by X-15-7 and LX-8b vaccination. Effect of vaccination of synthetic peptide on latency, incidence, and tumor size of MAT-LyLu. LX-8b is a mimic of MAT-LyLu gp96–peptide complex used as a positive control

Protective effect of X-15 peptide as a vaccinating agent

Figure 8 and Table 1 show that vaccination with MAT-LyLu-derived gp96 has a significant tumor protective effect. In a vaccination study, the animals were vaccinated with 100 μ g peptide/rat on day 0 and 50 μ g peptide/rat as a booster dose on days 14 and 21. MAT-LyLu cells were challenged on day 14 with 10,000 cells per rat. A very pronounced delay in tumor incidence, latency, and rate of tumor growth was noted in peptide-vaccinated groups. Two of four animals vaccinated with peptides were tumor free after 6 weeks, and untreated control animals showed 100% tumor. This is very significant result considering that 10,000 MAT-LyLu cells injected subcutaneously were able to give rise to palpable tumors in 10 days. The differences of gp96–peptide complexes and peptide in their antitumor effects may be reflective on antigen differences, dose, and large number of antigens associated with gp96.

Discussion

Currently, HSP-based vaccines are being evaluated for immunotherapy of cancers and have demonstrated success and promise in clinical trials. The HSP–peptide complex confers specific immunogenicity to the cancer from which they are derived, and the immunogenicity is ascribed to the unique repertoire of antigenic peptides that survive in different cancers. Of the three major HSPs including HSP96, HSP90, and HSP70, the most studied are gp96 (GRP94) which was the first autologous HSP-based

Table 1 Effect of vaccination of synthetic peptides on MAT-LyLu tumor incidence

Group	Percent of tumor-bearing animals post-tumor cell challenge			
	2 weeks	3 weeks	4 weeks	6 weeks
Control	100 (4/4)	100 (4/4)	100 (4/4)	100 (4/4)
BTE6-LX-8b	25 (1/4)	25 (1/4)	50 (2/4)	50 (2/4)
BTE6-X-15-7	25 (1/4)	25 (1/4)	50 (2/4)	50 (2/4)

vaccine, purified from resected tumor, and got translated from murine to clinical trials (Janetzki et al. 2000). Major studies in cancer patients demonstrated that vaccination with gp96–peptide complex is safe and apparently rendering clinical and immunological responses via inducing an efficient T cell response in cancer patients with almost no signs of autoimmunity to date (Cohen et al. 2002; Hoos et al. 2004; Belli et al. 2002; Richards et al. 2006; Pilla et al. 2006; Testori et al. 2008). Further, in spite of the tremendous and significant outcomes of tumor-derived gp96–peptide complex in immunotherapy of various cancers, the prerequisite of sufficient amount of tumor-derived HSPs after surgery limits the accessibility of these vaccines in clinical trials (Wang et al. 2006). Accordingly, to overcome this problem, the present study is focused on the use of mimotopes as vaccines against tumors, specifically tumors of the prostate.

Several reports have demonstrated that mimotopes selected from phage display libraries could be synthesized as synthetic peptides with similar individuality of specificity and reactivity (Jolivet-Reynaud et al. 2001, 2004). The gp96–peptide complex isolated from the MAT-LyLu prostate cancer cell line was panned against the single-chain antibody library. The resultant clone E6 obtained after consecutive screening was found to be highly specific and reactive to gp96–peptide complex isolated from MAT-LyLu cell line. This highly reactive clone E6 was then panned against X-15mer peptide phage display library, and the consequential highly reactive phage clone to HSP96 antibody was isolated and sequenced, and a linear X-15mer peptide was synthesized that serves as a mimotope of gp96–peptide complex.

The immunogenicity of synthesized gp96 mimotope was observed in normal BALB/c mice. Further, for standard vaccination and to drive a long-term, potent, and desired immune response against cancer antigens, an optimal, safe formulation of the adjuvants is required. Thus, the animals were vaccinated with gp96 mimotope in combination with different adjuvants such as CFA, ALUM, GM-CSF, and liposomes, of which CFA serves as positive control.

Our results showed that the antibody response was higher in mice vaccinated with gp96 mimotope in combination with ALUM, which is followed by GM-CSF and liposomes. Additionally, as several studies have demonstrated that HSP–peptide complexes either purified from tumor cells or reconstituted in vitro assist the antigen-specific representation of MHC-I and MHC-II restricted epitopes and subsequent stimulation of CD4⁺ and CD8⁺ T cells (Srivastava 2002; Doody et al. 2004; Sengupta et al. 2004), the effect of mimotope immunization on the immune cell population was analyzed. In consistent with these reports, the T cell markers both CD8⁺

and CD4⁺ were increased in gp96 mimotope vaccinated in combination with ALUM, which is followed by GM-CSF and liposomes combinations. Further, of both the populations, a significant increase in CD4⁺ cells than CD8⁺ cells was observed, which may suggest the critical role of costimulated CD4⁺ T cell responses in the generation of proficiently competent antitumor response and possibly a memory response as well (Shedlock and Shen 2003; Sun and Bevan 2003).

Thus, the capability of gp96 mimotope to elicit an enhanced antibody response and presumably a T_H2 response, as evidenced by enhanced cytokine IL-4, is tumor specific and robust enough to produce tumor rejection. The precise interphase with the T cell-mediated response needs further investigation. In addition, mimotope-induced autoimmunity needs to be monitored, although mimotope vaccination exhibited no signs of adverse effect in cancer patients (Sharav et al. 2007). Further, the present study suggests that vaccination of gp96 mimotope in combination with ALUM and GM-CSF may be better choices of adjuvants for future studies.

In conclusion, the study laid a source for the use of gp96 mimotopes in adjuvant setting as tumor vaccines for immunotherapy in future preclinical trials specifically for prostate cancer and that HSPs such as gp96 are one of the most powerful tumor rejection antigen whose utility cannot be overlooked.

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