[PHAR07] Development of a DNA Vaccine against human breast cancer

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Introduction

Breast cancer is the most common malignancy among women in most parts of the world. The lack of effective conventional treatments for breast cancer has attracted substantial attention in the search for new treatment modalities. A variety of immunotherapeutic regimens have been evaluated in recent years (Moingeon, 2001). While the specific etiology of breast cancer remains unknown, DNA vaccines directed at eliciting an immune response toward tumors appear to offer promise for both the prophylactic and therapeutic treatment of cancer. The recent identification and cloning of several breast cancer-associated antigens make it possible to develop antigen-specific cancer vaccines. Among them, MUC-1 is an attractive candidate for use in vaccine development because it displays lower sequence variability in the human breast and plays critical roles in mammary tumorgenesis (Spicer *et al.,* 1995).

The ultimate goal of cancer immunotherapy is to induce effective tumor-specific cellular immunity that can inhibit or block the growth and metastasis of tumor cells. Therefore the development of a protocol to induce Th1 response may be required to develop more efficient immunotherapy. Interleukin (IL) 18, a well identified Th 1-biasing cytokine, mediates many important biological functions including inducing of interferon (IFN)-γ production, stimulation of T cell proliferation and regulation of FasL-dependent apoptosis (Okamura *et al*., 1995; Hashimoto *et al*., 1999). In this study, the strategy of using IL-18 and MUC-1 genes was used as potential candidates for the DNA vaccine development against human breast cancer.

Materials and Methods

Cloning and sequencing

The cDNAs of human MUC-1 with 22 tandem repeats and IL-18 were cloned into mammalian expression plasmid, pVAX1 (Invitrogen) and transformed in DH5-α. Positive clones were inoculated into PCR reaction mixture to screen for the insert. Clones with the correct insert were then grown and the recombinant plasmids were purified using Wizard® Plus SV Minipreps DNA Purification System (Promega) before being sent for sequencing. The sequences obtained were analyzed using BLAST, NCBI. Subsequently, IL-18 cDNA was subcloned into pTrcHis2-TOPO® plasmid (Invitrogen) for protein expression in *Escherichia coli*.

Transfection

COS-7 cells, an African green monkey kidney cell line (ATCC) were transfected with pVAX1/LacZ (Invitrogen) using Lipofectamine™ 2000 Transfection Reagent (Invitrogen). Initial transfection assay was designed to optimize the ratio of DNA(μg) to Lipofectamine™ (μl). Twenty-four hours after transfection, COS-7 were assayed for *in-situ* β-galactosidase staining to evaluate transfection efficiency. The DNA: Lipofectamine™ 2000 ratio which gave the highest transfection efficiency was used in subsequent transfections.

Expression of clones

To express the MUC-1 protein, pVAX1/MUC-1 clones were transfected into COS-7 cells. Translated proteins were detected by SDS-PAGE followed by Western blotting. Indirect immunofluorescence staining of fixed COS-7 cells was also performed using the monoclonal antibody mouse anti-human MUC-1 (Chemicon) and the binding visualized using a fluorescein isothiocynate (FITC)-conjugated goat anti-mouse secondary antibody (Chemicon).

pTrcHis2-TOPO/IL-18 clones were expressed in *E. coli*. Positive clones were cultured in the presence of Isopropyl-β-D-thiogalactopyranoside (IPTG) to induce expression of hIL-18 recombinant protein.

Mixed Leukocyte Reaction

Dendritic cells (Mattek) that were untransfected or transfected with pVAX1, pVAX1/IL-18, and/ or pVAX1/MUC-1 were inactivated by 50μg/ ml mitomycin C (Amnesco) for 30 min and added to 10,000 in fixed number of autologous T cells (Mattek). Cells were co-cultured in phenol-red free RPMI 1640 medium containing 5% fetal calf serum at 37°C and 5% CO_2 for 96 h. The cultures were incubated for an additional 4 h in the presence of CellTiter 96® Aqueous One Solution Reagent (Promega) and the OD_{490nm} was determined using microplate reader (Dymex). Control wells contained either DC alone or T cells alone. T cells activated with phytohemagglutin (PHA; 10 μg/ ml) were used as a positive control. Responses are reported as mean OD+SEM from triplicate samples.

In vitro Cytotoxicity Assay

Varying number of stimulated T cells as prepared above in mixed leukocyte reaction act as effector cells were co-cultured with target cells T47-D and MDA-MB -231, human breast carcinoma (ATCC). Cytotoxicity activity was determined by lactate dehydrogenase release assay with CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega). The reaction was measured at $OD_{490\ nm}$. Calculations were carried out according to the following formula: % specific lysis=100 x (experimental-effector spontaneous-target spontaneous)/ (target maximum-target spontaneous).

Immunization

A hundred micrograms of endotoxin-free purified pVAX1, pVAX1/ IL-18, pVAX1/ MUC-1, pVAX1/MUC-1 + IL-18 and PBS were intramuscularly injected into 6-8 weeks old female BALB/C mice. The same immunization was repeated twice at 3 week intervals. Mouse sera were collected for use in following experiments.

Humoral Response

An ELISA test was performed by coating 96-well plate (Immunlon) with 5μg/ ml well of purified MUC-1 recombinant protein (Xema), suspended in carbonate buffer, pH 9.6 and incubated overnight at 4°C. Diluted mouse sera were used as primary antibody and HRP-conjugated goat anti-mouse IgG (Chemicon) as secondary antibody. Color development was achieved by incubating the plate with TMB substrate. The absorbance was measured at 450 nm.

Assay of cytokine production

The amounts of cytokines (IL-18 and IL-4) in mouse sera were determined using a standard sandwich ELISA technique with corresponding kit from MBL (for IL-18 assay) and Biosource (for IL-4 assay).

Results

Cloning and sequencing

Human MUC-1 (3 kb) and 471 bp of IL-18 cDNAs (Figure 1) were successfully cloned into mammalian expression plasmid, pVAX1.
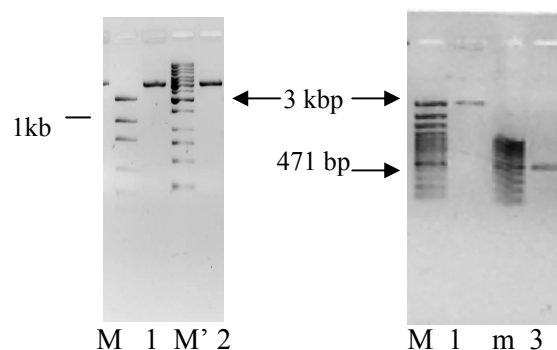

FIGURE 1 Gel electrophoresis of purified MUC-1& IL-18 with digested pVAX1 plasmid prior to ligation in cloning.
M': 1 kb marker; M: 100 bp plus marker; m: 100 bp marker; 1: Digested pVAX1 plasmid; 2: Purified MUC-1; 3: Purified IL-18.

Transfection

Different ratios of pVAX1/lacZ DNA to Lipofectamine™ complexes at 1:1, 1:2.5 and 1:5 were set up and transfected into COS-7 cells. The transfected cells were then stained for β-galactosidase activity 24 h after transfection. The results showed transfection using ratio 1:2.5 yielded the highest transfection efficiency of 70% (Figure 2). This ratio was used in subsequent transfection.
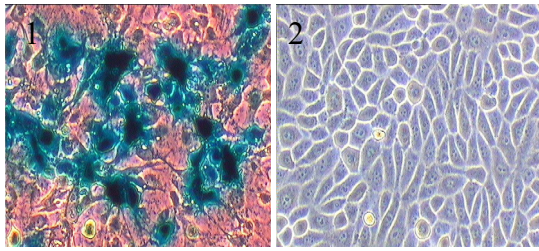
FIGURE 2 β-galactosidase staining of COS-7 cells transfected with pVAX1/*lacZ* using Lipofectamine 2000 (Invitrogen)
1: pVAX1/X-gal: Lipofectamine 2000=1:2.5
   Highest Transfection Efficiency: 70%
2: Negative control (pVAX1)

Expression of clones

Protein Expression of IL-18:

Recombinant IL-18 was expressed by induction of pTrcHis2-TOPO/ IL-18 cultures with 1mM IPTG for 3 h. The SDS-PAGE and western blot analysis revealed a distinct band of increased intensity with the expected size of approximately 23 kDa (Figure 3).
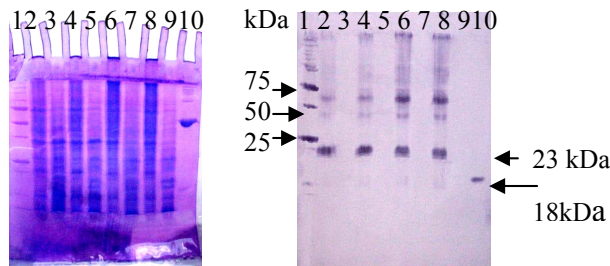
FIGURE 3 Protein analysis of transformed *E. coli* containing pTrcHis2-TOPO/ IL-18 plasmid, subcloned from pVAX1/IL-18.
1: Precision Plus Protein Standards (BIO-RAD); 2,4,6 & 8: Positive transformants with the induction of 1 mM IPTG exhibiting a prominent band of ~23kDa; 3,5,7& 9: Positive transformants without the IPTG induction as -ve controls; 10: Recombinant human IL-18 (MBL International) as +ve control.

Expression of MUC-1

In Western blot analysis, a major band of 120 kDa appeared in COS-7 cells transfected with pVAX1/MUC-1 and pCDNA3/MUC-1, and T47-D cell lysates (Figure 4). MCF-7 cell lysate showed a lower band of 68 kDa. It has been previously shown that the lower molecular weight refers to MUC-1 core protein.
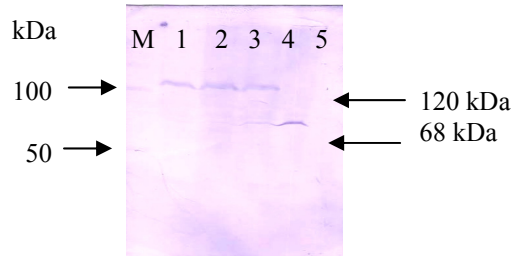

FIGURE 4 Western blot showed a major molecular mass of ~120 kDa MUC-1 in pVAX1/MUC-1 transfected COS-7.
M: Protein Marker;1: pVAX1/MUC-1 transfected COS-7; 2: pCDNA3/MUC-1 transfected COS-7; 3 & 4: T47D and MCF-7 cell lysates as +ve controls; 5: pVAX1 transfected COS-7 as –ve control.

MUC-1 was expressed on the membrane of COS-7 cells after transfection with pVAX1/MUC-1. The results revealed the presence of MUC-1 in about 40 % of the transfected cells, demonstrating that MUC-1 was indeed expressed on the membrane (Figure 5).
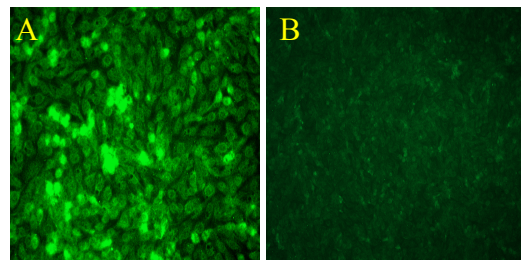

FIGURE 5 Immunofluorescence staining of fixed COS-7 cells transfected with pVax1/MUC-1 using FITC-conjugated goat anti mouse antibody revealed ~40% of the cells stained.
A: pVAX1/MUC-1 transfected COS-7
B: pVAX1 transfected COS-7 (-ve control)

T cell Proliferation Assay

Autologous T cells were stimulated *in vitro* with untransfected DC or with DC transfected with plasmid expressing various genes to investigate the capacity of gene-modified DC to prime naive T cells, as measured by T cell proliferation response in mixed leukocyte reaction. The most significant T cell proliferation was observed after stimulation of DC transfected with pVAX1/MUC-1-+IL-18 (Figure 6).
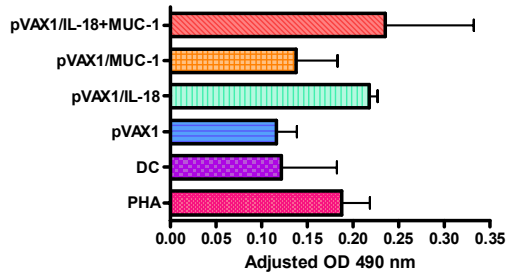
FIGURE 6 Proliferation of T cells (TC) induced by autologous dendritic cells (DC) transfected with various plasmid constructs, treated with mitomycin C (50 μg/ ml) for 30 min, and washed 2X with PBS. Varying treatments of DC were then used to stimulate a fixed number of TC(100,000). The DC/TC mixture was cocultured for 96 h and then incubated for an additional 4 h in the presence of CellTiter 96® Aqueous One Solution Reagent (Promega). TC proliferation was determined by the level of MTS formazan production as determined by the optical density at 490nm. Results are expressed as mean $OD_{490nm}\pm SD$ of triplicate cultures.

Cytotoxicity Assay

T cells were stimulated *in vitro* with inactivated DC transfected with various plasmids for induction of specific cytotoxicity. Consistent with the cytotoxicity induction, highest response was observed in T cells co-cultured with pVAX1/ MUC-1 and IL-18 transfected DC on both the T47-D and MDA-MB-231 human breast cancer cell lines (Figure 7). A certain cytotoxicity induction was observed in T cells stimulated with pVAX-1/DC or DC.
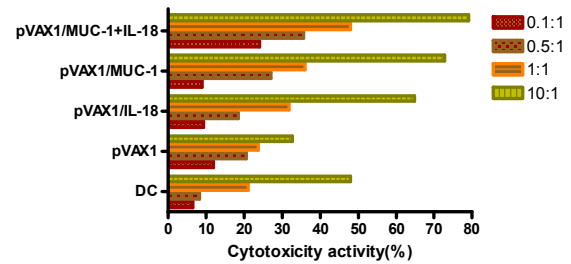

A

B

FIGURE 7 Cytotoxicity response in T cells cocultured with inactivated DC transfected with various plasmids as effector cells. The T47-D (A) and MDA-MB-231 (B) were used as target cells. The cytotoxicity was determined at E: T ratio of 10:1, 1:1, 0.5:1 and 0.1:1 by lactate dehydrogenase release assay.

MUC-1 specific humoral response

As shown in Figure 8, immunization with pVAX-1/ MUC-1 and IL-18 led to highest production of MUC-1 specific humoral response (about 22,200 ng/ ml) at day 42.
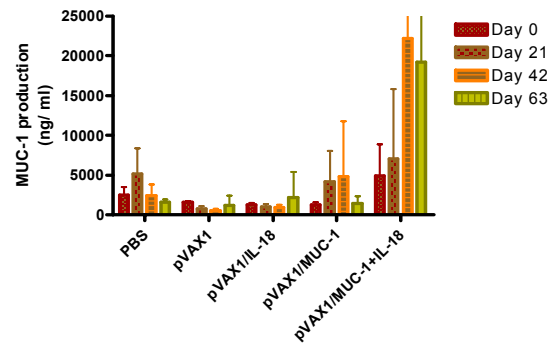

FIGURE 8 MUC-1 production from mice injected with various plasmid constructs
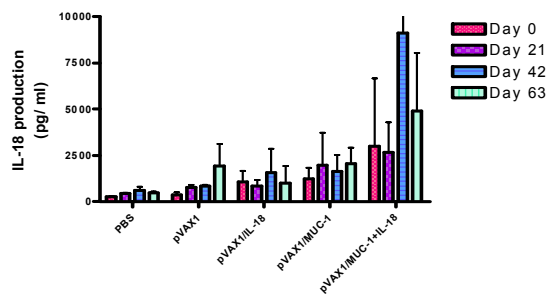
Cytokine Production

The secretion of Th1 (IL-18) and Th2 (IL-4) cytokines from mice immunized with different plasmid constructs were determined by the corresponding ELISAs. The results showed that about 1575 ρg/ ml and 1975 ρg/ ml IL-18 could be detected at day 42 and day 21 in mice injected with pVAX1/IL-18 and pVAX/MUC-1, respectively (Figure 9). Most importantly, administration with pVax1/MUC-1 and pVax1/IL-18 led to the potent generation of IL-18 of about 9100 ρg/ ml at day 42. Less than 1000 ρg/ ml was detected in sera from PBS and pVAX-1 injected mice except a remarkable increase of
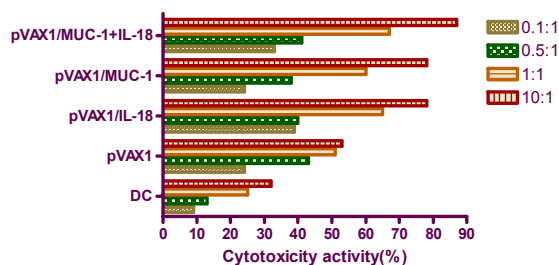
about 1900 ρg/ ml in PVAX1 injected mice at day 63. This showed that the pVAX1 plasmid itself may pose some immunostimulatory effects. However, in contrast to secretion of IL-18 cytokine, immunization of these plasmids resulted in a decrease of IL-4 production in mice injected with pVAX1/ MUC-1 and pVAX1/ MUC-1 and IL-18.
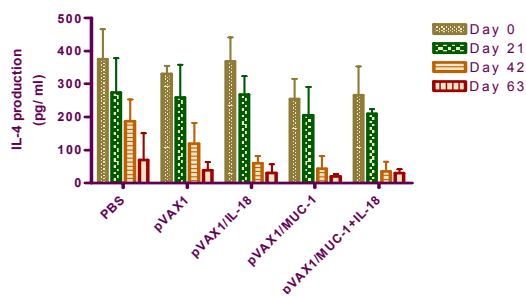
1

2

FIGURE 9 Production of Cytokines derived from mice injected with plasmids expressing various genes. 1: IL-18 production and 2: IL-4 production.

Discussion

Treatment available now for breast cancer is far from satisfactory. Current efforts are now directed towards novel strategies for the treatment of breast cancer. One of the promising approaches is to design DNA vaccine for effective induction of antitumor immunity. In this study, pVAX1 plasmid encoding human MUC-1 and IL-18 were used as immunogens to evaluate the *in vitro* antitumor response in human breast cancer cell lines and its immunogenicity in mice.

An effective cancer immunotherapy approach requires the activation of host T cells capable of recognizing tumor target antigens, and T cells activation needs appropriate antigen presentation by antigen-presenting cells (APC). Dendritic cells are the most potent professional APC, with exquisite

capacity to interact with T cells and initiate immune response (Steinman, 1991). Apart from this, gene modified DC to express tumor antigen has been documented to be efficient for inducing antitumor immunity (Kirk *et al*., 2000). Therefore, the protective and therapeutic effects of DC transfected with pVAX1/MUC-1 and pVAX1/IL-18 were investigated by stimulation of naïve T cells with these gene-modified DC. The results showed that stimulation of DC transfected with pVAX1/MUC-1-+IL-18 led to generation of the most significant T cell proliferation and the most potent cytotoxicity activity on human breast cancer cell lines T47-D and MDA-MB-231.

Consistent with the *in vitro* study in dendritic cells, immunization with pVAX1/MUC-1 and pVAX1/IL-18 in BALB/c mice enhanced MUC-1 antigen specific humoral response more potently than immunization with pVAX1/MUC-1 or PVAX1/IL-18 alone. The results also showed that BALB/c mice immunized with pVAX1/MUC-1 and IL-18 promote the production of Th1 cytokine (IL-18) significantly compared with those immunized with the other plasmid constructs.

In summary, we have demonstrated that the combination application of pVAX1/ MUC-1 and PVAX1/ IL-18 elicit a strong and specific *in vitro* antitumor immune response, indicating that DNA vaccine coexpressing tumor antigens MUC-1 and cytokine IL-18 can be used as immunogens and their immunization may be an effective strategy for a successful therapeutic vaccination.

Acknowledgements

References

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