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Publication Information

Chablani, Lipika; Mulla, Nihal S.; Selvaraj, Periasamy; and D'Souza, Martin J. (2015). "Mucosal Delivery of Particulate Breast Cancer Vaccine." *Nanoparticulate Vaccine Delivery Systems* , 171-184.

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Mucosal Delivery of Particulate Breast Cancer Vaccine

Abstract

Vaccination has been widely used as a mode of protection against various diseases by taking advantage of host's immune system. Even though vaccination has provided relief from many infectious diseases, vaccination for cancer still remains a challenge. Cancer is caused by mutated cell functioning leading to uncontrolled growth in the organ of genesis and further possible metastasis worsens the situation. In spite of various current therapies such as surgery, chemotherapy and radiation therapy, we are still lacking behind in the race with this evolving disease. There are two major approaches for vaccination: prophylactic or therapeutic. Prophylactic vaccines find their applications in the prevention of viral, bacterial, or parasitic infectious diseases such as influenza, HIV, tuberculosis, malaria, pneumonia, polio, small pox, etc., which are caused by foreign antigens. However, in the case of cancer, which is caused by mutated self-cells, vaccine formulation is a challenging task as it requires immune response against self-cell antigens without causing auto-immune response.

Keywords

fsc2015

Disciplines

Pharmacy and Pharmaceutical Sciences

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Chapter 9

Mucosal Delivery of Particulate Breast Cancer Vaccine

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9.1 Introduction

Vaccination has been widely used as a mode of protection against various diseases by taking advantage of host's immune system. Even though vaccination has provided relief from many infectious diseases, vaccination for cancer still remains a challenge. Cancer is caused by mutated cell functioning leading to uncontrolled growth in the organ of genesis and further possible metastasis worsens the situation. In spite of various current therapies such as surgery, chemotherapy and radiation therapy, we are still lacking behind in the race with this evolving disease. There are two major approaches for vaccination: prophylactic or therapeutic. Prophylactic vaccines

Nanoparticulate Vaccine Delivery Systems

Edited by Martin J. D'Souza

Copyright © 2015 Pan Stanford Publishing Pte. Ltd.

ISBN 978-981-4613-30-9 (Hardcover), 978-981-4613-31-6 (eBook)

www.panstanford.com

find their applications in the prevention of viral, bacterial or parasitic infectious diseases such as influenza, HIV, tuberculosis, malaria, pneumonia, polio, small pox, etc., which are caused by foreign antigens. However, in case of cancer, which is caused by mutated self-cells, vaccine formulation is a challenging task as it requires immune response against self-cell antigens without causing auto-immune response. Few prophylactic cancer vaccines are available on market, such as Gardasil® (Merck) and Cervarix® (GSK) vaccine for human papilloma virus infection causing cervical cancer. Prophylactic cancer vaccines can prevent tumor growth based on the use of over-expressed or mutated proteins, mutated oncogenic growth factor receptors, heat-shock proteins or other tumor-associated antigens as vaccines [1]. In case of therapeutic approach, vaccines are administered in order to trigger immune response against existing residual tumor cells mostly in combination with surgery or chemotherapy and thus aiming at preventing or prolonging the relapse [2]. Currently, there is only one therapeutic cancer vaccine, Provenge® (Dendreon) approved recently by FDA for treatment of prostate cancer. Various other clinical trials have been reported utilizing DNA/dendritic cell (DC)/viral vector based vaccines depicting the continuous growth in the field of cancer immunotherapy [3,4].

With the development of new type of vaccines, there is a critical need for an efficient delivery system. This is where the nanotechnology or microparticles can come into effect. This delivery system may be able to facilitate targeting and/or provide controlled release of the antigen to the antigen presenting cells. Particulate system in the order of micron size provides numerous benefits for vaccine delivery as they mimic the invading pathogens [5]. However, larger particles provide longer duration of antigen release compared to the smaller counterparts and hence have dramatic effect on immunogenicity.

Particulate delivery system in general provides a lot of advantages compared to traditional methods of drug delivery. Some of them are the following:

- capability of delivery via oral, transdermal and parenteral routes
- ability to accommodate small and large molecules
- multi-drug therapy using one particle

- stable delivery system for bioactive molecules
- easy manufacturing and scale-up

With the above strategies in mind, our group explored the use of the microparticulate platform technology to deliver breast cancer vaccines via oral route.

9.2 Formulation and in vivo Evaluation of Particulate Breast Cancer Vaccines

9.2.1 Oral Vaccines

Oral vaccine delivery is an attractive mode of immunization because of its ease of administration, low manufacturing cost, and patient compliance. However, the major hurdle in oral vaccine delivery is the protection of the antigen from acidic and enzymatic degradation in the gastrointestinal tract. One of the ways to avoid these issues is to formulate microparticles by using enteric coating polymers. Intestinal Peyer's patches are the predominant sites for uptake of such particles upon oral administration [6]. The particle uptake depends on various factors such as size, charge, and hydrophobicity [7,8]. For oral delivery, it has been reported that particles of size less than 5 μm with positive charge and hydrophobic nature can preferentially enter Peyer's patch of small intestine [9]. Orally delivered vaccines, especially particulate antigens are recognized and sampled by microfold (M) cells in Peyer's patches. This is followed by transport of the particles to underlying follicles and to professional antigen presenting cells (APCs) such as dendritic cells and macrophages. These APCs can phagocytose the particles, process them and present them on both MHC Class I, through cross priming, and MHC Class II molecules due to which both T and B cells can be triggered [10,11].

Several studies have been performed involving enteric coating polymers such as Eudragit L 100, S 100, L 100-55, cellulose acetate phthalate, and hydroxyl propyl methyl cellulose phthalate/acetate succinate as a polymer of choice for a particulate delivery vehicle [12–15]. Microparticles containing these enteric polymers are protected from gastric pH and can be delivered to the intestine for further uptake. Oral delivery of vaccine antigens using such

polymeric microparticles offers remarkable advantages over others, such as induction of mucosal as well as systemic immune response, protection of antigen from gastric degradation, prolonged presentation of antigen to immune system, and obviation of the need of vaccine adjuvants because microparticles themselves can act as self-adjuvants [16,17].

The present work explores the formulation and evaluation of oral murine breast cancer vaccine, which can prevent the proteolytic degradation of antigens and retain the particles intact until they reach the M cells in the small intestine. For this purpose, enteric polymers such as Eudragit® FS 30 D and hydroxyl propyl methyl cellulose acetate succinate (HPMCAS) were used. In an attempt to enhance the targeting capability of our vaccine formulation to the M cells, the M cell targeting agent, *aleuria aurantia* lectin (AAL) was added to the particulate matrix. These microparticles were formulated by using a single-step spray drying process. Spray drying involves atomization of aqueous antigen containing polymeric suspension, followed by short drying period. The process limits the exposure of the antigens to high temperatures thus causing minimal or no degradation of proteins [18–21]. Our group investigated the potential of these particles to target M cells in Peyer's patches upon oral administration, leading to activation of underlying immune cells. Thus, we aim to trigger both humoral and cell-mediated immune response through this prophylactic breast cancer vaccine, which can impart resistance against tumor challenge later.

9.3 Methods

9.3.1 Preparation of Lectin-Containing Microparticles for M-Cell Targeting

9.3.1.1 Whole-cell lysate preparation

The murine breast cancer cell line 4T07 was used for preparation of whole-cell lysate. The cells were lysed using hypotonic lysis buffer (10 mM Tris and 10 mM NaCl) and further subjected to five freeze thaw cycles at -80 and 37°C for 10 min each. At the end of last freeze thaw cycle, cell lysis was confirmed using trypan blue

dye exclusion assay; presence of dead cells confirmed the end point. The whole-cell lysate (WCL) thus obtained was stored at -80°C for further use. The total protein content of WCL was quantified by Bio-Rad DC protein assay.

9.3.1.2 Vaccine microparticle preparation

The 4T07 antigen-loaded vaccine particles were formulated using the WCL, β -cyclodextrin, ethyl cellulose, albumin, trehalose, hydroxyl-propyl methylcellulose acetate succinate (HPMCAS) and targeting agent AAL dissolved in de-ionized water. The formulation also contained trehalose to increase protein stability. AAL was added to target the particles to M cells in the intestinal lumen. The resulting microparticles were stored at -20°C in a desiccant chamber until further use.

9.3.2 Physical Characterization of Microparticles

9.3.2.1 Characterization of size, shape, and charge of microparticles

The 4T07 vaccine particles were irregular shaped with smooth edges as seen in the scanning electron micrograph in Fig. 9.1a. Particle size analysis using Spectrex laser counter indicated an average particle size of $1.5\ \mu\text{m}$, with particles of size range of $1\text{--}4\ \mu\text{m}$ as shown in Fig. 9.1b. The zeta potential was neutral, ranging from $+4$ to $+7\ \text{mV}$.

9.3.2.2 In vitro antigen release from microparticles

Antigen release from microparticles was studied to evaluate antigen protection in gastric pH conditions. Amount of antigen released was plotted against square root of time. The linearity for Higuchi release profile of these microparticles shows $10.0 \pm 6.9\%$ antigen release in first 2 h in pH 1.2 (gastric simulated fluid) and $51.6 \pm 12.8\%$ release at the end of 24 h in pH 6.8 (intestinal simulated fluid) (Fig. 9.1c). Amount of antigen remaining in the microparticles post release study was evaluated to confirm the results. The antigen content of remaining particles was also analyzed to confirm these results.

Figure 9.1 Physical characterization of microparticles: (a) SEM image; (b) particle size distribution; (c) release of antigen from microparticles.

9.3.2.3 Particle cytotoxicity

MTS cytotoxicity assays were performed to test for cytotoxic properties of microparticles. RAW 264.7 cells were plated in 96-well plates and incubated with various concentrations of antigen-loaded microparticles (2, 1, 0.5, 0.25, 0.125, 0.0625 mg/mL). Cells treated with DMEM and cells treated with benzalkonium chloride served as the negative and positive control respectively. After 24 h, MTS and phenazine methosulfate (PMS) solution was added to the wells. The plate was incubated for 4 h at 37°C in a humidified, 5% CO₂ chamber and absorbance was recorded at 490 nm using a UV plate reader (BioTek instruments Inc., VT). Cytotoxic effect of particles at given concentrations is depicted in Fig. 9.2. Particles were non-cytotoxic within the tested concentration range (0.0625–2 mg/mL). Percentage cytotoxicity is relative to negative controls of cells treated with DMEM media only. Thus, the combination of formulation polymers in the given ratio at tested concentrations was not cytotoxic and thus was used for further studies. This test confirms that the polymers used in

preparation of microparticles were non-toxic at the concentrations tested.

Figure 9.2 MTS cell cytotoxicity assay performed with various concentrations of both vaccine microparticles, cells alone as negative control and benzalkonium chloride (10 mg/mL) treated cells as positive control tested in triplicates. Vaccine particles were non-cytotoxic for tested concentrations. Results are expressed as mean $\pm$ SE.

9.3.3 In vivo Evaluation

For in vivo studies, four- to six-week-old Balb/c female mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Animals were divided into following groups: (a) control animals and (b) vaccinated animals receiving vaccine microparticles via oral route. Animals were primed with 5 mg of vaccine microparticles (equivalent to 250 μ g antigen) suspension in 200 μ L of citrate buffer (10 mM, pH 4.0) using oral feeding needle. Seven booster doses were given in a two-week interval with respect to previous dose.

9.3.4 Tumor Challenge

Animals were vaccinated for 10 weeks and then challenged with 1×10^6 live 4T07 cells subcutaneously to determine the efficacy of microparticulate vaccine. Briefly, cells were suspended in 100 μ L of serum-free DMEM media and injected subcutaneously using a

25-gauge needle at the back and between the ears of each animal. Each animal was monitored for tumor growth. Tumor growth was measured using Vernier calipers and tumor volume was calculated according to the equation given below. Animals were observed for any signs of discomfort and tumor volumes were monitored for four weeks after challenge. Vaccinated animals had significantly smaller tumor volumes than controls $^*(p < 0.05)$ ($n = 6$) as shown in Fig. 9.3.

$$\text{Tumor Volume} = \frac{1}{2} \{ \text{Length} \times (\text{Width})^2 \}$$

Figure 9.3 Tumor volumes of vaccinated and control animals as measured post challenge with live 4T07 cancer cells. Vaccinated animals had significantly smaller tumor volumes than controls $^*(p < 0.05)$ ($n = 6$). Results are expressed as mean $\pm$ SE.

9.3.5 Flow Cytometry Analysis to Elucidate Role of Immune Cells

Animals were euthanized at the end of the study. Abdominal cavities were excised open and spleens of animals were isolated aseptically. Spleen cells were processed to evaluate immune cell population distribution. Fluorescently labeled antibodies for various T cell and B cell population were added to the sample. Following cell populations were analyzed. (a) Anti-CD4+ PE (for T-helper cell) and anti-CD8+ FITC (for T-cytotoxic cell) markers, (b) anti-CD45R

(B220) marker (for B cells) and (c) anti-CD161 marker (for NK cells). All the markers were added as per manufacturer's protocol. All the samples were incubated on ice for 30 min and subjected to analysis using BD Accuri® C6 flow cytometer (BD Accuri Cytometers, MI) to analyze various cell populations in vaccinated and control groups.

CFlow software by Accuri Inc. was used to analyze immune cell populations in the control and vaccinated animals. The CD4⁺ cells (Q2-UL) were significantly higher in vaccinated animals ($27.17 \pm 0.69\%$) compared to the controls ($21.73 \pm 1.23\%$) ($p < 0.05$). There was no significant difference observed in the CD8⁺ cells (Q2-LR) in vaccinated ($4.50 \pm 0.47\%$) and control ($4.33 \pm 0.70\%$) animals (Fig. 9.4). CD4⁺ cells help formation of cytotoxic T cells and aid the formation of antibodies against antigens.

Figure 9.4 Flow cytometry analysis showing cell population of CD4⁺ and CD8⁺ cells in (a) animals administered placebo microparticles orally, (b) animals administered 4T07 vaccine microparticles orally. CD4⁺ cells were marked with anti-CD4⁺ PE marker, read in FL2-A channel and are denoted by the percentage of cell population as seen in Q2-UL, while the CD8⁺ cells were marked with anti-CD8⁺ FITC marker read in FL1-A channel and designate the percentage of cells present in Q2-LR. Vaccinated animals had significantly higher, $27.17 \pm 0.70\%$ of CD4⁺ cells (Q2-UL) versus control animals having $21.73 \pm 1.23\%$ of them ($p < 0.05$) ($n = 3$). No significant difference in CD8⁺ cell population (Q2-LR) was observed. Results are expressed as mean $\pm$ SE.

9.4 Discussion

To develop a breast cancer vaccine, 4T07 breast cancer cells were lysed and used as the source of antigen. Breast cancer-associated specific antigens are not well established. Hence, we used whole-cell lysate as the vaccine. Using whole-cell lysate is more advantageous than using individual proteins in many ways. Since there is a pool of unknown antigens, it can lead to a higher immune response compared to individual tumor-associated antigen. The cell lysate was characterized for its protein content and was further delivered orally using polymer microparticles. AAL was used to enhance the uptake of microparticles by M cells. Several studies prove that M cell uptake of antigen is better if administered along with AAL [22,23]. The vaccine microparticles were prepared using a novel one-step spray drying process [13,19,24–28]. The polymers used in manufacturing these microparticles are GRAS listed. Organic solvents that can degrade the protein of interest were avoided while manufacturing microparticles. Particles size obtained is optimum for cellular uptake [19]. M cells transport these microparticles across the epithelium and present to the antigen presenting cells. Uptake of vaccine microparticles is higher than vaccine solution, which ultimately leads to a higher immune response. Peyer's patch is a rich source of antigen presenting cells. Therefore, we chose the oral route to deliver vaccine microparticles. The particles are non-toxic and do not induce a specific immune response. This finding can be well supported by the fact that the components of these particles are GRAS substances and have been used previously in various marketed formulations such as Sporanox® (Itraconazole), Brexin® (Piroxicam), and Nitrophen® (Nitroglycerin).

One of the major challenges in oral delivery is protein stability. Protein delivery via oral route requires protection using enteric coating polymers. In this study, we use pH-dependent polymers to prevent protein degradation in the acidic stomach pH conditions. The sustained release of antigen followed the Higuchi release pattern suggesting diffusion-controlled antigen release from the matrix of microparticle. It is vital for these vaccine particles to have sustained release and maintain their particulate nature in order to be taken up by M cells and recognized by antigen presenting cells

(APCs) for further processing of breast cancer antigens. It has been shown that particulate antigens result in improved antigen presentation than their soluble counter-parts [29]. Especially if soluble peptide antigens are given orally, they can lead to tolerance and fail to produce immune response. As these particles only release ~30% of antigen payload by the end of 8 h (which is the gastro-intestinal transit time), most of the antigen was still available in the particulate form to function as a vaccine particle. The immune response elicited by these particles was further analyzed by the *in vivo* immune response obtained by them. *In vivo* vaccine efficacy was evaluated by measuring both the arms of adaptive immune system: (a) humoral response by measuring the serum antibody titers obtained after successive boosters administered and (b) cellular response by analyzing the difference in total number of CD4+, CD8+ T cell populations, B cells, and NK cells of treated animals.

Flow cytometry analysis was performed to elucidate the role of immune cells. A significantly higher number of CD4+ T cells were found in vaccinated animals as compared to controls. We also observed that CD4+ T cells were expanded to a greater extent than the CD8+ T cells in response to vaccination. CD4+ T cells have proven to be effective in rejecting tumors and have outperformed CD8+ cells in few reported instances [30,31]. As these vaccine particles elicited both humoral and cellular immune response, they can serve as a potent vaccine for long-term protection against the antigens of interest.

9.5 Conclusions

Mucosal delivery of particulate vaccines holds immense potential as a mode of immunization. Individualized vaccine microparticles can be prepared using patients own cancer cells. Studies have been planned to explore therapeutic benefits of cancer vaccines. This study will mimic clinical scenario, wherein the patient will have an existing tumor. In a therapeutic vaccine study, the mouse will be challenged with breast cancer cells and vaccinated with particulate vaccine.

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