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Formulation and Evaluation of Oral Microparticulate Ovarian Cancer Vaccines

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Keywords

Microparticles; ovarian cancer; oral delivery; M-cells; ID8 cells; spray drying

1. Introduction

Ovarian cancer is the most lethal gynecological cancer in the US. Only about 10% of ovarian cancers are usually found in early stages. Patients with epithelial tumors, which account for approximately 90% of ovarian cancer, generally have poor overall survival [1]. First-line treatment for advanced cancer involves surgery followed by chemotherapy. However, cancer relapses within relatively short periods of time even after treatment. [2]. Therefore, alternative approaches, immunotherapy is being investigated to prevent relapse of cancer.

Several vaccines are underway in clinical trials and most of them have not progressed beyond phase I/II studies. Even though antigen-specific response is obtained with different approaches of antigen specific immunization, there is no consistency in clinical benefit [3,4]. In this study, we have investigated whether oral vaccination with microparticles containing the ovarian cancer antigens can prevent/ retard ovarian cancer growth. A murine ovarian cancer cell line, ID8 was used as a source of antigens as it correlates closely to human ovarian cancer cell lines in signaling pathways and results in development of tumor in mice models similar to human ovarian cancer. [5]. Despite of advancement in vaccine technology, the whole cell lysate vaccine still remains a very promising approach as it can overcome the

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Conflict of Interest statement

The authors report no conflict of interest.

demerits associated with a single antigen/epitope vaccine. Whole cell lysate provides a pool of tumor-associated antigens (TAAs) which can induce both CD8⁺ and CD4⁺ T cells. [6]. Therefore, we proceeded with a whole cell lysate of ID8 cells to prepare the vaccine for this study.

Oral vaccine delivery is an attractive mode of immunization because of its ease of administration, low manufacturing costs and patient compliance. However, for an oral vaccine to be effective, it must withstand gastro-intestinal degradation. For this purpose, enteric polymers such as methacrylic copolymer Eudragit® FS 30 D and hydroxyl propyl methyl cellulose acetate succinate (HPMCAS) were used. Ano et al. have reported a formulation of oral vaccine of inactivated *Vibrio cholerae* by spray drying using Eudragit® FS30D [7]. This polymer was found to be a good candidate for controlled release of antigens and resulted in vibriocidal antibody titers. We prepared the particles utilizing this polymer along with other excipients by using an optimized microparticulate formula for oral vaccine delivery by spray drying [8]. To target our vaccine formulation to M-cells in the Peyer's patches of the intestine, M-cell targeting agent, Aleuria aurantia lectin (AAL) was used in the formulation [9,10]. In addition, inclusion of immunostimulatory molecules such as IL-2 and IL-12 was evaluated in order to enhance the overall potency of the formulated vaccines [11,12]. It has been observed that specific immune response by tumor cell vaccines is often impaired due to deficiency of co-stimulatory signals. Vaccines prepared with cancer cells secreting IL-2 or IL-12 have resulted in better immune response when compared to non-secreting cell lines. IL-2 has been efficiently used in immunotherapy against cancers [13]. Moreover, higher anti-tumor effects were reported when IL-2 was delivered as an adjuvant in a slow-releasing depot form rather than a free form [11,13]. Incorporation of IL-2 in particle addresses this issue seen with free form of IL-2.

In the present study, we demonstrate the efficacy of oral vaccine formulations which was evaluated *in vivo* in mouse tumor model, using the ID8 murine ovarian cancer cell line as a solid tumor model.

2. Materials and Methods

2.1 Materials

ID8 cell line was kindly provided by Dr. Katherine Roby, Kansas University Medical Center, Kansas City, KS. Six to eight week-old C57BL/6 female mice were purchased from Charles River Laboratories, Wilmington, MA. HPMCAS was purchased from AQOAT, FMC Biopolymers, Philadelphia, PA. Eudragit® FS 30 D was generously gifted by Evonik industries, Parsippany, NJ. Mouse plasma was obtained from Biochemed, Winchester, VA. AAL was obtained from Vector Labs, Inc., Burlingame, CA. Recombinant murine interleukins, IL-2 (5×10^6 units/mg) and IL-12 (1×10^7 units/mg) were purchased from Peprotech Inc., Rocky Hill, NJ. Flow cytometry cell markers were purchased from eBioscience, San Diego, CA. Goat anti- mouse HRP-IgG and anti-IgG subtypes were purchased from Bethyl Laboratories, Montgomery, TX and Sigma, St. Louis, MO respectively. All other materials used were of analytical grade.

2.2 Preparation of whole cell lysate

The murine ovarian cancer ID8 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite (Sigma, St. Louis, MO) [14]. The confluent cells were washed with cold phosphate buffered saline (PBS). The flasks were then treated with hypotonic buffer (10mM Tris and 10mM NaCl) and subjected to five 15 min freeze-thaw cycles at temperatures of -80° C and 37° C respectively to obtain the cell lysate [15,16].

2.3 Characterization of the lysate

The protein content of lysate was analyzed using Bio-Rad DC™ protein assay. The lysate was screened for presence of the only known antigen, SP17 by western blot analysis [17]. Briefly, 25 µg of lysate protein was resolved by SDS-PAGE gel electrophoresis and subjected to overnight wet-transfer on to a PVDF membrane. After blocking, the membrane was treated with primary mouse anti-SP17 antibody (kindly provided by Dr. Chiriva-Internati, Texas Tech University Health Science Center, Lubbock, TX), followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Finally, the membrane was incubated with Enhanced Chemiluminescence (abcam, Cambridge, MA), then exposed to imaging film.

2.4 Preparation of vaccine microparticles

The vaccine formulation was prepared by spray drying technique [8–10,18–24]. Briefly, HPMCAS and Eudragit® FS 30D were dissolved in an alkaline solution, followed by addition of chitosan glycol. Mouse plasma was added to the polymeric solution at pH 7.0 as a source of albumin. Trehalose, tween 20 and AAL were added to the solution, followed by addition of the lysate (5% w/w). In case of vaccine with interleukins formulations, 4×10^5 U of IL-2 and 8×10^5 U of IL-2 were added to this feed mixture [11,12]. This aqueous solution was spray dried using Buchi B-191 Mini Spray Dryer (Buchi Corporation, New Castle, DE).

2.5 Characterization of microparticles

Microparticles were visualized by using scanning electron microscope (JEOL JSM 5800LV, Tokyo, Japan) at EM Core facility, Emory University, Atlanta. The particles were characterized for size and charge, using laser particle counter (Spectrex PC -2000) and Malvern zeta sizer (ZEN 1600) respectively. Loading efficiency was determined by extracting the proteins in PBS and analyzing them using Biorad DC™ protein assay in comparison to placebo particles.

2.6 Immunization

The immunogenicity of microparticulate vaccine was evaluated using C57BL/6 female mice model. Animal experiments were carried out as per approved protocols by Mercer University's Institutional Committee for the care and Use of Laboratory animals. Animals (n=8) were administered with microparticles as one prime dose followed by one booster after one week and thereafter by 8 boosters with an interval of two weeks. Briefly, 5mg of microparticles were suspended in citrate buffer (pH 4.0) and administered orally using oral gavage. Three different formulations such as placebo, vaccine and vaccine with interleukins were evaluated for this purpose.

2.7 Tumor challenge study

One week after the last vaccination, the mice were challenged s.c. with 1×10^7 live ID8 cells [25]. The cells were suspended in incomplete media and injected into the right back flank of mice. Tumor development was monitored using digital vernier calipers. The mice were euthanized whenever the tumor ulcerated or tumors exceeded a size of 15 mm in any of the perpendicular diameters. The tumor volume (V) was determined by using the formula, $V = 1/2(\text{Length})(\text{Width})^2$ [26,27].

2.8 Assessment of humoral (B-cell mediated) immune response in serum

The blood samples were collected prior to each dose of vaccination. Serum was analyzed by ELISA. Briefly, overnight coating of the lysate (100µg/well) was done in a 96 well plate. The plate was blocked with non-fat dry milk. After washing, the plate was incubated with 1:10 dilution of serum samples. HRP-tagged secondary anti-mouse goat IgG was then added

to each well, and incubated at 37°C for 1 h. TMB substrate reagent (3,3', 5,5''-tetramethyl benzidine) (BD OptEIA™, BD Biosciences, CA) was added and the plate was again incubated at 37°C for 30min. The reaction was stopped by addition of 4N H₂SO₄. The plate was read using microplate reader (BioTek instruments Inc., Winooski, VT) at 450 nm. In case of IgG subtypes analysis, serum samples were analyzed in dilution of 1:50. The plate was then incubated with goat anti-mouse IgG subtype IgG1 or IgG2a, followed incubation with HRP-conjugated anti-goat IgG. Thereafter, same procedure as described above for IgG titers analysis was followed.

2.9 Determination of T-cell and B-cell based immune response in lymphatic organs

A separate group of mice was vaccinated in similar way as described in section 2.6. At the end of vaccination, mice were euthanized and lymphatic organs such as the spleen, lymph nodes (axillary and inguinal) and bone marrows were harvested. The single cell suspension of these organs were stimulated for 5 days at 37°C with mitomycin-treated tumor cells in a ratio 10:1 with 10 U/ml of recombinant murine IL-2 [28]. At the end of 5-days, the cells were washed with Hank's balanced salt solution and labeled with anti-mouse CD4 PE, anti-mouse CD8a FITC and anti-mouse/human CD45R (B220) FITC (for B-cells). The cells were analyzed for the specific cell populations by flow cytometric analysis using BD accuri® C6 flow cytometer.

2.10 Statistical analysis

Serum IgG subtype titers and flow-cytometry results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Tumor volume measurements and serum IgGs were analyzed by two-way ANOVA followed by Bonferroni multiple comparisons. All statistical analyses were performed using GraphPad Prism5 (trial version 5.04, GraphPad Software, Inc., La Jolla, CA). For each test, p value less than 0.05 was considered significant. Data are expressed as mean $\pm$ standard error.

3.0 Results and Discussion

3.1 Preparation and characterization of the lysate

The lysate obtained was a turbid extract and the total protein concentration of lysate was 1.56 ± 0.5 mg/ml. The only protein which has been studied is the sperm protein (SP17, molecular weight 15kD) which is expressed by ID8 cells [17]. It is one of the cancer/testis antigens, a sub-class of TAAs. This protein has been extensively studied and it is found to be expressed or over-expressed in multiple myeloma and ovarian cancers. In a study by Chiriva-Internati et al., this protein has been evaluated as an ovarian cancer vaccine in murine models, where the mice were immunized intramuscularly with SP17 in ten doses of vaccination. It has shown to be a promising approach prophylactically as well as therapeutically. Moreover, human SP17 shows 70% homology with murine SP17 and is expressed in ovarian cancer. Thus, SP17 may be a promising antigen for immunotherapy in ovarian cancer [17]. Therefore, we performed western blot analysis for SP17 on the lysate prepared. We could trace the presence of this protein as shown in figure 1(A). The presence of SP17 in the lysate ensured that the protein cocktail obtained was antigenic when given as a vaccine.

3.2 Preparation and characterization of vaccine microparticles

One of the major challenges of oral vaccination is the avoidance of immune tolerance. However, this issue can be avoided by entrapping vaccine antigen into particles [29–31]. This can be due to the efficient and enhanced uptake of particulate antigen by M cells in the Peyer's patches by phagocytosis, when compared to minimal antigen uptake in solution

form by pinocytosis. Moreover, protein antigens taken up as particles are more potent in activating antigen presenting cells (APCs) than soluble antigens [32–34]. Orally delivered vaccines, especially particulate antigens are sampled by M-cells in the Peyer's patches of the intestine. Further these antigens are processed by professional APCs such as dendritic cells and macrophages that reside in the Peyer's patches [35].

The morphological characterization of particles showed crumbled, collapsed and irregular shaped microparticles as shown in figure 1(B). Eudragit® FS30D microparticles have shown to result in such a shape upon spray drying by others as well [7]. The production yield was 72.58 ± 3.41 % w/w. The particles obtained were of 1.58 ± 0.62 μm size with a charge of 12.48 ± 2.32 mV. There was no significant change in size and charge upon loading these particles with the lysate and interleukins. The loading efficiency of the particles was found to be 92.68 ± 4.77 % w/w.

3.3 Immunization suppresses tumor growth

In case of mice treated with placebo particles, the tumor developed rapidly. However, vaccinated mice showed around six-fold tumor suppression when compared to non-vaccinated/placebo group. The tumor volume measurements obtained are shown in figure 2. However, there was no significant difference between vaccine and vaccine with interleukin groups in terms of tumor volumes. This can be due to the interleukin concentration used in vaccine which was not high enough to retard tumor growth more than that seen with vaccine alone. Higher concentration of interleukins might be needed to show additional tumor suppression.

3.4 Immunization generates humoral immune response and orally administered interleukins influence Th1/Th2 pathway

In order to determine B-cell response, the serum samples collected before each dose were analyzed by ELISA. As shown in figure 3(A), at end of vaccination, immunized mice showed elevated IgG titers as compared to non-vaccinated ones ($p < 0.05$). Mice treated with vaccine microparticles with interleukins showed even further elevated response as compared to mice receiving placebo microparticles ($p < 0.0001$). When serum IgG1 titers were analyzed, there was an elevation in titers in mice treated with vaccine alone when compared to placebo group ($p < 0.01$) as shown in figure 3(B). The response was even further elevated in case of vaccine with interleukins group when compared to placebo ($p < 0.001$). In case of IgG2a titers, the titers were elevated in case of vaccine alone when compared to placebo ($p < 0.01$) as shown in figure 3(C). Thus, both subtypes of IgGs indicate that mixed Th1 and Th2 immune response was generated in case of vaccine alone and Th2 type response was triggered in case of vaccine with interleukin group. The reason behind the difference in the immune response can be attributed to IL-12 effect in the Peyer's patches which has been reported to be a possible strategy for targeted manipulation of immune responses to oral vaccines. Such response was reported by Marinaro et al., when IL-12 was given orally to mice orally immunized with tetanus toxoid and cholera toxin and the effect was compared with intraperitoneal administration of IL-12. They found that oral IL-12 resulted in the Th2 response to oral vaccine, while intraperitoneal IL-12 caused Th1 response [12].

3.5 Immunization generates T-cell and B-cell based immune response

Lymphatic organs such as spleen and lymph nodes were analyzed for T-cell populations. The CD8+ as well as CD4+ T-cell populations were found to be elevated in vaccinated mice when compared to placebo group as shown in figure 4. The CD4+ T-cell population was found to be elevated in case of spleen cells of vaccine with interleukin group (figure 4(C)) when compared to spleen cells of vaccine alone ($p < 0.01$). On the other hand, B-cell population was determined in spleen, lymph nodes and bone marrow. In case of B-cell

population in spleen cells (figure 5(A)), there were elevated levels in vaccine with interleukins group when compared to placebo ($p<0.001$) and vaccine alone ($p<0.01$). B-cell populations in lymph node cells as shown in figure 5(B) were found to be elevated in case of vaccinated groups when compared to non-vaccinated mice ($p<0.001$). B-cells in bone marrows (figure 5(C)) were found to be elevated in case of vaccine with interleukins group than placebo as well as vaccine alone ($p<0.001$).

The overall results obtained are summarized in table 1. When data was analyzed for vaccinated and non-vaccinated mice, humoral response study showed higher antibody titers in vaccinated mice. Vaccine alone resulted in mixed Th1 and Th2 response, while vaccine with interleukins showed predominant Th2 response. The B-cell population was found to be elevated in bone marrow and spleen cells of vaccine with interleukins group than vaccine alone, while it was higher in lymph nodes in case of vaccine alone as well as vaccine with interleukins groups when compared to placebo. We found CD8+ and CD4+ T-cells were expanded in mice treated with vaccine with and without interleukins when compared to non-vaccinated mice. However, when the comparison was done between vaccine with and without interleukins groups, B-cell populations in bone marrow and spleen along with CD4+ T-cells in spleen were found to be elevated in case of interleukins group. The overall stimulation of humoral and cellular response upon vaccination indicates the efficacy of oral vaccine microparticles. Moreover, the immune stimulation in terms of humoral and cellular response obtained correlated with tumor volume retardation.

However, the anti-tumor effect was expected to be enhanced by interleukins, which was not observed in this study. The interleukin concentration used might need to be increased to result in enhanced anti-tumor activity. Another reason can be the activation of regulatory T-cells (T-regs) by IL-2, which can be a demerit for vaccine efficacy. It has been reported that low-dose of IL-2 can result in expansion of T-regs. The overall effect of T-helper cells and NK-cells versus Tregs in response to IL-2 depends on IL-2 concentration, dosing time and duration of exposure, which ultimately determines anti-tumor activity [13].

4. Conclusion

We have demonstrated the efficacy of vaccine microparticles containing whole cell lysate of ID8 ovarian cancer cells in retarding tumor growth in murine models. Thus, the oral microparticulate vaccine described provides a promising approach in terms of cost-effectiveness, ease of production and patient compatibility. This study was performed in prophylactic setting to check the efficacy of particles, which forms the basis for further studies to evaluate therapeutic efficacy of the particles in tumor bearing animals.

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Highlights

- We prepared microparticulate vaccine containing cell lysate using spray dryer.
- Vaccine efficacy was evaluated in mice via oral administration.
- Vaccinated mice showed retardation of tumor volume after tumor challenge.
- Humoral and cellular responses were elevated in case of vaccinated mice.
- This oral vaccine provides a promising approach to ovarian cancer treatment.

(A) **(B)**

Fig. 1. Characterization of lysate and microparticles prepared (A) Western blot analysis of ID8 lysates for SP17 with BSA as a negative control, indicating the presence of SP17 (15kD) in the two different batches of lysates prepared (B) Scanning electronic microscopy photograph of microparticles obtained by spray drying technology, showing crumbled, collapsed and irregular shaped microparticles

Fig. 2.

Immunization with vaccine microparticles suppresses tumor growth: Mean tumor volumes for mice groups treated orally with vaccine microparticles with and without interleukins along with data with mice treated with placebo microparticles. The tumor volume was monitored with the aid of vernier calipers on a weekly basis. Vaccinated mice showed higher tumor suppression as compared to non-vaccinated/placebo treated mice ($p < 0.0001$), **** $p < 0.0001$

Fig. 3. Immunization with vaccine microparticles generates humoral immune response and orally administered interleukins influence Th1/Th2 pathway: (A) Serum IgG titers determined by ELISA, showing higher titers at the end of final vaccination in case of mice receiving vaccine orally with ($p < 0.0001$) and without interleukins ($p < 0.05$) when compared to mice treated with placebo microparticles, (B) Serum IgG1 titers determined by ELISA, showing higher titers in case of vaccinated mice as compared to placebo treated/ non-vaccinated mice ($p < 0.05$). Vaccine alone showed elevated titers ($p < 0.01$) and vaccine with interleukins showed even further elevation in IgG1 titers ($p < 0.001$), (C) Serum IgG2a titers determined by ELISA, showing higher titers in case of mice treated with vaccine alone as compared to placebo treated/ non-vaccinated mice ($p < 0.01$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Fig. 4.

Immunization with vaccine microparticles generates CD8+ and CD4+ T-cell based immune response: CD8+ T-cells population in (A) spleen (B) lymph nodes, CD4+ T-cell population in (C) spleen (D) lymph nodes, (E) CD8+ T-cells population in spleen determined by flow cytometry, FL1-H being the count detected by filter FL1 for FITC-tagged CD8+ T-cells (shown in gate P5). The results are summarized in (A) indicating the elevated CD8+ T-cell population in vaccinated mice when compared to non-vaccinated mice ($p < 0.05$) (F) CD8+ T-cells population (gate P1) in lymph nodes determined by flow cytometry. The results are summarized in (B) indicating the elevated CD8+ T-cell population in mice receiving vaccine alone ($p < 0.001$) and in mice receiving vaccine with interleukins ($p < 0.01$) when compared to non-vaccinated mice (G) CD4+ T-cells population (gate P7) in spleens determined by flow cytometry. The results are summarized in (C) indicating the elevated CD4+ T-cell population in mice receiving vaccine alone ($p < 0.05$) and in mice receiving vaccine with interleukins ($p < 0.001$) when compared to non-vaccinated mice (H) CD4+ T-cells population (gate P3) in lymph nodes determined by flow cytometry. The results are summarized in (D) indicating the elevated CD4+ T-cell population in vaccinated mice when compared to placebo treated/ non-vaccinated mice ($p < 0.001$), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Fig. 5.

Immunization with vaccine microparticles generates B-cell based immune response: (A) B-cells population in (A) spleen (B) lymph nodes and (C) Bone marrow, (D) B-cells population in spleen determined by flow cytometry, FL1-A being the count detected by filter FL1 for FITC- tagged B-cells (shown in gate P8). The results are summarized in (A) indicating the elevated B- cell population in mice receiving vaccine with interleukin when compared to non-vaccinated mice ($p < 0.001$), (E) B-cells population in lymph nodes (gate P23) determined by flow cytometry. The results are summarized in (B) indicating the elevated B-cell population in vaccinated mice when compared to non-vaccinated mice ($p < 0.001$) (F) B-cells population (gate P23) in bone marrow determined by flow cytometry. The results are summarized in (C) indicating the elevated B-cell population in mice receiving vaccine with interleukin when compared to non-vaccinated mice ($p < 0.001$), ** $p < 0.01$, *** $p < 0.001$

Table 1

Summary of results obtained from *in-vivo* study of ovarian cancer vaccine microparticles when administered orally to mice

In-vivo study	Organ/Tissue analyzed	Placebo	Oral Vaccine	Oral Vaccine + Interleukins
Tumor volume suppression	Tumor	-	****	****
IgG titers at the end of vaccination	Serum	-	*	****
IgG subtype IgG1 titers (Th2 type response)	Serum	-	**	***
IgG subtype IgG2a titers (Th1 type response)	Serum	-	**, <i>a</i>	-
B-cell population	Bone Marrow	-	-	***, <i>aaa</i>
	Spleen	-	-	***, <i>aa</i>
	Lymph Nodes	-	***	***
CD8+ T-cells	Spleen	-	*	*
	Lymph Nodes	-	***	**
CD4+ T-cells	Spleen	-	*	***, <i>aa</i>
	Lymph Nodes	-	***	***

*
p<0.05,

**
p<0.01,

p<0.001,

p<0.0001, results expressed in comparison to placebo group

a
p<0.05,

aa
p<0.01,

aaa
p<0.001, results expressed for comparison between vaccine and vaccine with interleukins groups.