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Intranasal immunization with a flagellin-adjuvanted peptide anticancer vaccine prevents tumor development by enhancing specific cytotoxic T lymphocyte response in a mouse model

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Purpose: Human papillomavirus (HPV) is a significant cause of cervical cancer-related deaths worldwide. Because HPV is a sexually transmitted mucosal pathogen, enhancement of antigen-specific mucosal immune response likely serves good strategy for vaccination. However, mucosal vaccines generally do not induce strong enough immune responses. Previously we proved that a bacterial flagellin, *Vibrio vulnificus* FlaB, induce strong antigen-specific immune responses by stimulating the Toll-like receptor 5. In this study, we tested whether FlaB could serve as an effective mucosal adjuvant for a peptide-based HPV preventive cancer vaccine.

Materials and Methods: Mice were intranasally administered with a mixture of FlaB and E6/E7 protective peptides in 5-day interval for a total of two times. Five-days after the last vaccination, cellular immune responses of the vaccinated mice were analyzed. Tumor growth was also observed after a subcutaneous implantation of TC-1 cells bearing E6/E7 antigens.

Results: Intranasal administration of the E6/E7 peptide mixture with FlaB elicited a strong antigen-specific cytotoxic T lymphocyte activity and antigen-specific interferon- γ production from splenocytes and cervical lymph node cells. Furthermore, FlaB, as a mucosal adjuvant, conferred an excellent protection against TC-1 tumor challenge with high survival rates in E6/E7 immunized animals.

Conclusion: These results indicate that FlaB can be a promising mucosal adjuvant for nasal HPV vaccine development.

Keywords: Flagellin, Adjuvant, Neoplasms, Vaccines

Introduction

The prevalence of cervical cancer is maintained at high levels in women worldwide and approximately 270,000 cases of cervical cancer associated death are reported annually [1]. It has been known that human papillomaviruses (HPV) are the primary causative agent of cervical cancers [2]. Recently, to control the outburst of HPV related cervical cancers, two types of vaccines (Gardasil and Cervarix) have been licensed for use in young boys and girls [3,4]. These preventive vaccines are based on the induction of neutralizing antibodies against major protein such as L1 of HPV and the vaccine shows significant safety profile and clinical efficacy against HPV genotypes contained within the vaccine formulation [3-5]. However, HPV are highly diverse with respect to

their DNA sequences of L1 [6] and no protection has been anticipated against the strains not included in the vaccine formulation. Moreover, these vaccines are too expensive for widespread use in the developing world, where cervical cancer is the second leading cause of death in women [1,4].

Efficacy of any vaccine largely depends on the type of antigens incorporated in the vaccine formulations [3]. E6 and E7 are HPV proteins that are expressed during early phases of viral infections. These proteins have been found to be expressed stably in HPV related carcinomas and also are essential for the induction of cell transformations [7]. Thus, it is believed that targeting these proteins on cell surface can modulate the course of infection and development of transformed cancer cells. Several approaches have been tried to induce E6/E7 specific immune responses [8]. Peptide based vaccines have been found to be safe, stable and easy to produce. Moreover, administered E6/E7 peptides have been found to induce antigen-specific T cells in animal subjects [8-11]. However, the poor immunogenicity of peptide-based vaccines has limited the widespread development of E6/E7 peptides as vaccine candidates.

Previously, we have shown that FlaB, a flagellin protein from *Vibrio vulnificus*, exerts strong immune reactivity in the mucosal compartments through Toll-like receptor 5 (TLR5) activation [12-15]. Taking these observations into consideration, in this study, we demonstrate that intranasal co-administration of E6/E7 peptides with FlaB potently induced strong antigen-specific immune responses in vaccinated animals. Furthermore we show that co-administration of FlaB and E6/E7 peptides induced protective antitumor activity in mouse challenge model.

Materials and Methods

Peptides

Peptides from HPV-16 E6, the E6 cytotoxic T lymphocyte (CTL) peptide (amino acids 50-57: YDFAFRDL), and HPV-16 E7, the E7 CTL peptide (amino acids 49-57: RAHYNIVTF) containing major histocompatibility complex class I epitopes [16] were synthesized by the AnyGen company (Gwangju, Korea) with the purity of >97%.

Purification of a recombinant flagellin, *V. vulnificus* FlaB, and evaluation of its TLR5 stimulatory activity

Recombinant FlaB was prepared as previously described [12, 13].

Cell line

TC-1 cells were kindly provided by Dr. Young-Chul Sung (Pohang University of Science and Technology) and maintained as previously described [17]. The cells were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (Life Technologies).

Animal model

Five- to six-week-old female C57BL/6 mice were purchased from the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). All animal experimental procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee of Chonnam National University.

Mice were intranasally immunized twice with 20 μ L phosphate buffered saline (PBS) only (control group), PBS containing 4 μ g FlaB (F group), 50 μ g each of E6 and E7 peptides (P group), or 50 μ g each of E6 and E7 peptides along with 4 μ g FlaB (P+F group) [8,12] under anesthesia (intraperitoneal injection of 100 μ L of PBS containing 2 mg of ketamine and 0.2 mg of xylazine) at an interval of 5 days. Five days after the final immunization, spleen and cervical lymph nodes were collected from five mice per group to assess CTL responses and interferon- γ (IFN- γ) production. Five immunized mice per group were subcutaneously challenged with 5×10^5 TC-1 cells each in 200 μ L PBS injected into the right mid-flank. Tumor growth and mouse survival were monitored every 3 days. The tumor volume was calculated as follows: tumor volume (mm^3) = $(L \times H \times W)/2$, where L is the length, W is the width, and H is the height of the tumor in millimeters [18].

CTL assay

CTL assay was performed as previously described [7,19]. Briefly, to obtain the effector cells, splenocytes from vaccinated animals were stimulated *in vitro* with 1 μ g/mL each of E6/E7 peptides and 25 IU/mL of interleukin-2 (BD Biosciences, San Jose, CA, USA) for 5 days. TC-1 cells were treated with 1 μ g/mL cisplatin for 24 hours and then used as the target cells. The CTL assay was performed with effector (E) and target (T) cells mixed at various E:T cell ratios (25:1, 50:1, and 100:1). After 5 hour incubation at 37°C, 50 μ L cell supernatant was collected to quantify the lactate dehydrogenase activity using the CytoTox96 Non-Radioactive Cytotoxic Assay kit, according to the manufacturer's instructions (Promega, Madison, WI, USA).

Measurement of IFN- γ production by ELISPOT assay

Cells isolated from the spleen and cervical lymph nodes (cLN) of vaccinated mice were added to a BD ELISPOT plate (BD Biosciences Pharmingen, San Diego, CA, USA). Cells were then stimulated with 1 $\mu\text{g}/\text{mL}$ each of E6 and E7 peptide, as described previously [20]. After the incubation for 3 days at 37°C in 5% CO₂, antigen-specific IFN- γ -producing cells were quantified using an ELISPOT kit in accordance with the manufacturer’s instructions. The spots represent individual IFN- γ -producing cells as spot-forming cells (SFCs) on the membrane. The SFCs were measured using a CTL-Immunospot Analyzer and analyzed with the ImmunoSpot Professional Software version 5.0 (Cellular Technology, Shaker Heights, OH, USA). The responses were considered positive above 50 SFC/well and when at least double the numbers of SFCs were obtained in comparison to cells cultured in the culture medium alone. A non-specific negative peptide was included as an additional control [8].

Statistical analysis

The results are expressed as the means \pm SEM unless otherwise noted. Statistical comparisons were performed by the one-way analysis of variance (ANOVA) and the unpaired Student’s t-test. The difference in survival rates among various experimental groups was tested by the Kaplan-Meier analysis. $p < 0.05$ were considered statistically significant. All experiments were repeated more than three times, and results from the representative experiments are shown.

Results

FlaB potentiated antigen-specific CTL responses

As cytotoxic T lymphocyte responses to E6 and E7 are important for tumor eradication in the mouse TC-1 cell cervical cancer model [7,19], we first evaluated the effect of FlaB on TC-1 cell-specific CTL responses *in vitro*. As can be seen in Fig. 1, the lytic activity of E6/E7 peptide-specific CTLs against TC-1 tumor cells in P + F group was significantly higher than the peptide only group (P). This result indicated that FlaB significantly potentiates the E6/E7 peptide-specific CTL activity.

FlaB enhanced antigen-specific IFN- γ production

We next investigated the number of peptide specific IFN- γ producing cells generated by the intranasal vaccination. Five days after last immunization, immune cells from spleen and cLN were isolated and re-stimulated with E6/E7 peptides. Af-

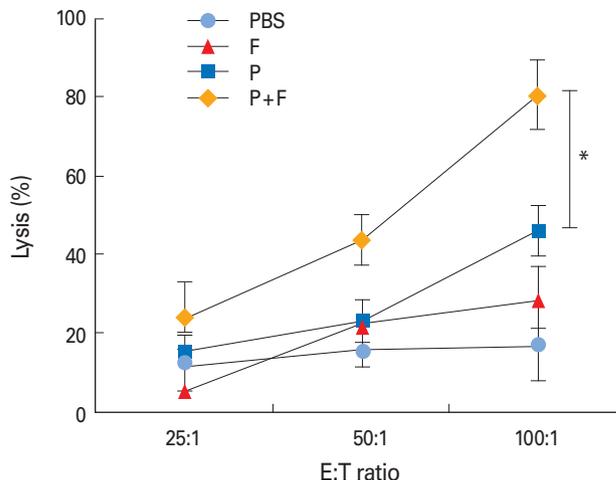


Fig. 1. FlaB enhances antigen-specific cytotoxic T lymphocyte (CTL) responses. Each group of vaccinated mice (n = 5) was sacrificed at day 10. The splenocytes (SPL) were prepared to determine the CTL activity *in vitro*. To prepare the effector cells, SPLs were stimulated *in vitro* with E6/E7 peptides (1 $\mu\text{g}/\text{mL}$ each) in the presence of interleukin-2 (25 IU/mL) for 5 days. The TC-1 cells were treated with cisplatin (1 $\mu\text{g}/\text{mL}$) for 24 hours and then used as the target cells. The effector and target cells were co-cultured at various effector:target (E:T) cell ratios. After 5-hour incubation at 37°C, 50 μL of the cell supernatant was collected to measure the lactate dehydrogenase activity in the culture medium. The ANOVA was used to compare CTL responses. * $p < 0.05$.

ter 3 days of re-stimulation, the number of E6/E7 peptide-specific IFN- γ producing cells generated in vaccinated mice was determined by the ELISPOT assay. As shown in Fig. 2, the number of IFN- γ secreting cells observed in the spleen (Fig. 2A) or cLN (Fig. 2B) of P + F group was significantly higher than peptides only (P) group ($p = 0.006$ and $p = 0.027$, respectively). These results are consistent with the CTL activity result described above.

FlaB potentiated protective antitumor activity in a TC-1 cell challenge model

To test the protective antitumor effect of FlaB-adjuvanted peptide based vaccine, tumor protection experiments were performed by challenging vaccinated mice with TC-1 tumor cells. Five days after the last immunization, mice were injected subcutaneously with 5×10^5 TC-1 cells. As shown in Fig. 3A, three weeks after the challenge the tumor volume was increased to $1,048 \pm 191 \text{ mm}^3$ in PBS (control) group. The tumor volume in the FlaB only (F) group ($786 \pm 143 \text{ mm}^3$) and the peptide only (P) group ($913 \pm 146 \text{ mm}^3$) was comparable to that in PBS group ($p > 0.05$). However, the co-administration group (P + F) showed significantly reduced tumor sizes ($172 \pm$

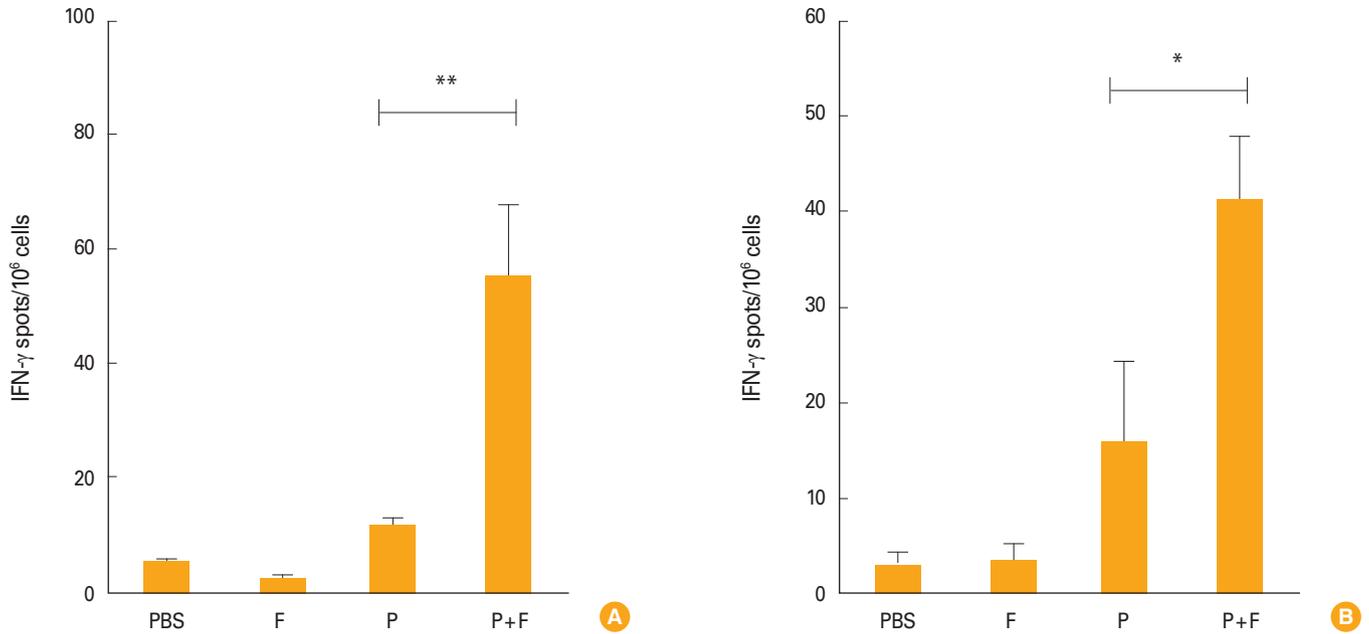


Fig. 2. Interferon- γ (IFN- γ) production from splenocytes and cervical lymph node cells. Each group of vaccinated mice ($n = 5$) was sacrificed at day 10. The splenocytes (A) and cervical lymph node cells (B) were prepared to determine IFN- γ production. Immune cells were stimulated *in vitro* with the E6/E7 peptides (1 $\mu\text{g}/\text{mL}$ each) for 3 days. The number of E6/E7 peptide-specific IFN- γ producing cells generated in vaccinated mice was determined by group by ELISPOT assay. The Student's t-test was used to compare the IFN- γ production between two groups. * $p < 0.05$ and ** $p < 0.01$.

112 mm³) compared to the P group. In some animals in P + F group, tumor development was not observed at all.

We also evaluated the long-term survival of vaccinated mice after challenge with TC-1 cells. Sixty five days after the TC-1 cell challenge, mice in P + F group survived with a cumulative survival rate of 80% ($p = 0.037$ in P + F group as compared to the P group) (Fig. 3B). Interestingly, P group enhanced the survival of mice till 48 days after which a sudden fall in the survival rate was observed. These results indicate that FlaB potentiates E6/E7-mediated protective antitumor activity and serves as a potent adjuvant to the peptides even through the mucosal vaccination. These challenge study results correlated closely with the CTL activity and IFN- γ production.

Discussion

Cervical cancer is one of the most common cancers of women, especially in the developing countries [1,2]. An ideal preventive cervical cancer vaccine should be able to prevent infection and at least eliminate symptoms of preexisting infections, if not totally eradicate the related disease. Moreover, it should be safe and cheap enough for the widespread use amongst masses. E6/E7 peptide-based anticancer vaccines

have been proposed and tested by many groups. Some of the advantages of these vaccines include safety and the ease of production and stability [8-11]. However, the E6/E7 peptides themselves are not often antigenic enough to induce an effective antitumor immune response. Adjuvant must be administered simultaneously [8,9,11].

FlaB is one of very few TLR agonists that can be genetically engineered [12-14] and mediates signal transduction in mammalian cells via TLR5 [21,22]. In previous studies, we have found that FlaB could efficaciously potentiate protective immunity when administered intranasally as a mixture with either tetanus toxoid, influenza antigens (trivalent influenza vaccine) or fused with PspA [12-14]. So, we hypothesized that intranasal co-administration of E6/E7 peptides as a mixture with FlaB should be more effective in stimulating the protective immunity compared with E6/E7 peptides alone. To test our hypothesis, we intranasally co-administered HPV-16 E6 and E7 peptides along with FlaB, a flagellin of *V. vulnificus* origin [12]. The results clearly showed that the mice that received E6/E7 peptide along with FlaB manifested a strong antigen-specific CTL activity and the levels of antigen-specific-IFN- γ producing cells in spleen and cLN were significantly higher as compared to the group that received peptide only

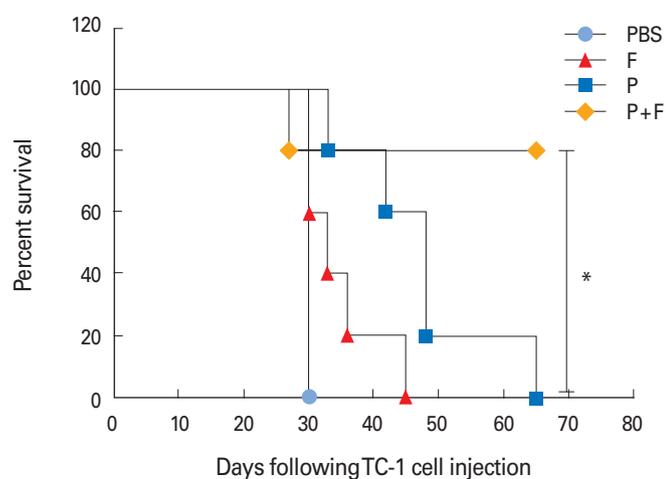
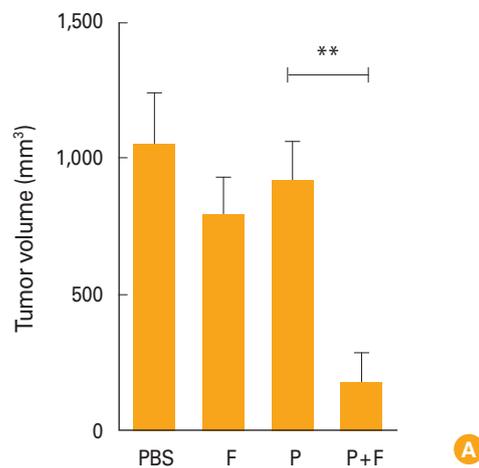
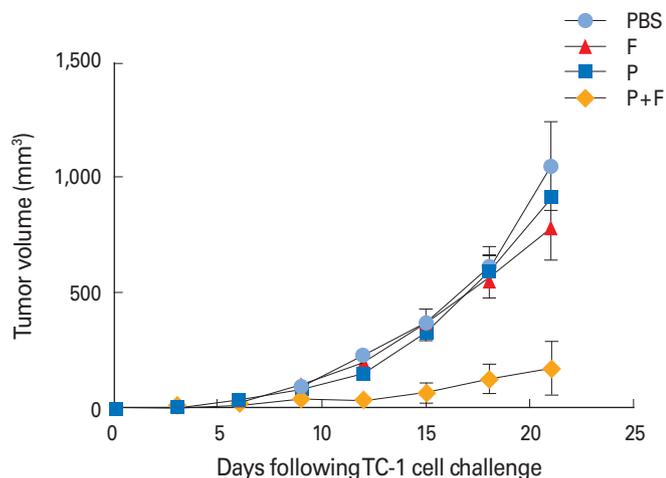


Fig. 3. Prophylactic antitumor effect of flagellin-adjuvanted peptide based vaccine. Groups of 5 vaccinated mice were subcutaneously challenged with 5×10^5 TC-1 tumor cells at 5 days after the last vaccination. Changes in tumor sizes (A) and mouse survival rates (B) were monitored at 3-day intervals. The statistical significance of tumor volume at 21 days after TC-1 cell challenge was determined by the Student's t-test. The survival curve was constructed according to the Kaplan-Meier method, and the statistical significance between P and P + F group was determined by the log-rank test. * $p < 0.05$ and ** $p < 0.01$.

as antigen. These results led us to check if animals immunized with the FlaB-adjuvanted peptide vaccine could withstand the challenge with tumor cells. For this we immunized the animals with various peptide preparations (for details see methods section) and challenged with TC-1 cells as reported earlier [8,23]. We found that P+F immunized animals had 80% protection from tumor while animals in other groups died over the duration of the study. These results went well in parallel with protective CTL and IFN- γ production. Even though, the protection rate in peptide only group also remained marginally significant till 48 day, protection efficacy in P group was drastically decreased in long term observation. These results suggest that flagellin helps not only the antigen-specific CTL responses but also the maintenance of a significantly sustainable cellular immune responses.

Recently, it has been demonstrated that vast array of innate immune system could be activated by bacterial flagellins [24-27]. According to the studies, those researchers demonstrated that flagellin itself has significant antitumor activity [24-26].

In the present study, we found that FlaB alone does not induce antitumor activity in TC-1 cell implantation model. These results are in contradiction to previously published reports where direct activation of TLR5 on tumor cells has been demonstrated to mediate flagellin specific anti-tumor effects [26]. It is likely that TLR5 expression pattern on the tumor cells might have led to different outcomes obtained in our study and the previous studies (data not shown) [26]. Nonetheless, these results converge at one point that FlaB has strong immunomodulatory effects and that flagellin could be used as an adjuvant to increase the efficacy of concomitantly administered weak antigens in a preventive cancer vaccine model.

Based on these results, use of flagellin-adjuvanted E6/E7 peptides as a mucosal vaccine is envisaged to significantly contribute to lower the morbidity associated with HPV infections and in particular HPV associated cervical cancers. However, contrasting reports in literature add a caution and require universally controlled experimental setups to check the efficacy of FlaB as an adjuvant in conjunction to various antigens.

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