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ORIGINAL ARTICLE

EphA2-derived peptide vaccine with amphiphilic poly(γ-glutamic acid) nanoparticles elicits an anti-tumor effect against mouse liver tumor

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Abstract The prognosis of liver cancer remains poor, but recent advances in nanotechnology offer promising possibilities for cancer treatment. Novel adjuvant, amphiphilic nanoparticles (NPs) composed of L-phenylalanine (Phe)-conjugated poly(γ-glutamic acid) (γ-PGA-Phe NPs) having excellent capacity for carrying peptides, were found to have the potential for use as a peptide vaccine against tumor models overexpressing artificial antigens, such as ovalbumin (OVA). However, the anti-tumor potential of γ-PGA-Phe NPs vaccines using much less immunogenic tumor-associated antigen (TAA)-derived peptide needs to be clarified. In this study, we evaluated the effectiveness of immunization with EphA2, recently identified TAA, derived peptide-immobilized γ-PGA-Phe NPs (Eph-NPs) against mouse liver tumor of MC38 cells (EphA2-positive colon cancer cells). Immunization of normal mice with Eph-NPs resulted in generation of EphA2-specific type-1 CD8+ T cells. Immunization with Eph-NPs tended to provide a degree of anti-MC38 liver tumor protection more than that observed for immunization with the mixture of EphA2-derived peptide and complete Freund's adjuvant (Eph + CFA). Neither Eph-NPs nor Eph + CFA vaccines inhibited tumor growth of BL6, EphA2-negative melanoma cells. Splenocytes isolated from MC38-bearing mice treated with Eph-NPs showed strong and specific cytotoxic activity against MC38 cells. Immunization with Eph + CFA induced liver damage as evidenced by elevation of serum alanine aminotransferase, while Eph-NPs vaccination did not exhibit any toxic damage to the liver. These results demonstrated that immunization with Eph-NPs displayed anti-tumor effects against liver tumor by generating acquired immunity equivalent to the toxic adjuvant CFA, suggesting that safe γ-PGA-Phe NPs could be applied clinically for the vaccine treatment of liver cancer.

Keywords Peptide vaccine · EphA2-derived peptide · Acquired immunity · Liver tumor

Abbreviations

IFA	Incomplete Freund's adjuvant
NPs	Nanoparticles
γ-PGA	Poly(γ-glutamic acid)
Phe	L-Phenylalanine
CFA	Complete Freund's adjuvant
PBS	Phosphate buffered saline
i.p.	Intraperitoneal
ALT	Alanine aminotransferase
DCs	Dendritic cells

Introduction

Immunotherapies using peptide vaccine combined with immunologic adjuvants, such as incomplete Freund's

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adjuvant (IFA), saponin QS-21, and several cytokines, could enhance the anti-tumor immune response after immunization [1, 2]. To date, these therapies have been clinically applied to patients with several types of cancer and have shown limited anti-tumor effects [3–7]. This is because dose-limiting toxicities of the adjuvant were often observed or the adjuvant effects of the peptide vaccine were too weak to induce a sufficient anti-tumor effect. At present, only aluminum salt has been approved as an immunological adjuvant for clinical use; it appears to have weak activity as an adjuvant [8]. Thus, a new strategy using strong and safe immunologic adjuvant is needed to improve their clinical efficacy in cancer treatment. Recently, advances in nanotechnology have offered promise for application in medical science. Some investigators have reported testing various kinds of nanoparticles (NPs) using efficient antigen-carriers for their biological potential [9–11]. We previously demonstrated the efficacy of immunotherapies using HIV-capturing non-biodegradable polystyrene NPs in an animal model [12–15]. However, non-biodegradable polystyrene NPs would not be applicable in clinical situations as vaccine material due to their safety issues. To improve NP-based vaccines, we have successfully generated biodegradable NPs composed of poly(γ-glutamic acid) (γ-PGA) and hydrophobic amino acid, L-phenylalanine (Phe) [16]. γ-PGA is a naturally occurring poly(amino acid) that is synthesized by certain strains of *Bacillus*. The polymer is made of D- and L-glutamic acid units linked through the α-amino and the γ-carboxylic acid groups, respectively. γ-PGA is water soluble, biodegradable and edible. Therefore, the potential applications of γ-PGA and its derivatives have been of interest in a broad range of fields, including the medical field [17–19]. γ-PGA-Phe NPs can be degraded by γ-glutamyl transpeptidase [20], which is widely distributed in the entire body, and various molecules such as proteins and peptides can be immobilized on the surface or encapsulated into γ-PGA-Phe NPs [21]. We demonstrated that γ-PGA-Phe NPs have an excellent capacity for carrying various proteins and peptides into antigen-presenting cells such as dendritic cells (DCs) and macrophages [22]. However, previous reports were studies that examined the potential of vaccines with γ-PGA-Phe NPs using artificial antigens, such as OVA, which are much more immunogenic than tumor-associated self-antigens. The anti-tumor potential of tumor-associated antigen (TAA)-derived peptide vaccine must be examined in order to establish peptide vaccine therapy using γ-PGA-Phe NPs.

The liver is the most common site of distal metastasis for tumors developing in distal organs, such as the colon, stomach and pancreas, and the physiological status of this organ correlates with the survival of patients with advanced disease, even if the primary tumor site has been resected curatively [23, 24]. We demonstrated that the recently identified TAA EphA2 is overexpressed in colon cancer tissues and that EphA2-derived peptide pulsed DCs showed the high potential as a cancer vaccine in a mouse tumor model [25, 26], suggesting that EphA2-derived peptide could be applicable to evaluate the potential of peptide vaccines with γ-PGA-Phe NPs.

In the present study, we demonstrated that immunization with EphA2-derived peptide-immobilized γ-PGA-Phe nanoparticles (Eph-NPs) displayed anti-tumor effects against EphA2-expressing liver tumor by eliciting EphA2 antigen-specific acquired immunity equivalent to peptide vaccine using the strongest but very toxic adjuvant, complete Freund's adjuvant (CFA). These results indicate that peptide vaccine using γ-PGA-Phe NPs could be a promising candidate for a vaccine adjuvant against liver cancer.

Materials and methods

Mice

Female C57BL/6 mice were purchased from Clea Japan Inc. (Tokyo, Japan) and were used at 6–8 weeks of age. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care and our study protocol complied with the institution's guidelines.

Cell lines

MC38 as EphA2-positive cell, a mouse colon carcinoma cell derived from C57BL6/J mice, was generously provided by Dr. Kazumasa Hiroishi (Showa University School of Medicine, Tokyo) [25]. BL6 as EphA2 negative cell, a melanoma cell line, and YAC-1, a sensitive cell line to NK cells were purchased from American Type Culture Collection (Rockville, MD) [25]. These cell lines were maintained in Complete Medium (RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2.

Preparation of peptide-immobilized γ-PGA-Phe NPs

Nanoparticles composed of γ-PGA-Phe were prepared as previously described [27]. To prepare EphA2-derived peptide-immobilized NPs (Eph-NPs), a carboxyl group of the γ-PGA-Phe NPs (10 mg/ml) was first activated by water-soluble carbodiimide (1 mg/ml in 20 mM phosphate buffer, pH 5.8) for 20 min. The NPs (5 mg) obtained by centrifugation were mixed with 1 ml EphA2-derived peptide (0.5 mg/ml) in phosphate buffered saline (PBS)

and the mixture was incubated at 4°C for 24 h. After the reaction, the centrifuged NPs were washed three times with PBS and resuspended at 10 mg/ml in PBS. Eph-NPs immobilizing 20 μg of EphA2-derived peptide per 1 mg of NPs were prepared. The particle size distribution and the surface charge of NPs were measured by a dynamic light scattering (DLS) and zeta potential measurement using a Zetasizer Nano ZS (Malvern Instruments, UK). The mean diameters of NPs and Eph-NPs were 219 ± 78 and 246 ± 88 nm (mean ± SD), respectively. The NPs and Eph-NPs had a strongly negative zeta potential (−20 to −25 mV) in PBS.

IFN-γ ELISPOT assays for peptide-reactive CD8+ T cells responses

Splenocytes were harvested 5 days after intraperitoneal (i.p.) immunization of normal mice with various amounts of Eph-NPs or equal amounts of EphA2-derived peptide alone twice with a 1-week interval. In another experiments, splenocytes were harvested 5 days after i.p. immunization of normal mice with 10 μg of Eph-NPs or a mixture of 10 μg of EphA2-derived peptide with CFA (Eph + CFA), 10 μg of Eph peptide only (Eph), the γ-PGA-Phe NPs only (NPs) or PBS twice with a 1-week interval. CD8+ T cells were selectively isolated from splenocytes by magnetic cell sorting using CD8 MicroBeads (Miltenyi Biotec, Gladbach, Germany). Mouse IFN-γ ELISPOT assays were performed using a mouse IFN-γ ELISPOT kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. IFN-γ-secreting cells appeared as blue spots. The data are represented as mean IFN-γ spots ± standard deviation (SD) per 100,000 CD8+ T cells analyzed.

Animal experiments

C57BL/6 mice were immunized intraperioneally with Eph-NPs, Eph + CFA, Eph, NPs or PBS twice a week as above. On day 0, at the time of the second injection with these vaccines, mice were lightly anesthetized by isoflurane and 1×10^6 MC38 cells (EphA2-positive) or 1×10^6 BL6 cells (EphA2-negative) were injected under the capsule of the left medial liver lobe by using a 30-gauge needle as previously described [26]. To prevent leakage, a cotton swab was held over the injection site for 2 min. Skin and peritoneum were closed in a single layer using a nylon suture. The procedure was well tolerated by all animals and no intraoperative or anesthesia-related deaths occurred. Mice were killed 14 days after tumor inoculation and the liver weight was measured. Data are reported as the average liver weights ± SD. All the protocols of animal experiments were approved by Institutional Animal Care and Use Committees of Osaka University Graduate School of Medicine.

Cytolytic assays

Splenocytes were harvested 14 days after tumor inoculation. After 5 days of in vitro stimulation with mitomycin C(MMC) (Kyowa Hakko, Tokyo, Japan)-treated MC38 cells, lymphocytes were analyzed for their ability to kill MC38 tumor cells in 4-h ^{51}Cr release assays as previously described [28]. In some experiments, liver lymphocytes were isolated 1 day after immunization of Eph-NPs into MC38-bearing mice as previously described [26], and subjected to 4-hr ^{51}Cr release assays against NK-sensitive YAC-1 target cells.

In vivo depletion of immune cells

The procedure used in this study was described previously [25]. The efficiency of specific subset depletions (CD4+, CD8+ T cell or NK cell) was confirmed by flow cytometric analysis. In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

Blood biochemistry test

Blood samples were obtained 7 days after final immunization. Levels of serum alanine aminotransferase (ALT), total bilirubin (TBil), albumin (Alb), and creatinine (Crnn) were measured with a standard UV method using a Hitachi type 7170 automatic analyzer (Tokyo, Japan).

Statistical analyses

Statistical differences between the groups were determined by applying Student's *t* test with Welch correction or one-way ANOVA after each group had been tested with equal variance and Fisher's exact probability tests. Statistical significance was defined as $P < 0.05$.

Results

Detection of EphA2-derived peptide-specific CD8+ T cells after immunization with Eph-NPs into normal mice

We performed IFN-γ ELISPOT assays to examine whether i.p. injection of Eph-NPs into normal mice could generate CD8+ T cells specific for EphA2-derived peptide. As shown in Fig. 1a, the frequencies of EphA2-derived peptide-specific CD8+ T cells in mice treated with the NPs immobilized with 10 or 50 μg of EphA2-derived peptides were significantly higher than those observed for mice

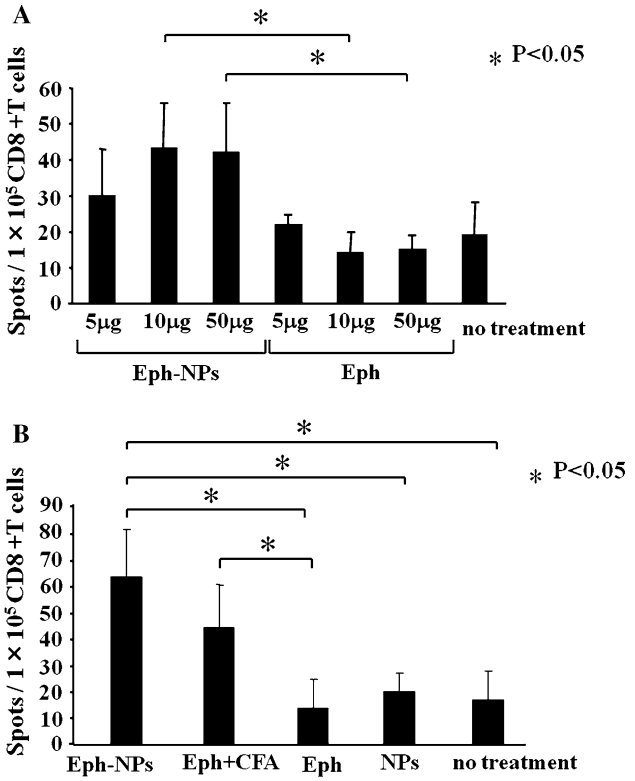

Fig. 1 IFN-γ ELISPOT assays for peptide-reactive CD8+ T cells responses. Normal mice ($N = 3$) were immunized with the indicated dose of Eph-NPs, Eph + CFA, Eph peptide only (*Eph*) or NPs only (*NPs*), and killed on day 5 post-immunization. Spleen cells were harvested and CD8+ T cells isolated using CD8 MicroBeads as described in "Materials and methods". CD8+ T cells were then subjected to IFN-γ ELISPOT assays to detect EphA2-derived peptide-specific CTLs. The data are represented as mean IFN-γ spots ± SD per 100,000 CD8+ T cells analyzed. Similar results were obtained in three independent experiments. *$P < 0.05$

treated with equal amounts of peptides alone. The frequency of EphA2-derived peptide-specific cytotoxic T lymphocytes (CTLs) from mice immunized with the NPs immobilized with 10 μg of EphA2-derived peptides was equal to that from mice treated with NPs immobilized with 50 μg of EphA2-derived peptides. Thus, we used NPs immobilized with 10 μg of EphA2-derived peptides as Eph-NPs vaccines in the following experiments. As shown in Fig. 1b, the frequency of EphA2-derived peptide-specific CD8+ T cells in mice treated with the NPs immobilized with 10 μg of EphA2-derived peptides (Eph-NPs) was significantly higher than that observed for mice treated with NPs alone or EphA2-derived peptides alone. The frequency of EphA2-derived peptide-specific CTLs from mice immunized with Eph-NPs was equal to that from mice treated with mixture of 10 μg of EphA2-derived peptide with CFA (Eph + CFA). These results demonstrated that EphA2-specific type-1 CD8+ T cells (i.e. Tc1) are effectively generated by in vivo immunization with Eph-NPs.

Immunization with Eph-NPs prevents progression of EphA2-expressing liver tumors

We examined whether immunization with the Eph-NPs would promote protective anti-tumor effects against the EphA2-positive MC38 or EphA2-negative BL6 liver tumors. C57BL/6 mice were immunized on day −7 and 0 with Eph-NPs, Eph + CFA, EphA2-derived peptide only (Eph), NPs only (NPs) or PBS. On day 0, at the time of the second injection with these vaccines, mice were lightly anesthetized by isoflurane and 1×10^6 MC38 cells or 1×10^6 BL6 cells were injected under the capsule of the left medial liver lobe. Mice were killed 14 days after tumor inoculation and the liver weight was measured. As shown in Fig. 2a, the liver tumor from mice treated by Eph-NPs tended to be smaller than those from mice treated by Eph + CFA, Eph, NPs or PBS. The liver weights bearing MC38 tumor in mice immunized with Eph-NPs were significantly lighter than those in mice treated with Eph, NPs or PBS. In contrast, those in mice treated with Eph + CFA were not significantly lighter than those in control mice. The liver weights bearing MC38 tumor in mice treated with Eph-NPs tended to be lighter, but not significantly, than those with Eph + CFA (Fig. 2a). Neither Eph-NPs nor Eph + CFA inhibited BL6, EphA2 negative melanoma, tumor growth (Fig. 2b). These results suggest that immunization with Eph-NPs provides specific anti-tumor effects against EphA2-positive MC38 tumors. We also examined the liver weights of mice treated with Eph-NP, Eph + CFA, Eph, NPs or PBS without tumor injection. Mice were treated twice a week with each treatment without tumor injection and evaluated the liver weights 14 days after treatment. The liver weights from all treated mice without tumor injection were almost similar (data not shown), suggesting that each treatment did not affect the liver weight.

Induction of specific CTLs against MC38 cells after immunization with Eph-NPs into MC38 bearing mice

We examined whether immunization of Eph-NPs would induce tumor-specific cytolytic activity against MC38. As shown in Fig. 3a, splenocytes isolated from mice treated with Eph-NPs or Eph + CFA displayed stronger cytolytic activity against MC38 cells when compared with those immunized with EphA2-derived peptide alone, NP alone or PBS. Furthermore, splenocytes harvested from mice treated with Eph-NPs displayed a degree of anti-MC38 cytolytic activity equivalent to those immunized with Eph + CFA. On the other hand, the cytolytic activity was not observed against EphA2-negative BL6 cells in all treatment groups. We next examined whether lymphocytes isolated from the liver 1 day after tumor inoculation displayed cytolytic activity against a NK-sensitive cell, YAC-1 in vitro.

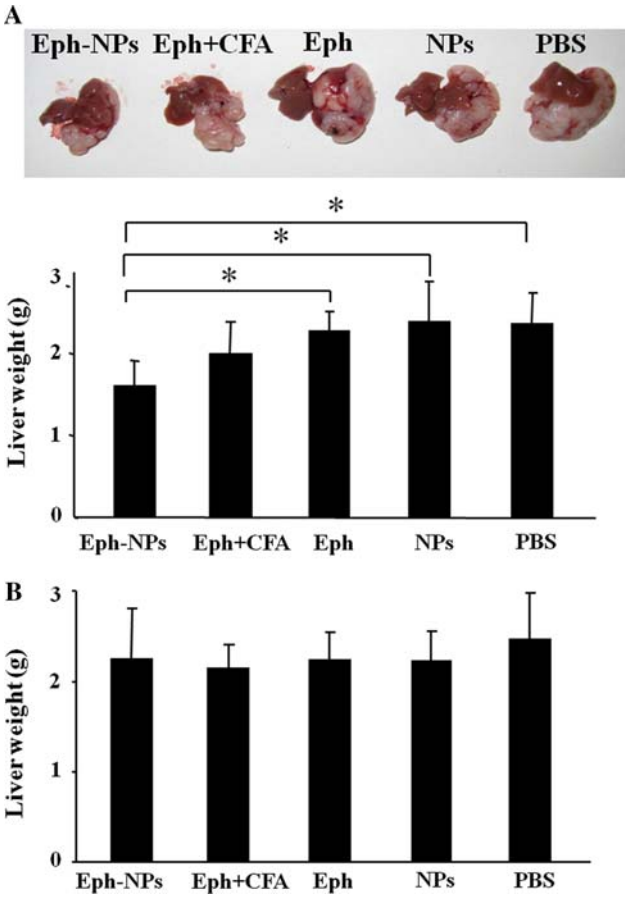

Fig. 2 Anti-tumor effects of immunization with Eph-NPs against liver tumor. C57BL/6 mice were immunized on day −7 and 0 with Eph-NPs, Eph + CFA, EphA2-derived peptide only (*Eph*), NPs only (*NPs*) or PBS. On day 0, 1×10^6 MC38 cells (**a**) or 1×10^6 BL6 cells (**b**) were injected intrahepatically. Fourteen days after immunization, mice were killed and liver weight was examined (**a** *upper panel*). Representative liver macroscopic view of each treatment group (**a** lower panel, **b**). Comparison of liver weight of each group. $^*P < 0.05$. $N = 8$/group. Each data point represents the mean liver weight ± SD

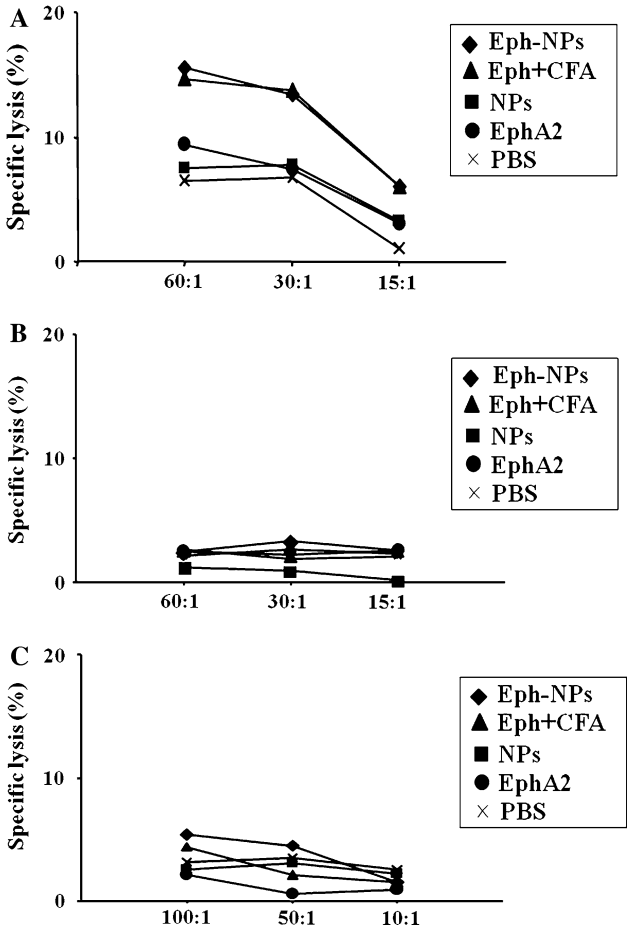

Fig. 3 Eph-NPs vaccines generated tumor-specific CTLs. Splenocytes were harvested from MC38 tumor-bearing mice 14 days after final treatment with Eph-NPs, Eph + CFA, NPs, Eph or PBS. Splenocytes were stimulated in vitro with MMC-treated MC38 cells for 5 days. The cytolytic activity of spleen cells was evaluated using 4-h ^{51}Cr release assays against MC38 (**a**) or irrelevant BL6 (**b**) tumor target cells at the indicated E:T ratios. **c** Liver lymphocytes were harvested 1 day after immunization into MC38-bearing mice. Liver lymphocytes were subjected to 4-h ^{51}Cr release assays against the NK-sensitive cells, YAC-1 as target cells at the indicated E:T ratios. Similar results were obtained in three independent experiments

No cytolytic activity was observed against a YAC-1 target cell in any of the control/treatment protocols (Fig. 3c). These results suggest that immunization using Eph-NPs or Eph + CFA effectively generated MC38-specific CTLs in vivo, which played essential roles in the liver tumor rejection.

Depletion of CD8+ T cells impairs the anti-tumor effects of immunization with Eph-NPs

To prove whether the therapeutic benefit associated with Eph-NPs vaccine in the MC38 liver tumor was dependent on CD4+, CD8+ T cells or NK cells, we performed selective cell subset depletion studies and C57BL/6 mice were immunized intraperioneally with Eph-NPs or PBS twice a week. On day 0, at the time of the second injection with these vaccines, mice were lightly anesthetized by isoflurane and 1×10^6 MC38 cells (EphA2-positive) were injected under the capsule of the left medial liver lobe as above. Mice were killed 14 days after tumor inoculation and the liver weight was measured. The anti-tumor efficacy of Eph-NPs immunization tended to be reduced in CD8+ T cell-depleted mice, while the liver weights of CD4+ T cell or NK cell-depleted mice were similar to those of non-depleted mice if the animals received Eph-NPs vaccines (Fig. 4). These results suggest that CD8+ T cells, but not CD4+ T cells or NK cells, tended to be required for optimal anti-tumor effects associated with Eph-NPs vaccines against liver tumor.

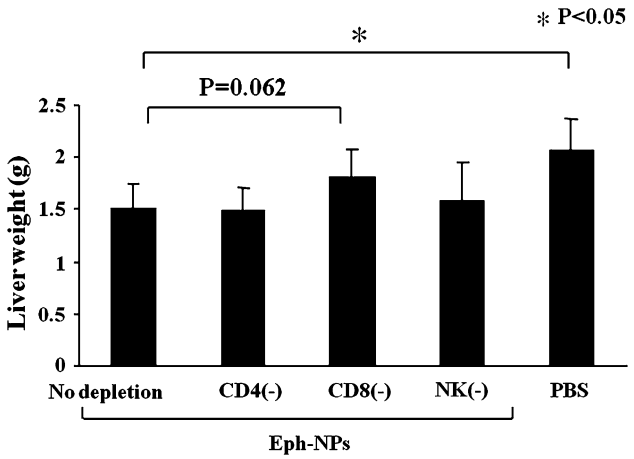

Fig. 4 Eph-NPs immunization tended to require CD8+ T cells, but not CD4+ T cells and NK cells in preventing liver tumor. Ab-mediated in vivo depletion of CD4+, CD8+ T cells, NK cells were performed (as described in "Materials and methods"), with the depleted mice then receiving Eph-NPs intraperitoneally (on day −7, 0) and 1×10^6 MC38 cells intrahepatically (day 0). Mice were killed 14 days after tumor inoculation and the liver weight was measured. $^*P < 0.05$. $N = 8$/group. Each data point represents the mean liver weight ± SD

Safety of Eph-NPs versus Eph + CFA

To evaluate the safety of Eph-NPs vaccine, the serum ALT, TBil, Alb and Crnn were examined for mice immunized with Eph-NPs, Eph + CFA, Eph, NPs or PBS as above. Immunization with Eph + CFA induced liver damage as evidenced by elevated serum ALT levels compared with those for mice treated with PBS. In contrast, other treatments did not lead to liver damage. There was no toxic effect on TBil, Alb and Crnn in all treatment groups (Fig. 5). Immunization with CFA induced granulomatous peritonitis in all of the mice, but immunization with the other regimens did not. These results demonstrated that the Eph + CFA vaccine is toxic to hepatocytes but the Eph-NPs vaccine does not harm the liver or kidney.

Discussion

We created new biodegradable γ-PGA-Phe NPs for use as a new adjuvant [16]. Uto et al. [22] reported that γ-PGA-Phe NPs could activate DCs in vivo and cellular immunity

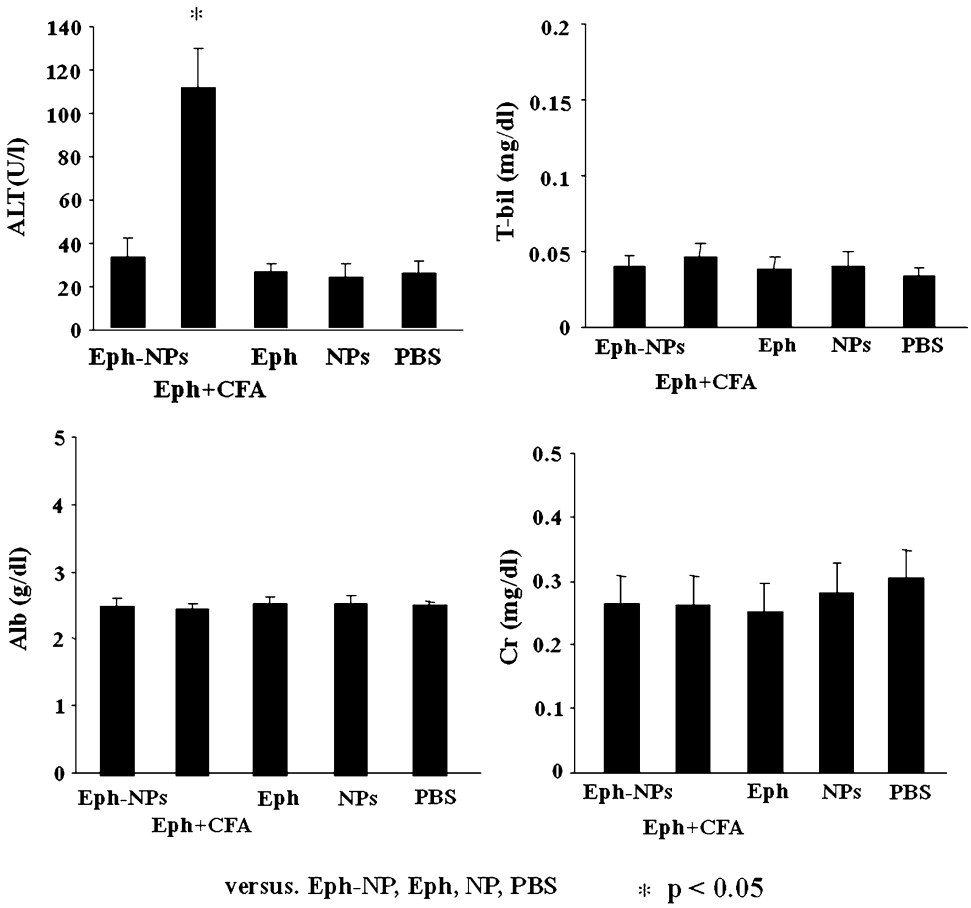

Fig. 5 Safety of Eph-NPs vaccine. Blood samples were obtained 7 days after final immunization of Eph-NPs, Eph + CFA, NPs, Eph or PBS. Levels of serum ALT, TBil, Alb or Crnn were examined. $N = 5$/group. $^*P < 0.05$ versus PBS group

against tumor cells expressing artificial antigen OVA. All previous reports using γ-PGA-Phe NPs as a vaccine adjuvant were evaluated with OVA artificial antigen models [22, 29–31]. Dhodapkar et al. [32] reported that the immunogenicity of peptides derived from self-melanoma antigens were very weak compared with viral protein-derived peptides. Although many TAA-derived peptides may be applicable to clinical use as peptide-based vaccines, most TAAs are self-antigens and not or weakly immunogenic, which is inferior to elicit enough anti-tumor immunity. Thus, the anti-tumor effect of γ-PGA-Phe NPs vaccines should be reevaluated by using self-TAA-derived peptides. In this study, we used EphA2-derived peptide [25] as a self-TAA. EphA2 is of particular interest due to evidence suggesting its involvement in carcinogenesis. EphA2 is a 130 kDa protein normally localized to sites of cell-to-cell contact, where it plays a role in contact growth inhibition [33]. However, cellular overexpression of EphA2, either as a result of its constitutive dysregulation or ectopic gene insertion, results in the disruption of cell-to-cell contacts, and enhancement of cell-to-extracellular matrix attachments [33]. As a result, tumor cells that overexpress EphA2 exhibit increased motility and invasive properties, consistent with a pro-metastatic phenotype [33]. Overexpression of EphA2 has been observed in numerous cancer types [34], including melanoma [35] and carcinomas of the breast [36, 37], lung [38], pancreas [39] and prostate [40]. We demonstrated the usefulness of Eph-NPs vaccine therapy, which revealed the future potential of clinical applications of this treatment in various cancers.

Complete Freund's adjuvant is an emulsion of water and mineral containing killed mycobacteria and has highly potent activity as an adjuvant. However, CFA administration induces adverse effects such as weight loss, neutrophilia and granulomatous peritonitis [41–43]. Consistent with earlier observations, immunization with Eph + CFA induced liver hepatocyte damage evidenced by elevation of ALT levels and granulomatous peritonitis in all of the mice. We demonstrated that immunization with Eph-NPs revealed anti-tumor effects against liver tumor via the generation of acquired immunity equal to the strongest but very toxic adjuvant, CFA, suggesting that our biodegradable γ-PGA-Phe NPs could be a promising candidate for a vaccine adjuvant against liver cancer.

IFN-γ ELISPOT assays revealed that immunization with Eph-NPs into normal mice resulted in induction of EphA2-derived peptide-specific CD8+ T cells at a level equivalent to Eph + CFA vaccine. Based on these results, we examined the anti-tumor effect of Eph-NPs vaccines in the EphA2-positive MC38 liver tumor model. The Eph-NPs vaccines resulted in eliciting anti-tumor effects against EphA2-positive MC38 liver tumor, but not against EphA2-negative BL6 melanoma, suggesting that EphA2-specific anti-tumor immunity was generated by Eph-NPs vaccines, which is consistent with our IFN-γ ELISPOT assay data. These results suggested that the anti-tumor potential of γ-PGA-Phe NPs vaccine is similar to that of CFA as an adjuvant in peptide-based vaccine. Importantly, Eph-NPs vaccine showed no toxic side effect on liver and kidney function. In contrast, CFA + Eph vaccine caused liver damage. γ-PGA-Phe NPs vaccine is safe and should be clinically applicable. This supports the clinical potential of γ-PGA-Phe NPs vaccine in cancer treatment.

In vitro cytotoxicity assays revealed that the anti-tumor effector cells for killing MC38 cells were CD8+ T cells, and possibly CTLs. This cytolytic activity was specific for MC38 cells because splenocytes did not kill BL6 cells. These results suggested that Eph-NPs vaccines could efficiently generate specific CTLs that recognize and kill relevant EphA2-positive, but not irrelevant EphA2-negative tumor targets. The liver uniquely contains an abundance of not only T cells, but also NK cells and NKT cells when compared with other organs [44, 45]. We have previously reported that not only CD8+ T cells, but also NK cells are required for optimal anti-tumor effects associated with EphA2-derived peptide pulsed DCs vaccines in liver tumors [26]. In this study, liver NK cells were not activated by Eph-NPs vaccination. Spleen NK cells were also not activated by Eph-NPs vaccine, and naïve spleen cells co-cultured with γ-PGA-Phe NPs or Eph-NPs could not display cytolytic activity against YAC-1 targets (S. Yamaguchi et al., unpublished data). These results suggested that the Eph-NPs vaccine could activate acquired immunity specifically. Our in vivo lymphocyte depletion studies demonstrated that CD8+ T cells, not CD4+ T cells and NK cells, tended to contribute to the inhibition of liver tumor growth in Eph-NPs vaccine, although we could not deny the possibility that humoral immune responses against EphA2 may also be generated by Eph-NPs vaccine. Previous reports demonstrated that biodegradable NPs were taken up by dedicated professional antigen-presenting cells, such as DCs, which resulted in their subsequent migration to lymph nodes, increased production of cytokines, and enhanced expression of costimulatory molecules followed by antigen-presentation to T cells [22, 29, 30]. Eph-NPs taken by DCs were directly presented to T cells and the generated Eph-specific CD8+ CTL could serve as effector cells against EphA2 expressing MC38 tumor.

In spite of recent progress and early success reported for adjuvant peptide vaccine trials in the prevention of liver cancer, there remains a great need to develop novel and effective treatment modalities. In this study, we demonstrated that immunization with Eph-NPs vaccines revealed anti-tumor effects against liver cancers via acquired immunity equivalent to the strongest CFA and that Eph-NPs vaccines did not lead liver or kidney damage. These results

suggest that γ-PGA-Phe NPs could be a promising candidate for a vaccine adjuvant against liver cancer. We are now preparing for the clinical application of γ-PGA-Phe NPs-peptide vaccine against liver cancer.

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