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

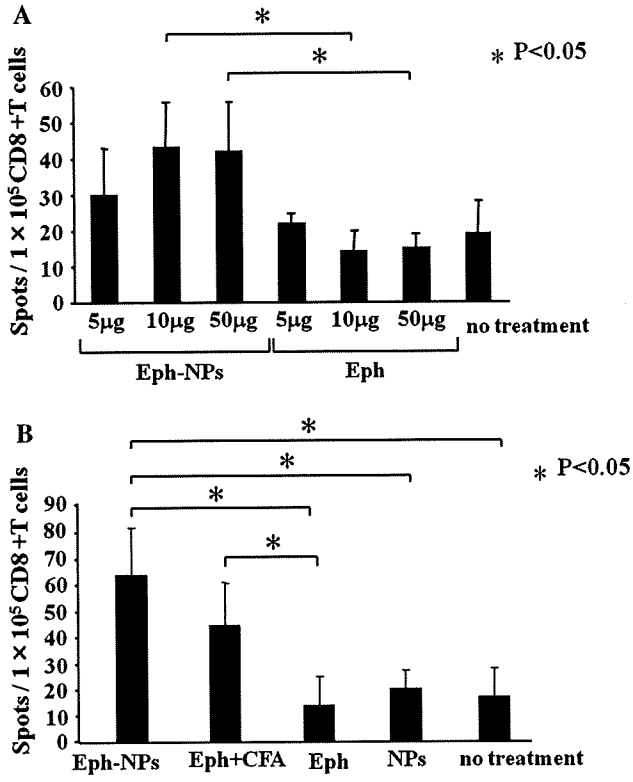

Fig. 1 IFN-γ ELISPOT assays for peptide-reactive CD8+ T cells responses. Normal mice ($N = 3$) were immunized with the indicated dose of Eph-NPs, Eph + CFA, Eph peptide only (*Eph*) or NPs only (*NPs*), and killed on day 5 post-immunization. Spleen cells were harvested and CD8+ T cells isolated using 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.

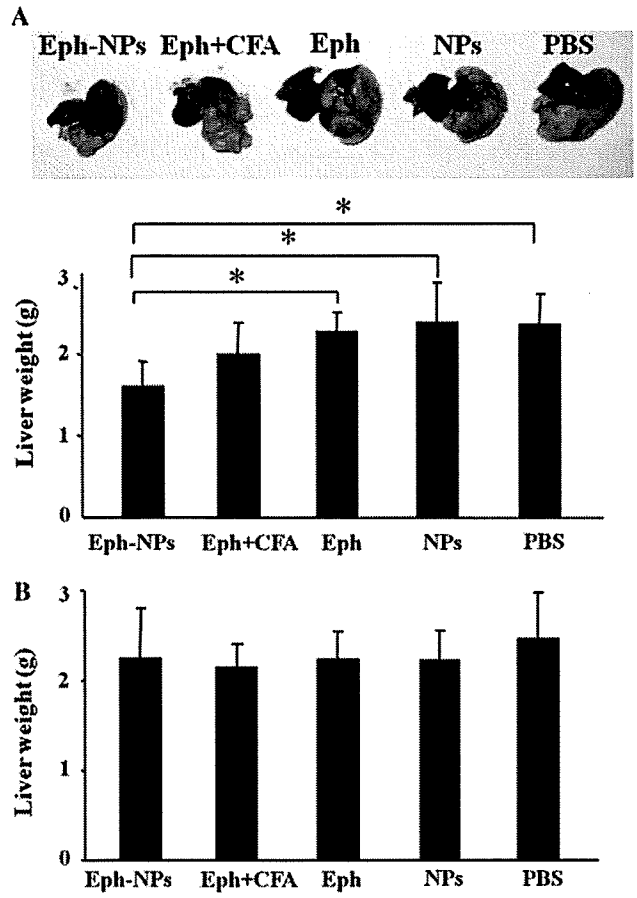

Fig. 2 Anti-tumor effects of immunization with Eph-NPs against liver tumor. C57BL/6 mice were immunized on day −7 and 0 with Eph-NPs, Eph + CFA, EphA2-derived peptide only (*Eph*), NPs only (*NPs*) or PBS. On day 0, 1×10^6 MC38 cells (**a**) or 1×10^6 BL6 cells (**b**) were injected intrahepatically. 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

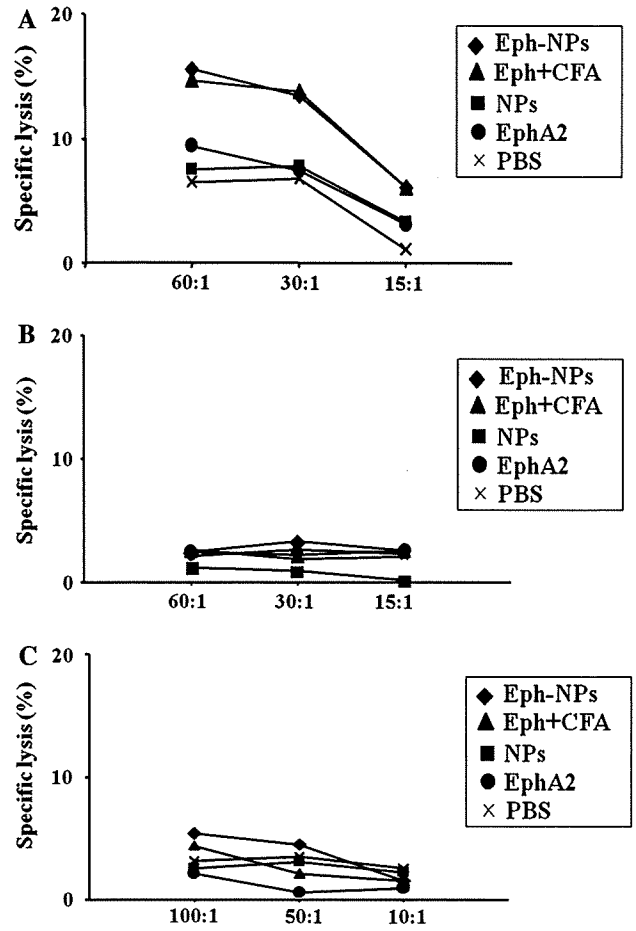

Fig. 3 Eph-NPs vaccines generated tumor-specific CTLs. Splenocytes were harvested from MC38 tumor-bearing mice 14 days after final treatment with Eph-NPs, Eph + CFA, NPs, Eph or PBS. Splenocytes were stimulated in vitro with MMC-treated MC38 cells for 5 days. The cytolytic activity of spleen cells was 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.

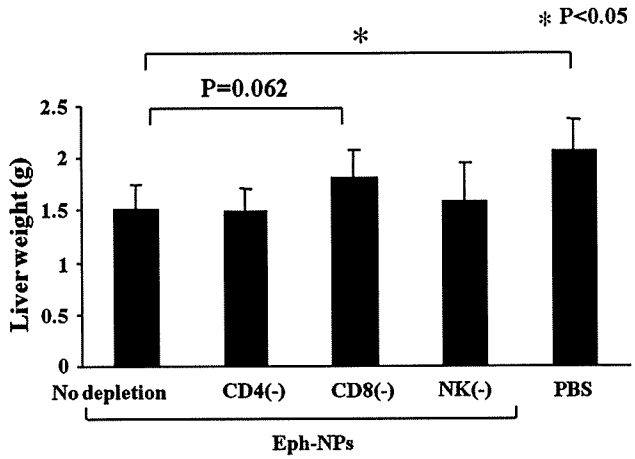

Fig. 4 Eph-NPs immunization tended to require CD8+ T cells, but not CD4+ T cells and NK cells in preventing liver tumor. Ab-mediated in vivo depletion of CD4+, CD8+ T cells, NK cells were performed (as described in "Materials and methods"), with the depleted mice then receiving Eph-NPs intraperitoneally (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

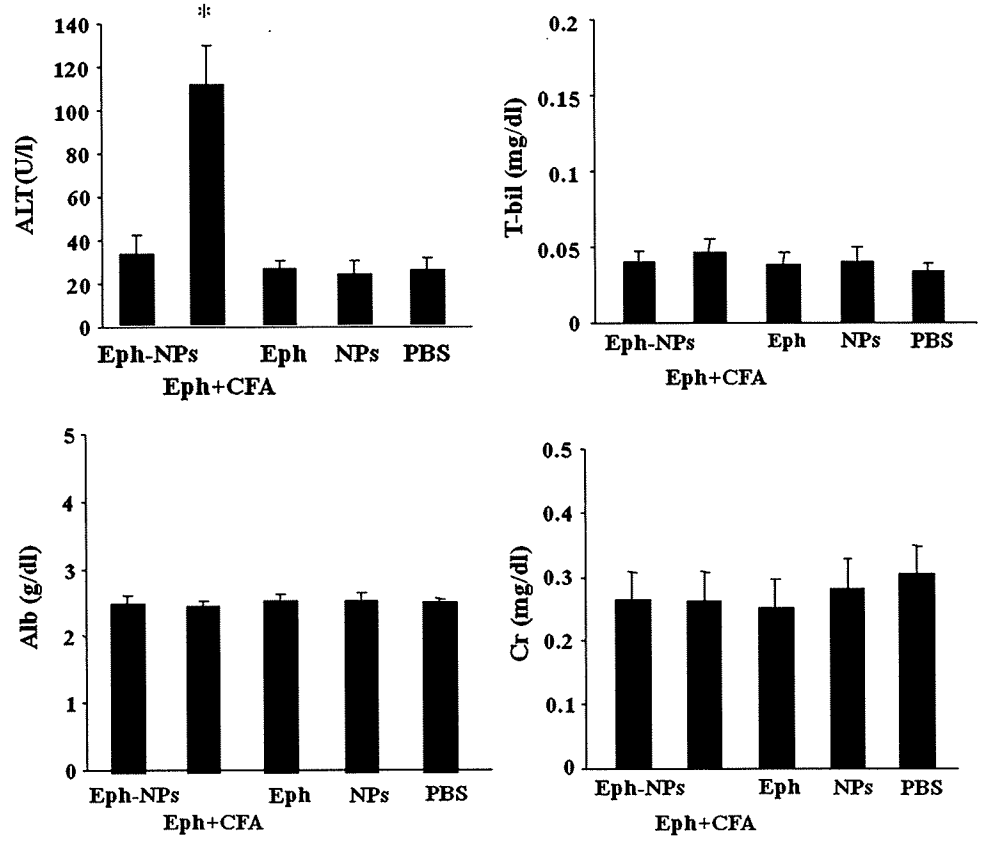

Fig. 5 Safety of Eph-NPs vaccine. Blood samples were obtained 7 days after final immunization of Eph-NPs, Eph + CFA, NPs, Eph or PBS. Levels of serum ALT, TBil, Alb or Crnn were examined. $N = 5$/group. $*P < 0.05$ versus PBS group

versus. Eph-NP, Eph, NP, PBS * $p < 0.05$

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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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The complement component C3a fragment is a potential biomarker for hepatitis C virus-related hepatocellular carcinoma

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Abstract
Background Hepatocellular carcinoma (HCC) has a high mortality rate, and early detection of HCC improves patient survival. However, the molecular diagnostic markers for early HCC have not been fully elucidated. The aim of this study was to identify novel diagnostic markers for HCC.
Methods Serum protein profiles of 45 hepatitis C virus infection (HCV)-related HCC patients (HCV-HCC) were compared to 42 HCV-related chronic liver disease patients without HCC (HCV-CLD) and 21 healthy volunteers using the ProteinChip SELDI system. One of the identified proteins was evaluated as a diagnostic marker for HCC in patients with HCV.
Results Five protein peaks (4067, 4470, 7564, 7929, and 8130 m/z) had p-values less than 1×10^{-7} and were significantly increased in the sera of HCV-HCC patients compared to HCV-CLD patients and healthy volunteers. Among these proteins, an 8130 m/z peak was the most differentially expressed and identified as the complement component 3a (C3a) fragment. For HCV-HCC and HCV-CLD, the relative intensity of this C3a fragment had the best area under the ROC curve [0.70], followed by des-γ-carboxy prothrombin (DCP) [0.68], lectin-bound alpha fetoprotein (AFP-L3) [0.58] and AFP [0.53] for HCC. A combined analysis of the C3a fragment, AFP and DCP led to a 98% positive identification rate. In addition, the measurable C3a fragment in some HCC patients was not only significantly higher in the year of HCC onset compared to the pre-onset year, but also decreased after treatment.
Conclusions The 8130 m/z C3a fragment is a potential marker for the early detection of HCV-related HCC.

Keywords Hepatocellular carcinoma · Complement component C3a · Serum proteomics · Serum biomarkers · Proteinchip SELDI system · Hepatitis C virus

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Introduction

Hepatocellular carcinoma (HCC) is reportedly the third most frequent cause of global cancer-related deaths, and the incidence of HCC is increasing worldwide [1, 2]. The clearly established risk factor for HCC is chronic hepatitis C virus (HCV) infection [3].

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To date, both ultrasonography and serum tumor markers such as the alpha fetoprotein (AFP), and des-γ-carboxy prothrombin (DCP) assay are the principle methods for screening and detecting HCC. Routine screening is the best method to detect early HCC and improve patient survival; however, elevated serum AFP and DCP levels have insufficient sensitivity and specificity, respectively. The sensitivity and specificity of serum elevated AFP levels were reported to range from 39–64% and 76–91%, while those of the serum elevated DCP levels were 41–77% and 72–98%, respectively [4–9]. In addition, it was recently reported that only a small percentage of small HCC tumors were diagnosed based on AFP and DCP [6, 10]. The lens culinaris agglutinin-reactive fraction of AFP (lectin-bound AFP or AFP-L3) has been reported to be elevated in the serum of HCC patients. Although AFP-L3 has a high range of specificity for detecting HCC, the sensitivity is low [11, 12]. The ability to detect early HCC, prior to the onset of clinical symptoms, leads to curative treatment and significantly improves the disease prognosis. Thus, additional biochemical markers are necessary for the specific detection of early HCC.

Serum profiling using a proteomic approach is thought to be a useful technique to detect or predict early HCC in chronic liver disease patients. Studies using the ProteinChip SELDI system, which is a powerful tool to discover new biomarkers, have shown that this method may be successfully used to diagnose HCC. Zinkin et al. [13], Schwegler et al. [14] and our research group [15] previously detected early HCC using the profile of several protein peaks that were identified by the ProteinChip SELDI system. Paradis et al. [16] reported the highest discriminating peak (8900 Da), which was identified as the V10 fragment of vitronectin. Furthermore, Lee et al. [17] described complement 3a, which had a molecular weight of approximately 8900 Da, as a novel marker of HCC. Therefore, using this proteomic approach to identify specific proteins may not only help establish simple methods to detect HCC, but also further our understanding of the molecular mechanisms of hepatocarcinogenesis and facilitate the development of novel cancer therapies. Therefore, this study assessed and compared the protein expression profiles in the sera of HCC patients in order to identify a more useful biomarker of HCC-associated HCV infection using proteomic approach.

Materials and methods

Samples

Eighty-seven patients [45 HCC patients and 42 patients with chronic liver diseases without HCC (CLD)] with HCV infection were selected to participate in this study (Table 1). These patients provided informed consent. Serum samples were collected by the Faculty of Medicine, University of Miyazaki (Miyazaki, Japan), and some patients were in a hyperendemic HCV area with a cohort study in Miyazaki [18]. The sera of all patients with and without HCC, which was confirmed by abdominal ultrasonography or computed tomography, were obtained prior to treatment. All of the sera samples from HCV-infected patients were analyzed in a previous study [15]. In addition, sera from 10 HCV-HCC patients who were diagnosed with HCC within 1 or 2 years and sera from five patients who had received radiofrequency ablation (RFA), percutaneous ethanol injection therapy (PEIT) and/or transarterial chemoembolization (TACE) for HCC were collected through a cohort study in Miyazaki. We also analyzed the sera of 21 healthy volunteers without HCC as controls. After freezing and thawing once, all samples were separated into 50–100 μl aliquots and refrozen at −80°C. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Miyazaki, Kagoshima University Graduate School of Medical and Dental Sciences, and Harvard School of Public Health and Boston University School of Public Health.

Table 1 Patient characteristics

	HCC[a]	CLD[b]	*p* value
Patients (male/female)	45 (40/5)	42 (40/2)	–
Age	73.6 [63–85]	61.8 [41–83]	<0.0001
PLT[c] ($\times 10^4$/ul)	12.5 ± 5.8	8.4 ± 4.6	0.001
Albumin (g/dl)	3.8 ± 0.8	4.2 ± 1.6	0.8
ALT[d] (IU/l)	57.7 ± 28.3	52.8 ± 37.5	0.7
AFP[e] (ng/ml)	311 ± 1144	51.6 ± 36.1 (38)	0.008
DCP[f] (mAU/ml)	235 ± 605 (44)	37.1 ± 59.8 (39)	<0.0001
HA[g] (ng/ml)	388 ± 446 (40)	280 ± 272 (27)	0.6
Diameter of HCC (mm)	23.2 [10–40]	–	–
TNM stage[h] (I/II/III/IV)	24/18/3/0	–	–

Data are shown as the means ± SD or means [range] (numbers)

[a] Hepatocellular carcinoma

[b] Chronic liver disease

[c] Platelet counts

[d] Alanine aminotransferase

[e] Alpha fetoprotein

[f] Des-γ-carboxy prothrombin

[g] Hyaluronic acid

[h] TNM; primary tumor/lymph node/distant metastasis

SELDI-TOF/MS analysis of sera

Expression difference mapping analysis profiles of the samples were obtained using weak cation-exchange (CM10) ProteinChip Arrays (Bio-Rad Laboratories). Arrays were analyzed by ProteinChip reader as previously reported [15]. In addition, the laser intensity ranged from 220 to 245, with a detector sensitivity of 8, and spectra ranging from 1300 to 150000 m/z were selected for analysis in this study.

Separation of candidate biomarker (8.1 k m/z)

The purification strategy was determined by the ProteinChip Arrays. Two hundred microliters of sera from HCV-HCC patients were diluted 5-fold into 50 mM Na-phosphate buffer, pH 7.0, and loaded onto a CM-Ceramic HyperD F spin column (Bio-Rad Laboratories). After equilibrating with the same buffer, the samples were eluted with a stepwise sodium chloride gradient from 0, 200, 300, and 1000 mM. The elution was desalinated and concentrated using a centrifugal concentrator (VIVASPIN, Vivascience, Hannover, Germany), and the purification progress was monitored using NP20 arrays. The flow-through fraction was dialyzed and then separated by 16.5% tricine one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The SDS–PAGE samples were run in tricine sodium dodecyl sulfate buffer according to the manufacturer's instructions and then stained with Coomassie brilliant blue (CBB).

Identification of the candidate biomarker (8.1 k m/z)

Gel pieces containing the target 8.1 k m/z protein were excised. The excised bands were reduced and alkylated for 30 min at room temperature, and then digested with trypsin (Modified Sequence Grade, Roche Diagnostics, Basel, Switzerland) in Tris–HCl, pH 8.0, for 20 h at 35°. The reaction solution was applied to NP20 arrays and allowed to air dry. To identify the protein, the digested peptides were purified by high-performance liquid chromatography (HPLC; MAGIC 2002; Michrom Bioresources Inc., Auburn, CA) and analyzed by Q-Tof2 (Micromass; Waters Ltd., Hertsfordshire, UK). The HPLC solvent consisted of solvent A (2% acetonitlile/0.1% formic acid) and B (90% acetonitlile/0.1% formic acid). The digested peptides were separated with a linear gradient from 10 to 50% solvent B with a flow rate of 400 nl/min using HPLC [19]. Mass spectral data were searched with Mascot (http://www.matrixscience.com) to identify proteins based on the peptide mass [20, 21].

Immunodepletion assay

For immunodepletion, serum samples were prepared as follows. Sera (250 μl) from HCC patients were diluted 5-fold in 50 mM Tris–HCl buffer, pH 8.0, and loaded onto a CM-Sepharose Fast Flow spin column (GE Healthcare Bio-Sciences Corp., NJ). After equilibration with the same buffer, the samples were eluted with a stepwise sodium chloride gradient from 0, 500, and 1000 mM. The elution from each NaCl concentration was monitored using NP20 arrays. To prepare the antibodies for immunodepletion, 6 μl anti-human C3 antibody, which detected C3 and C3a expression, or anti-C4a antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 20 μl Interaction Discovery Mapping (IDM) affinity beads (Bio-Rad Laboratories) and Protein A (Sigma Chemical Co, St. Louis, MO) over night at 4° with shaking. These beads were centrifuged, and the supernatant was discarded. The beads were washed with 50 mM phosphate buffer (pH 7.0), and 3 μl of the prepared serum sample was incubated with 15 μl IDM affinity beads with shaking for 2 h at 4°. As a negative control, 3 μl sample was incubated with IDM affinity beads and Protein A with an anti-C4a antibody or without antibody. After the incubation, the samples were cleared by centrifugation, and 5 μl of each supernatant was analyzed on NP20 ProteinChip arrays in a PBS II reader.

Cell culture and SELDI-TOF/MS analysis of culture supernatants

The human hepatocarcinoma cell line HuH-7 and human hepatoblastoma cell line HepG2 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin G, and 100 mg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA). Before starting the experiments, the cells were cultured on 96-well microplates in medium without FBS for 24 h. After washing with FBS-free media, the cells were cultured for 24 h with FBS-free media with or without 500 μg/ml of C3a (Calbiochem, San Diego, CA). The supernatants were collected by centrifugation and analyzed for the expression of 8.1 k m/z using the ProteinChip system.

Statistical analysis

Values are shown as the means ± SD. Statistical differences, including laboratory data and individual peaks in SELDI TOF/MS, were determined using the Mann–Whitney U test. Values of $p < 0.05$ were considered statistically significant. The discriminatory power for each putative marker was described via receiver operating characteristics

(ROC) area under the curve (AUC). These statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA), SPSS software (SPSS Inc., Chicago, IL), JMP software, or Ciphergen ProteinChip Software, version 3.0.2.

Results

Profiling sera from HCC patients and healthy controls

We analyzed the sera of all patients with HCV-HCC or HCV-CLD and healthy controls without HCC using the CM10 ProteinChip array to identity the most differential protein peak. Peaks were automatically detected using the Ciphergen ProteinChip Software 3.0.2. following baseline subtraction as described previously [15, 22]. This analysis identified 178 protein peak clusters, as seen in the spectrum representations from the three groups (HCV-HCC, HCV-CLD, and healthy control) in the 3000- to 15000-m/z range. Peak expressions were increased for 18 proteins and decreased for 14 proteins in sera from HCV-HCC patients compared to HCV-CLD patients. Compared to healthy subjects, 68 protein peaks were increased, and 16 protein peak intensities were decreased in the sera of HCV-HCC patients. Five protein peaks (4067, 4470, 7564, 7929, and 8130 m/z) had a p-value less than 1×10^{-7} and were significantly increased in the sera of HCC patients compared to the sera of HCV-CLD patients and healthy volunteers. In particular, an 8130 m/z peak was the most significantly different peak and had the most differential expression profile between patients with HCV-HCC and with HCV-CLD.

Purification and identification of the 8.1 k m/z peak

We optimized the adsorption and desorption conditions on the arrays using an HCV-HCC patient serum sample and healthy volunteer serum sample in order to determine a procedure to purify the target 8.1 k m/z protein. The optimal pH for retention of the 8.1 k m/z protein was a *pI* value of approximately 7.0 on the CM10 arrays, which indicates that weak cation-exchange sorbents and buffer pH should be fixed for further experiments. The target protein was eluted by increasing the sodium chloride concentrations in a Na-phosphate buffer and was eluted in the 1000 mM sodium chloride fraction. The concentrated serum protein that was eluted with 1000 mM sodium chloride was applied to SDS–PAGE for further separation. The 8.1 k m/z protein was identified and excised by in-gel trypsin digestion for identification. The peptide sequences were analyzed using liquid chromatography (LC)-MS/MS and then examined by a database search with Mascot. The digested peptides matched human complement C3a (Fig. 1).

After reacting the HCC sera with anti-complement C3a or anti-C4 antibodies or without antibody, the supernatants were analyzed by the SELDI ProteinChip system for immunodepletion. Analysis of the supernatant showed that only the 8.1 k m/z peak corresponding to complement C3a

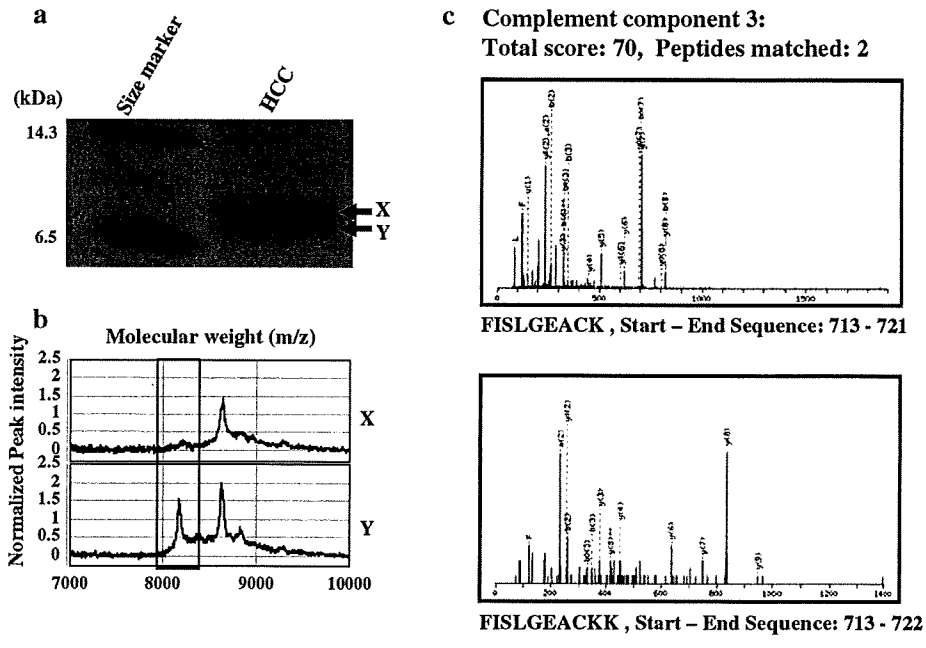

Fig. 1 **a** Partially purified proteins were separated by SDS–PAGE using serum samples from HCV-HCC patients. The Coomassie-stained SDS-PAGE gel shows two clear bands at approximately 8 kDa (*X* and *Y*). **b** After each band (*X* and *Y*) was excised from the gel, the proteins were extracted and analyzed using the ProteinChip system. The target protein in the excised band was detected, and the 8.1 k m/z peak corresponded only to the "*Y*" band contained in gel. **c** The excised "*Y*" band was alkylated and digested using trypsin. The peptides were collected and subjected to LC–MS/MS analysis. The proteins, which were derived from complement C3a, were identified using a database search

was reduced. On the other hand, immunodepletion with a control anti-C4 antibody or without antibody did not reduce the 8.1 k m/z peak (Fig. 2).

Profiling the C3a of sera from patients with HCC and without HCC

The 8.1 k m/z peak was confirmed as the complement C3a fragment using an immunodepletion assay. However, C3a was stabilized as C3adesArg with a molecular weight of approximately 8.9 k m/z. Figure 3a, b compares the expression of the 8.1 k m/z peak in the sera of HCV-HCC or HCV-CLD patients and healthy controls. The intensities

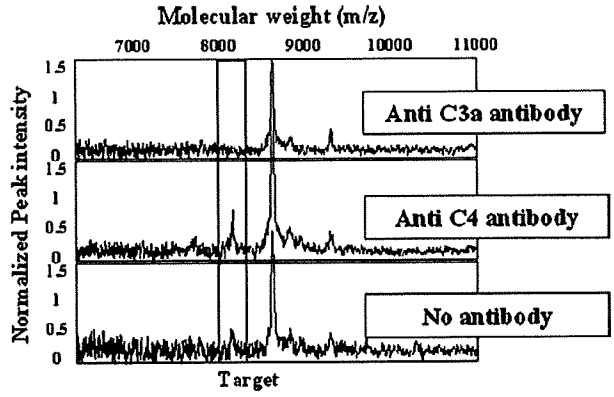

Fig. 2 Immunodepletion assay of the C3a fragment. Analysis of supernatant that had been immunodepleted with an anti-C3a antibody showed that only the 8.1 k m/z peak corresponding to complement C3a was reduced. Supernatants that had been immunodepleted with either a control anti-C4 antibody or without antibody did not have reduced 8.1 k m/z peaks by the ProteinChip system

in HCC patient sera were significantly higher than those in the HCV-CLD patients or healthy controls. The expression of the 8.9 k m/z peak in HCV-HCC patients was also higher than that in HCV-CLD patients or healthy controls (Fig. 3c, d). Although the 8.9 k m/z peak was not identified as C3adesArg, it is possible that both the 8.1 and 8.9 k m/z peaks were specific tumor markers for HCC. Furthermore, we analyzed sera from 10 HCV-HCC patients who were diagnosed with HCC within 1 or 2 years and sera from five patients who had received curative treatments using RFA, PEIT, and TACE for HCC. The 8.1 k m/z C3a fragment in the HCV-HCC patients was significantly increased in the year of disease onset compared to the pre-onset year. After treatment, expression of the C3a fragment significantly decreased in all five of the patients who had measurable samples after treatment (Fig. 4a). In contrast, the 8.9 k m/z peak did not change regardless of the occurrence of HCC over time (Fig. 4b). Thus, the 8.1 k m/z C3a fragment appears to be the most discriminatory tumor marker for HCV-HCC.

Relationship between the C3a fragment and other tumor markers

AFP and DCP levels were measured in sera from 83 of 87 patients with HCV-associated liver disease. The recommended cutoff levels for these tumor markers, AFP and DCP, are 20 ng/ml and 40 mAU/ml, respectively. AFP-L3 in 26 patients with HCV-associated liver disease was also investigated among measurable samples in which AFP in a total 35 patients was higher than 20 ng/ml, The cutoff level of AFP-L3 was set at 10%. When samples from patients

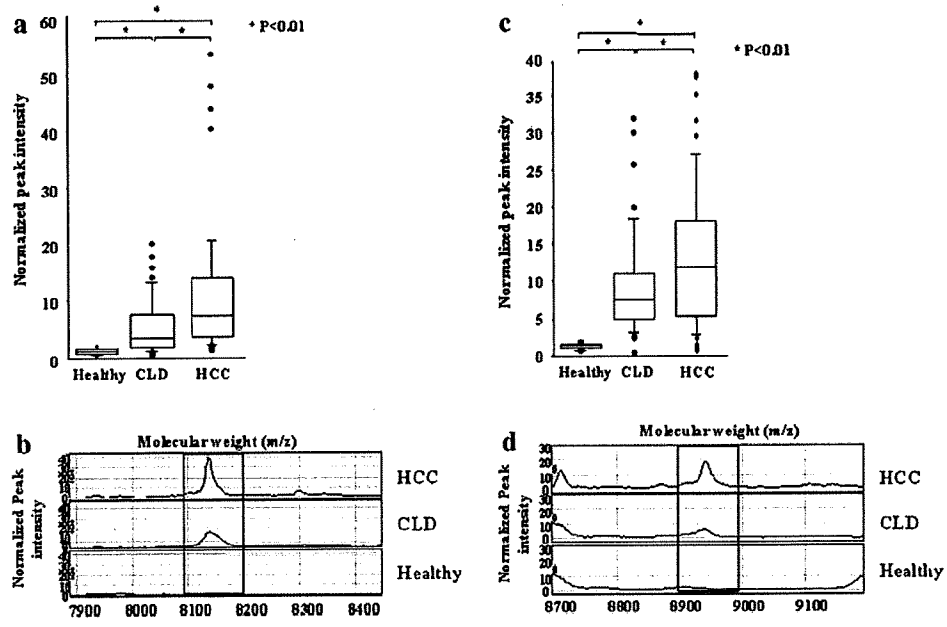

Fig. 3 **a** and **c** Comparisons of the expression profiles of the 8.1 and 8.9 k m/z peaks in HCV-HCC, HCV-CLD, and healthy sera. *Boxes* indicate the median ± 25th percentile. The *lower* and *upper bars* represent the 10th and 90th percentiles, respectively. **b** and **d** Representative spectra of the 8.1 and 8.9 k m/z peaks from patients in each group. The *horizontal axis* indicates the protein molecular weight, while the *vertical axis* designates the relative intensity

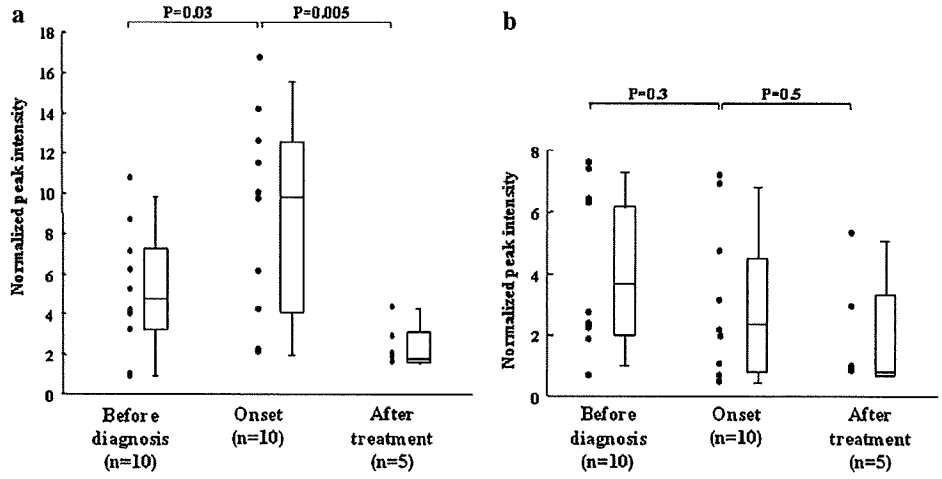

Fig. 4 Comparisons of the expression profiles of the 8.1 k m/z (**a**) and 8.9 k m/z (**b**) peaks in sera from HCV-HCC patients before diagnosis, during disease onset, and after treatment. The samples in the before diagnosis group included sera collected 1 or 2 years before the onset of HCC. *Boxes* indicate the median ± 25th percentile, the *lower bar* indicates the 10th percentile and the *upper bar* indicates the 90th percentile

Table 2 Diagnostic rates for hepatocellular carcinoma in the HCV infected patients

Markers	Sensitivity (%)	Specificity (%)	ROC AUC
AFP[a] (>20 ng/ml)	38 (17/45)	47 (18/38)	0.53
DCP[b] (>40 mAU/ml)	45 (20/44)	74 (29/39)	0.68
AFP-L3[c] (>10%)	58 (8/14)	50 (6/12)	0.58
C3a fragment (>3.5)	78 (37/45)	52 (22/42)	0.70
C3a fragment + AFP	91 (41/45)	26 (10/38)	0.72
C3a fragment + DCP	93 (41/44)	33 (13/39)	0.77
AFP + DCP	64 (28/44)	34 (12/35)	0.70
C3a fragment + AFP + DCP	98 (43/44)	20 (7/35)	0.80

[a] Alpha fetoprotein

[b] Des-γ-carboxy prothrombin

[c] Alpha fetoprotein, lectin lens culinaris agglutin-bound fraction

with HCV-HCC and HCV-CLD without HCC were compared, the sensitivity and specificity of AFP were 38 and 47%, whereas those of DCP were 45 and 74% and those of AFP-L3 were 58 and 50%, respectively. When the cutoff level for the relative intensity of the C3a fragment was set at 3.5, the sensitivity and specificity were 78 and 52%, respectively; the C3a fragment had the most sensitivity for the diagnosis of HCC. Furthermore, the ROC AUC of the C3a fragment, AFP, DCP, and AFP-L3 was 0.70, 0.53, 0.68, and 0.58, respectively (Table 2). There was no relationship between the C3a fragment and several other tumor and inflammation markers [AFP, DCP, AFP-L3, alanine aminotransferase (ALT), and high-sensitivity C-reactive protein (hs-CRP)], and each of these markers was independent of the diameter and number of tumors. The ROC AUC using AFP and DCP was highly similar to the ROC AUC with the C3a fragment alone. In addition, we investigated a combination assay that included the C3a fragment, AFP and DCP. This combination test, in which at least AFP, DCP, or the C3a fragment was positive, had a positive identification rate of 98%, although the specificity of this assay was too low at 20%. The ROC AUC of the combination test using AFP, DCP, and the C3a fragment was higher than those of any other markers. This result indicates that this combination assay using three markers is more useful than the combination assay using AFP ± DCP, which are measured worldwide to detect HCC (Table 2).

Profiling C3a expression in culture medium

C3a reacted with HCC cell lines, and the C3a peak in the culture medium was monitored by the ProteinChip system. The C3a fragment (approximately 8.1 k m/z) was not detected in the supernatants of HuH-7 and HepG2 cell cultures. However, the 8.9 k m/z peak was detected in the culture medium. This 8.9 k m/z peak was considered to be a stabilized form of C3a. This result indicated that the stabilized form of C3a (8.9 k m/z) was not undergoing proteasome-mediated degradation to yield the C3a fragment (8.1 k m/z) in these HCC cell lines.

Discussion

Because the HCC disease-associated mortality rate remains high, it is highly important to develop early diagnostic tools and treatments for HCC. Our study indicates that an 8.1 k m/z peak, which was identified as the C3a fragment by both peptide sequencing and an immunoassay, is up-regulated in the serum of HCC patients, 93% (42/45) of whom were TNM stage I or II. The C3a fragment in some HCC cases was also significantly higher in the year of HCC onset compared to the pre-onset year and decreased after curative treatment. Therefore, the C3a fragment appears to

be a promising simple tumor marker for the diagnosis of early HCC. In addition, a combination serum HCC diagnostic test that included AFP, DCP, and the C3a fragment had higher sensitivity than each individual marker. These results suggest that this combination test may be a useful HCC screening method, although the low specificity may pose challenges. Further examinations are needed to determine whether the C3a fragment or a combination test can be used to detect early HCC.

The results of our study demonstrated that the C3a fragment (8.1 k m/z) is a highly expressed novel tumor marker that is abundant in the sera of early HCC patients but not in the sera of healthy volunteers or HCV-CLD patients. A similar study by Lee et al. [17] used the ProteinChip SELDI system to show that C3a is a potential candidate biomarker for HCV-HCC. However, Lee et al. found that the molecular weight of C3a was represented by an approximately 8.9 k m/z peak. C3a has a very short half-life and is immediately cleaved into the more stable C3adesArg (8.9 k m/z), which is the anaphylatoxin C3a that lacks the C-terminal arginine and is stable state in the serum [23]. In our study, the 8.9 k m/z peak was also significantly different among HCV-HCC patients, HCV-CLD patients, and healthy volunteers (Fig. 3c, d). However, the discriminatory power of the 8.9 k m/z peak (ROC AUC was 0.60) was lower than the 8.1 k m/z peak (ROC AUC was 0.70) to distinguish between HCV-HCC and HCV-CLD. In addition, unlike the 8.1 k m/z peak, the levels of the 8.9 k m/z peak did not significantly increase with time as HCC progressed in 10 HCV-HCC cases (Fig. 4b). In contrast, Li et al. identified two proteins (8926 m/z and 8116 m/z) as complement component C3adesArg and a C-terminal truncated form of C3adesArg; the latter was a C-terminal truncation of C3adesArg that lacked the C-terminal sequence RASHLGLA (referred to as C3adesArgΔ8) in breast cancer patients [24]. However, these two biomarkers cannot be used to discriminate between breast cancers and benign tumors, and there were minimal differences in the peak intensities between breast cancer patients and healthy controls. Therefore, the C3a fragment with a molecular weight of 8.1 k m/z appears to be a potential diagnostic marker for HCC, although we cannot explain why the 8.1 k m/z fragment of C3a is overexpressed in HCC patients and did not confirm whether our C3a fragment (8.1 k m/z) is C3adesArgΔ8.

C3a, including C3adesArg, was also previously identified as a tumor marker for lymphoid malignancies, breast and colorectal cancers using the ProteinChip SELDI system [24–26]. Complement activation and subsequent deposition of complement components on tumor tissues has been demonstrated in cancer patients [27]. Malignant ovarian cells isolated from ascitic fluid samples had C3 activation products deposited on their cell surface [28]. Complement components are important mediators of inflammation and help regulate the immune response. C3a is biologically active and binds to mast cells and basophils, triggering the release of their vasoactive contents [29]. We investigated C3a expression by immunochemical examination of HCC tissues and Western blot analysis of proteins extracted from human HCC cell lines, including HepG2 and HuH-7. However, specific C3a expression, including the C3a fragment (8.1 k m/z), was not detected.

The complement system can be activated after exposure to tumor antigens [30]. It is speculated that small tumors can trigger a systematic reaction. Therefore, elevated C3a (8.9 k m/z) levels in the serum of HCV-HCC patients may reflect both a systematic immune response to HCV infection and non-specific tumor antigens rather than a specific immune response to HCC [24–26, 31]. In contrast, it is possible that overexpression of the C3a fragment (8.1 k m/z) is specific for HCC in addition to non-specific C3 activation.

In contrast to our results, Steel et al. [32] searched for HCC biomarkers using HCC-associated HBV-infected patient sera and found that the C-terminal fragment of complement C3 was down-regulated. Kawakami et al. [33] searched for characteristic alterations in the sera of HBV- and HCV-HCC-infected patients who had undergone curative radiofrequency ablation treatment and showed that C3 was up-regulated after treatment. In these studies, C3 was separated and identified using 2-DE of a mixture of proteins from a small number of patient sera samples, and this process identified various molecular weights for C3. In addition, we analyzed the sera of 25 patients with HCC-associated HBV infections, and the profile of several proteins was different between HCV- and HBV-infected patients. Although 35 protein peaks, including the C3a fragment, were overexpressed in the sera of both HCV-HCC and HBV-HCC patients compared to sera from healthy volunteers, the C3a fragment (8.1 k m/z) was particularly overexpressed in the sera of HCV-HCC patients and was not significantly different between HBV-HCC patients and HCV-CLD patients without HCC (data not shown). The biologic and pathogenic activities of HCV and HBV are different, and the molecular mechanisms underlying the development of hepatitis and hepatocarcinogenesis may differ between HBV and HCV infections [34–36]. Although the number of samples, cause of liver disease, and method of protein identification may affect these results, we speculate that the C3a fragment with a molecular weight of 8.1 k m/z is a candidate tumor marker for HCV-HCC but not HBV-HCC.

AFP, which is a commonly used HCC tumor marker, is elevated not only during HCC, but also during hepatocyte regeneration following liver damage. Previous reports revealed that AFP was abnormally elevated in the sera of patients with acute hepatitis, chronic hepatitis, and liver

cirrhosis. This lack of specificity for HCC means that AFP has a comparatively high false-positive rate [37]. The C3a fragment may also be elevated during hepatocyte regeneration following liver damage [38], and early diagnosis of small HCC tumors may be difficult with one marker alone. Therefore, the false-positive rates for HCC must be carefully considered [39–41]. Also, a combination of markers, including AFP, DCP, and the C3a fragment, in the serum should be verified to improve the diagnostic rate.

The ProteinChip SELDI system can separate and partially characterize multiple proteins in tissue and serum samples. Our previous report used a panel of proteins to diagnose early HCC with the ProteinChip SELDI system [15]. This panel diagnosis of seven protein peaks included a discriminant peak of 4060 m/z. This 4060 m/z peak may be a double-charged 8130 m/z peak, although the C3a fragment (8130 m/z) was not used to develop this diagnostic method. These results suggest that the C3a fragment is a useful HCC biomarker, regardless of whether this fragment carries a single or double charge. In addition, the panel diagnosis method is more useful than measuring the C3a fragment alone to diagnose and predict the occurrence of HCC. However, this method must be performed using the ProteinChip SELDI system, which is expensive and does not detect putative interactions between various proteins. Identifying a specific HCC protein such as the C3a fragment will also further our understanding of the molecular mechanisms of hepatocarcinogenesis. Therefore, the C3a fragment should not only be considered a simple HCC tumor marker, but should also be evaluated for its contribution to HCC carcinogenesis.

In conclusions, serum profiling with the ProteinChip SELDI system may be used to distinguish HCC from chronic liver disease without HCC and to detect early HCC in HCV-infected patients. Because we identified the C3a fragment (8.1 k m/z) in serum samples from HCC patients, the C3a fragment is a promising marker that can be used to screen for HCV-HCC and to develop new therapeutic targets.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinical significance of alanine aminotransferase levels and the effect of ursodeoxycholic acid in hemodialysis patients with chronic hepatitis C

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Abstract
Background The natural history of hepatitis C virus (HCV) carriers and the effect of ursodeoxycholic acid (UDCA) have not been fully elucidated among hemodialysis (HD) patients.
Methods Eighty-four anti-HCV antibody- and HCV RNA-positive and 154 anti-HCV antibody-negative HD patients who were retrospectively observed for at least 3 years were analyzed. We investigated the factors associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) and decreased platelet count (PLT) (more than 20% decrease during the follow-up period), which were considered to be indicators of hepatic fibrosis. In addition, another 16 HD patients with HCV who received 300 mg/day UDCA orally for at least 6 months were investigated. Changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT) and PLT were assessed.
Results After the 60.3-months mean follow-up period, HCV infection was independently associated with both thrombocytopenia [odds ratio (OR) 2.589] and decreased PLT (OR 2.339) in 238 HD patients. In 84 HD patients with HCV, the average ALT levels ($\geq$ 15 IU/L) during the follow-up period was associated with thrombocytopenia (OR 3.882) and decreased PLT (OR 4.470). In addition, ALT, AST and GGT significantly decreased at 6 months after starting UDCA, but PLT did not change in 16 HD patients with HCV.
Conclusions These results indicate that HCV infection is a risk for thrombocytopenia which should be associated with hepatic fibrosis in HD patients. In addition, the clinical course of ALT levels predicts the progression of thrombocytopenia, and UDCA may effectively lower ALT levels in HD patients with HCV.

Keywords Hemodialysis · HCV · Thrombocytopenia · ALT · Ursodeoxycholic acid

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Introduction

Chronic kidney disease (CKD) patients who are on hemodialysis (HD) continue to have a higher prevalence of hepatitis C virus (HCV) infection than the general population [1–4]. The prevalence of anti-HCV seropositivity among patients undergoing regular dialysis in developed countries ranges between 7 and 40% [5–8].

HCV infection in HD patients is usually recognized as asymptomatic and cirrhosis is infrequent in this population [9]. One of the reasons for these findings is that the clinical course of chronic hepatitis C extends over decades and dialysis patients generally have higher morbidity and mortality rates than the general population, making the long-term consequences of HCV infection with HD difficult to establish [6]. However, more recently, the prognosis of HD patients has been improving, so addressing HCV infection in these patients is becoming more important [10].

The strong association between serum alanine aminotransferase (ALT) levels and the fibrosis progression rate or occurrence of hepatocellular carcinoma has been well documented in HCV carriers without HD [11–13]. HD

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patients with persistent HCV infection also have higher ALT levels than those patients without HCV, and ALT values may predict the outcome of HCV infection in patients with HD [14]. In contrast, ALT values are still typically within the normal range in HCV carriers with HD and ALT values are lower in HCV carriers with HD than those without HD. Recently, the risk of liver disease-related deaths is higher in chronic hepatitis C patients with ALT levels closer to the upper limit of the normal range (ULN) (20–29 IU/L) compared to patients with lower ALT levels (< 20 IU/L) [15, 16]. In addition, it has been proposed that the cut-off for serum ALT levels should be reduced by half to screen for hepatic damage in HCV carriers with HD [17]. However, the association between serum ALT levels in those patients with HCV and fibrosis progression has not been fully elucidated.

Platelet count (PLT) is a simple biomarker of hepatic fibrosis in HCV carriers [18]. PLT is also lower in HCV RNA-positive HD patients than in HD patients with HCV RNA-negative serum [16]. In addition, severe hepatic fibrosis is independently associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) in HCV carriers with end-stage renal disease [19]. This study evaluated the association of ALT status over a long period and changes in PLT, which was considered an indicator of hepatic fibrosis, in HD patients.

Several trials have examined the efficacy of interferon monotherapy or interferon plus ribavirin combination therapy in HD patients with HCV, and some of these patients obtained a sustained virological response [20]. However, the virological response was limited and side effects may occur more frequently in patients with HD than in those without HD [21, 22]. Therefore, other therapies should be considered for these patients. For chronic hepatitis C patients with or without HD, ursodeoxycholic acid (UDCA) has already been used up to 150 mg/day as routine care in Japan. In addition, the effect of UDCA up to 900 mg/day in HCV carriers who are not undergoing HD was investigated [23], and the use of UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients after April 2007 in Japan. However, the effect of UDCA was not fully elucidated in HCV carriers with HD. Therefore, in this retrospective study we investigated the clinical significance of biochemical markers in the natural course of disease with particular emphasis on PLT and assessed the effect of oral UDCA on serum biomarkers in those patients with HCV.

Materials and methods

Study population

The patients in this study were retrospectively recruited. This study was approved by the Kagoshima University Graduate School of Medical and Dental Sciences. The study population consisted of patients who were on HD in August 2008 and whose data were obtained at least 3 years before August 2008 at 17 HD facilities in Kagoshima, Japan. Their alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total cholesterol (TC) and PLT were monitored once or twice each month. In 2539 patients, 243 patients were positive for anti-HCV, 143 patients were excluded because they were positive for hepatitis B virus surface (HBs) antigen, they were positive for anti-HCV antibody (anti-HCV) but were not examined for HCV RNA, they had received antiviral treatment or they had hepatocellular carcinoma (HCC). The final population enrolled in this study consisted of 100 patients. Among this cohort of 100 HD patients who were both anti-HCV- and HCV RNA-positive, 84 subjects had not received UDCA and were enrolled in study 1 (HD + HCV Group) and 16 subjects had already received 300 mg/day UDCA for at least 3 months after April 2007 when UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients in Japan and were enrolled in study 2 (UDCA Group). The control subjects in study 1 were 154 HD patients who were anti-HCV-negative (HD Group), and the controls in study 2 were the 84 HD patients among the study 1 population who were both anti-HCV- and HCV RNA-positive but had not received previous treatments including UDCA and were observed until August 2008 (non-UDCA Group). Of the 84 HD patients who were controls in study 2, 2 patients died before November 2008. Blood samples were obtained before routine HD procedures and then were used to assay for ALT, AST, GGT, TC and PLT. The relationship of these markers to PLT and the percent change in PLT were examined, and the percent change in PLT was calculated according to the formula: $\Delta\%PLT = [PLT \text{ (at the end of study)} - PLT \text{ (at enrollment)}]/PLT \text{ (at enrollment)} \times 100$.

Serum HCV markers

Serum anti-HCV and HBsAg were determined using a commercially available third-generation enzyme-linked immunosorbent assay and anti-HBs assay, respectively. For anti-HCV antibody-positive patients, HCV RNA was quantified using the COBAS TaqMan HCV kit (COBAS AmpliPrep/COBAS TaqMan HCV assay, Roche Diagnostics, Tokyo, Japan) during the follow-up period. The serologically defined HCV genotype (HCV serotype) was also determined with a serological genotyping assay kit (Immunocheck F-HCV Grouping, International Reagents Co., Tokyo, Japan). In some patients, the HCV genotype was examined (HCV Core Genotype, SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b with serotype II. No other HCV genotype was detected in this study population.

Study 1

The HD + HCV Group, which contained 84 HD patients with HCV, was compared to the HD Group, which contained 154 HD anti-HCV-negative patients. We compared the basal characteristics at enrollment and the changes in PLT during the follow-up period between the two groups. In addition, we divided the HD + HCV patients into the following four groups according to the average ALT level of all available ALT levels during the follow-up period: Group A, ALT < 15; Group B, 15 ≤ ALT < 20; Group C, 20 ≤ ALT < 30; and Group D, 30 ≤ ALT. Clinical characteristics at baseline or average ALT levels and change in PLT during the follow-up period were compared between these four groups.

Study 2

Sixteen patients with HD and HCV had been treated with 300 mg/day UDCA orally for at least 3 months after April 2007, when UDCA up to 900 mg/day was approved for chronic hepatitis C patients, until August 2008 (UDCA Group). These patients were observed every month for at least 6 months before the administration of UDCA and then monitored for the efficacy of UDCA for more than 3 months until August 2008. Then, these patients were observed for a total of at least 6 months until November 2008. We compared the basal characteristics between the UDCA Group just before UDCA treatment and the non-UDCA Group in May 2008. In addition, the changes in ALT, AST, GGT and PLT during the follow-up period were compared between the two groups. For example, the percent of ALT was calculated according to the formula: %ALT = [ALT (−6, 0, 1, 2, 3 or 6 M]/ALT[0 M] × 100).

Statistical analysis

When appropriate, χ^2 test, Fisher's exact test, Student's *t* test and Mann–Whitney *U* test were used to compare the frequencies or means. Logistic regression models were used for calculating the odds ratios (ORs), 95% confidential intervals (CIs) and *P* values. Statistical analyses were performed using STATVIEW (version 5.0; Abacus Concepts, Berkeley, CA), or SPSS (SPSS Inc., Chicago, IL) software programs. A *P* value less than 0.05 was considered statistically significant.

Results

Demographic characteristics of study 1 subjects

As shown in Table 1, 84 HD patients among the anti-HCV-positive patients were HCV carriers (positive for HCV RNA). One hundred fifty-four HD patients were anti-HCV-negative. On average, the frequency of males, levels of AST, ALT and GGT were higher and TC and PLT were lower at baseline in patients with HCV than those in patients without HCV. The follow-up period was also shorter in patients with HCV than those in patients without HCV. In contrast, there were no significant differences between the two groups with respect to age and duration of dialysis.

Table 1 Baseline characteristics of hemodialysis patients

	HCV (+)[a]	HCV (−)[b]	*P* value
Number	84	154	
Age (year)	64.4 ± 10.3	62.2 ± 12.5	0.165
Sex (male/female)	54/30	77/77	0.034
Duration of HD (years)	13.5 ± 9.6	11.8 ± 7.4	0.669
Follow-up period (months)	56.8 ± 15.8	62.2 ± 7.9	0.039
HCV RNA (Log IU/mL)[c]	4.9 ± 1.4	–	
Serotype (I/II/undetermined)[c]	59/21/4	–	
AST (IU/L)	19.7 ± 8.5	14.9 ± 6.7	<0.001
ALT (IU/L)	18.5 ± 9.3	13.2 ± 7.1	<0.001
GGT (IU/L)	41.5 ± 43.0	30.1 ± 42.1	0.002
TC (mg/dl)	153.7 ± 41.0	167.1 ± 35.0	0.003
PLT (× 10^5/μl)	1.59 ± 0.53	1.93 ± 0.73	<0.001

Unless otherwise indicated, data are given as the mean ± SD or number of patients

HD hemodialysis, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *TC* total cholesterol, *PLT* platelet count

[a] HCV (+), both anti-HCV antibody and HCV RNA positive

[b] HCV (−); anti-HCV antibody negative

[c] HCV RNA and serotype were examined during follow-up period

Predictors of thrombocytopenia in HD patients

Table 2 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT < 1.3 × 10^5/μl) at the end of study 1 (August 2008) using 9 baseline characteristics in all HD patients with or without HCV. Older age, HCV viremia, elevated AST, ALT, and GGT levels were significantly associated with thrombocytopenia. In addition, a multivariate analysis revealed that HCV viremia was independently associated with thrombocytopenia (Table 2). Furthermore, after the 60.3-month mean follow-up period (mean of HD + HCV Group, 56.7 months; HD Group, 62.2 months), PLT in the HD + HCV Group had decreased (from 1.59 × 10^5/μL to 1.22 × 10^5/μL) significantly compared to that in the HD Group (from 1.93 × 10^5/μL to 1.77 × 10^5/μL) (average Δ%PLT in each patient: −22.4 vs. −5.3%, $P < 0.001$). Variables that were statistically significant by a univariate analysis were further analyzed to identify variables that