Developmental and Comparative Immunology 41 (2013) 68–76

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Humoral immune responses to CTL epitope peptides from tumor-associated antigens are widely detectable in humans: A new biomarker for overall survival of patients with malignant diseases

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ARTICLE INFO

Article history:
Received 12 December 2012
Revised 19 March 2013
Accepted 3 April 2013
Available online 11 April 2013

Keywords:
Immunoglobulin
Tumor-associated antigen
CTL epitope peptide
Prognostic marker
Malignant diseases

ABSTRACT

Both cellular and humoral immune responses are crucial to induce potent anti-tumor immunity, but most of currently conducted peptide-based cancer vaccines paid attention to cellular responses alone, and none of them are yet approved as a therapeutic modality against cancer patients. We investigated humoral immune responses to CTL epitope peptides derived from tumor-associated antigens in healthy donors and patients with various diseases to facilitate better understanding of their distribution patterns and potential roles. Bead-based multiplex assay, ELISA, and Western blotting were used to measure immunoglobulins reactive to each of 31 different CTL epitope peptides. Importantly, the sums of anti-peptide IgG levels specific to 31 CTL epitope peptides were well correlated with better overall survival (OS) in patients with malignant diseases. Our results suggested that humoral immune responses to CTL epitope peptides were widely detectable in humans. Measurement of immunoglobulins specific to CTL epitope peptides may provide a new biomarker for OS of patients with malignant diseases, although it still remains to be determined whether the correlations between humoral immune responses to epitope peptides and OS are observed only for the CTL epitopes used, or also for other panels of peptides. Quantity of circulating IgG reactive to these peptides was also discussed.

1. Introduction

Peptide-based cancer vaccines have been extensively studied following the discovery of human tumor-associated antigens (TAA) and cytotoxic T lymphocyte (CTL) epitope peptides (Rosenberg et al., 2004; Mellman et al., 2011). However, none of them are yet approved as a therapeutic modality. There might be at least two important hurdles to obtain clinical benefits from the peptide-based cancer therapies currently in practice. One of these hurdles, the negative signaling against CTL activation through check point molecules, such as CTLA-4 and PD-1, was recently overcome by developing blocking antibodies against these molecules (Hodi et al., 2010; Topalian et al., 2012; Brahmer et al., 2012). The second potential hurdle is that no or little humoral immune responses can be induced by the vaccination using most of currently available CTL epitope peptides, although it has been well recognized that both cellular and humoral immune responses are

Abbreviations: TAA, tumor-associated antigen; HD, healthy donor; Flu, influenza virus; HCV, hepatitis C virus; Ig, immunoglobulin; OS, overall survival.

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http://dx.doi.org/10.1016/j.dci.2013.04.004

crucial to induce potent anti-tumor immunity in animal models (Hu et al., 2005; Bequet-Romero et al., 2012; Zeng et al., 2009). In fact, most of currently conducted peptide-based cancer vaccines have paid attention to cellular immune responses alone. To our knowledge, exception is "personalized peptide vaccination" that we have developed (Terasaki et al., 2011), in which CTL epitope candidates for therapeutic cancer vaccines were at first screened based on not only their ability to induce CTL but also reactivity to IgG responses in pre-vaccination samples.

Although humoral immune responses against whole proteins of TAA have been well investigated (Yuan et al., 2011; Toh et al., 2009; Zhang and Tan, 2010), those against CTL epitope peptides derived from TAA, which have been used for therapeutic cancer vaccines, have rarely been studied. We hypothesized that a CTL epitope peptide possessing a B cell epitope could provide more effective clinical benefits than a CTL epitope peptide without it. In fact, we reported potential clinical benefits in advanced glioblastoma multiforme or prostate cancer patients under personalized peptide vaccines using such peptides (Terasaki et al., 2011; Noguchi et al., 2010, 2011a,b; Yajima et al., 2005). In addition, IgG responses were identified as an excellent prognostic marker for predicting overall survival (OS) of the vaccinated patients, although CTL responses also showed a prognostic correlation (Noguchi et al., 2011b; Mine et al., 2004). However, it remains to be fully studied whether anti-peptide immunoglobulins (Igs) are detectable in healthy donors (HD) and patients with various diseases. The current study has addressed this issue to facilitate better understanding of humoral immune responses to CTL epitopes and better designing of cancer vaccine protocols. The results suggest that humoral immune responses are widely detectable in humans and have potential as a new biomarker for overall survival (OS) of patients with malignant diseases.

2. Materials and methods

2.1. Patients and sample collection

Plasma or sera were collected from HD ($n = 74$, 43 ± 20 years old) and from patients with rheumatoid arthritis ($n = 20$, 67 ± 7 years old), IgA nephropathy ($n = 20$, 34 ± 13 years old), influenza virus (Flu) infection ($n = 20$, 34 ± 17 years old), hepatitis C virus (HCV) infection ($n = 20$, 55 ± 8 years old), hematological malignancies ($n = 55$, 61 ± 14 years old; 24 leukemia, 27 lymphoma, and 4 myeloma), or non-HCV hepatocellular carcinoma (HCC, $n = 55$, 60 ± 11 years old; 26 non-B non-C hepatocellular carcinoma, 23 hepatitis B associated hepatocellular carcinoma and six alcoholic hepatic carcinoma) (Supplementary Table 1). HD were categorized into the following four age groups according to the Ministry of Health, Labor and Welfare in Japan; 15–24 ($n = 20$), 25–44 ($n = 19$), 45–64 ($n = 23$), and ≧65 ($n = 12$) years old (Supplementary Table 2). This study was approved by the Kurume University Ethical Committee. After informed consent was obtained from all subjects, blood samples (plasma or sera) were obtained and frozen at −80 °C until use (Noguchi et al., 2011b; Mine et al., 2004).

2.2. Peptides

Thirty-one different peptides employed in the current study were prepared under conditions of Good Manufacturing Practice by Poly Peptide Laboratories (San Diego, CA) or American Peptide Company (Vista, CA), and dissolved in DMSO (Wako, Osaka, Japan). Detailed information on these peptides, including the original protein, peptide position, amino acid sequence, HLA class I A restriction, and references, are given in Supplementary Table 3. Twenty-four of 31 peptides were derived from TAA that were identified by the cDNA expression cloning method, followed by determination of CTL epitopes. The remaining seven peptides including PAP-213, PSA-248, PSMA-624, and PAP-248 were identified by the reverse-immunology method (Kobayashi et al., 2003; Matsueda et al., 2005; Inoue et al., 2001). CTL epitope peptides were determined to be cancer vaccine candidates, based on both their ability to induce CTL activity from peripheral blood mononuclear cells *in vitro* as well as the IgG levels against them in plasma of un-vaccinated cancer patients, and these peptides have been used in clinical trials of personalized peptide vaccine for advanced cancer patients (Terasaki et al., 2011; Noguchi et al., 2010, 2011a,b; Yajima et al., 2005; Mine et al., 2004; Terazaki et al., 2012).

2.3. Measurement of Igs reactive to each of 31 different peptides

The levels of Igs reactive to each of 31 different peptides were measured by multiplex bead suspension array using the Luminex system (Luminex Corp., Austin, TX) as reported previously (Komatsu et al., 2004). In brief, plasma or serum was incubated with 100 μL of peptide-coupled color-coded beads for 1.5 h at 30 °C. To detect IgG or IgM, after washing, the beads were incubated with 100 μL of biotinylated goat anti-human IgG (gamma chain-specific; Vector Laboratories, Burlingame, CA) or biotinylated goat anti-human IgM (mu chain-specific; Vector Laboratories) Abs for 1 h at 30 °C. To detect IgG1, IgG2, IgG3, or IgG4, the beads were incubated with 100 μL of sheep anti-human IgG1, IgG2, IgG3, or IgG4 Abs (Binding Site, Birmingham, UK) for 1 h at 30 °C, followed by washing and incubation with 100 μL biotin-rabbit anti-sheep IgG Ab for 1 h at 30 °C. After washing, the beads were incubated with 100 μL of streptavidin-PE (Life Technologies, Carlsbad, CA) for 30 min at 30 °C, followed by washing and detection of fluorescence intensity unit (FIU) on the beads using the Luminex system (Komatsu et al., 2008). The cut-off values of anti-peptide IgG were set to 10 FIU in 100-time diluted samples, as reported previously (Komatsu et al., 2008). In brief, the calibration curves of FIU were obtained with serially diluted samples. The plasma samples from cancer patients were two times diluted from 160 to 1,310,720. The minimum detectable level of anti-peptide IgGs was 2 FIU when the samples were diluted at 40,960 times as shown by an arrow in Supplementary Fig. 1. However, the levels of anti-peptide IgGs at the minimum detectable range were not reliable since the standard deviations were high. Therefore, we set 10 FIU of the 100-time diluted sample, which was considered to be a reliable value, as a cut-off level as reported previously (Komatsu et al., 2008). There were no significant differences between plasma and serum with regard to the levels of anti-peptide Igs (data not shown).

The specificities of IgG against these peptides were confirmed by competition assay. Plasma was incubated with 100 μL of peptide-coupled color-coded beads and 5 μL of each of the corresponding peptides for 1.5 h at 30 °C. The binding of anti-peptide IgG was detected by same method as described above.

Plasma from frequently vaccinated (12–18 vaccinations) cancer patients who were enrolled in clinical trials of personalized peptide vaccine (data not shown) were used for estimation of anti-peptide IgG levels by other methods, Western blotting and ELISA. To isolate anti-peptide IgGs for Western blotting, plasma was incubated with 100 μL of peptide-coupled color-coded beads for 1.5 h at 30 °C. After washing, the beads were incubated with 6 μL of sample buffer (NuPAGE LDS Sample buffer; Life Technologies, Carlsbad, CA) at 70 °C for 15 min prior to loading onto the SDS–PAGE gel. The separated proteins were transferred to nitrocellulose membrane (Life Technologies, Carlsbad, CA), and IgG gamma chain was detected by using Goat $F(ab')_2$ Fragment anti-human IgG(H+L)-peroxidase (IM0837; Beckman Coulter, Fullerton, CA) Ab and an ECL system (GE Healthcare, Uppsala, Sweden). As a