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Identification of erythropoietin receptor-derived peptides having the potential to induce cancer-reactive cytotoxic T lymphocytes from HLA-A24$^+$ patients with renal cell carcinoma ☆

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ABSTRACT

Molecular targeting therapy with anti-angiogenic agents, including sunitinib and sorafenib, has been proven to be the first- and second-line standard treatments for metastatic renal cell carcinoma (mRCC) worldwide. Despite their significant antitumor effects, most of the patients with mRCC have not been cured. Under such circumstances, anti-cancer immunotherapy has been considered as a promising treatment modality for mRCC, and cytotoxic T lymphocytes (CTLs) are the most powerful effectors among several immune cells and molecules. Therefore, we previously conducted anti-cancer vaccine therapy with peptides derived from carbonic anhydrase-9 and vascular endothelial growth factor receptor-1 as phase-I/II trials for mRCC patients and reported their clinical benefits. Alternatively, up-regulated expression of erythropoietin (Epo) and its receptor (EpoR) in RCC has been reported, and their co-expression is involved in tumorigenesis. In order to increase options for peptide-based vaccination therapy, we searched for novel EpoR-peptides for HLA-A24$^+$ RCC patients. Among 5 peptides derived from EpoR, which were prepared based on the binding motif to the HLA-A24 allele, EpoR$_{52-60}$ peptide had the potential to induce peptide-specific CTLs from peripheral blood mononuclear cells of HLA-A24$^+$ RCC patients. Cytotoxicity toward HLA-A24$^+$ and EpoR-expressing RCC cells was ascribed to peptide-specific CD8$^+$ T cells. These results indicate that the EpoR$_{52-60}$ peptide could be a promising candidate for a peptide-based anti-cancer vaccine for HLA-A24$^+$ mRCC patients.

1. Introduction

Renal cancer comprises about 1–2% of all adult cancers, and at the time of diagnosis, 20–30% of renal cell carcinoma (RCC) patients have already been associated with metastasis [1]. In recent years, molecular targeting therapy has been recognized as the standard therapy for metastatic RCC (mRCC). However, despite their significant antitumor effects, most of the patients with mRCC have not been cured [2], highlighting a need to develop more effective therapies.

Significant advances in cancer immunology have enabled for the development of various types of anti-cancer immunotherapies, many of which are considered to be promising treatment strategies against mRCC [2]. Among the various types of immunotherapies developed, cancer vaccines against RCC have been evaluated for over 30 years. Cytotoxic T lymphocytes (CTLs) are the most powerful effectors among several immune cells and molecules. Thus, we have identified several peptide candidates derived from carbonic anhydrase (CA)-9 antigen capable of inducing HLA-A24-restricted RCC-reactive CTLs and applied them in phase I/II clinical trials for HLA-A24$^+$ RCC patients [3,4]. Furthermore, we performed a phase I clinical trial using an HLA-A2-binding R1-770 peptide and an HLA-A24-binding R1-1084 peptide, both of which were derived from vascular endothelial growth factor receptor-1, and reported that this trial was well-tolerated and that antigen-specific immunity was induced in two-thirds of vaccinated patients [5].

Clear cell RCC is the most common RCC and is often associated with *von Hippel–Lindau* (*VHL*) gene mutations. *VHL* is a tumor suppressor

Abbreviations: CA, carbonic anhydrase; CTLs, cytotoxic T lymphocytes; EBV, Epstein–Barr virus; Epo, erythropoietin; EpoR, erythropoietin receptor; HIF, hypoxia-inducing factor; HIV, human immune deficiency virus; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; mAb, monoclonal antibody; mRCC, metastatic RCC; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; RCC, renal cell carcinoma; VHL, von Hippel–Lindau.

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gene located on chromosome 3p25–26 [6]. Inactivation of VHL protein results in failure of ubiquitination of hypoxia-inducible factor (HIF), resulting in up-regulation of HIF proteins and activation of the downstream pathways. As a consequence, the expression of erythropoietin (Epo) and VEGF is increased, thereby promoting angiogenesis and other changes in cellular metabolism [7]. Additionally, recent data on VHL-associated tumors including RCC suggest that co-expression of Epo and its receptor (EpoR) is involved in tumorigenesis [8]. These lines of evidence suggest that Epo and EpoR could be targets of anti-cancer vaccines. In this study, we identify novel EpoR peptides as potential candidates for the development of anti-cancer vaccines for HLA-A24$^+$ RCC patients.

2. Materials and methods

2.1. Patients

Peripheral blood mononuclear cells (PBMCs) were obtained from RCC patients and healthy donors who had provided a written informed consent. The subjects included HLA-A24$^+$ patients. None of the participants was infected with human immunodeficiency virus (HIV). Thirty milliliters of peripheral blood was obtained, and PBMCs were prepared by Ficoll–Conray density gradient centrifugation. All samples were cryopreserved until further use. The Institutional Ethics Review Board of Kinki University approved the study protocol (Approval No. 18–51).

2.2. Cell lines

C1R-A24 is a C1R lymphoma subline that was stably transfected with the HLA-*A*24:02* gene. SKRC-1, SKRC-44, SKRC-52, KPK-13, and KK-RCC6 are all RCC cell lines. The former three RCC lines were kindly provided by Dr. K. Yoshikawa (Aichi Medical University). The latter two RCC lines were kindly provided by Dr. H. Mimata (Oita University Faculty of Medicine) and Dr. Y. Kakehi (Kagawa University Graduate School of Medicine), respectively. All cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) with 10% FCS.

2.3. Peptides

All EpoR-derived peptides were prepared based on the binding motifs to the HLA-A24 molecule [9]. In brief, the peptide-binding score was calculated based on the predicted half-time of dissociation from HLA class I molecules as obtained from BIMAS (Bioinformatics and Molecular Analysis Section, Computational Bioscience and Engineering Laboratory, Division of Computer Research & Technology, NIH, bimas.cit.nih.gov), and 5 peptides with the highest scores were selected. Epstein–Barr virus (EBV)-derived peptide (TYGPVFMSL) and HIV-derived peptide (RYLRDQQLL) were used as controls for binding to the HLA-A24 molecule. All peptides were purchased from OPERON (Tokyo, Japan) and were dissolved in DMSO at a dose of 10 mg/ml.

2.4. Induction of peptide-specific CTLs from PBMCs

Assays for the detection of peptide-specific CTLs were performed according to a previously reported method with several modifications [10]. PBMCs (5×10^4 cells/well) were incubated with 10 μg/ml of each peptide in a U-bottom-type 96 well microculture plate (Nunc, Roskilde, Denmark) in 100 μl of culture medium. The culture medium consisted of 45% RPMI 1640, 45% AIM-V medium (Gibco BRL, Gaithersburg, MD, USA), 10% FCS, and MEM nonessential amino acid solution (×100 dilution, Gibco BRL). Eight wells were used for each peptide. On the next day, 100 μl of the culture medium containing 50 U/ml IL-2 was added. On day 8, 100 μl of the culture medium was removed and replenished with fresh culture medium containing the corresponding peptide (10 μg/ml), and, on day 9, 100 μl of the culture medium containing 50 U/ml IL-2 was added. On the 17th day of culture, fresh culture medium was replaced and the cultured cells were separated from one well into four wells: two wells for the stimulation with the corresponding EpoR peptide-pulsed C1R-A24 cells and the other two wells for the stimulation with control HIV peptide-pulsed C1R-A24 cells. After 18-h incubation, the supernatant was collected and the level of IFN-γ was determined by enzyme-linked immunosorbent assay. The induction of peptide-specific CTLs was judged to be positive only when the following two criteria were satisfied: (1) both the IFN-γ levels in corresponding EpoR peptide-stimulated two wells were >50 pg/ml, and (2) both the IFN-γ levels in corresponding EpoR peptide-stimulated two wells were >1.2 times more than the mean of IFN-γ levels in control HIV peptide-stimulated two wells.

2.5. RT-PCR analysis

Total RNA was extracted from RCC cell lines using PureLink RNA Mini Kit (Invitrogen) and reverse transcribed using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. Template cDNAs were subjected to 30 cycles of the PCR using Platinum *Taq* DNA polymerase (Invitrogen). The following PCR primers were chosen based on a previous report [11]. Their primer sequences are as follows: 5′-TCGTGGTCATCCT GGTGCTGCTGA-3′ (forward) and 5′-ACCTTCAGGAGAGTCTCGCGACGA-3′ (reverse). The PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and photographed. We qualitatively evaluated the expression level of EpoR normalized to the expression of GAPDH. The ratio of the expression of EpoR to that of GAPDH was evaluated by densitometric analysis using ImageJ analysis software (http://rsbwed.nih.gov/ij/).

2.6. Flow cytometric analysis

To examine the expression of HLA molecules, cells were stained with either anti-HLA class I mAb (hybridoma: W6/32), or anti-HLA-A24 mAb (One Lambda, Canoga Park, CA, USA), followed by the staining with FITC-conjugated goat anti-mouse IgG (H + L) antibody (KPL, Gaithersburg, MD, USA). In examining the cell surface expression of EpoR, RCC cells were harvested from culture plates using a scraper to avoid cleavage and/or degradation of cell surface EpoR by EDTA/trypsin treatment. Thereafter, cells were stained with anti-EpoR mAb or isotype-matched mouse IgG2b, followed by Alexa Fluor 488-conjugated anti-mouse IgG $F(ab')_2$ fragment (Cell Signaling Technology, Danvers, MA, USA). To stain intracellular EpoR, RCC cells were treated with 3% formalin, followed by 0.2% Triton-X before staining with the antibodies. Analysis was performed using the FACSCalibur (BD Biosciences, San Jose, CA, USA).

2.7. Confocal imaging

Cells were cultured on round microscope cover glasses in 24-well plates for 1 day. After incubation with Hoechst 33342 (5 μg/ml) for 30 min, cells were stained with anti-EpoR mAb or control mouse IgG2b, followed by Alexa Fluor 488-conjugated anti-mouse IgG F(ab′)2 fragment. After staining, cover glasses with cells were placed on slide glasses with 4 μl of mounting medium for fluorescence (Vectashield; Vector Laboratories, Inc., Burlingame, CA, USA). Confocal imaging was performed using an Olympus FV1000-D laser scanning microscope (Olympus, Tokyo, Japan).

2.8. Immunohistochemistry

Tissue specimens were cut into 5-μm-thick sections and deparaffinized. Antigen retrieval was performed using Target Retrieval Solution (DAKO, Glostrup, Denmark). After incubation in 0.3% hydrogen peroxide for 30 min, tissue slides were reacted with an anti-pan HLA class I heavy chain mAb (EMR8-5) (Abcam, Cambridge, UK) for 1 h, washed with PBS-T buffer, incubated with biotin-conjugated anti-mouse IgG (H + L) horse polyclonal antibody (VECTOR, Burlingame,