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Aspartate-β-hydroxylase induces epitope-specific T cell responses in hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) has a poor prognosis due to high recurrence rate. Aspartate-β-hydroxylase (ASPH) is a highly conserved transmembrane protein, which is over expressed in HCC and promotes a malignant phenotype. The capability of ASPH protein-derived HLA class I and II peptides to generate antigen specific $CD4^+$ and $CD8^+$ immune responses is unknown. Therefore, these studies aim to define the epitope specific components required for a peptide based candidate vaccine. Monocyte-derived dendritic cells (DCs) generated from the peripheral blood mononuclear cells (PBMCs) of HCC patients were loaded with ASPH protein. Helper $CD4^+$ T cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) were co-incubated with the DCs; T cell activation was evaluated by flow cytometric analysis. Immunoinformatics tools were used to predict HLA class I- and class II-restricted ASPH sequences, and the corresponding peptides were synthesized. The immunogenicity of each peptide in cultures of human PBMCs was determined by IFN-γ ELISpot assay. ASPH protein-loaded DCs activated both $CD4^+$ and $CD8^+$ T cells contained within the PBMC population derived from HCC patients. Furthermore, the predicted HLA class I- and class II-restricted ASPH peptides were significantly immunogenic. Both HLA class I- and class II-restricted peptides derived from ASPH induce T cell activation in HCC. We observed that ASPH protein and related peptides were highly immunogenic in patients with HCC and produce the type of cellular immune responses required for generation of anti-tumor activity.

1. Introduction

Hepatocellular carcinoma, the third most common cause of cancer-related death worldwide, is characterized by a very poor prognosis and a high mortality rate [1,2]. Available therapeutic modalities are largely inadequate. Currently, surgical resection is considered the optimal treatment approach. Only a small proportion of patients qualify for treatment, however, and a recurrence of disease is common following surgery [3,4]. Therefore, the development of new treatment strategies is a priority.

Aspartate-β-hydroxylase (ASPH, also known as aspartyl-asparaginyl-β-hydroxylase) is a type 2, 758 amino acid, transmembrane protein that belongs to the α-ketoglutarate-dependent dioxygenase family [5]. It is a highly conserved enzyme, which catalyzes the hydroxylation of aspartyl and asparaginyl residues in epidermal growth factor-like domains of proteins including Notch and homologs [6–8]. ASPH is over expressed in HCC compared to normal liver tissue; over expression produces a malignant phenotype characterized by increased cell motility, invasion and metastasis [6,9]. The level of ASPH protein expression in HCC correlates significantly with postoperative prognosis [10]. ASPH is not expressed in normal liver or regenerating nodules. Indeed, most if not all HCC cells within a given tumor highly express ASPH and not the surrounding uninvolved liver [11].

Previous studies demonstrated that immunization with ASPH protein-loaded dendritic cells (DCs) exerted a substantial anti-tumor effect in an experimental murine model [12,13]. Moreover, recombinant ASPH protein stimulated a significant increase in antigen-specific $CD4^+$ T cells in PBMC cultures derived from both

Abbreviations: HCC, hepatocellular carcinoma; ASPH, aspartate-β-hydroxylase; DCs, dendritic cells; PBMCs, peripheral blood mononuclear cells; CTLs, cytotoxic T lymphocytes; ICS, immunogenic consensus sequences; T_{reg} cell, regulatory T cell; AFP, α-fetoprotein.

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healthy volunteers and HCC patients [12]. These findings suggest that ASPH may serve as a potential target for immunotherapy since it is expressed on the cell surface. The response of human, cytotoxic $CD8^+$ T lymphocytes to ASPH protein, however, remains to be determined. In the present study, we first demonstrated that full-length ASPH protein induced significant activation of CTLs, as well as $CD4^+$ helper T cells, obtained from individuals with HCC before and after depletion of T_{reg} cells. Next, immunoinformatics tools were used to predict 15 HLA-DRB1-restricted immunogenic consensus sequences (ICS, each composed of multiple epitopes) contained within ASPH. These predicted ICS were synthesized as peptides and their capacities to bind multiple HLA-DRB1 alleles were determined. Thirty HLA class I-restricted ASPH epitopes were also predicted and synthesized. Subsequently, each HLA class I- and class II-restricted peptide was evaluated for immunogenicity in HCC patients.

2. Materials and methods

2.1. Recombinant ASPH and ASPH peptides

Full-length human ASPH (GenBank Accession No. 583325) was cloned into the EcoRI site of the pcDNA vector (Invitrogen, Carlsbad, CA). Recombinant protein was produced in a Baculovirus system (Invitrogen) according to the manufacturer's instruction [12,13].

ASPH ICS were predicted using methods previously described [14,15]. Briefly, the entire ASPH protein was parsed into overlapping nine amino acid frames (9-mers), which constitute the length of the peptide chain that fits into the binding grooves of HLA class I and class II molecules. Each frame was then evaluated using EpiMatrix for its potential to bind a panel of eight common HLA-DRB1 alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301, and DRB1*1501), which represent >95% of the MHC diversity in the human population [16]. HLA-DRB1-restricted ICS were constructed from those frames that exhibited potential binding activity using EpiAssembler, an algorithm that maximizes epitope density by assembling immunogenic 9-mers into 18–25 amino acid stretches (ICS) [17]. Each "promiscuous" ICS that resulted was comprised of multiple epitopes capable of binding more than one HLA-DRB1 allele. Additionally, all parsed 9-mers were scored for their potential to bind a panel of six common class I "supertype" alleles; A*0101, A*0201, A*0301, A*2402, B*0702, and B*4403, which cover over 95% of the human population [18]. Based upon these predictions, the peptide sequences were synthesized using FMOC chemistry and purified >85% by HPLC (21st Century Biochemicals, Marlboro, MA); each was dissolved in 100% DMSO (100 mg/ml) and stored at −80 °C. Stock peptide solutions were diluted 1:1000 in medium just prior to culture; 0.1% DMSO in medium alone served as the negative control. The amino acid sequences of the HLA-DRB1-restricted peptides and HLA class I-restricted peptides are shown in Tables 1 and 2, respectively.

2.2. Healthy blood donors and HCC patients

Blood samples were obtained from 12 HCC patients (HCC #1–12) and 5 healthy blood donors (HD #1–5). The peripheral blood mononuclear cells (PBMCs) were purified from whole blood of the HCC patients as previously described [12]. Hartford Hospital Transplantation Research Laboratory (Hartford, CT) performed HLA typing on DNA extracted from each sample.

De-identified whole-blood leukocyte reduction filters (blood filters; Sepacell RZ-2000, Baxter Healthcare Corporation, Irvine, CA), obtained after use from the Rhode Island Blood Center (Providence, RI), served as the source of PBMCs derived from blood donated with informed consent by healthy volunteers. The PBMCs were recovered by back-flushing the filters according to the methods of Meyer et al. and purified by centrifugation on Ficoll-Paque Plus (1.077; Pharmacia, Uppsala, Sweden) gradient as we described previous [19,20]. The Rhode Island Hospital Institutional Review Board approved this study.

2.3. Epitope-specific T cell induction

Epitope-specific T cells were induced according to methods we described previously [21]. Briefly, 2.5 × 105 PBMCs/200 μl X-VIVO 15 medium supplemented with 1 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 U/ml recombinant human IL-2 (R&D Systems) in round-bottom 96-well plates were cultured for 2 weeks with 10 μg/ml individual peptide.

Alternatively, ASPH-specific T cells were generated by co-culturing purified T lymphocytes with protein-pulsed DCs in accordance with methods we also reported previously [12]. Briefly, monocytes were isolated from PBMCs using anti-CD14 microbeads (Miltenyi Biotec, Auburn, CA) and cultured for 5 days in X-VIVO 15 medium (Lonza, Walkerville, MD) supplemented with human GM-CSF (R&D Systems, Minneapolis, MN) and IL-4 (R&D Systems). ASPH protein (1 μg/ml) was added on day 5; TNF-α (R&D Systems) was added on the following day to stimulate DC maturation, and the cells were incubated for another 48 h. DCs incubated with α-fetoprotein (AFP; Zynaxis Cell Science, Malvern, PA) or alone served as the control. Mature, epitope-expressing DCs were collected at the end of the incubation period. T cells were isolated from PBMCs by negative selection using the Pan T Cell Isolation Kit II (Miltenyi Biotec). Regulatory $T_{(reg)}$ cells were removed by the addition of anti-CD25 microbeads (Miltenyi Biotec) where indicated. T_{reg} cell-depleted or non-depleted T lymphocytes (2.4×10^6) were co-cultured for 8 days with 4×10^4 mature DCs loaded with relevant antigen in 24-well plates [12].

2.4. Enzyme-linked immunospot (ELISpot) assay

Human IFN-γ ELISpot assays were performed as we described previously using a kit purchased from eBioscience (San Diego, CA) to determine T cell immune-reactivity [21]. Cells (5×10^4/well) collected after induction were added to ELISpot plates (Millipore, Bedford, MA) pre-coated with anti-IFN-γ capture antibody and incubated with peptides (10 μg/ml) for 20 h. Subsequently, the plates were washed and incubated sequentially with biotinylated IFN-γ detection antibody then avidin-HRP. The plates were developed by adding substrate, 3-amino-9-ethyl carbazole, and the number of spots/well was quantified using a CTL-immunospot S5 UV Analyzer (Cellular Technology Limited, Shaker Heights, OH).

2.5. Blocking of T cell response

To demonstrate the contribution of HLA molecules to ASPH peptide-dependent T cell activation, the cells were incubated with antibodies specific for HLA class I (clone W6/32; BioLegend, San Diego, CA) or HLA-DR (clone L432; BioLegend) (15 μg/ml) for 1 h at 37 °C prior to analyses.

2.6. Flow cytometric analysis

Flow cytometric analysis was conducted as previously described [12]. Intracellular cytokine staining was performed to evaluate T cell activation. Conjugated mouse monoclonal antibodies specific for the following determinants were used: CD4 (clone OKT4; BioLegend, San Diego, CA), CD8a (clone RPA-T8; BioLegend), CD137 (clone 4B4-1; BD Biosciences, San Diego, CA), CD154 (clone TRAP1;