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Enhanced anti-colon cancer immune responses with modified eEF2-derived peptides

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ABSTRACT

Eukaryotic elongation factor-2 (eEF2) is overexpressed in many human cancers and is an attractive target for cancer immunotherapy. The eEF2 derived polypeptides have been shown to be able to induce cytotoxic T lymphocytes from healthy donor. Here, we demonstrate the evidence indicating that modification of a segment of peptides from wild type eEF2-derived immunogenic peptides is able to further enhance its capacity of inducing antigen-specific cytotoxic T lymphocytes (CTLs) against colon cancer cells. Using peptide-MHC binding algorithms, potential HLA-A2.1-restricted epitopes capable of inducing specific $CD8^+$ CTLs were identified. By analyzing HLA-A2.1 affinity and immunogenicity, we further identified one novel immunogenic peptide, P739–747 (RLMEPIYLV), that elicited specific CTL responses in HLA-A2.1/K^b transgenic mice and culture with peripheral blood lymphocytes from colon cancer patients. Furthermore, replacing certain amino acids (at positions 1, 3, 7) within the P739–747 sequence improved the immunogenicity against eEF2. Several analogs containing the auxiliary HLA-A*0201 anchor residues were able to stably bind to HLA-A*0201 and enhance CTL responses compared with the native sequence; two of them showed increased anti-tumor effects during the adoptive immunotherapy *in vivo*. Thus, these results support that modified immunogenic analogs are promising candidates for peptide-based cancer vaccination and immunotherapy.

Introduction

Cytotoxic T cell (CTL)-based specific immunotherapy is considered one of the most promising strategies for tumor therapy. The $CD8^+$ CTLs are capable of lysing tumor cells by recognizing peptides derived from tumor-associated antigens (TAAs) presented on MHC class I molecules [1,2]. Indeed, encouraging clinical responses have been observed in some cancer patients receiving peptide-based tumor immunotherapy [3,4]. As most TAAs are self-antigens, antigen-specific CTL repertoires may be significantly reduced in the host during the negative selection process, leaving behind a T-cell repertoire that is poorly effective at mounting productive antitumor responses [5]. As a potential approach to enhance the immunogenicity of epitopes recognized by CTLs, modifications to the peptide sequences are applied to improve binding to MHC class I molecules [6–9]. However, replacing residues that are directed toward the TCR (T cell receptor) can also improve epitope immunogenicity [10–12]. Some of these peptides with enhanced immunogenicity have been successfully used to immunize cancer patients, improve detection of antitumor immune responses, and reverse non-responsiveness to tumor antigens [10].

Eukaryotic elongation factor-2 (eEF2) is previously identified as a novel tumor-associated antigen [13,14]. Clinical specimens of cancer tissues show that eEF2 protein is highly expressed in human breast, prostate, lung, gastric and colorectal carcinoma tissues, but not in normal tissues, as examined by immunohistochemical analysis [13,14], indicating eEF2 being an effective TAA target for immunotherapy. Oji et al. [14] have shown that eEF2-derived 9-mer peptides, EF786 (eEF2 786–794 aa) and EF292 (eEF2 292–300 aa), elicited cytotoxic T lymphocyte (CTL) responses in peripheral blood mononuclear cells (PBMCs) from an HLA-A*24:02- and an HLA-A*02:01-positive healthy donor, respectively, in an HLA-A-restricted manner. Following this line of study, we investigated whether additional eEF2-derived peptides could elicit CTL response, and whether

Abbreviations: eEF2, eukaryotic elongation factor-2; TAAs, tumor-associated antigens; PBLs, peripheral blood lymphocytes; Tg, transgenic; TCR, T cell receptor; GrB, granzyme B; CTL, cytotoxic T lymphocyte; PBMCs, peripheral blood mononuclear cells; DC, dendritic cell; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; mAb, monoclonal antibody; OVA, ovalbumin; FI, fluorescence index; MFI, mean fluorescence intensities; FITC, fluorescein isothiocyanate; CAP-1, carcinoembryonic antigen peptide-1; E:T, effector:target.

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the CTL response by such eEF2-derived peptides could be further enhanced by peptide modifications. Here, we identified a nonamer peptide derived from the eEF2 protein, designated as P739–747 (RLMEPIYLV), which possessed the ability to provoke a peptide-specific, HLA-A2.1-restricted CTL response in HLA-A2.1/K^b transgenic (Tg) mice *in vivo* as well as in peripheral blood lymphocytes (PBLs) from HLA-matched colon cancer patients using dendritic cells (DCs) pre-pulsed with the peptides *in vitro*. In addition, amino acid substitution at HLA-A*0201-binding anchor positions in the native peptide enhanced the HLA-A*0201-binding affinity and immunogenicity of these modified peptides. These results provide the first evidence indicating the potential clinical application of this eEF2-derived analog in peptide-mediated immunotherapy for eEF2-expressing tumors.

Materials and methods

Synthetic peptides

All peptides utilized in this study were synthesized by GL Biochem (Shanghai, China) using fluorenylmethoxycarbonyl chemistry and purified to more than 95% by reversed phase high-performance liquid chromatography (HPLC), as confirmed by mass spectrometry. The lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO), diluted with phosphate-buffered saline (PBS; pH 7.4) at a concentration of 10 mM, and stored in aliquots at –80 °C. The amino-acid sequences and the predicted score for binding to HLA-A*0201 were generated by four databases available online (*ProPred-I*, http://www.imtech.res.in/raghava/propred1/; *SYFPEITHI*, http://www.syfpeithi.de/0-Home.htm).

Animals and cell lines

HLA-A2.1/K^b Tg mice, 5–6 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were bred and maintained under specific pathogen-free conditions. Mice were fed with an autoclaved laboratory rodent diet. The research was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Animal Subject of Qingdao University. Cell surface HLA-A*0201 expression was assessed by flow cytometry using fluorescein isothiocyanate (FITC)-labeled HLA-A2-specific monoclonal antibody (mAb) BB7.2 (Serotec, Oxford, UK). The human TAP-deficient T2 cell line (human B and T-cell hybrid expressing HLA-A2.1 molecules), the human breast adenocarcinoma MCF-7 ($eEF2^-$/HLA-$A2.1^+$), and the human colorectal adenocarcinoma SW480 ($eEF2^+$/HLA-$A2.1^-$) and Caco-2 cell ($eEF2^+$/HLA-$A2.1^+$) lines were obtained from American Type Culture Collection (ATCC, Manassas, VA).

Peptide-binding assay

A peptide-induced stabilization assay of the HLA-A*0201 molecule expressed by the TAP-deficient T × B-cell hybrid T2 cells [15] was performed according to a previously described protocol [16,17]. The HLA-A2.1-restricted carcinoembryonic antigen peptide-1 (CAP-1, YLSGANLNL) served as a positive control peptide and the ovalbumin (OVA)-derived H-2b-restricted peptide $OVA_{257-264}$ (SIINFKEL) was used as a negative control peptide. The fluorescence index (FI) was calculated as follows: FI = (mean FITC fluorescence with the given peptide – mean FITC fluorescence without peptide)/(mean FITC fluorescence without peptide). Peptides with an FI > 1 were regarded as high-affinity epitopes.

*Measurement of the peptide-HLA-A*0201 complex stability*

T2 cells were incubated overnight at 37 °C with 100 μM of each peptide in serum-free RPMI 1640 medium supplemented with 100 ng/mL β2-microglobulin as described [18]. The cells were then washed four times to remove free peptides, incubated with 10 μg/mL brefeldin A (Sigma-Aldrich, USA) for 1 hour to block cell-surface expression of newly synthesized HLA-A*0201 molecules, washed, and further incubated at 37 °C for 0, 2, 4, and 6 hours. Subsequently, the cells were stained with mAb BB7.2 to evaluate HLA-A*0201 molecule expression. Mean fluorescence intensity (MFI) measured at 0 hour was considered at 100%. MFI at all other time points are expressed relative to the MFI at 0 hour and calculated as follows: [MFI (0 hour) – MFI (2, 4, or 6 hour)/MFI (0 hour)] × 100%.

eEF2 RNA-interference assay

Transient knockdown of eEF2 using an shRNA assay with a chemically synthesized shRNA duplex and empty shRNA vector control was performed as previously described [13].

Generation of CTL in HLA-A2.1/K^b Tg mice

Bone marrow-derived DCs were generated from transgenic mice as previously described [19]. Vaccination of HLA-A2.1/K^b Tg mice was performed as previously described [20] with minor modifications. Briefly, on day 7, DCs were pulsed with 20 μM peptides in the presence of 3 μg/mL β2-microglobulin at 37 °C for 4 h and then washed. HLA-A2.1/Kb transgenic mice (5 mice per group for each peptide) were immunized intraperitoneally thrice at 1-week interval with 1×10^6 DCs per mouse. A control group was set simultaneously to receive PBS-treated DCs. According to a previously described protocol [21–23], on day 7 after the last immunization with peptide-pulsed DCs, total immune splenocytes from the pooled samples from the same group were cultured at a density of 1×10^7 cells per well in 6-well plates and stimulated with peptides (20 μM) for 7 days *in vitro*. Then, induced cells were harvested and functionally tested for IFN-γ and granzyme B by enzyme-linked immunospot (ELISPOT), ELISA, and intracellular staining assays as well as for cytotoxicity by ^{51}Cr-release cytotoxicity assays.

CTL induction in PBLs of colon cancer patients in vitro

We obtained 1.5×10^7 peripheral blood lymphocytes (PBLs) from 20 mL of heparinized peripheral blood collected from each HLA-$A2.1^+$ patient with colon carcinoma (who gave written informed consent and the protocol was reviewed and approved by the Ethic Committee for Application of Human Samples, Qingdao University Medical College) and human peripheral blood monocyte-derived DCs were generated as previously described [24–26]. Fifteen HLA-$A2.1^+$ patients with colon carcinoma were recruited in this study. Peptide-specific CTLs were generated as previously described [20] with minor modifications. After 7 days of co-culturing with peptide-pulsed autologous DCs, lymphocytes were re-stimulated with peptide-pulsed autologous DCs in medium containing 10 ng/mL IL-7, which was then supplemented with 20 IU/mL rhIL-2 (Sigma-Aldrich) 72 hours later. Lymphocytes were restimulated each week in the same manner. Half of the media volume was changed every 3 days in the presence of recombinant human rhIL-2 (20 IU/mL) and expanded as necessary. On day 7 after the third stimulation, 7×10^8 $CD8^+$ T lymphocytes were enriched by positive selection using magnetic immunobeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the procedure recommended by the manufacturer. IFN-γ secretion of these $CD8^+$ T cells was then examined by ELISPOT and ELISA, and the cells were evaluated for cytotoxic ability using both the granzyme B ELISPOT and ^{51}Cr-release assays.

ELISPOT assay

ELISPOT assays were performed using a commercially available kit (R&D Systems, Minneapolis, MN, USA) as described [20]. Splenocytes (1×10^5) from the immunized HLA-A2.1/K^b mice described above and purified $CD8^+$ T cells (more than 95% pure) were used as effector cells. Peptide-pulsed or unpulsed T2 cells or tumor cells (1×10^4) were used as stimulator cells. The data in the figures represent the mean of triplicate assays. Standard deviation (SD) was generally within 10% of the mean.

Intracellular detection of IFN-γ

Intracellular IFN-γ secretion was detected as described [27]. Briefly, effector T cells from immunized mice or colon cancer patients were re-stimulated in the presence of corresponding peptides or tumor cell lines (Caco-2, MCF-7, and SW480) for 48 hours. Brefeldin A (20 μg/mL) (Sigma-Aldrich) was added during the last 8 hours of culture to block protein secretion. Cells were harvested, washed, and stained with a FITC-conjugated anti-CD8 monoclonal antibody (BD Pharmingen, San Diego, CA). PE-conjugated anti-IFN-γ monoclonal antibody (BD Pharmingen, San Diego, CA) was added after cells were fixed and permeabilized using saponin (Sigma-Aldrich). After washing with PBS, stained cells were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry (FASCcan or FACSVantage SE; BD Biosciences). Data analysis was performed using CellQuest software (BD Biosciences).

IFN-γ ELISA

ELISA was performed using a commercially available kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Peptide-pulsed or unpulsed T2 cells or tumor cells were used as stimulator cells. Splenocytes from immunized HLA-A2.1/K^b mice or purified $CD8^+$ T cells were used as effector cells. The amount of cytokine presented in the effector-cell culture supernatant was calculated based on the IFN-γ standard curve. The data in the figures represent the mean of triplicate assays. SD was generally within 10% of the mean.

Cytotoxicity assay

Cytotoxicity assays were performed using a standard 4-hour ^{51}Cr-release assay as previously described [20]. Percent specific lysis was determined according to the following formula: percent specific lysis = [(mean experimental cpm – mean spontaneous cpm)/(mean maximum cpm – mean spontaneous cpm)] × 100%. SD of triplicate wells was less than 15%.

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