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Original Articles

Chimeric peptide containing both B and T cells epitope of tumor-associated antigen L6 enhances anti-tumor effects in HLA-A2 transgenic mice

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ABSTRACT

Synthetic peptides are attractive for cancer immunotherapy because of their safety and flexibility. In this report, we identified a new B cell epitope of tumor-associated antigen L6 (TAL6) that could induce antibody-dependent cellular cytotoxicity (ADCC) in vivo. We incorporated the B cell epitope with a cytotoxic T lymphocyte (CTL) and a helper T (Th) epitope to form a chimeric long peptide. We formulated the chimeric peptide with different adjuvants to immunize HLA-A2 transgenic mice and evaluate their immunogenicity. The chimeric peptide formulated with an emulsion type nanoparticle (PELC) adjuvant and a toll-like receptor 9 agonist (CpG ODN) (PELC/CpG) induced the greatest ADCC and CTL responses. The induced anti-tumor immunity inhibited the growth of TAL6-positive cancer cells. Moreover, we observed that immunization with the chimeric peptide inhibited cancer cell migration in vitro and metastasis in vivo. These data suggest that a chimeric peptide containing both B and T cell epitopes of TAL6 formulated with PELC/CpG adjuvant is feasible for cancer immunotherapy.

Introduction

Tumor associated antigen L6 (TAL6) is a cell surface protein of the transmembrane-4 superfamily (TM4SF) also known as TM4SF1. TM4SF proteins are over-expressed in different types of human cancers including lung, breast, colon, prostate and liver cancer [1–4]. TM4SF1-, TM4SF4- and TM4SF5-specific monoclonal antibodies can inhibit colon cancer growth, indicating that TM4SF proteins are crucial targets for cancer therapy [3,5–7]. TAL6 is over-expressed in more than 80% of human lung, breast, colon and ovarian tumors but not normal tissues [1,8,9]. Recently, TAL6 was found to play critical roles in cancer cell motility, invasion, metastasis and angiogenesis [2,8,10,11]. We previously demonstrated that an HLA-A2-restricted CTL epitope, A2-5, of TAL6 is capable of inducing CTL responses against cancer cells that express TAL6 [12]. In addition, the induced-CTL responses can be adoptively transferred to inhibit human lung cancer growth in immunocompromised mice [13].

TAL6 is an ideal antigen for cancer immunotherapy because it may be targeted by both humoral and cellular immunity. In this report, we not only identify the major B cell epitope of TAL6 but also combined it with a TAL6 CTL epitope to increase cancer killing potency. To enhance both B and T cell function, the adjuvant formulation was designed to contain both immunostimulatory molecules and an antigen delivery system. We found that TLR9 agonist CpG ODN with the emulsion type adjuvant PELC induced strong B and CTL responses against TAL6-expressing cancers in HLA transgenic mice. Furthermore, the induced anti-sera inhibited tumor cell migration and tumor metastasis to the lung.

Materials and methods

Animal and cell line

Female 6-week-old C57BL/6 mice were obtained from the National Laboratory Animal Center, Taiwan. The HLA-A2 transgenic mice were purchased from Jackson Laboratory which express HLA-A2.1 in C57BL/6 mice [14], and housed in the

Abbreviations: IFA, incomplete Freund's adjuvant; PELC, poly(ethylene glycol)-block-poly(lactideco-e-caprolactone) with squalene and Span®85; TAL6, tumor-associated antigen L6; TM4SF1, transmembrane-4 superfamily 1; ADCC, antibody-dependent cellular cytotoxicity; CTL, cytotoxic T lymphocyte; Th, helper T cell; Tc, cytotoxic T cells; HLA, human leukocyte antigen; TLR, toll-like receptor; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt); CpG, unmethylated CpG.

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Laboratory Animal Center of the National Health Research Institutes, Taiwan. All animal experiments were performed in specific pathogen-free (SPF) conditions under protocols approved by the Animal Committee of the National Health Research Institutes (NHRI).

The B16F1 cells were stably express TAL6 (B16-L6), and then were transfected with the HLA-A2 gene to generate a stable cell line B16-L6-A2. The B16-L6-A2 and MCF-7-TAL6 [12] cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin/streptomycin, 0.5 mM sodium pyruvate, 20 mM HEPES (Biological industries, Beit Haemek, Israel) at 37 °C in 5% CO_2 [12]. The EL4-L6-A2 and EL4-L6 cells were cultured in RPMI-1640 medium supplemented with 10% FBS [13].

Preparation of monoclonal antibodies

Hybridoma producing anti-TAL6 antibody (L6) was obtained from the American Type Culture Collection. 1F4 and 9C7 anti-TAL6 monoclonal antibodies were generated from BALB/c mice immunized with human TAL6 plasmid DNA as described. Monoclonal antibodies were purified by Protein A affinity chromatography using high salt conditions as described [2]. Antibody concentrations were determined by Micro BCA protein Assay Kit (PIERCE).

ELISA

For antibody epitope mapping L6, 9C7 and 1F4 Mabs were assayed by ELISA. For the assay of EP1 titers of the specific antibodies, antisera were collected from the mice immunized with EP1 peptide, and the titers of the specific antibodies were assayed by ELISA. The 96 well assay plates were coated with peptide (1 μg/ml) or cell (2×10^6). After blocking with 5% BSA-PBS, the antisera (1:1000 v/v) were diluted with 5% BSA-PBS and added to the plate for 1 h. HRP-conjugated goat anti-mouse IgG (1:4000 v/v) was used to detect EP1 antibody titer. The TMB peroxidase EIA substrate was added, which was stopped with 1N H_2SO_4. Absorbance was measured at 450 nm.

Animal study

HLA-A2 Tg mice were injected subcutaneously (s.c.) twice at a 2-week interval with peptide (50 μg/mouse) formulated in IFA [12], DOTAP liposome [15], PELC nanoparticles or TLR9 agonist CpG [16]. The CpG sequence used was 5′-TCC ATG ACG TTC CTG ACG TT-3′ with a phosphorothioate backbone. Seven days after the second immunization, the B16-L6-A2 or B16-L6 cells (2×10^4) were inoculated s.c. on the opposite site of the peptide injection. Tumor sizes were measured 3 times per week. Tumor volume was calculated using the formula: tumor volume = length × width × width/2.

Antibody dependent cell-mediated cytotoxicity (ADCC)

Mice spleen cells were used as effector cells for the ADCC assay. Spleen cells were adjusted to a concentration of 8×10^6 cells/ml in LCM medium. Cells were added in tubes and then divided into aliquots (100 μl/well in 96-well plates). EL4-L6 or EL-4 target cells (2×10^7/ml) were labeled with 100 μCi of ^{51}C ($Na_2{}^{51}CrO4$, PerkinElmer, MA) at 37 °C for 1 h. The ^{51}Cr-labelled EL4-L6 or EL4 cells were adjusted to a concentration of 2×10^5 cells/ml in LCM medium and then TAL6 antiserum or naïve mouse serum (1:10) was added. After 6 h, supernatants were harvested to measure the radioactivity using a gamma counter. Spontaneous release was measured in wells containing target cells alone. Triton X-100 (2%) was used to lyse the target cells to estimate maximal release. Percent cytotoxicity was determined according to the formula: percent lysis = 100 × (experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(maximal ^{51}Cr release- spontaneous ^{51}Cr release).

ELISPOT assay

HLA-A2 Tg mice were injected subcutaneously (s.c.) twice at a 2-week interval with peptide (50 μg/mouse) and TLR9 agonist CpG (10 μg/mouse) formulated in each adjuvant. At day 7 after the second vaccination, mice were sacrificed and spleen cells were collected. Spleen cells (5×10^5) were mixed with 10 μg/ml of the indicated peptides and added to a 96-well PVDF-membrane plate coated with anti-IFN-γ antibody. The plates were then incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 48 hours. After incubation, cells were removed by washing the plates 6 times with 0.05% (w/v) Tween 20 in PBS. A 50 μl aliquot of biotinylated secondary anti-IFN-γ antibody (clone R46A2; eBioscience, San Diego, CA) was added to each well. After 2 hours, the plate was washed and streptavidin-HRP (eBioscience) was added. Spots were developed using a 3-amine-9-ethyl carbazole (AEC, Sigma) solution. The reaction was stopped after 4–6 minutes by running the plate under tap water. The spots were then counted using an ELISPOT reader (Cellular Technology Ltd., Shaker Heights, OH).

CD107a cytotoxicity assay

HLA-A2 Tg mice were injected s.c. twice with the indicated peptides (50 μg/ml) emulsified in IFA, DOTAP or PELC in the absence or presence of CpG ODN (10 μg/mouse). On day 7 after the second immunization, splenocytes were harvested and then resuspended to 2×10^7 cell/ml in medium that contained 10 μg/ml of the indicated peptides (50 μg/ml) or cells (2×10^6 cell/ml) and PE-conjugated anti-mouse CD107a antibody (1:100) in 96-well round-bottom plates. After 2 hours at 37 °C, brefeldin A (10 μg/ml) and monensin (0.66 μg/ml) were added for another 2–6 hours. The plates were washed with PBS containing 0.1% FBS, and anti-mouse Fc antibody (1:100) was added for 5 minutes, followed by addition of the FITC-conjugated anti-mouse CD8 antibody (1:100) for 30 minutes. The cytotoxic CD107a+ CD8+ cells were detected using flow cytometer (FACS Calibur, BD Bioscience) and data were analyzed by FlowJo software.

Wound healing assay

Wound healing was investigated using a Culture-Insert (500 μm) (Ibidi). A 100 μl suspension of B16-L6 cells in DMEM-10% FBS (5×10^6 cells/ml) was seeded into each well of the insert. After cell attachment for 24 h, the culture inserts were removed and the cells were incubated with antiserum (1:100 v/v) in DMEM-10% FBS. The cell migration into the defined cell free gap was observed for 48 h as indicated under an inverted microscope. For assay analysis, cells were tracked using the manual tracking software component of the ImageJ programmer.

Statistical analysis

The statistical significant of differences between the mean values of the experimental groups was determined using the student t test and ANOVA. The differences were considered statistically significant if the P value was < 0.05.

Results

Identification of B cell epitopes of TAL6

Three purified anti-TAL6 monoclonal antibodies (1F4, 9C7, L6) [2] were used to detect the surface TAL6 expressed on EL-4 (EL-4-L6) cells. Serial diluted monoclonal antibodies bound EL-4-L6 cells but not negative control EL-4 cells (Fig. 1B), confirming that these three monoclonal antibodies recognize an extracellular domain of TAL6. To further map the antibody-binding epitopes, five peptides (EL1 and EP1-4) that cover the EL1 and EL2 extracellular loops were used to determine the linear B cells epitopes using ELISA [6]. Monoclonal antibody 1F4 and 9C7 cannot recognize these peptides. By contrast, EP1 peptide could be detected by L6 mAb (Fig. 1C). To test if the EP1 peptide can raise antibodies that bind native TAL6, the EP1 peptide or MCF-7-L6 cells (as positive control) were formulated with IFA/Th and immunized into C57BL/6 mice. Sera from immunized mice were collected and the anti-TAL6 antibody titers were analyzed using cell-based ELISA. The sera (1:500) of mice immunized with EP1 or MCF-7-L6 cells had significantly higher amount of antibody than mice immunized with vehicle (PBS) (Fig. 2A). We further investigated whether EP1 immunization could induce antibody-dependent cellular cytotoxicity (ADCC). Sera (1:100) from mice immunized with either EP1 or MCF-7-L6 cells could kill TAL6-expressed EL4 (EL4-L6) cells but not EL4 parental cells (61.82% ± 6.12% vs. 0.36% ± 0.96% and 65.54% ± 1.77% vs. 11.41% ± 3.094%, Fig. 2B). To investigate if the EP1 peptide could induce anti-tumor activity, mice were immunized with EP1 peptide and then challenged with B16-L6 cells (2×10^4/mouse) at 7 days after the final immunization. Immunization of mice with either EP1 or MCF-7-L6 cells significantly inhibited tumor growth (Fig. 2C). We conclude that the EP1 peptide is a linear B cell epitope of TAL6 that can produce antitumor activity against cancer cells that express TAL6.

A chimeric peptide containing B and T cells epitopes induces greater anti-tumor activity than B or T cell epitopes alone

To investigate whether incorporation of a cytotoxic T cells (Tc) epitope and a Helper T epitope (Th) can enhance the anti-tumor effects of B-cell epitope-based approaches, the synthetic peptides Th-A2-5, Th-EP1 or Th-A2-5-EP1 (Table 1) were formulated with incomplete Freund's adjuvant (IFA) and immunized in HLA-A2 transgenic (Tg) mice. Seven days after the final immunization, mice