Article

Construction and Functional Test of HLA-A*2402-Peptide Tetramer

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HLA-A*2402 is one of the most frequent HLA-A allele in Asian population. To construct HLA-A*2402-peptide tetramers, the transmembrane and intracellular segments of HLA-A*2402 cDNA were replaced with BSP sequence to form a fusion gene of sHLA-A*2402-BSP. The sHLA-A*2402-BSP fusion protein and β2m were high-level expressed as insoluble aggregates in *E.coli*, and refolded to form an HLA-A*2402-peptide monomeric complex by dilution method in the presence of an antigenic peptide. The HLA-A*2402-peptide monomeric complex was biotinated and tetramized to prepare HLA-A*2402-peptide tetramer. Then using the HLA-A*2402-peptide tetramers to detect antigen-specific cytotoxic T lymphocyte (CTL) induced by artificial antigen presenting cell (aAPC) *in vitro*. The results showed that HLA-A*2402-peptide tetramer was prepared correctly, and functional in detecting antigen-specific CTL *in vitro*, HLA-A*2402-peptide monomeric and its multimeric complexes are expected to provide a powerful tool for studying mechanisms of immune-related diseases in Asian populations. *Cellular & Molecular Immunology*. 2005;2(2):145-149.

Key Words: tetramer, HLA-A*2402-peptide monomeric complex, CTL

Introduction

Multimeric major histocompatibility complex (MHC) class I-peptide complexes, including dimer, tetramer and pentamer, acting as a high-affinity and specific ligand for TCR complex, are capable of visualizing antigen-specific cytotoxic T lymphocyte (CTL) with flow cytometry and other techniques, significant information about the generation of *in vivo* immunity can be obtained. Many allelic MHC molecules and their corresponding peptides have been applied to prepare multimeric peptide-MHC molecules, and applied to study the function of T lymphocytes in infectious diseases and cancer, and to find epitopes of T lymphocytes. For example, they were used to reveal how infectious agents shape the memory profile of the immune system (1), in addition to offering information on the affinity of T cell populations (2) and the relationship between T cell repertoire and tolerance (3). MHC multimers were reported to be sensitive and practical tools to monitor the survival of adoptively transferred tumor-specific CTL over time in the peripheral blood(4), as well as to assess the therapeutic effects of vaccination with peptide or peptide pulse dendritic cells (DC) by determination of the frequence of specific CTL *in vivo* (5). Tetrameric peptide-MHC complex was first reported by Altman et al. in 1996 (6), which was produced by combining soluble peptide-MHC tagged with biotin and bound *via* fluorescently labled streptavidin. HLA-A*2402 is one of the most common HLA class I allele in East Asian populations, especially in the Japanese (allelic frequency is 58.1%) and Chinese populations (allelic frequency is 32.9%) (7). In order to study the ethnic-specific immune related diseases in these populations, HLA-A*2402-peptide multimeric complex is of importance. In this study, HLA-A*2402-peptide [NH2-TYPVLEEMF-COOH of EB virus BRLF1] (8) complex was prepared and tetramerized into HLA-A*2402-peptide tetramer, the latter was applied to detect antigen-specific CTL induced by artificial antigen presenting cell (aAPC) (9, 10) *in vitro*. The results showed HLA-A*2402-peptide monomeric complex and its tetramer can work as the specific ligand for TCR, which can be used to generate and detect the specific T cells.

Materials and Methods

Plasmids

Plasmid pET21d-HLA-A*2402 was kindly provided by professor Sahara (Medical University of Sapporo, Japan). The secretive prokaryotic shuttle pression plasmid β2m was constructed previously in our laboratory (11).

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Cells

E003 cell line was an EBV-transformed B lympholoid cell line from a normal individual, which was kept in our Department. K562 is leukimic cell line with no HLA class I or class II expressed on its surface.

Peptides

The peptides NH2-TYPVLEEMF-COOH were synthesized at Meilian Biotechnology Company (Xi'an, China), purified to purity > 95%.

Reagents

Mouse anti-human HLA class I mAb W6/32 was prepared with the supernatant of hybridoma HB-95 (ATCC). T4 DNA ligase and PCR kit were purchased from Beckman Company (St Louis, MO, USA). Goat anti-rabbit IgG-HRP, CD28-specific antibody were purchased from eBioscience (Camarillo, CA, USA). PE-Cy5-CD8 antibody was purchased from BD PharMingen (San Diego, CA, USA). Phycoerythrin-labeled streptavidin was from Sigma Co. (St Louis, MO, USA).

Construction of the prokaryotic expression plasmid sHLA-A 2402-BSP-pET21d*

The construction of sHLA-A*2402-BSP fusion gene was carried out by PCR as following: starting at 95°C for 1 min for denaturation followed by 30 cycles at 95°C for 45 s, at 40°C for 1.5 min, at 68°C for 1 min and then at 72°C for 10 min. The sequences of primers were as follows: sense primer, 5'-CAT ACC ATG GGC AGC CAT TCT ATG CGC TAT TTT TCT ACC TCC GT-3'; anti-sense primer, 5'-TAA AGC GGC CGC TTA ACG ATG ATT CCA CAC CAT TTT CTG TGC ATC CAG AAT ATG ATG CAG GGA TCC TGC TCC CAT CTC AGG GTG AGG GGC TTG GGC AGA CCC TC-3' (including BSP sequences). The fusion gene was recombined into pET-21d plasmid by Nco I and Not I digested, and transformed into the *E.coli* strain BL21 (DE3) (Strategene, La Jolla, CA). The recombinant plasmid was verified with DNA sequencing.

*Expression and purification of sHLA-A*2402-BSP fusion protein and β2m*

Expression and purification of sHLA-A*2402-BSP fusion protein and β2m were carried out according to the protocol (12). The plasmids containing HLA-A*2402-BSP heavy chains and human β2m were introduced into *E.coli* strain BL21. Heavy chain transformants were cultured at 37°C when an OD_{600} reached 0.5 with induction by adding 1 mmol/L isopropyl β-D-thiogalactopyranoside (IPTG). Transformants with plasmids coding human β2m were grown and induced by adding 0.4 mmol/L IPTG. These host bacteria were harvested by centrifugation at 4,000 rpm for 20 min and resuspended with sonication buffer containing 50 mmol/L Tris/HCl (pH 8.0), 50 mmol/L NaCl, 1 mmol/L ethylene-diaminetetra-aceticacid (EDTA) and 1 mmol/L dithiothreitol (DTT). After adding Triton-100, the insoluble inclusion bodies collected, washed to remove contaminating proteins, and dissolved in 8 M Urea, 50 mmol/L Tris/HCl (pH 8.0), 1 mmol/L EDTA, 1 mmol/L DTT.

*Construction of monomeric and tetrameric HLA-A*2402-peptide complexes*

Construction of monomeric and tetrameric HLA-A*2402-peptide complexes were carried out according to the protocol of Altman et al. (6). Briefly, HLA-A*2402-BSP heavy chain and human β2m were refolded with synthetic peptide (NH2-TYPVLEEMF-COOH of EB virus BRLF1) in refolding buffer, consisting of 100 mmol/L Tris-HCl (pH 8.0), 400 mmol/L L-arginine, 2 mmol/L EDTA, 5 mmol/L reduced glutathione, 0.5 mmol/L oxidized glutathione, 0.5 mmol/L phenylmethysul-fonylfluoride (PMSF) per 500 ml. The refolding mixture was stired and incubated at 4°C for 72 h and then concentrated using PEG20000 dialysis. Subsequently, the mixture was desaulted and the buffer was changed in 20 mmol/L Tris-HCl by ultra-filtration with Centrecon-30 units (Centrecon). The refolding product was stored in phosphate-buffered saline (PBS) plus a cocktail of protease inhibitors: i.e., 1 mM pepstatin, 0.1 M phenylmethysul-fonylfluoride (PMSF) and 1 mmol/L EDTA. The folded product was then subjected to enzymatic biotinylation by BirA enzyme (Avidity, Denver Co.) at 25°C for 12 h. The tetrameric complexes of biotinylated HLA-A*2402-peptide were produced by mixing purified, biotinylated monomer with phycoerythrin-labeled streptavidin at a molar ratio of 4:1.

*Identification of the conformation of HLA-A*2402-peptide monomeric complex*

The conformation of the refolded HLA-A*2402-peptide monomeric complex was determined by sandwich ELISA (13). The ELISA plates were coated by mAb W6/32 and blocked by 30 g/L BSA with the following addition of refolded complexes, cell membrane protein with HLA I molecule from E003 (positive control), cell membrane protein without HLA I molecule from K562 (negative control) and PBS respectively, heavy chain, β2m, were also added for quality control. After incubating at 37°C for 1 h, rabbit anti-human β2m antibody was used as the primary antibody and horseredish peroxidase (HRP) labeled goat anti-rabbit IgG antibody (1:1,000) was used as the secondary antibody.

*Induction of specific CTL in vitro and CTL detection with HLA-A*2402-peptide tetramer*

In vitro CTL generation was performed as described previously (9, 10). Briefly, PBMCs from HLA-A*2402-positive healthy donors (from the Blood Center of Wuhan, China) were co-cultureded with aAPC prepared by coupling multimeric HLA-A*2402-peptide complex and CD28-specific antibody onto 5 μm sulfate polystyrene latex microbeads (Interfacial Dynamics, Portland, OR, USA) *in vitro* three times at weekly intervals. PBMCs with or without aAPC were stained with the appropriate PE-labeled tetramer at 37°C for 30 min, and FITC-conjugated anti-CD8 antibody at 4°C for 30 min. Cell were washed twice with phosphate buffer saline (PBS) before fixation in 1% formaldehyde. Stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The fluorescence intensity (FI) of cells was analyzed with CELLQuest software (Becton Dickinson).

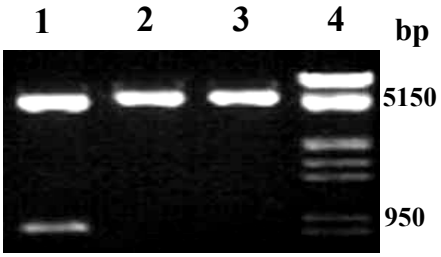

Figure 1. Restriction analysis for recombinant pET-HLA-A*2402-BSP. Lane 1, digest of Nco I and Not I, which showed a sHLA-A*2402-BSP fusion gene of 900 bp and a plasmid band; Lanes 2 and 3, digests of Nco I, Not I, respectively, which gave a single band; Lane 4, λDNA Hind III/EcoR I marker. It indicated that the recombinant plasmid was constructed successfully.

Statistical analysis

Data were presented as $x \pm s$. Group differences were analyzed with one-way ANOVA, using SNK test for multiple comparison, and $p < 0.05$ was considered as significant.

Results

*Verification of pET-HLA-A*2402-BSP recombinant plasmid*

The recombinant plasmid was verified with restriction analysis and DNA sequencing. The plasmid was digested by Nco I and Not I, digests were analyzed with 1% agarose gel electrophoresis (Figure 1). Both the digested fragments and DNA sequence analysis (data not shown) indicated that the recombinant plasmid was constructed correctly.

*Determination of the conformation of HLA-A*2402-peptide complexes by mAb W6/32*

The heavy chain and light chain were expressed in inclusion body with the form of denaturalization. During the procedure of renaturation, denatural β2m can almost renature completely, but heavy chain can renature and be refolded only in the presence of β2m and corresponding peptide, forming the HLA-A*2402-peptide monomeric complex (14). The human HLA class I specific monoclonal antibody W6/32 binds to a discontinuous epitope within the α 2 domain of HLA class I, but can not bind with β2m alone and can bind sole heavy chain very weakly (15). We used W6/32 and anti-β2m to detect the conformation of HLA-A*2402-peptide monomeric complex by sandwich ELISA (Figure 2). The OD_{450} values of HLA-A*2402-peptide complex monomer and positive control (membrane protein of LCL E003) are significantly increased when compared with those of blank control and negative control (membrane protein of K562) ($p < 0.01$). Meanwhile, the OD_{450} values of sole β2m and sole heavy chain are similar to that of blank control ($p > 0.05$). The results show that the HLA-A*2402-peptide monomeric complex shares the conformational epitope with natural HLA class I molecule.

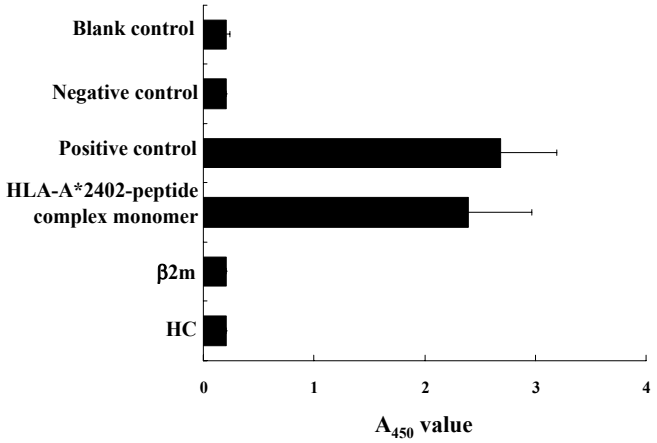

Figure 2. Identification of HLA-A*2402-peptide monomeric complex in the refolded complex (n = 8). The OD_{450} values of HLA-A*2402-peptide monomeric complex (2.68 ± 0.508) and positive control (membrane of LCL) (2.39 ± 0.575) are significantly increased when compared with those of blank control (0.20 ± 0.005) and negative control (membrane protein of K562) (0.21 ± 0.004) ($p < 0.01$). Meanwhile, the OD_{450} values of β2m (0.20 ± 0.005) and heavy chain (0.21 ± 0.032) are similar to that of blank control ($p > 0.05$). It shows that the HLA-A*2402-peptide monomeric complex shares the conformational epitope with natural HLA class I molecule.

*Detection of CTL with soluble HLA-A*2402-peptide tetramer in vitro*

The monomer of HLA-A*2402-peptide complex was biotinylated, and tetramerized with phycoerythrin-labeled streptavidin at a molar ratio of 4:1. In order to confirm the prepared tetramer is able to bind to specific CTL, aAPC coated with HLA-A*2402-peptide complex and CD28-specific antibody was used to propagate the specific CTL. The PBMCs of HLA-A*2402 positive individual were co-cultured with the aAPC. Then the culture cells were stained with the prepared tetramer, and subjected to FCM analysis. The results were shown in Figure 3, the tetramer-stained CTL frequency increased from 0.21% (A) to 10.44% (B) after three times stimulation with the aAPC. The results demonstrated that the prepared tetramer can be used for detection of specific CTLs.

Discussion

Classical HLA class I and class II molecules are essential to present antigenic peptide (including viral peptide) for T cell recognition and initiate adaptive immune response. The epitopes recognized by T cells are formed by antigenic peptide associated with MHC class I or class II molecules, which are recognized by $CD8^+$ T and $CD4^+$ T cells respectively. Determination of frequencies of antigen-specific T cells is the key to understanding the cellular immune response. Since recognition of antigen-specific T cells is initiated by the specific binding of TCR to peptide-MHC, it

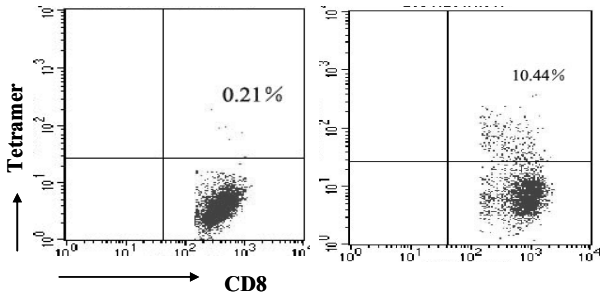

Figure 3. Determination of specific CTL. Specific CTL was detected by HLA-A*2402-EBV tetramer and FITC-conjugated anti-CD8 mAb in PBMC of an HLA-A*2402$^+$ individual before co-culture with the aAPC (A) and after co-culture with the aAPC (B). CTL frequency increased from 0.21% (A) to 10.44% (B) after three times stimulation with the aAPC. The results demonstrated that the prepared tetramer can be used for detection of specific CTLs.

is theoretically possible to study specific TCR-peptide-MHC interactions using recombinant soluble monomeric MHC molecules, these molecules do not bind T cells in a stable and predictable manner, and therefore cannot be used for analysis of antigen-specific T cells by flow cytometry (16, 17). As multimeric peptide-MHC complexes acting as high-affinity and specific ligand for TCR, they are recently applied to identify antigen-specific T cells. The forms of multimeric peptide-MHC complexes includes dimer, tetramer and pentamer, the essential advantage of these multimeric variations is displaying a significantly increased avidity for their specific TCR as they bind with multiple arms independently to the same cell, which results in stable and reproducible binding. Therefore, multimeric peptide-MHC complexes provide sensitive and practical tools to visualize antigen-specific T cells, since the assay is more rapid and permits an assessment of the total number of peptide-specific T cells in the peripheral blood without the need for *in vitro* manipulation.

In order to make a population survive, the genetic cluster of HLA loci showed a striking polymorphism to protect host from any pathogen mutation eluding host immune surveillance. Antigenic peptides binding to different alleles of HLA molecules vary in affinity, therefore the allotype of HLA is supposed to affect the host immune response to a given antigen. As the polymorphism of MHC molecules, the choice of MHC allele for preparation of multimeric complex was an essential step. Although there are many peptide-MHC complexes already prepared, most of them are HLA-A2 based, for HLA-A2 is the most common allele in every ethnic populations. HLA-A*2402 is one of the most common HLA class I allele in East Asian populations, especially in the Japanese and Chinese (7). HLA-A*2402 based multimeric complexes are specifically important to study the T cell response and its related pathological involvement in these populations.

Assembling of MHC-peptide monomeric complex is the initial step to prepare MHC-peptide tetramer. Although MHC I-peptide complexes are expressed by nearly all types of nucleated cells, and can be collected by enzymatic cleavage from cell surface, it is not a practical method for the polymorphism and polygeny of MHC molecules and constellation of antigenic peptides. The most practical way to produce MHC-peptide monomeric complex seems to be the refolding of heavy and light chains by dilution method in the presence of an antigenic peptide. In order to prepare HLA-A*2402-peptide tetramers, the cDNA encoding the extracellular domain of sHLA-A*2402 was linked with BSP (BirA Substrate Peptide) at its C terminal, then recombined into pET21d to construct a recombinant plasmid (sHLA-A*2402-BSP-pET21d), the sHLA-A*2402-BSP fusion protein and β2m were high-level expressed as insoluble aggregates in *E.coli.* Then the two subunits refolded to form an HLA-A*2402-peptide monomeric complex by dilution method in the presence of an antigenic peptide. The monomeric complex was tetramerized by binding to fluorescently labled streptavidin to generate HLA-A*2402-peptide tetramer. The HLA-A*2402-peptide monomeric complex binds to mAb W6/32, and shares the same antigenic determinant (or conformation) as its natural counterpart.

The essential biological function of MHC-peptide complex is acting as a specific ligand for TCR. T cell is activated by dual signals provided by APC, the first signal is antigen-specific, generated by the binding of TCR to MHC-peptide complex, the second signal is due to the interactions of co-stimulatory molecules, such as CD28-B7. Based on this principle, a cell-sized microbead coated with the MHC-peptide complex and anti-CD28, which provides the antigen-specific and costimulatory signals required for T cell activation, is called artificial APC (9, 10). Artificial APC turns to be an effective tool to generate MHC-peptide specific T cell, for the convenience in manipulating the MHC-peptide complex "expressed" on the surface of the "cell". HLA-A*2402-peptide monomeric complex prepared in this study, together with anti-CD28 were coated on microbead to form an artificial APC, and to propagate specific CTL by co-culture with PBMC of HLA-A*2402$^+$ individual.

After co-culture of the artificial APC with the PBMC, the frequency of MHC-peptide specific CD8$^+$ T cells was increased dramatically, as shown the tetramer-stained CD8$^+$ T cells in the cultural bulk with FCM assay. The results indicate HLA-A*2402-peptide monomeric complex prepared in this study can act as a specific ligand for TCR, which can be used both in stimulation and detection of antigen-specific CTL. The generation of HLA-A*2402-peptide complex and its tetramer lay the foundation to further study on the characteristic of antigenic peptide presented by HLA-A*2402, and provide a forcible tool for the research on mechanism of immune-related diseases in the Asian populations, including Chinese.

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References

1. Kern F, Bunde T, Faulhaber F, et al. Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. J Infect Dis. 2002; 185:1709-1716.
2. Yee C, Savage PA, Lee PP, Davis MM, Greenberg PD. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramer. J Immunol. 1999;162: 2227-2234.
3. Hernandez J, Lee PP, Davis MM, Sherman LA. The use of HLA2.1/p53 peptide tetramer to visualize the impact of self tolerance on the TCR repertoire. J Immunol. 2000;164:596-602.
4. Meidenbauer N, Marienhagen J, Laumer M, et al. Survival and tumor localization of adoptively transferred Melan-A-specific T cells in melanoma patients. J Immunol. 2003;170:2161-2169.
5. Mackensen A, Herbst B, Chen JL, et al. Phase I study in melanoma patients of a vaccine with peptide-pulsed dentritic cells generated *in vitro* from $CD34^+$ hematopoietic progenitor cells. Int J Cancer. 2000;86:385-392.
6. Altman JD, Moss PAH, Goulder PJR, et al. Phenotypic analysis of antigen-specific T lymphocytes. Science. 1996;274:94-96.
7. Nakamoto Y, Kaneko S, Takizawa H, et al. Analysis of the CD8-positive T cell response in Japanese patients with chronic hepatitis C using HLA-A*2402 peptide tetramers. J Med Virol. 2003;70:51-61.
8. Kuzushima K, Hayashi N, Kudoh A, et al. Tetramer-assisted identification and characterization of epitopes recognized by HLA-A*2402-restricted Epstein-Barr virus-specific $CD8^+$ T cells. Blood. 2003;101:1460-1467.
9. Oelke M, Maus MV, Didiano D, June CH, Mackensen A, Schneck JP. *Ex vivo* induction and expansion of antigen-specific cytotoxic T cells by HLA-Ig-coated artificial antigen-presenting cells. Nat Med. 2003;9:619-624.
10. Tham EL, Jensen PL, Mescher MF. Activation of antigen-specific T cells by artificial cell construcs having immobilized multimeric peptide-class I complexes and recombinant B7-Fc protein. J Immunol Methods. 2001;249:111-119.
11. Chen H, Weng XF, Wu XW, et al. Cloning and expression of human β2m gene. J Huazhong Univ Sci Tech [Health Sci]. 2004;33:1-4.
12. Recommendations for soluble MHC/peptide tetramer use. Available at: http:// www.niaid.nih.gov/reposit/tetramer/protocol. html. Last updated February 2, 2004. Accessed February 4, 2004.
13. Weng XF, Chen H, Wu XW, et al. *In-vitro* refolding and biotinylation of soluble HLA-A2-peptide complex. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2004;20:265-268.
14. Parker KC, Silver ML, Wiley DC. An HLA-A2/β_2m/peptide complex assembled from subunits expressed separately in *E.coli*. Mol Immunol. 1992;29:371-378.
15. Maziarz RT, Fraser J, Strominger JL, Burakoff SJ. The human HLA-specific monoclonal antibody W6/32 recognizes a discontinuous epitope within the α2 domain of murine $H\text{-}2D^b$. Immunogenetics. 1986;24:206-208.
16. Corr M, Slanetz AE, Boyd LF, et al. T cell receptor-MHC class I peptide interactions: affinity, kinetics, and specificity. Science. 1994;265:946-949.
17. Matsui K, Boniface JJ, Reay PA, et al. Low affinity interaction of peptide-MHC complexes with T cell receptors. Science. 1991; 254:1788-1791.