Cytotoxic T Cell Induction against Human Malignant Melanoma Cells Using HLA-A24-restricted Melanoma Peptide Cocktail

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Abstract. Many human leukocyte antigen (HLA)-class I (mainly A*0201)-restricted peptide-specific cytotoxic T cells (CTLs) have been derived from peripheral blood lymphocytes (PBLs) of melanoma patients. However, few studies regarding HLA-A*2402-restricted melanoma-associated peptides have been performed, because HLA-A24 is not a common allele in Caucasians. In this study, we investigated the specific CTL-inducing activity of 5 HLA-A*2402-restricted peptides derived from gp100, tyrosinase, MAGE1, MAGE2 and MAGE3. A CTL induction culture was performed using PBLs and cultured dendritic cell (DC) pulsed with HLA-A*2402-restricted melanoma peptide cocktail. The CTLs derived from volunteers killed the A24 peptide-pulsed TISI cells and even HLA-A*2402-positive melanoma cells, but not HLA-A*0201-positive cells. IFN-γ levels produced by the melanoma patients’ CTLs were obviously low in each peptide group compared with those produced by the volunteers’ CTLs, which indicated the presence of immunosuppressive factors in metastatic melanoma. These results suggested that polyvalent immunotherapy using multiple epitopes from melanoma antigens might be a better way of improving the efficacy of treatment.

In the last few years, though many immunotherapeutic attempts to target cancer-specific antigens have been made, a breakthrough in terms of clinical response has not yet been achieved mainly because of a scarcity of effective genuine cancer antigens, immunological evasion, or an immunosuppressive state.

As for cancer antigens which primed T cells can recognize in the context of major histocompatibility antigen (MHC), Renkvist et al. demonstrated 6 different groups of human tumor antigens (1). Melanoma-associated antigens are categorized as class I human leukocyte antigen (HLA)-restricted cancer/testis antigens which are considered to be tolerable to the immune system because they are also expressed on normal tissues like testis. However, malignant melanoma is the most well known cancer in which multiple tumor-specific antigens have been defined and utilized in vaccination strategies as peptide vaccines (2-9). So far, no adverse effects regarding damage to normal tissue have been reported, except for vitiligo in some cases.

From a clinical point of view, few vaccination strategies for stage IV melanoma using a combination of several (more than 3) peptides have been reported to date. Notably, little immunotherapeutic research using human leukocyte antigen (HLA)-A24-restricted multiple peptides has been conducted because HLA-A24 is not a common allele in Caucasians. As far as peptides are efficiently immunogenic, there may be certain advantages of multiple peptide-based vaccinations over single peptide-based vaccinations in terms of generating polyclonal cytotoxic T cells (CTLs) targeting tumors. In the present study, we investigated the feasibility of using a combination of 5 melanoma-associated peptides (peptide cocktail) as a specific cancer vaccine.
**Table I. Melanoma-associated peptide binding profiles.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>AA</th>
<th>Protein Position</th>
<th>HLA-A24 binding NIH Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWKTWGQYW</td>
<td>gp100</td>
<td>152-160</td>
<td>0.12</td>
</tr>
<tr>
<td>AFLPWHRLF</td>
<td>Tyrosinase</td>
<td>206-214</td>
<td>18.0</td>
</tr>
<tr>
<td>NYKHCPEI</td>
<td>MAGE1</td>
<td>135-143</td>
<td>66.0</td>
</tr>
<tr>
<td>EYLQLVFGI</td>
<td>MAGE2</td>
<td>156-164</td>
<td>90.0</td>
</tr>
<tr>
<td>IMPKAGLLI</td>
<td>MAGE3</td>
<td>195-203</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*NH Score was shown as the estimate of half time of a dissociation of peptides containing motif from HLA-A24 molecules.

**Materials and Methods**

Reagents and cell lines. Recombinant human (rh) granulocyte macrophage colony-stimulating factor (GM-CSF), rh-interleukin (IL)-2, rhIL-4 and rhIL-7 were purchased from Pepro Tech Inc. (Rocky Hill, NJ, USA). GM-CSF and IL-4 were used at 10 ng/ml for dendritic cell (DC) culture. Mouse monoclonal antibodies (MoAbs) to human CD1a, CD8, CD11c, HLA-ABC (class I), HLA-DR (class II), CD80, CD83 and CD86 were all purchased from Pharmingen (San Diego, CA, USA). Mouse MoAbs to human HLA-A24 were obtained from One Lambda, Inc. (Canoga Park, CA, USA). TISI, a human B-lymphoblastoid cell line showing a high level of expression of homozgyous HLA-A24 (A*2402), was supplied by Takara Bio Inc., Ltd. (Kusatsu, Shiga, Japan). The human chronic myelogenous leukemia cell line, K562, was obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). Human melanoma cell lines established from Japanese patients were kindly supplied as follows; MMG-1 and MMG-3 from Dr. Takagi (Gifu University School of Medicine, Gifu, Japan), SMTK-1 from Dr. Matsumoto (Shinshu University School of Medicine, Matsumoto, Japan), NCC-KT from Dr. Hamanaka (Yamaguchi University School of Medicine, Ube, Japan), TDMM1 from Dr. Takahashi (Japanese Red Cross Medical Center, Tokyo, Japan) and KU-MELTC-1 from Dr. Ikeda (Keio University School of Medicine, Tokyo, Japan). The melanoma cell line, RPMI7951, was purchased from the American Type Culture Collection (Manassas, VA, USA).

Synthetic peptides. The sequences of melanoma-associated peptides used in the present study are shown in Table I. These peptides were chosen as HLA-A24-restricted peptides according to past studies; gp100 (VWKTWGQYW), tyrosinase (AFLPWHRLF) (10), MAGE1 (NYKHCPEI) (11), MAGE2 (EYLQLVFGI) (12) and MAGE3 (IMPKAGLLI) (13). The gp100 peptide was selected from 3 candidate peptides using a MHC peptide-binding assay (14) performed by Takara Co. Ltd. CEA (TYACFVSNL) was used as a negative control peptide. Peptides were synthesized according to a manual solid phase synthesis method using TBTU as a coupling reagent. The binding NIH score of each peptide with HLA-A24 molecules was measured at the web site of Bioinformatics and molecular analysis section (BIMAS), HLA peptide binding predictions (http://bimas.dcrt.nih.gov/molbio/hla bind/index.html). Five kinds of HLA-A24-restricted melanoma-associated peptides were mixed together and used as a peptide cocktail for DC pulsing in the CTL induction experiment.

CTL induction cultures. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque plus (Pharmacia, Sweden) density centrifugation of venous blood from HLA-A24+ melanoma patients or healthy donors (Institutional Review Board No. 12-93 and 12-94). Informed consent regarding investigational research using each donor’s blood was obtained. DCs were generated in vitro as described previously (15). Briefly, PBMCs were placed in 6-well culture plates (Corning Inc., Corning, NY, USA) at 4 x 10^6 cells/ml in RPMI1640 medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 U/ml) and 5% (v/v) AB human serum, referred to as DC medium, and incubated for 90 min at 37°C. After the incubation, the non-adherent cells were removed by gentle washing, and the adherent monocyte-enriched population was cultured in the presence of 10 ng/ml of GM-CSF and 10 ng/ml of IL-4. After 7 days culture, on day 0, harvested DCs were washed with Dulbecco’s PBS with calcium and magnesium [referred to as PBS(+)] containing 1% human serum albumin (Kaketsuken, Kumamoto, Japan) and incubated at 1 x 10^6 cells/ml with a cocktail of HLA-A24- binding peptide (each 25 µg/ml) and 5 µg/ml of β2-microglobulin (Sigma, St. Louis, MO, USA) for 4 hours at room temperature with gentle shaking. After being washed, DCs were irradiated (50 Gy) and incubated with non-adherent autologous PBMCs at a ratio of 1:20 in the presence of 10 ng/ml of IL-7. On days 7 and 14, the PBMC cultures were restimulated with irradiated peptide-pulsed DC at a ratio of 1:40. Human IL-2 suspended in DC medium was added to PBMC cultures every 2-3 days at a final dose of 2.5 ng/ml. A cytotoxicity assay was performed on day 21.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from melanoma cancer cells using Rneasy total RNA kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized using 1 µg of total RNA, Moloney murine leukemia virus reverse transcriptase and an oligo (dT) primer (Stratagene, CA, USA) mixed in a volume of 50 µl containing 50mM Tris-HCl (pH 8.3), 75 mM KCl, 3mM MgCl2, 10mM dithiothreitol and 1mM of each dNTP. The cDNA from each mRNA was amplified by PCR with specific primers and methods. The primer sequences used were 5’-ATTCAGGTGGTATTCTGGG-3’ and 5’-CAGGAGAATTTC TCTGAACCT-3’ for MART-1, 5’-GAGGAGCATTTGTG GCC-3’ and 5’-TGTTGCAACATCAGGGAAAA-3’ for tyrosinase, 5’-TGGTGAAACCCTGTACTCTCTC-3’ and 5’-TCAGGGAATAGG TAGCTCTCT-3’ for gp100, 5’-GGCCGCCGAGAACTCGTACCAAG-3’ and 5’-GCTGGAAACCTCCTGCTTGGC-3’ for MAGE-1, 5’- AAGTGAAGCCGCGGACCTG-3’ and 5’-GAAGAGGAGAGA GGCCATGG-3’ for MAGE-2, 5’-TGGGAGGACGAGGCCCCC CCC-3’ and 5’-GGCCGCCGAGAACTCGTACCAAG-3’ for MAGE-3, 5’-GGAGGAGCACCGGACCAAG-3’ and 5’-AAGGAGCTCTGGTTGC-3’ for MAGE-4, 5’-TGTTGAAACCCTGTACTCTCTC-3’ and 5’-TCAGGGAATAGG TAGCTCTCT-3’ for gp100, 5’-GGCCGCCGAGAACTCGTACCAAG-3’ and 5’-GCTGGAAACCTCCTGCTTGGC-3’ for MAGE-1, 5’-AAGTGAAGCCGCGGACCTG-3’ and 5’-GAAGAGGAGAGA GGCCATGG-3’ for MAGE-2, 5’-TGGGAGGACGAGGCCCCC CCC-3’ and 5’-GGCCGCCGAGAACTCGTACCAAG-3’ for MAGE-3, 5’-GGAGGAGCACCGGACCAAG-3’ and 5’-AAGGAGCTCTGGTTGC-3’ for MAGE-4, 5’-TGTTGAAACCCTGTACTCTCTC-3’ and 5’-TCAGGGAATAGG TAGCTCTCT-3’ for gp100, 5’-GGCCGCCGAGAACTCGTACCAAG-3’ and 5’-GCTGGAAACCTCCTGCTTGGC-3’ for MAGE-1, 5’-AAGTGAAGCCGCGGACCTG-3’ and 5’-GAAGAGGAGAGA GGCCATGG-3’ for MAGE-2, 5’-TGGGAGGACGAGGCCCCC CCC-3’ and 5’-GGCCGCCGAGAACTCGTACCAAG-3’ for MAGE-3, 5’-GGAGGAGCACCGGACCAAG-3’ and 5’-AAGGAGCTCTGGTTGC-3’ for MAGE-4, 5’-TGTTGAAACCCTGTACTCTCTC-3’ and 5’-TCAGGGAATAGG TAGCTCTCT-3’ for gp100, 5’-GGCCGCCGAGAACTCGTACCAAG-3’ and 5’-GCTGGAAACCTCCTGCTTGGC-3’ for MAGE-1, 5’-AAGTGAAGCCGCGGACCTG-3’ and 5’-GAAGAGGAGAGA GGCCATGG-3’ for MAGE-2, 5’-TGGGAGGACGAGGCCCCC CCC-3’ and 5’-GGCCGCCGAGAACTCGTACCAAG-3’ for MAGE-3, 5’-GGAGGAGCACCGGACCAAG-3’ and 5’-AAGGAGCTCTGGTTGC-3’ for MAGE-4, 5’-TGTTGAAACCCTGTACTCTCTC-3’. The cytotoxicity assay was performed on day 21.
20 µg/ml or anti-HLA-A24 MoAb at a dilution of 1:10, respectively, for 4 hours at 37 °C before a 4-hour coincubation.

IFN-γ production by CTLs restimulated with melanoma peptide-pulsed TISI cells. CTLs (2 x 10⁵), derived from 3 healthy volunteers and 3 melanoma patients, were co-incubated with TISI cells (2 x 10⁵) pulsed with each melanoma peptide or untreated TISI cells in 96-well culture plates for 24 hours. Supernatants were collected and stored at -20 °C until use. The amounts of IFN-γ in the supernatant were measured with a human IFN-γ ELISA kit (R and D Systems, Minneapolis, MN, USA).

Statistical analysis. Statistical differences were analyzed using the paired two-tailed Student’s *t*-test. Values of *p* < 0.05 were considered significant.

Results

Expression of various tumor antigens in Japanese melanoma cell lines. The expression of MART-1, gp100, tyrosinase, MAGE-1, MAGE-2 and MAGE-3 mRNA was analyzed in 7 Japanese melanoma cell lines (Figure 1). Four of the cell lines were positive for MART-1, 3 for gp100, 5 for tyrosinase, 5 for MAGE-1 and all for MAGE-2 and MAGE-3. Two cell lines, NCC-KT and KU-MELTC-1, expressed all the tumor antigens analyzed. More interestingly, the expression of MAGE4, MAGE6 and MAGE12 mRNAs was verified in almost all melanoma cell lines.

HLA-A24-binding melanoma peptides. Four out of 5 peptides, tyrosinase, MAGE-1, MAGE-2 and MAGE-3, have been reported to have an ability to stimulate CTLs (10-13). MHC binding scores showed that 3 peptides had high affinity and 2 had low affinity (Table I).

Generation of DCs from blood monocytes. Flow cytometric analysis revealed that CD1a as a DC marker was expressed in 40-60% of cultured DCs. Other molecules including CD11c, HLA-class I, HLA-DR, CD40, CD54, CD80 and CD86 were seen on most of the cultured DCs. The frequencies of surface markers on DCs seemed not to be different between volunteers and melanoma patients (data not shown).

Killing activity of CTLs induced by peptide cocktail-pulsed DC against TISI cells or HLA-A24⁺ melanoma cell lines. The bulk of cultured CTLs exposed to melanoma peptide-cocktail-pulsed DCs showed killing activity against MAGE1, MAGE2, MAGE3 or tyrosinase peptide-treated TISI cells (Figure 2). In addition, they exhibited the most killing activity against peptide cocktail-treated TISI cells (Figure 3A). In contrast, they did not recognize TISI cells treated with gp100 or with CEA peptide as a control. The killing activity of CTLs against TISI cells pulsed with a cocktail of A24-restricted peptides was significantly inhibited by anti-HLA-A24 or anti-CD8 MoAb, but not anti-HLA-DR MoAb (Figure 3B). Furthermore, CTLs from one representative case out of 3 healthy volunteers exhibited potent HLA-A24⁺ melanoma cell line-specific killing activity (Figure 4A and B). Most interestingly, CTLs derived from one of the 3 metastatic melanoma patients also showed potent killing activity similar to that in the healthy volunteer described above (Figure 4C). However, the CTLs of both individuals did not recognize HLA-A2⁺ melanoma cells or K562 cells.
Figure 2. Killing activity of volunteer-derived CTLs against melanoma peptide-pulsed TISI cells. Cultured monocyte-derived DCs were incubated with non-adherent autologous PBMCs in the presence of IL-7 in 6-well culture plates. On days 7 and 14, the PBMC cultures were restimulated with irradiated peptide-pulsed DC. Human IL-2 was added to PBMC cultures every 2-3 days. A cytotoxicity assay was performed on day 21. Each point shows the mean±S.D. of triplicate samples from donor No.1, one of 3 healthy volunteers. HLA-A24 CEA peptide (TYACFVSNL) was used as a control peptide.

Figure 3. Inhibitory effect of anti-HLA-A24 MoAb on the killing activity of CTLs against TISI cells pulsed with a cocktail of A24-restricted peptides. (A) Killing activity of CTLs from volunteer No.2 against TISI cells pulsed with a cocktail of A24-restricted peptides. (B) Significant inhibition of CTL killing activity by anti-HLA-A24 or anti-CD8 MoAb against TISI cells pulsed with HLA-A24-restricted peptide cocktail. Each point or column shows the mean±S.D. of triplicate samples from donor No.2. **; p<0.01, statistically significant compared with the control MoAb (mIgG1) group.
Figure 4. Killing activity of volunteer or melanoma patient-derived CTLs against HLA-A24+ melanoma cell lines. (A) and (B): killing activity of CTLs from volunteer No.1. (C) killing activity of CTLs from a HLA-A*2402+ melanoma patient. Each point shows the mean±S.D. of triplicate samples. HLA-A24+ melanoma cell lines (TDMM1, KU-MELTC-1, MMG-1), HLA-A2+ melanoma cell line (RPMI7951).

Figure 5. INF-γ production by CTLs restimulated with melanoma peptide-pulsed TISI cells. CTL cells (2x10⁵) derived from volunteers or melanoma patients and peptide-treated TISI cells (2x10⁵) were co-cultured in 96-well culture plates for 24 hours. The amounts of IFN-γ in supernatant were measured using an ELISA kit specific for human IFN-γ. Each column represents the mean±S.D. of triplicate samples from 3 healthy donors or 3 melanoma patients. The IFN-γ level in the peptide (-) (responders alone) group was set at 100% of control. HLA-A24 CEA peptide (TYACFVSNL) was used as a negative control peptide.
IFN-γ production by CTLs from melanoma patients restimulated with peptide-pulsed TISI cells. The mean levels of IFN-γ produced by the volunteers’ CTLs were significantly higher in the peptide cocktail, MAGE1, MAGE2, MAGE3 and tyrosinase-stimulated groups than unstimulated or CEA peptide-treated group (Figure 5). In contrast, IFN-γ levels produced by the CTLs of melanoma patients were obviously low in all groups compared with those produced by the volunteers’ CTLs, and even in the highest group that was stimulated with a peptide cocktail, the IFN-γ level was approximately one-third of the control value.

**Discussion**

Since the MAGE1 gene was cloned and its product was demonstrated to be the first cancer antigen recognized by specific T cells, intensive and collective research into the molecular identification and characterization of tumor antigens has been done using samples from melanoma patients. Renkvist et al. (1) classified human tumor antigens recognized by T cells into 6 categories: (i) HLA class I-restricted cancer/testis antigens, (ii) differentiation antigens, (iii) widely expressed antigens, (iv) tumor-specific antigens, (v) HLA class II-restricted antigens, and (vi) fusion proteins. Melanoma-associated antigens are allocated to the cancer/testis antigen (MAGE, GAGE family, NY-ESO) and melanoma differentiation antigen (MART-1, gp100, tyrosinase) groups.

Based on immunological observations regarding melanoma-associated antigens, many HLA-class I (mainly A*0201)-restricted peptide specific CTLs have been cloned and characterized (2-6). Some peptides including MART-1, gp100, tyrosinase and MAGE3 with or without dendritic cells have been subjected to clinical immunotherapeutic trials in metastatic melanoma patients (7-9) and obvious anti-tumor effects resulting in a beneficial effect on survival time were obtained in some cases.

Considering these findings together, we focused on the immunological activity of HLA-A24 (A*2402)-restricted melanoma-associated peptides because the HLA-A24 allele is most common in Japanese people. In the present study, we investigated the specific CTL-inducing activity of 5 A*2402-restricted peptides derived from gp100, tyrosinase, MAGE1, MAGE2 and MAGE3 using DCs pulsed with a cocktail of 5 peptides. Each of these 5 A*2402-restricted peptides was demonstrated to be able to activate peptide-specific CTLs (10-13). We made a specific CTL line using DCs pulsed with a cocktail of 5 peptides and showed, in Figures 2 and 4, that the CTLs killed each of the peptide-pulsed TISI cells and even HLA-A*2402-positive melanoma cell lines, though not HLA-A*0201-positive cells. There were two reasons why we used a peptide cocktail for CTL induction. First, our RT-PCR analysis using Japanese melanoma cell lines in Figure 1 revealed that all 5 melanoma antigens were expressed in more than half of the melanoma cell lines. Moreover, in a larger study, Zedman et al. (16) reported that more than 90% of 47 metastatic lesions of cutaneous melanoma expressed at least one of a total of 11 melanoma antigens, including cancer/testis antigens and melanoma differentiation antigens. These results suggested that a polyvalent immunotherapy using multiple epitopes from melanoma antigens might improve the efficacy of the treatment. Second, using a peptide cocktail for DC pulsing is much easier and efficient. In fact, some clinical trails using DCs pulsed with more than three kinds of HLA-A*0201-restricted melanoma peptides have been successful in terms of peptide-specific CTL induction (17-19). However, there is still some controversy over the efficacy of multiple epitope-based vaccinations and Smith et al. (20) demonstrated that, although polyepitope vaccines are an effective way of priming polyvalent CTLs, continual stimulation with polyepitope vaccines might restrict CTL induction as a result of immunodominance. Therefore, it would be worth trialing the peptide cocktail vaccine in melanoma patients.

Next, our finding that IFN-γ production levels by CTLs derived from the PBLs of metastatic melanoma patients were significantly lower than those derived from healthy volunteers suggested the presence of an immunosuppressive mechanism in the process of CTL induction. Two other cases showed poor CTL killing activity and low IFN-γ production in the present experiment. There have been several findings explaining the immunosuppressive state in advanced melanoma. Harada et al. (21) reported that immunoglobulin-bound transforming growth factor (TGF)-β at a late tumor-bearing stage impairs antigen-specific responses of CD4+ T cells. As to the contribution of IL-10, it was demonstrated that human DCs exposed to IL-10 at early tumor sites could induce the generation of anergic CD8+ T cells or CD4+ regulatory T cells resulting in the systemic collapse of anti-tumor immunity (22-24).

Finally, a clinical immunotherapeutic trial using DCs pulsed with a HLA-A*2402-restricted peptide cocktail is being planned at the moment. While the use of peptide cocktail vaccines is an efficient way of priming polyvalent CTLs, the immunosuppressive state has to be overcome. A clinical trial will provide important information leading to the improvement of vaccination protocols. Therefore it is worth while waiting for a positive feedback from clinic.

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References


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