**CD4+ T Cells from Peripheral Blood of a Melanoma Patient Recognize Peptides Derived from Nonmutated Tyrosinase**

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**ABSTRACT**

Tyrosinase is an antigen that is expressed by normal melanocytes as well as melanoma cells, against which responses by autologous T cells have been detected. Although CD4+ T cells play an important role in tumor immunity in animal tumor models, little information about CD4+ T-cell immunity against human tumors exists. Here, we report that CD4+ T cells from the peripheral blood of a patient with melanoma respond to synthetic peptides derived from nonmutated tyrosinase. T-cell clones were generated that recognized the tyrosinase p386–406 peptide when it was presented by the HLA-DR15 (DRB1*1501) molecule. The CD4+ T-cell clone also recognized autologous EBV-transformed B-lymphoblastoid cell lines that had been pulsed with the lysate of melanoma cells. The synthetic tyrosinase p386–406 peptide was capable of binding to HLA-DR15 (DRB1*1501) molecules on cell surface of DR15 homozygous cells. Thus, the finding that nonmutated tyrosinase peptides are immunogenic in a melanoma patient may provide the basis for the development of cancer immunotherapy, based on knowledge of synthetic tumor-associated peptide antigens.

**INTRODUCTION**

CD4+ T-cell responses play an essential role in immunologically mediated tumor regressions in most animal studies (1–4). In a recent clinical study, the in vivo persistence of adoptively transferred Ag-specific CD8+ TCCs was dependent upon an endogenous CD4 response (5). CD4+ T cells serve cooperative and effector functions in immunity against tumors, not only by inducing CD8+ tumor-specific CTLs but also by maintaining immune memory (6). Moreover, it can be observed that patients with some cancers, such as pancreatic or colon cancer, have antibodies directed against ras protein, indicating that the protein is recognized by the immune system and can be processed by the class II pathway for interaction with CD4+ helper T cells (7).

To date, the research concerning T-cell immunity against human tumors has focused mainly on CD8+ HLA class I-restricted CTL responses (8). In recent years, several melanoma-associated Ags have been identified that are recognized by CD8+ CTLs present in tumor-infiltrating lymphocytes and PBLs of melanoma patients (9–16). Among the epitopes recognized, most were wild type and derived from melanocyte lineage-specific proteins, including tyrosinase, Pmel17/gp100, and MART-1/Melan-A (11, 17–20).

Tyrosinase, a differentiation Ag that is a unique enzyme in melanin synthesis, is a normal self-protein expressed in skin, mucous membrane, and retinal and choroidal melanocytes, as well as in leptomeninges and substantia nigra (21, 22). The expression of tyrosinase has been demonstrated in >80% of melanoma samples, thus making this protein an attractive target Ag for cellular adoptive therapy (10, 23).

Knowledge of the mechanisms and specificities of CD4+ T-cell responses against human tumors and their regulation would contribute decisively to immunization strategies (24–27). However, there is little information available about CD4+ T-cell responses against human tumors. Here, we demonstrate that CD4+ T cells from PBLs of a melanoma patient recognize tyrosinase peptide and that tyrosinase-derived synthetic peptide can bind to the HLA-DR molecule.

**MATERIALS AND METHODS**

**Cells.** PBMCs from melanoma patient SA [HLA-A2; B39, 46; DR4 (DRB1*0405), DR15 (DRB1*1501)] were isolated by Ficoll-Hypaque gradient centrifugation. The melanoma cell lines, MM8 and MM33.1, were a kind gift from M. Nakashima (Kyushu University, Fukuoka, Japan). The melanoma cell line colo679 was kindly provided by Y. Matsuo (Hayashibara Biochemical Laboratories, Okayama, Japan). The melanoma cell line G361 (28) was purchased from the Japan Health Sciences Foundation (Osaka, Japan). The melanoma cell line SK-Mel28 was purchased from American Type Culture Collection (Rockville, MD).

**Synthesis of Peptides.** Forty-six 21-mer overlapping peptides, corresponding to the amino acid sequence of tyrosinase, were synthesized in an AMS 422 multiple peptide synthesizer (Abimed, Langelfeld, Germany) using the N-(9-fluorenyl)methoxycarbonyl solid-phase method. Peptides were analyzed by reversed-phase high-performance liquid chromatography and electroscopy mass spectrometry. Biotinylated peptide was purchased from Wako Pure Chemical Industries (Osaka, Japan).

**Generation of an Ag-specific TCC.** PBMCs (1.5 × 10⁷), in a volume of 1 ml of tissue culture medium (α-MEM; Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated human male AB serum, antibiotics, and 2-mercaptoethanol (2 × 10⁻³ M), were stimulated with a mixture of the 46 overlapping peptides. Each peptide was used at a final concentration of 1 μg/ml. Lymphocytes were restimulated weekly with 5 × 10⁵ autologous, mitomycin C-treated (Kyowa Hakko Kogyo, Tokyo, Japan) PBMCs, preincubated with the same peptide mixture (1 μg/ml) for at least 2 h at 37°C in conical tube (5-ml volume), in the presence of 5 units/ml IL-2, in a 24-well flat-bottomed plate. Seven days after the fifth stimulation, T-cell blasts from this bulk culture were cloned by limiting dilution as follows. Specific reactive CD4+ T cells were isolated from bulk culture with anti-CD4-coated magnetic beads (Dyna beads; Dynal, Oslo, Norway), seeded at one T-cell blast per well, and restimulated with the peptide mixture (1 μg/ml) in the presence of 5 × 10⁵ mitomycin C-treated autologous PBMC feeder cells. Growing microcultures were then transferred to a 24-well plate and stimulated at weekly intervals with mitomycin C-treated autologous PBMCs (5 × 10⁵well) pulsed with peptide (1 μg/ml) in medium containing 20 units/ml IL-2.

**T-Cell Proliferation Assay.** Mitomycin C-treated autologous EBV-BLCLs were used as APCs for peptide and protein Ag (29). EBV-BLCL (2 × 10⁶ cells/well in a 96-well plate) were incubated for 2 h at 37°C in the presence of peptides (20 μM) or freeze-thaw lysates of melanoma cells before T cells were added at 2 × 10⁵ cells/well for 72 h. Cultures were pulsed with 1 μCi/well of [³H]thymidine for the last 16 h, and incorporated radioactivity was measured by liquid scintillation counting. Blocking of the proliferative responses was investigated by adding anti-HLA-A,-B, or -C, mAb W6/32, or anti-HLA-DR mAb L243 or anti-HLA-DQ mAbs SPV13 throughout the culture period. All assessment of proliferations were carried out at least in triplicate. The S.I. was calculated by dividing the mean radioactivity (cpm) obtained from autologous EBV-BLCL presentation of each Ag by the mean radioactivity (cpm) from autologous EBV-BLCLs incubated with no Ag.

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3 The abbreviations used are: Ag, antigen; TCC, T-cell clone; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; IL, interleukin; EBV-BLCL, EBV-transformed B-lymphoblastoid cell line; APC, Ag-presenting cell; mAb, monoclonal antibody; S.I., stimulation index.
RESULTS

Generation of Bulk-cultured T-Cell Lines and TCC Responses against Synthetic Tyrosinase Peptides. We first incubated PBMCs from melanoma patient SA in a primary culture with peptide pools containing 46 overlapping synthetic peptides, corresponding to the tyrosinase amino acid sequence. The primary stimulated PBMCs were repeatedly (five times) stimulated with tyrosinase-derived peptide mixtures and freshly prepared mitomycin C-treated PBMCs. After 7 days of rest, they were examined for their ability to proliferate in response to specific Ag. They proliferated in response to peptide mixtures containing the 46 overlapping peptides and were, at this point, designated as S1 (Fig. 1). The S1 cells were further tested for their reactivity to individual peptides. As shown in Fig. 2, S1 had positive responses to four tyrosinase peptides (tyrosinase p386–406, p408–428, p452–472, and p485–505), with S.I.s of >3. To determine

Analysis of Cytokine Production. TCC (1 × 10⁶ cells/well) was cultured in a 24-well flat-bottomed plate with mitomycin C-treated autologous EBV-BLCLs (3 × 10⁶ cells/well) in the presence of tyrosinase p386–406 peptide (10 μg/ml) or freeze-thaw lysates of melanoma cells (MM8, MM33.1, and SK-Mel28) in a final volume of 1 ml for 48 h, and IL-2, IL-4, and IFN-γ levels in the supernatants were evaluated using commercial solid-phase immunoenzymatic assay (SRL Inc., Tokyo, Japan).

**Peptide-binding Assay.** Aliquots of 100 μl of biotinylated peptide (100 μM final concentration) were added to EBV-BLCLs (3 × 10⁶) in 100 μl of RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% FCS and incubated at 37°C for 16 h (30). Similarly, biotinylated mAb L243 (5 ng) was added and incubated to estimate the expression of HLA-DR molecules. After further incubation with 50 ng of phycoerythrin-streptavidin (Becton Dickinson, Mountain View, CA) for 30 min, specific binding was measured by flow cytometry. Each step was followed by two to five washes at 4°C with PBS containing 0.1% BSA and 0.1% sodium azide.
The fine specificity of the responding T cells, we cloned the activated T cells present in the culture that were responding to tyrosinase-derived peptide mixtures. One TCC, S11 (CD4+, CD8+, CD3+, and HLA-DR+; data not shown), showed specificity for the tyrosinase p386–406 (Phe-Leu-Leu-His-His-Ala-Phe-Val-Asp-Ser-Ile-Phe-Glu-Gln-Trp-Leu-Gln-Arg-His-Arg-Pro; Fig. 3). When this peptide was added to TCC S11 cultures at concentrations ranging from 3.7 to 80 μM, a dose-dependent response could be observed (Fig. 4).

**Ag-presenting HLA Molecules Recognized by TCC S11.** Peptide-induced proliferation of the CD4+ TCC S11 was inhibited by anti-HLA-DR mAb (L243) but not by anti-HLA class I mAb (W6/32) and anti-HLA-DQ mAb (SPV3L3), indicating that TCC S11 recognized the tyrosinase p386–406 peptide presented by the HLA-DR molecule of the melanoma patient SA (Fig. 5). Patient SA is HLA-DR15 (DRB1*1501) restricted CD4+ TCC that recognizes nonmutated tyrosinase p386–406 peptide or lysate mixture from MM8, MM33.1, and SK-Mel28. After 48 h, the supernatants were examined for cytokines, including IL-2, IL-4, and IFN-γ, by ELISA (Table 1). CD4+ TCC S11 secretes IL-2 and IFN-γ, but IL-4 is found in low or undetectable amounts. These results suggest that CD4+ TCC S11 show a Th1-like cytokine profile.

**DISCUSSION**

Tyrosinase is one of the melanocyte lineage-specific proteins that, including its signal sequence, contains 529 amino acids. Identifying the epitopes, recognized by both CD4+ and CD8+ T cells, constituting tumor-rejection Ags, in patients with melanoma may help in the design of a tumor vaccine for immunotherapy. Here, we isolated a HLA-DR15 (DRB1*1501)-restricted CD4+ TCC that recognizes nonmutated tyrosinase p386–406 peptide from the peripheral blood of a patient with melanoma by repeated stimulation of PBLs in vitro with synthetic peptides and have demonstrated that tyrosinase-specific T cells can be isolated from the peripheral blood of a patient with melanoma, despite the fact that tyrosinase is a normal self-protein.

To isolate a tyrosinase-specific T-cell line or TCC, we used peripheral blood as a source for both responder T cells and APCs under conditions involving Ag stimulation by synthetic peptide mixtures, followed by a period of IL-2-induced growth. The relatively low dose of 

- Table 1: Cytokine Profile of CD4+ TCC S11

<table>
<thead>
<tr>
<th>Peptide</th>
<th>mAbs</th>
<th>[3H]-thymidine incorporation (cpm × 10^-3)</th>
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<tbody>
<tr>
<td>p386-406</td>
<td>anti-HLA-class I</td>
<td>20</td>
</tr>
<tr>
<td>p386-406</td>
<td>anti-HLA-DR</td>
<td>20</td>
</tr>
<tr>
<td>p386-406</td>
<td>anti-HLA-DQ</td>
<td>20</td>
</tr>
</tbody>
</table>
of IL-2 used (5 units/ml) before cloning and repeated weekly stimulation (five times) with synthetic peptides may help to reduce the number of nonspecific killer T cells that respond to EBV Ags or abnormally expressed autoantigens during bulk culture, suggesting that effective cloning of tyrosinase peptide-specific T cells by limiting dilution can be performed. Tyrosinase-specific bulk-cultured T-cell lines and TCC S11 have been maintained for up to 5 months in culture while retaining Ag specificity.

Extensive studies in animal models have shown that T cells specific for malignant cells can cure advanced malignancy and that the generation and persistence of a CTL response is dependent on the influence of the presence of a CD4+ helper T-cell response (5, 6, 31). Walter and coworkers (5) showed that CD8+ cytomegalovirus reactivity could be reconstituted following adoptive transfer of T cells but that the long-term in vitro persistence (>12 weeks) of these transferred CTL clones was dependent on the development of an endogenous CMV-specific CD4+ T-helper response. The same group demonstrated that tyrosinase-specific CD4+ TCCs were isolated from five of six patients with melanoma and tyrosinase-specific CD8+ CTL clones could not be isolated from those individuals whose peripheral blood did not mount a CD4+ T-cell response following in vitro stimulation with recombinant vaccinia virus expressing tyrosinase (32). Thus, with its capacity to generate both CD4+ and CD8+ T-cell responses, tyrosinase may provide optimal immunization against melanoma if used as a whole-protein or multivalent peptide vaccine.

MHC class II molecules, in principle, present antigenic peptides derived from the extracellular milieu, whereas MHC class I molecules usually present peptides derived from endogenous cellular compartments. However, it has been recently shown that the MHC class II pathway can present endogenous cellular peptides and that endogenous peptides may be potential dominant Ags presented by MHC class II molecules (33–37). The transmembrane proteins tyrosinase, gp75, and gp100 are located within melanosomes. Melanosomes are believed to belong to the lysosomal lineage of organelles, by virtue of expressing LAMP-1 (38). It seems that the melanosomal proteins, tyrosinase, and the other members of the pigment-related proteins degrade into peptides. The immunogenicity of melanosomal proteins may stem from their ability to directly bind MHC class II molecules transported to the melanosomal/lysosomal compartment, thus generating a CD4+ T-cell response that ultimately leads to CD8+ T-cell immunity. Human melanomas commonly express MHC class II molecules on their cell surface, and the relative abundance of the pigment-related proteins in melanoma cells might favor their presentation by MHC class II molecules. Alternatively, one may speculate that some melanoma cells fall into necrosis, are engulfed into endosomes, usually by phagocytic cells such as macrophages, and enter intracellular vesicles and that MHC class II molecules deliver peptides derived from pigment-related proteins to the cell surface, where they are recognized by CD4+ T cells. The isolated TCC S11 was able to recognize both synthetic tyrosinase-derived peptides and melanoma-associated Ags that are naturally processed Ags derived from lysates of allogeneic melanoma cells, suggesting that TCC S11 may respond to live melanoma cells. The five melanoma cell lines have equal tyrosinase activity, and therefore, the incomplete ability of G361 lysate to stimulate TCC S11 may be explained by the mutation in the region of p386–406 of G361 tyrosinase, although we have not known the amino acid sequence of G361 tyrosinase. At this moment the precise mechanism of difference among the five is not clear.

Here, we have identified tyrosinase-derived peptide p386–406, which is specifically recognized by a CD4+ TCC from the peripheral blood of a patient with melanoma. This peptide was presented by the HLA-DR15 (DRB1*1501) molecule. Stimulation of the CD4+ TCC by the peptide or protein Ag resulted in the significant production of IL-2 and IFN-γ, whereas low or undetectable production of IL-4 was

Fig. 6. Presentation of synthesized tyrosinase p386–406 peptide by HLA-DR15 (DRB1*1501) molecule. The homozygous DR4 (DRB1*0405) and DR15 (DRB1*1501) EBV-BLCLs were used as APCs for the tyrosinase p386–406 peptide-specific TCC. S11. T cells (2 × 10⁵) were cultured in triplicate with peptide (20 μg)-pulsed EBV-BLCLs (2 × 10⁵) in a 96-well plate for 72 h. Proliferation was determined by [³H]-thymidine incorporation. Columns, S.I.s (cpm, with Ag/ cpm in the absence of Ag).

<table>
<thead>
<tr>
<th>APC</th>
<th>HLA classII</th>
<th>Stimulation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous EBV-BLCL</td>
<td>DR4/15</td>
<td></td>
</tr>
<tr>
<td>EBV-BLCL DR4</td>
<td>DR4/4</td>
<td></td>
</tr>
<tr>
<td>EBV-BLCL DR15/15</td>
<td></td>
<td></td>
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Fig. 7. Reactivity of tyrosinase p386–406 peptide-specific TCC S11 with allogeneic melanoma cell lysates. T cells (2 × 10⁵) were cultured in triplicate in the presence of autologous EBV-BLCLs (2 × 10⁵) pulsed with freeze-thaw lysates of allogeneic melanoma cells in a 96-well plate for 72 h. Columns, proliferations determined by [³H]-thymidine incorporation (cpm).
Table I  Cytokine profile of the tyrosinase p386–406-specific TCC, SI

<table>
<thead>
<tr>
<th>Ag</th>
<th>IL-2 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td>Tyrosinase p386–406</td>
<td>114</td>
<td>&lt;2.0</td>
<td>1360</td>
</tr>
<tr>
<td>Lysate mixture (MM8, MM33.1, and SK-Mel28)</td>
<td>114</td>
<td>2.4</td>
<td>1580</td>
</tr>
</tbody>
</table>

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REFERENCES


observed, suggesting that TCC belonged to Th1-type T-cell. CD4+ T cells that secrete Th1 cytokines are capable of enhancing the cytolytic activity of natural killer cells and monocytes and macrophages. Thus, tyrosinase-reactive CD4+ T cells may have an important role for tumor rejection in melanoma individuals. These results have significant implications because the identification of normal self-proteins as immunogenic proteins is becoming an increasingly common finding in tumor immunotherapy.
T-CELL IMMUNITY TO NONMUTATED TYROSINASE PEPTIDE


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