Identification of Cyclophilin B-derived Peptides Capable of Inducing Histocompatibility Leukocyte Antigen-A2-restricted and Tumor-specific Cytotoxic T Lymphocytes

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We recently suggested that cyclophilin B (Cyp-B) is a tumor antigen recognized by histocompatibility leukocyte antigen (HLA)-A24-restricted and tumor-specific cytotoxic T lymphocytes (CTLs). In this study, we tried to identify Cyp-B-derived epitopes, which can induce HLA-A2-restricted and tumor-specific CTLs in cancer patients. The tumor-infiltrating lymphocytes (TILs) from an HLA-A0207 patient with colon cancer were found to respond to COS-7 cells when co-transfected with the Cyp-B gene and either HLA-A0201, -A0206, or -A0207 cDNA. These TILs contained CTLs capable of recognizing either the Cyp-B129–138 or the Cyp-B172–179 peptide among 28 different peptides, all of which were prepared based on the HLA-A2 binding motif. Both Cyp-B peptides possessed the ability to induce tumor-specific CTLs in HLA-A2+ cancer patients. Cyp-B172–180 (V), which is a 9-mer peptide with valine added at the C terminus, showed no clear superiority over the parental Cyp-B172–179 peptide in an in vitro sensitization experiment. In vitro-sensitized T cells with these peptides responded to cancer cells in an HLA-A2-restricted manner. These two Cyp-B peptides could be useful for specific immunotherapy of HLA-A2+ cancer patients.

Key words: Cyclophilin B — Cytotoxic T lymphocytes — Peptide — HLA-A2

Many peptide antigens capable of inducing histocompatibility leukocyte antigen (HLA) class I-restricted cytotoxic T lymphocytes (CTLs) have been identified from melanoma cDNA. Specific immunotherapy utilizing these peptides has been under investigation for treatment of HLA-A1+ or -A2+ metastatic melanoma patients, and has resulted in partial clinical responses in the initial trials. However, there are few peptides useful for specific immunotherapy for patients with adenocarcinoma or squamous cell carcinomas, histologically the two major malignancies in the world. We have found several genes coding antigenic peptides of these cancers capable of inducing HLA class I-restricted CTLs in the peripheral blood mononuclear cells (PBMCs). One such antigen, cyclophilin B (Cyp-B), was identified using HLA-A24-restricted and tumor-specific CTLs established from a patient with lung adenocarcinoma. Although the HLA-A24 allele is most commonly expressed in Japanese (60%), HLA-A2 is also expressed in Japanese at a relatively high frequency (40%), and appears in other ethnic populations (e.g., with an estimated frequency of 50% in Caucasians). In this study, we investigated whether the Cyp-B antigen possesses antigenic peptides available for HLA-A2+ cancer patients as cancer vaccines, and we present evidence that the Cyp-B antigen contains immunogenic epitopes capable of inducing HLA-A2-restricted and tumor-specific response in HLA-A2+ cancer patients.

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MATERIALS AND METHODS

Generation of HLA-A2-restricted CTLs The HLA-A2-restricted and tumor-specific CTL (OK-CTL) lines were established from tumor-infiltrating lymphocytes (TILs) of a patient (HLA-A0207/31, -B46/51, -Cw1) with colon adenocarcinoma by incubation with interleukin-2 (IL-2, 100 U/ml) alone for more than 50 days, by the method reported previously. The anti-tumor specificity of these CTL lines was previously reported.

Typing of HLA-class I alleles Genotypes of HLA-class I alleles of the tumor cells were reported previously. HLA-class I alleles of PBMCs were serotyped by the conventional method, whereas HLA-A2 subtypes were determined by a sequence-specific oligonucleotide probe method and direct DNA sequencing, as previously reported.

In vitro transfection To determine whether the Cyp-B gene encodes antigenic epitopes recognized by the OK-CTLs, both the Cyp-B gene and one of the HLA-class I cDNAs (HLA-A0201, -A0206, -A0207, -A2402, or -A2601) were co-transfected into COS-7 cells followed by a test of their activity to stimulate interferon (IFN)-γ production by the OK-CTLs by methods reported previously.

IFN-γ ELISA To determine the level of IFN-γ, both anti-human IFN-γ coating monoclonal antibody (mAb) and anti-human IFN-γ rabbit polyclonal antibody, which was prepared in our laboratory and used as a detecting antibody, were used. Thereafter, DAKO EnVision™ Peroxidase-conjugated anti-rabbit antibody (DAKO Corp., Carpinteria, CA) was used. The development was carried...
out using the TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD). After stopping the enzyme reaction with \(1 M\) \(H_3PO_4\), absorbance at 450 nm was determined with an ELISA spectrophotometer.

**Peptides** The Cyp B-derived peptides capable of binding to the HLA-A2 molecules were determined based on the HLA-A2-binding motifs,\(^{15, 16}\) and 28 different peptides were synthesized. The peptides (>95% purity) were kindly provided by Dr. Takasu (Research Division of Sumitomo Pharmaceutical Co., Osaka). To determine which Cyp-B peptides could be recognized by the OK-CTLs, prepared peptides were loaded onto T2 cells at a concentration of \(10 \mu M\) for 2 h. Thereafter, the culture supernatants were harvested and the level of IFN-\(\gamma\) was determined by ELISA.

**CTL induction by the peptides** For induction of tumor-specific CTLs by the peptides, PBMCs from 5 HLA-A2\(^+\) patients with adenocarcinoma were incubated with a peptide (10 \(\mu M\)). These PBMCs were re-stimulated at days 7, 14, and 21 with irradiated (30 Gray) autologous PBMCs, as antigen-presenting cells, that had been pulsed with the same peptide at the same dose for 2 h. These cultured cells at day 27 were tested for their tumor-specific IFN-\(\gamma\) production and cytotoxicity by a standard 6 h \(^{51}\)Cr-release assay. The assay of cytotoxicity was performed, as previously reported.\(^6\) The surface phenotypes of the cultured cells were determined by indirect staining with anti-CD8 mAb, followed by staining with FITC-conjugated goat anti-mouse IgG antibody. For inhibition of IFN-\(\gamma\) production by the cultured cells, 10 \(\mu g/ml\) of anti-class I (W6/32, mIgG2a), anti-class II (H-DR1, mIgG2a), anti-CD4 (Nu-Th/I, mIgG1), anti-CD8 (Nu-Ts/c, mIgG2a), anti-A2 (BB7.2, mIgG2b), and anti-A23/24 (DU41HA, mIgG2a) as anti-HLA-A24 mAb antibodies were used as reported previously.\(^5\)

**RESULTS**

**Recognition of the Cyp-B-derived antigen by HLA-A2-restricted OK-CTLs** Several HLA-A2-restricted and tumor-specific CTL lines were established from the TILs of a patient (HLA-A0207/31, -B46/51, -Cw1) with colon adenocarcinoma by incubation with 100 U/ml IL-2. These CTL lines showed a response to several types of cancer cells expressing HLA-A2 molecules, as previously reported.\(^13\) One (OK-CTL) of these CTL lines was utilized to determine which Cyp-B peptides could induce HLA-A2-restricted and tumor-specific CTLs in cancer patients. As shown in Fig. 1A, the OK-CTL cells produced a higher level of IFN-\(\gamma\) when the COS-7 cells were co-transfected with both Cyp-B and HLA-A0207 cDNAs than when co-transfected with both control cDNA and HLA-A0207 cDNA. The OK-CTL cells failed to produce IFN-\(\gamma\) when the COS-7 cells were co-transfected with both Cyp-B and HLA-A2402 cDNAs. These results indicate that the OK-CTL line contains T cells recognizing Cyp-B-derived antigens in an HLA-A0207-restricted manner.

HLA-A2 subtypes share similar binding motifs.\(^12, 18\) For example, HLA-A0201 and -A0207 have a dominant anchor leucine at position 2, and HLA-A0201 shares dominant and strong anchors leucine and valine at position 9 with -A0206 and -A0207, respectively. In addition, these OK-CTL lines possess HLA-A2-restricted and tumor-specific CTL activity toward antigenic epitope(s) expressed on HLA-A0201, -A0206, -A0207 molecules of epithelial cancer cells.\(^13\) Therefore, we next determined whether the OK-CTL line could recognize Cyp-B-derived antigens in association with HLA-A0201, -A0206, or -A0207 molecules. Fig. 1B shows that the OK-CTL line responded to the COS-7 cells when transfected with Cyp-B cDNA in
Next, the 28 Cyp-B peptide candidates were prepared, based on the HLA-A2-binding motif, and we investigated whether these peptides could be recognized by the OK-CTLs (Fig. 2). Although 8-mer and 11-mer peptides were not common as peptides binding to class I molecules and it was impossible to determine the score (an estimated half-time of dissociation of the peptide binding to HLA-A2 molecules) by computer search, these were also included. Among them, two Cyp-B peptides at positions 129–138 and 172–179 were found to be recognized by the OK-CTLs. The score of the Cyp-B129–138 peptide was 6.1 (low), but that of the Cyp-B172–179 peptide was undetermined (it was an 8-mer peptide). Because the Cyp-B172–179 peptide was an 8-mer and because the optimal C-terminal anchor residue for HLA-A2 binding peptides has been shown to be valine, a modified Cyp-B peptide, which had valine added at position 180 and was designated as Cyp-B172–180 (V), was prepared. Although the score of the modified peptide was 3.1 (low), this peptide was also tested in the following experiments.

**Induction of tumor-specific CTLs by peptides** These Cyp-B peptides were examined for the ability to induce tumor-specific CTLs from 5 cancer patients (Table I). The PBMCs of patients #1 and #2 were repeatedly stimulated with the Cyp-B129–138, Cyp-B172–179, and Cyp-B172–180 (V) peptides, respectively, and the in vitro-sensitized PBMCs were examined for IFN-γ production in response to HLA-A2+ or HLA-A2− tumor cell lines. Sensitized cells stimulated with any of the three peptides produced a higher level of IFN-γ in response to the HLA-A2+ tumor cell lines (Panc-1, SW620 and CA9-22) than to the HLA-A2− tumor cell lines (QG-56, RERF-LC-MS and colo-320). PBMCs of patients #3, from whom the OK-CTLs were established, and patients #4 and #5 were repeatedly stimulated with either the Cyp-B129–138 or Cyp-B172–179 peptide, respectively, and these sensitized PBMCs were also examined for IFN-γ production. In these cases, although the difference was not as apparent as that in the cases of patients #1 and #2, the sensitized cells also produced a higher level of IFN-γ in response to the HLA-A2+ tumor cell lines than to the HLA-A2− tumor cell lines. These results suggest that these three Cyp-B peptides could be useful for specific immunotherapy.

Next, the cytotoxicity of these in vitro-sensitized PBMCs was examined (Fig. 3). In patient #1, the in vitro-sensitized PBMCs with the Cyp-B172–179 peptide showed a higher cytotoxicity to HLA-A2+ tumor cell lines (Panc-1 and 1-87) than to HLA-A2− tumor cell lines (QG-56 and RERF-LC-MS). However, no difference in cytotoxicity was observed when PBMCs were in vitro-sensitized with either the Cyp-B129–138 or the Cyp-B172–180 (V) peptide (data not shown). On the other hand, in patient #3, the in vitro-sensitized PBMCs with the Cyp-B129–138 peptide showed a higher cytotoxicity to HLA-A2+ SW620 than to other
tumor cell lines. In patient #4, the *in vitro*-sensitized PBMCs with the Cyp-B_{129–138} peptide showed a higher cytotoxicity to HLA-A2+ tumor cell lines (Panc-1 and SW620) than to HLA-A2- tumor cell lines (QG-56 and RERF-LC-MS). However, no difference in cytotoxicity was observed when PBMCs from patients #3 and #4 were *in vitro*-sensitized with either the Cyp-B_{172–179} or the Cyp-B_{172–180 (V)} peptide, respectively (data not shown). Overall, although peptides to induce tumor-reactive CTLs with cytotoxicity varied among cancer patients, these results suggest that both Cyp-B_{129–138} and Cyp-B_{172–179} peptides appear to be effective for specific immunotherapy of HLA-A2+ cancer patients, and that the Cyp-B_{172–180 (V)} peptide was not necessarily superior to the parental Cyp-B_{172–179} peptide.

**HLA-A2 restriction of the peptide-induced CTLs**

Finally, we tried to confirm HLA-A2 restriction of the CTLs that had been *in vitro*-sensitized by the Cyp-B_{129–138} and the Cyp-B_{172–179} peptides. PBMCs from patients #5 and #3 were repeatedly stimulated with these peptides. As

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**Table I. Induction of HLA-A2-restricted CTLs by *in vitro* Sensitization with Cyp-B Peptides**

| Donors   | Disease/stage | Peptides       | CD8 (%) | Stimulator cells          |
|----------|---------------|----------------|---------|---------------------------|
|          |               | Cyp-B_{129–138}| 45.8    | Panc-1 (A0201/1101)       |
| Patient #1 | Lung cancer/T4N2M0 |               |         | 122                       |
|           | (adenocarcinoma)| Cyp-B_{172–179}| 64.5    | SW-620 (A0201/2402)       |
|           |               | Cyp-B_{172–180 (V)} | 70.5    | CA9-22 (A0207/2402)       |
| Patient #2 | Lung cancer/T4N2M0 | Cyp-B_{172–179}| 33.8    | QG-56 (A2601/)            |
|           | (adenocarcinoma)|               |         | RERF-LC-MS (A1101/)       |
|           |               | Cyp-B_{172–180 (V)} | 48.9    | colo-320 (A2402/)         |
| Patient #3 | Colon cancer/T2N0M0 | Cyp-B_{129–138}| ND      | Panc-1 (A0201/1101)       |
|           | (adenocarcinoma)| Cyp-B_{172–179}| ND      | SW-620 (A0201/2402)       |
|           |               | Cyp-B_{172–180 (V)} | ND      | CA9-22 (A0207/2402)       |
| Patient #4 | Gastric cancer/T2N1M0 | Cyp-B_{129–138}| ND      | QG-56 (A2601/)            |
|           | (adenocarcinoma)| Cyp-B_{172–179}| ND      | RERF-LC-MS (A1101/)       |
| Patient #5 | Gastric cancer/T2N1M0 | Cyp-B_{129–138}| ND      | colo-320 (A2402/)         |
|           | (adenocarcinoma)| Cyp-B_{172–179}| ND      | Panc-1 (A0201/1101)       |
|           |               | Cyp-B_{172–180 (V)} | ND      | SW-620 (A0201/2402)       |
|           |               | Cyp-B_{172–180 (V)} | ND      | CA9-22 (A0207/2402)       |

PBMCs from five HLA-A2+ patients with adenocarcinoma were stimulated 4 times with the indicated peptides, as described in “Materials and Methods.” On day 27, the cultured cells were harvested and cultured with the indicated tumor cell lines at an E/T ratio of 5/1 for 20 h. The level of IFN-γ in the culture supernatant was determined by ELISA. Values represent the means of triplicate determinations. The background production of IFN-γ by the effector cell alone was subtracted from the experimental values. Percentage of CD8+ T cells in the cultured cells was examined by flow cytometry. These 5 cases were classified according to the TNM classification of UICC. ND: not determined.

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**Fig. 3. Induction of CTLs by the peptides.** PBMCs from 3 cancer patients were repeatedly stimulated *in vitro* with the Cyp-B_{129–138}, CypB_{172–179}, and Cyp-B_{172–180 (V)} peptides, respectively, as described in “Materials and Methods.” Therafter, their respective cytotoxic activity against HLA-A2+ and HLA-A2- tumor cell lines was determined by a 6 h 51Cr-release assay. Values represent the means of triplicate determinations. The following target cell lines were used: (A) ○ Panc-1 (A0201/1101), △ 1-87 (A0207/1101), ● QG-56 (A2601/), and ▲ RERF-LC-MS (A1101/). (B and C) ○ Panc-1 (A0201/1101), △ SW620 (A0201/2402), ● QG-56 (A2601/), and ▲ RERF-LC-MS (A1101/).
In this study, we identified two Cyp-B-derived peptides capable of inducing HLA-A2-restricted and tumor-specific CTLs in cancer patients. In screening by IFN-γ, both Cyp-B peptides possessed the ability to induce HLA-A2-restricted and tumor-reactive CTLs in all 5 HLA-A2+ cancer patients (Table I), although not all peptide-induced CTLs showed cytotoxicity against HLA-A2-expressing tumor cells, as shown in Fig. 3. These results suggest that tumor-specific CTLs, which could recognize Cyp-B peptides in association with HLA-A2 molecules and subsequently produce IFN-γ, could not necessarily lyse the tumor cells. Each tumor cell line might have a unique machinery by which to show resistance to CTL-mediated cytolysis. Alternatively, cytokine production by CTLs might not necessarily be associated with their cytolysis activity. We can not explain the discrepancy. In addition, in the cytotoxicity assay, the ability to generate T cells with cytotoxicity against HLA-A2-expressing tumor cells varied among the peptides. These findings may imply that CTL precursor frequency varies among cancer patients, and that confirmation of the presence of CTL precursors prior to immunotherapy would greatly improve the efficacy of a subsequent vaccination with the relevant peptides.

There are several major subtypes of HLA-A2 alleles. The frequencies of HLA-A0201, -A0206, and -A0207 among HLA-A2+ Japanese are about 45%, 36%, and 17%, respectively, whereas HLA-A0201 is the predominant subtype among HLA-A2+ Western Caucasians (96%), African Blacks (62%), and Sardinian Caucasians (56%). Interestingly, the OK-CTLs (HLA-A0207) not only recognized the two Cyp-B peptides presented on T2 cells (HLA-A0201), but also responded to HLA-A0201-expressing tumor cell lines. In this study, the in vitro-sensitized PBMCs from HLA-A0206 or -A0207 cancer patients showed a response to HLA-A0201-expressing tumor cell lines. These results suggest that the Cyp-B129-138 and Cyp-B172-179 peptides would be appropriate for use in specific immunotherapy of a vast majority of cancer patients with different HLA-A2 subtypes.

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