Particulate Naturally Processed Peptides Prime a Cytotoxic Response against Human Melanoma in Vitro

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Abstract

For an efficient antitumor cytotoxic response, tumor antigenic peptides need to be presented by professional antigen-presenting cells in association with MHC class I molecules. We established in vitro short-term human CTL lines from healthy and melanoma-bearing subjects, using as antigen-presenting cells autologous adherent cells after phagocytosis of latex beads coated with melanoma peptides. Melanoma peptides were obtained by acid extraction of melanoma cells that matched with donor peripheral blood mononuclear cells, at least for one HLA-A allele. The cytotoxic activity of the lines was specific for the melanoma from which peptides were obtained and for melanoma sharing HLA alleles. These results demonstrate that a complex mixture of naturally processed melanoma peptides conjugated to a phagocytic substrate that targets them into the MHC class I pathway of adherent cells can prime a CTL response in healthy subjects in vitro, and that peptides from allogeneic tumors may be used to propagate CTL in melanoma patients. Our data support the feasibility of active and passive vaccination procedures with nonliving vaccines in cancer patients.

Introduction

Many Ags from human cancer cells have been described that are able to stimulate a CTL response in vitro (reviewed in Refs. 1 and 2); however, tumor rejection seldom occurs in vivo. Precursor CTLs are activated when they recognize short peptides (9–10 mer) in the context of MHC class I molecules, in the presence of costimulatory molecules. We established in vitro short-term CTL lines from healthy and melanoma-bearing subjects, using as antigen-presenting cells autologous adherent cells after phagocytosis of latex beads coated with melanoma peptides. Melanoma peptides were obtained by acid extraction of melanoma cells that matched with donor peripheral blood mononuclear cells, at least for one HLA-A allele. The cytotoxic activity of the lines was specific for the melanoma from which peptides were obtained and for melanoma sharing HLA alleles. These results demonstrate that a complex mixture of naturally processed melanoma peptides conjugated to a phagocytic substrate that targets them into the MHC class I pathway of adherent cells can prime a CTL response in healthy subjects in vitro, and that peptides from allogeneic tumors may be used to propagate CTL in melanoma patients. Our data support the feasibility of active and passive vaccination procedures with nonliving vaccines in cancer patients.

Materials and Methods

Subjects and Cell Lines. PBMCs were obtained from a healthy subject (C. F.) and a melanoma patient (G. F.). Melanoma cell lines were purchased from the American Type Culture Collection; mAb, monoclonal antibody; HLA, human leukocyte antigen; TCM, tissue culture medium.

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Previous studies by others (5, 6) and by us (7) have demonstrated that the acid peptide fraction extracted from tumor cells is immunogenic in murine systems. This peptide pool should include all naturally processed peptides bound to MHC class I molecules at the cell surface (8) as well as larger (Mr, >5000) peptides (8, 9).

MHC class I molecules present peptides derived primarily from endogenous proteins processed into peptides in the cytosol. The peptides are transported into the endoplasmic reticulum to bind nascent MHC class I molecules (10). Different systems have been used to generate human CTL in vitro. Most of them are based on the presence of "empty" class I molecules on the APC surface (reviewed in Refs. 1 and 2). However, only a small fraction of the total class I molecules on the cell surface are empty (11), and therefore, large amounts of high-affinity exogenous peptides are needed to obtain an efficient peptide-class I binding that is able to trigger a CTL response.

It has been suggested that macrophages may initiate CTL responses (12, 13), i.e., may function as professional APCs. This function may be related to the phagocytic activity of macrophages. An alternative phagocytic pathway for delivery of peptides to MHC class I molecules has been suggested recently (14–18). In those studies, murine macrophages, upon phagocytosis of particulate exogenous Ags, even at low (nanomolar) peptide concentrations, could present peptides in association with MHC class I molecules and prime a CTL response in vitro and in vivo (14–19).

We describe here the in vitro priming of human CTL able to recognize melanoma cells from healthy and melanoma-bearing subjects. The CTLs were obtained using autologous adherent cells as APCs after phagocytosis by these cells of latex beads coated with peptides obtained by acid extraction of melanoma cells matched with donor PBMC for at least one HLA-A allele.

1The abbreviations used are: Ag, antigen; APC, Ag-presenting cell; PHAB, phytohemagglutinin blast; PBMC, peripheral blood mononuclear cell; HPLC, high-performance liquid chromatography; RaMlg, conjugated Fab2 rabbit antimouse immunoglobulin; ATCC, American Type Culture Collection; mAb, monoclonal antibody; HLA, human leukocyte antigen; TCM, tissue culture medium.
according to the manufacturer's instructions (Polyscience, Trinitital s.r.l., Milan, Italy). The amount of peptides bound to the beads was determined on the basis of the difference in absorbance (210 nm) between the solution of peptides before and after the adsorption; in different preparations, it was 70–190 µg/100 beads/500 µl for HT144p and 85–140 µg/100 beads/500 µl for SK-Mel 5p.

**HPLC Analysis.** Melanoma peptides were analyzed on an ODS-RP 18 (150 x 4.6 mm; 5 µm) column (Beckman, Palo Alto, CA) with a mobile phase. Eluent A was 0.1% trifluoroacetic acid, and eluent B was 0.1% trifluoroacetic acid in CH₃CN (80%) at a flow rate of 0.5 ml/min, performing a gradient from 10 to 100% eluent B in 95 min. The sample was injected in the HPLC, and the presence of protein in the eluate was monitored at 210 nm.

**Transfection of RMA-Shβ2A1.** RMA-S cells were transfected sequentially with 10 µg of human β₂-microglobulin cDNA (a kind gift of Dr. Agresti, Dipartimento Biotecnologie) subcloned into the pREP-7 hygro vector (Invitrogen, San Diego, CA) and 10 µg pRVS-5-Neo vector containing the whole HLA-A1 cDNA (a kind gift of Dr. Malnati, Dipartimento Biotecnologie). Cells were electroporated at 260 V, 960 µF using a Bio-Rad apparatus (Richmond, CA). Transfectants resistant to hygromycin (Boehringer Mannheim, Mannheim, Germany) were sorted on FACStarPlus for surface expression of human β₂-microglobulin using the mAb BBM1 (purified from a hybridoma purchased from ATCC) and FITC-RaMg antiserum (DAKO A/S, Glostrup, Denmark). To select for HLA-A1 surface-positive RMA-S cells, double-transfected cells were incubated overnight at 25°C, stained with the anti-HLA-A1 mAb 131 (kindly donated by Dr. A. Moretta, University of Genoa, Genoa, Italy), followed by FITC-RaMg antiserum, and sorted as above.

**HT144p Binding to RMA-Shβ2A1.** Transfected RMA-Shβ2A1 cells were incubated overnight at 25°C to induce expression of empty HLA-A1 on the cell surface and then stained with mAb 131, followed by FITC-RaMg antiserum, and analyzed by flow cytometry using a FACStarPlus. Different binding conditions were tested: cells alone; cells in the presence of 125 µg/ml of p2 (a synthetic peptide of MAGE 3; sequence: EVDPIGHLY; Primm s.r.l., Milan, Italy); cells in the presence of 125 µg/ml of p1 (a synthetic peptide of HIV; sequence: ILKEPVHGV; Primm s.r.l.); cells in the presence of 500 µg/ml of HT144p; and cells in the presence of 500 µg/ml of SK-Mel5p.

**Propagation of Short-Term CTL Lines.** PBMCs were seeded at 4 x 10⁶/ml in a 24-well plate in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated pooled human serum (Technogenetics, Milan, Italy), 2 mM l-glutamine, 100 units/ml penicillin, and 50 mg/ml streptomycin (TCM; Biowittaker, Walkersville, MD) for 1.5 h. Nonadherent cells were removed and used to purify CD8⁺ cells using microbeads coated with anti-CD8 mAb and a miniMACS magnetic cell sorter (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany). Adherent cells, to be used as APCs, were incubated in TCM with the HT144p-coated latex beads (10 µl) for 4 h. CD8⁺ cells were added to the APCs and cultured in TCM for 1 week. At day 5, recombinant interleukin 2 (Euro-Cetus, Amsterdam, the Netherlands) was added at a final concentration of 20 IU/ml. The cells were restimulated at weekly intervals for 3–4 weeks with autologous APCs obtained as described above. Phytomemagglutinin blasts were obtained from CD8⁺-depleted PBMC by stimulation with phytomemagglutinin (10 µg/ml) for 3 days and further expanded with T cell growth factor (Lymphocult-T; Biotest Diagnostic, Inc., Dreieich, Germany).

**Cytotoxicity Assays.** CD8⁺-stimulated cells were tested for specific lytic activity after 1, 2, 3, or 4 weeks of culture using a cytotoxicity assay (7). Target cells were M14, HT144, SK-Mel 24, SK-Mel 5, SK-Mel 28, and PHAb. In inhibition experiments, different concentrations of mAbs specific for MHC class I molecules (W6/32, purified by protein A affinity chromatography from a hybridoma purchased from ATCC) or MHC class II molecules (anti-DR; Becton Dickinson) were added to the wells. The percentage of specific cytotoxicity and inhibition were determined as described (7).

**Results**

**Propagation of Short-Term CTL Lines.** HT144p and SK-Mel 5p were obtained from HT144 (HLA-A1) and SK-Mel 5 (HLA-A11) cells, respectively. HT144p and SK-Mel 5p were characterized by HPLC analysis and HT144p by binding to RMA-Shβ2A1 to verify the presence of peptides able to bind to HLA-A1. The HPLC profiles included a number of peaks (data not shown) consistent with the presence of a complex peptide mixture (5, 6, 9). Table 2 shows the results of the binding of HT144p and of control peptides to RMA-Shβ2A1. Binding to HLA-A1 was present only when p2 (the positive control peptide) and HT144p were incubated with RMA-Shβ2A1 cells. Binding of HT144p was dependent on the amount of Ag used, and it was detectable at concentrations equal to ≥500 µg/ml (data not shown). The specificity of the binding we observed was confirmed by the negative controls. Peptide p1 (a synthetic HIV sequence, ILKEPVHGV, that is a HLA-A2 binder), and SK-Mel 5p, peptides derived from HLA-unrelated melanoma cells, did not bind to HLA-A1 (Tables 1 and 2). These results support the notion that the peptides of the optimal length for MHC class I binding present in whole-cell extracts are MHC dependent (8).

HT144p-coated latex beads were used to generate CTLs from donor C. F., whose HLA-A1 allele matched the HT144 line (Table 1). Previous attempts to generate CTL with the same Ag peptides not conjugated to latex beads were unsuccessful; stimulation of PBMC with nonparticulate HT144p resulted in activation of mostly CD4⁺ cells with minimal nonspecific killing activity, if any.⁴

We established five short-term CTL lines using different particulate Ag preparations. The enrichment in the lines of CD8⁺ cells, as verified by flow cytometry (data not shown), was 70–90% and was consistent during the culture. Cytolytic activity could be detected after 1–2 weeks of culture and lasted for 3–4 weeks, i.e., the time of propagation of the lines. Fig. 1A shows a typical cytotoxicity assay. Lytic activity could be detected only against the HT144 cells from which the Ag peptides were obtained. No lytic activity was present against melanoma cells that did not match the donor or against autologous cells (PHAb). Lytic activity was occasionally detectable against the SK-Mel 24 cells, which share the alleles A1 and B14 with the donor, suggesting that the HT144 and SK-Mel 24 lines might share peptides bound to HLA-A1 (Fig. 1B1). The lytic activity of CTL against HT144 cells was MHC I restricted, but it was not affected by anti-DR mAb (data not shown). Fig. 1B2 shows a representative experiment of three inhibition experiments with mAb W6/32 (anti-HLA class I).

To verify that this method could induce antimalanoma CTL from

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Table 1  **HLA types of cells used in this study**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Cell type</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-C</th>
<th>HLA-DR</th>
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<tr>
<td>CF</td>
<td>PBMC</td>
<td>1,19</td>
<td>5,14</td>
<td>1,5</td>
<td>ND³</td>
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<tr>
<td>GF</td>
<td>PBMC</td>
<td>11,24</td>
<td>35,51</td>
<td>w6</td>
<td>ND³</td>
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<tr>
<td>Cell lines</td>
<td>HT144</td>
<td>Melanoma</td>
<td>12,4</td>
<td>13,15</td>
<td>w3</td>
</tr>
<tr>
<td>M14</td>
<td>Melanoma</td>
<td>11,24</td>
<td>40,64</td>
<td>w1</td>
<td>3,4</td>
</tr>
<tr>
<td>SK-Mel 24</td>
<td>Melanoma</td>
<td>1,2</td>
<td>12,14</td>
<td>w5</td>
<td>ND²</td>
</tr>
<tr>
<td>SK-Mel 5</td>
<td>Melanoma</td>
<td>2,11</td>
<td>w1B40</td>
<td>NE¹</td>
<td></td>
</tr>
<tr>
<td>SK-Mel 28</td>
<td>Melanoma</td>
<td>11,26</td>
<td>40</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

³ ND, not determined.
⁴ NE, not expressed.

Results are expressed as Δ-mo-fluorescence-intensity (ΔΔMFI), calculated as the difference of MFI between sample and negative control (FITC-RaMg), expressed in linear arbitrary units. p1 (ILKEPVHGV) is a high-affinity HLA-A2 binder, p2 (EVDPIGHLY) is high-affinity HLA-A1 binder, and HT144p and SK-Mel5p are defined in "Materials and Methods."

<table>
<thead>
<tr>
<th>ΔΔMFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No p</td>
</tr>
<tr>
<td>p1(125 µg/ml)</td>
</tr>
<tr>
<td>p2(125 µg/ml)</td>
</tr>
<tr>
<td>HT144p (500 µg/ml)</td>
</tr>
<tr>
<td>SK-Mel 5p (500 µg/ml)</td>
</tr>
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</table>
melanoma patients, we used SK-Mel 5p-coated latex beads to propagate a short-term line from a patient whose HLA-A11 allele partially matched the SK-Mel 5 line (Table 1). Fig. 2 shows that the CTL line obtained had cytolytic activity against the SK-Mel 5 cells, from which the Ag peptides were obtained, and against the M14 cells, which share the allele A11 with SK-Mel 5 cells and the alleles A11 and A24 with the PBMC of the donor. The HLA-unrelated SK-Mel 24 cells were not killed. These results demonstrate the existence of peptides able to bind HLA-A11, shared by SK-Mel 5 and M14 cells.

Discussion

In this report, we show that minute amounts (10–30 ng/50,000 adherent cells/well) of a complex mixture of naturally processed tumor peptides are highly effective in priming human CTL against melanoma in vitro, when conjugated with a particulate carrier that targets them to the MHC class I pathway of adherent cells.

This Ag was chosen: (a) to avoid bias due to selection of high-affinity MHC class I molecules binders, thus increasing the number of tumor-specific peptides potentially able to stimulate CTL precursors; (b) to overcome the limits of using tumor-specific synthetic sequences available for a very limited number of HLA alleles; and (c) toward future in vivo studies, to reduce the risk of selection of Ag-loss tumor variants.

The peptide pools we used should comprise the naturally processed peptides bound to MHC class I molecules at the cell surface (8, 9) as well as larger (up to M, 5000) precursor peptides. The binding experiments using RMA-Shβ2A1 cells demonstrate the presence among HT144p of peptides able to bind HLA-A1. The stimulating peptides may be the peptides of the optimal length (8–10 mer) present in the pool, and possibly peptides derived from further processing of larger precursors bound to the beads, if leaking from the phagosome into the cytosol had occurred (17).

Priming of CTL from healthy donors has been described for melanoma Ag using high doses of synthetic peptides (21–23). Here we demonstrate that, if targeted properly into the MHC class I pathway of professional APCs, a very low amount of peptides can prime a CTL response in vitro in a "semi-autologous" system. The CTL showed a cytolytic activity not only against tumor cells from which the peptides were obtained but also against tumor cells that share the same allele with the PBMC of the donor. The cross-reactivity was especially strong for the line we obtained from the melanoma patient, suggesting that allogeneic tumor peptides may be used to stimulate precursor CTL in cancer patients. Although HT144p-stimulated CTL did not kill autologous PHAb or HLA-unrelated melanoma cells, we cannot exclude the possibility that other normal autologous cells might be the targets of autoimmune reactions. Experiments are in progress to verify the specificity of the CTL for the melanoma peptides used to propagate the lines.
The strict requirement of the phagocytic alternative pathway for delivery of tumor-associated Ag onto MHC class I molecules agrees with the lack of spontaneous tumor rejection despite the existence of several tumor Ags (1, 2). The proposed "indirect" presentation of tumor peptides by macrophages or dendritic cells that would occur in vivo (4) may not target efficiently the tumor peptides onto the MHC class I molecules.

The system we describe overcomes the need for immunotherapeutic protocols to define tumor-specific sequences and the restrictions posed by the number of HLA alleles for which tumor-specific sequences are available. Our results raise the possibility that active and passive vaccination procedures might be devised, based on the use of naturally processed peptides derived from autologous and from allogeneic, partially matched tumor cells.

References

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