Major Histocompatibility Complex Class I Restricted Cytotoxic T Cells Specific for Natural Melanoma Peptides Recognize Unidentified Shared Melanoma Antigen(s)  

Maria Adele Imro, Simona Manici, Vincenzo Russo, Giuseppe Consongno, Matteo Bellone, Claudio Rugarli, Catia Traversari, and Maria Pia Potti


Abstract

CTLs were generated in vitro from two healthy donors and one melanoma patient by stimulation of CD8⁺ T cells with autologous dendritic cells pulsed with natural melanoma peptides (NMPs), obtained by acid treatment of HLA-matched melanoma cells. CTLs showed MHC class I-restricted melanoma-specific cytolytic activity. Importantly, CTLs from the patient, induced with NMPs obtained from an allogeneic HLA-A1-matched melanoma, killed the autologous tumor. COS-7 cells cotransfected with the cDNA of 13 melanoma antigens and the HLA-A1-restricting allele did not induce cytokines release from NMP-specific CTLs, suggesting that they recognize unidentified shared melanoma antigens and that they may be valuable for identification of new tumor antigens. These results strongly support the use of autologous and/or allogeneic NMP-pulsed dendritic cells as cancer vaccines in patients whose neoplasms do not express or have lost expression of known tumor antigens.

Introduction

Several TAAs and related epitopes, recognized by autologous CTLs, have been identified (reviewed in Refs. 1 and 2). On the basis of their pattern of expression, TAAs are classified as: (a) tumor-specific antigens shared by tumors of different histotypes; (b) tissue-specific antigens expressed in melanomas and melanocytes; or (c) unique tumor antigens, resulting from point mutations (1, 2). The existence of a fourth class of TAAs, shared among melanomas but not expressed in melanocytes or tumors of different histotypes has been suggested (3), and a melanoma antigen belonging to this class has been cloned recently (4).

The identification of TAAs has opened new perspectives in the treatment of cancer patients and clinical trials based on the use of synthetic peptides corresponding to sequence segments of known TAAs are ongoing. However, several requirements/concerns may limit their use as cancer vaccines.

(a) The expression of TAAs by the patients’ tumors and their role/immunodominance in the induction of tumor immunity must be demonstrated (5). Indeed, a study (3) in which the antigen specificity of a panel of CTL clones from melanoma patients was evaluated showed that most of them were not directed against tissue or tumor-specific antigens. This observation suggests that still unidentified antigens could be effective in eliciting tumor immunity in those patients and that the repertoire of melanoma antigens may be larger than that which is currently known.

(b) Antigen loss tumor variants during progression of the disease and/or vaccination (6, 7) may occur, making multiple determinations of antigen expression over time necessary.

(c) The TAAs are applicable to a restricted number of patients expressing HLA alleles for which synthetic sequences are available. This limitation may be overcome in the future because an increasing number of epitopes and their HLA-restricting alleles are being identified (reviewed in Ref. 8).

An alternative source of tumor antigens is the mixture of NMPs obtained by acid treatment of tumor cells (9). Preclinical studies, both in vitro in humans and in vivo in animals, have documented the efficacy of natural tumor peptides in inducing antitumor responses (reviewed in Ref. 9). Recently, melanoma patients were treated with autologous DCs pulsed with tumor lysate, obtained from the supernatants of autologous melanoma cells by freeze/thaw cycles (10). Although the number of patients was very small, this is the first report of treatment of cancer patients with unfractioned tumor antigens.

Here, autologous DCs pulsed with NMPs extracted from allogeneic HLA-matched melanoma cells were used in vitro to stimulate CD8⁺ T cells from two healthy donors and one melanoma patient. The induced CTLs were characterized for killing and tumor antigen specificity. The results demonstrate that NMPs obtained from an allogeneic melanoma are able to activate an antitumor response against the autologous tumor in the melanoma patient. Moreover, the study of the tumor antigen specificity shows that NMPs contain still unidentified melanoma peptides and supports their use as cancer vaccines and for identification of new TAAs.

Materials and Methods

Subjects and Cells. PBMCs were obtained from two healthy donors and one melanoma patient (subject 1, subject 2, and patient 1). The melanoma cell lines SK-Mel-24, HT144, SK-Mel-28, and SK-Mel-5 were from the American Type Culture Collection (Manassas, VA). The melanoma cell lines SK23-Mel, LB33-Mel, and MZ2-Mel.2.2ET.1 (hereafter referred to as ET1) were kindly provided by Prof. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). The OI-TC and VC-TC cell lines were established in our laboratory from melanoma cells isolated from cutaneous metastases. ME-180, a cervix epidermoid carcinoma cell line, was a generous gift from Interlab Cell Line Collection (CBA, Genoa, Italy). VR-LCL and FR-LCL (from patient 1) are EBV-transformed LCLs. All cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY), containing 2 mM L-glutamine, 100 units/ml penicillin, 50 mg/ml streptomycin (BioWhittaker, Walkersville, MD), and 10% FCS (Life Technologies, Inc., Paisley, Scotland). The HLA types of the donors and the cells used in this study, identified by standard serologic or molecular typing, are reported in Table 1.

Flow Cytometry. Cytofluorimetric analyses were performed on a FAC StarPlus (Beckton Dickinson, Sunnyvale, CA). The following mAbs were used: anti-DR-FTTC, anti-CD4-PE, anti-CD8-FTTC, anti-CD14-PE, anti-
CD33-PE, anti-CD83-FITC (Becton Dickinson), anti-CD1a-FITC (Serotec, UK), and anti-MHC class I (purified by protein A affinity chromatography from supernatant of W6/32 hybridoma purchased from American Type Culture Collection; FITC-rabbit antimouse immunoglobulin antibody (DAKO A/S, Glostrup, Denmark) was used as second-step reagent in indirect immunofluorescence staining.

Preparation of Melanoma Peptides and Generation of DCs. NMPs were obtained by acid treatment of SK-Mel-24 cells, followed by gel filtration on a Sephadex G-25 column, as described previously (Ref. 11; 28–30 mg from ~5 × 10^6 cells). DCs were generated as described previously (12, 13). Briefly, PBMCs obtained from 50–100 ml of blood were resuspended in RPMI 1640 supplemented with 10% heat-inactivated pooled HS, 2 mM l-glutamine, 100 units/ml penicillin, and 50 mg/ml streptomycin (TCM; Technogenetics, Milan, Italy) and plated at the concentration of 5 × 10^6 cells/ml in six-well plates for 2 h at 37°C. Nonadherent cells were then removed, and the adherent cells were cultured for 7 days with TCM (1% HS), in the presence of granulocyte macrophage colony-stimulating factor (800 units/ml; Mielogen, Schering Plough, Milan, Italy) and IL-4 (500 units/ml; kindly provided by Dr. Latini, Milan, Italy). Half of the supernatant was replenished with fresh TCM and cytokines every other day. At day 7, DCs were retrieved on a Percoll (Pharmacia, Milan, Italy) gradient (1–5 × 10^6 cells), as described previously (14), and used for phenotypic analysis and for pulsing with melanoma peptides. DCs were incubated with NMPs (75–125 μg/ml) for 3–4 h at 37°C before they were admixed with CD8+ T lymphocytes.

CTL Induction. CD8+ T cells were purified from the nonadherent fraction of PBMCs, using microbeads coated with anti-CD8 mAb and a miniMACS magnetic sorter (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), mixed with peptide-pulsed DCs at a 10:1 ratio, and cultured in TCM for 7 days. At day 7, activated cells were isolated on a Percoll gradient, as described previously (14), and expanded in the presence of TGFi (Lymphocult-T; Biotest Diagnostic, Inc., Dreieich, Germany) for a further 5–7 days. At day 14 and then at weekly intervals, CD8+ T cells were restimulated with irradiated (4000 rad) SK-Mel-24/B7-2 cells (i.e., SK-Mel-24 cells transfected with the B7-2 molecule, described in Ref. 15). The next day, effector cells were cleaned on a Ficoll gradient and re-expanded in TCGF. CD8+ T-cell clones were obtained by limiting dilution as described previously (16). Clones were restimulated every week until irradiated (4000 rad) allogeneic PBMCs and LCLs.

Chromium-Release Assay. Effector CD8+ T cells were tested for lytic activity in standard 4-h ⁵¹Cr release assays as described previously (15). In inhibition experiments, different concentrations (final dilutions, chosen on the basis of surface staining, were 1:100,000; the mouse mAbs specific for the MHC class I molecules (W6/32) and the MHC-class II molecules (anti-DR; Becton-Dickinson) were added to the wells. The percentages of specific lysis and inhibition were calculated as described previously (15).

RT-PCR Analysis. Total RNA was extracted by the use of RNAzolTM (Biotech, Houston, TX), according to manufacturer’s instructions. Single-stranded cDNA was synthesized from 2 mg of total RNA, by Moloney murine leukemia virus-derived reverse transcriptase (Life Technologies, Inc.), in the presence of 20 units of RNasin (Promega, Madison, WI). After 1 h of incubation at 42°C, reverse transcription products were stored at −80°C. cDNAs coding for tumor antigens were detected by PCR amplification. Reaction mixture contained 5 ml of cDNA, corresponding to 100 ng of total RNA, 4 ml of a 10 mm dNTPs mixture (containing each dNTP at 2.5 nm), 5 ml of 10× DNA polymerase buffer (Finnzymes Oy, Espoo, Finland), 2 units of DNAzyme DNA polymerase (Finnzymes Oy), and sterile water up to a 50-ml total reaction volume. For oligonucleotide primer sequences and PCR amplification programs, see Dalera et al. (Ref. 17; MAGE-1, MAGE-3, BAGE, GAGE, tyrosinase, and Melan-A/MART-1) and Lupetti et al. (Ref. 5; TRP-2, TRP-2-INT2, and gp100). Samples were scored as positive when a band of the appropriate size was visible on a agarose gel in the presence of ethidium bromide.

Transfection of COS-7 Cells. Transfections were performed by the DEAE-dextran-cloroquine method (18). In brief, 1.2 × 10⁶ COS-7 cells were cotransfected with 100 ng of plasmid pcDNA-SRas containing the HLA-A1 gene (19) and 100 ng of pcDNA/Amp (Invitrogen, San Diego, CA) or pcDNA-SRas plasmids containing the cDNA of one of the following melanoma antigens: Melan-A/MART-1, tyrosinase, gp100; MAGE-1, -2, -3, -4, -6, or -12; BAGE-1; GAGE-1 (kindly provided by Dr. P. van der Bruggen, Ludwig Institute for Cancer Research, Brussels, Belgium); TRP-1 (kindly provided by Dr. R. S. Wang, National Cancer Institute, NIH, Bethesda, MD); and TRP-2 (cloned as described in Ref. 5).

CTL Stimulation Assay. Transfectants or melanoma cell lines were tested for their ability to induce the production of TNF-α or IFN-γ by NMP-specific CTLs. Two thousand and 6000 CTLs for TNF-α and IFN-γ release assays, respectively, were added in wells containing target cells in 100 μl of Iscove’s modified Dulbecco’s medium (BioWhittaker) supplemented with 10% HS and 30 units/ml IL-2 (EuroCetus, Amsterdam, the Netherlands). TNF-α and IFN-γ release were measured after 20 h of incubation, using the TNF-sensitive murine fibrosarcoma line WEHI-164 clone, as described previously (20), and a standard ELISA (BMB, Mannheim, Germany), respectively.

Results and Discussion

NMPs were obtained from SK-Mel-24 cells that share the HLA-A1 allele with subject 1, the HLA-A1 and -B14 alleles with subject 2, and the HLA-A1 and -A2 alleles with patient 1 (see Table 1). DCs were generated by culture of adherent cells in the presence of granulocyte macrophage colony-stimulating factor and IL-4. Their surface phenotypic analysis showed high levels of CD33, MHC class I and class II, CD80, CD86, and CD40 molecules; variable expression of the CD1a molecule; low-level expression of the CD83 molecule, and no expression of the CD14 molecule (data not shown). CD8+ T cells were mixed with NMP-pulsed DCs and cultured for 1 week; activated cells were then expanded in the presence of TCGF for further 5–6 days. CD8+ T cells were propagated in culture for several months by
activity was tested in a 51Cr release assay against the following targets: respectively. The percentage of lysis against RF-TC (E

T staining, were 1D, percentage of inhibition of the lytic activity of CTLs from subjects 1 and 2 against

T cells (SK-Mel-28, SK-Mel-5, and M14). Target cells symbols, along with their

HLA cells (SK-Mel-28, SK-Mel-5, and M14) and -related melanoma cells (SK-Mel-24, HT144, OI-TC, VC-TC, and RF-TC); and non-

HLA -related melanoma cells (SK-Mel-28, SK-Mel-5, and M14). Target cells symbols, along with their

HLA type, are indicated at the bottom. HLA types of donors' lymphocytes are reported in Table 1. Right, percentage of inhibition of the lytic activity of CTLs from subjects 1 and 2 against SK-Mel-24 (D (n = 4) and E (n = 2)) and of CTLs from patient 1 against the autologous tumor [F (n = 3)] with W6/32 mAb. Final dilutions, chosen on the basis of surface staining, were 1:100 and 1:1000. The percentages of lysis against SK-Mel-24 (E:T = 10:1), in the absence of mAb, for CTLs of subjects 1 and 2 were 17 and 33%, respectively. The percentage of lysis against RF-TC (E:T = 10:1), in the absence of mAb, for CTLs of patient 1 was 26%.

weekly restimulations. The percentage of CD8+ T cells in the CTL lines, tested after the first stimulation and periodically thereafter during propagation, was always >90%. CD8αdim NK cells were almost undetectable in CTL lines obtained from the healthy donors and were found in frequencies of 13–15% in CTL lines from the patient (data not shown).

We first verified the induction of CTLs by testing, at day 4 or 5 after the first restimulation, their killing activity against autologous DCs, unpulsed or pulsed with the NMPs (Fig. 1). The CD8+ T cells strongly recognized NMP-pulsed DCs, thus demonstrating that DCs, after incubation with the NMP, express MHC-NMP complexes that are able to activate a specific CTL response.

We next evaluated whether: (a) the CTLs recognized melanoma cells from which the NMP were extracted; (b) the killing activity was

MHC restricted; (c) the CTLs preferentially recognized melanoma-specific epitopes; and (d) the epitopes recognized were shared among HLA-matched melanomas, and in particular, in the case of the patient, the autologous tumor.

CTLS from all subjects recognized SK-Mel-24 from which the NMPs were extracted. Moreover, CTL-lysed melanoma cells sharing HLA alleles with donors' lymphocytes (HT144 sharing the HLA-A1 allele with all donors, OL-TC sharing the HLA-A1 allele with subject 1 and the HLA-A1 and -B14 alleles with subject 2; (Fig. 2, A–C). Importantly, CTLs from the patient recognized the autologous tumor (RF-TC; Fig. 2C), demonstrating that NMPs contained peptides shared between the allogeneic HLA-matched melanoma and the autologous tumor. This is a relevant finding of this study; in fact, a limitation in the use of NMPs as cancer vaccines is the need for a large number of tumor cells from which to extract the peptides. The possibility to use allogeneic NMPs instead or in combination with the autologous NMPs would make the use of natural peptides for vaccination protocols more widely applicable. Indeed, strong evidence is now available on CTL priming across allogeneic barriers by engaging DCs in situ (21, 22). Furthermore, the use of allogeneic NMP might favor the activation of precursor CTL specific for epitopes that are dominant in the allogeneic but subdominant or cryptic in the autologous tumor and therefore eventually more relevant in antitumor immunity.

CTLS from subject 1 (Fig. 2A) and subject 2 (Fig. 2B) killed, at background levels (<5%), non-HLA-related melanoma cells (SK-Mel-28, SK-Mel-5, and M14) and HLA-A1+ cells of nonmelanoma origin (ME-180 and VR-LCL). CTLs from the patient had some nonspecific lytic activity toward non-HLA-related melanoma cells and HLA-A1+ LCL (SK-Mel-28, VR-LCL and RF-LCL; Fig. 2C), probably because of the presence of NK cells in the polyclonal CTL population. The lytic activity against SK-Mel-24 of CTL from subjects 1 and 2 (Fig. 2, D and E, respectively) and the lytic activity against RF-TC (the autologous tumor) of CTL from patient 1 (Fig. 2F) was inhibited by addition in the assay of an anti-MHC class I mAb (W6/32). Collectively, these data demonstrate that NMP-specific CTLs recognize melanoma epitopes shared among HLA-matched melanomas but not HLA-matched cells of nonmelanoma origin.

SK-Mel-24 expresses, albeit mainly at low levels, most of the known tumor- and tissue-specific tumor antigen genes, as detected by RT-PCR analysis (Table 2). To identify the antigen(s) recognized by NMP-specific CTLs, we cotransfected COS-7 cells with the cDNAs encoding the melanoma antigens and the

HLA-A1

TRP-2-INT2

Table 2 Expression of tumor antigen genes in SK-Mel-24 melanoma cells

\[\text{Antigens} \quad \text{Gene} \quad \text{Expression}\]

\begin{tabular}{|l|l|l|}
\hline
\text{Shared tumor antigens} & MAGE-1 & + \\ 
& MAGE-3 & ± \\ 
& GAGE-1/2 & ± \\ 
& BAGE & - \\ 
\hline
\text{Differentiation tumor antigens} & gp100/Pmel & + \\ 
& TRP-2 & + \\ 
& tyrosinase & ± \\ 
& Melan-A/MART-1 & ± \\ 
& TRP-2-INT2 & + \\ 
\hline
\end{tabular}

\[\text{As determined by RT-PCR analysis.}\]
HLA-A1 and COS-7 cells transfected with the cDNA of HLA-A1 were used. The production of IFN-γ or TNF-α by CTLs was measured after 20 h of coculture with stimulator cells (i.e., melanoma cell lines or COS-7 cells cotransfected with the cDNA of HLA-A1 and the indicated melanoma antigens), as described in “Materials and Methods.” As controls, CTLs alone, CTLs in the presence of K562, COS-7 cells alone, and COS-7 cells transfected with the HLA-A1 cDNA were used. The data are representative of three experiments.

Fig. 3. NMP-specific CTLs do not recognize known melanoma antigens. Stimulation of CTL polyclonal lines from subjects 1 (A and D) and 2 (B and E) and of CTL clone E7 from patient 1 (C and F). The release of IFN-γ or TNF-α by CTLs was measured after 20 h of coculture with stimulator cells (i.e., melanoma cell lines or COS-7 cells cotransfected with the cDNA of HLA-A1 and the indicated melanoma antigens), as described in “Materials and Methods.” As controls, CTLs alone, CTLs in the presence of K562, COS-7 cells alone, and COS-7 cells transfected with the HLA-A1 cDNA were used. The data are representative of three experiments.

References


Major Histocompatibility Complex Class I Restricted Cytotoxic T Cells Specific for Natural Melanoma Peptides Recognize Unidentified Shared Melanoma Antigen(s)

Maria Adele Imro, Simona Manici, Vincenzo Russo, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/10/2287

Cited articles
This article cites 23 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/10/2287.full.html#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
/content/59/10/2287.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.