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A new tumor-specific antigenic peptide encoded by *MAGE-6* is presented to cytolytic T lymphocytes by HLA-Cw16

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Abstract

"Cancer-germline" genes such as those of the *MAGE* family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They encode shared tumor-specific antigens, which have been used in therapeutic vaccination trials of cancer patients. *MAGE-6* is expressed in more than 70% of metastatic melanomas and more than 50% of carcinomas of the lung, esophagus, bladder, and head and neck. We report here the identification of a new MAGE-6 antigenic peptide, which is recognized by a tumor-specific cytolytic T lymphocyte clone isolated from a melanoma patient. The peptide, ISGGPRISY, corresponds to positions 293 to 301 of the MAGE-6 protein sequence and is presented by HLA-Cw1601 molecules.

Introduction

Patient MZ2 was diagnosed with a metastatic melanoma in 1982. A cell line, MZ2-MEL, was established from an adrenal metastasis. After ineffective cytostatic chemotherapy, multiple lymph nodes and visceral metastases were resected in 1982 and 1983. From 1983 onwards, the patient received multiple injections of mutagenized and lethally irradiated autologous melanoma cell clones (1). She has been free of detectable melanoma for eighteen years.

A panel of stable cytolytic T lymphocyte (CTL) clones directed against the autologous MZ2-MEL cells has been obtained by mixed lymphocyte-tumor cell cultures (1, 2). These CTL clones showed specificity for the tumor cells, insofar as they did not lyse autologous fibroblasts, autologous Epstein Barr virus-transformed B cells, or the natural killer target cells K562 (3). They were used to select variants of MZ2-MEL cells that resisted lysis by some of the CTL clones. We concluded from the analysis of these antigen-loss variants that several distinct antigens were recognized on MZ2-MEL cells by autologous CTLs (3). Gene transfection approaches led to the

identification of seven of these antigens: MAGE-1 peptides presented by HLA-A1 or Cw16, a MAGE-3 peptide presented by A1, a BAGE-1 peptide presented by Cw16, GAGE peptides presented by A29 or Cw6, and a tyrosinase peptide presented by B44 (4, 5, 6, 7, 8, 9, 10). We report here the identification of an eighth antigen recognized on MZ2-MEL cells by autologous CTLs.

Results

Isolation of an anti-tumor CTL restricted by HLA-Cw*1601

Blood lymphocytes from melanoma patient MZ2 were stimulated with the autologous tumor cell line MZ2-MEL.43 and anti-tumor CTL clones were obtained by limiting dilution of the responder T cell population. Several clones failed to lyse the MZ2-MEL.3.1 subline, which had been passaged *in vitro* for more than 150 generations (Figures 1 and 2). These CTL clones, including CTL 82/21, were labeled anti-MZ2-B, under the assumption that they were directed against a single antigen lost by MZ2-MEL.3.1 (3). However subsequent analysis indicated that subline MZ2-MEL.3.1 had lost the genes coding for HLA-A29, B4403, and Cw1601 (5) (Figure 1). CTL 82/21 recognized MZ2-MEL.3.1 cells when they were transfected with the autologous HLA-Cw*1601 cDNA (data not shown). This suggested that the antigenic peptide recognized by this CTL clone was presented by Cw1601 molecules.

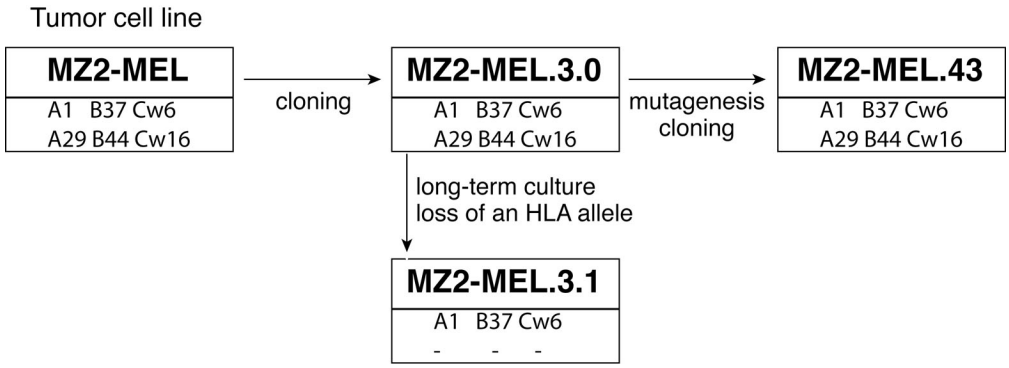

Figure 1. Sublines of melanoma cell line MZ2-MEL.

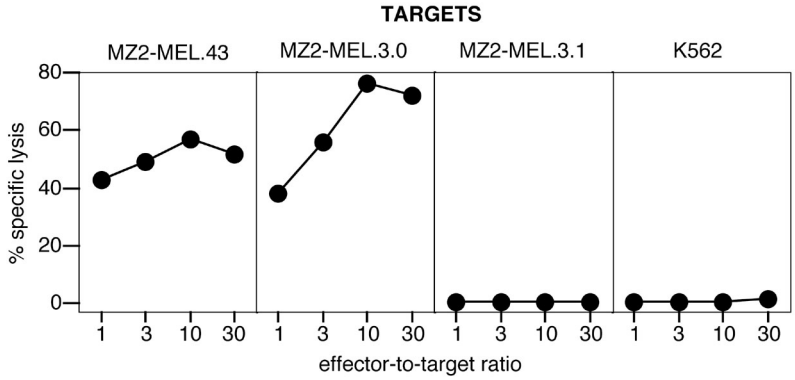

Figure 2. Lysis of melanoma sublines by MZ2-CTL 82/21. The different melanoma sublines of patient MZ2 are described in Figure 1. Chromium release was measured after 4 h.

Identification of the gene encoding the antigenic peptide

A cDNA library prepared with RNA extracted from MZ2-MEL.43, and containing approximately 66,000 inserts, was divided into pools of 200-400 bacteria (7). Plasmid DNA from each pool was transfected together with the Cw*1601 construct into duplicate microcultures of COS-7 cells. The transfected cells were screened for expression of the antigen by adding CTL 82/21 and measuring TNF production. Two positive cDNA pools were identified. One of them was cloned to obtain cDNA GEP3/317/2B7, which transferred the expression of the antigen (Figure 3C). The sequence of this cDNA corresponded to that of *MAGE-6*.

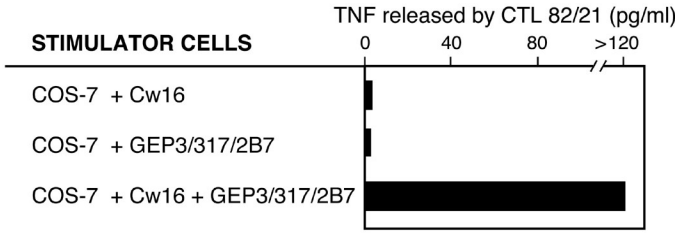

Figure 3. Identification of the presenting HLA molecules and of the gene encoding the antigenic peptide. COS-7 cells were transiently transfected by the DEAE-dextran method with 50 ng of HLA-Cw*1601 cDNA and 50 ng of cDNA GEP 3/317/2B7, which was isolated from a cDNA library screened with CTL 82/21. Each cDNA was inserted into pCD-SRalpha. After 24 h, 1,500 CTLs were added to the transfected cells. TNF production was measured 24 h later.

Identification of the MAGE-6 antigenic peptide

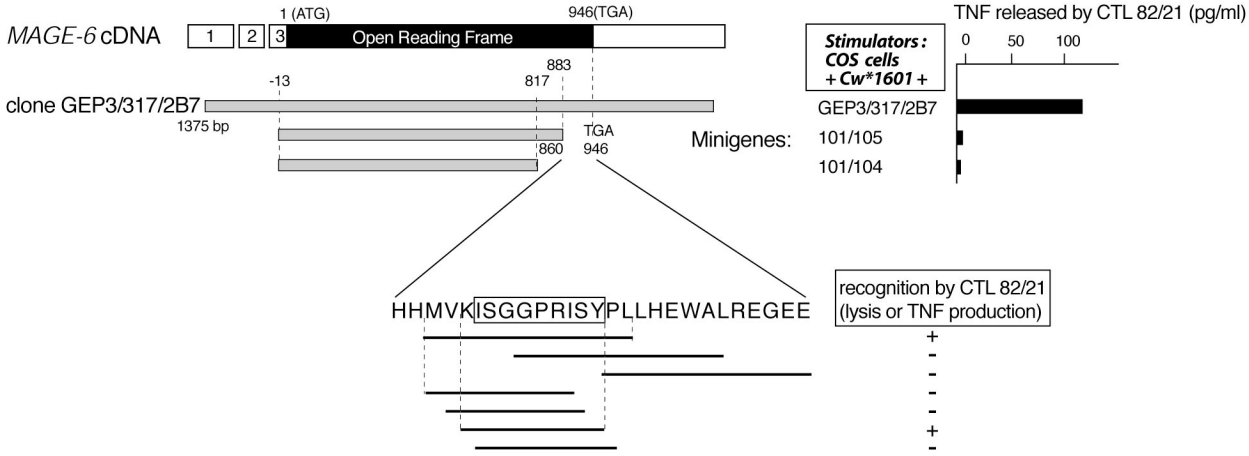

Figure 4. Overview of the strategy used to identify the antigenic peptide. The *MAGE-6* cDNA is shown in the upper part of the figure with the three exons appearing as open boxes and the open reading frame as a black box. The *MAGE-6* cDNA isolated from the library, GEP3/317/2B7, and truncated cDNA constructs were cotransfected into COS-7 cells together with an HLA-Cw*1601 cDNA. The transfected cells were tested for recognition by the CTLs. Three long peptides (underlined) were synthesized on the basis of the sequence of the region coding for the antigenic peptide, and tested for recognition by the CTLs. A 12 amino acid-long peptide tested positive and shorter peptides were subsequently tested. The shortest peptide recognized by CTL 82/21 is boxed.

To localize the peptide-coding sequence, we prepared several truncated *MAGE-6* cDNA sequences that were cloned into expression plasmids and transfected into COS-7 cells together with the Cw*1601 construct (Figure 4). Transfected cells were tested for their ability to stimulate CTL 82/21. We concluded that the antigenic peptide was encoded by the last 86 nucleotides of the *MAGE-6* open reading frame. Peptides encoded in this region were synthesized and tested for recognition by the CTLs (Figures 4 and 5). Nonapeptide ISGGPRISY was the shortest peptide recognized, with half-maximal lysis obtained at 20 nM. Nonapeptide ISGGPRISY corresponds to positions 336 to 344 of the MAGE-6 protein sequence. The homologous MAGE-3 peptide, which differs from the MAGE-6 peptide only at position 6, was not recognized by CTL 82/21 (Figure 5).

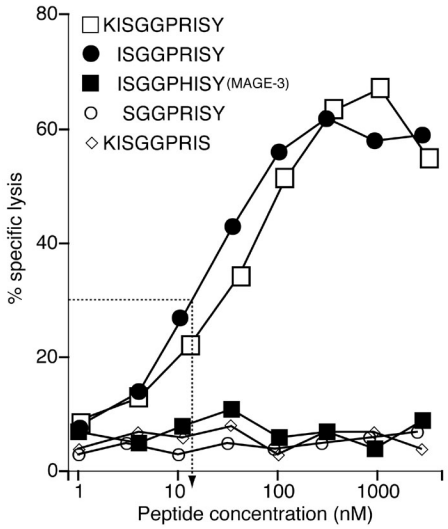

Figure 5. Identification of the antigenic peptide. ^{51}Cr-labeled HLA-Cw*1601 LB678 EBV-B cells were incubated with peptides for 15 min before addition of CTL clone 82/21 at an effector to target ratio of 5 to 1. Chromium release was measured after 4 h. The concentrations indicated in the figure are the concentrations during the 4-h incubation. The arrow indicates the concentration at which half-maximal lysis was observed.

Discussion

The anti-tumor T cell response of patient MZ2 is almost exclusively directed against antigens encoded by "cancer-germline genes". The MAGE, BAGE, GAGE antigenic peptides are presented by HLA-A, B, and C molecules. HLA-C molecules are considered to be expressed at the cell surface at about 10% of the levels of the HLA-A and B molecules and their importance for antigen presentation has been questioned (11). Of note, out of the eight peptides that have been identified with anti-tumor CTLs from patient MZ2, three are presented by HLA-Cw*1601 molecules and a fourth by HLA-Cw*0601. The relevance of HLA-C for antigen presentation should perhaps be reconsidered.

HLA-Cw16 molecules cannot be detected by serology and there is limited information about their population distribution. They seem to be expressed by 7-10% of Caucasians (5, 12, 13). Six alleles have been described, and Cw*1601 represents 90% of the expression. No peptide-binding motif has been proposed yet and only three Cw16-binding peptides have been described: the MAGE-1 peptide SAYGEPRKL, the BAGE-1 peptide

AARAVFLAL, and this MAGE-6 peptide ISGGPRISY. The Cw16 binding motif may therefore comprise a hydrophobic residue, L or Y, at the C-terminus, as is the case for many other HLA class I molecules.

MAGE-6 is expressed in 72% of metastatic melanomas, 68% of esophageal carcinomas and more than 50% of carcinomas of the head and neck, lung and bladder (14). Two MAGE-6 peptides presented by HLA class I molecules have been described previously: REPVTKAEML (MAGE-$6_{127-136}$), which was recognized on B3701 by anti-tumor CTLs isolated from a melanoma patient, and VKISGGPR (MAGE-$6_{290-298}$), which was recognized on A34 by CTLs infiltrating a spontaneously regressing human primary melanoma (15, 16). These results indicate that some MAGE-6 antigenic peptides are immunogenic in cancer patients and can therefore be used as cancer vaccine targets.

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Materials and methods

Cell lines and T cells

Patient MZ2 was typed HLA-A1, A29, B37, B*4403, Cw*0601, Cw*1601. Melanoma cell line MZ2-MEL was derived from an abdominal metastasis of patient MZ2 and a number of sublines were obtained. Subclone MZ2-MEL.3.0 was obtained by limiting dilution (Figure 1). Subline MZ2-MEL.3.1 was obtained by extending the culture of subclone MZ2-MEL.3.0 for more than 150 generations. Subline MZ2-MEL.43 was derived by limiting dilution from MZ2-MEL.3.0 cells that had survived a mutagen treatment (2, 3). Melanoma cell lines were grown as previously described (3, 17). Blood lymphocytes collected in 1984 from patient MZ2 were stimulated with MZ2-MEL.43 cells in standard mixed lymphocyte-tumor cell culture conditions and MZ2-CTL clone 82/21 was obtained by limiting dilution (3). It was grown in conditions similar to those described previously (17). CTL clone 82/21 expresses a TCRBV5-8*01/J2-7*01 rearrangement. Lymphoblastoid cell line MZ2-EBV was derived from patient MZ2 by standard techniques.

cDNA library

The construction of the cDNA library has already been described in Boël *et al*. (7). Poly-A^+ RNA was extracted from MZ2-MEL.43 cells using the FastTrack® mRNA extraction kit (Invitrogen Corp., Oxon, UK). mRNA was converted to cDNA using random primers, ligated to adaptors as described in the SuperScript plasmid system kit (Life Technologies, Gaithersburg, MD, USA), and inserted into the *Eco*RI site of expression vector pCD-SRalpha (18). Recombinant plasmids were electroporated into *E. coli* JM101 and selected with ampicillin (50 µg/ml). The library contained 66,000 inserts and was divided into 87 pools of 400 bacteria and 297 pools of 200 bacteria. Each of these pools comprised approximately 280 or 140 different cDNAs respectively, as about 70% of the plasmids carried an insert. Each pool of bacteria was amplified to saturation and plasmid DNA was extracted by the alkaline lysis method.

Transfection of COS-7 cells

Transfection experiments were performed by the DEAE-dextran-chloroquine method (18, 19, 20). Briefly, $1.5x10^4$ COS-7 cells were transfected with 50 ng of plasmid pCD-SRalpha containing an HLA-Cw*1601 cDNA insert, and 50 ng of a pool of the cDNA library or 50 ng of a cDNA clone. The HLA-Cw*1601 cDNA was isolated from a cDNA library prepared with RNA extracted from subline MZ2-MEL.43 (5).

CTL stimulation assay

Transfectants were tested for their ability to stimulate the production of Tumor Necrosis Factor (TNF) by CTLs 24-48 h after transfection (17). Briefly, 1,500 CTLs were added in microwells containing target cells in 100 µl of Iscove medium (Life Technologies) containing 10% human serum and 20 U/ml r-hu-IL2. After 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on WEHI-164 clone 13 cells in an MTT colorimetric assay (17, 21, 22).

Truncated cDNA

To identify the region of the *MAGE-6* sequence that codes for the antigenic peptide presented by HLA-Cw*1601, we constructed several truncated cDNA clones that were obtained by PCR. The PCR products were inserted in vector pCR3 (Invitrogen, Paisley, UK).

Antigenic peptides and CTL assay

Lyophilized peptides were dissolved first in 1 volume DMSO, and 9 volumes of 10 mM acetic acid were then added. Diluted peptides were stored at -20°C. Lysis of target cells by CTLs was tested by chromium release as previously described (23). In the peptide sensitization assay, target cells were labeled with ^{51}Cr for one hour at 37°C and washed extensively. One thousand target cells were then incubated in 96-well microplates in the presence of various concentrations of peptide for 15 min at room temperature. The CTLs were then added and chromium release was measured after 4 h at 37°C.

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