A MAGE-A3 Peptide Presented by HLA-DP4 Is Recognized on Tumor Cells by CD4+ Cytolytic T Lymphocytes

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Abstract

Antigens encoded by MAGE-A3 and recognized by T cells are interesting targets for tumor immunotherapy because they are strictly tumor specific and shared by many tumors of various histological types. A number of MAGE-A3 antigenic peptides presented by HLA class I molecules have been used in clinical trials, and regressions of melanoma metastasis have been observed. We report here the identification of a MAGE-A3 epitope, TQHFVQENYLEY, presented to CD4+ T lymphocytes by HLA-DP4 molecules, which are expressed in ~76% of Caucasians. This new epitope may be useful both for therapeutic vaccination and for the evaluation of the immune response in cancer patients. Interestingly, the CD4+ T cells lysed HLA-DP4 tumor cells expressing MAGE-A3, indicating that this epitope, in contrast to other class-II MAGE-A3 epitopes, is presented at the surface of tumor cells. The study of this disparity in the presentation of two epitopes from the same protein may lead to a better understanding of the endogenous class II presentation pathway.

Introduction

Studies on T-cell responses against human tumors focused initially on the identification of epitopes presented to CD8+ CTLs by HLA class I molecules. In several animal models, however, a critical role for CD4+ T cells in inducing and maintaining antitumor immunity has clearly been demonstrated (1, 2). If the lessons learned from these murine models apply to cancer patients, the identification of tumor antigens presented by HLA class II molecules to CD4+ T cells might be of great benefit in optimizing tumor immunotherapy.

Human tumor antigens recognized by CD4+ T cells are being identified with increasing frequency. Some of them are the result of point mutations in ubiquitously expressed genes, whereas others are fusion gene products. Several melanoma antigens recognized by CD4+ T cells are encoded by genes expressed specifically in cells of the melanocytic lineage (3). Another category of antigens recognized by T cells comprises those encoded by “cancer-germ line genes,” such as MAGE genes (4). These genes are silent in normal cells, except male germ-line cells, which do not express HLA molecules. These antigens are interesting targets for tumor immunotherapy because they are strictly tumor specific and shared by many tumors of various histological types. The first two HLA class II-restricted epitopes of this category were identified for MAGE-A3 (5, 6), which is frequently expressed in metastatic melanomas (76%) and in carcinomas of the esophagus (47%), the head and neck (49%), the lung (47%), and the bladder (36%). NY-ESO-11MAGE-2 peptides were also reported to be recognized on HLA-DR molecules by CD4+ T cells (7).

We report here the isolation of a CD4+ T-cell clone, specific for another MAGE-A3 peptide presented by HLA-DP4 molecules. Interestingly, this new epitope is presented at the surface of HLA-DP4 tumor cells, which are lysed by the CD4+ T-cell clone.

Materials and Methods

MAGE-A3 Proteins. Two different MAGE-A3 proteins were used. One was produced in our laboratory in Spodoptera frugiperda (SF9) insect cells, hereafter referred to as protein MAGE-A3insect, using a baculovirus expression system (PharMingen, San Diego, CA). The other MAGE-A3 protein, hereafter referred to as protein MAGE-A3bacteria, was produced in Escherichia coli by Smithkline Beecham Biologicals (Rixensart, Belgium), as reported previously (5).

The pMFG Retrovirus Encoding II-MAGE-A3. A MAGE-A3 cDNA was ligated downstream of the first 80 amino acids of the human invariant chain (Ii80; Ref. 5), followed by an internal ribosomal entry site sequence derived from the encephalomyocarditis virus and by the sequence encoding a truncated form of the human low-affinity nerve growth factor receptor (ΔLNNGFr; kindly provided by Dr. C. Traversari, Istituto Sciﬁco H. S. Raffaele, Milan, Italy). The procedure for transducing cell lines has been described previously (5).

The Recombinant Adenoviruses. Adeno-MAGE-A3 was constructed according to the procedures described for adeno-MAGE-A4 (8). To construct adeno-II-MAGE-A3, a MAGE-A3 cDNA was ligated downstream of the first 80 amino acids of the human invariant chain (Ii80). The Ii80 template was kindly provided by Dr. J. Pieters (Basel Institute for Immunology, Basel, Switzerland). A recombinant adenovirus was obtained using the system described by He et al. (9). The vectors and bacterial strains were kindly provided by Dr. T-C. He (Johns Hopkins Oncology Center, Baltimore, MD).

Mixed Lymphocyte/Dendritic Cell Culture. Media, cytokines, peptides, and protocols were described previously (5). Briefly, stimulator dendritic cells were incubated with protein MAGE-A3insect (10 μg/ml) for 20 h in medium supplemented with IL-4 (200 units/ml), granulocyte/macrophage-colony-stimulating factor (70 ng/ml), and tumor necrosis factor-α (5 ng/ml). Cells were washed and added at 104 per well of a round-bottomed microwell to 105 CD4+ T lymphocytes in 200 μl of IMDM (Life Technologies, Inc., Gaithersburg, MD) supplemented with AAG (5%) and 1% autologous plasma in the presence of IL-6 (1000 units/ml) and IL-12 (10 ng/ml). The CD4+ T lymphocytes were restimulated on days 7, 14, and 21 with autologous dendritic cells freshly loaded with protein MAGE-A3insect and grown in IMDM supplemented with AAG and 10% human serum (hereafter referred to as complete IMDM) supplemented with IL-2 (10 units/ml) and IL-7 (5 ng/ml). Aliquots of each microwell (~4000 cells) were assessed on day 36 for their capacity to produce IFN-γ when stimulated with ~10,000 autologous EBV-B cells and loaded for 20 h with 20 μg/ml of protein MAGE-A3insect or ovalbumin (Sigma Chemical Co., Bornem, Belgium). After 20 h of coculture in round-bottomed microwells...
and in 100 μl of complete IMDM medium supplemented with IL-2 (25 units/ml). IFN-γ released in the supernatant was measured by ELISA using reagents from Medgenix Diagnostics-Biosoftware (Fleurus, Belgium).

**CD4**+ **T Cell Clones.** Microcultures that were tested positive were cloned by limiting dilution, using irradiated autologous EBV-B cells loaded for 20 h with 20 μg/ml of protein MAGE-A3 bacteria (5 × 10^5 to 10^6 cells/round-bottomed microwells) as stimulator cells. Irradiated allogeneic LG2-EBV (5 × 10^5-10^6 cells/well) were used as feeder cells. CD4**+** T-cell clones were restimulated once per week with 0.5 μg/ml purified PHA (HA16; Murex Diagnostics, Dartford, United Kingdom) in complete IMDM supplemented with IL-2 (50 units/ml) and feeder cells.

**Recognition Assays with Peptides and Tumor Cells.** EBV-B or tumor cells were distributed at 20,000 cells per round-bottomed microwell and incubated (or not) for 2 h at 37°C in the presence of the different peptides, the indicated concentrations representing their concentrations during the incubation step. CD4**+** T lymphocytes (3000) were added in 100 μl of complete IMDM medium supplemented with IL-2 (25 units/ml). Supernatants were harvested after 20 h, and IFN-γ production was measured by ELISA. For cytotoxicity assays, target cells were labeled with 100 μCi of Na(15)CrO_4 for 1 h, washed, and incubated with the CD4**+** effector cells at various E:T ratios. Chromium release was measured after incubation at 37°C for 4 h.

**Results**

To identify new MAGE-A3 epitopes presented by HLA class II molecules, we cultured monocyte-derived DCs from blood donor LB1981 in autologous plasma and incubated them overnight with a recombinant MAGE-A3 protein and with tumor necrosis factor-α to induce their maturation. These cells were then used to stimulate autologous CD4**+** T lymphocytes. In previous experiments, a large number of the CD4**+** T-cell clones obtained with the same method were apparently directed against bacterial contaminants in the batch of protein (5). Therefore, we have used here a MAGE-A3 protein produced in *S. fragip erda* insect cells (MAGE-A3_insect) to stimulate the lymphocytes and a MAGE-A3 protein produced in *E. coli* (MAGE-A3_bacteria) to test the specificity of the responder lymphocytes. Their specificity was further tested on presenting cells in which the MAGE-A3 protein was targeted into the endosomal compartments. This was achieved by transducing cells with a retroviral construct, retro-li.MAGE-A3, which encodes a truncated human invariant chain (Ii) fused with MAGE-A3 (5, 10).

**A CD4**+ **T-Cell Clone Directed against a MAGE-A3 Antigen.** A total of 96 microcultures were set up, each containing 10^5 CD4**+** T cells and 10^4 autologous stimulator DCs loaded with protein MAGE-A3_insect as stimulator cells. Responder cells were restimulated three times at weekly intervals with DCs loaded with the protein. After a resting period of 2 weeks, responder cells of each microculture were tested for IFN-γ production after stimulation with autologous EBV-B cells loaded with protein MAGE-A3_bacteria, or with ovalbumin as a negative control. Only one microculture specifically produced a high level of IFN-γ after stimulation with protein MAGE-A3_bacteria (Fig. 1). It was cloned by limiting dilution, using autologous EBV-B cells loaded with protein MAGE-A3_bacteria as stimulator cells. Several positive clones were obtained, including clone 22 that recognized autologous EBV-B cells loaded with protein MAGE-A3 or transduced with retro-li.MAGE-A3 (Fig. 1).

**Clone 22 Recognized Peptide TQHFVQENLEY.** A set of peptides of 16 amino acids, which overlapped by 12 and covered the entire MAGE-A3 protein sequence, was screened initially with another clone derived from the microculture. This CD4**+** T cell clone produced IFN-γ after stimulation with two overlapping peptides, namely MAGE-A3_243–255 and MAGE-A3_247–262. These two peptides also stimulated clone 22 (Fig. 2). We tested a number of shorter peptides of different lengths and the shortest peptide that was well recognized by clone 22 was TQHFVQENLEY (MAGE-A3_247–255, Fig. 2).

**The Peptide Is Presented by HLA-DP4 Molecules.** The recognition by clone 22 of autologous EBV-B cells loaded with peptide MAGE-A3_243–258 was abolished by an anti-HLA-DP antibody but not by antibodies against HLA-DR or HLA-DQ (data not shown). Blood donor LB1981 was typed HLA-DPB1*0401. Peptide TQHFVQENLEY was loaded on several EBV-B cell lines, and those expressing DPB1*0401 were able to present the peptide to clone 22 (Table 1). The peptide was also tested for recognition on cells expressing DPB1*0402, the other DP4 allele. These cells had to be incubated with five time more peptide than DPB1*0401 cells to stimulate clone 22 to produce the same amount of IFN-γ (data not shown). Importantly, clone 22 was stimulated by DPB1*0402 cells transduced with retro-li.MAGE-A3 (Fig. 3).

**Recognition of Tumor Cell Lines.** Clone 22 lysed melanoma cell line LB1622-MEL and autologous EBV-B cells transduced with retro-li.MAGE-A3 (Fig. 4A). Cold target inhibition experiments were performed that showed that the lytic activity of CD4**+** T cells against...
Direct recognition of tumor cells by clone 22 was unexpected, but only if they were pretreated with IFN-γ for 20 h. IFN-γ production in the supernatant was measured by ELISA.

**Table 1 HLA-DPB1*0401 cells present the MAGE-A3 peptide to clone 22**

<table>
<thead>
<tr>
<th>EBV-B cell line</th>
<th>HLA DPB1</th>
<th>IFN-γ production (pg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB 1981 (autologous)</td>
<td>DPB1*0401</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>LB 1622</td>
<td>DPB1*0401</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>LB 1841</td>
<td>DPB1*0401/0501</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>LB 1532</td>
<td>DPB1*0401</td>
<td>128</td>
</tr>
<tr>
<td>LB 1857</td>
<td>DPB1*0401/0501</td>
<td>80</td>
</tr>
</tbody>
</table>

* EBV-B cells were incubated for 1 h with peptide TQHFWQENYLEY (1 μg/ml) and washed. Clone 22 (3,000 cells) was then incubated for 20 h with 20,000 peptide-pulsed EBV-B cells. IFN-γ production in the supernatant was measured by ELISA.

Discussion

Class II molecules of the MHC present peptides derived from proteins translated with a signal peptide in the presenting cell itself, such as membrane-associated or secretory proteins, which may also follow the exogenous presentation pathway (13, 14). In addition, there is an “endogenous class II presentation pathway” involving primarily proteins containing an endosomal targeting sequence, such as melanocyte-specific proteins (15).

In a previous report on MAGE-A3 antigens presented by class II molecules, we described two antigenic peptides, MAGE-A3_14–127 and MAGE-A3_21–134, that were not expressed at the surface of tumor cells expressing MAGE-A3 (5). One explanation for these results was that the MAGE-A3 protein was unable to follow the endogenous class II presentation pathway, because it contains neither a signal peptide nor an endosomal targeting sequence. However, Manici et al. (6) identified another epitope, MAGE-A3_281–295, that was recognized on tumor cells by a CD4+ T cell line. The results obtained in this study suggested that the entire MAGE-A3 protein was unable to follow the endogenous class II presentation pathway, because it contains neither a signal peptide nor an endosomal targeting sequence.
MAGE-A3 protein does not have access to endosomes. Otherwise, all antigen peptides would have been presented at the surface, as is the case for cells loaded with protein or transduced with retro-II-MAGE-A3. This disparity in the presentation of two epitopes from the same protein provides us with the opportunity of a further study of the presentation mechanisms that may improve our understanding of the endogenous class II presentation pathway.

Therapeutic vaccinations of cancer patients based on MAGE-A3 peptides presented by class I molecules have been tested (16–18). In one of these trials, 25 melanoma patients received s.c. injections of a MAGE-A3 peptide presented by HLA-A1 (17). Seven patients showed significant tumor regressions, three of which were complete. A major limitation of such a class I peptide-based approach could be that the peptide-specific CTLs reach the tumor but fail to be restimulated properly at the tumor site so that a massive CTL amplification does not occur. This could be attributable to the lack of help by tumor-specific CD4+ T cells. Vaccination strategies with MAGE-A3 products may benefit from the induction of anti-MAGE-A3 CD4+ T cells. Results obtained in mice support this concept (19, 20). Besides providing help for the induction and maintenance of tumor-specific CD8+ CTLs, CD4+ T cells may also play a broader role in mediating activation of dendritic cells, macrophages, and eosinophils (2, 21–23). Furthermore, direct killing of class II-expressing tumor cells by CD4+ T cells has been demonstrated (6, 24, 25). This is also confirmed by our results.

The new MAGE-A3 epitope described here is presented to the CD4+ T-cell clone by HLA-DPB1*0401 molecules, which are expressed by 64% of Caucasians. We demonstrated that the epitope can also be presented by the second allele of HLA-DPA4, HLA-DPB1*0402, which is expressed in 21% of Caucasians. Thus, ~76% of Caucasians could be vaccinated with this peptide. The choice of the optimal length of a class II-restricted peptide used in vaccination deserves further study because, in a murine model with hen egg white lysozyme, it was shown that the life span of peptide-MHC class II complexes is influenced by the length of the peptides (26).

In clinical trials using proteins, reliable monitoring of the anti-MAGE-A3 CD4+ T cell response will be important. To avoid possible misinterpretation of in vitro assays and of delayed-type hypersensitivity assays, a protein produced in another organism should be used for these assays. Another possibility for the monitoring, which narrows the analysis to certain epitopes, is the use of a set of relevant peptides, that can either be used to select and amplify peptide-specific T cells in vitro or to label directly T-cell receptors with soluble HLA class II tetramers presenting the relevant peptide (27). This approach requires prior identification of these antigenic peptides, as described in this report.

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References


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