Cancer Letters 276 (2009) 61–67

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Out of frame peptides from BCR/ABL alternative splicing are immunogenic in HLA A2.1 transgenic mice

C. Casnici [a], G. Volpe [b], D. Lattuada [a], K. Crotta [a], M. Kuka [b], C. Panuzzo [b], C. Mastrotto [a], G. Tonon [c], V.M. Fazio [d,e], G. Saglio [b], O. Marelli [a,*]

[a] Department of Pharmacology, School of Medicine, University of Milan, Milan, Italy
[b] Department of Clinical and Biological Sciences, University of Turin, Turin, Italy
[c] Bio-Ker, Polaris Scientific Park, Piscinamanna-Pula (Cagliari), Italy
[d] Interdisciplinary Center for Biomedical Research, Section of Molecular Medicine and Biotechnology, University Campus Bio-Medico, Rome, Italy
[e] U.O. Oncology, Istituto di Ricovero e Cura a Carattere Scientifico Casa Sollievo della Sofferenza, San Giovanni Rotondo (FG), Italy

ARTICLE INFO

Article history:
Received 6 August 2008
Received in revised form 23 October 2008
Accepted 24 October 2008

Keywords:
Chronic myelogenous leukemia
Cancer vaccine
Peptide vaccination
HLA A2.1 transgenic mice
BCR/ABL transcripts

ABSTRACT

New, potentially tumor-specific antigens have been described in Bcr/Abl positive leukemias. Besides the main BCR/ABL hybrid fusion transcripts, a small number of transcripts derived from alternative splicing between BCR exons 1, 13, and 14 with ABL exons 4 and 5 have been identified. These variants are expressed in chronic myelogenous leukemia and acute lymphocytic leukemia patients. The transcriptional products were characterized at their C-terminus by a large amino acid portion derived from out of frame (OOF) reading of the *ABL* gene. This OOF peptide is expressed only in leukemic cells and has no homology with known human proteins.

In order to study an *in vivo* model, three 39-amino acid peptides, each corresponding to a third of the whole human OOF peptide sequence, were tested for their capacity to elicit specific immune responses in HLA A2.1 transgenic mice. Peptides A and B, but not C, induced the production of specific antisera, while A and C induced the generation of specific cytotoxic T lymphocytes.

1. Introduction

Characterization of tumor associated antigens (TAAs) is an important step in the development of new immunotherapies for human cancers. Chronic myeloid leukemia (CML), a malignancy caused by the Bcr/Abl fusion oncoprotein, is an attractive candidate for immunotherapy because it is a slow growing malignancy that may allow time for an immune response to be generated against the malignant cells. Peptides derived from the junction region of the Bcr/Abl fusion protein have been considered as potential targets for an immune response against leukemic cells. Despite the intracellular location of these proteins, cellular processing of the fusion proteins results in peptides that can be presented on the leukemic cell surface in association with HLA molecules and be recognized by T cells [5]. The evidence that leukemic cells present HLA-associated immunogenic peptides derived from the Bcr-Abl fusion protein [1–3] suggests the potential for an immune response and vaccine development that may used in an attempt to completely eradicate the leukemic clones.

Currently, vaccination approaches are based essentially on the use of peptides derived from amino acid sequences spanning the e14a2 fusion junction between Bcr and Abl [4,5]. Other approaches have attempted to exploit the presence of proteins over-expressed by CML cells such as proteinase-3 and preferentially expressed antigen of melanoma (PRAME) [6,7].

* Corresponding author. Fax: +39 0250317000/0250317056.
E-mail address: ornella.marelli@unimi.it (O. Marelli).

Abbreviations: OOF, out of frame; HLA, human leukocyte antigen; TAA, tumor associated antigen; CML, chronic myeloid leukemia; ALL, acute lymphocytic leukemia; PRAME, preferentially expressed antigen of melanoma; CTL, cytotoxic T lymphocyte; PNPP, *para*-nitrophenyl phosphate; Igs, immunoglobulins; PBS, phosphate buffered saline.

0304-3835/$ - see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2008.10.032

Recently, potentially tumor-specific antigens in Bcr/Abl positive leukemias have been described. We identified novel hybrid BCR/ABL fusion transcripts arising from alternative splicing between BCR exons 1, 13, and 14 with ABL exons 4 or 5 [8]. Their corresponding proteins are very interesting from an immunological point of view since they contain an initial and correct Bcr peptide attached to a sequence of 112 amino acids arising from an out of frame (OOF) reading of either the entire *ABL* exons 4 and 5 sequence or only a portion of exon 5. This OOF portion is clearly detectable in more than 80% of Ph-positive CML (Philadelphia chromosome) and acute lymphocytic leukemia (ALL) patients independently of which main fusion transcript is present. As these OOF proteins are expressed only by the Philadelphia-positive cells and have no homology with other known human proteins, they could represent a new group of tumor-specific antigens suitable as immunological targets. Peptides arising from frame shifts have also been described in other tumors. Patients with acute myelogenous leukemia have been observed to express six sequence variants leading to an OOF C-terminus of the nucleophosmin protein and variant residues [9]. In addition, in both inherited and spontaneous colorectal cancer, T cell reactivity against several peptides derived from a frameshift mutation in the TGFbRII gene has been detected, suggesting that these newly out of frame epitopes could be attractive targets for cancer vaccines [10].

In our previous work [8] we demonstrated the presence of OOF-peptide-specific T cells in the peripheral blood of CML patients, and the ability of these cells to lyse primary autologous CML cells after *in vitro* sensitization. These observations suggest the importance of OOF fusion proteins as new leukemia antigens for vaccination approaches. In the current study, we evaluated the immunogenicity of three 39-amino acid peptides, each corresponding to a third of the whole human OOF amino acid sequence, in HLA A2.1 transgenic mice. We show that two OOF peptides can induce the generation of specific cytotoxic T lymphocytes (CTLs) able to kill human tumor cell lines in a class I HLA A2.1 restricted manner *in vitro*. Thus, immunization with peptides derived from OOF sequences appears to be an effective approach to generating multiple and potent CTL-mediated immune responses against OOF Abl peptides, suggesting that they may be candidates for CML-specific tumor antigens to be used in attempts at complete eradication of leukemic clones.

2. Materials and methods

2.1. HLA transgenic mice

C57 BL/6 transgenic mice expressing a chimeric class I molecule composed of the α1 and α2 domain of the human HLA A2.1 molecule and the α3 intracellular domain of the H-2b allele were used [11].

2.2. Peptides

Three synthetic peptides (Primm), each 39 amino acids long and corresponding to a third of the Abl OOF amino acid sequence, were used for immunization. The sequences were Peptide A: LRLLREPLQHPGRVGSSSFNGGRRAHHHAP LSSPKAQQA, Peptide B: QQAHCLWCVPQLRQVGDGTHGHH HEAQAGRGPVRGGVRG, and Peptide C: VRGRVEEIQPDG GREDLEGGHHGGGRVLERSCSHERDQTT. The purity of peptides was determined by analytical Reverse Phase-HPLC and is >95%.

2.3. Immunization protocols

Transgenic mice, 7–12 weeks old, were immunized in groups of 6. Immunization was done subcutaneously six times at 3-week intervals and consisted of peptides A, B or C at 100 μg/mouse. Peptides were emulsified in the same volume of Freund's Adjuvant (Sigma). For priming, the emulsion was prepared with Complete Freund's Adjuvant. Each immunization experiment was repeated at least three times.

2.4. Cells

The human chronic myelogenous leukemia cell line K562, positive for Philadelphia chromosome and also for the alternative hybrid BCR/ABL transcript e14a4 [8], was used. K562 cells transfected with the HLA A2.1 gene, in order to induce MHC class I expression on the cell surface, were kindly provided by Dr. C.M. Britten – Tumorvakzinationzentrum (TVZ) of the III Medical Department, Mainz University (Germany) [12].

Cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Fetalclone I – Hyclone – Euroclone), glutamine, and gentamicin (Sigma), and cultured under G418 selection (Sigma).

The expression of HLA A2.1 molecules on the surface of transfected cells was periodically analyzed with a flow cytofluorimeter (FACSCalibur, Becton Dickinson) by direct immunofluorescence using anti-human HLA class I FITC-labeled antibody (BD Pharmigen). Cells were incubated with the antibody for 30 min on ice and washed three times with FACS buffer (see below for protocol). For experimental assays we used cells with a high (90–95%) expression of HLA molecules. Human MCF-7 breast carcinoma cells were grown in DMEM medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin.

2.5. Cytotoxic assay

Splenocytes and lymph node cells from immunized mice were pooled, resuspended in RPMI with 10% FCS and 5×10^{-5} M 2-mercaptoethanol, and then cultured in 24-well plates (Costar) in the presence of K562 cells pretreated with Mitomycin-C (33 μg/mL) (Sigma) and transfected with HLA A2.1 (ratio 20:1). After 5 days, viable cells were collected and used as effector cells in a conventional ^{51}Cr-release assay. Target cells were labeled with $Na_2{}^{51}CrO_4$ (PerkinElmer) and incubated at 37 °C for 1 h. After washing with RPMI 1640 supplemented with 10% FCS, cells were plated in triplicate at a final concentration of 1×10^4 cells/well in 96-well V-bottom Microtiter plates (Greiner Bio-One). Effector cells were added to the ^{51}Cr-labeled target cells at effector to target cell ratios (E:T) ranging from