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Use of major histocompatibility complex class I tetramers to monitor tumor-specific cytotoxic T lymphocyte response in melanoma patients

Abstract There is now considerable evidence that human tumors often express antigens that render them susceptible to lysis by cytotoxic T lymphocytes (CTLs). These findings have raised hope for the development of cancer vaccines to trigger a tumor-specific immune response in cancer patients. To optimize the immunogenicity of cancer vaccines, it is important to improve the monitoring of the immune response. The use of tetrameric soluble major histocompatibility complex (MHC) class I/peptide complexes (''tetramers'') to identify tumor-specific CTLs has shown that these novel reagents allow rapid and accurate analysis of human CTL responses in cancer patients. We have used fluorescence-driven cell sorting to clone tumor-specific CTLs after staining with tetrameric MHC class I/peptide complexes. Analysis of melanoma-infiltrated lymph nodes revealed that strong CTL responses often occur in vivo, and that the reactive CTLs have substantial proliferative and tumoricidal potential.

Key words Melanoma · Vitiligo · Immunotherapy · Tumor immunity

Introduction

A central question in tumor immunology is whether a tumor-specific immune response is capable of controlling tumor progression in cancer patients. Although several animal models have established the role of tumor-specific $CD8^+$ and $CD4^+$ T cells in antitumor immunity, a definitive answer to this question in patients with cancer is still elusive.

Characterization of CTL activity against tumor antigens, whether endogenous or vaccine-induced, has previously been hampered by a lack of suitable assay systems. Previous data have revealed the presence of tumor-specific CTLs in patient samples only after antigenic stimulation and/or prolonged tissue culture, precluding the accurate quantification of these cells or their phenotypic characterization.

Now that a wide range of tumor antigens is available as immunotherapeutic targets [10], the adoptive transfer of CTLs of known specificity has become a research priority. We have previously reported the synthesis of reagents (tetrameric major histocompatibility complex [MHC] class I/peptide complexes, or ''tetramers''), which specifically stain CTLs recognizing melanoma antigens, enabling direct analysis of tumor-specific CTLs ex vivo from both tumor-infiltrated lymph nodes and peripheral blood [1, 3, 4, 7].

Methods

Synthetic peptides

Peptides were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer using F-moc for transient NH2-terminal protection and were analyzed by mass spectrometry. All peptides were >90% pure as indicated by analytical high-performance liquid chromatography (HPLC). Lyophilized peptides were diluted in dimethyl sulfoxide (DMSO) and stored at −20 °C.

Tetramers

Complexes were synthesized as previously described [1, 4]. Briefly, purified human leukocyte antigen (HLA) heavy chain and β2-microglobulin (β2M) were synthesized by means of a prokaryotic expression system (pET R + D). The heavy chain was modified by deletion of the transmembrane-cytosolic tail and COOH-terminal addition of a sequence containing the Bir enzymatic biotinylation site. Heavy chain, β2M, and peptide were refolded by dilution. The 45-kD refolded product was isolated by fast protein liquid chromatography (FPLC) and then biotinylated by BirA (Avidity,

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Denver, CO, USA) in the presence of biotin (Sigma, St. Louis, MO, USA), adenosine 5′-triphosphate (Sigma), and Mg^{2+} (Sigma). Streptavidin-phycoerythrin conjugate (Sigma) was added in 1:4 molar ratio, and the tetrameric product was concentrated to 1 mg/mL.

Patients and samples

Melanoma patients who underwent therapeutic surgical lymph node resection were selected for this study, as previously described [3, 8].

Flow cytometry immunofluorescence analysis

Thawed-cell suspensions were cultured overnight in Iscove's medium supplemented with 5% human serum and then stained at 37 °C with tetramers for 15–30 min. Cells were then washed and FITC monoclonal antibody (mAb) conjugates added at 4 °C for 40 min. Data acquisition and analysis were performed using CellQuest™ software (Becton Dickinson & Co., Mountain View, CA, USA).

Results

Characterization and cloning of tumor-specific CTLs

We have made reagents that specifically stain CTLs recognizing melanoma antigens and studied responses to these antigens by direct fluorescence-activated cell sorting (FACS) analysis of patient lymph node cells and peripheral blood leukocytes (PBLs) (Fig. 1). MHC class I complexes were biotinylated and bound to phycoerythrin-labeled streptavidin to form multimers of one streptavidin molecule bearing four refolded complexes (“tetramers”).

Tetramer-guided flow cytometric sorting was used to isolate melanoma-specific CTLs, allowing their functional characterization, including confirmation of specificity and the ability to lyse autologous tumor cells. CTLs specific for the melanoma antigens melan-A, tyrosinase, and MAGE-3 were cloned from the peripheral blood, tumor-infiltrated lymph nodes, and skin metastases [3, 9]. Tumor-specific CTLs were cloned from $CD8^+$ cells and incubated on plates pretreated with anti-CD3. Cloning efficiency was extremely efficient at between 2% and 13% of sorted clones, which is higher than the frequency obtained with previous cloning methods. CTL clones were isolated and characterized in as little as 6 weeks. Surface phenotyping of tetramer-isolated CTL clones confirmed that all were $CD8^+$ TCR-α,β^+ and $CD45RO^+$ $CD45RA^-$ $CD44^+$ and $CD62L^-$, consistent with previous antigenic stimulation. All tetramer-sorted CTL clones were tested in the chromium release assay and recognized the relevant peptide and tumor cells expressing the relevant target protein.

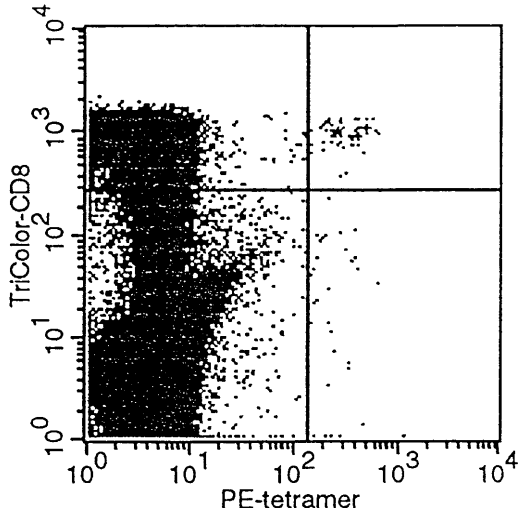

Fig. 1 FACS profile of a melanoma patient peripheral blood lymphocyte stained with melan-A tetramer (X-axis) and anti-CD8 Ab (Y-axis). Frequency of melan-A-specific CTLs is one in 344 $CD8^+$ lymphocytes

We showed that these CTLs express markers compatible with immunotherapeutic use in melanoma, including the cutaneous lymphocyte antigen (CLA) [5], which is associated with homing to skin. We showed that clones generated by this technique are likely to be useful for adoptive immunotherapy, as bulk expansion of CTL clones gave yields up to 2×10^9.

Monitoring of tumor-specific CTLs in melanoma patients

Tetramer staining revealed the presence of much higher numbers of tumor-specific CTLs in tumor-infiltrated lymph nodes than had previously been reported, and most of these cells were antigen-experienced by surface phenotype [8]. The frequency of CTLs specific for the Melan A HLA-A2 epitope ELAGIGILTV was between one in 50 and one in 500 $CD8^+$ LN cells, while that of melan-A tetramer plus $CD8^+$ cells in LN cells from a healthy individual was $<0.05\%$. The frequency of antigen-specific CTLs derived from tetramer staining was compared with frequencies generated using a limiting dilution assay (LDA), which detects CTLs with high proliferative potential. This comparison demonstrated that the frequency of melan-A-specific CTLs was five- to 20-fold lower than that obtained through direct staining with tetramers. During in vitro culture of lymph node cells with cytokines, the expansion of tumor-specific CTLs was directly visualized with tetramers. This CTL expansion could exceed three orders of magnitude over 3 weeks of culture, and was dependent on the presence of tumor cells in the lymph node cell suspension.

Monitoring of melanocyte-specific CTLs in vitiligo patients

We investigated the role of antigen-specific CTLs in the etiology of vitiligo, a discoloration of the skin due to destruction of melanocytes [6]. The observation that

melanocyte proteins are targets for anti-melanoma CTLs raises the possibility that destruction of epidermal melanocytes in vitiligo patients could be due to a melanocyte-specific CTL response. The spontaneous appearance of vitiligo has been associated with improved prognosis in individuals with metastatic melanoma [2].

We described the use of HLA A*0201-peptide tetrameric complexes to identify melanocyte-specific CTLs ex vivo in A*0201-positive patients with vitiligo [7]. No melanocyte-specific CTLs were observed ex vivo in four A*0201-negative patients with vitiligo or in five of six A*0201-positive healthy controls. CTLs from vitiligo patients expressed high levels of CLA [5], indicating that they were able to home to the skin. In contrast, none of the CTLs identified ex vivo from the A*0201-positive healthy controls expressed CLA antigen and therefore were unable to home to the skin. We went on to show a significant positive correlation between disease activity and the frequency of skin-homing, melanocyte-specific CTLs. These data are consistent with a role for such CTLs in causing the melanocytic destruction observed in vitiligo.

Discussion

Antigen-specific human CTL clones have previously been obtained by a laborious process involving the generation of polyclonal lines by repeated rounds of antigen stimulation, followed by the screening of large numbers of clones for antigen-specific effector function. The lack of reliable protocols to quantify CTL responses in vivo makes it difficult to characterize immune responses to tumor antigens in patients and to monitor the efficacy of vaccine trials.

We recently reported the use of tetrameric-soluble MHC class I/peptide complexes to characterize melanoma-specific CTLs ex vivo [3, 4, 6]. We developed reagents that specifically stain CTLs recognizing melanoma antigens and studied responses to these antigens by direct FACS analysis of patient lymph node cells and PBLs. MHC class I complexes were biotinylated and bound to phycoerythrin-labeled streptavidin to form multimers of one streptavidin molecule bearing four refolded complexes (''tetramers''). These tetramers specifically stain CTL clones raised against the appropriate peptide, and in CTL lines raised from patient samples stain an increasing proportion of $CD8^+$ cells with each peptide restimulation [4, 9]. We have shown that CTL specific for one melanoma epitope could be separated from a mixed CTL population using tetramer-driven cell sorting and that such polyclonal CTL lines can efficiently lyse autologous tumor cells [3, 4, 8, 9].

CTL lines sorted for tetramer staining demonstrate antigen-specific killing and γ-interferon release. Melanoma antigen-specific CTLs are detectable by tetramer staining in both patient PBLs and resected lymph nodes. Tetramer staining can be used to isolate and phenotype tumor-specific CTL clones rapidly, making the technique suitable for adoptive immunotherapy of melanoma. The advantages of using tetramer-isolated clones for immunotherapy rather than polyclonal lines include the complete assurance of antigen specificity and the ability to profile individual clones for their likely homing behavior after infusion.

Finally, we showed that vitiligo patients without melanoma have a significant number of skin-homing melan-A-specific CTLs. To our knowledge, this is one of the first descriptions of self-reactive CTLs in a human autoimmune disease and has implications for many related conditions. The lack of homing receptor on the surface of autoreactive CTLs in healthy controls is of importance because it is consistent with a mechanism to control peripheral tolerance in vivo.

These experiments are important to clarify the role of melanocyte-specific CTLs in the pathogenesis of vitiligo and shed light on the presence of the autoreactive immune response in healthy volunteers. Furthermore, they may show possible side effects of an autoreactive melanocyte-specific CTL response in melanoma patients undergoing vaccination trials based on peptides derived from melanocyte differentiation proteins.

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