A T-cell epitope determined with random peptide libraries and combinatorial peptide chemistry stimulates T cells specific for cutaneous T-cell lymphoma

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Summary

Background: Mycosis fungoides is the most frequent T-cell lymphoma of the skin. Despite numerous attempts, no tumour antigens have yet been identified. Only in one case has an idiotype-derived peptide been found to trigger CTL of the respective patient. The identification of natural antigens requires the cultivation of large amounts of tumour cells in vitro, which has been possible in two exceptional cases. The identification of synthetic epitopes for tumour-specific CTL with random peptide libraries can overcome this limitation and is a powerful tool for application in the development of immune therapies for a wide range of patients.

Materials and methods: The critical amino acids for the construction of epitopes for the CTCL-specific CTL clone My-La CTL were determined with synthetic peptide libraries in positional scanning OX8 format in a standard 61 chromium release assay. Sixteen different peptides could be synthesized from the combinatoric of these amino acids with the canonical anchor amino acids for MHC binding. These peptides were tested for their capacity to stimulate My-La CTL and PBMC of an HLA-matched CTCL patient.

Results: A synthetic epitope could be identified for My-La CTL, which was recognized in a HLA-restricted manner. The response towards this epitope was comparable to the response towards their natural target My-La. Using these synthetic epitopes, T cells of a HLA-matched patient could be induced in vitro and led to the establishment of different cell lines and clones. Some of these lines recognized the peptides as well as the allogenic but HLA-matched tumour cell line My-La, indicating that they are specific for a naturally expressed tumour antigen.

Conclusions: The identification of synthetic epitopes for tumour-specific CTL clones can be used for the development of vaccines for immune therapies of cancer; such peptides can be applied inter-individually. Synthetic epitopes must not correspond to the natural ones, but they can be even more potent as stimulation of specific T cells and can be fine-tuned to increase the success of the therapy.

Key words: cutaneous T-cell lymphoma, immunotherapy, peptide library, T-cell epitope, tumour-associated antigen

Introduction

Mycosis fungoides is the most frequent cutaneous T-cell lymphoma (CTCL). It is a low-grade malignancy of unknown aetiology and with a slow progression. Tumour cells and tumour infiltrating lymphocytes (TIL) are located in the dermis as well as in the epidermis in varying ratios [1]. The identification of natural antigens could reveal information about the pathophysiology of the tumour cells and provides tools for monitoring tumour-specific cytotoxic T cells (CTL) as well as for the development of therapeutic vaccination strategies.

In recent years, a number of tumour-associated T-cell epitopes have been identified. Most of them were found for melanoma, a few for renal cell carcinoma [2, 3]. Various attempts to identify tumour-associated antigens for CTCL have failed, and only in one case was an idiotype-derived peptide found to trigger CTL of the respective patient [4]. The identification of tumour antigens is still a major challenge.

Four different strategies are currently in use for the identification of tumour-associated T-cell epitopes:

1) direct isolation and sequencing of MHC-bound peptides by means of high resolution HPLC and mass spectrometry [5] (this approach requires large amounts of tumour cells); 2) preparation of cDNA expression libraries from tumour cells, transfection of the cDNA clones together with the genes coding for the MHC molecules of the patient into appropriate target cells, and subsequent identification of tumour antigen-specific cDNA clones with the patient’s CTL [6] (this approach requires several selection steps and is laborious and time-consuming); 3) epitope scanning of known tumour antigens with overlapping peptides [7]; and 4) scanning of the protein sequence of a known antigen for the dominant binding motifs of the expressed MHC molecules and prediction of putative epitopes [8]. For the latter two approaches, prior knowledge of the protein sequence of the tumour antigen is required; hence, only a restricted number of antigens is available for these techniques. As a consequence of the disadvantages of the above approaches, only a few tumour-associated antigens have been identified so far.

This paper presents a new approach for the identifica-
were prepared. The peptides with defined sequences were synthesized using the Fmoc technique, as described elsewhere [9]. The X amino acids were used for the construction of the molecular peptide library (Figure 1) [14].


designation of tumour-associated T-cell epitopes that overcomes the disadvantages of the above-mentioned techniques. By using synthetic combinatorial peptide libraries, it is possible to identify synthetic epitopes for tumour-specific CTL by positional scanning [9–11]. As CTL predominantly recognizes peptides 9 amino acids in length, nonapeptide libraries are used with one defined amino acid in one of the sequence positions combined with random mixtures of all proteinogenic amino acids in the remaining positions (Figure 1). The amino acids identified by the positional scanning are combined with the canonical MHC anchor residues. The thus designed peptides are synthesized and tested to determine the most potent epitope. This approach can be applied to all cases where tumour-specific CTL are available.

Materials and methods

Peptides and peptide libraries

The synthetic combinatorial peptide library Xg and the OXg sublibraries were synthesized with preixed activated amino acids in solid phase using the Fmoc technique, as described elsewhere [9]. The X positions contain all proteogenic amino acids with the exception of cysteine. thus 171 (19 x 9) sublibraries, each with 19 different peptides, were prepared. The peptides with defined sequences were synthesized using the same technique. The quality of the peptides and peptide libraries was analysed by HPLC and mass spectrometry.

Patients

For the analysis of peptide-specific CD8+ T cells, 16 HLA-B8+ patients suffering from cutaneous T-cell lymphoma were recruited regardless of the state of disease. As control, three healthy HLA-B8+ donors were chosen. All specimens were taken with the consent of the patients.

Cell culture and cell lines

The cells were cultured in media supplemented with 2 mM glutamine, 30 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C in a humidified atmosphere with 8% CO2. The cutaneous lymphoma-specific CTL clone My-La CTL was established from autologous PBMC by repeated stimulation with the tumour cell line My-La and cultured in Minimum Essential Medium α modification (MEMα, GIBCO-BRL, Karlsruhe, Germany) with 5% fetal calf serum (FCS, Serotec Linaris, Berlin, Germany), 5% human serum (PAN Systems, Panssa, Germany), 1000 U/ml recombinant interleukin-2 (IL-2), and 500 U/ml IL-4 [12]. The tumour cell line My-La was grown in Dulbecco minimum essential medium (DMEM, GIBCO, BRL, Karlsruhe, Germany) containing 5% FCS and 100 U/ml IL-2. PBMC from the CTCL patients were isolated by Ficoll-Paque (Pharmacia, Freiburg, Germany) density centrifugation, washed twice, and used directly for intracellular cytokine analysis.

T-cell assays

Combinatorial peptide scanning analysis was done with My-La CTL using a standard 5l chromium (Cr) release assay with 5Cr-labelled My-La as target cells which were pulsed with the peptides (100 μg/ml). Radioactivity released from the target cells was measured in solid scintillator plates in a 96-well plate β-counter (Packard, Dreieich, Germany). Results of at least threefold standard deviations above the background obtained without peptide were considered positive. All tests were done in duplicate.

The peptide-specific CD8+ T cells in the PBMC of the HLA-B8+ CTCL patients and healthy control donors were determined by flow cytometric analysis of intracellular IFN-γ as described in detail elsewhere [13]. Values below 0.03% were considered nonspecific.

Results

Synthetic T-cell epitopes associated with cutaneous T-cell lymphoma

Cytotoxic T cells recognize peptides in complex with MHC class I molecules which are mostly 9 amino acids long and conform to MHC allele-specific epitope motifs [14]. By using a completely randomized nonapeptide library Xg and 171 OXg combinatorial sublibraries, it is possible to analyse the impact of every amino acid at every position of the epitope [10, 11]. All proteinogenic amino acids were used for the construction of the libraries, except cysteine. The OXg sublibraries consist of one defined sequence position O in combination with eight randomized positions X [9]. These libraries were used to determine the synthetic epitope for the lymphoma-specific CTL clone My-La CTL. Since the autologous tumour cell line My-La is poorly lysed by My-La
CTL (10% specific $^{51}$Cr release), it could be used as the target cell\textsuperscript{12}. The critical amino acids for the recognition of My-La CTL were identified in a standard $^{51}$Cr release assay. At least one amino acid could be determined for every position, with the exception of position 9 where no response was detected. At positions 6 and 7, two different amino acids were active (Figure 1). Sixteen different putative epitopes could be designed combining the identified amino acids with the dominant anchor motifs of the expressed MHC molecules, HLA-A1 and B8 [14]. Since tyrosine was the most potent amino acid at position 5, it was included in the sequence of 4 peptides predicted to bind to HLA-B8, although it is not an associated motif amino acid. Two peptides were capable of inducing IFN-$\gamma$ secretion to My-La CTL: PVKTYDAKL (designated YDA) and PVKTYDIKL (YDI, Figure 1). These two peptides were tailored for binding to HLA-B8, indicating that My-La CTL is HLA-B8-restricted in its antigen recognition. The peptides differ only at position 7, where both alanine and isoleucine seem to be acceptable. The peptides with lysine at position 5 instead of tyrosine conform more closely to the canonical HLA-B8-epitope motif, but were less effective than the tyrosine-containing peptides. In sequence database analysis with the active epitope sequences, no match was found.

CD8+ T cells specific for the synthetic epitopes in HLA-B8+ CTCL patients

The two active peptides, YDA and YDI, and a third one, PVKTKDIKL (designated KDI), were used to analyse peptide-specific CD3+, CD8+, and CD69+ T cells in the peripheral blood of HLA-B8+ CTCL patients and healthy control donors. Specifically responding T cells were detected by flow cytometry for intracellular IFN-$\gamma$ induced by the synthetic epitopes [13]. In more than 80% (13 of 16) of the patients, peptide-specific CD8+ T cells could be detected, whereas only in 1/3 of the healthy donors was a slight response to peptide YDA seen [Linnemann et al., submitted]. The majority of reacting T cells (up to 1.42%) expressed CD8 at an intermediate level (Figure 2). CD8 high-expressing T cells specific for the synthetic peptides were less frequent (up to 0.17%, Figure 3). In the patients analysed, repertoire differences of the reacting T cells could be detected since the recognition patterns to the three peptides were different. The results indicate that there must be a natural tumour-associated antigen that is shared by different CTCL patients and which is mimicked by the synthetic T-cell epitopes.

Discussion

The determination of synthetic T-cell epitopes with combinatorial peptide libraries is a new approach for the identification of tumour-associated antigens. In this report, synthetic epitopes for the CTCL-specific CTL clone My-La CTL are presented which mimic a natural tumour-associated antigen. This approach does not require tumour material nor prior knowledge of the protein sequence of the tumour antigen [11]. The detection of peptide-specific CD8+ T cells in HLA-matched patients indicates that there must be a natural antigen that is shared by a high proportion of CTCL patients.
Figure 3 Frequency of synthetic peptide-specific CD8$^+$ T cells in 16 HLA-B8$^+$ patients and three healthy donors as detected by flow cytometry. Results for the healthy donors are represented by rows 17–19. KDI = PVKTKD, YDA = PVKTYD, YDI = PVKTDY; control peptide = peptides that do not bind to the expressed MHC molecules.

Peptides eluted from MHC molecules commonly show a restricted number of different amino acids at two to three positions. These positions are considered to be anchor positions for the binding to the MHC molecules as they are located deeply in the binding cleft and cannot be recognized by the T cells [14]. These binding motifs are frequently not identified correctly by positional scanning because the complexity of peptides competing for the binding is too high [15]. Therefore, it is crucial to determine the HLA-haplotype of the T-cell clones tested. In the past years, a high number of MHC-binding peptides and MHC allele-specific binding motifs have been identified which facilitates the prediction and design of tumour-associated epitopes [14, 16].

In earlier studies, combinatorial peptide libraries were used for the determination of epitopes for murine CTL clones and could be demonstrated to be very potent [17]. Since two T-cell assays are sufficient to define the sequence of the active peptide, this strategy can be performed much faster than other approaches for the identification of tumour-associated antigens. Although the synthetic epitopes mimic the natural ones, they can differ considerably from the cognate natural epitope [17]. However, they tend to be more potent [10, 11]. The synthetic epitopes presented here could be used for the development of vaccines or for the monitoring of tumour-specific T cells in tumour patients, and thereby are suitable for immunomonitoring in immunotherapies. The frequency of peptide-specific T cells in CTCL patients corresponds to the frequencies of tumour-specific T cells in melanoma patients, which are increased in tumour patients in comparison to healthy donors [18, 19]. In the case of melanoma patients, limiting dilutions were used to analyse the frequency of the tumour-specific T cells. Similar results were obtained with tetrameric MHC molecules in cases of virus infections [20]. As different techniques yield the same results, they can be considered reliable.

In conclusion, combinatorial peptide libraries have great potential for the identification of tumour-associated antigens which can be considered as tumour vaccines. Therefore, synthetic peptide libraries offer a powerful means for the development of new therapeutic strategies.

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References


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