Restricted T-Cell Receptor Repertoire in Melanoma Metastases Regressing after Cytokine Therapy

Martina Willauhck, Carmen Scheibenbogen, Michael Pawlita, Thomas Möhler, Eckhard Thiel, and Ulrich Keilholz


Abstract

One major rationale for using interleukin-2 and IFN-α in cancer immunotherapy is to activate tumor-specific T cells at the tumor site. To study the in situ T-cell response, we determined the T-cell receptor (TCR) repertoire in six melanoma metastases regressing after cytokine treatment obtained from five patients. Sequence analysis of overexpressed TCR β-chain variable regions revealed the presence of clonally expanded T cells and also of T cells with highly homologous complementarity determining regions in all five patients. This finding indicates that the T-cell response in regressing melanoma lesions is dominated by T cells directed toward a limited number of epitopes and that epitope-specific T cells frequently use a highly restricted TCR repertoire.

Introduction

T cell-directed therapy of tumors using either cytokines or antigen-specific immunization is able to mediate objective tumor regression in a limited number of patients. Although significant advances in understanding the principle mechanisms of T cell-mediated tumor destruction have been made, little is known which effects are critical in vivo. Although induction of vaccine-specific T cells is thought to play a central role in antigen-specific immunization, possible mechanisms attributed to cytokine-mediated tumor regressions are more complex. Recent studies of the transcriptional profiles within melanoma metastases after systemic IL-2 administration suggest as predominant early effects the activation of antigen-presenting monocytes, the activation of cytolytic mechanisms in monocytes and natural killer cells, and the massive production of chemoattractants which can recruit specific T cells (1). Induction of T cells recognizing melanoma-associated antigens has been shown in peripheral blood from patients after IL-2-based treatment, suggesting their participation in melanoma destruction (2).

More direct evidence supporting the importance of antigen-specific T cells in cytokine-mediated melanoma destruction comes from in situ analyses of the TCR repertoire in metastatic lesions after treatment with IFNα and IL-2. In a previous investigation using semiquantitative reverse transcription-PCR-based analysis of the TCRBV segments used by tumor-infiltrating lymphocytes, we found an association between response to therapy and overexpression of a limited number of TCRBV subfamilies indicative of clonal or oligoclonal expansion of tumor-reactive T cells (3). To further analyze the TCR repertoire of specific T cells, cDNA from overexpressed TCRBV subfamilies was cloned and sequenced, and the amino acid sequence of the CDR3 was deduced. The hypervariable CDR3 region of the TCR embedded between the TCR family-defining BV and variable-joining regions is critical for recognition of the antigenic peptide.

Patients and Methods

Study Population and Tissues. Twelve patients of a cohort of 65 patients we had treated with a regimen of IFNα and high-dose IL-2 had achieved objective tumor regression as described elsewhere (4). Six residual regressing tumor lesions which had been resected from 5 patients in an attempt to surgically complete the partial response have been studied previously for TCRBV distribution (3) and are subject of the extensive sequence analysis in this study.

Preparation of RNA and Synthesis of cDNA. Single-stranded cDNA was synthesized from total RNA extracted from tumor tissue as described previously (3).

TCRBV-PCR. The PCR was performed with 5′-primers specific for the TCRBV subfamilies and 3′-primers specific for the constant region of the BV gene as described previously (3). Briefly, the first strand cDNA/RNA heteroduplex was submitted to 30 cycles of amplification on a thermocycler. The PCR product bands were size purified on a 1% low melting agarose gel for subsequent cloning.

Cloning and Sequencing of PCR-amplified Products. The purified PCR products of the overexpressed TCRBV subfamilies were ligated to a pGEM-T/E vector (T/E Cloning Kit; Promega, Heidelberg, Germany), which was subsequently used to transform competent Escherichia coli DH5α cells. After blue/white screening, 10 white colonies were randomly selected for each overexpressed TCRBV subfamily, and the plasmid DNA was prepared using the plasmid DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s manual. Nucleic acid sequences were determined by the dideoxy chain termination method. Plasmid DNA (4 μg), 10 pmol of Cy5-labeled universal primer, and 8 units of T7 DNA polymerase were used (Cy5 Auto Read Sequencing Kit; Pharmacia, Freiburg, Germany). The sequencing reaction was performed according to the “quick annealing of primer” procedure of the manufacturer’s manual, and sequencing products were electrophoretically separated in an automated sequencer (ALFexpress; Pharmacia).

Results

Clonal Expansion of T Cells in Regressing Metastases. In our previous study, between one and four overexpressed TCRBV subfamilies had been found in each of seven metastases regressing after therapy with IFN-α and IL-2 and originating from 6 patients, whereas no TCRBV overexpression was observed in five progressing metastases from 5 patients (3). Here, we analyzed the CDR3 sequences of T-cell clones in a total of 10 overexpressed TCRBV subfamilies selected from six partially regressing metastases after cytokine therapy obtained from 5 patients (one to three overexpressed TCRBV subfamilies per metastasis were sequenced; Table 1). Ten clones from each overexpressed TCRBV subfamily (in total, 100 T-cell clones) were sequenced.

In 4 patients, overexpressed TCRBVs were found to consist of one or two dominant T-cell clones [≥3 of 10 clones with identical CDR3 sequences: patient 1(L), BV3; patient 3, BV11; patient 4, BV4; patient 5, BV3 of the CDR3 was deduced. The hypervariable CDR3 region of the TCR embedded between the TCR family-defining BV and variable-joining regions is critical for recognition of the antigenic peptide.


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Most clones were present in the lung metastasis twice and abdominal metastasis once. Patient 6, where a neutral amino acid was substituted by a polar amino acid, all other substitutions were by chemically similar amino acids. Identical clones occurring twice were frequently detected. Patient 1 had two metastases, abdominal (A) and in the lung (L). Interestingly, an identical clone (CASS FTASQNPQHG) was found in the lung metastasis twice and abdominal metastasis once.

Clones with Highly Homologous CDR3 Regions Are Frequently Present in Regressing Metastases. Several clones were present in overexpressed TCRBVs, which differed only in one amino acid position in the CDR3, whereas the junctional V/D/J sequences were identical (patient 2, BV11; patient 3, BV11; patient 4, BV4; patient 6, BV7; the amino acid sequences are shown in Table 2). With the exception of the substitution from prolin to glutamin in TCRBV7 in patient 6, where a neutral amino acid was substituted by a polar amino acid, all other substitutions were by chemically similar amino acids, suggesting that the resulting TCR protein structures and antigen-binding capabilities are very similar.

Discussion
The present analysis provides a first insight into the molecular composition of the T-cell response in effective cytokine therapy. Data from this study give evidence that the overexpression of TCRBV subfamilies, found in our previous analysis to be associated with cytokine-induced tumor regression (3), frequently results from clonal expansion of T cells in situ. Although no functional data from specific T-cell clones with identical sequences as those found overexpressed in the tumor lesions are available, the relevance of the overexpressed T-cell clones in the tumor tissue for the immune-mediated tumor regression is supported by the fact that: (a) these T cells are enriched and clonally expanded at the tumor site but are not overexpressed in peripheral blood; and (b) overexpressed or clonally expanded T-cell clones have not or rarely been found in progressing melanoma lesions (3, 5, 6). The presence of clonally expanded T cells indicates that the T-cell response in regressing melanoma lesions is dominated by T cells specific to a limited number of epitopes. In addition, T cells with highly homologous CDR3 regions, most likely recognizing the same epitope, were frequently found in our study, showing that epitope-specific T cells often use highly conserved TCR sequences.

Clonally expanded T cells have also been described in melanoma metastases of patients with spontaneous or vaccine-induced tumor regression (5, 7, 8). Ferradini et al. (7) showed clonally expanded T cells in a case of a spontaneously regressing melanoma. In their study, they succeeded to expand one tumor-specific cytotoxic T cell clone that was able to lyze the autologous tumor in vitro exhibiting the same transcripts as those found overexpressed in situ in the melanoma tissue (8). This finding further supports the relevance of amplified T-cell sequences identified in situ in regressing melanomas for the immune-mediated tumor regression. Manne et al. (5) analyzed inflamed melanoma metastases after vaccination with 2,4-dinitrophenol or 2,4-dinitrophenyl-modified autologous melanoma cells. In their study, dominant CDR3 peaks were found in the melanoma tissue in 9 of 10 patients after vaccination but only in 3 of 16 metastases from nonvaccinated patients. Sequence analysis of dominant peaks revealed in accordance to our results identical clones as well as clones with 50 to >90% homologies of CDR3 sequences in all seven tumors analyzed. Vaccination was associated with tumor regression or long-term freedom from recurrence in 6 of the 10 patients.

Using a similar methodological approach, other investigators have also reported clonal expansion of tumor-infiltrating T cells in untreated or progressing metastatic melanoma but at a much lower frequency (5, 6). In contrast to our results and those in patients after 2,4-dinitrophenol or 2,4-dinitrophenyl tumor vaccination (5), homologies of CDR3 sequences were, however, not (9) or very rarely found (6) in progressing melanoma. Results from other groups analyzing the TCR repertoire in melanoma lesions using CDR3 size distribution or denaturing gel electrophoresis (10), allowing to distinguish between

Table 1 Percentages of overexpressed TCRBV transcripts and number of clones with identical CDR3 regions

<table>
<thead>
<tr>
<th>Patient</th>
<th>TCRBV subfamily</th>
<th>Expression (%) in</th>
<th>Identical clones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tumor</td>
<td>Blood</td>
</tr>
<tr>
<td>1 (A)</td>
<td>3</td>
<td>10.1</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10.6</td>
<td>5.0</td>
</tr>
<tr>
<td>1 (L)</td>
<td>3</td>
<td>10.2</td>
<td>4.0</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>6.4</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>3.3</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>5.1</td>
<td>2.1</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>6.5</td>
<td>3.0</td>
</tr>
<tr>
<td>6</td>
<td>10.5</td>
<td>2.4</td>
<td>2 (J2.1)</td>
</tr>
<tr>
<td>7</td>
<td>10.6</td>
<td>1.5</td>
<td>2 (J1.5)</td>
</tr>
</tbody>
</table>

* Patient number, patient 1 had two metastases, abdominal (A) and in the lung (L).
* Data from reverse transcription-PCR-based analysis of the TCRBV chains as described in Ref. 3.
* Number of identical cDNA clones within 10 parallel clones analyzed per TCRBV subfamily.

Table 2 Amino acid sequences of identical or highly similar CDR3 regions

<table>
<thead>
<tr>
<th>Patient</th>
<th>TCRBV</th>
<th>Expression (%)</th>
<th>Deduced amino acid sequence of the CDR3 region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (L)</td>
<td>3</td>
<td>50</td>
<td>CASS LVN EDFQFGPGLRTLTVLE DLNK</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>10</td>
<td>CASS ESGRGLRE EKYQFGPGLRTLTVLE DLNK</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>50</td>
<td>CASS AKSSPGQGSTGLFQFGPGLRTLTVLE DLNK</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>40</td>
<td>CASS DSGIERQK PQLHFGPGLRTLTVLE DLNK</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>40</td>
<td>CASS LWGAGQTGAE QLHFGPGLRTLTVLE DLNK</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>10</td>
<td>CASS PDLFGST EAFQFGPGLRTLTVLE DLNK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>CASS QDLFGST EAFQFGPGLRTLTVLE DLNK</td>
</tr>
</tbody>
</table>

* Percentage of cDNA clones within 10 parallel clones analyzed per TCRBV subfamily.
* *β* joining region.
* *β* constant region.
oligoclonal and polyclonal T-cell infiltrates, cannot directly be compared with our results because of methodological differences. Schrama et al. (10) found oligoclonal T-cell responses in 4 patients after IL-2-based therapy and in 2 patients treated with chemotherapy, but no sequence analyses had been performed. In accordance with our results in 1 patient, in whom we found an identical clone in two metastases, Schrama et al. also detected identical TCR transcripts in different melanoma metastases from the same patients.

T-cell responses against the melanosomal antigens MelanA/MART-I or tyrosinase analyzed in specific T cells either isolated from patients or generated in vitro generally exhibit a broad and diverse TCR repertoire (11–13). A similar diverse TCR usage of her-2-specific T cells was found in patients vaccinated with her-2 peptide (14). In contrast, there are several reports describing usage of identical or highly conserved TCR sequences among T-cell clones specific for the cancer germ-line antigens MAGE and BAGE (15, 16) and for tumor-specific mutated antigens (17, 18). CDR3 sequence analysis has been performed, however, only in the small study by Farina et al. (15) describing identical and two highly homologous BAGE-specific T-cell clones differing at a single nucleotide. Similarly, identical or highly conserved TCR sequences have been frequently described to be used by T-cell clones specific for viral epitopes, a finding that was generally attributed to the selection of high-affinity T-cell clones (19–21). In one study, sequencing of various T-cell clones specific to two different EBV nuclear antigen peptides revealed a highly restricted TCR repertoire in response to one epitope and a diverse TCR repertoire in response to the other epitope. Functional analysis showed that the restricted T-cell clones used TCRs of high affinity. In contrast, the T-cell clones using diverse and nonoverlapping TCR sequences were of low and high affinity. This finding would be in accordance with the diverse pattern of TCR usage found in T-cell clones against melanosomal antigens, which are usually of low affinity.

Taken together, the results from our study show that the specific TCR repertoire frequently found in cytokine-induced melanoma regression is characterized by clonal expansion of T cells and epitope-specific T cells with highly homologous TCRs. On the basis of the structural and functional data available for T-cell clones against viral and tumor antigens, one may speculate that such a T-cell response pattern as observed in our study is predominantly mediated by high-affinity T cells and not directed against self-antigens (e.g., melanosomal antigens). The similarities of the TCR repertoire in patients responding to cytokine treatment and patients responding to a tumor cell vaccine (5) suggest that a restricted TCR repertoire may be a hallmark of effective antitumor immunity.

References

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