In Vivo Local Expansion of Clonal T Cell Subpopulations in Renal Cell Carcinoma

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ABSTRACT

Renal cell carcinoma (RCC) is one human tumor to which the immune response may control the growth of tumor cells. These tumors are infiltrated by a large mononuclear infiltrate mainly composed of T lymphocytes. To characterize the lymphocytes infiltrating RCC, we analyzed the molecular structure of the T cell receptor (TCR) α and β chains in tumor and paired peripheral blood lymphocytes from a series of 6 untreated patients. We first determined Va and Vβ gene segment usage by PCR using a panel of V specific oligonucleotide primers (Vα1-29 and Vβ1-24). A highly diverse usage of TCR Va and Vβ gene usage was observed in 5 of 6 tumors. In addition, the few tumor overexpressed Vβ specificities detected by reverse transcription-PCR were shown to contain minor T cell expansions. Strikingly, 1 of the 6 tumor studied displayed a skewed TCR repertoire with Vβ4 transcript representing 25% of the TCR signals. Clonality of the tumor overexpressed Vβ transcripts was analyzed by CDR3 size distribution analysis. In the particular tumor displaying a biased repertoire large expansions of T cell subpopulations were detected (particularly in Vβ4) specifically at the tumor site. Such T cells may be expanded locally in response to tumor antigens.

INTRODUCTION

The recent characterization of tumor specific antigens recognized by cytolytic T lymphocytes in melanoma (1–5) is an important step to better understand how the immune system interacts with tumor cells. Although much less studied than melanoma, RCC is another human cancer for which the immune system is thought to play a role in the control of malignant cell growth. Indeed, an overall response rate of about 20% is achieved with systemic interleukin 2 administration, which is now the first line treatment of metastatic RCC (6). Finally, human RCC are infiltrated by numerous lymphocytes, mainly CD3+, α/β+ T cells and few tumor specific T cell lines, have been recently described (7–9). These observations suggest that an antitumor response mediated by T cells may be taking place in RCC.

Mature T cells specifically recognize antigenic peptides presented by MHC molecules through their heterodimeric surface receptor (TCR) which associates the α and the β polypeptides. The specific recognition is dependent upon interaction between the MHC/peptide complex and the variable region of TCR molecules. During T cell differentiation, unique variable region genes are created by recombination of variable (V), diversity (D), and joining (J) segments for the α locus and V and J segments for the β locus. The expression of unique rearranged TCR gene products determines the specificity of a given T cell (12). Identification of recurrent TCR transcripts (same CDR3) in T cell populations indicates antigen driven expansion of the corresponding T cells. Thus, the direct analysis of the molecular structure of TCR polypeptides expressed by TIL is one way to study how tumor cells modulate the T cell repertoire.

Using different PCR approaches, we analyzed in situ the fine molecular structure of TCR α and β chains in TIL and paired PBL from 6 RCC patients. A highly diverse repertoire was found in 5 of 6 tumors, whereas it was strongly skewed in the last tumor. Dramatic clonal expansions of few T cell subsets were indeed identified in the last patient. Such data strengthen the view that antigen driven T cell expansion may occur locally and contribute to the control of the tumor evolution at least in some RCC.

MATERIALS AND METHODS

Patients and Samples. Six previously untreated patients with RCC were studied. There were 4 males and 2 females with a mean age of 55 years (range, 42 to 70 years). All patients underwent radical nephrectomy with regional lymphadenectomy. Clear cell carcinoma was the histological diagnosis in 5 cases (including 2 tumors with papillary structures), whereas the last tumor corresponded to a granular cell variant with eosinophilic staining cytoplasm (patient 5). Regional lymph node involvement by tumor cells was histologically demonstrated in 2 patients. At the time of nephrectomy, 4 patients presented with metastases to lungs (3 cases) and/or to the liver (2 cases). Patients 2 and 4 were free of metastases.

For each patient, samples were collected within the tumor, in normal kidney, and in blood. Tissue biopsies were washed with 0.9% NaCl and immediately frozen in liquid nitrogen for further RNA extraction. Blood samples (30 ml) were collected at the time of nephrectomy. In addition, lymph node tissues were available for patients 1 and 2. Informed consent for blood and tissue samples was obtained from all patients.

TCR Repertoire Analysis. TCR Va and Vβ gene segment usage was determined by reverse transcription-PCR using a panel of experimentally controlled primers specific for the 29 Va and 24 Vβ subfamilies (13, 14). In brief, total RNA was prepared from tumor, lymph nodes, and PBL (0.2–0.5 g tissue or 5 × 10⁶ cells) using RNAzol B and converted to cDNA by standard methods using reverse transcriptase and an oligodeoxynucleotide primer. cDNAs were amplified using the panel of 5′ sense primers as well as a 3′ antisense primer for the Cα and Cβ region. Identification of recurrent TCR transcripts (same CDR3) in T cell populations was assessed by the length of the PCR products after Southern blotting and hybridization with a labeled Cα and Cβ oligonucleotide (15). Comparative analysis of each V product between different samples was achieved by densitometric analysis of the signals on the autoradiographies (16).

Cloning and Sequencing of V Transcripts. The procedure used for cloning and sequencing of Vβ transcripts has been previously described (13, 14). Briefly, sequences of the primers for cloning were Vβ14 5′-GGGTCCG-GCTAAGGCAAGACTAC-3′, Vβ17 5′-CTGCTGAATTTCCCAAGGG-GCC-3′, and Vβ8 5′-CCATGATGCGGGGACTGGAGTTGC-3′. The amplified products were performed in 2 rounds of 30 cycles. After ethanol precipitation the amplified products were separated on a 2% agarose gel and purified by absorption on glass beads (Gene Clean Bio 101, Inc., La Jolla, CA).
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Patient 1

Patient 4

Patient 2

Patient 5

Patient 3

Patient 6

Fig. 1. Vß gene usage in 6 patients with RCC. Relative gene usage in tumor (tum), paired PBL, and tumor draining lymph nodes (ln). The autoradiographic spots were expressed as respective percentages of the sum of all Vß spots detected on the autoradiogram.

material was directly ligated into a EcoRV/SacII-cut PBS-SK+ cloning vector (Stratagene, La Jolla, CA). Competent XL-1 Blue Escherichia coli strains (Stratagene) were transformed and plated for blue/white color selection on media containing 5-bromo-6-chloro-3-indolyl-ß-D-galactopyranoside. The white colonies were screened by the dot blot technique and a Cß oligonucleotide probe. Plasmid DNA was extracted from positive colonies and sequenced by the deoxy chain termination procedure (Sequenase 2.0; United States DNA Chemical Corp., Cleveland, OH).

CDR3 Size Analysis of Vß Transcripts. To extend the analysis to a large number of different Vß specificities, a PCR-based runoff methodology was performed as described previously (17, 18). Briefly, cDNA copies of 0.1 µg RNA were amplified in 40 cycles of Vß1–24/Cß PCR in 50 µl, and aliquots (2 µl) were copied in 1–5-cycle runoff reactions primed with fluorescent (ABI fluorophore Fam)-labeled oligonucleotides specific for Cß or Jß primers. The runoff products were then subjected to electrophoresis on an ABI (Applied Biosystem) sequencer in the presence of fluorescent size markers and analyzed by Immunoscope software.4

RESULTS

TCR Vα and Vß Gene Usage in Tumor, Lymph Nodes, and PBL from Six Renal Cell Carcinomas. TCR V gene usage was analyzed by reverse transcription-PCR in tumor, paired PBL, and tumor draining lymph nodes (2 cases) in six RCC patients. Starting from RNA, cDNA were synthesized and used as a template of the DNA amplification reaction. The amplified material was revealed by autoradiography. Each Vα and Vß gene product was obtained with the expected size (deduced from the positions of the V and C primers on the cDNA sequence) varying from 250 to 535 base pairs. Reproducible results were obtained starting from tumor, lymph nodes, and PBL (data not shown). No reproducible amplifications of TCR V genes were obtained from normal kidney tissue, probably because it contains too few lymphocytes. Densitometer analysis was performed to facilitate comparative analysis from differently exposed autoradiograms. Each V signal was expressed as a percentage of the sum of all Vα or Vß signals detected on the autoradiogram.

It was possible to compare the relative representation of a given Vα or Vß gene in the different samples given that the same Vα/Cα or Vß/Cß primer pair was used in PCR reactions. Variations in signal intensities were estimated by comparing the ratio of each V signal observed in tumor to that of PBL or tumor draining lymph nodes. Ratios >2 on two separate experiments were considered to be relevant. Histograms in Fig. 1 summarize the results of TCRß gene usage obtained in PBL, tumor, and tumor draining lymph nodes. PBL from the 6 RCC patients express all the TCR Vα and β genes, as observed

4 C. Pannier, unpublished observation.
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Table 1 TCR β chain analysis expressed by tumor and PBL in patient 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of sequences</th>
<th>No. of recurrences (%)</th>
<th>Vβ</th>
<th>NDBN</th>
<th>JB</th>
<th>Recurrent transcript size (nt)</th>
<th>Jβ1 (123456)</th>
<th>Jβ2 (1234567)</th>
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<td>Tumor</td>
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<td></td>
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<tr>
<td>Vβ14</td>
<td>18</td>
<td>6 (33)</td>
<td>CASS</td>
<td>LPQGN</td>
<td>EOYFG (2.7)</td>
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<td>200000</td>
<td>1020100</td>
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<td></td>
<td></td>
<td></td>
<td>CASS</td>
<td>LQGRGD</td>
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<td></td>
<td></td>
<td>CASS</td>
<td>RNQGR</td>
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<td>Vβ17</td>
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<td>PGGD</td>
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<td>138</td>
<td>001000</td>
<td>0010000</td>
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<td>Vβ8</td>
<td>10</td>
<td>3 (30)</td>
<td>CASS</td>
<td>NQGR</td>
<td>TCEAPFG (1.1)</td>
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<td>1100002</td>
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<td>Vβ17</td>
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Identification of Recurrent TCR Transcripts. The presence of recurrent TCR transcripts in tumor and PBL was determined by cloning and sequencing PCR-amplified cDNA from overexpressed Vβ14, Vβ17, and Vβ8 transcripts in patient 1's tumor. V and J segments were assigned for each cloned transcript and the size of the transcripts was determined (Table 1). A predominant transcript with unique Vβ17-PGGD-Jβ1.1 rearrangement accounted for 71% (5 of 7) of Vβ17 in frame transcripts in the tumor. Vβ14 tumor transcripts used two different Vβ14 gene segment members. Six Vβ14 cloned cDNAs of 18 (33%) displayed the same rearrangement using Jβ2.7. Less striking recurrences were also observed (Vβ14/Jβ2.1, Vβ14/Jβ2.2 and Vβ8/Jβ1.1). In contrast, Vβ17 PBL transcripts were devoid of any junctional region recurrence. Overall, in spite of the limited number of sequences performed, recurrent TCR species are obviously transcribed in this tumor.

CDR3 Analysis of Vβ/Cβ PCR Products on Bulk cDNA. To extend the analysis to other Vβ specificities and to a second patient,
we examined CDR3 size distribution by runoff analysis, thus circumventing the cumbersome process of sequencing large numbers of TCR transcripts. The corresponding V\beta/C\beta products were copied with a nested fluorescent C\beta. The sizes of the fluorescent runoff products were determined by electrophoresis on an automated DNA sequencer as described previously (17, 18). Since the positions of 5' and 3' primers are fixed, size variations of the runoff products are due only to different CDR3 lengths reflecting the imprecise V/D/J joining mechanism and peaks spaced by 3 nt correspond to in frame transcripts (18, 19). The graphs representing CDR3 size patterns were normalized to 100% for the most intense peaks and data used to generate these graphs could be used to determine the intensity of each peak and to evaluate the background (Fig. 2).

Analysis of CDR3 size distribution of V\beta/C\beta PCR products in patient 1 showed that most tumor V\beta transcripts give intense peaks with a bell shaped pattern of about 8 peaks with the major peak corresponding to CDR3 sizes of 9–11 amino acids, specially V\beta8, V\beta14 and V\beta17. In PBL, all V\beta genes displayed bell shaped patterns with the exception of V\beta1 which displayed a prominent peak of 189 nt (Fig. 2A).

In patient 2's tumor, most of V\beta transcripts gave polyclonal patterns of low intensity whereas few V\beta/C\beta combinations give intense signals (>20,000) with monoclonal patterns such as V\beta4 (corresponding to 25% of the signals on autoradiography), V\beta13 (7%), and V\beta 21 (2%) (Fig. 2B). V\beta4/C\beta tumor transcripts contain a prominent peak of 192 nt and a shorter and hardly detectable one of 183 nt (Fig. 2B, arrow) which will be further defined (see below). In PBL and lymph nodes, all V\beta/C\beta displayed polyclonal patterns with few exhibiting prominent peak, such as V\beta17 in PBL (Fig. 2B).

**CDR3 Size Analysis of V\beta/J\beta PCR Products.** To strengthen the sequence analysis done on tumor 1, V\beta14, V\beta17, and V\beta8 PCR products were copied using fluorescent J\beta1.1, J\beta2.1, and J\beta2.7 primers according to sequencing results. Prominent and intense V\beta14/J\beta2.7, V\beta17/J\beta1.1 and V\beta8/J\beta1.1 rearrangements were detected in the tumor whereas other V\beta/J\beta combinations exhibited polyclonal patterns (Fig. 3). The size of the recurrent transcripts (Table 1) matched the size of the prominent runoff peaks, demonstrating once again the consistency of the sequencing and CDR3 size analysis approaches (19, 20). In paired PBL, these rearrangements were not standing out. In addition, a prominent peak was detected in PBL in V\beta1 (Fig. 2); it contains an intense V\beta1/J\beta1.2 rearrangement which was present in the tumor (data not shown).

In patient 2, it was particularly interesting to refine the clonality of V\beta4, V\beta13, and V\beta21 transcripts which displayed intense monoclonal patterns when copied with a C\beta fluorescent primer. PCR products from tumor, PBL, and lymph nodes were copied with the 13 different
fluorescent Jβ runoff primers and the main results are shown in Fig. 4. For Vβ4 tumor transcript, single peaks in Vβ4/Jβ2.2 and Vβ4/Jβ2.3 (5 times more intense than Jβ2.2) runoff copies were obtained; the respective sizes and relative intensities of these rearrangements matched with the corresponding peaks in the V-C runoff taking into account the position of the corresponding nested Jβ primers. Runoff using other Jβ primers give background fluorescent signals as illustrated for Jβ2.6 suggesting that tumor Vβ4 transcripts included mainly clonal expansions rearranged with Jβ2.2 and Jβ2.3. Vβ4/Jβ2.2 and Vβ4/Jβ2.3 runoff gave polyclonal patterns in paired PBL and in lymph nodes. However, in PBL as well as in lymph nodes, we detected a clonal Vβ4/Jβ2.6 rearrangement absent from the tumor (Fig. 4). Tumor Vβ13 transcripts contained three clonal rearrangements using Jβ2.1, Jβ2.6, and Jβ2.7 with Vβ13/Jβ2.6 and Vβ13/Jβ2.7 accounting for more than 90% of the total intensity. These rearrangements have almost similar sizes and thus could not be distinguished in Vβ/Cβ runoff analysis (Fig. 3). They displayed polyclonal patterns in PBL and lymph nodes (Fig. 4). The Vβ21 transcript give one clonal rearrangement using Jβ1.2 (not prominent in PBL and lymph nodes) whereas other Jβ transcripts gave background signals (data not shown).

In PBL, Vβ17 (Fig. 2B) contained a Vβ17/Jβ2.3 rearrangement which is also prominent in the tumor but not in lymph nodes.

**DISCUSSION**

Using different PCR approaches, we analyzed here the fine molecular structure of TCR α and β chains in TIL in situ and paired PBL from 6 RCC patients. A highly diverse repertoire was found in 5 of 6 tumors, whereas it was strongly skewed in the last tumor.

Several aspects of our approach deserve discussion. Here, the molecular structure of the TCR Vα and Vβ chains was directly analyzed in tumor and paired PBL without any cell culture, thus avoiding in vitro manipulation which may skew the initial V gene representation (21). Although the initial Vβ/Cβ PCR reactions are not strictly quantitative since primer efficiency may vary from one primer pair to another, conditions of amplifications have been carefully validated to avoid saturated amplifications before densitometric analysis (15). This first level of TCR repertoire analysis is appropriate to select in situ overexpressed V specificities. Indeed, such selected V transcripts were previously found to contain recurrent rearrangements in melanoma (22, 23), head and neck tumors (20), chronic lymphoid leukemias (24), and acute graft versus host disease (19). Furthermore, previous studies on several tumor tissues using the two approaches (cloning/sequencing and runoff analysis) showed that sequence recurrences corresponded to prominent peaks in runoff reactions (19, 20). The high consistency of results obtained independently with the same PCR products by these two methods was also evidenced by the present study. Since cloning and sequencing experiments are
extremely tedious, it is clear that runoff methodology with its powerful resolution level allowing extensive and precise characterization of T cell repertoire is useful to study a large number of samples.

In the 5 patients exhibiting an unrestricted TCR repertoire, some overexpressions of few Vβ gene segments were detected by quantitative densitometric analysis and were further shown to contain recent Vβ transcripts. Minor expansions of clonal T cells may thus be detected within tumor even when Vα and Vβ gene usage is apparently not skewed. In contrast, patient 2’s tumor contained a large overexpression (25%) of Vβ4 transcript consisting of two major rearrangements (Vβ4/Jβ2.2 and Vβ4/Jβ2.3). Several other clonal expansions were found within Vβ13 and Vβ21 tumor transcripts. The dramatic intensity of T cell expansions detected in this tumor may be related to its particular clinical presentation. It was a small clear cell renal carcinoma, accompanied by several enlarged lymph nodes that were tumor free and in adipsic regression, suggesting that tumor growth has been partially controlled by immune mechanisms. In addition, rare tumor regression occurring spontaneously or after nephrectomy has been documented although the role of the immune response in this phenomenon is not known (25). The results obtained in this tumor are reminiscent of those obtained in a case of regressive melanoma where a drastically increased expression of clonal Vβ16 transcripts was evidenced (22) in the tumor. Such cells selected in vitro with the use of a specific Vβ mAb were found cytotoxic for the tumor cells (26).

In contrast to melanoma, RCC are difficult to grow in culture and few specific T cell lines have been yet described (8, 9). Worth mentioning is the work of Schendel et al. (9) who derived a tumor specific T cell line from TIL by in vitro stimulation with tumor cells. This specific T cell line displayed a limited TCR heterogeneity suggesting that TIL with restricted TCR repertoire expand upon tumor cell stimulation, although TCR repertoire had not been determined before stimulation. In addition, in a patient with von Hippel Lindau disease receiving autologous tumor vaccine, in situ expansion of Vβ13.1 T cells (up to 30%) was found in a regressive lung metastasis but not in the progressive renal tumor (27). Our present results showing large in situ expansions of clonal T cells in some RCC tumors constitute additional data suggesting the presence of tumor specific T cell effectors at least in some RCC. However, before any firm conclusions can be drawn, the biological response of these expanded T cell clones to tumor antigens must be evidenced.

REFERENCES


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