Heterogeneous Expression of the Tumor-associated Antigens RAGE-1, PRAME, and Glycoprotein 75 in Human Renal Cell Carcinoma: Candidates for T-Cell-based Immunotherapies

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Abstract

It has recently been shown that tumor-associated antigens (TAAs) can evoke tumor-specific T-cell-defined immune responses in cancer patients, thereby offering the possibility of treating patients with such antigens. To develop T-cell-based immunotherapeutic approaches for renal cell carcinoma (RCC), we studied the mRNA expression profile of the TAAs RAGE-1, tyrosinase, MAGE-1, MAGE-2, NY-ESO-1, Melan-A/MART-1, glycoprotein (gp) 75, gp100, β-catenin, PRAME, and MUM-1 in 14 human RCC cell lines and in tissue specimens of 37 primary RCCs, 2 related metastases, and 33 specimens of normal renal epithelium. Reverse transcription-PCR was performed with TAA-reactive primers, and the specificity of the PCR products was confirmed by Southern blot and/or direct sequencing. PRAME (10 of 14 cell lines), RAGE-1 (7 of 14 cell lines), and gp75 (4 of 14 cell lines) antigens were expressed in a high percentage of RCC cell lines, although the level of TAA expression varied among the different RCC cell lines. However, low levels of TAA expression in RCC cells are sufficient for recognition by TAA-specific CTLs. Transcription of tyrosinase, Melan-A/MART-1, MAGE-1, MAGE-2, NY-ESO-1, gp100, β-catenin, and MUM-1 was not detected in any RCC cell line. Approximately 50% of surgically removed neoplasias expressed at least one TAA. RAGE-1 mRNA expression was found in 8 of 39 (21%) RCC samples, PRAME mRNA expression was found in 15 of 39 (40%) RCC samples, and gp75 mRNA expression was found in 4 of 39 (11%) RCC samples, but the expression levels of these TAAs were heterogeneous in the different RCC lesions. One RCC specimen expressed MAGE-2, whereas transcription was not detected in any RCC specimen for MAGE-1, NY-ESO-1, tyrosinase, Melan-A/MART-1, gp100, β-catenin, and MUM-1. The normal kidney epithelium samples were negative for any TAA tested. Thus, RAGE-1, PRAME, and gp75 expression is found with a different frequency in surgically removed lesions and in RCC cell lines, suggesting that a subgroup of RCC patients could be selected for immunotherapeutic strategies that may benefit from immunization against the RAGE-1, gp75, and/or PRAME antigens. However, additional targets for T-cell-based immunotherapy of RCC have yet to be identified.

Introduction

In recent years, a number of TAAs recognized by CTLs in the context of appropriate MHC class I molecules have been characterized, giving new impetus to the treatment of patients with immunotherapy (1, 2). Thus far, three main categories of CTL-defined determinants exist (1, 3): (a) differentiation-specific antigens that are expressed only in melanoma and in normal melanocytes, such as tyrosinase (4, 5), Melan-A/MART-1 (6), gp75 (7), and gp100 (8); (b) cancer/testis antigens, such as the MAGE gene family, BAGE, GAGE, and NY-ESO-1, which are expressed in a significant proportion of tumors of distinct histological origin but are silent in normal adult tissue, except in the testis (9–14); and (c) antigens caused by point mutations of ubiquitously expressed genes that are present in a variety of individual tumors (15–17). Due to their expression pattern in cancers and the existence of antigen-mediated autoimmunity, TAAs represent attractive targets for antigen-specific immune responses. Indeed, a number of melanoma-associated antigens are currently being tested in clinical trials for the treatment of metastatic melanoma (18–20). Immunization with peptides derived from gp100, Melan-A/MART-1, and tyrosinase-induced delayed-type hypersensitivity reactions, peptide-specific CTL responses, and, in some cases, objective tumor regressions (19, 20).

RCC represents the most common malignancy of the kidney. The morphological classification according to Thoenes et al. (21), which was confirmed by cytogenetic analysis (22), distinguishes between clear cell RCC and chromophilic/papillary RCC, both of which originate from the proximal tubulus, and chromophobe RCC, which originates from the distal tubulus. In contrast to chromophobe tumors, clear cell RCC and chromophilic/papillary RCC possess a high metastatic potential, and the prognosis of RCC patients in these subgroups is very poor. Until now, the benefit of conventional therapies, such as surgical, radiological, or chemotherapeutic approaches, has been quite limited. Due to a remarkable spontaneous regression rate, the existence of lymphocytic infiltrates, and some objective remission in clinical trials with immune response modifiers, RCC seems to represent an attractive target for immunotherapeutic strategies using TAAs (2, 23, 24).

The main prerequisites for the successful implementation of TAAs are: (a) adequate expression of the target antigen(s) in tumor lesions; (b) a tumor antigen concentration adequate for efficient CTL recognition; and (c) conservation of the MHC class I restriction element(s) throughout tumor progression (25–27). The absence or low expression of these genes can abrogate or lead to poor immune responses (28). Therefore, it is of interest to evaluate the expression pattern of TAAs in human tumors (29, 30). To gain insight into the suitability of TAA-based immunotherapy for RCC patients, it is essential to evaluate the abundance of TAA expression in an appropriate number of RCC cell lines and tumor specimens of RCC. Here we determine the expression of MAGE-1, MAGE-2, NY-ESO-1, tyrosinase, Melan-A/MART-1, gp75, gp100, MUM-1, β-catenin, RAGE-1, and PRAME in samples of primary RCC, metastases, and normal kidney epithelium. Data from studies like this one will be mandatory to estimate the potential of specific RCC immunotherapy.
the chronic myelogenous leukemia cell line K562; and (?) the acute T-cell gastric carcinoma cell line MKN-45: (c) the RCC cell line LE921 IRC (33); (d) Germany. Table 2).

4 Unpublished observations.  

The following cell lines were used as positive controls in PCR analyses; (a) the melanoma cell lines Mel-624, MZ2-Mel (10), and SK29Mel-l (32); (b) the RCC cell lines designated with the prefix LE were a kind gift of Prof. P. Schrier (University Hospital of Leiden, Leiden, the Netherlands). The RCC cell lines were kindly provided by E. Hilmes (University Hospital, Mainz, Germany).

4 The sequences of the different primers were derived from the published sequences using the Primer Designer Program (Version 2.01; Scientific & Educational Software). 

f. forward; b. backward.

Table 2 TAA mRNA expression in various RCC cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>HLA phenotype</th>
<th>Expression</th>
<th>CTL lysis</th>
<th>PRAME expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>MZ1257RC</td>
<td>A2, A3, B7, B44, CW5, CW7</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MZ1851RC</td>
<td>A1, A2, B7, CW4, CW7</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MZ1940RC</td>
<td>A1, A23, B37, B50, CW6</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MZ1971RC</td>
<td>A11, A30, B13, B18, CW5, CW6</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>MZ1771RC</td>
<td>A1, B2, B8, B40, CW7, CW10</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MZ1795RC</td>
<td>A3, A29, B7, CW7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LE8915RC</td>
<td>A1, B37, CW6</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>LE8901RC</td>
<td>A1, B13, B37, CW6</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>LE8903RC</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>LE8904RC</td>
<td>A1, B27, CW7, CW10</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>LE9104RC</td>
<td>A2, B7, CW4, CW5</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>LE9211RC</td>
<td>A3, A11, B7, B35, CW4, CW7</td>
<td>+</td>
<td>+</td>
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<tr>
<td>NW232RC</td>
<td>A1, B51, BW4, CW2</td>
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<td>ND</td>
<td></td>
</tr>
<tr>
<td>NW2514RC</td>
<td>A1, B2, B7, CW6, CW7</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

a Specific PCR product detectable/lysis by RAGE-1-specific CTLs; - no detectable PCR amplification product/no detectable anti-RAGE CTL mediated lysis.

Materials and Methods

Cell Lines and Tissue Culture. Fourteen cell lines derived from primary RCCs and designated with the prefix MZ or NW were established by Knuth et al. (31). The RCC cell lines designated with the prefix LE were a kind gift of Prof. P. Schrier (University Hospital of Leiden, Leiden, the Netherlands). The HLA phenotype of the different RCC cell lines, as determined by standard PCR analyses, was kindly provided by E. Hilmes (University Hospital, Mainz, Germany).

The following cell lines were used as positive controls in PCR analyses: (a) the melanoma cell lines Mel-624, MZ2-Mel (10), and SK29Mel-l (32); (b) the gastrin carcinoma cell line MKN-45; (c) the RCC cell line LE9211RC (33); (d) the chronic myelogenous leukemia cell line K562; and (e) the acute T-cell leukemia cell line Jurkat. MKN-45, Jurkat, and K562 cells were obtained from the American Type Culture Collection (Rockville, MD).

All cell lines were maintained in RPMI 1640 (Biochrom KG, Berlin, Germany) supplemented with 10% (v/v) FCS (Greiner GmbH Frickenhausen, Germany), 2 mm l-glutamine, 20 units/ml penicillin, and 200 units/ml streptomycin. The cells were harvested by trypsinization and washed with PBS before RNA extraction.

Patients and Tissue Samples. Representative tissue samples from RCC tumors and normal epithelial kidney tissue at a site distant from the tumor were obtained from patients who had undergone radical nephrectomy. In total, 37 primary RCCs, 2 metastases, and 33 normal kidney specimens were collected at resection. Histopathological classification of each tumor was performed according to the criteria proposed by Thoenes et al. (21). These data, including gender, stage of disease, tumor invasion, and lymph node involvement according to the TNM classification system, are listed in Table 3. All tissue samples...
were immediately frozen in liquid nitrogen after surgical resection and stored at \(-70^\circ\text{C}\) until RNA extraction.

**RT-PCR Analysis and Sequencing.** Total cellular RNA was extracted by the guanidinium isothiocyanate/cesium chloride procedure, as described previously (31). All oligonucleotides used for PCR amplification and sequence analysis were generated using the Primer Designer program (Version 2.01; Scientific & Educational Software), synthesized by the triester method, and purchased from MWG Biotech GmbH (Ebersberg, Germany). The TAA-specific nucleotide primers and the PCR conditions used are listed in Table 1.

For the classical RT-PCR analysis, 1 \(\mu\)g of RNA was reverse-transcribed to cDNA with 10 units of avian myeloblastosis virus reverse transcriptase (United States Biochemicals, Bad Homburg, Germany) in the presence of 40 units of RNasin (Boehringer Mannheim, Mannheim, Germany) in the guanidinium isothiocyanate/cesium chloride procedure, as described previously (31). All oligonucleotides used for PCR amplification and sequence analysis were generated using the Primer Designer program (Version 2.01; Scientific & Educational Software), synthesized by the triester method, and purchased from MWG Biotech GmbH (Ebersberg, Germany). The TAA-specific nucleotide primers and the PCR conditions used are listed in Table 1.

For the classical RT-PCR analysis, 1 \(\mu\)g of RNA was reverse-transcribed to cDNA with 10 units of avian myeloblastosis virus reverse transcriptase (United States Biochemicals, Bad Homburg, Germany) in the presence of 40 units of RNasin (Boehringer Mannheim, Mannheim, Germany), 2 \(\mu\)l of hexanucleotide mix [0.5 \(\mu\)M Tris-Cl (pH 7.2), 0.1 \(\mu\)M MgCl\(_2\), 1 \(\mu\)M DTT, 2 mg/ml BSA, 62.5 units/ml random primer; Boehringer Mannheim], and 250 \(\mu\)M of each deoxynucleotide triphosphate (Boehringer Mannheim) in the guanidinium isothiocyanate/cesium chloride procedure, as described previously (34). For PCR amplifications, 100 ng of reverse-transcribed cDNA was used as a template in 50 \(\mu\)l of 1X PCR buffer [20 \(\mu\)M Tris-Cl, 2.5 \(\mu\)M MgCl\(_2\), and 50 mM KCl (pH 8.3; Boehringer Mannheim)], 50 pmol of each specific oligonucleotide primer, 250 \(\mu\)M of each deoxynucleotide triphosphate, and 1.5 units of Taq DNA polymerase (Boehringer Mannheim). Amplifications were performed with a hot start of 5 min at 94°C, and the PCR was terminated with an end extension for 10 min at 72°C. Primers for the housekeeping gene \(\beta\)-actin were used as a control for the amplifiability of the RNA used.

One-step PCR was performed using the Titan kit (One Tube RT-PCR System; Boehringer Mannheim) according to the manufacturer’s instructions. \(\beta\)-actin- and TAA-specific PCR was performed in the same reaction using the following conditions: 10 cycles of 20 s at 94°C, 30 s at the annealing temperature, and 1 min at 68°C followed by 25 cycles of 1 min at 94°C, 1 min at the annealing temperature, and 1 min at 68°C with a cycle elongation of 5 s for each cycle.

The PCR products were fractionated by size and visualized on a 1% agarose gel containing ethidium bromide. To confirm specificity, the gels were blotted onto Hybond-N membranes (Amersham, Braunschweig, Germany), and filters were then processed with digoxigenin-labeled oligonucleotides using the digoxigenin chemiluminescence detection kit (Boehringer Mannheim) and exposed to Reflection autoradiography film (New England Nuclear Life Science Products, Cologne, Germany). Samples were tested at least twice. In addition, the PCR products of tumor samples expressing gp75 and/or MAGE-2 were directly sequenced as described previously (35).

**Cytotoxicity Assays.** The sensitivity of target cells to lysis by the HLA-B7-restricted anti-RAGE-1 CTLs was evaluated by a standard 4-h \(^{51}\)Cr release assay (36, 37).

**Results**

**Expression of RAGE-1, PRAME, and gp75 in RCC Cell Lines.** Fourteen cell lines derived from primary RCCs were analyzed for the mRNA expression of tyrosinase, MAGE-1, MAGE-2, Melan-A/MART-1, gp75, gp100, NY-ESO-1, \(\beta\)-catenin, PRAME, RAGE-1, and MUM-1. As shown in Table 2, a high percentage of RCC cell lines express RAGE-1 (7 of 14 cell lines) and PRAME (10 of 14 cell
Expression of TAAs in Surgically Removed RCC Lesions. The results and the clinical and pathological features of the cases studied are summarized in Table 3. Based on the immunohistogenetic classification according to Thoenes et al. (21) and Stöcker et al. (22), 39 surgically removed RCCs have been characterized as 25 primary clear cell RCCs, 2 metastases, 2 chromophobe RCCs, 6 chromophobe/papillary RCCs, 2 mixed RCCs, and 2 nonclassified RCCs. Samples of corresponding (23 samples) and unrelated (10 samples) normal kidney epithelium served as controls. These tissue samples were analyzed for mRNA expression of the different T-cell-defined TAAs. As shown in Table 3, more than 50% of primary RCCs expressed at least 1 TAA, 5 of 39 RCC lesions simultaneously expressed 2 TAAs, and 2 of 39 RCCs expressed 3 TAAs. RAGE-1 mRNA was expressed in 8 of 39 (21%) RCC specimens, and PRAME mRNA was expressed in 15 of 39 (40%) RCC specimens. MAGE-2 expression was only found in one RCC lesion (MZ2572T), which also expressed the antigens RAGE-1 and PRAME. Expression of the gp75 antigen was detected in 4 of 39 (11%) RCCs. False positive RT-PCR results could be excluded by direct sequencing of the gp75 PCR amplification products. PRAME, RAGE-1, MAGE-2, and gp75 were not expressed in any of the matched and nonmatched control samples of normal renal tissue. Furthermore, none of the tumors or normal kidney epithelium expressed MAGE-1, tyrosinase, Melan-A/MART-1, gp100, β-catenin, and MUM-1 (Table 3). A distinct TAA expression profile as well as heterogeneous levels of TAA mRNA expression are found in RCC lesions. This is shown for PRAME and gp75 in selected tumor specimens (Fig. 2). In all cases, the level of PRAME and gp75 expression was significantly higher than that of RAGE-1.

Discussion

With the exception of RAGE-1 and NY-ESO-1, the T-cell-defined TAAs analyzed in this study have been isolated from human melanoma cell lines (1, 2). In contrast to the differentiation-specific antigens tyrosinase, Melan-A/MART-1, gp100, and gp75 (40), the cancer/testis antigens PRAME, RAGE-1, NY-ESO-1, and MAGE as well as antigens caused by point mutations (MUM-1, β-catenin) are expressed in tumors of distinct histology including various carcinomas and sarcomas as well as lymphocytic leukemias (14, 41–44). Little information was available on the expression of these TAAs in RCC.

The present study demonstrates that individual TAAs were expressed both in surgically removed primary lesions and in RCC cell lines. A heterogeneous expression pattern of the PRAME, gp75, and RAGE-1 antigens was detected in a subset of RCCs, but in all cases, the expression of PRAME and gp75 was more pronounced compared with that of RAGE-1. In addition, the level and incidence of PRAME, gp75, and RAGE-1 expression were higher in RCC cell lines than they were in fresh tumors. For example, RAGE-1 transcription is present in >50% of RCC cell lines and in approximately 20% of the RCC tumors analyzed, and RAGE-1 mRNA expression is more pronounced in cell lines than it is in tumor lesions. The discrepancy in TAA expression observed between RCC cell lines and freshly isolated tumor specimens has not yet been classified. It could be due to clonal selection or a rapid turn-over or short half-life of TAA-specific mRNA in surgical specimens. Preliminary data using a RAGE-1-specific polyclonal antibody revealed RAGE-1 protein expression in RT-PCR-positive tumors, thereby arguing for clonal selection during the tissue culture of RCC.

3 B. Seliger, personal communication.
protein expression in RCC lesions demonstrate that the immunohistochemical data perfectly matched the RT-PCR results for RAGE-1 expression. A comparison of mRNA and protein expression of the antigens gp100 and Melan-A/MART-1 in melanoma revealed similar results (45).

The frequency of RAGE-1-expressing RCCs described here is in contrast to the findings of Gaugler et al. (36), who reported only 1 RAGE-1-positive RCC specimen of 57 tumor samples. This discrepancy could be explained by differences in sensitivity of the RT-PCR conditions, e.g., a different primer set and Southern blotting, which was able to detect very low levels of RAGE-1 transcription in RCC tumors. Expression of the melanoma-associated antigens PRAME and gp75 was detected in a relatively high percentage of RCCs. The latter antigen is of particular interest, because gp75 expression has mainly been found in melanoma lesions or melanocytes and was found very recently in normal brain tissue and glioblastoma multiforme (46). However, the role and function of gp75 expression in RCC have yet to be defined. It is noteworthy that MAGE-2 expression has been found in 1 of 39 RCC tumor specimens, but not in any RCC cell line analyzed. These overall results indicate that RCCs may exhibit a more significant lysis by different Melan-A/MART-1-specific CTL clones. Whether low levels of TAA expression are sufficient to induce T-cell response and to constitute the most critical factor for optimal T-cell activation, the requirement for an efficient antitumor response is (48).

An important question for T-cell-based immunotherapies is whether low levels of TAA expression are sufficient to induce T-cell responses. Autologous and allogeneic HLA-B7+ RCC cell lines expressing minimal levels of RAGE-1 were susceptible to lysis by anti-RAGE-1 CTLs (Table 2). This is in accordance with a recent report by Labarriere et al. (47) demonstrating that minimal expression of the Melan-A/MART-1 antigen in melanoma is sufficient to induce significant lysis by different Melan-A/MART-1-specific CTL clones. However, a higher threshold of antigen expression is required for efficient cytokine release by CTLs. Although antigen density seemed to constitute the most critical factor for optimal T-cell activation, the expression of adhesion molecules and costimulatory factors is also required for an efficient antitumor response (48).

To date, no effective standard treatment for RCC is available, and the survival times of the patients are very short. It is therefore of importance to develop new treatment strategies for these patients, such as immunizations with tumor antigens. Pretreatment monitoring of tumor lesions for TAA expression may help in selecting patients for an individually tailored antigen-specific vaccine therapy. Due to the relatively high frequency of RAGE-1, gp75, and PRAME expression in RCC lesions, these antigens alone or in combination may represent possible candidates for the specific immunotherapy of patients with kidney tumors.

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References


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