Immunologic and Clinical Responses after Vaccinations with Peptide-Pulsed Dendritic Cells in Metastatic Renal Cancer Patients

Jan Wierecky, Martin R. Müller, Stefan Wirths, Edith Halder-Oehler, Daniela Dörfel, Susanne M. Schmidt, Maik Häntschel, Wolfram Brugger, Stephen Schröder, Marius S. Horger, Lothar Kanz, and Peter Brossart

Departments of 1Hematology, Oncology, Rheumatology, and Immunology, 2Radiology, and 3Cardiology, University of Tübingen, Tübingen, Germany

Abstract

A phase I trial was conducted to evaluate the feasibility, safety, and efficacy of a dendritic cell–based vaccination in patients with metastatic renal cell carcinoma (RCC). Autologous mature dendritic cells derived from peripheral blood monocytes were pulsed with the HLA-A2-binding MUC1 peptides (M1.1 and M1.2). For the activation of CD4+ T-helper lymphocytes, dendritic cells were further incubated with the PAN-DR-binding peptide PADRE. Dendritic cell vaccinations were done s.c. every 2 weeks for four times and repeated monthly until tumor progression. After five vaccinations were done s.c. every 2 weeks for four times with the PAN-DR-binding peptide PADRE, dendritic cell helper lymphocytes, dendritic cells were further incubated with the PAN-DR-binding peptide PADRE. The induction of vaccine-induced T-cell responses was monitored using enzyme-linked immunospot and Cr release assays. Twenty patients were included. The treatment was well tolerated with no severe side effects. In six patients, regression of the metastatic sites was induced after vaccinations with three patients achieving an objective response (one complete response, two partial responses, two mixed responses, and one stable disease). Additional four patients were stable during the treatment for up to 14 months. MUC1 peptide–specific T-cell responses in vivo were detected in the peripheral blood mononuclear cells of the six patients with objective responses. Interestingly, in patients responding to the treatment, T-cell responses to antigens not used for vaccinations, such as adipophilin, telomerase, or oncofetal antigen, could be detected, indicating that epitope spreading might occur. This study shows that MUC1 peptide–pulsed dendritic cells can induce clinical and immunologic responses in patients with metastatic RCC. (Cancer Res 2006; 66(11): 5910-8)

Introduction

Renal cell carcinoma (RCC) is a relatively rare tumor, accounting for ~3% of malignancies in adults. When diagnosed at an early stage of disease, most of the patients can be successfully treated by radical nephrectomy. However, in ~30% of cases, patients with RCC develop metastatic spread with a 5-year survival rate of <10% (1). Treatment of metastatic RCC is still challenging because of its resistance to conventional therapies, such as radiation or chemotherapy (2). In view of the observed spontaneous remissions of advanced RCC and infiltration of cancer tissue with dendritic cells and lymphocytes able to recognize malignant cells, immune mechanisms have been suggested to play a role in the natural disease course of RCC (3, 4). These findings led to the attempts to develop novel immunotherapeutic strategies using cytokines or dendritic cell–based vaccines. Interleukin-2 (IL-2) and IFN-α containing therapies are the most commonly used agents to treat patients with advanced disease. However, application of these cytokines is often only moderately tolerated, and response rates remain unsatisfactory (5).

Dendritic cells play a central role in the presentation of antigens to naïve T cells and the induction of primary immune responses and thus represent an attractive adjuvants for therapeutic immunization of cancer patients (6). Preclinical studies have established that dendritic cells loaded with antigens induce potent antitumor immune responses in vitro and in vivo (7, 8). This has led to a proliferation of clinical trials testing their effectiveness in humans, particularly in patients with advanced malignancies (9-11). Reports from several phase I/II trials were encouraging and showed the safety of immunotherapy with dendritic cells. The broad usage of such vaccines in RCC was, however, limited due to the lack of appropriate numbers of tumor-associated antigens (TAA) and the insufficient definition of T-cell epitopes presented on a multitude of HLA molecules. Thus, studies using whole-tumor approaches, including tumor cell lysates, fusions of dendritic cells with tumor cells, or whole-tumor RNA were done in RCC patients, and remissions of tumor lesions were reported in some of these trials (12-15).

In the last years, several human TAAs expressed in RCCs and recognized by antigen-specific CTLs have been defined and characterized using expression cloning, reverse immunology approach, or by applying DNA microarray technology (16, 17). We recently identified two HLA-A2-binding peptides derived from the MUC1 protein (M1.1 and M1.2) and have shown that these T-cell epitopes are presented on different human malignancies, including RCC (18). In a previous phase I study done in patients with metastatic breast and ovarian cancers, we were able to show that injection of these peptides when pulsed on dendritic cells is safe and can elicit cytotoxic T cells in vitro capable of recognizing tumor cells endogenously expressing MUC1 in an antigen-specific and HLA-restricted manner (9).

In the present study, we modified the previously used clinical protocol and analyzed the efficacy and feasibility of a MUC1
peptide–pulsed dendritic cell vaccination in patients with metastatic RCC. For the activation of CD4+ T-helper lymphocytes, dendritic cells were further incubated with the PAN-HLA-DR-binding peptide PADRE (19). We could previously show in vitro studies that the addition of PADRE as a T-helper epitope resulted in a higher cytotoxicity of the in vitro–generated MUC1-specific CTL (18).

Materials and Methods

Patient characteristics and clinical protocol. This is a nonrandomized phase I/II trial, in which monocyte-derived dendritic cells pulsed with HLA-A2-binding MUC1 and PADRE peptides were administered s.c. in patients with metastatic RCC. The primary end point was to assess feasibility and safety. The secondary end points were the analysis of immunologic effects and antitumor activity.

Patients with histologically confirmed metastatic RCC that expressed HLA-A2 and MUC1 were eligible to participate in the study either as primary therapy or after failure of one or two other therapies. Inclusion criteria were bidimensionally measurable lesions, willingness and ability to give informed consent, and an Eastern Cooperative Oncology Group score of <3. Exclusion criteria were other immunotherapy or chemotherapy within 8 weeks before the start of vaccination, severe heart or lung disease, neurologic or psychiatric disorders, brain metastases, pregnancy, and history of second malignancies. The protocol has been approved by the local institutional ethics committee of the University of Tübingen (Tübingen, Germany). Written informed consent has been given by each patient before the study inclusion. Before the first vaccination, all patients underwent an extended clinical evaluation, including physical examination, hematologic and biochemical variables, and computed tomography (CT) scans. Clinical response was defined according to the WHO criteria.

Peptide-pulsed dendritic cells generated from peripheral blood monocytes were injected s.c. on days 1, 14, 28, and 42 (Supplementary Fig. S1). Between days 49 and 56, evaluation of clinical response was done, and CT scans were taken. In case of tumor regression or stable disease, vaccination was repeated monthly until progression. After the fifth vaccination, patients additionally received three s.c. injections weekly of low-dose IL-2 (1 million IE/m²) for 3 weeks. For evaluation, the WHO definitions of clinical responses and adverse effects were applied. CT scans were reviewed by a senior faculty radiologist not otherwise involved in the study.

Cell isolation and cultures. Generation and characterization of patients’ monocyte-derived dendritic cells was done as described previously (9, 18). Patient-derived peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (Life Technologies, Inc., Grand Island, NY) density gradient centrifugation of 100 mL heparinized blood. Isolated PBMCs were plated (1 × 10⁷/mL) in six-well plates (Costar, Cambridge, MA) in serum-free X-VIVO 20 medium. After 2 hours of incubation at 37°C, nonadherent cells were removed, and the adherent cells (12-19% of the incubated cells) were cultured in X-VIVO 20 medium supplemented with IL-4, granulocyte macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α).

IL-4 (1,000 IU/mL) and TNF-α (10 ng/mL) were purchased from Genzyme (Cambridge, MA). Human recombinant GM-CSF (Leucomax or Leukine, 100 ng/mL) was from Novartis (Basel, Switzerland) or Immunex (Seattle, WA), respectively. The cultures were fed with fresh medium and cell differentiation was monitored by light microscopy. The viability, phenotype, and sterility of dendritic cell preparations were analyzed after 7 days of culture before the application.

The HLA-A2-binding peptides derived from MUC1 (M1,1, STPPHVHN; M1,2, LLLLVTLV) and the T-helper epitope PADRE were synthesized using standard Fmoc chemistry on a peptide synthesizer (432A, Applied Biosystems, Weiterstadt, Germany) and analyzed by reverse-phase high-performance liquid chromatography and mass spectrometry. Dendritic cells were separately pulsed for 2 hours with 50 μg/mL of each peptide and washed thrice with PBS before the application. PADRE peptide (25 μg/mL) was added to both dendritic cell preparations.

Immunostaining. Cell staining was done using FITC- or PE-conjugated mouse monoclonal antibodies against CD86 (PharMingen, Hamburg, Germany), CD3, CD19, CD20, CD80, HLA DR, CD14 (Becton Dickinson, Heidelberg, Germany), CD83 (Coiler-Immunotech Diagnostics, Hamburg, Germany), CD1A (OKT6, Ortho Diagnostic Systems, Germany, and T6-RI, Coulter Immunology, Miami, FL), and mouse IgG1 isotype controls. Samples were analyzed on a FACScan Calibur (Becton Dickinson, Seattle, WA). Dendritic cells were positive for CD83, CD1A, CD80, CD86, and HLA class II expression but negative for CD14 expression (data not shown).

Enzyme-linked immunospot assay. The analysis of vaccine induced expansion of MUC1-specific T cells was assessed in an IFN-γ enzyme-linked immunospot (ELISPOT) as described recently (20). PBMCs obtained before and after the vaccinations were isolated by Ficoll gradient centrifugation and cryopreserved. After completion of the treatment, samples from prevaccination and after one and more applications of the vaccine were evaluated simultaneously. Lymphocytes were assayed after a single sensitization in vitro for 1 week with peptide-pulsed autologous irradiated PBMCs. A HLA-A2-binding HIV peptide (pol HIV-1 reverse transcriptase peptide, amino acids 476-484, and ILKEPVHGV) or DMSO (used for the peptide dilution) was used as a negative control. In addition, HLA-A2-binding peptides derived from adipophilin, telomerase, oncofetal antigen, G250, and c-Met were used in the assays for epitope spreading analysis.

Analysis of antigen-specific CTL responses after in vitro restimulation. Autologous PBMCs were incubated with 10 ng/mL synthetic peptides for 2 hours, washed, and incubated with 2.5 × 10⁶ autologous PBMCs in RP10 medium. Cells were restimulated after 7 days of culture, and 1 ng/mL human recombinant IL-2 (Genzyme) was added every other day. The cytolytic activity of the CTLs was analyzed on day 5 after the last restimulation in a standard ⁵¹Cr release assay (9). PBMCs obtained before and after the vaccinations were isolated by Ficoll gradient centrifugation and cryopreserved. After completion of the treatment, samples from prevaccination and after one and more applications of the vaccine were evaluated simultaneously.

The standard ⁵¹Cr release assay was done with some modifications as described (9). A498 (RCC, HLA-A2+, and MUC1+), SKOV3 (ovarian cancer, HLA-A2+, and MUC1+), and Croft cell lines (immortalized B-cell line, HLA-A2+, and MUC1+, kindly provided by O.J. Finn, University of Pittsburgh, Pittsburgh, PA) were used as target cells in the assay. Croft cells were pulsed with 25 μg/mL peptide for 2 hours and labeled with sodium chromate in RP10 medium for 1 hour at 37°C. Cells (10⁴) were transferred to a well of a round-bottomed 96-well plate. Dendritic cells that were used as targets in CTL assays were generated in RP10 medium (RPMI 1640 with glutamax-I, 1% FCS, and glutathione, 1-mM mercaptoethanol, 10% fetal calf serum, and antibiotics) or cryopreserved. After completion of the treatment, samples from prevaccination and after one and more applications of the vaccine were evaluated simultaneously.

The percentage specific lysis was calculated as 100 × (experimental release – spontaneous release)/(maximal release – spontaneous release). Spontaneous and maximal release were determined in the presence of either medium or 1% Triton X-100, respectively.

Generation of mRNA by in vitro transcription. Enhanced green fluorescent protein (EGFP) in vitro transcript was synthesized from the plasmid pSP64-Poly(A)-EGFP-2 (g enerously provided by V.F.I. Van Tendeloo, University of Antwerp, Antwerp University Hospital, Edegem, Belgium) as described previously (21, 22). Full-length cDNA sequences of MUC1 were excised from p858-PEM-tm (generously provided by S. Gendler, Imperial Cancer Research Fund, London, United Kingdom) and subcloned into pSP64-Poly(A). For generation of MUC1, the plasmids pSP64-Poly(A)-MUC1 were linearized using the restriction enzyme PsII. The in vitro transcription was done using the SP6 Cap Scribe kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Purification of in vitro transcript was done with RNeasy Mini anion-exchange spin columns (Qiagen, Hilden, Germany). Quantity and purity of generated in vitro transcript was determined by UV spectrophotometry and formaldehyde/bisulfite treatment.

www.aacrjournals.org
agarose gel electrophoresis. RNA was stored at −80°C in small aliquots. Electroporation of dendritic cells that were generated from frozen blood samples obtained during the vaccinations in RP10 medium (RPMI 1640 with glutamax-I, supplemented with 10% inactivated FCS, and antibiotics) using GM-CSF and IL-4 with in vitro transcript was done as described previously (21). In brief, before the electroporation, immature dendritic cells were washed twice with serum-free X-VIVO 20 medium (Cambrex, Belgium) and resuspended to a 2 × 10^7/mL final concentration. Subsequently, 200 μL cell suspension was mixed with 10 μg in vitro transcript and electroporated in a 4-mm cuvet using an Easyject Plus device (Peqlab, Erlangen, Germany). The physical variables used were as follows: (a) voltage of 300 V, (b) capacitance of 150 μF, (c) resistance of 1540 V, and (d) pulse time of 231 ms. After electroporation, cells were transferred immediately into RP10 medium supplemented with the cytokines GM-CSF (100 ng/mL) and IL-4 (20 ng/mL) and returned to the incubator.

**Proliferation assay.** A total of 2 × 10^5 PBMNCs were cocultured in flat-bottomed 96-well microplates (Nunc, Wiesbaden, Germany) with 1 × 10^5 autologous mononuclear cells pulsed with the PADRE peptide (25 μg/mL) or treated with the solvent (DMSO) in X-VIVO 20 medium. Thymidine incorporation was measured on day 5 by a 16-hour pulse with [3H]thymidine (1 μCi/well, 0.037 MBq/well; Amersham Life Sciences, Braunschweig, Germany). Assays were done at least in triplicates or quadruplicates. A stimulation index of >2 was considered as significant.

**Statistical analysis.** Comparisons between individual data points were made using a χ^2 test. P < 0.05 was considered significant. The correlation between the detection of a vaccine-induced immune response to MUC1 peptides and clinical response [responder (stable disease and complete, partial, and mixed responses) versus nonresponder (progressive disease)] was analyzed using the Spearman rank analysis.

**Table 1. Characteristics of patients enrolled in the clinical trial**

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (y)</th>
<th>Previous therapy</th>
<th>Site of metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61</td>
<td>Surgery</td>
<td>Lung, skin</td>
</tr>
<tr>
<td>2</td>
<td>69</td>
<td>Surgery</td>
<td>Lung</td>
</tr>
<tr>
<td>3</td>
<td>74</td>
<td>Surgery</td>
<td>Lung</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>Surgery</td>
<td>Liver, bone</td>
</tr>
<tr>
<td>5</td>
<td>46</td>
<td>Surgery, radiotherapy</td>
<td>Lung, mediastinal lymph nodes, bone</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>Surgery</td>
<td>Lung</td>
</tr>
<tr>
<td>7</td>
<td>46</td>
<td>Surgery, chemotherapy</td>
<td>Lung, mediastinal lymph nodes, contralateral kidney</td>
</tr>
<tr>
<td>8</td>
<td>38</td>
<td>Surgery</td>
<td>Lung, mediastinal lymph nodes, contralateral kidney, bone</td>
</tr>
<tr>
<td>9</td>
<td>49</td>
<td>Surgery</td>
<td>Lung</td>
</tr>
<tr>
<td>10</td>
<td>47</td>
<td>Surgery</td>
<td>Lung, retroperitoneum, skin</td>
</tr>
<tr>
<td>11</td>
<td>55</td>
<td>Surgery, chemotherapy</td>
<td>Lung</td>
</tr>
<tr>
<td>12</td>
<td>56</td>
<td>Surgery</td>
<td>Lung, liver, lymph nodes</td>
</tr>
<tr>
<td>13</td>
<td>77</td>
<td>Surgery</td>
<td>Lung, adrenal gland</td>
</tr>
<tr>
<td>14</td>
<td>47</td>
<td>Surgery</td>
<td>Lung, pancreas, thyroid gland</td>
</tr>
<tr>
<td>15</td>
<td>45</td>
<td>Surgery</td>
<td>Bone lymph nodes, liver</td>
</tr>
<tr>
<td>16</td>
<td>52</td>
<td>None</td>
<td>Lung, mediastinal lymph nodes</td>
</tr>
<tr>
<td>17</td>
<td>52</td>
<td>Surgery, chemotherapy</td>
<td>Parotid gland, pancreas, contralateral kidney, bone</td>
</tr>
<tr>
<td>18</td>
<td>58</td>
<td>None</td>
<td>Lung, liver, adrenal gland</td>
</tr>
<tr>
<td>19</td>
<td>35</td>
<td>Surgery</td>
<td>Lung, lymph nodes, pancreas</td>
</tr>
<tr>
<td>20</td>
<td>42</td>
<td>Surgery, chemotherapy</td>
<td>Lung, bone</td>
</tr>
</tbody>
</table>

**Table 2. Vaccination induced immunologic and clinical responses**

<table>
<thead>
<tr>
<th>No.</th>
<th>Course before vaccination</th>
<th>Mean no. of dendritic cells × 10^6 (range)</th>
<th>PADRE response</th>
<th>CTL response</th>
<th>Clinical response</th>
<th>Duration of response (mo)</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PD</td>
<td>2.67 (6.3-1.1)</td>
<td>+</td>
<td>+</td>
<td>PR</td>
<td>4</td>
<td>AWD</td>
</tr>
<tr>
<td>2</td>
<td>PD</td>
<td>2.49 (5.6-0.2)</td>
<td>+</td>
<td>+</td>
<td>PR</td>
<td>14</td>
<td>AWD</td>
</tr>
<tr>
<td>3</td>
<td>PD</td>
<td>1.19 (2.7-0.4)</td>
<td>+</td>
<td>+</td>
<td>CR</td>
<td>24</td>
<td>AWD</td>
</tr>
<tr>
<td>4</td>
<td>PD</td>
<td>4.12 (5.5-0.6)</td>
<td>–</td>
<td>–</td>
<td>PD</td>
<td>–</td>
<td>AWD</td>
</tr>
<tr>
<td>5</td>
<td>PD</td>
<td>1.59 (2.2-1.4)</td>
<td>+</td>
<td>+</td>
<td>MR</td>
<td>10</td>
<td>DOD</td>
</tr>
<tr>
<td>6*</td>
<td>PD</td>
<td>2.3 (5.3-0.6)</td>
<td>–</td>
<td>(+)</td>
<td>PD</td>
<td>–</td>
<td>AWD</td>
</tr>
<tr>
<td>7</td>
<td>PD</td>
<td>2.02 (3.6-0.6)</td>
<td>+</td>
<td>+</td>
<td>SD</td>
<td>8</td>
<td>DOD</td>
</tr>
<tr>
<td>8</td>
<td>PD</td>
<td>4.14 (7.3-1)</td>
<td>+</td>
<td>+</td>
<td>SD</td>
<td>10</td>
<td>AWD</td>
</tr>
<tr>
<td>9</td>
<td>PD</td>
<td>3.23 (6.5-0.9)</td>
<td>–</td>
<td>+</td>
<td>PD</td>
<td>–</td>
<td>DOD</td>
</tr>
<tr>
<td>10</td>
<td>PD</td>
<td>2.52 (5.5-0.4)</td>
<td>–</td>
<td>–</td>
<td>PD</td>
<td>–</td>
<td>DOD</td>
</tr>
<tr>
<td>11</td>
<td>PD</td>
<td>2.75 (6.3-0.7)</td>
<td>+</td>
<td>–</td>
<td>PD</td>
<td>–</td>
<td>AWD</td>
</tr>
<tr>
<td>12</td>
<td>PD</td>
<td>5.4 (6.8-3.8)</td>
<td>–</td>
<td>–</td>
<td>PD</td>
<td>–</td>
<td>DOD</td>
</tr>
<tr>
<td>13</td>
<td>–</td>
<td>2.47 (4.06-5)</td>
<td>–</td>
<td>–</td>
<td>PD</td>
<td>–</td>
<td>DOD</td>
</tr>
<tr>
<td>14</td>
<td>PD</td>
<td>1.59 (2.2-0.8)</td>
<td>+</td>
<td>+</td>
<td>SD</td>
<td>4</td>
<td>AWD</td>
</tr>
<tr>
<td>15</td>
<td>PD</td>
<td>3.2 (3.2, 3.2)</td>
<td>ND</td>
<td>ND</td>
<td>PD</td>
<td>–</td>
<td>DOD</td>
</tr>
<tr>
<td>16</td>
<td>PD</td>
<td>2.7 (4.1-4)</td>
<td>ND</td>
<td>ND</td>
<td>PD</td>
<td>–</td>
<td>DOD</td>
</tr>
<tr>
<td>17</td>
<td>SD</td>
<td>2.56 (11.2-0)</td>
<td>+</td>
<td>+</td>
<td>SD</td>
<td>14</td>
<td>AWD</td>
</tr>
<tr>
<td>18</td>
<td>–</td>
<td>6.12 (8.7-4.2)</td>
<td>+</td>
<td>+</td>
<td>MB</td>
<td>8</td>
<td>DOD</td>
</tr>
<tr>
<td>19</td>
<td>PD</td>
<td>14.5 (15-1)</td>
<td>+</td>
<td>+</td>
<td>SD</td>
<td>12</td>
<td>AWD</td>
</tr>
<tr>
<td>20</td>
<td>PD</td>
<td>4.92 (8.9-2.4)</td>
<td>–</td>
<td>–</td>
<td>PD</td>
<td>–</td>
<td>DOD</td>
</tr>
</tbody>
</table>

Abbreviations: PD, progressive disease; SD, stable disease; MR, mixed response; PR, partial response; CR, complete response; AWD, alive with disease; DOD, died of disease; ND, not done.

*Patient 6 had preexisting MUC1 peptide–reacting CTLs that were unable to recognize HLA-matched MUC1-expressing tumor cells.
Results

Clinical responses to dendritic cell vaccinations. Between September 2000 and April 2003, 20 HLA-A2+ patients with metastatic RCC were enrolled in the study. All patients had histology of a clear cell carcinoma. The mean age of the 15 men and 5 women was 53.9 years (range, 35-77 years). Eighteen patients had nephrectomy before entering the study. Four patients had received chemotherapy and one palliative radiotherapy. Characteristics of patients are presented in Table 1. Of the 20 patients initially enrolled, 16 patients completed the first four vaccinations. Three patients were removed from the study after two vaccinations because of rapidly progressive disease.

Another patient developed grade 3 anemia after two vaccinations, and the therapy was discontinued. Hematologic and biochemical evaluation of blood and bone marrow samples revealed an anemia of chronic disorder. This patient responded to the treatment with a regression of lung and liver metastases (without meeting the criteria of a partial response and was therefore declared as stable disease) while being progressive before vaccinations.

In six patients, some kind of regression of metastatic lesions have been observed. One patient had a complete regression of all lung metastases. There was one mediastinal lymph node of 12 to 14 mm in size remaining unchanged during the treatment for >18 months without clear relationship to metastatic disease. Therefore, this patient was classified as complete response. Two patients had a partial response. Two additional patients showed a mixed response with regression of some metastatic lesions and progressive diseases of other sites. Another five patients had a stabilization of a progressive disease. The remaining 10 patients progressed after four vaccinations. Duration of response was >18 months for the complete response, 4 and 14 months for the partial response, and 8 and 10 months for the mixed response. Data of clinical outcome are presented in Table 2 and Supplementary Fig. S2.

PADRE peptide–specific T-cell proliferation in patients after dendritic cell immunizations. The induction of PADRE-specific responses in vivo after dendritic cell vaccinations was assessed by doing proliferation assays. The generation of PADRE-specific proliferative responses in vaccinated patients was determined after one in vitro sensitization (in RP10 medium without addition of cytokines) of patients PBMNCs with irradiated PADRE-pulsed mononuclear cells. In 10 of 18 tested patients, PADRE-specific proliferation was detected in some patients already after the first two dendritic cell administrations. The proliferation of PADRE-reactive cells in general increased and reached a maximum during the first four to six injections (Fig. 1). The proliferative response to PADRE was considered as significant when a stimulation index of >2 was detected at least at two different time points after vaccinations.

Induction of MUC1-directed responses in vaccinated patients. The vaccination-induced MUC1 antigen–specific responses in patients were determined in Cr release assays and by analysis of IFN-γ production by T lymphocytes after stimulation with the cognate MUC1 peptides in vitro.

After three to four vaccinations, in 11 of 20 patients (1 patient with progressive disease, 6 patients with tumor regressions, and 4 patients with stable disease), antigen-specific cytotoxic T-cell responses could be detected in peripheral blood of treated patients by doing 51Cr release assays. Results from experiments obtained with CTL derived from two patients are presented in Fig. 2 and Table 2. The CTL obtained after in vitro restimulation efficiently lysed target cells pulsed with the antigenic MUC1 peptides as well as the allogeneic HLA-A2+ tumor cell lines naturally expressing the TAA (A498 cells). No lysis was detected when tumors missing either HLA-A2 (SKOV3 cells, MUC1+) or MUC1 (Croft cells, HLA-A2+) expression were used as target cells in the assay (Fig. 2A). To exclude an ex vivo priming during the restimulation, blood samples obtained before and after the treatment were evaluated simultaneously. The cytotoxic response to MUC1 peptides was considered as significant when the cytolytic activity of CTL was detected at least at two different time points after vaccinations.

The induction of an immune response to MUC1 was significantly higher in the group of patients that responded to the treatment with stabilization of the disease (stable disease) or shrinkage of metastatic lesions (complete, partial, and mixed responses; \( P = 0.0459 \)). Furthermore, there was a significant correlation between the vaccine-induced immune and clinical responses (\( r = 0.7906 \)).

Interestingly, in one patient (patient 6) who was progressive during the treatment, CTLs recognizing target cells pulsed with the antigenic MUC1 peptides could be detected in Cr release assays (Fig. 2C). However, these CTLs were unable to lyse the A498 tumor cells endogenously expressing MUC1, indicating that, in these patients, low-affinity T cells were present. We have, thus far, no explanation for this observation, as there were no differences in the phenotype and numbers of injected dendritic cells as well as the amount of peptides used.

In two patients, we had the opportunity to analyze the elicited T-cell responses against the MUC1 peptides more extensively (Fig. 3). Peripheral blood lymphocytes obtained after five vaccinations were restimulated with each peptide separately to confirm that the induced CTLs are recognizing both peptides used in the trial. These in vitro restimulated CTLs efficiently lysed
autologous dendritic cells pulsed with the cognate peptide or transfected with \textit{in vitro} transcribed MUC1 RNA.

To further confirm the induction and expansion of \textit{in vivo}–elicited MUC1 responses and to extend the analysis of their effector functions, ELISPOT assays to determine IFN-\(\gamma\) production were done after one \textit{in vitro} sensitization. Results from four different patients representing the variations in the response patterns in the ELISPOT assays are shown in Fig. 4A.

Immunization with MUC1 peptide–pulsed dendritic cells can induce epitope spreading with T-cell responses against epitopes not used for vaccination. In our previous vaccination trial using peptide pulsed dendritic cells, we observed that, in a small proportion of analyzed patients, epitope spreading occurred after immunization with a single tumor antigen probably as a result of cross presentation of killed and engulfed malignant cells by antigen-presenting cells. To analyze this, we used a set of

![Image of Figure 2](image.png)

Figure 2. Induction of MUC1-specific CTL responses after vaccinations with peptide-pulsed dendritic cells. PBMCs obtained before and during the vaccinations were restimulated with irradiated MUC1 peptide–pulsed autologous PBMCs, and the cytotoxic activity of CTL responses was analyzed in a standard Cr release assay as described in Materials and Methods. From two patients responding to the treatment (A and B) and one patient with progressive disease (C). Samples from prevaccination and after one or more vaccinations were evaluated simultaneously. C, in patient 6 with progressive disease, low-affinity T cells only recognizing the antigenic peptides were detected before and after vaccinations.
several HLA-A2-binding peptides derived from antigens known to be expressed in RCC. This analysis has been done by IFN-γ ELISPOT in eight patients who showed some kind of tumor regression or stabilization and peptide-specific reactivity against the MUC1 peptides used for vaccination. In six of eight patients, T cells specific for HLA-A2-binding peptides derived from oncofetal antigen, G250, adipophilin, survivin, or telomerase were detected after several vaccinations, suggesting that epitope spreading might indeed occur in vivo after successful vaccination (Fig. 4B).

**Discussion**

Dendritic cells play a central role for the initiation and maintenance of primary immune responses. The discovery of TAAs and the development of protocols for the in vitro generation of dendritic cells for clinical use resulted in development of vaccination protocols currently evaluated in patients with malignant diseases. The first clinical application of antigen-pulsed dendritic cells was done in follicular non-Hodgkin's lymphoma, resulting in remission induction in two of four treated patients (11, 23). The following trials were mainly exploited in malignant melanoma, and several studies reported the induction of variable clinical and immunologic responses in a small proportion of patients with minimal side effects (10, 24–27).

The incidence of RCC is rising, with 62% of estimated cases occurring in men and ~38% in female population. RCC constitutes ~3% of all solid malignancies and ranks 10th as the leading cause of death (28). When detected at early stage of disease, RCC can often be successfully treated by radical nephrectomy. However, 20% to 30% of patients that underwent this procedure develop metastatic disease, and the 5-year survival rate of these patients is <2% (29). In contrast to many other malignancies, the treatment for metastatic RCC is limited, as RCC is generally resistant to chemotherapy (30).

RCCs like the malignant melanomas are recognized as immunogenic tumors based on the observation of spontaneous remissions and identification of tumor-infiltrating lymphocytes capable of recognizing the malignant cells (31). With the introduction and approval of nonspecific biological response modifiers, single-agent IL-2 and IFN-γ treatment resulted in response rates of 10% to 20%. These clinical responses were clearly superior to that achieved with chemotherapy, and, in a small proportion of RCC patients, long-term survival was reported (5, 32, 33).

In the recent years, the development of immunotherapeutic treatment strategies in RCC has been driven by the definition of protocols to generate clinical grade dendritic cells and the discovery of several TAAs, including HER-2/neu, telomerase, MUC1, survivin, adipophilin, c-Met, G250, oncofetal antigen, or members of the cancer-testis antigens, found to be expressed in RCC (24, 34, 35). Furthermore, clinical trials using dendritic cells fused to tumor cells (12, 14) or pulsed with lysates from RCC cells have proved the feasibility of these whole-tumor approaches and resulted in the generation of antigen-specific immune responses. In the study by Holtl et al. where cell lysates were used to stimulate anti-RCC immunity, 3 of 27 patients responded to the treatment (2 complete responses and 1 partial response) with additional 7 patients experiencing stabilization of the disease (15). Su et al. conducted a clinical trial to evaluate the efficacy of a vaccine consisting of dendritic cells transfected with renal tumor RNA. The vaccine-induced T-cell responses were directed against G250, telomerase, and oncofetal antigen, showing that this approach can generate a polyclonal T-lymphocyte response specific for a broad variety of RCC-associated antigens (13).

In our study, we analyzed the feasibility, safety, and efficiency of autologous monocyte-derived dendritic cells pulsed with the HLA-A2-binding MUC1-derived peptides in patients with metastatic RCC. In our previous in vitro studies, we showed that MUC1-derived T-cell epitopes are expressed by RCC cell lines, and CTLs specific for these HLA-A2-binding peptides were able to lyse tumor cells expressing MUC1 in an antigen-specific fashion (18).

Results from our initial vaccination trial in breast and ovarian cancer indicate that a dendritic cell–based treatment approach is
safe and results in the induction of antigen-specific T-lymphocytes (9). MUC1-specific CTL responses capable of recognizing in an antigen-specific and HLA-A2-restricted manner target cells pulsed with MUC1-derived peptides or tumor cells endogenously expressing MUC1 were found in the peripheral blood after several in vitro restimulations in all but one patient who responded to the treatment with tumor regressions or disease stabilization. This observation was further confirmed in ELISPOT assays done after one in vitro sensitization. To exclude an ex vivo priming during the restimulations, blood samples obtained before and after the treatment were evaluated simultaneously. We could not detect or expand any MUC1-specific cytotoxic T-cell response in six of eight progressive patients. One patient developed MUC1-reactive T cells unable to recognize tumor cells constitutively expressing MUC1.

In two patients, we had the opportunity to monitor and analyze the elicited T-cell responses against the MUC1 peptides more extensively. Peripheral blood lymphocytes obtained after several peptide vaccinations were restimulated with each peptide separately to confirm that the induced CTLs are recognizing both peptides used in the trial. These in vitro–restimulated CTLs efficiently lysed autologous dendritic cells pulsed with

---

**Figure 4.** Analysis of vaccine-induced T-cell responses in ELISPOT assays. A, representative of several different T-cell response patterns for each of four presented patients. Samples from prevaccination and representative samples after one or more vaccinations were evaluated simultaneously by an IFN-γ ELISPOT assay. In general, peripheral blood samples (PBMNCs) were collected 1 week after vaccine administration. The samples obtained after the fourth vaccine were collected at an average of 21 days after the dendritic cell application. Blood samples were assayed after a single sensitization in vitro with synthetic peptide (50 μg/mL). Each sample was tested in triplicate. HIV peptide or DMSO was used as a negative control. Number of these T-cell responses were considered as background activity and subtracted from values obtained with the tested MUC1 epitopes. B, epitope spreading after vaccinations with MUC1 peptide–pulsed dendritic cells was analyzed in responding patients. Samples from prevaccination and samples after one or more vaccinations were evaluated simultaneously by an IFN-γ ELISPOT assay using autologous irradiated PBMNCs pulsed with HLA-A2-binding peptides derived from the indicated antigens. From two different patients.
the cognate peptide or transfected with in vitro–transcribed MUC1 RNA.

Based on the results from our previous study (9) where the induction of T-cell responses specific for epitopes not used for vaccinations were detected in a proportion of treated patients on vaccinations with peptide-pulsed dendritic cells, we analyzed this phenomenon called epitope spreading in patients responding to the treatment. To accomplish this, we used a set of HLA-A2-binding peptides deduced from antigens known to be expressed in RCC in an IFN-γ ELISPOT assay. By applying this approach, we were able to detect T-cell responses after 4 to 12 vaccinations specific for the oncostiral antigen, G250, survivin, adiopophilin, and/or telomerase-derived epitopes that were either not present before the treatment or after the first dendritic cell injections or increased during the immunizations with MUC1 peptides.

At the time when the trial was designed, only a few HLA class II–binding epitopes were defined for some TAAs, and they were restricted by a minority of HLA class II molecules. Therefore, we decided to include a nonspecific T-helper epitope (PADRE peptide) that have been reported to be immunogenic when used as adjuvant (19). Furthermore, in our in vitro analysis, we found that the addition of this peptide during T-cell priming might increase the cytotoxic activity of antigen-specific T lymphocytes. In our study, PADRE-specific proliferative response was found in 10 of 18 analyzed patients in some cases already after the first or second injection supporting the immunogenicity of this helper epitope. The PADRE-specific proliferation increased during the first four to six vaccinations and could not be further boosted by additional applications or IL-2 administrations.

It is encouraging that among 20 patients treated in our protocol, tumor regression was observed in 6 patients with 3 patients meeting the criteria of an objective response, 1 patient with stable disease, and 2 patients with mixed responses. Another 4 patients had a stabilization of the disease while being progressive before vaccinations. Some remissions lasted for >1 year, and 9 patients were still alive at the time of article preparation. Regression of the metastatic lesions was not only restricted to pulmonary site but included visceral and chest wall tumors. Interestingly, remissions of tumor sites or stabilization of the disease significantly correlated with the T-cell responses to MUC1 (Table 2).

It is important to note that all patients received low-dose IL-2 injections after the fifth vaccination. However, all tumor regressions were detected already after the first four to five injections (patient #3 experienced tumor regression that started after the fifth dendritic cell vaccine), indicating that the observed clinical effects are not mediated by unspecific effects of IL-2 applications. It is still unclear whether the addition of IL-2 to dendritic cell vaccinations might result in improved or prolonged vaccine-induced T-cell responses. Based on our results obtained by monitoring the proliferative responses to PADRE or CTL activity directed to MUC1, we do not have the impression that IL-2 adds any additional positive effects to observed immune responses. In contrast, as shown in Fig. 1, it seems that IL-2 might even have adverse effects on the induced T-cell responses. Therefore, in our following trial, we decided to exclude IL-2 injections.

Dendritic cell applications were well tolerated with no relevant side effects with the exception of one patient who developed anemia of chronic disease after two vaccinations. The toxicities were mainly associated with IL-2 injections and consisted of moderate fever increase up to 38°C in few patients and local reactions with induration and inflammation.

In our study, we show that vaccination therapies containing autologous monocyte-derived dendritic cells pulsed with HLA-A2-binding MUC1 peptides can elicit clinical and immunologic responses in patients with metastatic RCC. Induction of epitope spreading by a single antigen that results in the generation of T-cell responses able to target a plethora of different TAAs could represent an important mechanism by which the immune system may efficiently eliminate malignant cells.

Acknowledgments

Received 10/28/2005; revised 3/1/2006; accepted 3/21/2006.

Grant support: AKF programme of the University of Tübingen.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

17. Michael A, Pandula HS. Renal cell carcinoma: tumour markers, T-cell epitopes, and potential for new thera-


Immunologic and Clinical Responses after Vaccinations with Peptide-Pulsed Dendritic Cells in Metastatic Renal Cancer Patients

Jan Wierecky, Martin R. Müller, Stefan Wirths, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/11/5910

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2006/06/05/66.11.5910.DC1

Cited articles
This article cites 35 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/11/5910.full.html#ref-list-1

Citing articles
This article has been cited by 20 HighWire-hosted articles. Access the articles at:
/content/66/11/5910.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.