Vaccination of colorectal cancer patients with CEA-loaded dendritic cells: antigen-specific T cell responses in DTH skin tests


Departments of 1Medical Oncology, 2Tumor Immunology, 3Pediatric Oncology and 4Surgery, Nijmegen Centre for Molecular Life Sciences and Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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Background: Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system. As such they are currently used in clinical vaccination protocols in cancer patients.

Patients and methods: We evaluated the ability of mature DCs pulsed with carcinoembryonic antigen (CEA)-peptide to induce CEA-specific T cell responses in patients with resectable liver metastases from colorectal cancer. CEA-specific T cell reactivity was monitored in peripheral blood, biopsies of vaccination sites and post-treatment DTH skin tests, and when available also in resected abdominal lymph nodes and tumor tissue.

Results: Ten patients were vaccinated intradermally and intravenously with CEA-peptide pulsed mature DCs three times prior to resection of liver metastases. High numbers of CEA-specific T cells were detected in post-treatment DTH biopsies in seven out of 10 patients, which produced high amounts of interferon (IFN)-γ upon stimulation with CEA-loaded target cells. These responses were not found in biopsies of first vaccination sites, indicating a de novo T cell induction or at least a strong potentiation by the vaccine. In addition, CEA-specific T cells were detected in a resected lymph node in one patient, but not in peripheral blood or tumor tissue.

Conclusions: Vaccination with CEA-peptide loaded mature DCs induced potent CEA-specific T cell responses in advanced colorectal cancer patients. In this study, antigen-specific T cell responses were readily detected in DTH skin tests, much less in abdominal lymph nodes, and not in peripheral blood and tumor tissue.

Key words: carcinoembryonic antigen, colorectal cancer, delayed type hypersensitivity reaction, dendritic cells, immunomonitoring, cancer vaccine

Introduction

Although progress has been made in the treatment of metastatic colorectal cancer using chemotherapy and targeted therapies, clinical outcome remains poor [1]. Immunotherapy forms an alternative approach for the treatment of colorectal cancer [2, 3]. Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system. They are essential for the induction of antigen-specific T cell immunity and as such they have great potential for cancer immunotherapy [4]. DC vaccination has shown to be feasible and safe, and both immunological and clinical responses have been reported [5]. Most clinical studies using DCs have been performed in melanoma patients [6]. Only a few small studies investigated the immunogenicity of DC vaccines in colorectal cancer patients with variable results [7–13].

The majority of clinical studies in cancer patients have been carried out using immature rather than mature DCs. We, as well as others, have demonstrated in comparative studies that only mature DCs are able to stimulate T cell responses [14–16]. These findings should be considered when interpreting recent negative clinical trials comparing immature DCs with other vaccination modalities [17, 18]. Other pivotal questions, including the optimal DC culture method, dose, route of administration, etc., remain unanswered to date. To start to address these questions in small cohorts of patients, there is a need of validated assays that can monitor immunological outcome, since clinical responses occur in only a minority of patients. Most studies so far have focused on the monitoring of antigen-specific T cell responses in peripheral blood which, however, often requires in vitro restimulation due to low precursor frequencies. Tumor tissue and lymph nodes
would be more interesting compartments to monitor these responses, but these tissues are often not available. We recently described a novel method of monitoring antigen-specific T cell responses: a short culture of biopsies of post-treatment delayed type hypersensitivity (DTH) reaction sites allowed a detailed analysis of tumor antigen-specific CD8+ T cell responses in stage IV melanoma patients [19]. We found that the presence of vaccine-induced melanoma-specific T cells in these biopsies significantly correlated with an improved clinical outcome.

Carcinoembryonic antigen (CEA) is expressed in almost all colorectal cancers, making it an attractive antigen for immunotherapy in patients with this tumor type [20]. Here, we investigated the immunogenicity of CEA-peptide loaded mature DCs in colorectal cancer patients with resectable liver metastases. This model provided the unique opportunity to rigorously monitor and compare immune responses after vaccination in several relevant compartments of the body: peripheral blood, lymph nodes, tumor tissue and biopsies of DTH skin tests and vaccination sites.

patients and methods

study design

This was an open-label, single-institution, single-arm exploratory study in which monocyte-derived mature DCs loaded with CEA-peptide were administered to patients with resectable liver metastases of colorectal cancer (Figure 1A). Approval from the local regulatory committee was obtained.

objectives

The primary end point was to assess the immunogenicity of the vaccine. Secondary end points were the toxicity and the feasibility of CEA-specific vaccination in colorectal cancer patients and, specifically, the feasibility of using DTH-infiltrated lymphocytes in the monitoring of T cell responses in comparison with other relevant body compartments.

patients

Inclusion criteria included: patients with liver metastases from CEA-expressing colorectal cancer scheduled for surgical resection, HLA-A0201 phenotype, Eastern Cooperative Oncology Group (ECOG) performance status 0–1, age ≥18 years, no clinical signs of extra-hepatic metastases, no prior chemotherapy, immunotherapy or radiotherapy within 2 months before planned surgery.

DC preparation

DCs were generated as described previously [15, 21] with several modifications (Figure 1A). Patients donated 0.5 l blood, from which auffy-coat was made (Sanquin Bloodbank Nijmegen, the Netherlands). Peripheral blood mononuclear cells (PBMC) were isolated by PureCell (Medicult, Denmark) density gradient centrifugation (30 min, 4°C, 2100 rpm), adherent monocytes were cultured in Cellgro® medium enriched with 500 U/ml interleukin (IL)-4 and 800 U/ml granulocyte macrophage colony stimulating factor (GM-CSF, all CellGenix, Freiburg, Germany).

KLH (10 μg/ml; Calbiochem, USA) was added at day 3 of culture, and 2 days before harvesting we added the maturation cocktail [IL-4 (500 U/ml), tumor necrosis factor alpha (TNF-α), 10 ng/ml), IL-1β (5 ng/ml) and IL-6 (15 ng/ml); all CellGenix)].

Cells were harvested at day 7 and approximately 25% of the cells were put in a syringe for immediate vaccination; the remaining cells were frozen for the second and third vaccination and the DTH [22]. From each batch of

Figure 1. (A) Treatment schedule. (B) KLH-specific proliferation of PBMC before and after vaccination. In all patients a clear KLH-specific proliferative response was observed after vaccination. One representative patient (patient 1) who received three vaccination cycles is shown.

patient DCs, a sample was used for quality control. Release criteria were as previously described [5].

DCs were pulsed with the wild type CEA-peptide CAP-1 (CEA_A21-3796, YLSGANLNL, Clinalfa, Switzerland) [23] directly after harvesting or after thawing [15, 22].

treatment schedule

Vaccinations were administered three times at day 0, 7 and 14 (Figure 1A). The patients received 5 × 10⁶ DCs intradermally (i.d.) in the upper leg, 5–10 cm from an inguinal lymph node. The remaining cells were given intravenously (i.v.) as a bolus injection at the same time point. If the patient consented, a biopsy was taken from the first vaccination site at day 7. On day 26 a post-treatment DTH test was performed (see below). At day 28, surgical resection of the liver metastases was performed. During the operation an accessible abdominal lymph node was excised and 6 mm punch biopsies were taken from the post-treatment DTH reaction sites. Before and after vaccination PBMCs were obtained.

The same schedule of 3 weekly i.v./i.d. vaccinations followed by a post-treatment DTH was repeated twice at intervals of 6 months in the absence of recurrent disease.

immunologic monitoring

CD8+ T cell responses against KLH were measured using a ³H-thymidine incorporation proliferation assay with PBMCs of the patients before and
after vaccination [15]. The index was calculated as the counts ratio between KLH-stimulated PBMC and non-stimulated PBMC.

Post-treatment DTH reactions were performed as described previously [19]. Briefly, CEA-peptide only (100 µg in 100 µl), DCs pulsed with CEA-peptide, DCs pulsed with KLH and CEA-peptide, and DCs pulsed with KLH only (0.4–5 × 10⁶ DCs each in approximately 100–200 µl) were injected i.d., 5–10 cm from an inguinal lymph node at different sites, in the upper leg contralateral from the leg in which the DC vaccinations were performed. The maximum diameter of induration was measured after 48 h. T cell culture from DTH biopsies was performed in low dose IL-2 (100 U/ml; Proleukin®, Chiron, the Netherlands) for approximately 2 weeks without ex vivo restimulation with antigen as described before [19].

Resected lymph nodes were disrupted and cell suspensions were made by gentle squeezing in a sterile open filter chamber (NPBI; Amsterdam, the Netherlands) in IMDM medium (Invitrogen; Paisly, UK) supplemented with 5% human serum (Sanquin; Nijmegen, the Netherlands). The cell suspension (2 × 10⁶) was plated on a 24-well plate (Costar Badhoevedorp, the Netherlands; 5 × 10⁵ cells/well), preincubated with sterile anti-CD3 antibody (Immunotech, Marseille, France) in 1 ml RPMI/7% human serum was added. After 1 day medium was added, containing IL-2 (100U/µl), which was repeated every 3 days. T cells were tested after 1–2 weeks of culture.

Tumor-infiltrating lymphocytes (TILs) were obtained and cultured according to the same protocol as the lymph node cell suspension (see above).

DTH-derived cells (1 × 10⁶ cells in 10 µl) or PBMC (1 × 10⁶ cells in 10 µl) were incubated with PE-labeled CEA and cytomegalovirus (CMV) tetrameric-MHC complexes (Sanquin; Amsterdam, the Netherlands) for 60 min at room temperature. In the last 20 min of this incubation, fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies directed against HLA-A2.1 binding peptide (tyrosinase or G250). Cytokine production was measured in response to T2 cells pulsed with CEA-peptide or an irrelevant peptide, DCs pulsed with KLH and CEA-peptide, and DCs pulsed with KLH only (1×10⁵ DCs each in approximately 100–200 µl) were injected i.d., 5–10 cm from an inguinal lymph node at different sites, in the upper leg contralateral from the leg in which the DC vaccinations were performed. The maximum diameter of induration was measured after 48 h. T cell culture from DTH biopsies was performed in low dose IL-2 (100 U/ml; Proleukin®, Chiron, the Netherlands) for approximately 2 weeks without

Table 1. Number of DCs injected per vaccination and induration (mm) of vaccination sites and post-treatment DTH skin tests

<table>
<thead>
<tr>
<th>Patient</th>
<th>Number of vaccinated DCs</th>
<th>Induration (mm)</th>
<th>Post-treatment DTH skin tests</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>of vaccinated DCs</td>
<td>Vaccination sites</td>
<td></td>
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<tr>
<td>I</td>
<td>II</td>
<td>III</td>
<td></td>
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<tr>
<td>1</td>
<td>5/8</td>
<td>5/0</td>
<td>5/0</td>
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<td>2</td>
<td>5/3</td>
<td>5/2</td>
<td>5/0</td>
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<td>3</td>
<td>5/14</td>
<td>5/10</td>
<td>5/10</td>
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<tr>
<td>4</td>
<td>5/7</td>
<td>5/9</td>
<td>5/10</td>
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<td>5</td>
<td>3/0</td>
<td>3/0</td>
<td>4/0</td>
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<td>6</td>
<td>5/5</td>
<td>5/0</td>
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<td>7</td>
<td>5/3</td>
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<td>10</td>
<td>4/0</td>
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*Cell numbers are given in 10⁶, intradermally/intravenously.

**In some cases the exact induration was not noted in the chart: if a palpable induration was found, this is indicated with a +.

CEA-pep, CEA-peptide; ND, not done.
DC vaccine characteristics
In all patients sufficient amounts of buffy-coat monocyte-derived mature DCs could be obtained for three i.d. vaccinations (Table 1). However, the yield was not always sufficient for simultaneous i.v. injections (see, for example, patient 1 or 5 in Table 1). Final DC vaccine products met the criteria of a mature phenotype [5, 15]. All patients had a proliferative peripheral blood CD4+ T cell response against the control protein KLH, as expected for a mature DC vaccine [15], indicating that the DCs were able to induce de novo T cell responses. The results of a representative patient who received three vaccination cycles are shown in Figure 1B.

CEA-responses in blood, tumor tissue and lymph nodes
In none of the patients could an increase in CEA-specific T cells be detected in unstimulated peripheral blood by direct tetramer analysis (Table 2). CEA-specific T cells were observed in one patient, however, these cells were also present before vaccination (Supplementary Figure 1D, E; patient 5). In five out of the nine patients who underwent resection of the metastases, low numbers of TILs were obtained. No specific anti-CEA or anti-tumor reactivity was observed by tetramer analysis and cytokine secretion (Table 2). In five patients an abdominal lymph node was resected: a coeliac node (patient 1), a mesenteric node (patients 2 and 4) and a node of the hepatoduodenal ligament (patients 3 and 6). In one of the hepatoduodenal ligament nodes, which are considered the tumor-draining lymph nodes in case of liver metastases [24], a clear population of CEA-specific T cells was detected (patient 3, Table 2, Figure 2C). These T cells were able to produce IFN-γ and IL-2 when co-cultured with CEA-peptide loaded T2 target cells (Figure 2C). In the other four patients, in whom a lymph node could be resected, no CEA-specific T cell reactivity could be observed (Table 2).

post-treatment DTH: induration
The induration sizes of the vaccination sites and the post-treatment DTH reaction skin tests that were performed with CEA-peptide alone or DCs loaded with CEA-peptide, KLH or both, are listed in Table 1. Several trends are noteworthy. The first vaccination already induced induration in eight of 10 patients, suggesting that in these patients some form of immunity against CEA already existed before vaccination. Secondly, in all patients induration size increased after subsequent vaccinations. Thirdly, the induration at the third vaccination site was usually larger compared with the induration at the post-treatment DTH sites, which were performed with lower numbers of the exact same CEA and KLH-loaded DCs (5 × 10^6 versus 0.05–0.5 × 10^6 DCs, respectively). Fourthly, post-treatment DTH skin tests that were performed with CEA-peptide alone induced induration in only two of 10 patients. These results suggest that the (amount of) DCs are of importance for induration to occur.

post-treatment DTH: CEA-specific T cell immunity
In seven out of 10 patients, CEA-specific DTH-infiltrated T cells were detected after vaccination by tetramer analyses. Of all patients that had specific T cells in at least one of their DTH-sites an example is shown in Figure 2A–H. In Table 2 the presence or absence of CEA-specific T cells after vaccination is given per DTH-site for each patient. We found 0.3% (Figure 2E) up to 98% (Figure 2G) of CD8+ T cells to be CEA-specific in these DTH biopsies. When these T cells were co-cultured with CEA-peptide loaded T2 target cells they were able to produce large amounts of IFN-γ and IL-2 (Figure 2A–H), but not IL-4 or IL-10 (not shown).

Table 2. CEA-specific T cell responses after vaccination

<table>
<thead>
<tr>
<th>Patient</th>
<th>First vaccination</th>
<th>Post-treatment DTHs</th>
<th>LN</th>
<th>Blood</th>
<th>Tumor</th>
<th>Clinical outcome (duration)</th>
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<tr>
<td></td>
<td></td>
<td>CEA-pep</td>
<td>DC+KLH</td>
<td>DC+CEA</td>
<td>DC+CEA+KLH</td>
<td>ND</td>
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<td>1</td>
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</table>

The presence of CEA-specific T cells was scored positive (+) if both tetramer analysis and IFN-γ release after co-incubation with CEA-loaded target cells were positive. Data from the first vaccination cycle are given. The criteria for determination of positive immune responses are described in patients and methods. Shaded cells denote results of which the details are given in Figure 2.

*In this patient data from the DTH of the second cycle are given. The first post-treatment DTH-culture was not evaluable.

*bThis vaccination site.

*In this patient data from the DTH of the second cycle are given. The first post-treatment DTH-culture did not show CEA-specificity.

LN, lymph node; ND, not done (no biopsy taken); NE, not evaluable (no tissue excised); NED, no evidence of disease.
of induration in four of these patients (Table 1; patients 5–7 and 9). This suggests that a possible pre-existing CEA-specific T cell reactivity did not have the potency to result in T cell outgrowth from the biopsy. However, in two of these patients CEA-specific T cells were readily detected in DTH skin tests after treatment (patients 5 and 6; Table 2, Supplementary Figure 1).

In a patient with a history of CMV-infection, we found high numbers of CMV-specific T cells in peripheral blood, but not in a biopsy of a positive DTH reaction skin test (Supplementary Figure 2A, B). In this patient, no CEA-specific T cells could be detected in peripheral blood, but a large population was readily detected in the DTH biopsy (Supplementary Figure 2C, D), demonstrating the specificity of the DTH assay.

**discussion**

We vaccinated 10 patients with liver metastases of colorectal cancer with CEA peptide-pulsed mature monocyte-derived DCs prior to liver metastasectomy. Antigen-specific T cells responses were monitored in peripheral blood, T cell cultures from biopsies of vaccination sites and post-treatment DTH skin tests that were performed in skin sites remote from the injection sites, and when available also in abdominal lymph nodes and tumor tissue. We here demonstrate that (1) mature CEA-peptide pulsed DCs induce potent CEA-specific T cell responses, (2) vaccination with buffy-coat derived, monocyte-derived DCs is feasible and (3) monitoring antigen-specific T cells in post-treatment DTH skin tests proved more sensitive when compared with blood, abdominal lymph nodes and tumor tissue.

Functional CEA-specific T cells in short cultures of post-treatment DTH biopsies without *in vitro* restimulation could be demonstrated in seven of 10 patients. No CEA-specific T cells were present in biopsies of first vaccination sites, indicating that specific T cell reactivity in the post-treatment DTH was induced by the vaccine. In previous reports spontaneous low-grade CEA-specific responses have been found infrequently in metastatic colorectal cancer patients [25]. The local induration that we observed after the first vaccination, which we have never observed in our melanoma studies using the same DC vaccine [15], suggests that some form of pre-existing immunity against CEA was also present in our patients, although apparently this immunity was not strong enough to result in T cell outgrowth from this vaccination site. We cannot, therefore, be certain that *de novo* induction of anti-CEA immunity is caused by the vaccine; however, it is evident that at least a strong potentiation takes place.

We did not observe a correlation between the induration size of DTH reactions and CEA-specific T cell reactivity. This confirms our previous observations in melanoma patients [19]. Here, we found that antigen-specific T cell reactivity can even be detected in the absence of induration in a DTH reaction that was performed with CEA-peptide only. The absence of induration in this DTH reaction could be explained by a lack of CD4+ T cells in the absence of DC-produced chemokines and cytokines [26]. Together these data show that the DTH reaction is a complex assay. Yet, these findings also indicate that for the detection of antigen-specific T cells in cancer
immunotherapy it may be beneficial to always obtain biopsies of DTH-reaction sites irrespective of induration size.

We show here that adequate numbers of DCs can be cultured fromuffy-coat derived monocytes. Compared to leukapheresis, which is so far the predominantly used method to obtain DCs for clinical vaccination studies, this is a more simple and less laborious procedure with little burden for the patient, although cell yields are lower. Nevertheless we observed CEA-specific IFN-γ producing T cells after three i.d. injections of $3 \times 10^6$ DCs only, indicating that low numbers of DCs may suffice. If the migration efficiency of these low numbers of DCs can be improved, this response may be further enhanced [27]. Therefore, the use of buffy-coat derived DCs may facilitate clinical DC vaccination trials.

In other studies with CEA-peptide pulsed DCs no CEA-specific immune responses were found [7] or only in a minority of patients [9–10], which may be explained by differences in DC maturation state, the culture protocol, patient selection or immunomonitoring methods (peripheral blood only). Fong et al. [8] investigated the immunogenicity of another type of DCs (Flt-3-Ligand expanded blood DCs) pulsed with an altered CEA peptide in patients with CEA-expressing colorectal and non-small-cell lung tumors. In this study, a correlation was found between clinical response and the post-vaccination expansion of CEA-specific T cells in peripheral blood. However, in contrast to our study, several restimulation steps in vitro were necessary for evaluation of the functionality of these cells.

In our study, CEA-specific T cells were detected slightly above background in peripheral blood of only one out of 10 patients by direct tetramer staining. However, in this patient these cells were already present before vaccination. From these results it can be concluded that CEA-specificity could be detected in one in 10 000 CD8+ T cells by tetramer staining of peripheral blood mononuclear cells. Therefore, the fact that we could not find CEA-specific T cells in more patients may be due to lower frequencies of these cells in the circulation after vaccination. Perhaps a short in vitro culture period would have allowed the detection of lower CEA-specific T cell frequencies. Nevertheless, very low numbers of high-affinity T cells that are not detectable by means of direct tetramer-staining of peripheral blood may suffice for rejecting tumors in cancer patients, as shown by others [28, 29]. In another study these authors showed higher frequencies of vaccine- and tumor-specific T cells in metastases compared with peripheral blood after MAGE-3 vaccination in a melanoma patient [30]. In our study we did not detect CEA-specific T cell responses in tumor samples. This may be explained by the limited amount of available tumor tissue, the short interval between vaccination and surgical resection or immunomodulatory mechanisms in the tumor microenvironment that may preclude adequate T cell infiltration [31].

Regional lymph nodes at the vaccination site may be another relevant compartment to monitor antigen-specific T cell responses, as has been shown in murine studies as well as in human melanoma studies [17, 27, 32]. In our study an easily accessible abdominal lymph node was resected in five patients. In one patient functional CEA-specific T cells were found. In most patients no detectable CEA-response was found in lymph nodes, although they were present in biopsies of the DTH skin tests. This may be because the excised lymph nodes were not draining either the DC vaccination sites or the tumor [24]. The small patient numbers and short follow-up of our series does not allow any correlation between immunological results and clinical outcome. Given the positive correlation between specific T cell reactivity in DTH biopsies and clinical outcome in our previous study with melanoma patients vaccinated with peptide-pulsed DCs [19], our current results provide further support for the potential of DTH skin testing to monitor specific T cell reactivity upon antigen-specific cancer immunotherapy.

In conclusion, CEA-peptide pulsed mature DCs derived from buffy-coats can induce potent CEA-specific T cell reactivity in advanced colorectal cancer patients. In this study, DTH skin testing provided superior results in the monitoring of antigen-specific T cell responses compared with peripheral blood, abdominal lymph nodes and tumor tissue. These results warrant further studies with CEA-loaded DC vaccines in colorectal cancer.

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