Natural T-cell Response against MHC Class I Epitopes of Epithelial Cell Adhesion Molecule, her-2/neu, and Carcinoembryonic Antigen in Patients with Colorectal Cancer

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ABSTRACT

The antigens epithelial cell adhesion molecule (Ep-CAM), her-2/neu, and carcinoembryonic antigen (CEA) are potential T-cell targets in antigen-specific vaccination-based cancer therapy. We performed this study to evaluate whether a natural specific T-cell response against these tumor-associated antigens (TAAs) already exists in patients with colorectal carcinoma (CRC). We used the IFN-γ ELISPOT assay to detect circulating TAA-reactive T cells directly ex vivo in unstimulated peripheral blood mononuclear cells. We analyzed the T-cell response in peripheral blood mononuclear cells of 22 HLA-A2-positive patients with CRC and 8 HLA-A2-positive healthy subjects against 3 HLA A2-restricted peptide epitopes of the TAAs Ep-CAM (GLKAGVIAV), her-2/neu (IISAVVGIL), and CEA (YLSTGANNLNL). Seven of 22 patients but none of the 8 healthy subjects had T cells specifically secreting IFN-γ in response to one to three of these antigens (n = 4, Ep-CAM; n = 5, her-2/neu; n = 6, CEA). In three of the seven responding patients, TAA-reactive T cells were further characterized by flow cytometry. In all three patients, the majority of these T cells have a CD3+CD8+IFN-γ+/CD69+CD45RA+ phenotype, resembling activated effector-type T cells. T-cell responses occurred only in patients with metastatic disease (Dukes’ stages C and D). The results of this study indicate that natural T-cell responses against TAAs occur in approximately one-half of CRC patients with involvement of lymph nodes or distant metastases, but not in CRC patients with disease confined to the intestinum.

INTRODUCTION

There is increasing evidence that a tumor-directed T-cell response exists in patients with CRC.3 Tumor-reactive T cells have been expanded from tumor-infiltrating lymphocytes and PBMCs in CRC patients (1–3). However, these tumor-reactive T cells have been expanded in vitro by repeated stimulation with antigen and IL-2, which results in quantitative and qualitative changes. From these studies, the question of whether functionally active colon cancer-reactive T cells exist in CRC patients remains unresolved.

Thus far, little is known about possible natural target antigens for cytotoxic T cells in CRC patients. Recently, some antigens have been identified as potential targets of cytotoxic T cells including Ep-CAM, her-2/neu, and CEA. Ep-CAM (17-1A, EGP-2, GA733-2) is a cell surface glycoprotein that is expressed in over 90% of CRCs (4). An Ep-CAM-derived, HLA-A2-restricted peptide reproducibly induced peptide-specific cytotoxic T cells in HLA-A*0201 Kb transgenic mice (5). However, whether this peptide is recognized by T cells of CRC patients has not yet been shown. A natural humoral immune response against Ep-CAM has been described in 15% of patients with CRC (6). Another potential T-cell target in CRC is her-2/neu (C-erbB2, p185), a protein found to be overexpressed in a number of adenocarcinomas including CRC. More than 80% of CRCs are her-2/neu positive (7). her-2/neu was identified as a target of cytotoxic T-cell lines in patients with breast and ovarian tumors (8, 9). Brossart et al. (10) showed that her-2/neu-specific T cells were able to lyse her-2/neu-positive CRC cell lines as well. However, whether her-2/neu-reactive T cells do exist in CRC patients is not known. A humoral immune response against her-2/neu is described in 14% of patients with CRC (11). The TAA CEA is expressed in up to 85% of CRCs (12). Cytotoxic T-cell lines reactive with CEA were generated from the PBMCs of CRC patients vaccinated with a recombinant CEA vaccine (13). The same group showed that human tumor cells can process CEA and present a 9-mer peptide (CAP-1) by MHC class I molecules and, in doing so, may generate and activate CTLs.

We have performed this study to evaluate whether circulating T cells reactive to the above-mentioned TAA-derived peptides are detectable in peripheral blood lymphocytes from patients with CRC. To study the T-cell response directly ex vivo, we have used the ELISPOT assay. This assay is a sensitive, reproducible, and reliable technique to detect T cells by their antigen-induced secretion of cytokines on a single cell level (14–16). In this study, we demonstrate a natural T-cell response against tumor antigens in one-half of the patients with metastatic CRC. Further characterization of the TAA-reactive T cells was performed by flow cytometric analysis in CRC patients with a strong T-cell response.

MATERIALS AND METHODS

PBMCs. Blood samples were obtained from HLA-A2-positive patients with colorectal adenocarcinoma and healthy subjects after obtaining informed consent. PBMCs were isolated from heparinized blood by density gradient centrifugation using Ficoll-Hypaque 1.077 (Biochrom, Berlin, Germany). Cells were washed twice with PBS (Biochrom) and cryopreserved at −196°C in FCS (Biochrom) containing 10% DMSO (Merck, Berlin, Germany). Before assaying, the frozen cells were thawed, and analysis of peptide-reactive T cells was performed after overnight incubation in basal Iscove’s medium (Biochrom) supplemented with 10% human AB serum and antibiotics.

HLA Typing. HLA typing of the healthy subjects was performed serologically by the standard NIH microlymphocytotoxicity test. Expression of HLA-A2 in patients was determined by using monoclonal mouse antihuman IgG antibodies specific for HLA-A2 (One Lambda, Krefeld, Germany). Fluorescein-conjugated goat antimouse IgG was used as a second antibody (Immunotech, Hamburg, Germany). Data acquisition was performed on FACS-Calibur and analyzed using CellQuest software (Becton Dickinson, Heidelberg, Germany).

Peptides. The peptides were synthesized using an Applied Biosystems (Foster City, CA) 432 A peptide synthesizer following a standard protocol according to the published sequences for Ep-CAM p263–271 [GLKAGVIAV (5)], her-2/neu p654–662 [IISAVVGIL (8, 17)], and CEA p571–579 [YLSTGANNLNL (CAP-1; Ref. 13)]. The peptides were purified by reverse high-performance liquid chromatography and checked by mass spectrometry. They
were dissolved in DMSO (Merck, Darmstadt, Germany) at a concentration of 5 mg/ml and further diluted in PBS. The peptides were kindly provided by Dr. Stefan Stevanovic (University of Tuebingen, Tuebingen, Germany).

**IFN-γ-ELISPOT Assay.** The 96-well nitrocellulose plates (Millititer; Millipore, Bedford, MA) were coated overnight with 50 μl/well of 8 μg/ml antihuman IFN-γ mAb (catalogue number 1598-00; Genzyme, Rüsselsheim, Germany). Wells were then washed and blocked with Iscove’s modified DMEM (Biochrom) and 10% AB serum for 2 h at 37°C. PBMCs (1 × 10^6) were incubated in a concentration of 1.67 × 10^5 cells in 200 μl of basal Iscove’s medium (supplemented with 10% AB serum and antibiotics) per well, admixed with peptides in a concentration of 10 μg/ml. PBMCs incubated with or without pokeweed mitogen served as positive or negative controls, respectively. After 24 h of incubation in the antibody-coated plates at 37°C and 5% CO₂, the plates were washed six times with PBS + 0.05% Tween 20. Wells were incubated for 24 h at 4°C with 50 μl/well biotinylated mouse antihuman IFN-γ mAb (clone 4S.B3; PharMingen, Hamburg, Germany) at a concentration of 2.5 μg/ml. After washing four times with PBS, 100 μl of streptavidin-alkaline phosphatase (Bio-Rad, Munich, Germany) diluted 1:1000 were added for 2 h at room temperature. After another washing step with PBS, 100 μl/well 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Bio-Rad) was added to each well for 10–20 min. Color development was stopped by washing under running tap water. After drying at room temperature, IFN-γ-secreting T cells were counted using the automated image analysis system ELISPOT Reader (AID, Strassberg, Germany).

**Flow Cytometric Analysis.** PBMCs (1 × 10^6) were stimulated with 10 μg/ml peptide (Ep-CAM or CEA). PBMCs incubated with or without pokeweed mitogen served as positive or negative controls, respectively. After 1 h, 20 μg of brefeldin A (Sigma, Deisenhofen, Germany) were added. After 5 additional h of incubation, PBMCs were washed once with PBS and incubated in PBS containing 1 mM EDTA for 10 min. Two additional washing steps with PBS and 2% polyclonal human immunoglobulin (a-globin; Grifols, Langen, Germany) were performed. CD3, CD8, and CD45RA were stained by incubation with fluorescein-conjugated mAbs for 15 min on ice in the dark. Afterward, lysing solution and permeabilization solution were added (Becton Dickinson, San Jose, CA) according to the manufacturer’s instructions. IFN-γ and CD69 were then stained by incubation with fluorescein-conjugated mAbs for 30 min on ice in the dark. After another washing step, cells were fixed with 1% formaldehyde in PBS. Data acquisition was performed on FACScalibur and analyzed using CellQuest software (Becton Dickinson). The following antibodies from Becton Dickinson were used: (a) peridinin-chlorophyll-protein-conjugated mouse antihuman CD3; (b) allophycocyanin-conjugated mouse antihuman CD8; (c) phycoerythrin-conjugated mouse antihuman CD69; (d) phycoerythrin-conjugated mouse antihuman CD45RA; and (e) FITC-conjugated mouse antihuman IFN-γ.

**Statistical Analysis.** We assumed a potential T-cell response against a peptide if more than 10 T cells per 1 × 10^6 PBMCs secreted IFN-γ in response to the peptide (background subtracted). In these cases, the Wilcoxon signed-rank test was performed to determine whether there was a statistically significant difference between the number of IFN-γ-secreting T cells in peptide-stimulated and unstimulated wells (background). To test whether there is a statistically significant difference in the number of T-cell responses between patients with metastatic and limited disease, data were analyzed using χ² statistics. Because of multiple testing, these Ps are descriptive.

**RESULTS**

**Patient Characteristics.** PBMCs of 9 patients with limited disease (Dukes’ stages A and B) and 13 patients with metastatic disease (Dukes’ stages C and D) were investigated. All patients except P2, P13, and P19 had undergone surgery at least 1 month before we took the blood samples. Nine of 22 patients had received 5-fluorouracil combined with folinic acid [limited disease (Dukes’ stages A and B), P3, P4, and P6; metastatic disease (Dukes’ stages C and D), P11, P12, P18, P20, P21, and P22]. Patient P22 had also been treated with irinotecan. Three patients (P4, P11, and P15) had been irradiated. No patient had received immunotherapy by anti-17-1A antibody, IL-2, or vaccination before our analyses (see Table 1).

**T-cell Responses in Healthy Subjects.** As a control, we first tested PBMCs from healthy subjects. We analyzed the T-cell reactivity of eight healthy HLA-A2-positive subjects against the 3 HLA-A2-positive restricted peptide epitopes of the TAAs Ep-CAM, her-2neu, and CEA. None of the healthy individuals had a frequency of T cells secreting IFN-γ significantly above background for any of the three peptides (see Figs. 1–3). The mean response against pokeweed mitogen as positive control was 261 IFN-γ-secreting T cells per 1 million PBMCs (range, 144–374 IFN-γ-secreting T cells in peptide-stimulated and unstimulated wells). To test whether there is a statistically significant difference in the number of T-cell responses between patients with metastatic and limited disease, data were analyzed using χ² statistics. Because of multiple testing, these Ps are descriptive.

**T-cell Responses in Patients with CRC.** We tested the T-cell response of 22 HLA-A2-positive patients with CRC against the three HLA-A2-positive restricted peptide epitopes. Patient data are shown in Figs. 1–3 and Table 1. Seven of 22 patients (P10, P13, P15, P16, P19, P20, and P22) did not have a positive T-cell response. (a) Patient P13 had received 5-fluorouracil, 5-fluorouracil, and folinic acid.

**Table 1 Patient characteristics**

<table>
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<tr>
<th>Patient no.</th>
<th>Dukes’ stage</th>
<th>Sex</th>
<th>Previous therapy</th>
<th>Time since surgery (mo)</th>
<th>Ep-CAM</th>
<th>Her2/neu</th>
<th>CEA</th>
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<td>P1</td>
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<td>M</td>
<td>Surgery</td>
<td>102</td>
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<td>B</td>
<td>M</td>
<td>Surgery, chemo²</td>
<td>17</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>P4</td>
<td>B</td>
<td>M</td>
<td>Surgery, chemo, radiation</td>
<td>19</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P5</td>
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<td>M</td>
<td>Surgery</td>
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<td>–</td>
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<td>–</td>
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<td>P6</td>
<td>B</td>
<td>M</td>
<td>Surgery, chemo</td>
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<td>P7</td>
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<td>–</td>
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<td>M</td>
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<td>D</td>
<td>F</td>
<td>Surgery, chemo</td>
<td>21</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P22</td>
<td>D</td>
<td>M</td>
<td>Surgery, chemo²</td>
<td>21</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

², positive T-cell response.

², chemo, 5-fluorouracil and folic acid.

² This patient had received 5-fluorouracil, folic acid, and irinotecan.
patients with metastatic disease (Dukes’ stages C and D), we could detect a T-cell response against one to three of the TAAs. The proportion of responders among patients with metastatic disease is significantly increased ($P < 0.05$) when compared with the expectation for the whole group of patients with CRC.

**T-cell Response before and after Treatment with Anti-17-1A Antibody (Anti-Ep-CAM).** In one patient (P12), we were able to obtain PBMCs before and after therapy with anti-17-1A antibody (anti Ep-CAM) and low dose IL-2. Whereas this patient had no specific T-cell response before therapy with anti-17-1A antibody, T cells specifically secreting IFN-$\gamma$ could be detected by ELISPOT assay in response to the Ep-CAM peptide as well as the other two peptides after three cycles of therapy (see Fig. 5).

**DISCUSSION**

This study provides evidence for the existence of an in vivo T-cell response to TAA in CRC patients. Furthermore, our study supports two other points. It demonstrates for the first time that her-2/neu is a tumor antigen recognized by T cells in CRC patients, as described previously in breast and ovarian tumors (8, 9). Second, our results show that the Ep-CAM peptide 263–271 characterized in HLA-A2 transgenic mice (5) is recognized by T cells derived from CRC patients, providing further support that this peptide is a human T-cell epitope.

Our data contrast with the common hypothesis that CRC is of poor immunogenicity. Several authors have shown that CRC cell lines...
secrete immunosuppressive cytokines and that stimulation of T-cell responses is hampered due to low expression of HLA class I molecules and the lack of intercellular adhesion molecule and HLA-DR (18, 19). Anergic or functionally deficient T cells have also been demonstrated in CRC patients (20, 21).

We do not know whether the TAA-reactive T cells we detected in our patients are able to destroy tumor cells. However, we postulate that these antigen-reactive T cells are functionally active because they were characterized by the ability to secrete IFN-γ in response to tumor antigen peptides, a characteristic usually associated with memory and effector T cells. Furthermore, we were able to demonstrate in three patients that most of the TAA-reactive CD3+CD8+ T cells express CD69 and CD45RA. CD3+CD8+IFN-γ+CD45RA+ T cells were shown to belong to the effector-type T-cell subset that is able to directly secrete perforin and granzyme upon stimulation (22).

Our results demonstrate T-cell responses against TAA only in patients with metastatic stages of disease (Dukes’ stage C and D disease). In accordance with our results, a higher frequency of autoantibodies against Ep-CAM (GA 773-2) was also reported among patients with metastatic CRC (6). This higher frequency of humoral immune responses in metastatic CRC is similar to observations in breast cancer (her-2/neu) and melanoma [tyrosinase and NY-ESO-1 (23–25)]. One hypothesis based on our observation is that the evasion of tumor cells, especially in lymph nodes, is a prerequisite for the induction of TAA-specific T-cell responses. An alternate explanation might be that peptide-specific T cells are at the tumor site in patients with limited disease, whereas they are in the periphery in patients with metastatic disease because the tumor does not attract them anymore. This may be due to “tumor escape” mechanisms such as HLA loss, antigen loss, or anergy induction.
Immunotherapy using mAb 17-1A directed against Ep-CAM in CRC patients is associated with an increased survival (26). T cells proliferating in response to Ep-CAM after mAb 17-1A therapy were described in patients with evidence of tumor regression (27). We could demonstrate the induction of a CD8+ T cells against Ep-CAM in a patient (P12) treated with mAb 17-1A. Furthermore, this patient also developed a T-cell response against the two other peptides derived from her-2/nu and CEA after antibody treatment. One possible explanation for a T-cell response against all three peptides might be that antibody-mediated tumor cell destruction favors cross-presentation of MHC class I epitopes with the consequent activation of tumor-reactive CD8+ T cells (28).

In summary, our study shows the existence of T cells that are reactive with TAA in nearly one-third of patients with CRC. T-cell responses appeared only in patients with metastatic disease. Our findings have profound implications for immunotherapy in CRC patients. The antigens Ep-CAM, her-2/nu, and CEA are immunogenic in CRC patients and may be suitable for vaccination therapy aimed at the induction of cytotoxic T-cell responses. Vaccination may be especially valuable in patients with limited stages of CRC, when no natural systemic T-cell response has developed, and therefore escape tumor cell variants are less likely to have developed.

REFERENCES


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