Increased Expression of CD40 Ligand on Activated T Cells of Patients with Colon Cancer

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ABSTRACT

Purpose: Proper function of T lymphocytes is crucial for an effective destruction of cancer cells in vivo. Identifying the cell surface molecules on the T cells that may be involved in this antitumor response may help to elucidate immunological factors influencing the biology of human tumors.

Experimental Design: Differences in the antigen expression profile of unstimulated and stimulated peripheral blood T-lymphocytes from patients with colon cancer and from normal controls were determined using flow cytometry.

Results: Freshly isolated peripheral blood T cells of patients with colon cancer did not differ in their phenotype significantly from those of patients with nonmalignant diseases. In contrast, in vitro stimulated T cells of patients with colon cancer had a significantly increased expression of CD40 ligand (CD40L, CD154) compared with activated T cells of the control group; increased CD40L expression was also found in the CD4+ and CD8+ T-cell subpopulations.

Conclusions: The data presented support additional studies investigating the role of CD40L in the immune response against colon carcinoma.

INTRODUCTION

Within the tumor-host interface (1), intact T cell–mediated immunity is a key determinant for the cellular defense mechanisms against colon carcinoma in vivo. To boost this antitumor immunity, much effort has been put into the study of ex vivo activated peripheral blood T cells as a source for adoptive immunotherapy of human malignancies. Defining the antigen repertoire of peripheral blood T cells of patients with colon cancer may help to describe the immune response against this tumor and may also be relevant for immunotherapeutic strategies.

To date, differential antigen repertoire of peripheral T cells of patients with colon cancer compared with T cells of patients with nonmalignant diseases has not been studied extensively. Studies of Takii et al. (2) and Okada et al. (3) demonstrated an increased proportion of T cells expressing the natural killer (NK) cell markers CD56 and CD57 in the peripheral blood of patients with colon cancer.

Yet, T cells contain subpopulations with significant antitumor effect, as has been shown in numerous murine (4–8) and clinical studies (9, 10) using T cells activated with anti-CD3 and IL-2.

In the present study, we addressed the question whether freshly isolated or in vitro stimulated T cells from patients with colon cancer differ in their antigen repertoire from those cells of patients with nonmalignant diseases.

Defining the differential antigen repertoire of peripheral blood T cells in patients with colon cancer may provide further insight into the pathophysiology of this malignancy and may help to design new immunotherapeutic strategies.

MATERIALS AND METHODS

Patients. Twenty patients (ages 43 to 86 years) before elective surgery for primary diagnosed colorectal cancer were included in this study after obtaining informed consent. This study was approved by the Ethics Committee of the Charité, Virchow Hospital of the Humboldt University of Berlin. None of the patients had received prior chemotherapy. Twenty age- and sex-matched patients on whom surgery was performed for nonmalignant diseases, e.g., cholecystolithiasis or appendicitis, were used as controls. In all cases, peripheral blood was obtained the day before surgery and processed immediately.

Isolation of Cells and Cell Culture. PBMCs were isolated from heparinized venous blood by centrifugation over Ficoll-Hypaque (1500 rpm, 30 min, 4°C). Isolated PBMCs cells were washed three times with 1% PBS Dulbecco (Seromed/ Biochrom kG, Berlin, Germany). Cells were cultured at a concentration of 3 × 10^6 cells/ml. Culture medium contained RPMI 1640 with 1-glutamine (PAA Laboratories, Linz, Austria) supplemented with 10% heat-inactivated FCS (Life Technologies GmbH, Karlsruhe, Germany), 0.1mol/liter sodium pyruvat (Life Technologies, Germany), 1% penicillin/streptomycin (Seromed, Germany) and 1mol/liter HEPES (Life Technologies, Germany). Cells were incubated at 37°C in a humidified 5% CO2 atmosphere.
T cell growth was stimulated by addition of monoclonal anti-CD3 (clone: UCHT1, 50 ng/ml; Immunotech, Marseille, France) and rIL-2 (Life Technologies) with a specific activity of 300 IU/ml for the first 24 h after initiation of culture. Thereafter, cells were cultured with rIL-2 alone (300 IU/ml) until day 7. Fresh medium containing rIL-2 was added every 48 h.

**Immunofluorescent Analysis.** Expression of a large panel of cell surface antigens (n = 38) on T cells was examined on day 0 and on day 7 of cell culture. T cells were phenotyped using coexpression of CD3. Furthermore, T-cell subpopulations (n = 13) were analyzed by coexpression of CD4 and CD8. Two- and three-color flow cytometry was performed using a FACScan (Becton Dickinson Company, San Diego, CA). Isotype controls were used to define positive or negative antigen expression.

**Antibodies.** FITC-conjugated MoAbs against CD3 (clone UCHT1), CD4 (13B8.2), CD8 (B911), CD11a (25.3.1), CD18 (B9.11), CD45RA (ALB11), CD45RO (UCHL1), CD48 (J4-57), CD49b (Gi9), CD49d (HP2/1), CD51 (AMF7), CD54 (84H10), CD62L/Ligand (DReg56), CD90 (Thy1.23), CD95L/Ligand (4H9), phycoerythrin-conjugated CD3 (UCHT1), CD8 (B9.11), CD45RA (ALB11), CD45RO (UCHL1), and CD56 (B159), and unconjugated MoAbs against CD35 (clone J3.D3), CD49a (HP2B6), CD49f (GoH3), CD50 (HP2/19), and mouse IgG1 (isotype control) with high (90%), medium (10–90%), or low expression (≤10%; Figs. 1 and 2). When analyzed for significant differences in their expression pattern unstimulated T cells from patients with colon cancer (n = 20) and with nonmalignant diseases (n = 9). Marked by the diagram are the means and the SDs. Antigens were combined into groups with high (+ +), medium (+), and low expression (−) according to the percentages of T cells that were positive for the antigen under investigation. No significant differences were detected (Mann-Whitney U test).

**RESULTS AND DISCUSSION**

**Expression Pattern of Unstimulated T Cells.** To define a disease-related phenotype of peripheral blood T cell subsets, it was crucial to avoid sampling errors. The antigen repertoire of peripheral blood lymphocytes may be influenced by numerous factors, such as age (11), general anesthesia (12), type of anticoagulant that was used, and time lag between blood draw and processing of cells.4 We minimized these artifactual risks by obtaining blood from age- and sex-matched patient groups the day before surgery and by preparing PBMCs under standardized conditions.

Differential antigen expression of peripheral T cells of patients with colon cancer and patients with nonmalignant diseases was determined using a large panel of MoAbs against functionally important markers. Defined by differing percentages of T cells positive for the respective antigens, groups could be designed with high (+++, >90%), medium (+, 10–90%), or no expression (−, <10%; Figs. 1 and 2).

In both patient groups, a number of cell surface markers was expressed on almost all unstimulated T cells, e.g., CD2 (LFA-2), CD11a/CD18 (LFA-1), CD48, CD50 (ICAM-3), CD98 (4F2), CD102 (ICAM-2), and CD162 (PSGL-1). In contrast, unstimulated T cells in both groups failed to express CD134 (OX40), CD152 (CTLA-4), and CD40 ligand (CD40L; CD154).

When analyzed for significant differences in their expression pattern unstimulated T cells from patients with colon can-
cer did not differ from those of patients with nonmalignant diseases (Fig. 1). Similarly, T-cell subpopulations defined by the markers CD4 and CD8 did not show a significant difference between the two patient groups (data not shown).

These findings were in contrast to the data presented by Takii et al. (2), who reported an increased expression of the natural killer cell marker CD56 on unstimulated T cells of patients with colon cancer. Instead, in our study, the percentage of unstimulated peripheral blood T cells whose phenotype pattern may be influenced by the malignant disease might have been too small to be detected by immunophenotyping.

Modulation of T-cell Expression Pattern by in Vitro Stimulation. To increase the proportion of those peripheral blood T cells subsets that may be related to the immunological response against colon cancer, T-cell growth was induced with anti-CD3 and IL-2. CTLs generated by this method were used in clinical studies (9, 10) and exerted significant antitumor effects in murine models (4–8). Stimulated peripheral blood T cells lead to the up-regulation of the adhesion molecules CD11a/CD18 (LFA-1) and CD102 (ICAM-2), which mediate in situ tissue-binding of T cells (13).

In both patient groups in vitro stimulation modified the antigen pattern of the T cells to a similar degree. The CD8 marker was expressed on more cultured T cells than on unstimulated T cells (Figs. 1 and 2), which indicated that in vitro stimulation enriched for activated cytotoxic T cells that might also exert in vivo immune effector functions. Within the CD4+ T-cell subsets of both groups, in vitro stimulation lead to a significantly higher increase of the CD4+/CD45RO−-subset compared with the CD4+/CD45RA+—subgroups (P = 0.001; data not shown). Furthermore, compared with unstimulated T cells, cultured T cells of both patient populations contained higher percentages of cells expressing CD25 (IL2-receptor), CD49d (integrin α4), CD134 (OX40), and CD40L. On the other hand, stimulated T cells contained fewer percentages of cells positive for CD4 and CD62L (L-selectin) than freshly isolated T cells did (Figs. 1 and 2).

A number of antigens were not affected by in vitro cell stimulation, such as CD35 (CR1), CD51, CD56 (N-CAM), CD90 (Thy-1), CD104 (β2 integrin), CDw137 (4-IBB), CD152 (CTLA-4), and CD158a/b (p58.1/p58.2; Figs. 1 and 2).
Increased Proportion of CD40L+ Cells among Activated T Cells in Patients with Colon Cancer. Among all of the antigens examined, CD40L was the only marker for which the expression differed in both groups significantly (Fig. 3). Stimulated T cells of patients with colon cancer compared with the control cells contained a significantly increased percentage of T cells marked by anti-CD40L (P < 0.0001). Whereas 19.1 ± 4.3% of the T cells of colon cancer patients expressed CD40L, only 5.3 ± 5.2% of the T lymphocytes of nonmalignant controls were positive for CD40L. Activated T cells of colon cancer patients had significantly increased percentages of CD40L-positive cells also in the CD4+ and CD8+-fractions (P < 0.002; Fig. 3). Consistent with prior data (14–16), this indicates that increased expression of CD40L may not be restricted to the T-cell helper or suppressor fraction. For the Wilcoxon-Mann-Whitney test used, we calculated a power of 76–92% when the location difference between the colon cancer and the control group is about one SD. Given these relatively high power and significant Ps, we conclude that the differential expression pattern of CD40L was significant. Yet, because this altered phenotype affected only a minority of all T cells, it is difficult to hypothesize the biological impact of these antigenic changes.

We further investigated whether the increased CD40L expression on activated T cells of patients with colon cancer may correlate with the stage of the disease. As shown in Fig. 4, T cells of patients in stages Bukes D and C showed a trend toward containing higher percentages of cells positive for CD40L than T cells of patients in stages A and D.

The finding that elevated CD40L expression on activated T cells was found only in patients with advanced but not metastatic colon cancer, can be interpreted only cautiously. Possibly, colon cancers with limited stages may be too small to mount an immune response that may be detectable in the peripheral blood. On the other hand, metastatic colon tumors may perturb important T-cell functions. Support for this latter thesis comes from multiple studies demonstrating that several tumor types can abrogate critical antitumor mechanisms of T cells (17–21).

Increased expression of CD40L on stimulated T cells of patients with colon cancer has not yet been described. Support for the biological relevance of these findings comes from a large set of data demonstrating that the binding of CD40L to its receptor, CD40, is a prime event during the antitumor immune response. Blockade of the CD40/CD40L complex, for example, has been shown to diminish priming of CD8+ cytotoxic T cells (CTLs; Ref. 22). In addition, CD4+ T-helper cells can activate antigen-presenting cells by binding with CD40L to CD40 on antigen-presenting cells, which subsequently stimulate CD8+ CTLs (23–26). Finally, the blockade of the CD40/CD40L interaction in vivo has been shown to specifically prevent the priming of Th1 cells (27), whose cytokine production is necessary for optimal antimtumor function (28, 29). Consequently, several immunotherapeutic studies used the CD40/CD40L system to generate CTLs leading to enhanced tumor regression (30–32).

Our finding that CD40L is expressed on higher percentages of activated T cells of patients with colon carcinoma supports additional studies investigating the role of CD40L in the immune response against this human malignancy.
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