Characterization of Intracellular Cytokine Profile of CD4(+) T Cells in Peripheral Blood and Tumor-draining Lymph Nodes of Patients with Gastrointestinal Cancer

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Background: Analysis of serum cytokine levels has shown that cancer-bearing hosts have lower levels of IL-2 and IFN-γ, suggesting that Th1-type immunity is impaired by cancer. However, the mechanisms of the Th1 dysfunction are not clearly understood.

Method: The frequencies of Th1 cells in CD4(+) helper T cells were evaluated with an intracytoplasmic cytokine staining method in peripheral blood lymphocytes (PBL) and lymph node lymphocytes (LNL) of patients with gastrointestinal cancer.

Results: Activation of lymphocytes with PMA + Ionomycin induced the expression of IL-2 and IFN-γ in each lymphocyte population. Compared with PBL of non-malignant donors, PBL in cancer patients contained significantly lower frequencies of CD4(+) T cells that produced IL-2 and IFN-γ. LNL in cancer patients also contained lower levels of IL-2- and IFN-γ-producing CD4(+) T cells, although the percentages did not show significant differences from those of PBL in the same patients.

Conclusion: Our data suggest that suppression of Th1 cytokine in cancer patients is, at least in part, due to the decreased frequency of Th1 cells with CD4(+) phenotype.

Key words: Th1/Th2 – helper T cell – intracytoplasmic cytokine – gastrointestinal cancer

INTRODUCTION

Cytokine secretion of T lymphocytes has important roles in the immune response and the susceptibility to disease. On the basis of their profiles of cytokine production, fully differentiated T helper (Th) lymphocytes are divided into at least two distinct subsets. Th1 cells produce type I cytokines such as IL-2, IFN-γ (1–4) and TNF-β (4), which are responsible for cell-mediated inflammatory reactions, delayed-type hypersensitivity and tissue injury in infectious and autoimmune diseases. Th2 cells, on the other hand, secrete type II cytokines such as IL-4, IL-5, IL-6 (1–3), IL-9 (5) and IL-10 (5), which are associated with helper function for antibody production by B cells. Th1- and Th2-type cytokines are mutually inhibitory for differentiation and effector functions of the reciprocal phenotypes. Imbalance of Th1/Th2 cytokines has been reported to be closely related to the pathogenesis of various diseases. Th1 predominance is reported in sarcoidosis (6) and tuberculosis (7), whereas Th2 predominance is seen in asthma (8,9), atopic dermatitis (10) and some neoplastic diseases such as Sezary syndrome (11,12), basal cell carcinoma (13–15), multiple myeloma (16) and colon carcinoma (17).

It is generally believed that cancer development and metastasis require the suppression of the host immune system. Various mechanisms have been proposed to cause cancer-mediated immune suppression, including the down regulation of T cell activity by immunosuppressive factors (18–21), the induction of Fas-mediated apoptosis (22,23) and anergy via the B7-CTLA-4 system (24). Especially the impairment of helper function in cancer tissue has been shown to be the main reason why the immune system cannot eradicate cancer cells (19,25). In fact, many studies have shown that IL-2 and IFN-γ are systematically or locally down regulated in cancer patients (12,13,17), suggesting that Th1-type immunity is impaired in cancer-bearing hosts. However, most of these studies analyzed serum cytokine levels of patients and the mechanisms of the Th1 dysfunction are not clearly understood. One possible mechanism is that cancer cells affect the differentiation of Th cells and decrease the total number of Th1-type compared with Th2-type cells. Another possibility is that the capacity of cytokine production per cell is specifically down regulated in each Th1 cell. To assess this question, we used flow cytometry to detect the intracellular cytokine in each T cell and determined the
frequency of Th1 cells in lymphocytes of tumor-draining lymph nodes (LNL) as well as peripheral blood (PBL) in cancer patients. In this study, we focused on the CD4(+) T lymphocytes, since the main source of these helper cytokines has the CD4(+) phenotype.

MATERIALS AND METHODS

PATIENTS

Sixteen patients with advanced gastrointestinal cancer (four gastric cancers and 12 colorectal cancers) who underwent surgical resection at the Department of Surgery, Tokyo University Hospital, and 10 healthy blood donors were enrolled in this study. Lymph nodes (LN) were obtained from resected specimens of gastrointestinal cancer patients. Peripheral blood was also obtained from each patient preoperatively. Patients with severe liver cirrhosis, inflammation, asthma and atopic dermatitis and who received blood transfusions were excluded because their immune system was likely to be altered. Informed consent was obtained from all patients before these studies were performed.

MONOCLONAL ANTIBODIES (MAB)

Fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 mAb (mIgG1) and phycoerythrin (PE)-conjugated anti-CD69 mAbs (mIgG1) were purchased from Becton Dickinson (San Jose, CA). PE-conjugated anti-human IL-2, IL-4 mAbs (mIgG1), anti-human IFN-γ mAbs (mIgG2a) and their isotype control mAbs were also from Becton Dickinson.

CELL CULTURES

PBL were isolated from heparinized whole blood by Ficoll-Hypaque density centrifugation and washed twice with RPMI-1640 (Sigma, St Louis, MO) as described elsewhere (21). Lymph node lymphocytes (LNL) were isolated by mincing the resected lymph nodes and filtering through 100 µm nylon mesh (Biotech, Tokyo, Japan) and Ficoll-Hypaque centrifugation. The cells were suspended in 5% FCS + RPMI-1640 containing 10 µg/ml Brefeldin A (Sigma) to stop the transport of the cytokine product in the Golgi apparatus (26). The cells were then incubated with 25 ng/ml PMA (Sigma) and 1 µg/ml of Ionomycin (Sigma) in 5% CO2 at 37°C. After 4 h of incubation, the lymphocytes were harvested and used for staining experiments.

STAINING AND FLOW CYTOMETRY

For immunostaining of intracellular cytokines, cell membrane was permeabilized prior to cell staining as described elsewhere (27,28). Briefly, cells were suspended in FACs permeabilizing solution (Becton Dickinson) at room temperature for 10 min and then suspended in FACs permeabilizing solution (Becton Dickinson) at room temperature for 15 min. After permeabilization, the cells were washed once with phosphate-buffered saline (PBS) and stained with mAb at room temperature for 30 min. To confirm that all procedures were successful, both activated and non-activated materials were stained with FITC-conjugated anti-CD4 mAb and PE-conjugated anti-CD69 mAb. These cells were analyzed by flow cytometry (FACS Calibur, BD). Lymphocytes were gated by the region determined by FSC and SSC, then CD4+ cells were gated by FITC intensity. In the gated CD4(+) mononuclear cells, a histogram was plotted according to PE intensity, to determine the positive cell ratio. Only when CD69 expression of an activated material was above 85% compared with a non-activated material were all procedures considered to be successful and data about cytokine expression were adopted.

STATISTICS

Data were analyzed with Statview J4.02 and statistical studies were performed using the Mann-Whitney non-paired non-parametric test.

RESULTS

PBL were stimulated with PMA + Ionomycin and their staining patterns were examined in CD4(+) cell populations. Fig. 1(A) and (B) show a representative dot plot analysis in PBL of a normal donor. Few of the CD4(+) T cells without stimulation contained IL-2 or IFN-γ as well as activation marker, CD69. However, a large number of the CD4(+) PBL expressed IL-2 and IFN-γ in their cytosol after 4 h of activation with PMA and Ionomycin. Some of the CD4(+) PBL were stained with anti-IL-4 mAb, although the percentage of IL-4 positive cells was much less than that of IL-2- and IFN-γ-positive cells (data not shown). The frequency of positive cells reached a maximum after 4 h of stimulation and gradually decreased thereafter for both cytokines (Fig. 2). Therefore, we examined the cytokine expression at the time point of 4 h stimulation in subsequent experiments.

The pattern of intracellular cytokine expression in CD4(+) PBL and LNL in gastrointestinal cancer patients is shown in Fig. 1 (C) and (D) and Table 1. Compared with control donors, PBL from the patients contained significantly lower percentages of CD4(+) T cells that produced IL-2 (14.2 ± 10.9% vs 32.3 ± 17.0%, p = 0.014). The frequency of CD4(+) T cells that expressed another Th1 cytokine, IFN-γ- tended to decrease in PBL of cancer patients (11.1 ± 10.7% vs 16.0 ± 12.0%, p = 0.13), although the difference in IFN-γ positivity was not statistically significant between normal donors and cancer patients. The number of Th2 cells recognized as positive for IL-4 in PBL did not show the significant difference between cancer patients and non-malignant donors (data not shown).

Lymphocytes in tumor-draining lymph nodes (LNL) are considered to be more directly exposed to various immunomodulatory factors derived from tumor tissue. As compared with normal PBL, the percentage of CD4(+) LNL that expressed Th1 cytokines was decreased to the same level as that in PBL of cancer patients (IL-2, 16.5 ± 6.36% vs 32.3 ± 17.0%, p = 0.0041; IFN-γ, 7.89 ± 4.79% vs 16.0 ± 12.0%, p = 0.038)
Table 1. Percentage of cytokine-positive cells in CD4(+)-cell population after the stimulation with PMA and Ionomycin

<table>
<thead>
<tr>
<th>Lymphocyte population</th>
<th>Percentage of positive cells</th>
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<tbody>
<tr>
<td></td>
<td>IL-2</td>
</tr>
<tr>
<td>PBL of healthy donor</td>
<td>32.3 ± 17.0</td>
</tr>
<tr>
<td>PBL of cancer patients</td>
<td>14.2 ± 10.9**</td>
</tr>
<tr>
<td>LNL of cancer patients</td>
<td>16.5 ± 6.4**</td>
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Each lymphocyte population was stimulated with PMA and Ionomycin, and intracytokine expression was analyzed by FACS as described in Materials and Methods. In the dot plot profiles shown in Fig. 1, the CD4(+) area was gated and the percentage of positive cells for each cytokine was calculated in the gated area. Data are means ± S.D. of cells positive for each cytokine in CD4(+) cells of 10 healthy donors and 16 cancer patients.

* p < 0.05 and ** p < 0.02 compared with data for PBL of healthy donors.

Table 2. Percentage of cytokine-positive cells in CD4(+) T cells in metastatic and non-metastatic lymph nodes after the stimulation with PMA and Ionomycin

<table>
<thead>
<tr>
<th>Lymphocyte population</th>
<th>Percentage of positive cells</th>
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<tr>
<td></td>
<td>IL-2</td>
</tr>
<tr>
<td>LNL in metastatic nodes</td>
<td>13.0 ± 6.6</td>
</tr>
<tr>
<td>LNL in non-metastatic nodes</td>
<td>17.9 ± 6.0</td>
</tr>
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Resected lymph nodes were macroscopically divided into metastatic and non-metastatic nodes and the cytokine profiles in CD4(+) LNL derived from these two nodes were compared as in Table 1. Data are means ± S.D. in seven metastatic and nine non-metastatic nodes.

DISCUSSION

Since the two subtypes of Th cells were first found by Mosmann et al. in 1986 (1), many diseases have been characterized as having Th1/Th2 imbalance. In neoplastic diseases, many studies have suggested that cancer patients have helper T cell dysfunction locally or systemically (12,13,17,19,25). In colorectal cancer, Pellegrini et al. reported decreased IL-2 and increased IL-4 and IL-6 serum levels in colorectal cancer patients (17). They stimulated PBL and LNL with PHA and showed significantly decreased IL-2 production and increased...
IL-4 production in PBL of cancer patients. However, most of the previous studies have detected type 2 cytokine dominance in serum and did not reflect pure Th cell function, because CD8(+) T cells and monocytes have also been shown to secrete type 1 and type 2 cytokines.

In our experiments, we applied a double staining method using mAbs to CD4 and each cytokine and determined the intracytoplasmic cytokine expression in each CD4(+) T cell. Our results clearly indicated that the frequencies of IL-2- and IFN-γ-producing cells in CD4(+)+ PBL were reduced in patients with gastrointestinal cancer. LNL in regional lymph nodes also contained significantly lower percentages of CD4(+) cells producing these Th1 cytokines. Recently, Sato et al. used the same methods and reported decreased frequencies of IFN-γ-producing subsets in PBMC of various cancer patients (29). Their and our results are consistent with previous reports showing Th2 predominance in malignant diseases and suggested that the decreased serum IL-2 and IFN-γ levels were, at least in part, due to the decreased frequency of IL-2 producing CD4(+) cells in PBL of cancer patients. The exact reason for the decreased number of Th1 cells remains unknown. Many cytokines have been shown to be important for the differentiation of Th1 cells. Especially IL-12 produced from macrophages or dendritic cells has recently been reported to play a critical role in Th1 differentiation (30,31). Deficiency of IL-12 is often present in tumor-bearing hosts and the administration of IL-12 has been reported to have a suppressive effect on tumor growth. These facts raise the possibility that the decreased number of Th1 cells may be the result of impairment of IL-12 production.

In contrast to Th1, the frequency of IL-4-positive CD4(+) cells was not increased in either PBL or LNL of cancer patients (data not shown). In the study by Sato et al., they showed that the frequency of IL-4-producing cells was only slightly increased in CD4(+) PBMC of cancer patients and the serum IL-4 level did not show the significant difference between control and cancer patients (29). Recently, Lissoni et al. also reported that the serum level of IL-4 measured by the ELISA method showed no significant differences between controls and patients with lung or gastrointestinal cancer (32). These data suggest that the IL-4 level may not be always elevated in the serum of patients with such solid cancers. The ability to produce IL-4 in other cells such as macrophages, interstitial cells or cancer cells to produce IL-4 should be examined in cancer-bearing hosts.

Tumor-draining lymph nodes are primary sites for the anti-tumor immune responses and it has been shown that the adoptive transfer of activated LNL in these nodes suppressed tumor growth in a murine model (33–35). These studies have shown that LNL elicits cytotoxicity for syngenic tumors after activation with anti-CD3 and/or IL-2. However, the cytokine production of these LNL has not yet been fully examined. In humans, it has been shown that the number of CD8(+) T cells was decreased and, moreover, LFA-1 on CD8(+) T cells was functionally down regulated in LNL of tumor-draining lymph nodes in cancer patients, that may cause impaired recognition of cancer cells (36). This led us to speculate that the LNL in these regional nodes might produce different types of cytokines from PBL. In this study, we found that CD4(+) LNL showed basically the same cytokine profiles as CD4(+) PBL in cancer patients and the number of Th1-type CD4(+) T cells was decreased compared with PBL of control donors. In mice, Okamoto et al. showed that the production of IFN-γ was decreased in LNL in B16-F10 melanoma-draining nodes as compared with LNL in control mice, and was restored by local injection of streptococcal preparation OK-432 (35). We also examined the intracellular cytokine expression in some non-malignant LNL that were accidently resected in surgery. Those LNL with CD4(+) phenotype tended to express higher levels of IFN-γ than tumor-draining LNL (data not shown), although this did not reach statistical significance since the examination of a larger number of normal LNL was hampered by ethical factors. Taken together, tumor-draining lymph nodes contained a lower number of Th1 cells and thus cannot provide enough type1 cytokine to induce a proper cellular immune response to reject tumor cells.

In summary, the frequencies of IL-2- and IFN-γ-producing cells were reduced in CD4(+) T cells, both in PBL and LNL of patients with gastrointestinal cancer. This indicates that the Th2 predominance in cancer-bearing hosts is mainly due to the decreased number of Th1 cells with CD4(+) phenotype. This method appears to be useful for determining the cytokine production in each cell type and will provide important information for understanding the role of specific cell types in tumor biology.

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References


