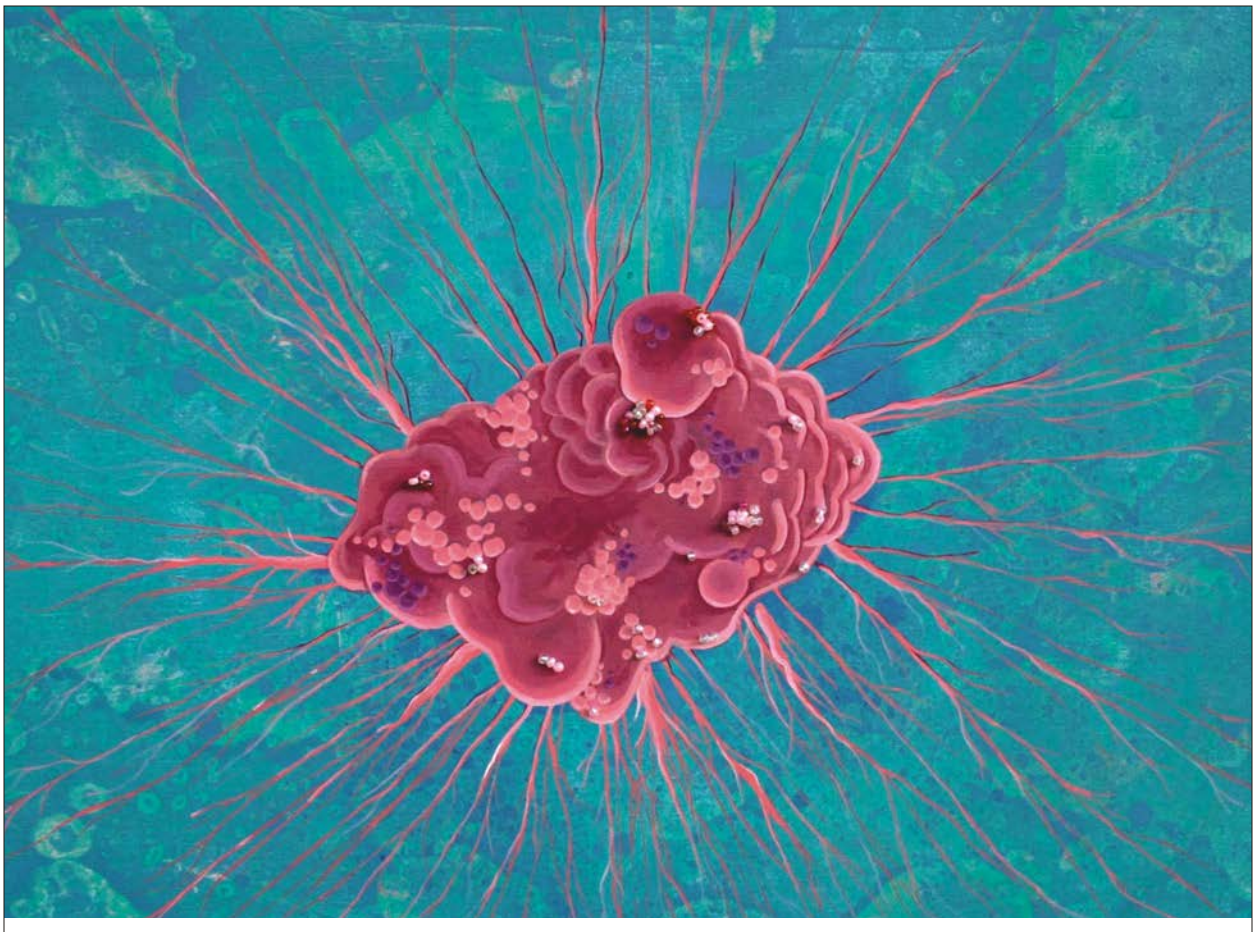

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Flow Cytometric Analysis of Peripheral Blood T-lymphocyte Subsets in Colon Cancer

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Abstract: The changes in lymphocyte subsets from 50 patients with colon cancer (25 patients with grade I and 25 patients with grade II) and 50 normal individuals were determined using flow cytometry. Flow cytometric immunophenotyping by direct and indirect immunoflourescence method was conducted using monoclonal antibodies to CD3 (T-lymphocytes), CD4 (helper/inducer T-cells) and CD8 (suppressor/cytotoxic T-cells) cell surface markers. Immunophenotyping of patients with colon cancer showed a significant decrease ($p<0.001$) in blood T-lymphocytes CD3 and helper/inducer T-cells CD4. Percentage of CD8 T-cells in colon cancer patients increased but did not reach to a statistical significant. A difference in lymphocyte subsets (CD3 and CD4) in GI and GII with high significance $p<0.001$ was shown compared with the control. CD4/CD8 ratio for healthy controls was (1.44±0.88) and decreased in colon cancer (1.36±0.93) without significance differences, however the difference between GII colon cancer and the control was highly significance $p<0.001$. In conclusion, colon cancer may associate with Dysregulation of cellular immune response and that T-lymphocytes subsets may play a role in the immunopathogenesis of colon cancer.

Key words: Flow cytometry, T-cell subsets, colon cancer, T-helper cells, T-suppressor cells

Introduction

There has been for a number of years, considerable interest in the hypothesis that impaired immunity is common in cancer patients (Zbar, 2004). This tumor-induced immunosuppression includes diminished responses to recall antigens (Young *et al*., 1972), decreased proliferative T-cell responses and loss of cytokine production (Horiguchi *et al*., 1999) and defective signal transduction in T cells and natural killer cells (Kiessling *et al*., 1999). There is also evidence for increased apoptosis among CD8 T cells in PBLs from cancer patients and mice with experimental tumors (Takahashi *et al*., 2001). Indeed a number of studies have reported that in patients with solid neoplasms, there is a reduction in both the number of peripheral blood T-lymphocytes and proportions of CD4 and CD8 T-lymphocytes (Tsutsui *et al*., 1992) which are thought to play an important role in cell mediated immunity (Dillman *et al*., 1984; Wu *et al*., 2006). In the present study, flow cytometric analysis was conducted on cell surface markers of blood T-lymphocytes CD3, helper/inducer cells CD4 and Suppressor/Cytotoxic cells CD8 from patients with colon cancer and healthy volunteer controls.

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Materials and Methods

Patients and Controls

Peripheral blood samples were collected from 50 patients with colon cancer (aged 28-51 years) and 50 healthy volunteers as controls (aged 28-48 years) using EDTA as anticoagulant for cell surface marker analysis. Diagnosis of patients was carried out according to clinical, ultrasound and pathological examination. None of the tested patients had received immunosuppressive drugs. The patients were classified according to their pathological examination into: grade-I colon cancer (n = 25) and grade-II colon cancer (n = 25).

Flow Cytometric Immunophenotyping Analyses

The following analyses were performed according to Attallah *et al*. (2003). The percentages of T-cell subsets (CD3, CD4 and CD8) were assessed in peripheral blood lymphocytes of patients and controls. Whole blood (100 μL) of was incubated at 4°C for 30 min with 10 μL of conjugated mouse monoclonal antibodies including: anti-CD3 against human total T-cells, anti-CD4 against human helper/inducer T-cells and anti-CD8 against human cytotoxic/suppressor T-cells (Sigma Chemical Co., St Louis, MO). Five mL of red blood cell lysing solution (8.9-g NH_4Cl, 1-g $KHCO_3$ and 37-mg tetra-sodium EDTA per one liter of distilled water, pH = 7.2) were added, mixed well and incubated at 4°C for 5 min. Samples were centrifuged and washed with phosphate buffered saline (PBS, pH = 7.2). Cells washed with PBS and resuspended in 0.5 mL of PBS in dark until flow cytometric analysis. Mouse monoclonal antibodies against CD3 total T-cells, CD4 helper/inducer T-cells and CD8 suppressor/cytotoxic T-cells were also used by indirect immunoflouroscence staining. One hundred μL of whole blood were incubated at 4°C for 30 min with 100 μL of diluted mouse monoclonal antibodies. Five mL of red cell lysing solution were added, mixed well and incubated at 4°C for 5 min. Samples were centrifuged and washed with PBS. One hundred micro liter of fluorescence isothiocyanate (FITC) conjugated with anti-mouse polyvalent (Sigma) diluted 1:100 in 2% bovine serum albumin in PBS was added. Samples were incubated at 4°C in dark for 30 min. Cells were washed with PBS and resuspended in 0.5 mL of PBS in dark until flow cytometric analysis. Results were expressed in the mean percentage for each group. Analysis was performed using an EPICS PROFILE II flow cytometry (Coulter Corporation, Hialeah, FL, USA). Flow cytometry optical alignment was performed and was periodically confirmed each day using 200 μL of Immuno-Check EPICS polystyrene fluorospheres (Coulter Corporation).

Statistical Analyses

All parameters were transferred to an IBM PC-AT-compatible computer for analysis using statistical analysis program Instate Software for Science, version 2.3 (Graphpad Software Inc., San Diego, USA). Mann-Whitney U test was used to compare the means of two distributions. Fisher's exact test was used to compare the differences between two proportions. *P* values (two-tailed test) of less than 0.05 were considered significant.

Results

Flow cytometry has the ability to differentiate blood leukocytes into three populations, lymphocytes, monocyctes and granulocytes. Figure 1 showed the histograms of lymphocyte subsets (total T-lymphocytes (CD3), helper/inducer T-cells (CD4) and suppressor/cytotoxic T-cells (CD8) from patients with colon cancer and health control. Flow cytometric analysis of blood lymphocyte subsets from colon cancer showed a significant decrease ($p<0.001$) in percentage of CD3 T-cells and

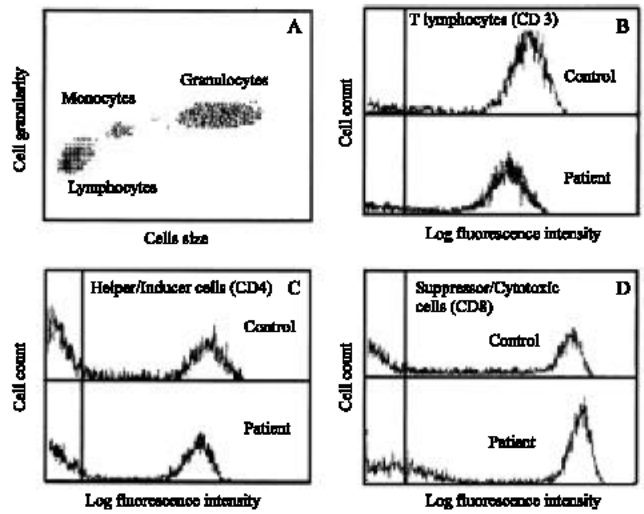

Fig. 1: Flow cytometric analysis of blood leukocytes. A. Flow cytometry differentiated blood leukocytes into three populations, lymphocytes, monocytes and granulocytes. B, C and D. Histograms showing the lymphocyte subsets (total T-lymphocytes (CD3), helper/inducer T-cells (CD4) and suppressor/cytotoxic T-cells (CD8), respectively of a patient with colon cancer in comparison with a healthy control

Table 1: The mean percentage of lymphocyte subsets measured by flow cytometry in patients with different grades of colon cancer (grade I and grade II) compared with controls

Status	CD3* (Mean±SD)	CD4* (Mean±SD)	CD8* (Mean±SD)
Control (n = 50)	75±11	44.6±5.4	31±8
Colon cancer (n = 50)	62±13**	36±8**	32±8
Grade I (n = 25)	60±19**	35.5±10.5**	29.5±9
Grade II (n = 25)	57.7±17.3**	30.2±10.8**	31.2±13

*Total T-lymphocytes (CD3); helper/inducer cells (CD4); Suppressor/cytotoxic cells (CD8). **Showed a highly significant difference ($p<0.001$) compared with control group

Table 2: CD4/CD8 ratio in patients with different grades of colon cancer compared with controls

Status	CD4/CD8 Ratio±SD	p-value*
Control (n=50)	1.44±0.88	
Colon cancer (n = 50)	1.36±0.93	p>0.05
Grade I (n = 25)	1.23±1.00	p>0.05
Grade II (n = 25)	0.96±0.67	p<0.001

*$p<0.05$ considered significance

CD4 T-cells. Percentage of CD8 T-cells in colon cancer increased but did not reach to a statistical significant in comparison with controls. There was a significant decrease ($p<0.05$) in the percentage of CD3 cells and CD4 cells ($p<0.01$) and no significant difference in CD8 cells of patients with G-I or G-II colon cancer in comparison with controls. A difference in lymphocyte subsets (CD3 and CD4) in GI and GII with high significance $p<0.001$ was shown compared with the control as shown in Table 1. CD4/CD8 ratio for healthy controls was (1.44±0.88) and decreased in colon cancer (1.36±0.93) without significance differences, however the difference between GII colon cancer and the control was highly significance ($p<0.001$) as shown in Table 2.

Discussion

The area of tumor immunology is often characterized as one of sustained optimism, yet although this field has enriched our knowledge of basic immunology, little has emerged to benefit the cancer

patient. Impaired immunity is common in cancer patients and unless reversed together with anti cancer treatment, tumor recurrence may result (Kaszubowski *et al*., 1980; Zbar, 2004, Park *et al*., 2005; Wu *et al*., 2006). Indeed in patients with solid neoplasms, there is a reduction in both the number of peripheral blood T-lymphocytes (Greenstein *et al*., 1991) which are thought to play an important role in cell-mediated immunity (Dillman *et al*., 1984). It is known that neoplastic diseases affect the immunity in cancer patients (Broder *et al*., 1978; Voiculescu *et al*., 1997). Colon cancer is one of the commonest cancers occurring worldwide today. Despite standard treatment modalities, approximately 40% of patients with colon carcinoma will die of their disease (Sophias *et al*., 1993, McMillan *et al*., 1995) and Voiculescu *et al*. (1997) indicated characteristic models of immunodeficiency for both groups of colon tumors. Cases belonging to the low aggressive tumor group exhibited a significant decrease in CD3+ cell subsets compared with controls, while a strong immunodeficiency model was present among highly aggressive tumor group patients. This is in agreement with the results of the present study in which the percentage of total lymphocyte percentage (CD3) in G-I and G-II colon cancer was significantly lower than that of control group. Arista *et al*. (1994) demonstrated that, progression of cancer is associated with changes in immune function and more specifically a reduction in the relative and absolute numbers of CD4 T-lymphocytes and either an increase (Dillman *et al*., 1984), or no change (Tsutsui *et al*, 1992). McMillan *et al*. (1995) reported that the magnitude of reduction CD4+ T-lymphocytes may be an important early factor in tumor progression in colon cancer. Therefore, in colon cancer patients with progressive development of disease following surgery, it may be of therapeutic value to use agents that maintain or increase the numbers of CD4+ T-lymphocytes. In the present study, the percentage of blood CD4+ lymphocytes in G-I and G-II was significantly lower than that of control group and these results agreed with Tancini *et al*. (1990) and Arista *et al*. (1994). Tancini *et al*. (1990) and Arista *et al*. (1994) reported that the progression of cancer is associated with changes in the immune function and more specifically a reduction in the percentage of CD4+ lymphocytes. The prolonged depletion of CD4+ populations would be predicted to result in a prolonged impairment of immune function limiting the ability of the immune system to eradicate minimal residual neoplastic disease (Tancini *et al*., 1990; Salgame *et al*., 1991). However, Attallah *et al*. (2003) found a significant decrease in CD3 and CD4 T-cells in patients with liver cirrhosis and hepatocellular carcinoma. The phenotypic analysis of the present work also demonstrated an evident but not significant increase of CD8+ cells in G-I and G-II in relation to healthy control group, which are consistent with other studies exhibiting either an increase or no change in the percentage of CD8 + T-lymphocytes (Young *et al*., 1972; Tancini *et al*., 1990; Greenstein *et al*., 1991). Furthermore, Voiculescu *et al*. (1997) found an increase in the T suppressor (CD8/CD11b) cell percentages in aggressive colon tumor. The changes in T-lymphocyte subsets may occur in a reasonable number of patients with solid tumors and that a decrease in the CD4/CD8 ratio may be frequently identified in patients with disseminated disease (Tancini *et al*., 1990). The shift in the CD4/CD8 ratio in cancer patients can be due to a decline in the T-helper subset and/or to an increase in T suppressor lymphocytes. The diminished T-helper/suppressor ratio is mainly due to increase in T suppressor cells in-patients with early disease whereas it is related to a decline in T-helper sub-populations in most patients with disseminated neoplasm and these results are consistent with our study. However, the clinical significance of these changes is difficult to understand since the mean percentage of CD3 and CD4 T-cells falls within a value currently considered normal. We found a difference between blood lymphocyte subsets of GI and GII colon cancer, however, it does not reach a significant value. This may be due to low number of the studied cases in each group (n = 25). In conclusion, the results of our study suggested that colon cancer is associated with dysregulation of cellular immune response and that T-lymphocyte subsets (CD3, CD4, or CD8) and may play a role in the immunopathogenesis of colon cancer. Functional activity resulting from changes in lymphocyte subsets will be investigated in the future.

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