Peripheral Foxp3+ regulatory T cells and natural killer group 2, member D expression levels in natural killer cells of patients with colorectal cancer

YAJUAN SHEN1, QIAN WANG1, YUANYING QI1, BIN CU1, ZHIFEN ZHANG1, JINGRAN SU3, XIAOWEN LIU2, CHAO LU1, HUI YE1, YING JU1 and ZHIMING LU1

Departments of 1Clinical Laboratory and 2Central Laboratory, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021; 3Department of Clinical Medicine, School of Medicine, Shandong University, Jinan, Shandong 250012, P.R. China

Received January 1, 2014; Accepted April 14, 2014

DOI: 10.3892/mmr.2014.2229

Abstract. Foxp3+ regulatory T cells (Tregs) and natural killer group 2, member D (NKG2D)-positive natural killer (NK) cells are considered to be important in the immune escape of colorectal cancer (CRC). However, the association between these two variables remains obscure. Therefore, in the present study, the levels of peripheral Tregs and NKG2D expression in NK cells and the associations in CRC patients were investigated. A total of 35 CRC patients and 16 healthy controls were enrolled in this study. Flow cytometry was performed to assay Treg numbers and NKG2D expression levels in NK cells in peripheral blood samples. Serum carcino-embryonic antigen (CEA) protein was assayed by electrochemiluminescence. Peripheral Treg numbers were significantly increased (P<0.05), while NKG2D expression levels in NK cells were significantly reduced (P<0.01) in CRC patients compared with healthy controls. However, no significant differences were identified in Treg numbers between CRC patients with and without lymph node metastases and between CRC patients with different clinical stages of CRC. Similarly, no significant differences were detected in NKG2D expression levels in NK cells between the different patient groups. Statistical analysis revealed that increased Treg numbers were not significantly correlated with the downregulation of NKG2D expression in NK cells in peripheral blood from CRC patients.

Introduction

CD4+CD25+ regulatory T cells (Tregs) are a specific subset of CD4+ T cells, and it has been suggested that Tregs are important in maintaining immune self-tolerance and in tumor immune escape (1). Forkhead box protein 3 (Foxp3) has been identified as a CD4+CD25+ T cell-specific transcription factor and has been shown to be widely expressed in this cell type. Notably, Foxp3 regulates the development and function of Tregs. A number of studies have observed that Tregs may suppress the functions of CD8+ T cells, natural killer (NK) cells, natural killer T (NKT) cells and dendritic cells by cell contact and transforming growth factor-β-dependent mechanisms (1-8) and that depletion of Tregs may result in effective antitumor immune responses (9-12). Patients with different types of cancer, including colorectal, ovarian, prostate and hepatocellular cancer, exhibit increased numbers of Tregs in the peripheral blood and tumor microenvironment; notably, the number of Tregs may have significant prognostic importance (13-18). Thus, Treg-targeting agents are currently being investigated in clinical trials and are a promising approach for cancer treatment (19-21).

NK cells are important innate effectors that defend the host against viruses, bacteria, parasites and tumor cells. The NK cells respond to immune stimuli by killing or ignoring target cells, depending on the balance between activating and inhibitory signals. These signals are produced by the interactions of activating and inhibitory receptors expressed on NK cells with the corresponding ligands expressed on target cells. Natural killer group 2, member D (NKG2D) is a crucial activating receptor located on NK cells and possibly on the surface of CD8+ T cells, γδ T cells, NKT cells and certain activated CD4+ T cells. NKG2D recognizes and binds corresponding ligands, including major histocompatibility complex class I-related antigens A and B and UL16-binding proteins expressed on tumor cells, and subsequently generates activating signals, triggering NK cells to kill the target cells.
A number of studies have demonstrated that NKG2D is critical for tumor surveillance (22-26). In a previous study, NKG2D expression levels were found to be significantly downregulated in NK cells collected from the peripheral blood of patients with colorectal cancer (CRC) and inhibition of the NKG2D signaling pathway with anti-NKG2D antibodies resulted in notably reduced cytotoxicity as well as CD107a degranulation in ex vivo experiments. Thus, reduced NKG2D expression levels may be associated with the suppression of NK cell activity in CRC (27).

Although the key role of Tregs as an immunosuppressive cell population is widely accepted, the association between Tregs and NKG2D expression levels in NK cells in CRC requires evaluation. In the present study, peripheral Treg numbers and NKG2D expression levels in NK cells were assayed in parallel using flow cytometry and the association between these variables in CRC patients was determined.

Materials and methods

Patients and controls. A total of 35 patients (18 males and 17 females) with primary CRC and 16 healthy donors (8 males and 8 females) were enrolled in this study. Patients were hospitalized in the gastrointestinal surgery ward of Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China). The CRC diagnosis in these patients was performed according to the Colorocctal Cancer Diagnosis Standard (2010) issued by the Ministry of Health, China. The patients were classified as limited CRC or metastatic CRC according to whether lymph node metastases were present. As determined by clinical classification, the patients were stratified into early and advanced CRC. No patients had received chemotherapy and/or radiotherapy prior to sample collection. The clinical characteristics of the enrolled subjects are summarized in Table I. Informed consent was obtained from each participant. The protocol was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University.

Flow cytometry. Tregs were assayed with a regulatory T cell kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. For surface antigen staining, fluorescein isothiocyanate-conjugated anti-human CD4 and allophycocyanin (APC)-conjugated anti-human CD25 antibodies (eBioscience) were added to 100 µl whole blood and incubated for 30 min in the dark at 4˚C. Subsequently, 1X red blood cell (RBC) lysis buffer (eBioscience) was added to whole blood samples at room temperature in order to lyse RBCs. For intracellular antigen staining, fixation/permeabilization working solution (eBioscience) was added to the cells and incubated for 60 min in the dark prior to staining the cells with phycoerythrin (PE)-conjugated anti-human Foxp3 antibodies (eBioscience). Isotype controls were also run in parallel.

For detecting the NKG2D expression levels in NK cells, PE-conjugated anti-human CD3 (BD Biosciences, Missisauga, ON, Canada), APC-conjugated anti-human CD56 (BD Biosciences) and Alexa Fluor 488-conjugated anti-human NKG2D (R&D Systems, Minneapolis, MN, USA) antibodies were used. Isotype controls were concurrently run.

Table I. Clinical characteristics of enrolled subjects.

<table>
<thead>
<tr>
<th>Group</th>
<th>Patients with CRC</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>18 (51%)</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>58.7±2.3</td>
<td>55.2±4.5</td>
</tr>
<tr>
<td>Limited CRC</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Metastatic CRC</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Early stage (I, II)</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Advanced stage (III, IV)</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>CEA&gt;10 ng/ml</td>
<td>10/29</td>
<td></td>
</tr>
</tbody>
</table>

*Median ± standard deviation; CEA, carcino-embryonic antigen.

At least 10,000 cells were analyzed using a BD FACS Canto II flow cytometer (BD Biosciences).

Serum carcino-embryonic antigen (CEA) assay. Serum CEA protein in CRC patients was routinely assayed using the electrochemiluminescence method with a Roche Cobas e601 system (Roche Diagnostics, Mannheim, Germany) at the Department of Medical Biochemistry, Shandong Provincial Hospital Affiliated to Shandong University. In healthy subjects, CEA<10 ng/ml was considered to indicate a normal level of CEA.

Statistical analysis. The data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Student's t-test was used for comparing quantitative variables between two groups. Spearman correlation analysis was performed to determine associations between two variables. P<0.05 was considered to indicate a statistically significant difference.

Results

CD4⁺CD25⁺Foxp3⁺ Tregs are significantly upregulated in peripheral blood from CRC patients. To evaluate the numbers of Tregs in peripheral blood, flow cytometric analysis was performed on samples from 35 CRC patients and 16 healthy controls. A significant increase was detected in the CD4⁺CD25⁺Foxp3⁺ Treg population in the CRC group (7.389±0.5810 in CRC patients vs. 5.169±0.4370 in healthy controls; P<0.05; Fig. 1A and B). While no statistically significant difference was observed in Treg numbers between patients with limited and metastatic CRC, the data revealed a tendency for the number of Tregs to be higher in metastatic CRC than in limited CRC (8.233±1.008 vs. 6.435±0.4896, respectively; P>0.05; Fig. 1C). Similarly, no significant difference was identified in the Treg numbers between early and advanced CRC patients (data not shown).

CD4⁺CD25hiFoxp3⁺ cells are upregulated in peripheral blood samples from CRC patients. Since CD4⁺CD25hiFoxp3⁺ cells have been commonly identified as Tregs, the numbers of this subpopulation of cells were also analyzed. The
numbers of CD4+CD25hiFoxp3+ cells in CRC patients were significantly increased compared with those in healthy controls (4.211±0.2793 vs. 3.131±0.3063, respectively; P<0.05; Fig. 2A). However, no significant differences in the numbers of CD4+CD25hiFoxp3+ cells between limited and metastatic CRC patients were identified (3.924±0.2855 vs. 4.483±0.4712, respectively; P>0.05; Fig. 2B). Thus, the data demonstrate that peripheral CD4+CD25hiFoxp3+ cells were increased in CRC patients, while no significant differences were observed between CRC patients with and without lymph node metastases.

CD4+CD25hiFoxp3+ cells are more frequent than CD4+CD25loFoxp3+ cells in CRC patients and healthy controls. As CD4+CD25Foxp3+ cells may be divided into CD4+CD25hiFoxp3+ and CD4+CD25loFoxp3+ subsets, the numbers of cells in each of these subsets were compared in peripheral blood samples from CRC patients and healthy controls. The numbers of CD4+CD25hiFoxp3+ cells were significantly higher than those of CD4+CD25loFoxp3+ cells in CRC patients (P<0.001) and healthy controls (P<0.001; Fig. 3). However, the levels of CD4+CD25loFoxp3+ cells did not differ significantly between CRC patients and healthy controls (1.483±0.1357 vs. 1.238±0.1455, respectively; P>0.05; Fig. 3). These data reveal that CD4+CD25hiFoxp3+ cells were the dominant cell type in the CD4+CD25Foxp3+ population in CRC patients and healthy controls.

Peripheral NKG2D expression is significantly downregulated in NK cells from CRC patients. NKG2D is an important activating receptor in NK cells and may be critical in the process of NK cytotoxicity. To investigate the expression levels of NKG2D in NK cells, flow cytometric analysis of samples from 20 CRC patients and 10 healthy controls was performed. NKG2D expression levels in NK cells were significantly lower in CRC patients than in healthy controls (73.14±1.758 vs. 82.56±1.569, respectively; P<0.01; Fig. 4A and B). However, no differences in NKG2D expression levels were observed between NK cells collected from limited and metastatic CRC patients (Fig. 4C).

CD4+CD25Foxp3+ and CD4+CD25hiFoxp3+ Tregs are not correlated with NKG2D expression levels in NK cells collected...
Peripheral CD4⁺CD25⁺Foxp3⁺ and CD4⁺CD25⁺⁺Foxp3⁺ Tregs are not correlated with serum CEA protein in CRC patients. Serum CEA protein is detected at a high frequency in CRC patients at Shandong Provincial Hospital Affiliated to Shandong University. Among 29 CRC patients analyzed prior to surgery, 10 exhibited increased serum CEA levels (>10 ng/ml) and the highest CEA level was 1,000 ng/ml. Spearman correlation analysis was performed to analyze the association between Tregs and CEA. No statistical significance was observed in the full dataset (P>0.05; Fig. 6). However, in advanced CRC patients, elevated Treg numbers and CEA levels were observed (data not shown). Due to the small sample size of the present study, the possibility that these two variables are correlated in advanced CRC patients cannot be ruled out.

Discussion

Since Foxp3 is considered to be the best marker of naturally occurring Tregs (2-4), the two CD4⁺CD25⁺Foxp3⁺ and CD4⁺CD25⁺⁺Foxp3⁺ populations were defined as Tregs. In the present study, a significant increase in Treg numbers was observed in peripheral blood from CRC patients compared with the numbers of Treg cells in healthy controls (P<0.05), which is in accordance with a previous study (13). However, no statistically significant difference in Treg numbers between CRC patients with and without lymph node metastases was identified, although increased Treg numbers in CRC patients with lymph node metastases were observed. Similarly, no significant difference was found in Treg numbers from patients with different clinical stages of CRC (data not shown). However, certain studies have reported that increased frequency of Tregs in peripheral blood and enhanced numbers of tumor-filtrating lymphocytes have marked prognostic significance in CRC (13-15). Another study has demonstrated that accumulation of Tregs in draining lymph nodes is correlated with disease progression in CRC (28).

CD4⁺CD25⁺Foxp3⁺ Tregs are divided into two subpopulations: CD4⁺CD25⁺⁺Foxp3⁺ cells and CD4⁺CD25⁺⁺⁺Foxp3⁺ cells (29). Notably, in the present study, Foxp3 expression levels were found to be markedly higher in CD4⁺CD25⁺⁺Foxp3⁺ cells than in CD4⁺CD25⁺⁺⁺Foxp3⁺ cells in CRC patients and healthy controls. As Foxp3 mediates the function of regulatory T cells (2,3), CD4⁺CD25⁺⁺Foxp3⁺ cells have commonly been investigated as Tregs exhibiting suppressive functions (17,29). Furthermore, CD4⁺CD25⁺⁺⁺Foxp3⁺ cells have been found to function differently from CD4⁺CD25⁺⁺⁺Foxp3⁺ cells (29,30).

NKG2D is a predominant activating receptor in NK cells. As previously reported, the NKG2D receptor is critical for immunosurveillance of primary tumors in mouse models (24,26). A previous study suggested that decreased expression levels of NKG2D may be involved in the suppression of NK activity in CRC (27). Another study observed that depletion of Tregs enhanced NK cell-mediated suppression of tumors expressing NKG2D ligands in a Rae1⁺ experimental metastatic mouse model (31). The Treg cells were able to suppress NK cell-mediated cytolysis of Rae1⁺ target cells in vitro. Therefore, Tregs may inhibit the cytotoxicity of NK cells via an NKG2D-NKG2D ligand pathway. However, in the present study, no significant correlation was identified between peripheral Treg numbers and NKG2D expression levels in NK cells collected from CRC patients.

Figure 3. Comparison of numbers of peripheral CD4⁺CD25⁺Foxp3⁺ cells and CD4⁺CD25⁺⁺Foxp3⁺ cells from patients with colorectal cancer (CRC) and healthy controls (A) Percentages of CD4⁺CD25⁺⁺Foxp3⁺ cells/CD4⁺ cells and CD4⁺CD25⁺⁺⁺Foxp3⁺ cells/CD4⁺ cells were compared from CRC patients (n=35) and healthy controls (n=16). (B) Representative plots of CD4⁺CD25⁺⁺Foxp3⁺ cells and CD4⁺CD25⁺⁺⁺Foxp3⁺ cells from CRC patients and healthy controls.

from the peripheral blood of CRC patients. The above results indicate that increased Treg numbers and reduced NKG2D expression levels in NK cells are found in CRC patients in comparison with healthy controls. Therefore, the association between these two variables in CRC patients was evaluated. Spearman correlation analysis identified no significant correlations between CD4⁺CD25⁺Foxp3⁺ Treg numbers and NKG2D expression levels in NK cells (P>0.05; Fig. 5A) or between CD4⁺CD25⁺⁺Foxp3⁺ Treg numbers and NKG2D expression levels in NK cells (P>0.05; Fig. 5B) in CRC patients. In conclusion, these results indicate that upregulation of Tregs was not correlated with downregulation of NKG2D expression in NK cells from the peripheral blood of CRC patients.
As a nonspecific tumor marker, serum CEA protein is commonly increased in CRC patients and regularly serves as a treatment response and prognostic indicator for CRC (32,33). Nevertheless, in the present study, no correlation between Treg numbers and serum CEA protein levels in CRC patients was observed.

In conclusion, the results of the present study demonstrated that the number of peripheral Tregs was upregulated in CRC, but was not correlated with lymph node metastasis or clinical stage. Additionally, NKG2D expression was found to be downregulated in NK cells collected from patients with CRC, but was not correlated with lymph node metastasis. No
significant correlations were identified between increased Treg numbers and reduced NKG2D expression levels in NK cells in CRC patients. Similarly, no significant correlations between peripheral Treg numbers and serum CEA levels were observed. Therefore, further studies are required to clarify the mechanisms through which Tregs and NKG2D may regulate immune escape in CRC.

Acknowledgements

This study was supported by grants from Shandong Medicine and Health Technology Development Program (grant no. 2009Q2022) and Shandong Natural Science Fund (grant no. ZR2013HM057). The authors would like to thank the participants in the study for making this work possible.

References