Impact of Chemotherapy for Colorectal Cancer on Regulatory T-Cells and Tumor Immunity

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Abstract. Background: Regulatory T-cells (Tregs) actively engage in the maintenance of immunological self-tolerance and immune homeostasis. The purpose of the present study was to determine how oxaliplatin plus infusional 5-fluorouracil and leucovorin (FOLFOX) and irinotecan plus infusional 5-fluorouracil and leucovorin (FOLFIRI) affect Tregs and other immune effectors. Patients and Methods: A total of 27 patients with metastatic colorectal cancer received the FOLFOX (n=17) or FOLFIRI (n=10) chemotherapeutic regimen. Blood samples were collected from patients before and 7 days after chemotherapy. The prevalence of Tregs co-expressing CD4+FoxP3+ was analyzed with flow cytometry. Results: The percentage and the number of CD4+FoxP3+ Tregs were significantly reduced after FOLFOX and FOLFIRI in the patients who had high levels of Tregs before chemotherapy. On the other hand, the total number of lymphocytes and the population of CD4+ T lymphocytes were unchanged. Conclusion: FOLFOX and FOLFIRI may enhance antitumor immunity via suppression of Tregs.

Regulatory T-cells (Tregs) constitute, at most, 3%-5% of mature Cluster of Differentiation (CD4+) T-cells in the thymus and in the periphery (1-5). Tregs actively engage in the maintenance of immunological self-tolerance and immune homeostasis (6). The nuclear transcription factor Forkhead Box p3 (FoxP3) is specifically expressed on Tregs and regulates their development and function (7). FoxP3 is not simply a marker of activation since CD4+CD25− T-cells do not express FoxP3 after activation (7-9). Moreover, FoxP3 can identify Tregs not only under steady-state conditions but also in tumor-bearing hosts and in patients with inflammatory and allergic diseases. As such, it is thought that FoxP3 is the most specific Treg marker.

We previously reported that the prevalence of CD4+FoxP3+ Tregs in the peripheral blood of gastrointestinal cancer patients is significantly higher than these of healthy volunteers, even in the early stages of the disease. Moreover, Treg levels were significantly reduced after curative resection (10). In addition, increased populations of Tregs have been found in the tumors and peripheral blood of cancer patients (10-18). Tregs promote the down-regulation of dendritic cell CD80 and CD86 by a cytotoxic T-lymphocyte antigen (CTLA)-4-dependent mechanism and modulate dendritic cell function. Some Tregs may further differentiate to kill or inactivate responder T-cells by secreting granzyme/perforin or immunosuppressive cytokines such as IL-10 (19). Thus, it is very important to regulate Tregs in tumor immunology. Curiel et al. demonstrated that CD4+CD25−FoxP3+ Tregs suppressed tumor-specific T-cell immunity in ovarian cancer patients and contributed to tumor growth. Furthermore, increased levels of Tregs were associated with a high death hazard ratio and reduced survival (20). Recent studies have shown that chemotherapeutic agents such as cyclophosphamide, fludarabine and paclitaxel down-regulated the numbers and functions of Tregs in cancer patients (21-23). Although bi-weekly 5-fluorouracil, leucovorin and oxaliplatin (FOLFOX) and bi-weekly 5-fluorouracil, leucovorin and irinotecan (FOLFIRI) are standard chemotherapies for colorectal cancer, few reports have examined the effects of these chemotherapeutic regimens on the immune system (24-26). Our purpose in the present study was to investigate the effects of FOLFOX and FOLFIRI on the immune system by focusing on Tregs and other immune effectors such as natural killer (NK) cells and cytotoxic T lymphocytes (CTLs).
Patients and Methods

Patient recruitment. A total of 27 patients with metastatic colorectal cancer received FOLFOX (5-fluorouracil+L-leucovorin+oxaliplatin: n=17) or FOLFIRI (5-fluorouracil+L-leucovorin+irinotecan: n=10) based chemotherapeutic regimens. Patient characteristics are summarized in Table I.

Patients, who were treated at Yamaguchi University Hospital from December 2006 to February 2010 were enrolled in this study. Patients who had elevated inflammatory parameters, as denoted by a white blood cell count of more than 10x10^9/l or a C-reactive protein (CRP) of more than 5 mg/dl, viral infection or autoimmune diseases, were excluded from the study. None of the patients received radiotherapy, but 17 patients had received other chemotherapy regimens at least 4 weeks prior to study enrollment. To clarify the changes in immune parameters, such as CD4+FoxP3+ Tregs, in the peripheral blood after chemotherapy, the levels of immune cells before and 7 days after chemotherapy were compared.

Written informed consent was obtained from all patients, and the study protocol was approved by the Institutional Review Board of the Yamaguchi University School of Medicine.

Chemotherapy. The modified FOLFOX6 (mFOLFOX6) regimen comprised oxaliplatin (85 mg/m²; day 1), L-leucovorin (200 mg/m²; day 1), and bolus 5-fluorouracil (400 mg/m²), followed by continuous 5-fluorouracil (2,400 mg/m²/46 h) every two weeks. The FOLFIRI regimen consisted of irinotecan (150 mg/m²; day 1), L-leucovorin (200 mg/m²; day 1), and bolus 5-fluorouracil (400 mg/m²), followed by continuous 5-fluorouracil (2,400 mg/m²/46 h) every two weeks. Some patients received chemotherapy in combination with antibody-based therapies (bevacizumab, cetuximab) or peptide vaccination.

Isolation of peripheral blood mononuclear cells (PBMCs) and immunofluorescence labeling. Blood samples (10 ml) were collected from patients in sterile heparinized tubes before and 7 days after chemotherapy. PBMCs were isolated by centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). PBMCs were then harvested, washed with Dulbecco’s phosphate-buffered saline (D-PBS) and incubated with Energy-Coupled Dye Phycoerythrin-Texas Red (ECD)-labeled anti-human CD4, fluorescein isothiocyanate (FITC)-labeled anti-human CD25 antibodies, Phycoerythrin-Cy5 (PC5)-labeled anti-human CD8 antibodies, PC5-labeled anti-human CD3 antibodies, phycoerythrin (PE)-labeled anti-human CD56 antibodies, as well as the corresponding isotype control antibodies (all from Beckman Coulter, Miami, FL, USA), all incubated for 30 min at room temperature. Cells were then incubated for 15 min at 4°C with normal rat serum and permeabilization buffer to prevent nonspecific binding to Fc receptors before incubation with rat PE-labeled anti-human FoxP3 antibody (eBioscience, San Diego, CA, USA) and the appropriate isotype controls (Beckman Coulter) for 30 min at 4°C. Cells were then washed and resuspended in 1% paraformaldehyde (PFA) in D-PBS and stored at 4°C in the dark until flow cytometric analysis.

Flow cytometry. Triple-color flow cytometric analysis was performed by using an Epics-XL flow cytometer (Beckman Coulter). To identify Tregs, lymphocytes were gated on the basis of forward vs. side scatter profile followed by gating of CD4+ T-cells. CD4+ cells were then analyzed for CD25 and FoxP3 expression. Stringent gating criteria were used, setting gates at the 0.5% level of the respective isotype control to identify cells positive for Treg markers (Figure 1). Previously, we reported that the average percentage of CD4+FoxP3+ Tregs in healthy volunteers was 0.63% (10). Thus, percentages of CD4+FoxP3+ Tregs greater than 0.63% before chemotherapy, were defined as high.

Statistical analysis. All data are shown as the mean±standard error (SE). Comparisons between data before chemotherapy and after chemotherapy were performed by using Student’s two-tailed t-test for paired samples. A p-value of less than 0.05 was defined as statistically significant. The statistical analysis was performed with Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

Results

The flow cytometry data are presented in Table II and Table III. In the FOLFOX group, lymphocyte numbers were unchanged 7 days after chemotherapy (Table II). On the other hand, in the FOLFIRI group, lymphocyte numbers decreased after chemotherapy. In both groups, the percentages of CD4+ cells did not change after chemotherapy (Tables II and III). The percentage and number of FoxP3+ Tregs in CD4+ cells (CD4+FoxP3+ Tregs) was somewhat reduced after FOLFOX administration, but the decrease was not statistically significant (Figure 2a and b).

In the FOLFIRI group, the percentage of CD4+FoxP3+ Tregs was significantly lower after chemotherapy (pre-chemotherapy vs. post-chemotherapy: 1.44±0.38 vs. 0.91±0.24, p=0.024). The number of CD4+FoxP3+ Tregs was also slightly reduced (pre vs. post: 7.80±2.46/mm³ vs. 4.55±1.27/mm³, p=0.077) (Figure 3a and b).
In patients with high Tregs before FOLFOX, the percentage of CD4+FoxP3+ Tregs was significantly reduced after chemotherapy (pre vs. post: 1.64±0.28 vs. 0.87±0.24, p=0.019). The absolute number of CD4+FoxP3+ Tregs also significantly decreased after chemotherapy (pre vs. post: 8.6±2.3/mm3 vs. 5.1±2.08/mm3, p=0.011) (Figure 4a and b).

In the patients with high Tregs before FOLFIRI, the percentage of CD4+FoxP3+ Tregs significantly decreased after chemotherapy (pre vs. post: 1.84±0.48 vs. 1.10±0.32, p=0.021). The number of CD4+FoxP3+ Tregs tended to be suppressed (pre vs. post: 10.15±3.14/mm3 vs. 5.54±1.69/mm3, p=0.076) (Figure 5a and b).

In both groups, there was no change in the percentage of CD8+ cells and CD3–CD56+ cells after chemotherapy (Tables II and III).

Discussion

This is the first report to show the influence of FOLFOX and FOLFIRI regimens on the tumor immune system including Tregs. We found that FOLFOX and FOLFIRI have the potential to deplete Tregs without severe suppression of the host’s antitumor effector T-cells.

In general, chemotherapy has been considered immunosuppressive because of its common toxicity of neutropenia. However, immunological benefits induced by chemotherapy have been reported. Cyclophosphamide (CTX), fludarabine, and paclitaxel have been found to selectively deplete Tregs (21-23, 27). Ghiringhelli et al. showed that oral administration of low-dose cyclophosphamide to advanced cancer patients induces a profound and selective reduction in circulating CD4+CD25+ Tregs, associated with a suppression of their inhibitory functions on conventional T-cells and NK cells, leading to a restoration of peripheral T-cell proliferation and innate killing activities (21). Hong et al. reported that after high-dose CTX injection, the proportion of Tregs among CD4+ T-cells decreased due to an expansion of memory and other CD4+ T-cell subtypes. They postulated that the CTX-induced change in T-cell balance may increase anti-tumor immunity (27). Alvino et al. reported that gemcitabine (2’-2’-difluorodeoxycytidine) markedly inhibits lymphokine-activated killer (LAK) cells or CTL generation, but does so less efficiently on ‘mature’ LAK cells or CTL lymphocyte function and only slightly on NK cell activity in vitro (28). Plate et al. showed that there were no significant changes in the percentages of CD86 and CD80 antigen presenting cells (APCs) or CD4+, CD25+ T-cells after treatment with gemcitabine infusion (29). Therefore, these regimens might not only directly affect tumors but also support immune responses to cancer cells, favoring better control of tumor progression.

In the present study, we examined the impact of FOLFOX and FOLFIRI regimens on immune effectors such as lymphocytes, NK cells, CTLs and Tregs in patients with colorectal cancer after chemotherapy.

Table II. Profiles of peripheral blood leukocytes before and after administration of FOLFOX regimen.

<table>
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<tr>
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<th>Pre</th>
<th>Post</th>
<th>p-Value</th>
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<tbody>
<tr>
<td>Leukocytes (cells/mm3)</td>
<td>5671.2±512.6</td>
<td>4950.0±394.3</td>
<td>0.033**</td>
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<td>Neutrophils (cells/mm3)</td>
<td>3453.8±378.0</td>
<td>2947.2±349.4</td>
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<td>Lymphocytes (cells/mm3)</td>
<td>1577.8±170.1</td>
<td>1548.7±142.3</td>
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<tr>
<td>CD4+ Lymphocytes (%)</td>
<td>36.1±2.53</td>
<td>37.8±2.56</td>
<td>0.272</td>
</tr>
<tr>
<td>CD4+CD25+ (%)</td>
<td>6.36±0.76</td>
<td>6.96±1.47</td>
<td>0.626</td>
</tr>
<tr>
<td>Over 0.63% (n=8)</td>
<td>1.64±0.28</td>
<td>0.87±0.24</td>
<td>0.019**</td>
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<td>CD4+FoxP3+ (%)</td>
<td>0.97±0.20</td>
<td>0.67±0.13</td>
<td>0.107</td>
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<tr>
<td>Over 0.63% (n=8)</td>
<td>8.6±2.3</td>
<td>5.1±2.08</td>
<td>0.011**</td>
</tr>
<tr>
<td>CD8+ (%)</td>
<td>25.4±3.71</td>
<td>26.0±3.64</td>
<td>0.851</td>
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<tr>
<td>CD3–CD56+ (%)</td>
<td>11.8±3.56</td>
<td>13.0±2.48</td>
<td>0.627</td>
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</tbody>
</table>

*p<0.1, **p<0.05.

Table III. Profiles of peripheral blood leukocytes before and after administration of FOLFIRI regimen.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (cells/mm3)</td>
<td>5964.0±884.2</td>
<td>3922.0±496.6</td>
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<td>Neutrophils (cells/mm3)</td>
<td>3292.1±392.8</td>
<td>2578.1±472.2</td>
<td>0.244</td>
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<td>Lymphocytes (cells/mm3)</td>
<td>1360.3±145.1</td>
<td>1193.0±151.3</td>
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<td>CD4+ Lymphocytes (%)</td>
<td>37.0±3.96</td>
<td>40.4±3.84</td>
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<tr>
<td>CD4+CD25+ (%)</td>
<td>7.00±1.31</td>
<td>4.83±0.74</td>
<td>0.074*</td>
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<tr>
<td>CD4+FoxP3+ (%)</td>
<td>1.44±0.38</td>
<td>0.91±0.24</td>
<td>0.024**</td>
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<tr>
<td>Over 0.63% (n=7)</td>
<td>1.84±0.48</td>
<td>1.10±0.32</td>
<td>0.021**</td>
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<tr>
<td>CD4+FoxP3+ (cells/mm3)</td>
<td>7.80±2.46</td>
<td>4.55±1.27</td>
<td>0.077*</td>
</tr>
<tr>
<td>Over 0.63% (n=7)</td>
<td>10.15±3.14</td>
<td>5.54±1.69</td>
<td>0.076*</td>
</tr>
<tr>
<td>CD8+ (%)</td>
<td>18.0±1.95</td>
<td>19.2±2.15</td>
<td>0.489</td>
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<tr>
<td>CD3–CD56+ (%)</td>
<td>10.6±1.49</td>
<td>12.1±2.40</td>
<td>0.521</td>
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</tbody>
</table>

*p<0.1, **p<0.05.
advanced colorectal cancer. Although the number of neutrophils was decreased, the number of lymphocytes was unchanged after FOLFOX administration (Table I). Moreover, the populations of CD4+ lymphocytes, CD8+ lymphocytes, and CD3–CD56+ mononuclear cells (NK cells) were also unchanged. These results suggest that FOLFOX may cause little damage to the host’s antitumor immune response. In a study of colorectal cancer patients treated with vaccination along with chemotherapy (5-fluorouracil, folinic acid and oxaliplatin), 10 out of 11 patients mounted antigen-specific antibody responses, as well as interferon (IFN)-gamma enzyme-linked immunospot responses specific for stimulated antigen (25). These results suggest that vaccination can be added to chemotherapy regimens without reducing immunologic efficacy.

Our study also revealed that patients with high levels of Tregs before chemotherapy had significantly lower percentages and numbers of CD4+FoxP3+ Tregs after treatment with the FOLFOX regimen. This suggests that FOLFOX chemotherapy may improve the status of immune suppression via tumor burden by depleting the high level of circulating Tregs. A previous clinical report (24) has indicated that combination chemo-immunotherapy with gemcitabine plus FOLFOX followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. A progressive increase in lymphocyte and eosinophil counts, amplification in central memory, a marked depletion of immunosuppressive Tregs, and activation of colon cancer-specific cytotoxic T-cells were observed. These reports...
suggest that the FOLFOX regimen may deplete Tregs and restore the host immune system against cancer.

In the FOLFIRI group (Table II), although the number of peripheral lymphocytes decreased significantly after chemotherapy, the level of lymphopenia was not so severe. Indeed, a previous report (26) showed that vaccination of colorectal cancer patients alongside chemotherapy (5-fluorouracil, leucovorin and irinotecan) induces potent immune responses. Of the 12 patients whose immune responses could be evaluated, 10 patients mounted antigen-specific antibody responses, and IFN-gamma ELISpot responses specific for loaded tumor antigen were detected in all patients.

The percentage of CD4+FoxP3+ Tregs decreased significantly after FOLFIRI in the analysis of all cases, as well as the high Treg group (Table II). Tregs were reported to be highly proliferative with a short doubling time as compared with other CD4+ subpopulations (30). This suggests that the FOLFIRI regimen could also restore the host immune system against cancer. In experimental models (31), vaccination with dendritic cells expressing carcinoembryonic antigen enhanced the antitumor effect after FOLFIRI treatment. Interestingly, although FOLFIRI treatment rather showed a rebound in the numbers of myeloid-derived suppressor cells and Tregs after 14 days, additional dendritic cell vaccines were able to inhibit the rebound of these immunosuppressive cells. These results demonstrate that immunotherapy in addition to the FOLFIRI regimen appears to improve antitumor effects through the inhibition of an immunosuppressive tumor environment in patients with colorectal cancer.

We conclude that the FOLFOX and FOLFIRI regimens induce not only direct cytotoxicity but also enhancement of antitumor immunity via Treg depletion. Thus, FOLFOX and FOLFIRI chemotherapy may be suitable to combine with immunotherapy.

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Disclosure Statement

The Authors have no conflict of interest.

References


