Suppressive Effects of Cyclophosphamide and Gemcitabine on Regulatory T-Cell Induction In Vitro

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Abstract. Background/Aim: Inhibition of CD4⁺FOXP3⁺ regulatory T-cell (Treg) activity may be important for successful cancer immunotherapy. We investigated the suppressive effects of several chemotherapeutic agents on Treg induction in vitro. Materials and Methods: Tregs were induced by incubating human peripheral blood mononuclear cells (PBMCs) with interleukin-2 (500 U/ml) and transforming growth factor-β (10 ng/ml) for four days. PBMCs were simultaneously treated with cyclophosphamide (CPA), gemcitabine (GEM), 5-fluorouracil, levofolinate, or oxaliplatin. Treated PBMCs were examined for CD4 and FOXP3 expression via flow cytometry. Results: Treg induction was significantly suppressed by treatment with CPA and GEM. The optimal concentration of CPA for Treg suppression was almost identical to the serum levels of patients with cancer, treated with low-dose CPA. Treatment with the other agents did not affect Treg induction. Conclusion: Chemotherapy using CPA or GEM may have the potential to augment the antitumor effects of cancer immunotherapy by suppressing Treg induction.

Regulatory T-cells (Tregs) possess immunosuppressive functions, which inhibit autoimmune responses and regulate various inflammatory reactions (1-3). There are three types of the well-identified Cluster of Differentiation (CD)-4⁺ Tregs: i) naturally-occurring, thymically selected CD4⁺CD25⁺Forkhead Box p3 (FOXP3)⁺ Tregs (nTregs); ii) peripherally induced CD4⁺CD25⁺FOXP3⁺ Tregs (iTregs); and iii) peripherally induced CD4⁺FOXP3⁻ type 1 Tregs (Tr1) (4). In particular, CD4⁺CD25⁺ Tregs specifically express the transcription factor FOXP3, which acts as a key molecule in their development and function (5-7), as well as eliciting their immunosuppressive functions through a cell–cell contact mechanism (1) and secretion of immunosuppressive cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)-β (8-10). CD4⁺CD25⁺ FOXP3⁺ Tregs can be induced from CD4⁺CD25⁻FOXP3⁻ T-cells in vitro via stimulation with IL-2 and TGF-β, and these induced Tregs are functionally similar to thymus-derived, naturally-occurring Tregs (4, 11-16).

Cancer cells are generated by a continuous accumulation of genetic mutations in normal cells, and it is likely that newly-developed cancer cells are recognized and eliminated by the immune system. However, cancer cells that have acquired immunosuppressive and immunoresistant abilities may grow selectively and potentially progress into advanced cancer. Cancer cells can escape T-cell recognition through a loss of tumor antigens and/or human leukocyte antigen (HLA), and suppress dendritic cell (DC) function and the induction of tumor-specific cytotoxic T-lymphocytes (CTLs) via the production of immunosuppressive cytokines, such as TGF-β, vascular endothelial growth factor (VEGF), IL-6, and IL-10 (17, 18). These factors may result in unsatisfactory outcomes in various clinical studies on cancer immunotherapy and vaccination. Tregs are thought to play a pivotal role in the induction of immune tolerance to tumor antigens because cancer cells actively generate Tregs by producing TGF-β and IL-10, which result in the suppression of CD4, CD8, and Natural killer (NK) cell-mediated antitumor immune responses (17, 19).

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Chemotherapeutic agents for cancer treatment kill not only target cancer cells, but also immune cells. However, it has been reported that the use of some conventional chemotherapeutic agents may modulate the immune function in hosts and inhibit the activity of immunosuppressive cells (20, 21). In particular, some chemotherapeutic agents that target rapidly-proliferating cells may reduce the accumulation of Tregs in the tumor environment (22, 23). Anticancer treatment of combined Treg-targeting chemotherapy and immunotherapy may also elicit a potent therapeutic effect by potentially inhibiting immune tolerance against tumor antigens in the tumor environment (16, 24).

In the present study, we investigated the suppressive effects of several chemotherapeutic agents on Treg induction in vitro. Our findings demonstrate that cyclophosphamide (CPA) and gemcitabine (GEM) suppress the induction of CD4^+FOXP3^+ Tregs.

Materials and Methods

Materials. The metabolite of cyclophosphamide, 40487s (CPA), was a gift from Shionogi & Co. (Osaka, Japan). GEM was purchased from Sigma Aldrich (St. Louis, MO, USA). 5-Fluorouracil (5-FU) and levofolinate (LV) were gifts from Kyowa Hakko Kirin Co. (Tokyo, Japan). Oxaliplatin (L-OHP) was purchased from Yakult (Tokyo, Japan). The metabolite of cyclophosphamide, 40487s (CPA), was purchased from Sigma Aldrich (St. Louis, MO, USA). 5-Fluorouracil (5-FU) and levofolinate (LV) were gifts from Kyowa Hakko Kirin Co. (Tokyo, Japan). Oxaliplatin (L-OHP) was purchased from Yakult Co. (Tokyo, Japan).

Treg induction in peripheral blood mononuclear cells (PBMCs) and treatment with chemotherapeutic agents. Human PBMCs were isolated via Ficoll-Paque (GE Healthcare Biosciences AB, Uppsala, Sweden) with density-gradient centrifugation, washed twice in Dulbecco's phosphate-buffered saline (D-PBS) (Nissui pharmaceutical, Tokyo, Japan), and stored at –80˚C. Treg induction was based on the methodology previously described (11, 13). PBMCs were seeded into 6-well culture plates (Falcon, Beckton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 1x10^6/ml in 2 ml of AIM-V medium (Invitrogen, Carlsbad, CA, USA) plus 5% heat-inactivated fetal bovine serum (FBS) (Invitrogen) containing recombinant human IL-2 (500 U/ml) (Shionogi & Co.) and recombinant human TGF-β1 (10 ng/ml) (Milenyi Biotec K.K., Bergisch Gladbach, Germany). Concurrently, various chemotherapeutic agents (CPA, GEM, 5-FU, LV, and L-OHP) were added to the culture at concentrations based on the serum concentrations of clinically treated patients with cancer. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO2 for four days. Cell numbers were then determined by counting the viable cells with Vi-CELL XR (Beckman Coulter, Miami, FL, USA).

Immunofluorescence labeling and flow cytometry. Cells were incubated with energy-coupled dye-phycocerythrin-Texas Red (ECD)-labeled anti-human CD4 (T4) or the corresponding isotype control antibodies (Beckman Coulter) for 30 min at 4˚C. After treatment with rat serum and permeabilization buffer for 15 min at 4˚C, cells were incubated with rat phycocerythrin (PE)-labeled human FOXP3 antibody (PCH101) (eBioscience, San Diego, CA, USA) or the appropriate isotype control (Beckman Coulter) for 30 min at 4˚C, then washed, re-suspended in 1% paraformaldehyde (PFA) in D-PBS, and stored at 4˚C in the dark until flow cytometric analysis. Two-color flow cytometry was performed with an Epics-XL flow cytometer (Beckman Coulter). To identify Tregs, lymphocytes were gated based on their forward- vs. side-scatter profile. CD4^+ lymphocytes were then analyzed for FOXP3 expression. Strict gating criteria were used, where the gates were set at the 0.5% level of the respective isotype control to identify cells positive for Treg cell markers.

Calculating the CD4^+ and CD4^+FOXP3^+ cell counts. The number of CD4^+ cells was determined by the following formula: [total cell number (viable cell number + dead cell number)] × [percentage of the gated cells in whole lymphocytes] × [percentage of CD4^+cells in the gated cells]. The number of CD4^+FOXP3^+ cells was determined by the following formula: [number of CD4^+cells] × [percentage of FOXP3^+cells in CD4^+cells].

Statistical analysis. All data are presented as the mean±standard error (SE). Comparisons between the untreated control and drug-treated groups were performed by the Student’s t-test. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed with Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

Results

CPA and GEM suppressed Treg induction in PBMCs in vitro. While the number of PBMCs decreased with increasing concentrations of CPA, approximately 70% of the cells remained viable following treatment with 0.5 μg/ml of CPA (Figure 1Aii). Additionally, although the percentage of CD4^+ T-cells in lymphocytes was not significantly altered (Figure 2Aii). After treatment with 0.5 μg/ml CPA, 58% of CD4^+ T-cells and only 32% of CD4^+FOXP3^+ Tregs were viable, expressing the cell number relative to that of the control (Table I). A marked decrease in CD4^+FOXP3^+ Tregs following CPA treatment was shown by flow cytometry (Figure 1B).

Although the incubation of PBMCs with 10 μg/ml of GEM resulted in a significant reduction of viable cells (p<0.01) (Figure 2Ai), the percentage of CD4^+ T-cells in lymphocytes was not significantly altered (Figure 2Aii). However, the percentage of CD4^+FOXP3^+ Tregs in lymphocytes was significantly reduced by the same treatment (p<0.01) (Figure 2Aii). After treatment with 10 μg/ml of GEM, 31% of CD4^+ T-cells, but only 8% of CD4^+FOXP3^+ Tregs were viable, expressing the cell number relative to control (Table II). A decrease in CD4^+FOXP3^+ Tregs was demonstrated by flow cytometry (Figure 2B). These results indicate that CPA and GEM may preferentially inhibit the induction of CD4^+FOXP3^+ Tregs that is mediated by IL-2 and TGF-β.

Treg induction was generally not significantly suppressed by treatment of PBMCs with 5-FU, LV, or L-OHP. We then
analyzed the effects of 5-FU, LV, and L-OHP on Treg induction in vitro. The number of viable PBMCs was significantly reduced following treatment with 0.5 and 5 μg/ml of 5-FU (p<0.05), but the percentage of CD4+ T-cells and CD4+FOXP3+ Tregs in lymphocytes were not changed by the treatment (Figure 3A). Although treatment with 100 μg/ml of LV reduced the number of viable PBMCs (p<0.05), the percentage of CD4+ T-cells and
CD4+FOXP3+ Tregs were not affected (Figure 3B). Treatment with 0.05, 0.2, and 0.8 μg/ml of L-OHP also reduced the number of viable PBMCs (p<0.05), but there was no significant reduction in the percentages of CD4+T-cells and CD4+FOXp3+ Tregs observed (Figure 3C). These results indicate that 5-FU, LV, and L-OHP may have little potential to suppress IL-2- and TGF-β-mediated Treg induction.
Figure 3. Effects of 5-FU, LV, and L-OHP on Treg induction in vitro. PBMCs were incubated with IL-2 and TGF-β to induce Tregs in the presence of 5-FU (A), LV (B), and L-OHP (C). i: Numbers of viable PBMCs after a four-day incubation are shown. ii: Percentage of CD4+ T-cells in lymphocytes and CD4+FOXP3+ T-cells in lymphocytes. Data are presented as the mean±S.E. of five independent experiments. *p<0.05 vs. untreated control. 5-FU, 5-Fluorouracil; LV, levofolinate; L-OHP, oxaliplatin; Treg, regulatory T-cell; PBMCs, peripheral blood mononuclear cells; IL-2, interleukin-2; TGF-β, transforming growth factor-β; CD4, Cluster of Differentiation-4; FOXP3, Forkhead Box p3.
Discussion

While cancer chemotherapy is considered to be immunosuppressive, with neutropenia and lymphopenia being common adverse effects, recent reports have demonstrated that some chemotherapeutic agents may have immunomodulating effects (23). CPA is a representative agent that elicits immunomodulating effects, but stimulation of immune cells via CPA is dependent on its dose. Motoyoshi et al. (22) compared the immunomodulatory effects of different doses of CPA on various T-cell subsets, including CD4+CD25+ T-cells in mice. They found that treatment with a low dose (20 mg/kg) of CPA selectively depleted the CD4+CD25+ T-cell population, which promoted the anti-tumor immune response to pre-existing tumors, whereas a high dose (200 mg/kg) of CPA was solely attributed to direct cytotoxicity. Ghiringhelli et al. (20) suggested that a metronomic regimen of low-dose CPA (50 mg orally, twice a day, one week on and one week off for one month or more), which selectively targets Tregs, enhances the function of effector T-cells and NK cells in patients with advanced cancer. Another mechanism for Treg suppression by CPA treatment was reported by Hong et al. (25). The number and percentage of CD4+CD25+FOXP3+ Tregs and expression of TGF-β1 in splenocytes increased following tumor establishment in mice, but a single injection of high-dose CPA reduced the proportion of Tregs among CD4+ T-cells. This phenomenon was closely associated with the proliferation of CD44hi memory T-cells and up-regulation of IL-15IL-15 receptor mRNA in the CD44hiCD8+ T-cell compartment (25). In the present study, we demonstrated that 0.5 μg/ml of CPA suppressed the IL-2- and TGF-β- mediated induction of CD4+FOXP3+ Tregs before and after FOLFOX treatment. Of note, 0.5 μg/ml of CPA is almost identical to the serum CPA levels of patients with colorectal cancer treated with low-dose CPA (2 mg/kg). This finding suggests that low-dose CPA treatment for cancer may contribute to the suppression of Treg induction and restore the impaired antitumor immune response, suggesting that this treatment has a synergistic effect with cancer immunotherapy.

In the present study, we reported that GEM inhibited the induction of CD4+FOXP3+ Tregs, but only at 10 μg/ml. Aside from the inhibition of Treg induction, GEM is known to elicit immunomodulatory effects by eliminating myeloid-derived suppressor cells (26), indicating that immunotherapy combined with GEM may be promising. It is probable that CPA and GEM may be cytotoxic to highly proliferative Tregs, but not to other subsets of CD4+cells. Van der Most et al. (27) demonstrated that CPA treatment of tumor-bearing mice depleted the CD4+FoxP3+ T-cell population, which expresses Ki-67, a marker of proliferating cells, better than GEM treatment. This may be because CPA treatment induces a long-lasting inhibition of the T-cell cycle, whereas GEM treatment allows for the recovery of T-cell proliferation earlier than CPA treatment. However, Rettig et al. (28) reported that treatment with GEM successfully reduced the population of CD4+FOXP3+Ki-67+ T-cells in patients with cancer.

In the present study, treatment of PBMCs with 5-FU, LV, and L-OHP in vitro did not suppress IL-2- and TGF-β-mediated Treg induction. We recently reported that treatment of patients with colorectal cancer with L-OHP plus an infusion of 5-FU and LV (FOLFOX) may suppress CD4+FOXP3+ Tregs in peripheral blood (29). It may be possible that these agents suppress Tregs in peripheral blood, but do not induce cytokine-dependent Tregs in the tumor environment. Correale et al. (30) reported that the tumor tissues of two patients did not show any differences in the number of tumor-infiltrating CD4+FOXP3+ Tregs before and after FOLFOX treatment.

In conclusion, we demonstrated that CPA (0.5 μg/ml) and GEM (10 μg/ml), at the same concentration as in the sera of clinically treated patients, inhibit the induction of CD4+FOXP3+ Tregs in vitro. We suggest that low-dose CPA and GEM may be promising agents for combined immunomodulatory effects by eliminating myeloid-derived suppressor cells (26), indicating that immunotherapy combined with GEM may be promising. No conflicts of interest were disclosed.

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