Abstract. Background: Vaccine therapy targeting tumor antigens recognized by cytotoxic T cells (CTL) has been tried extensively. However, in a cancer-bearing state, the Th1/Th2 balance shifts to Th2 dominance, and this has been the obstacle to vaccine therapy to induce the CTL. DC1/DC2 subsets have also been reported to control the differentiation of Th subsets. The key to tumor immunotherapy is how to activate the DC1-Th1 lineage. Patients and Methods: Six normal adults and 14 patients with gastric or colorectal cancers, who gave informed consent, were studied. The Th1/Th2 and DC1/DC2 ratios were determined by FACS. IL-12 and IL-10 production from PBMC were measured by ELISA. Results: The Th1/Th2 and DC1/DC2 ratios were all significantly lower in the patients with gastric or colorectal cancers compared to normal adults. After protein-bound polysaccharide K (PSK) therapy in cancer patients, the Th1/Th2 balance shifted to Th1 dominance and the DC1/DC2 balance to DC1 dominance. IL-10 production was significantly decreased by PSK therapy. Conclusion: In the cancer-bearing state, the Th1/Th2 and DC1/DC2 ratios were all significantly lower in the patients with gastric or colorectal cancers compared to normal adults. After protein-bound polysaccharide K (PSK) therapy in cancer patients, the Th1/Th2 balance shifted to Th1 dominance and the DC1/DC2 balance to DC1 dominance. IL-10 production was significantly decreased by PSK therapy. Conclusion: In the cancer-bearing state, the Th1/Th2 and DC1/DC2 balance becomes Th2- and DC2-dominant. PSK therapy results in a shift of the Th1/Th2 and DC1/DC2 balance towards Th1 and DC1 dominance. We plan to examine whether combining dendritic cells (DC) vaccination therapy with oral PSK enhances the induction of T cell and DC differentiation in cancer patients.

In tumor immunology, cytotoxic T cells (CTL) are considered to be the major effector cells against tumor cells. Many trials of vaccine therapies targeting tumor antigens recognized by CTL have been conducted. CTL are activated by helper T cells (Th). In 1986, Mosmann et al. advocated the existence of Th1 and Th2 subsets (1). It is known that Th1 cells are involved in cellular immunity and activate CTL, while Th2 cells are involved in humoral immunity and activate B cells. However, in the cancer-bearing state, Th2 becomes dominant and this situation is a major problem in vaccine therapy for cancer (2-4).

Apart from the Th subsets, other subsets of immune cells have also been reported. The dendritic cells (DC) involved in the differentiation of Th subsets are known to contain DC1 and DC2 subsets (5), and CTL activated by Th interacting with the cytokine network (6). Therefore, when designing means to eliminate antigen-specific cancer cells by the immune system, a method that effectively activates the DC1-Th1 cell lineage will be the key investigation to induce the CTL activation (7-9).

Protein-bound polysaccharide K (PSK) is extracted from the cultured mycelium of *Coriolus versicolor* (Fr.) Quél and a biological response modifier (BRM) that exhibits anti-tumor effects through activation of the immune system. In Japan, PSK has been mainly used to improve the patient's prognosis after surgery since the cell-mediated immune reaction of the cancer-bearing host is suppressed, especially after surgery. Many reports mentioned that PSK improved both disease-free and overall survivals on addition to standard oral adjuvant chemotherapy such as 5-FU (5-fluorouracil) or UFT (tegafur/uracil) in patients with gastric cancer (10), colorectal cancers (11) and small cell lung cancer (12).

In the present study, we compared the Th1/Th2, the DC1/DC2 ratios, and IL-12 and IL-10 values between patients with gastric or colorectal cancer and normal subjects, and evaluated the Th1/Th2 and DC1/DC2 ratio changes after adjuvant immunochemotherapy with PSK.

Patients and Methods

Patients. We studied 6 normal subjects (average age 53.8) and 14 patients (male 12, female 8, average age 56.8) with gastric or colorectal cancer from whom informed consent was obtained. The
patients underwent surgical resection of gastric cancer or colorectal cancer. From one month after surgery, they received 300mg/day of oral UFT (tegafur/uracil, Taiho Pharmaceutical Industry Co. Ltd., Japan) agent combined with 3g/day of oral PSK (Kureha Chemical Industry Co. Ltd., Tokyo, Japan).

Reagents. Brefeldin A (BFA), phorbol 12-myristate 13-acetate (PMA), ionomycin, phytohemagglutinin (PHA) and red blood cell lysing buffer were purchased from Sigma (Rockville, MD, USA). PSK was obtained from Kureha Chemical Industry. ELISA kits for IL-12 and IL-10 were obtained from R and D Systems (Minneapolis, MN, USA). CD4 PerCP reagents were from Immunotech Beckmann Coulter (Mvussella, France). Anti-IFNγ FITC, Anti-IL-4 PE, Lineage Cocktail 1 (CD3, CD14, CD16, CD19, CD20, CD56) FITC, Anti-HLA-DR PerCP, CD11c PE, FACS lysing solution and FACS permeabilizing solution were purchased from Becton Dickinson (San Jose, CA, USA).

Determination of the Th1/Th2 ratio. Five hundred µL of whole blood was diluted 1:1 in RPMI 1640 medium, and stimulated with 10 ng/mL of PMA and 1 µg/mL of ionomycin in the presence of 10 µg/mL of BFA. After incubating at 37°C for 4 hours, 10 µL of CD4 PerCP was added to 400 µL of the above mixture at room temperature for 15 minutes. Then, 2.5 mL of FACS lysing solution was added to lyse the red blood cells. The mixture was centrifuged at 500 x g for 10 minutes and the supernatant was decanted. 0.95mL of FACS permeabilizing solution was added to the residual cells and incubated at room temperature for 10 minutes. The cells were washed and the supernatant was removed. Twenty µL of Anti-IFNγ FITC and 20 µL of Anti-IL-4 PE were added and incubated at room temperature for 30 minutes. Then, the cell sample was analyzed in a fluorescence-activated cell sorter (FACS) Calibur (Becton Dickinson Immunocytometry, San Jose, CA, USA) (13-16).

Determination of the DC1/DC2 ratio. Twenty µL of Lineage Cocktail 1 FITC, 10µL of Anti-HLA-DR PerCP and 5µL of CD11c PE were added to 100µL of whole blood and incubated at room temperature for 15 minutes. After lysing the red blood cells with red blood cell lysing buffer, the sample was analyzed in the FACS Calibur. The cells stained Lineage Cocktail 1-negative and HLA-DR-positive were determined as the DC fraction and, among the DC fraction CD11c-positive cells were determined as DC1 and CD11c-negative cells were determined as DC2 (17).

Figure 1. Comparison between normal adults and patients in the Th1/Th2 ratio (upper left), the DC1/DC2 ratio (upper right), IL-12 production (lower left) and IL-10 production (lower right). Each number of measurements was shown in the figure.
Determination of IL-10 and IL-12. Peripheral blood mononuclear cells (PBMC) were isolated from 10 mL of heparinized peripheral blood sample using the Ficoll-Hypaque centrifugation method. The PBMC were suspended at a density of 1x10^6/mL in RPMI 1640 supplemented with 10% FCS and 30 μg/mL of PHA, and cultured at 37°C for 24 hours. After culture, the supernatant was collected and stored at -80°C until determination. After thawing, the IL-12 and IL-10 concentrations in the supernatant were measured using ELISA (18, 19).

Statistic analysis. All the data are presented as mean±standard deviation. The unpaired t-test was used to compare data of normal subjects and cancer patients. The paired t-test was used to compare time-related changes.

Results

Comparison of T cell differentiation between normal subjects and cancer patients. Figure 1 shows the comparison between normal adults and cancer patients in the Th1/Th2 ratio, the DC1/DC2 ratio, IL-10 production and IL-12 production.

The Th1/Th2 ratio was significantly lower in cancer patients (9.36±7.55, p=0.049) than in healthy adults (18.98±12.85). The decline of the Th1/Th2 ratio in cancer patients is mainly due to the elevation of Th2, because the Th2 percent was significantly higher in cancer patients (3.25±1.78%, p=0.026) than in healthy adults (1.42±0.55%). The Th1 percent did not significantly differ between cancer patients (21.30±9.46%) and healthy adults (24.27±13.80%).

The DC1/DC2 ratio was also significantly lower in cancer patients (1.25±0.69, p=0.003) than in healthy adults (3.09±1.76). The decline of the DC1/DC2 ratio in cancer patients is mainly due to the elevation of DC2, because the DC2 percent was significantly higher in cancer patients (0.14±0.07%, p=0.030) than in healthy adults (0.06±0.03%). The DC1 percent was lower in cancer patients (0.14±0.09%, p=0.055) than healthy adults (0.25±0.15%).

IL-10 production by PBMC was significantly increased in cancer patients (559.36±147.08 pg/mL, p=0.004) compared to healthy adults (311.63±208.19 pg/mL). There was no significant difference in IL-12 production by PBMC between healthy adults (22.01±10.94 pg/mL) and cancer patients (81.08±140.99 pg/mL).
Time course of the Th1/Th2 ratio during PSK therapy. Figure 2 shows the serial changes in the Th1/Th2 ratio during PSK administration in cancer patients. Fourteen patients, who received 300 mg/day of oral UFT combined with 3g/day of oral PSK for at least one month, were enrolled in this study. The Th1/Th2 ratio was 9.36±7.53 before administration of PSK, significantly increased to 12.31±10.11 at one month after PSK therapy (p=0.049), and the ratio at two months after PSK therapy was higher (10.93±8.08, p=0.074) compared to the ratio before administration of PSK. From 3 months after PSK therapy, the levels fluctuated almost at the pre-treatment level. The elevation of the Th1/Th2 ratio is mainly due to the decrease of the Th2 percent rather than increase of the Th1 percent, because the Th2 percent was significantly decreased to 2.83±1.75% at one month after PSK therapy from 3.25±1.78% before PSK administration (p=0.046), whereas the Th1 percent was not significantly changed (21.30±9.46% before PSK administration and 24.08±10.31% at one month after PSK therapy, respectively; p=0.072).

Table I. Changes of the Th1/Th2 ratio in patients before and one month after treatment with PSK.

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<th>Pre-treatment</th>
<th>1 Month</th>
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<td>&lt;10</td>
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<tr>
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<td>n</td>
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<td>25.13±10.19</td>
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<td>p-value</td>
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In cases with a pre-treatment Th1/Th2 ratio lower than 10, indicating that the patients are in immunosuppressive state, the Th1/Th2 ratio at one month after PSK therapy was significantly increased compared to the pre-treatment ratio (5.24±2.04 before PSK administration and 7.07±3.05 at one month after PSK administration, respectively; p=0.010). In cases with a pre-treatment Th1/Th2 ratio of 10 or above, there was no difference between the ratios in the pre-treatment period and one month after PSK therapy.

![Figure 3. Changes of the DC1/DC2 ratio (left; n=14), DC1 percent (center; n=14) and DC2 percent (right; n=14) between before administration of PSK and one month after PSK therapy.](image-url)
Time course of the DC1/DC2 ratio during PSK therapy. Figure 3 shows the changes of the DC1/DC2 ratio, DC1 percent and DC2 percent before administration of PSK to one month after PSK therapy in cancer patients receiving postoperative PSK therapy (n=14). The DC1/DC2 ratio was 1.25±0.69 before PSK administration and 1.62±1.16 one month after PSK therapy, showing a tendency to increase ($p=0.090$). The elevation of the DC1/DC2 ratio in cancer patients is due to the decrease of the DC2 percent, the DC2 percent being significantly decreased to 0.10±0.06% at one month after PSK therapy, showing a tendency to increase ($p=0.090$). The elevation of the DC1/DC2 ratio in cancer patients is due to the decrease of the DC2 percent, the DC2 percent being significantly decreased to 0.10±0.06% at one month after PSK therapy, showing a tendency to increase ($p=0.090$). The elevation of the DC1/DC2 ratio in cancer patients is due to the decrease of the DC2 percent, the DC2 percent being significantly decreased to 0.10±0.06% at one month after PSK therapy, showing a tendency to increase ($p=0.090$).

Time courses of IL-10 production, IL-12 production and the DC1/DC2 ratio during PSK therapy. Figure 4 shows the changes of IL-10 production (n=14), IL-12 production (n=14) and DC1/DC2 ratio (n=6) by PBMC before administration of PSK and one month after PSK therapy. The IL-10 production was 559.36±147.08 pg/mL before PSK administration and 422.14±219.29 pg/mL one month after PSK therapy, showing a significant decrease ($p=0.015$). The IL-12 production was 81.08±140.99 pg/mL before PSK administration and 60.29±125.67 pg/mL one month after PSK therapy, showing no significant difference.

Discussion

In 1986, Mosmann et al. (1) classified Th cells into two subsets; Th1 cells and Th2 cells, based on the differences in the cytokines produced. Th1 cells produce so-called Th1 cytokines including IFN-γ, IL-2 and TNF-α, and are associated with cellular immunity. Th2 cells produce Th2 cytokines including IL-4, IL-6 and IL-10, and are involved in humoral immunity. The Th1/Th2 balance has been reported to shift to one side in various disease conditions. Pellegrini et al. (3) used PHA to stimulate PBMC isolated from stage I to IV colorectal cancer patients, and compared IL-2 and IL-4 production. They found that with the progression of cancer stage, IL-2 production was reduced while IL-4 production was enhanced, and the Th1/Th2...
balance became Th2 dominant. In tumor immunology, since tumor cells are removed by CTL, the effector cells responsible for cellular immunity, a shift to Th2 dominance has been understood to be a mechanism of escape from the immune surveillance system in the cancer-bearing state.

Apart from the helper T cell lineage, the existence of other subsets of immune cells has been reported. Sato et al. (5) demonstrated that, within murine dendritic cells, there was a DC1 subset induced by the Th1 cytokines IL-12 and IFN-γ, and a DC2 subset induced by the Th2 cytokine IL-4. DC1 cells produced a high level of IL-12-inducing Th1 cells, while DC2 cells produced a low level of IL-12-inducing Th2 cells (20). Additionally, DC1 was more capable of inducing CTL than DC2 (9). Studies reported that, in humans as well, CD40L-induced monocyte-derived dendritic cells produced a high level of IL-12, enhancing differentiation of Th1 (7,8).

The above observations suggest that effective induction of the DC1-Th1 cell lineage is indispensable in tumor immunology. We first compared the Th1/Th2 and DC1/DC2 ratios between patients with gastric or colorectal cancer and normal adults. Then, we investigated the effects of PSK, a BRM used for the treatment of gastric cancer, colorectal cancer and small cell lung cancer in Japan, on the Th1/Th2 and DC1/DC2 ratios in the cancer patients.

Our results showed that the Th1/Th2 and DC1/DC2 ratios were all significantly lower in cancer-bearing patients. Thus, not only did the Th1/Th2 ratio change to Th2 dominance as reported previously, but DC2 ratios also shifted toward DC2 dominance.

We observed the serial changes of the Th1/Th2 ratio in the cancer patients who received postoperative PSK therapy. At one month after PSK therapy, the Th1/Th2 ratio shifted to Th1 dominance with a significant decrease of Th2 percent. Especially, cases exhibiting Th2 dominance in the Th1/Th2 balance before administration of PSK showed markedly significant improvement. Concerning the DC1/DC2 ratio, a tendency to shift to DC1 dominance was also observed one month after PSK therapy with a significant decrease of the DC2 percent. Therefore, concerning cellular immunity, PSK has the main effect of a shift to Th1, DC1 dominance by the decrease of Th2 and DC2, especially against an immunosuppressive host. It is considered to be reasonable as a normalization of cellular immunity because the Th1/Th2 and DC1/DC2 ratios were lower with a significant elevation of the Th2, DC2 percent in cancer-bearing patients compared to normal subjects. When IL-10 and IL-12 production by PBMC was examined, IL-10 production was significantly reduced one month after PSK therapy. Our present results confirmed that not only a Th1/Th2 balance shift to Th1 dominance, but also a DC1/DC2 balance shift to DC1 dominance, suggest a possibility that enhanced cellular immunity may act to eliminate tumor cells by the DC1-Th1 cell lineage, resulting in the augmentation of CTLs induction.

Shibata et al. (18) studied 10 patients with advanced colorectal cancers treated with immunotherapy using low-dose cisplatin, UFT and PSK for two months, and examined the changes of the Th1 and Th2 cytokines produced by PBMC before and after immunotherapy. They reported a tendency to increase in the Th1 cytokine IFN-γ and a significant decrease in the Th2 cytokine IL-10.

In the study of Yoshino et al. (21), 57 patients with colorectal cancer with curability grade A and B were administered PSK for 7 days before surgery, and the cytokines in CD4+ cells before and after administration were analyzed. They reported that approximately 60% of the patients showed reduced CD4+IL6+ and CD4+IL10+, showing resolution of the Th2-dominant state. In relapse cases, the Th2 dominance was not resolved.

Wada et al. (22) examined cytokine production in the spleen cells of Meth A cancer-bearing mice, and reported that PSK administration increased IFN-γ but did not change IL-4. On the other hand, PSK administration increased IFN-γ and lowered IL-4 in mesenteric lymph node cells of colon 26 cancer-bearing mice.

Comparing these previous findings with our present results, PSK may act via different mechanisms in humans and in mice. In mice, PSK administration enhances the production of Th1 cytokines and inhibits the production of Th2 cytokines, resulting in a shift of the Th1/Th2 balance to Th1 dominance. In humans, however, PSK treatment mainly suppresses the production of Th2 cytokines to shift the Th1/Th2 balance to Th1 dominance.

We are the first to report the phenomenon that PSK administration not only changes the Th1/Th2 balance to Th1 dominance, but also shifts the DC1/DC2 balance to DC1 dominance.

In our facility, we have been conducting DC vaccination therapy in patients with advanced cancers refractory to existing treatment. Since oral administration of PSK during DC therapy possibly induces DC1 and Th1 dominance resulting in enhancement of CTLs induction, the vaccination effect is expected to persist even in immunosuppressive state. We have planned further studies to examine whether combined oral PSK with DC therapy augments the effect of T cell and dendritic cell differentiation (23).

References


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