Effects of Preoperative Immunochemoradiotherapy and Chemoradiotherapy on Immune Responses in Patients with Rectal Adenocarcinoma

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Abstract. *Background: We previously reported that preoperative chemoradiotherapy (CRT) combined with intraoperative electron irradiation for cT3/T4 adenocarcinoma of the rectum reduced the local recurrence, with significant improvement of survival. Radiotherapy has been reported to reduce immune function. We examined the effects of PSK, a protein-bound polysaccharide, concomitant with preoperative CRT on immune responses. Patients and Methods: Thirty patients with cT3/T4 adenocarcinoma of the rectum were randomly assigned to 2 weeks' irradiation and 4 weeks' S-1 administration before surgery (control group), or the same CRT with simultaneous 4 weeks' PSK administration (PSK group). Both systemic and local immune responses were evaluated. Results: Significant increase of natural killer cell count in the peripheral blood and cytotoxic T-cell counts in the peri-tumoral and normal mucosa, and a significant decrease of serum immunosuppressive acidic protein level were observed in the PSK group. Conclusion: Combined use of PSK with preoperative CRT may improve immune function.*

Multimodal therapies, preoperative short-term intensive radiotherapy (RT), and conventional long-term radiotherapy with 5-fluouracil-based chemotherapy have gained wide acceptance for the treatment of locally advanced rectal adenocarcinoma (1-5). However, no significant improvements in the disease-free and overall survival rates have been achieved, although these treatments improve local control. We have used preoperative chemoradiotherapy (CRT) combined with intraoperative electron beam irradiation (IOR) for cT3/T4 or TxN+ adenocarcinoma of the middle or lower rectum, and reported a reduction in local recurrence to 3%, with significant improvement of survival (6, 7). Our regimen requires only 4 weeks between the start of RT and radical surgery.

RT has been reported to be associated with significant suppression of the immune system. Both B and T lymphocytes in peripheral blood decrease and inflammatory reaction in the irradiated field is suppressed (8-12). Local wound infection has been reported to increase after multimodal therapy (13). These results demonstrated that immune function of patients with rectal carcinomas treated with preoperative RT or CRT was suppressed during the perioperative period.

PSK, a protein-bound polysaccharide obtained from cultured mycelia of the *Basidiomycete coriolus versicolor*, activates various immunological functions including the activities of natural killer (NK) cells (14) and dendritic cells (15), and improves the prognosis of gastric (16) and colorectal cancer (17, 18). In the present study, we assessed the systemic and local immune functions of patients with rectal adenocarcinoma who received preoperative multimodal therapy, and also evaluated the effect of the immunomodulator PSK in preventing immunosuppression.

Patients and Methods

Patients. Patients with histologically confirmed adenocarcinoma of the middle or lower rectum (cT3-T4, Tx N+, M0) were enrolled. Additional eligibility criteria were as follows: no prior chemotherapy and/or pelvic radiation; Eastern Cooperative Oncology Group performance status ≤1; age between 20 and 80 years; and adequate organ function, defined as leukocyte count ≥4,000/μl and ≤12,000/μl, neutrophil count ≥2,000/μl, platelet count ≥100,000/μl, hemoglobin ≥9 g/dl, serum creatinine ≤1.1 mg/dl, serum bilirubin ≤1.5 mg/dl, and serum aspartate aminotransferase and alanine aminotransferase ≤100 U/l.

A total of 30 patients were registered. The patients were randomized before treatment into a control group (n=15) and a PSK group (n=15). Pretreatment stage was determined by computed tomography (CT), transrectal ultrasonography and magnetic

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resonance imaging (MRI). Pathological stage was based on histopathological findings of the resected specimen. The patient and tumor characteristics of the 30 patients in the current study cohort are shown in Table I. During therapy, toxicity was assessed weekly according to the Common Toxicity Criteria of National Cancer Institute version 2.0.

Preoperative chemoradiotherapy with or without PSK and surgical procedure. All patients received preoperative CRT. External beam RT for a total dose of 20 Gy was delivered in daily fractions of 2.0 Gy, 5 days a week for two weeks using a four-field box technique (6, 7). At the same time when RT was initiated, the control group also started to take oral S-1 (Taiho Pharmaceutical Co., Japan) 80 mg/m^2/day divided into 2 daily doses and the PSK group started to take the same dose of S-1 together with PSK (Kureha Corporation, Japan) 3.0 g/day divided into 3 daily doses, for 4 weeks until 3 days before surgery. Approximately 2 weeks after completion of RT, both groups underwent radical surgery. During surgery, electron beam irradiation of 15 Gy was delivered according to the methods we reported previously (6, 7, 19).

Evaluation of systemic immunity in the peripheral blood. Before preoperative treatment was started and one day before surgery, the white blood cell count (WBC), neutrophil count, lymphocyte count, NK cell count and immunosuppressive acidic protein (IAP) level in the peripheral blood were determined.

Peripheral blood mononuclear cells were isolated from heparinized blood by density gradient centrifugation using Isolymph (Gallard-Schlesinger, NY, USA) according to the manufacturer's instructions. The proportion of NK cells were measured by direct immunofluorescence with fluorescein-conjugated monoclonal antibodies specific for CD16 (Beckman Coulter, FL, USA) and for CD57 (BD Bioscience, CA, USA) using flow cytometry. The proportion of CD16$^+$/CD57$^+$ cells among lymphocytes is shown as percentages. Serum IAP levels were measured by a turbidimetric immunoassay using San test IAP-N kits (Sanko Junyaku Co., Tokyo, Japan), according to the manufacturer's instructions.

Evaluation of local immunity in the resected specimen. Resected specimens containing rectal cancer were fixed in 4% (v/v) phosphate-buffered formalin, dehydrated and embedded in paraffin. Tumor tissue, peri-tumoral mucosa, and normal mucosa which was 5 cm or greater apart from the tumor, but within the radiation field were collected. Tissue sections of 4 μm were cut, mounted onto glass slides pretreated with 2% 3-aminopropyltriethoxysilane and dried overnight. Serial sections were stained with hematoxylin and eosin or processed for immunohistochemistry. Various inflammatory cells were assessed by immunohistochemical investigations with the following antibodies: anti-CD3 (monoclonal mouse anti-human T-cell, 1:200; DakoCytomation, Glostrup, Denmark), anti-CD4 (monoclonal mouse anti-human CD4, 1:40; Novocastra/Vision BioSystems, Norwell, MA, USA), anti-CD8 (monoclonal mouse anti-human CD8, 1:40; Novocastra/Vision BioSystems), anti-CD56 (monoclonal mouse anti-human N-CAM-16, 1:200; BD Pharmingen, BD Bioscience, San Diego, CA, USA), anti-AA1 (monoclonal mouse anti-human mast cell tryptase, 1:200; DakoCytomation), anti-CD68 (monoclonal mouse anti-human macrophage, 1:100; DakoCytomation), and anti-elastase (monoclonal mouse anti-human neutrophil elastase, 1:200; DakoCytomation).

For every tissue sample, cells were counted in 15-high power fields (2.1 mm^2) according to the method of Nagtegaal *et al*. (20, 21), and expressed as number per mm^2. Cell counting was conducted independently by two investigators (SS, AT), and the mean numbers were used for analyses.

Table I. *Patient and tumor characteristics*.

Characteristic	Control (n=15)	PSK group (n=15)	*p*-Value
Female/male	4/11	2/12	0.68
Age (years, mean±SD)	65±6	60±10	0.29
Size (cm, mean±SD)	2.7±1.3	3.5±1.3	0.11
Histology			
Well-differentiated	6	6	1.0
Moderately differentiated	9	9	
ypT category			
1	3	1	0.15
2	5	2	
3	6	12	
4	1	0	
ypN category			
0	12	8	0.25
1/2	3	7	

This study was approved by the Institutional Review Board of Tokai University and all patients provided written informed consent.

Statistical analysis. Data are presented as the mean±SD or number of patients. All statistical analyses were performed with SAS software (version 9.1.3, SAS Institute Inc., Cary, NC, USA). For statistical comparisons of the patient characteristics and immunological response in peripheral blood before preoperative treatment between the two groups, the chi-square test or unpaired *t*-test was used. The immunological response in peripheral blood was compared before and after treatment in the same group using paired *t*-test. The immunological response in the irradiated tissue was compared between the two groups using unpaired *t*-test. Two-sided *p*-values of less than 0.05 were considered statistically significant.

Results

Patient and tumor characteristics. No differences in male/female ratio and age were observed between the PSK and control groups (Table I). There were also no significant differences between the two groups in histological type determined from biopsies conducted before treatment, tumor diameter of resected specimens, or TNM staging of resection specimens.

Adverse effects during chemoradiotherapy. Adverse events of grade 3 or above were observed in one patient (7%) in the PSK group. All other adverse events observed in both groups were mild (Table II).

Immunological response in peripheral blood. No significant differences in peripheral blood values before preoperative treatment were observed between the control and PSK groups. The numbers of WBC, neutrophils, and lymphocytes

Table II. *Adverse effects during preoperative chemoradiotherapy and immunochemoradiotherapy.*

	Control (n=15)				PSK group (n=15)			
	Gr 1	Gr 2	Gr 3	Gr 1-4 (%)	Gr 1	Gr 2	Gr 3	Gr 1-4 (%)
Leukopenia	0	2	0	13%	2	1	0	20%
Neutropenia	2	0	0	13%	1	0	0	7%
Anemia	7	1	0	53%	1	3	1	33%
Thrombocytopenia	2	0	0	13%	0	0	0	0%
Nausea	5	0	0	33%	5	0	0	33%
Vomiting	0	0	0	0%	0	0	0	0%
Diarrhea	2	1	0	20%	4	1	0	33%
AST/ALT	1	0	0	7%	1	0	0	7%
Fatigue	7	0	0	47%	7	0	0	47%
Stomatitis	0	0	0	0%	0	0	0	0%

AST, Aspartate aminotransferase; ALT, alanine aminotransferase.

Table III. *Changes of immunological parameter in peripheral blood during preoperative chemoradiotherapy and immunochemoradiotherapy.*

	Control			PSK group		
	Before Tx	After Tx	*p*-Value	Before Tx	After Tx	*p*-Value
WBC	7400±1794	4713±1770	<0.0001	6475±1308	5231±1567	<0.001
Neutrophils (%)	68±9	69±7	0.90	65±7	62±10	0.46
Neutrophils	5025±1468	3288±1543	0.0005	4228±1023	3247±1112	0.009
Lymphocytes (%)	25±8	20±7	0.04	27±7	25±17	0.55
Lymphocytes	1811±623	886±329	<0.0001	1730±556	1008±400	0.0002
NK cells (%)	13±8	15±6	0.17	11±8	16±10	0.003
IAP	475±332	405±276	0.19	408±123	323±190	0.03

WBC, White blood cells; NK, natural killer; Tx, treatment; IAP, immunosuppressive acidic protein.

were significantly reduced after chemoradiotherapy in both groups (Table III). The proportion of lymphocytes was significantly lower in the control group but did not change in the PSK group. No significant change in the proportion of NK cells was observed after chemoradiotherapy in the control group, while a significant increase in the proportion of NK cells was found in the PSK group. IAP level was significantly lowered in the PSK group.

Immunological response in irradiated tissue. The numbers of T-cells, helper T-cells, cytotoxic T-cells, NK cells, mast cells, macrophages and neutrophils in tumor tissues showed great individual differences, and were not significantly different between the control and PSK groups (Table IV). In the PSK group, the number of cytotoxic T-cells in the peri-tumoral mucosa (p=0.005) and normal mucosa 5 cm or greater away from the tumor (p=0.003) was significantly greater than that of the resected control tissue (Figure 1). Furthermore, the NK cell count in normal mucosa tended to be higher in the PSK group, but the difference was only marginally significant (p=0.06).

Discussion

Bone marrow stem cells are extremely radiosensitive (22), and lymphopenia occurs almost immediately after RT with or without chemotherapy. During long-term RT for cancer of the breast (8, 9), prostate (8), testicles (8, 9, 12), lung (10) and uterus (11), the numbers of both B-cells and T-cells in the peripheral blood decrease after RT, and the number of naïve T-cells does not completely recover to normal even after several years (9).

In human adults, 40% of the total active bone marrow is distributed in the sacrum, hip bone and proximal femur (23). The radiation field of patients with anal cancer has been reported to include up to 50% of a patient's total hematopoietically active bone marrow (24). The proportion of active bone marrow distributed in the radiation field of patients with rectal cancer is also relatively large. In our present study, the numbers of WBC, neutrophils and lymphocytes were significantly reduced by fractionated RT at a total dose of 20 Gy delivered to the rectal region.

Table IV. *Immune cell infiltration (n/mm²) in the resected specimen after preoperative chemoradiotherapy and immunochemoradiotherapy.*

Cell type	Antibody	Area	Control	PSK group	*p*-Value
T	(CD3)	Intratumor	466±472	199±299	0.10
		Peri-tumor	106±106	241±294	0.13
		Normal mucosa	167±96	229±172	0.26
T-helper	(CD4)	Intratumor	335±360	177±288	0.23
		Peri-tumor	62±61	58±76	0.86
		Normal mucosa	153±103	144±110	0.81
T-cytotoxic	(CD8)	Intratumor	177±250	183±228	0.94
		Peri-tumor	55±43	139±88	0.005
		Normal mucosa	74±20	186±114	0.003
NK	(CD56)	Intratumor	10±29	0.5±1.1	0.24
		Peri-tumor	33±46	26±35	0.67
		Normal mucosa	5±7	21±29	0.06
Mast	(AA1)	Intratumor	29±29	32±29	0.78
		Peri-tumor	33±23	48±31	0.17
		Normal mucosa	55±30	51±31	0.74
Macrophages	(CD68)	Intratumor	295±222	280±178	0.85
		Peri-tumor	98±76	158±103	0.09
		Normal mucosa	308±128	356±217	0.48
Neutrophils	(Elastase)	Intratumor	118±249	92±126	0.74
		Peri-tumor	3.0±3.6	4.3±6.5	0.51
		Normal mucosa	11±14	19±38	0.49

Values are means±SD of 15 high-power fields.

According to previous studies, although all subsets of peripheral lymphocytes decrease as a result of RT (10, 11), the degree of decrease, duration until recovery and degree of recovery differ among the subsets (9, 12). The decrease of NK cells has been reported to be relatively mild (11 12). In our study also, no significant change in the proportion of NK cells in peripheral blood was observed after CRT in the control group, while the proportion of NK cells significantly increased after therapy in the PSK group. The proportion of NK cells in peripheral blood before therapy did not reveal any significant differences between the two groups.

Although the mechanisms of action of the effects of PSK administration have not been elucidated, two possible mechanisms are speculated. The first possibility is that the decrease in NK cells is milder by PSK administration, based on the following findings: i) PSK alleviated leucopenia induced by chemotherapy in mice (25); and ii) PSK alleviated apoptosis of T-cells in peripheral blood induced by S-1 treatment in patients with gastric cancer (26). The second possibility is that PSK administration promotes the proliferation of NK cells, compensating for the decrease in the number of NK cells, based on the following reports: i) PSK showed *in vitro* mitogenic action against peripheral blood mononuclear cells of healthy individuals (27); ii) PSK promoted the proliferation of mouse splenic T-cells (28); iii) PSK promoted the proliferation of human NK cell line (29); iv) PSK promoted interleukin (IL)-2 production, although weakly, and increased IL-2 receptor expression in normal human lymphocytes (30, 31). Koda *et al*. (32) reported that rectal cancer patients with low NK cell activity before surgery had significantly lower metastasis-free survival, and that preoperative CRT impaired NK cell activity. The effect of any increase in the proportion of NK cells in the present study on clinical outcome has to be investigated in a larger scale randomized controlled study. Ohwada *et al*. (33) reported that PSK significantly prevented any decrease of the NK cell proportion in peripheral blood of colorectal cancer patients with curative resection, and the prognosis of patients with an increase of NK cell proportion was better than that of patients with a decrease or no change of NK cell proportion.

IAP is a protein that suppresses the immune response of lymphocytes against phytohemagglutinin (PHA) and other antigens. Serum IAP level has been reported to be elevated in cancer patients, and is related to cancer progression and prognosis (34, 35). Satomi *et al*. (36) reported a significant increase of IAP in the peripheral blood of patients with colorectal cancer compared to healthy controls. On the other hand, PSK has been reported to reduce IAP level (33, 37). In the present study, the IAP level was not changed in the control group but was significantly lowered by CRT with concomitant use of PSK. IAP level was reported to increase by IL-6 (38), and PSK has been shown to suppress IL-6 production (39). It is possible that the IAP level was reduced through suppression of IL-6 production. These results suggest that even under CRT, PSK is effective in improving the immunosuppressive state in cancer patients.

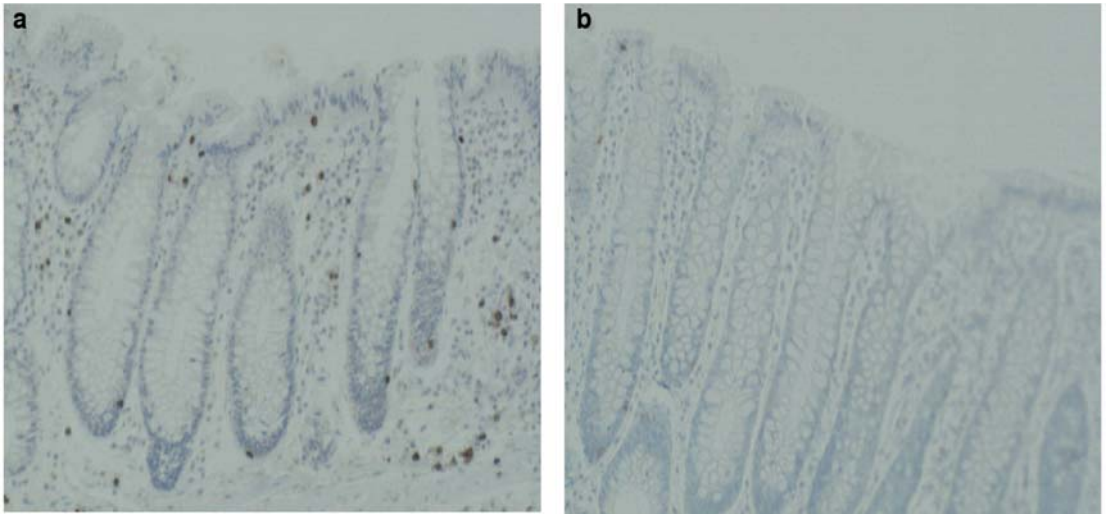

Figure 1. *Immunohistochemical staining for cytotoxic T-cells in the normal mucosa after preoperative chemoradiotherapy and immunochemoradiotherapy. CD8-positive cells (cytotoxic T-cells) in normal mucosa were assessed as described in the Patients and Methods. a: Immunochemoradiotherapy: Many positive cells (dark brown) can be seen in the normal mucosa of patients given PSK. b: Chemoradiotherapy: A few positive cells are observed in the normal mucosa of patients not given PSK.*

Mast cells (40-46), macrophages (41, 47-49), lymphocytes (50, 51), T-cells (52, 53) and NK cells (54) in cancer tissues have been investigated as biomarkers indicating proliferation and invasion of cancer cells, and angiogenesis. For colorectal cancer, a good prognosis is associated with a high lymphocyte count (50) and high NK cell count (54) in tumor tissue. The clinical significance of mast cells and macrophages has not been clarified.

Among patients enrolled in the total mesorectal excision (TME) trial, patients who underwent RT before surgery had a significantly lower numbers of T-cells ($CD3^+$), cytotoxic T-cells ($CD8^+$) and neutrophils in tumor tissue, as well as lower numbers of T-cells ($CD3^+$), helper T-cells ($CD4^+$), cytotoxic T-cells and neutrophils in peri-tumoral tissues compared to patients who underwent surgery alone (21). In patients who underwent abdominoperineal resection in the TME trial, the wound infection rate was 18% in patients who underwent surgery alone, and this increased to 29% in patients who had RT before surgery (13). This result may be associated with the impaired inflammatory reaction in patients who receive preoperative RT. In addition, patients with large numbers of macrophages, T-cells and cytotoxic T-cells in the tumor, and patients with large numbers of mast cells and basophils in peri-tumoral tissues have been reported to have good prognosis (20).

In the present study, the effect of concomitant use of PSK on lymphocyte counts within the tumor tissue was not clear due to large individual differences. However, the present study demonstrated that concomitant use of PSK significantly increased cytotoxic T-cells in peri-tumoral mucosa and normal mucosa within the irradiation field, as well as marginally increasing NK cells in the normal mucosa, although no increase in neutrophils was observed. Although we did not measure cytotoxic T-cells in peripheral blood in the present study, Nio *et al*. (55) reported that PSK treatment increased the proportion of cytotoxic T-cells in peripheral blood of patients with gastrointestinal cancer. As mentioned above, since PSK promotes lymphocyte proliferation and alleviates chemotherapy-induced leucopenia, the increase of cytotoxic T-cells or NK cells in peripheral blood may allow their migration to peri-tumor and normal mucosa. These findings suggest that PSK may promote local immunity in normal tissues within the radiation field.

Further clinical studies with larger samples will be needed to confirm the beneficial effects of combined use of PSK with preoperative CRT both on perioperative morbidity and prognosis in patients with rectal adenocarcinoma.

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