postoperative days. The time until the first flatus after the operation, the duration of the postoperative hospital stay, and the postoperative complications were also compared between the two groups. Any possible medications that might have affected gastrointestinal motility and functions, including prokinetics, antacids, and parasympathetic nerve blockers, were prohibited for 2 weeks before and during the study. All patients underwent a mechanical bowel preparation with magnesium citrate solution. During the operation, the incision was washed under high pressure while closing the abdomen. Only Loxoprofen was administered for postoperative pain, and the frequency of use was checked. This study was performed under the authors' institutional guidelines and after permission was obtained from the local institutional review board. Written informed consent was obtained from every patient.

DKT

DKT, from Tsumura & Co., Tokyo, Japan, is a traditional Japanese herbal medicine, containing 1.25 g of DKT extract powder, 10 g of malt sugar and 3.75 g of vehicle in a total amount of 15 g. DKT extract powder is a mixture of dried ginger root, ginseng and zanthoxylum fruit at a ratio of 5:3:2, respectively. D group took 7.5 g/day of DKT.

Statistical analysis

The results are expressed as the means ± SE, and an analysis of variance (ANOVA) was used to evaluate the results. The statistical analyses were performed using the Newman–Keuls method for multiple comparisons. Statistical significance was set at $P < 0.05$.

Results

Clinical findings

The surgical findings are shown in Table 2. Although there were no significant differences in the procedure performed, the inclusion of lymph node dissection was significantly different, with D3 dissection performed more frequently in D group. The intraoperative blood loss and length of the operation were not significantly different between D group (20 ± 6 ml; 204 ± 11 min) and C group (33 ± 12 ml; 224 ± 17 min). The frequency of the Loxoprofen use during this study was not different between the two groups (D group: 2.8 ± 0.7, C group 3.1 ± 0.6).

The postoperative outcomes are listed in Table 3. One patient in C group developed a complication (wound

Table 2 Operative data

	D group ($n = 15$)	C group ($n = 15$)	P value
Performed operation, n (%)			
Ileocecal resection	2	4	
Ascending colectomy	1	1	
Transverse colectomy	0	2	
Descending colectomy	1	0	
Sigmoid colectomy	4	2	
High anterior resection	1	1	
Low anterior resection	6	5	0.62
Lymph node dissection, n (%)			
D1	0	5	
D2	4	3	
D3	11	7	0.05
Intraoperative blood loss (mL), mean ± SE	20 ± 6	33 ± 12	0.37
Operation time (min), mean ± SE	204 ± 11	224 ± 17	0.51

Table 3 Postoperative outcomes

	D group ($n = 15$)	C group ($n = 15$)	P value
Wound infection, n (%)	0	1 (7)	
Ileus, n (%)	0 (0)	0 (0)	
Mortality, n (%)	0 (0)	0 (0)	
Frequency of NSAID	2.8 ± 0.7	3.1 ± 0.6	0.45
Time until start of flatus (day), mean ± SE	1.8 ± 0.5	2.7 ± 0.5	0.02
Duration of postoperative hospital stay (day), mean ± SE	12.2 ± 0.6	12.8 ± 0.9	0.75

infection). The incidence of ileus and the mortality were not significantly different between the two groups. The time until the first flatus was significantly shorter in D group (1.8 ± 0.5 days) than in C group (2.7 ± 0.5 days). However, the duration of the postoperative hospital stay was not significantly different between D group (12.2 ± 0.6 days) and C group (12.8 ± 0.9 days). The body temperature on the 3rd postoperative day was significantly lower in D group (36.2 ± 0.4°C) than in C group (36.9 ± 0.6°C). The heart rate was not significantly different between the two groups on any postoperative day (Fig. 1a, b).

Laboratory data analysis

The results of all of the laboratory tests are presented in Fig. 2a–c. The CRP levels of both groups reached a peak on the 3rd postoperative day and then gradually decreased. However, the CRP level was significantly lower in D group (4.6 ± 0.6 mg/dl) than in C group (8.3 ± 1.1 mg/dl) on the 3rd postoperative day. The WBC and lymphocyte counts were not significantly different between the two groups. β-D-glucan was positive in one patient in each group, and the Candida index was positive in 1 patient in D group and 2 patients in C group.

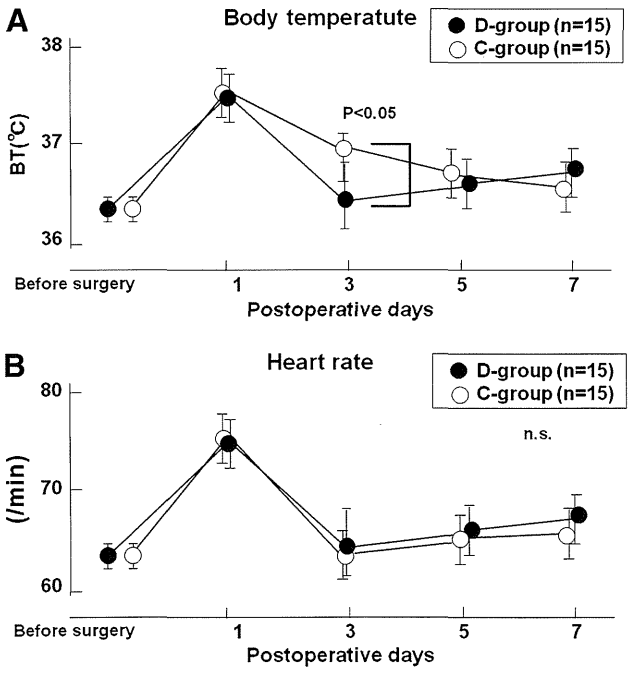

Fig. 1 A comparison of the body temperature (**a**) and heart rate (**b**) between the two groups. The Mann–Whitney *U* test was used for the analysis

Comments

In this study, we focused on patients undergoing laparoscopic surgery. This is the first report showing that DKT has anti-inflammatory effects following less invasive surgery. DKT is increasingly being used in the treatment of a variety of gastrointestinal diseases [13, 16]. Recent studies have been gradually clarifying the mechanism by which DKT stimulates the intestinal tract. One study suggested that DKT stimulates the gastrointestinal tract through cholinergic and non-cholinergic pathways [17–19]. Acceleration of the motility in the small intestine prevents endoluminal bacterial overgrowth [20]. An increase in the intestinal blood flow is also produced by one of the active components of DKT in a dose-dependent manner [10]. These phenomena were observed when the clinical symptom of feelings coldness in the abdomen improved after

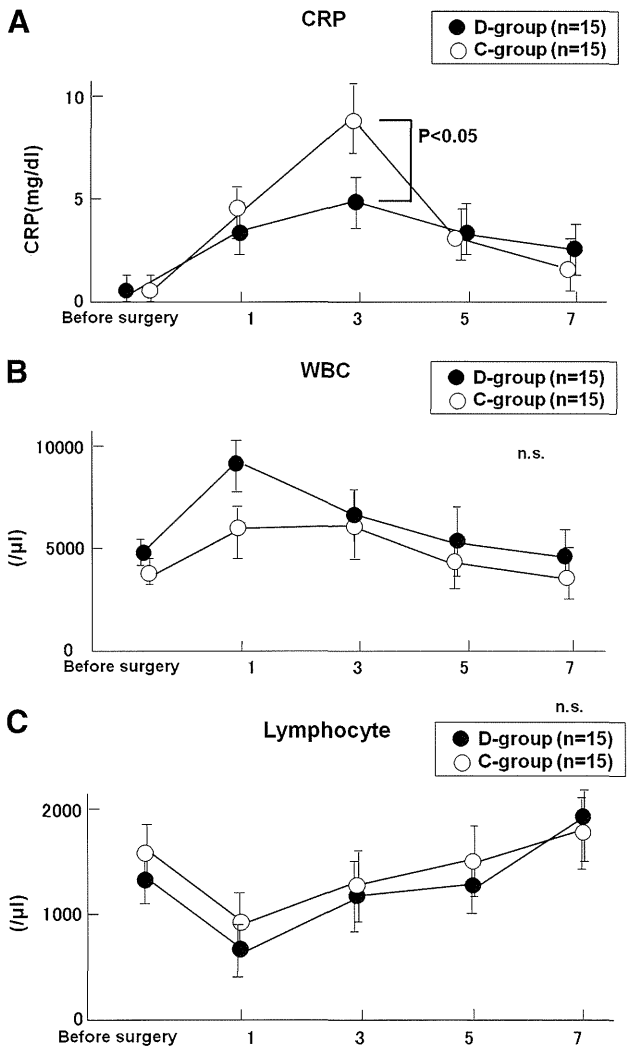

Fig. 2 A comparison of the CRP level (**a**), WBC count (**b**), and lymphocyte count (**c**) between the two groups. The Mann–Whitney *U* test was used for the analysis

DKT administration [13]. In previous studies, DKT induced acetylcholine release from intrinsic cholinergic nerves, mainly as a result of the zanthoxylum fruit [6, 21], and increased the intestinal blood flow as a result of the ginger rhizome, which may enhance intestinal motility [10, 13]. We have already reported that DKT exerted an anti-inflammatory effect by inhibiting the production of inflammatory cytokines, including IFN-γ, IL-6 and TNF-α, and significantly alleviated intestinal epithelial apoptosis in a rat model [15].

Furthermore, it is of clinical interest that a significant suppression of the postoperative elevation of body temperature on postoperative day 3 was found in D group. As the body temperature is one factor involved in the systemic inflammatory response syndrome (SIRS), DKT may have the potential to prevent SIRS and subsequent multiple organ failure (MOF). Compared to the control group, D group had decreased inflammation 2 days after the first DKT intake. Compared with D group, there were more elderly patients and less invasive surgery (D1 lymph node dissection) performed in C group. Although elderly patients and less invasive surgery are likely to be associated with a decreased inflammatory response, D group showed a better inflammation status after the surgery. As a result, it was concluded that DKT had anti-inflammatory effects even in patients with severe conditions. This result suggests that preoperative DKT intake may have an anti-inflammatory effect during the early postoperative period. Further investigations are needed to determine the most appropriate DKT regimen, including the starting day and the duration of treatment, in order to best control surgical stress.

Previous reports have indicated that DKT maintains the integrity of the intestinal epithelia and increases intestinal blood flow [12]. By maintaining epithelial integrity, lipopolysaccharide release may be suppressed. This mechanism also relates to the anti-inflammatory effects in postoperative patients. Although the WBC count was not significantly different between the two groups, the CRP level was lower in D group than in C group. This discrepancy is likely due to the fact that the CRP level is influenced by the presence of lipopolysaccharide.

In summary, the anti-inflammatory effects of DKT were evaluated in colorectal cancer patients following laparoscopic colectomy. This prospective, randomized, controlled study demonstrated that DKT accelerates intestinal motility, suppresses the CRP level and suppresses the body temperature postoperatively. Therefore, DKT has anti-inflammatory effects and this herbal medicine may help patients recover better following surgery.

Conflict of interest Drs. Kozo Yoshikawa, Mitsuo Shimada, Masanori Nishioka, Nobuhiro Kurita, Takashi Iwata, Jun Higashijima, Tomohiko Miyatani, Motoya Chikakiyo, Toshihiro Nakao, and Masato Komatsu have no conflicts of interest or financial ties to disclose.

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ORIGINAL ARTICLE

Kampo medicine "Dai-kenchu-to" prevents CPT-11-induced small-intestinal injury in rats

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Abstract
Purpose The key anticancer agent, CPT-11 (irinotecan hydrochloride), induces severe diarrhea clinically. We investigated the effect of a Kampo medicine, Dai-kenchu-to (DKT), on CPT-11-induced intestinal injuries in rats.
Methods Twenty-four male Wistar rats were divided into three groups: a control group; a CPT-11 group, given CPT-11 150 mg/kg intraperitoneally for 2 days; and a DKT group, given DKT 300 mg/kg orally for 5 days with CPT-11 150 mg/kg intraperitoneally on days 4 and 5. The rats were killed on day 6.
Results Interleukin (IL)-1β, IL-12, interferon (IFN)-γ, and tumor necrosis factor-α expression in the small intestine of the CPT-11 group was significantly higher than that of the control group. Interleukin-1β and IFN-γ expression was improved significantly by DKT ($P < 0.05$). The number and height of jejuna villi, injury score, and apoptosis index in the CPT-11 group were improved significantly by DKT ($P < 0.05$).
Conclusions DKT suppressed CPT-11 induced inflammatory cytokines and apoptosis in the intestinal mucosa and maintained the mucosal integrity.

Keywords Dai-kenchu-to · Intestinal injury · CPT-11 · Inflammatory cytokine · Apoptosis

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Introduction

The clinical drug CPT-11 (irinotecan hydrochloride) is well known as a key anticancer agent, used widely in the treatment of colorectal, lung, and breast cancer, and malignant lymphoma [1–3]. Its effectiveness against advanced gastric cancer has also been studied [1–4]. CPT-11 is converted to its active metabolite, SN-38, by the enzyme carboxylesterase [5], which has a cytotoxic effect by inhibiting DNA topoisomerase. Glucuronidation of SN-38 protects against irinotecan toxicity. Hepatic and intestinal uridine diphosphate glucuronosyl transferase (UGT), particularly the UGT1A1 isoform, play important roles in the glucuronidation of active metabolites [6, 7]. The intestinal microflora may also play an important role in the toxicity of this drug. Many resident bacteria in the gut have β-glucuronidase activity and are able to convert SN-38 glucuronide (SN-38G) back to the active metabolite, SN-38, within the intestine, potentiating toxicity of the drug and exacerbating side effects such as diarrhea [5, 8].

CPT-11 also induces severe side effects, such as severe diarrhea, which is the most clinically important toxic effect or dose-limiting factor. A few reports have described the effects of irinotecan on the structure of the small intestine mucosa, including increased apoptosis in the crypts, and inflammatory cytokines leading to loss of the normal histology of the intestinal tissue; however, the mechanism of the histological features of intestinal mucositis remains unclear.

Dai-kenchu-to (DKT) is a Kampo medicine, which increases bowel movement and intestinal blood flow in the intestinal mucosa. It is used widely for treating postoperative adhesive intestinal ileus [9] and eliminating the feeling of coldness. Several studies show that DKT accelerates the gastrointestinal transit [10] and has an

anti-inflammatory effect. We reported recently that DKT prevents bacterial translocation by reducing inflammatory reaction and maintaining intestinal integrity [11]. We conducted this study to examine the effect of DKT on CPT-11-induced small-intestinal injury in rats.

Materials and methods

Irinotecan (CPT-11) and Dai-kenchu-to

Irinotecan (kindly supplied by Daiichi-Sankyo, Japan) was freshly prepared prior to treatment and administered in saline. Control rats received saline only, whereas the rats in the CPT-11 group and the CPT-11+DKT group (DKT group) received CPT-11 at a dose of 150 mg/kg on consecutive days. This dose was previously reported to cause reproducible gastrointestinal mucositis [12]. The rats were given 0.01 mg/kg subcutaneous atropine to reduce any cholinergic reaction to irinotecan, immediately prior to each administration of irinotecan or saline control.

DKT, which has been used at a dose of 15 g to treat patients with adhesive ileus, was purchased from Tsumura (Tokyo, Japan). Based on 15 g DKT administered to a 50-kg patient, we estimated the appropriate dose of DKT for rats to be 300 mg/kg [13]. The DKT was diluted in sterile water and administered by oral gavage under light anesthesia each morning for the duration of the experiment.

Animals

The experiment was performed using male Wistar rats (Charles River, Yokohama, Japan) weighing 220–230 g. Animals were free of all pathogens and housed under standard conditions (room temperature 22°C, humidity 50 ± 5%, 12:12 h light/dark cycle). The study was approved by the Institute Animal Committee of Health Bioscience, Tokushima University Graduate School of Medical Science.

Experimental design

Twenty-four rats were divided into the following three groups: a control group, given saline intraperitoneally for 2 days ($n = 7$); a CPT-11 group, given CPT-11 150 mg/kg intraperitoneally for 2 days ($n = 8$); and a CPT-11+DKT (DKT) group, given DKT 300 mg/kg orally for 5 consecutive days, with CPT-11 150 mg/kg intraperitoneally on days 4 and 5. All rats were killed on day 6 of the experiment, by the administration of a high dose of anesthesia. Tissue samples from the distal small intestine of all rats were fixed immediately in 10% neutral buffered formalin and embedded in paraffin (Fig. 1).

Assessment of diarrhea

Severity of diarrhea was assessed in all rats on day 6 of the experiment and recorded according to previous gradings [12]. In brief, there were four grades: 0, no diarrhea; 1, mild diarrhea (staining of anus); 2, moderate diarrhea (staining over the top of the legs and lower abdomen); 3, severe diarrhea (staining over the legs and higher abdomen, often associated with continual oozing). All diarrhea assessments were conducted in a blinded fashion by two investigators.

Detection of inflammatory cytokine in ileal tissue

To validate gene expression changes, quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed with an Applied Biosystems Prism 7500 sequence detection system. We used TaqMan universal PCR master mix, according to the manufacturer's specifications (Applied Biosystems, Foster City, CA, USA), for the seven genes for which validated TaqMan gene expression assays are available. The TaqMan probes and primers for interleukin (IL)-1 (assay identification number Rn00580432 m1), IL-6 (assay identification number Rn00561420 m1), IL-8 (assay identification number Rn00570857 m1), IL-10 (assay identification number Rn00 563409 m1), IL-12 (assay identification number Rn00575112 m1), interferon (IFN)-γ (assay identification number Rn00594078 m1), and tumor necrosis factor (TNF)-α (assay identification number Rn00562055 m1) were assay-on-demand gene expression products (Applied Biosystems). We used β-actin gene as an endogenous control (Applied Biosystems, catalog number 4352931E). The gene-specific probes were labeled using the reporter dye FAM, and the β-actin internal control probe was labeled with a different reporter dye, VIC, at the 5′ end. A nonfluorescent quencher and the minor groove binder were linked at the 3′ end of the probe as quenchers. The thermal cycler conditions were as follows: hold for 10 min at 95°C, followed by two-step PCR for 50 cycles at 95°C for 15 s, followed by 60°C for 1 min. Amplification data were analyzed with an Applied Biosystems Prism sequence detection software (version 2.1; Applied Biosystems). To normalize the relative expression of the genes of interest to the β-actin control, standard curves were prepared for each gene mentioned above and the β-actin in each experiment. When the efficiency of the target gene amplification and the efficiency of β-actin amplification were approximately equal (proven by examining the absolute value (less than 0.1) of the slope of log input amount versus ΔC_T) the $\Delta\Delta C_T$ method recommended by the manufacturer was used to compare the relative expression levels between treatments.

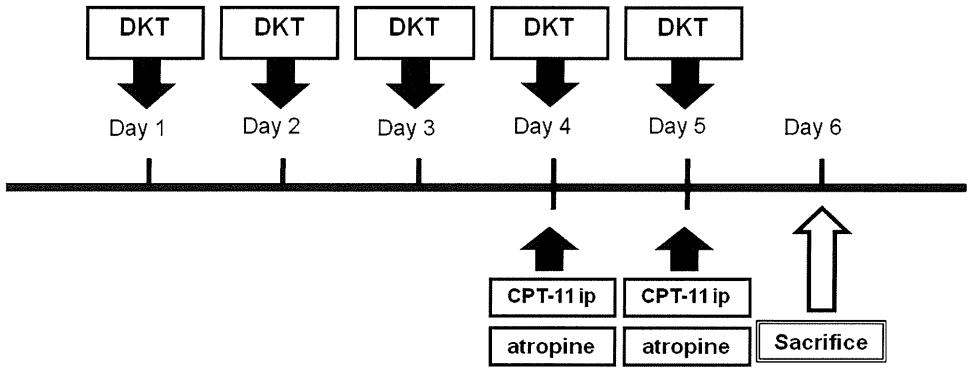

Fig. 1 Experimental design. The control group was given saline intraperitoneally for 2 days; the CPT-11 group was given CPT-11 (irinotecan hydrochloride) 150 mg/kg intraperitoneally for 2 days; and the DKT group was given DKT (Dai-kenchu-to) 300 mg/kg orally for 5 consecutive days with CPT-11 150 mg/kg intraperitoneally on days 4 and 5. The rats were killed on day 6 of the experiment

Histopathological examination

Two to four samples of ileum from all rats in each group were examined by light microscopy. Full-thickness sections of distal ileum were excised, fixed immediately in neutral buffered formalin, and embedded in paraffin.

Histological measurements of villi number and height

The sections of ileum were stained with hematoxylin–eosin and assessed for the degree of mucosal hyperplasia. Ileal sections were assessed for the number of villi. We used the method where the number of villi per 1 cm of the intestine was counted in five randomly selected areas, where villous height and morphology were best preserved [14]. Villous height was measured by micrometry using a microscope: At least five measurements from no overlapping well-oriented areas were taken from each sample and results expressed in micrometers. The villous height was defined as the micrometry from the base of the villous to the top [15, 16]. All assessments were conducted in a blinded fashion.

Evaluation of mucosal injury

Damage to the intestinal specimens was assessed according to microscopic criteria for the degree of damage based on the Chiu scale of mucosal injury [17]. The scale consists of values from 0 to 5: 0, normal mucosa; 1, subepithelial space at the villus tip; 2, extension of the subepithelial space with the epithelial layer lifting up in sheets from the lamina propria; 3, massive epithelial lifting down the slides of villi, possibly with a few denude tips; 4, denuded villi with exposed lamina propria, dilated and exposed capillaries with evidence of hemorrhage, and increased cellularity of the lamina propria; and 5, digestion and disintegration of the lamina propria in the villi with hemorrhage and ulceration. All assessments were conducted in a blinded fashion.

Apoptosis assessment

We performed terminal deoxynucleotidyl-transferase-mediated biotin-dUTP nick end labeling (TUNEL) histochemistry assay, using the DeadEnd colorimetric TUNEL system (Promega, Madison, WI, USA) as follows: After deparaffinization and rehydration, slides were immersed first in 0.85% NaCl for 5 min, then in phosphate-buffered saline (PBS) for 5 min, and then in 4% paraformaldehyde in PBS for 15 min. The next step was to immerse the slides twice in PBS for 5-min intervals. A solution of 100 μl of proteinase K 20 μg/ml was added, and the slides were incubated at room temperature for 10 min. Slides were then immersed in PBS for 5 min and then in 4% paraformaldehyde in PBS for 5 min. Following this step, the slides were immersed twice in PBS for 5-min intervals and a 100-μl equilibration buffer was added. They were then allowed to equilibrate at room temperature for 5 min, following which 100 μl of terminal deoxynucleotidyl transferase (TdT) reaction mix was added to the tissue sections of the slides, which were further incubated for 60 min at 37°C in a humidified chamber and immersed in 2 × standard saline citrate for 15 min. Slides were then immersed in PBS three times for 5 min, then in 0.3% hydrogen peroxide for 3 min, and then three times in PBS for 5 min again. Streptavidin horseradish peroxidase (100 μl) was added and the slides were incubated for 30 min at room temperature. Slides were then immersed three times in PBS for 5 min and 100 μl diaminobenzidine was added. Slides were developed until a light-brown background appeared, and were then immersed several times in deionized water before being mounted in a permanent mounting medium. Staining was then observed with a light microscope. The apoptotic cells showed cell shrinkage with condensed nuclei stained brown. For a negative control, slides were incubated with label solution not containing TdT. Ten villi and crypts of each section were observed under 200× magnification by bright-field microscopy. The average

number of apoptotic cells in 100 counted cells was assigned as the apoptotic index [18].

Statistical analysis

Results are expressed as mean ± standard deviation (SD), and the analysis of variance (ANOVA) was used to evaluate the results. Differences between two groups were analyzed using the Mann–Whitney's U test. Significance was set at $P < 0.05$. Statistical analyses were performed using StatView (version 5.0 for Windows, SAS Institute, Cary, NC, USA).

Results

Diarrhea

Diarrhea was not observed in any of the rats from the control group or the DKT group. Mild diarrhea was observed in two (25%) of the rats from the CPT-11 group.

Inflammatory cytokine in the ileum

The mean IL-1β/β-actin levels were 1.08 ± 0.27, 1.93 ± 0.73, and 1.35 ± 0.38 in the control, CPT-11, and DKT groups, respectively. CPT-11 significantly increased the level of IL-1β/β-actin versus that in the control group ($P < 0.05$). DKT significantly reverted the increased IL-1β level induced by the administration of CPT-11 ($P < 0.05$). A similar pattern was observed for the IFN-γ/β-actin level. The mean IFN-γ/β-actin levels were 0.85 ± 0.18, 1.16 ± 0.24, and 0.81 ± 0.13 in the control, CPT-11, and DKT groups, respectively (Fig. 2a).The mean IL-12/β-actin levels were 0.44 ± 0.21, 1.78 ± 0.79, and 1.39 ± 0.64 in the control, CPT-11, and DKT groups, respectively. The levels in the CPT-11 group were significantly higher than those in the control group ($P < 0.05$). DKT suppressed the increased level of IL-12/β-actin. A similar pattern was observed for the TNFα/β-actin level. The mean TNFα/β-actin levels were 0.15 ± 0.10, 0.75 ± 0.50, and 0.44 ± 0.24 in the control, CPT-11, and DKT groups, respectively (Fig. 2b).

Histological measurement of the number and height of villi

The architecture was destroyed and the number of villi was decreased in the CPT-11 group; however, this destruction was impaired in the DKT group (Fig. 3). The mean number of villi per cm in the small bowel was 92 ± 17, 68 ± 5, and 89 ± 10 in the control, CPT-11, and DKT groups, respectively. The number of villi in rats from the CPT-11 group was significantly lower than that in the control group ($P < 0.05$) or the DKT group ($P < 0.05$). A similar pattern was observed in the villous height. The mean small bowel villous height was 309 ± 56, 258 ± 42, and 292 ± 37 in the control, CPT-11, and DKT groups, respectively (Fig. 4a, b). DKT significantly reduced the histological alteration induced by CPT-11.

Injury score

The mean injury scores were 0.3 ± 0.6, 2.51 ± 1.1, and 0.6 ± 0.6 in the control, CPT-11, and DKT groups, respectively. CPT-11 alone caused a significant increase in the injury score. The injury score in the DKT group was significantly lower than that in the CPT-11 group (Fig. 5).

Apoptosis in intestinal mucosal epithelium and the apoptotic index

Apoptotic nuclei were increased significantly in the CPT-11 group (Fig. 6). The mean apoptotic index was 0.5 ± 0.2%, 30.4 ± 0.2%, and 3.0 ± 1.4% in the control, CPT-11, and DKT groups, respectively. CPT-11 alone caused a significant increase in the apoptotic nuclei. The apoptotic index in the DKT group was significantly lower than that in the CPT-11 group (Fig. 7).

Discussion

This study demonstrated that DKT significantly reduced the severe intestinal mucosal damage caused by treatment with CPT-11, maintaining the intestinal integrity and reducing inflammatory cytokines and apoptosis. The physiology and pharmacology of chemotherapy-induced mucositis is complex and likely to involve various mechanisms. The homeostatic balance between proliferation and apoptosis is essential for intestinal epithelium as a function of the gut barrier. Mucosal damage of the gastrointestinal tract is one of the main reasons for chemotherapy-induced disease. Anticancer agents induce epithelial apoptosis in the gastrointestinal tract. The mucosal damage reduces the water absorption capacity of the gastrointestinal tract, resulting in diarrhea [19–21].

Chemotherapy-induced diarrhea is a well-recognized side effect of cancer treatment. The major side effects of CPT-11, severe diarrhea and leukopenia, limit the dose able to be administered. Irinotecan causes severe diarrhea in approximately 60–80% of patients, with two distinct types recognized: an early secretory diarrhea that is cholinergic in nature and can be prevented by administering atropine prior to irinotecan; and a delayed diarrhea.

Cytokines secreted by the intestinal immune system are probably one of the key factors in maintaining the gut barrier. The balance between proinflammatory cytokines

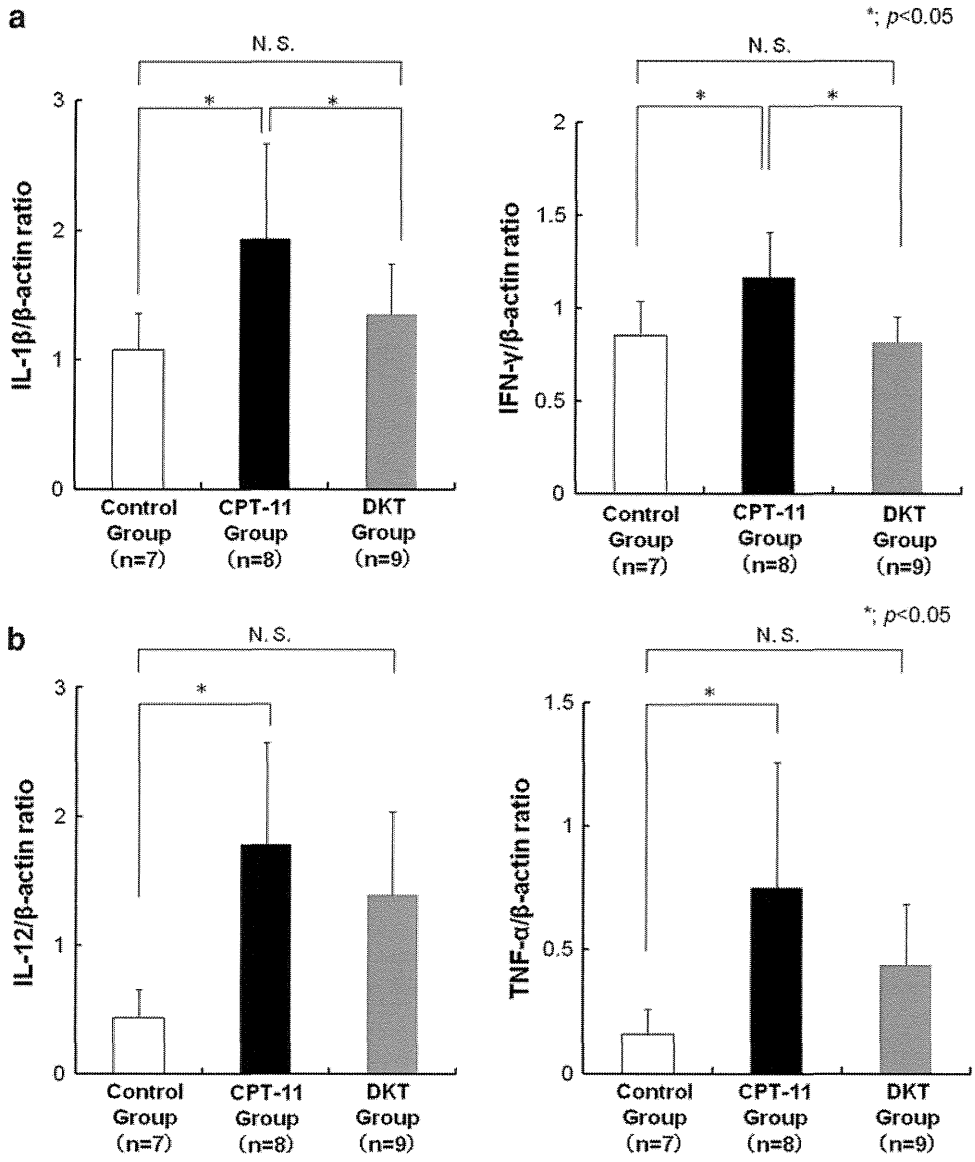

Fig. 2 Expression in real-time polymerase chain reaction on the small intestine. **a** The levels of interleukin-1β (*IL-1β*) and interferon-γ (*IFN-γ*) in the small intestine. CPT-11 significantly increased the level of IL-1β and IFN-γ versus that of the control group. Dai-kenchu-to significantly reverted the increase in the IL-1β level induced by CPT-11 ($^*P < 0.05$). **b** Levels of interleukin-12 (*IL-12*) and tumor necrosis factor-α (*TNF-α*) in the small intestine. The levels in the CPT-11 group were significantly higher than those of the control group. DKT suppressed the increased level in the CPT-11 group ($^*P < 0.05$). *N.S.* not significant

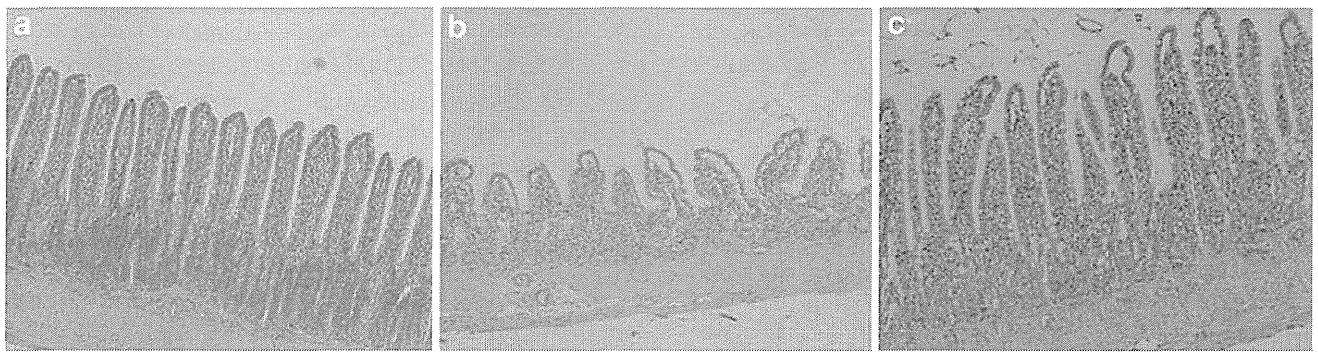

Fig. 3 Histological sections of the small intestine from the control group (**a**), the CPT-11 group (**b**), and the DKT group (**c**) The architecture was destroyed and the number of villi was decreased in the CPT-11 group. The destruction was less marked in the DKT group (H&E ×100)

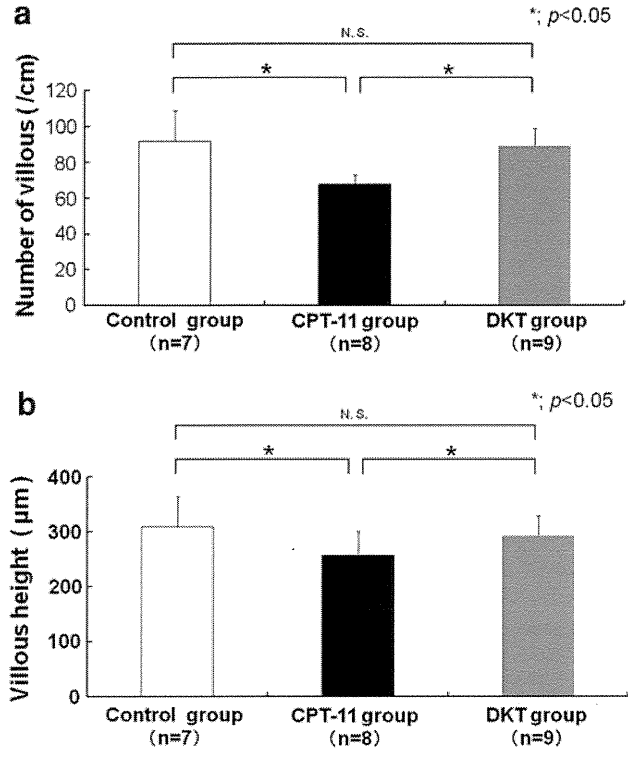

Fig. 4 Number and height of villi per centimeter in the ileum. The number (**a**) and height (**b**) of villi in the CPT-11 group were significantly lower than those in the control group. In the DKT group, the number and height of villi were significantly ameliorated versus the CPT-11 group (*$P < 0.05$). *N.S.* not significant

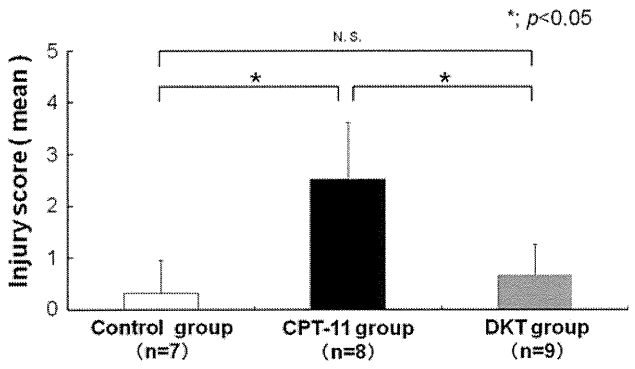

Fig. 5 Injury score. CPT-11 alone caused a significant increase in the injury score, which was significantly lower in the DKT group than in the CPT-11 group (*$P < 0.05$)

such as TNF-α, IL-1β, and IFN-γ, and regulatory cytokines such as IL-10 and transforming growth factor-β may ultimately determine whether an immune response is detrimental or innocuous to the gut [22–24].

Tumor necrosis factor-α is a critical cytokine that induces inflammatory responses by activating a wide range of cells, including neutrophils, macrophages, and NK cells. Activating these cells induces the production of inflammatory cytokine such as IL-1β, IL-6, and IL-8 [25]. Interleukin-1β plays a central role in mediating immune and inflammatory responses [26]. Along with TNF, IL-1β was reported to have a synergistic effect, for example by inducing endothelial adhesion molecules essential for the initial phases of the inflammatory response. Inappropriate or prolonged production of IL-1β has been implicated as playing a role in intestinal inflammatory damage. In this study, the increased intestinal tissue level of IL-1β caused by CPT-11 was reduced by DKT pretreatment. Interferon-γ can increase the production of IL-1β and, as a proinflammatory cytokine, sensitizes intestinal epithelial cells to physiological and therapeutic inducers of apoptosis [27]. In the present study, DKT suppressed significantly the expression of IFN-γ mRNA in the small intestine, which had been induced by CPT-11.

A few animal studies examined the histomorphological and histopathological changes in the jejunum and colon after treatment with varying doses of CPT-11. These studies found that CPT-11 causes severe intestinal damage with upregulation of the proinflammatory cytokines IL-1β, TNF-α, KC (the mouse ortholog of human IL-8), and IFN-γ, of intestinal tissues [22, 28] and tissue immunohistochemical staining [29]. Preventive and effective agents are needed to reduce CPT-11-induced intestinal injury and diarrhea, and many studies have been conducted in this regard.

Hange-shahsin-to, a Kampo medicine, alleviates diarrhea by decreasing colonic prostaglandin E2 (PGE2) significantly [20]. Palifermin prevents oral and intestinal mucosal barrier injury and reduces the mortality associated with intestinal mucositis [30]. Pentoxifylline inhibits the production of inflammatory cytokines (TNF-α, IL-1β, IL-8) [21]. The probiotic VSL#3 increases a crypt proliferation and inhibits apoptosis in both the small and large intestines [21, 24]. Glutamine induces heat shock protein and prevents upregulated activity of β-glucuronidase in the colon [31]. St. John's wort, *Hypericum perforatum*, inhibits the production of IL-1β, IL-6, TNF-α, and IFN-γ, thus diminishing crypt apoptosis [28].

DKT was prepared by mixing dried extract powder and malt sugar at a ratio of 1:8. Dried extract powder consists of ginger root, ginseng, and zanthoxylum fruit at a ratio of 3:2:5, respectively. DKT is being used increasingly to treat various gastrointestinal diseases [9, 10, 13, 32]. Recent studies show the mechanism by which DKT stimulates the gastrointestinal tract [33]. Acceleration of motility in the small intestine prevents endoluminal bacterial overgrowth. The intestinal blood flow is increased by one of the active components of DKT, 6-shogaol, in a dose-dependent manner [34]. DKT also increases the colonic blood flow by upregulation of the calcitonin gene-related peptide [35]. Studies at our institute found that DKT had an anti-inflammatory effect in a rat model subjected to fast stress,

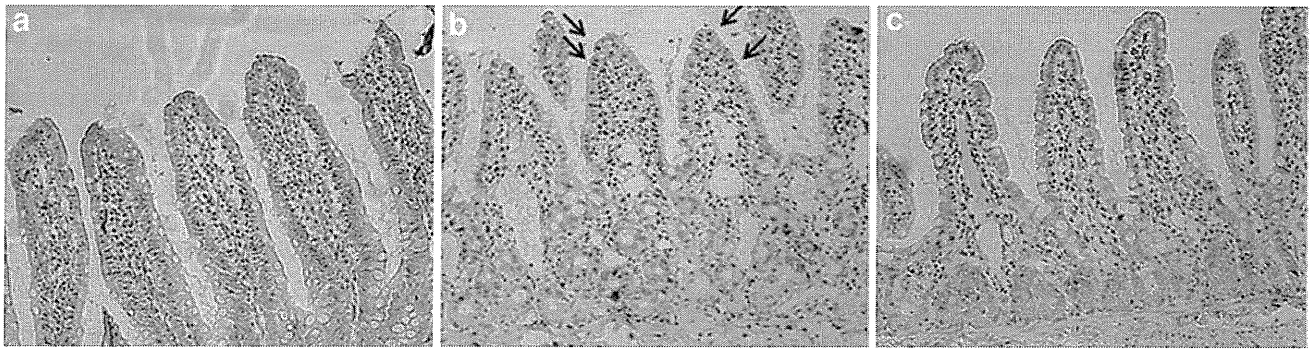

Fig. 6 Terminal deoxynucleotidyl-transferase-mediated biotin-dUTP nick end labeling (TUNEL) immunohistochemistry staining of the ileum from the control group (**a**), the CPT-11 group (**b**), and the DKT group (**c**). Apoptotic nuclei were increased significantly in the CPT-11 group (TUNEL staining ×200)

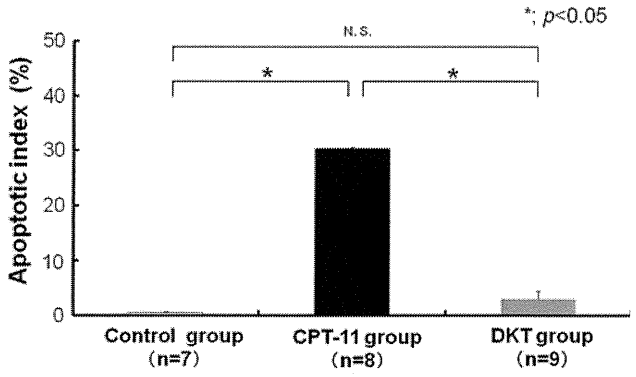

Fig. 7 Apoptotic index. CPT-11 alone caused a significant increase in apoptotic nuclei. The apoptotic index in the DKT group was significantly lower than that in the CPT-11 group (*$P < 0.05$). *N.S.* not significant

by inhibiting the production of inflammatory cytokines, including IFN-γ, IL-6, and TNF-α, and alleviated intestinal epithelial apoptosis significantly [11]. Although it has not yet been proved that DKT prevents the side effects induced by chemotherapy, it is inexpensive and has few side effects. In conclusion, we found that DKT reduced CPT-11 induced intestinal mucosal injury in rats by inhibiting the level of inflammatory cytokines including IFN-γ, TNF-α, IL-1β, and IL-12 and maintaining mucosal integrity.

Conflict of interest The authors have no conflicts of interest.

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REVIEW ARTICLE

Biological mechanism and clinical effect of protein-bound polysaccharide K (KRESTIN®): review of development and future perspectives

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Abstract The mechanism of action of protein-bound polysaccharide K (PSK; KRESTIN®) involves the following actions: (1) recovery from immunosuppression induced by humoral factors such as transforming growth factor (TGF)-β or as a result of surgery and chemotherapy; (2) activation of antitumor immune responses including maturation of dendritic cells, correction of Th1/Th2 imbalance, and promotion of interleukin-15 production by monocytes; and (3) enhancement of the antitumor effect of chemotherapy by induction of apoptosis and inhibition of metastasis through direct actions on tumor cells. The clinical effectiveness of PSK has been demonstrated for various cancers. In patients with gastric or colorectal cancer, combined use of PSK with postoperative adjuvant chemotherapy prolongs survival, and this effect has been confirmed in multiple meta-analyses. For small-cell lung carcinoma, PSK in conjunction with chemotherapy prolongs the remission period. In addition, PSK has been shown to be effective against various other cancers, reduce the adverse effects of chemotherapy, and improve quality of life. Future studies should examine the effects of PSK under different host immune conditions and tumor properties, elucidate the mechanism of action exhibited in each situation, and identify biomarkers.

Keywords PSK · Biological mechanism · Gastric cancer · Colorectal cancer · Biomarker

Introduction

Whether human immunity is effective against cancer, which originates from mutation of the host's normal cells, has long been a subject of skepticism. In 1991, van der Bruggen et al. [1] identified the tumor antigen in human melanoma that is recognized by cytotoxic T lymphocytes (CTLs) and proved at the molecular level that host immunity acts also against cancer. Thereafter, immunotherapy was developed mainly along the lines of vaccine therapy and cell therapy, with the aim of boosting specific immunity [2]. Research began to show that antigen-nonspecific innate immunity and antigen-specific acquired immunity are closely associated via the dendritic cells (DCs) that possess the important function of antigen presentation [3]. Also, antigen-presenting cells (APCs), including DCs, are known to be activated by recognizing various foreign pathogens via the Toll-like receptors (TLRs) [4]. These developments indicate that activation of nonspecific immunity plays certain roles in augmenting antitumor immunity. However, the antitumor effect of biological response modifiers (BRMs) is not necessarily potent [5]. For this reason, they have been used in combination with chemotherapy. The effect of chemotherapy is known to be affected by the performance status (PS) and nutritional and immune status of the patient [6–8]. Recently, Apetoh et al. [9] have presented basic research evidence that tumor cells damaged by chemotherapy release high mobility group box 1, which interacts with TLR4 to

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stimulate DCs and activates antitumor immunity, and these activities contribute to the success of chemotherapy. Clinically, these investigators also have reported earlier relapse after combined anthracycline-based chemotherapy and local radiotherapy in breast cancer patients with a functionally deficient TLR4 polymorphism, compared to breast cancer patients with normal TLR4. These findings show the importance of the host's immune capacity during chemotherapy, and suggest the importance of nonspecific immune activation in cancer patients whose immune functions are compromised.

Protein-bound polysaccharide K (PSK; KRESTIN®) is isolated and purified from the cultured mycelium of the Basidiomycete *Coriolus versicolor*, has an average molecular weight of approximately 100,000, and contains 18–38% protein. PSK exhibits antitumor activity against various experimental tumors, and nonspecific immunomodulatory activity is considered the principal mechanism of action of this agent [10]. PSK has been shown to regress tumors clinically, and was approved in 1976 for the treatment of cancers of the digestive organ, lung and breast. Clinical use in Japan was started in 1977. After reevaluation in 1989, PSK was approved for use in combination with chemotherapy to prolong survival of patients with gastric cancer (resected cases) or colorectal cancer (curatively resected cases), and to prolong remission of patients with small-cell lung carcinoma.

Along with advances in molecular biology and tumor immunology, many investigators have conducted research on the mechanisms of action of PSK and have built up substantial knowledge. Studies on clinical effects are still ongoing, mainly in accordance with the currently approved indications. The present article reviews the recent developments in research on the biological mechanisms of PSK and the major clinical results reported to date, to identify the challenges for the future.

Biological mechanism of PSK

Although the importance of BRMs in cancer treatment has been proven, better understanding of their mechanisms of action is essential for optimal application of these compounds. For PSK, three main mechanisms have been revealed (Fig. 1) [11]. First, PSK improves host immunocompetence by inhibiting the production of or neutralizing immunosuppressive substances that are increased in cancer. Second, PSK activates immune cells such as lymphocytes, either directly or by regulating the production of various cytokines. Third, PSK acts directly on cancer cells. These mechanisms are considered to support the clinical effectiveness of PSK in suppressing cancer relapse.

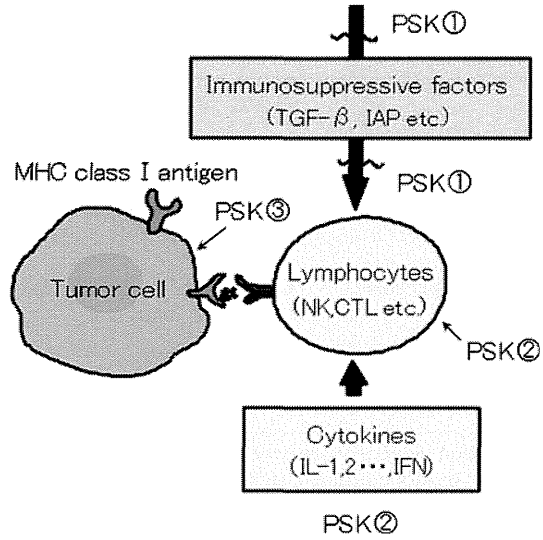

Fig. 1 Tumor microenvironment and actions of PSK. ① Suppressed production or neutralization of immunosuppressive factors. ② Activation of immune cells and regulation of cytokine production. ③ Direct action on tumor cells [induction of apoptosis, enhanced expression of major histocompatibility complex (MHC) class I antigen]. Adapted from Fujii [11]

Suppressed production or neutralization of immunosuppressive factors (Table 1)

It is well known that various humoral factors induce immunosuppression in cancer-bearing individuals. PSK restores or attenuates this suppression, as demonstrated by a larger number of reports showing abrogation of the immunosuppressive effects of serum obtained from cancer-bearing animals or cancer patients, inhibition of transforming growth factor (TGF)-β production or antagonism against TGF-β, and lowering of serum level of immunosuppressive acidic protein (IAP) and prostaglandin E_2 (PGE_2) production [12–17]. In addition, PSK has been reported to possess antioxidant activity [18]. No other drug exhibits such diverse actions as a single agent, and this is the characteristic of PSK.

In an in vitro study using peripheral blood mononuclear cells (PBMCs) from healthy volunteers, Yamaguchi et al. [19] have reported that PSK enhances interleukin-2 (IL-2)-induced proliferation of lymphokine-activated killer (LAK) cells and their cytotoxic activity, and abolishes the TGF-β-induced inhibition of LAK activity. They speculated that PSK acted on TGF-β receptors to block the association between TGF-β and its receptors. Thus, PSK seems to inhibit the effects of TGF-β through various mechanisms including suppression of TGF-β production, direct binding with TGF-β, and acting on TGF-β receptors.

IL-10 shows various immunosuppressive activities, and elevated serum levels of IL-10 have been reported as a negative prognostic factor. Shibata et al. [20, 21] have

Table 1 Suppressed production or neutralization of immunosuppressive factors

Immunosuppressive factors	Subjects/animals/cells	Key observations	References
Cytokine	PBMCs of healthy volunteers	Abrogation of inhibition of LAK activities by TGF-β	Yamaguchi [19]
	PBMCs of colorectal cancer patients	Suppression of IL-10 production	Shibata [20, 21]
Cancer cell	$CD8^+$ T cells in peripheral blood of healthy volunteers	Recovery of NKG2D expression downregulated by tumor cells	Tsujitani [22]
Tumor culture supernatant	$CD14^+$ cells in peripheral blood of healthy volunteers	Prevention of defective maturation of DCs by tumor culture supernatant	Okuzawa [23]
Surgical stress	Mice	Correction of Th1/Th2 imbalance	Ooshiro [25]
Chemotherapy	Gastric cancer patients	Partial prevention of S1-induced peripheral blood T-cell apoptosis	Kono [27]

PBMC peripheral blood mononuclear cell, *LAK* lymphokine-activated killer, *TGF-β* transforming growth factor-β, *NKG2D* natural killer (NK) group 2D, *DC* dendritic cell, *S-1* tegafur–gimeracil–oteracil potassium

studied the effect of PSK on IL-10. They found that IL-10 production was suppressed when PSK was added in vitro to phytohemagglutinin (PHA)-stimulated PBMCs from patients with advanced colorectal cancer, and that IL-10 production by PHA-stimulated PBMCs from advanced colorectal cancer patients was reduced after 2 months of immunochemotherapy with PSK, compared to that before treatment.

Tsujitani et al. [22] have examined the effect of PSK on the expression of natural killer group 2D (NKG2D), a receptor that activates $CD8^+$ T cells and natural killer (NK) cells. When PBMCs from healthy volunteers were co-cultured with the human gastric cancer cell line MKN-45, NKG2D expression on $CD8^+$ T cells was downregulated, while the addition of PSK restored NKG2D expression on $CD8^+$ T cells. The authors suggested that direct contact between $CD8^+$ T cells and gastric cancer cells was necessary for the downregulation of NKG2D expression. These findings raise interest on how PSK affects the interaction between tumor cells and T cells.

The suppressive effect of tumor-derived factors on DCs and the ability of PSK to overcome this effect have been studied. Okuzawa et al. [23] have studied the in vitro differentiation of peripheral blood $CD14^+$ cells into DCs and reported that the addition of the culture supernatant of human gastric cancer cells MKN-45P downregulated the surface markers of DC maturation, decreased IL-12 production, and increased apoptosis of DC. Addition of PSK restored all these changes to the levels observed in the absence of the culture supernatant. These findings suggest that, by reversing the defective DC differentiation and function, PSK is able to augment the subsequent immune response.

It is well known that surgical stress lowers the immune response. PSK has been reported to attenuate the immunosuppression due to surgical invasion [24]. Using a mouse laparotomy model, Ooshiro et al. [25] have found that concanavalin-A-stimulated interferon (IFN)-γ (Th1 cytokine) and IL-4 (Th2 cytokine) production by spleen cells was decreased, and the decrease in IFN-γ was especially marked, which resulted in a low IFN-γ/IL-4 ratio. In mice treated with PSK before laparotomy, the decreases in IFN-γ and IL-4 production were ameliorated, the recovery of IFN-γ production was especially marked, and the IFN-γ/IL-4 ratio was restored. The resultant Th1/Th2 balance shifted to Th1 dominance. The ability of PSK to correct the Th1/Th2 imbalance, to be described later, could also work during the perioperative period.

Many basic studies on the effects of the combined use of PSK with chemotherapy have been reported. While chemotherapy induces leukopenia and depresses immunity, PSK inhibits these adverse effects [26]. The ability of PSK to prevent chemotherapy-depressed immunity is one of the mechanisms that accounts for the benefit of PSK in combined therapy. Kono et al. [27] have examined apoptosis of peripheral blood T cells in gastric cancer patients treated with oral fluoropyrimidine agent tegafur–gimeracil–oteracil potassium (S-1; TS-1®), alone or in combination with PSK. While an increase in T-cell apoptosis concomitant with elevation of caspase-3 and Bax expression was observed in patients treated with S-1 alone, these increases were partially prevented in patients treated with S-1 and PSK (Fig. 2). The mechanism has been speculated as ensuing from the antioxidant activity of PSK, which counteracts the increase in reactive oxygen species resulting from chemotherapy.

Regulation of immune cells and cytokine production (Table 2)

Antitumor immune responses involve complex interactions among various immune cells and cytokines (Fig. 3). PSK exerts various effects on the immune cells, and its effects on lymphocytes, helper T cells, CTLs, NK cells, LAK

cells, and macrophages have been reported in numerous publications [28–36]. In addition, the effects of PSK on the production of various cytokines and nitric oxide (NO) have been reported [37, 38].

García-Lora et al. [39, 40] have used the human NKL cell line to study the intracellular signal transduction pathway involved in the activation of NK cells by PSK. PSK activates protein kinase C (PKC)δ, PKCε, and extracellular signal-regulated kinase (ERK)2 and ERK3, and increases DNA binding of activator protein-1 (AP-1) and cAMP response element (CRE) transcriptional factor. Asai et al. [41] have shown that the addition of PSK in the stimulation of mesenteric lymph node $CD4^+$ T cells with anti-CD3 antibody increased the production of IL-2, IFN-γ, and IL-4. They reported that the signaling pathway involved the activation of linker for activation of T cells (LAT), ERK1/2, nuclear factor of activated T cells, and AP-1. Although the actions of PSK on immune cells differ slightly depending on the cell type, these actions have started to unfold at the level of the signal transduction pathway. Further accumulation of data using different immune cells under various conditions might provide a comprehensive picture of the action of PSK.

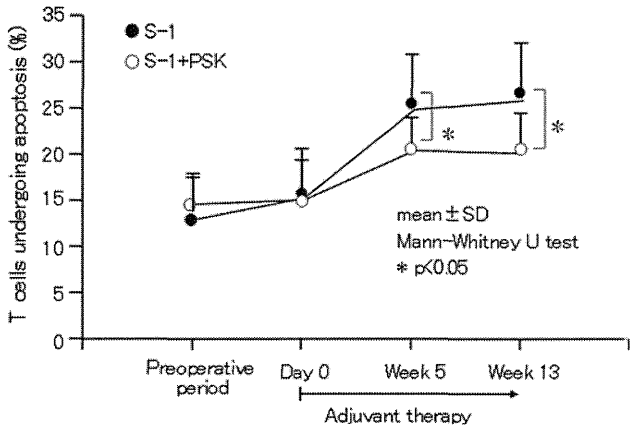

Fig. 2 Effect of oral administration of PSK on apoptosis of circulating T cells induced by the anticancer drug S-1 in gastric cancer patients. Adapted and modified from Kono et al. [27]

As mentioned earlier, DCs play an important role in the immune response. In the peripheral blood, DCs exist in an immature state. Upon maturation by the actions of inflammatory cytokines, they activate T cells and induce acquired immunity. Kanazawa et al. [42] have reported that the addition of PSK to cultures of peripheral blood $CD14^+$ mononuclear cells from healthy adults and advanced cancer patients with IL-4 and granulocyte-macrophage colony stimulating factor for DC differentiation potentiates the expression of maturation surface markers, such as CD83, production of IL-12, and induction of CTLs. Using a similar in vitro system, Ogihara et al. [43] have reported similar results with the addition of PSK.

Table 2 Regulation of immune cells and cytokine production

Immune cells	Subjects/animals/cells	Key observations	References
NK cells	Human NK cell line	Activation of PKCδ, PKCε, ERK2, ERK3, AP-1 and CRE transcriptional factor	García-Lora [39, 40]
T cells	$CD4^+$ T cells in murine mesentric lymph node	Activation of LAT, ERK1/2, NFAT and AP-1	Asai [41]
DCs	$CD14^+$ mononuclear cells in PBMC of healthy volunteers	Promotion of maturation of DCs	Kanazawa [42], Ogihara [43]
Th1/Th2	PBMC of human healthy volunteers	Increase of TNF-α production and decrease of IL-10 production by stimulation of human gastric cancer cell lysate	Sugiyama [45]
	Colorectal cancer patients	Decrease of proportion of IL-10^+ $CD4^+$ T cells (Th2) in peripheral blood	Yoshino [46]
B cells	Human B cell line	Enhancement of IgM production	Maruyama [49]
Monocytes	Human cord blood mononuclear cells	Activation of T cells and induction of CTLs through increase of IL-15 and LTB_4 production by monocytes in EBV-infected cultures	Liu [50–52], Klein [53]
NKT cells	Gastric cancer patients	Decrease of $CD57^+$ T cells in peripheral blood	Akagi [54]
Others	Murine peritoneal macrophages, etc.	Inhibition of binding of LPS to LBP, protection of LPS-induced lethality	Asai [55]
	Mouse model of DSS and DMH-induced inflammatory bowel disease and colorectal carcinogenesis	Antagonistic effects on inflammation and carcinogenesis	Tsutsumi [57]

NK natural killer, *PKC* protein kinase C, *ERK* extracellular signaling regulated kinase, *AP-1* activator protein-1, *CRE* cAMP-response element, *LAT* linker for activation of T cells, *NFAT* nuclear factor of activated T cells, *DC* dendritic cell, *PBMC* peripheral blood mononuclear cell, *TNF-α* tumor necrosis factor-α, *CTL* cytotoxic T lymphocyte, *LTB_4* leukotriene B_4, *EBV* Epstein–Barr virus, *LPS* lipopolysaccharide, *LBP* LPS-binding protein, *DSS* dextran sodium sulfate, *DMH* 1,2-dimethylhydrazine

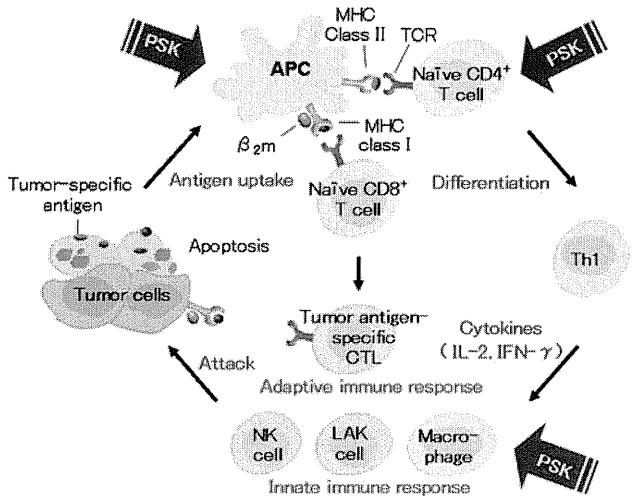

Fig. 3 Schematic representation of the antitumor immune responses and actions of PSK. Tumor cells or their fragments are taken up by APCs. Antigen peptides bind to MHC class I or II on the APCs. The antigen peptides on MHC class I molecules activate naïve $CD8^+$ T cell, whereas the antigen peptides on MHC class II molecules activate naïve $CD4^+$ T cells (T-helper cell precursors). T-helper cell precursors differentiate into Th1 and Th2 cells. Th1 cells produce cytokines, including IL-2 and IFN-γ. These cytokines induce proliferation and activation of tumor antigen-specific CTLs, activation of NK cells and macrophages, and induction of LAK cells

Through the actions of the APCs, T cells are activated and naïve $CD4^+$ T cells differentiate into Th1 or Th2 cells. In a cancer-bearing state, the Th1/Th2 balance shifts to Th2 dominance; therefore, cellular immunity, which is the main component of antitumor immunity, does not function efficiently [44]. Sugiyama et al. [45] have examined cytokine production of PBMCs from healthy adults stimulated with tumor antigen obtained by freezing and thawing a gastric cancer cell line after 1 week. Addition of PSK did not change IFN-γ production, but increased tumor necrosis factor-α (TNF-α) (Th1 cytokine) production and decreased IL-10 (Th2 cytokine) production. Yoshino et al. [46] treated colorectal cancer patients with PSK for 1 week before surgery and examined the peripheral blood Th1/Th2 balance by flow cytometry before and after treatment. After PSK treatment, although the proportion of IFN-γ-producing $CD4^+$ T cells (Th1) did not change, the proportion of IL-10-producing $CD4^+$ T cells (Th2) decreased. These studies suggested that PSK appeared to act mainly to suppress Th2, and PSK shifted the Th1/Th2 balance to Th1 dominance to augment antitumor immunity.

Various monoclonal antibodies have been developed for cancer treatment. PSK has been reported to augment antibody-dependent cell cytotoxicity [47]. The effect of PSK on antibody production is considered to act via helper T cells [48]. Maruyama et al. [49] have reported that PSK directly enhances the proliferation and IgM production of the human B cell line BALL-1, which suggests that PSK also acts directly on the humoral immunity pathway to potentiate acquired immunity.

Most of the studies on the effects of PSK on immune cells or cytokine production have been conducted by targeting individual immune cells or cytokines. In vivo, however, the immune response is a cascade reaction that involves many immune cells and cytokines. Liu et al. [50–52] have investigated the effect of PSK on the immune response against Epstein–Barr virus (EBV) infection, using human cord blood mononuclear cells (CBMCs) that contain various types of immune cells. The addition of PSK to EBV-infected CBMCs increases expression of signaling lymphocytic-activation molecule-associated protein (SAP), which is an indicator of T- and NK cell activation. PSK also induces EBV-specific $CD4^+$ CTLs, and inhibits proliferation of the potentially malignant EBV-infected B cells. Liu et al. have further demonstrated that the presence of monocytes and monocyte-derived IL-15 and leukotriene B_4 (LTB_4) is necessary for the upregulation of SAP expression by PSK. Klein et al. [53]. have reported that the interaction between T cells activated by EBV-infected B cells and monocytes activated by PSK occurs via CD40 ligand and CD40, and that these cells together with NK cells activated by EBV-infected B cells progress to a functionally activated state, which produces IL-15, IFN-γ, and LTB_4 to induce $CD4^+$ CTLs. IL-15 has been attracting the most attention as an antitumor agent with potential for clinical application (NCI Immunotherapy Agent Workshop, 2007), and studies have demonstrated that PSK induces IL-15.

Akagi et al. [54] have examined the effect of postoperative immunochemotherapy with PSK on immune cells in peripheral blood of gastric cancer patients at 3 months after surgery, and have reported that administration of PSK decreases the proportion of $CD57^+$ T cells that mediate suppressor T-cell functions.

On a slightly different note, Asai et al. [55] have examined the NF-κB activity (indicated by luciferase activity) when the pro-B cell line Ba/F3 transfected with TLR-4 and MD-2 was stimulated with LPS. They have reported that addition of PSK inhibited nuclear factor (NF)-κB activity, which was caused by inhibition of binding between LPS and LPS-binding protein (LBP) by PSK. Administration of PSK also protected mice from LPS-induced lethality, which indicates that PSK is useful for controlling sepsis, which is also interesting from a clinical viewpoint.

Association between chronic inflammation and cancer development has been well described, and various factors released from inflammatory cells have been reported to exacerbate cancer [56]. Using a dextran sodium sulfate (DSS) and 1,2-dimethylhydrazine-induced mouse model of inflammatory bowel disease and colorectal carcinogenesis,

Tsutsumi et al. [57] have shown that PSK administration reduced DSS-induced colitis-related mortality and inhibited colorectal carcinogenesis, which indicates that PSK possesses dual antagonistic effects against inflammation and carcinogenesis. This study offers a new perspective on the mechanism of the action of PSK.

Direct action on tumor cells (Table 3)

It has been demonstrated that PSK directly inhibits the proliferation of cancer cells and induces apoptosis [24, 58, 59]. Also, PSK has been reported to upregulate the expression of human leukocyte antigen (HLA) on human gastric cancer cells [60]. Hattori et al. [61] have examined the effects of PSK on 33 hematological malignant cell lines and have reported that PSK strongly inhibits the proliferation of Namalwa (Burkitt lymphoma) cells and increases apoptosis. It has been shown that inhibition of cell proliferation by PSK is antagonized by the addition of galactose. When Namalwa cells are treated with galactosidase, the effect of PSK is enhanced, which suggests that the galactose-containing structure of the Namalwa cell surface plays an important role in the action of PSK. Jiménez-Medina et al. [62] also have examined the effects of PSK on seven human and mouse cancer cell lines. The inhibitory effect of PSK on cell proliferation differs depending on the cell line. For the AGS (human gastric cancer cell line) cells in which a strong inhibitory effect is observed, arrest of the cell cycle at G1, and increased apoptosis and caspase-3 expression have been observed. From the above results, it is apparent that PSK induces apoptosis of cancer cells, but the effect seems to differ depending on the type of cancer. Further studies should examine the properties of cells in which apoptosis is induced by PSK.

PSK is well known to exhibit anti-metastatic activity in various experimental metastasis models, and the mechanism of action has been reported for each step of metastasis [63]. Two new and interesting reports on the mechanism of the anti-metastatic action of PSK, from the viewpoint of TGF-β suppression, have been published. Zhang et al. [64] have reported that PSK decreases the invasiveness of the human pancreatic cancer cell line, NOR-P1, and the gastric cancer cell line, MK-1P3, by multiple mechanisms that include direct suppression of matrix metalloproteinase (MMP) production, inhibition of the activation of latent TGF-β, and indirect suppression of MMP production through inhibition of TGF-β production. In recent years, the association of epithelial–mesenchymal transition (EMT) with invasion and metastasis of cancer cells has been speculated, and TGF-β has been shown to play an important role in the EMT process [65]. Hayashida et al. [66] have treated SW837 cells (human colorectal cancer cell line with normal TGF-β receptor and signal transduction) with TGF-β followed by PSK, and have found that nuclear translocation of the TGF-β signal transducer Sma- and Mad-related protein 2 (Smad2) was inhibited, and that the expression of genes including the TGF-β target gene SERPINE-1 was also inhibited. In addition, they performed

Table 3 Direct actions on tumor cells

Targets of PSK action	Animals/cells	Key observations	References
Apoptosis	Burkitt lymphoma	Induction of apoptosis	Hattori [61]
	Human gastric cancer cell line	Induction of G_1 arrest and apoptosis	Jiménez-Medina [62]
Metastasis	Human pancreas or gastric cancer cell line	Direct or indirect inhibition of MMP production through TGF-β production and suppression of latent TGF-β activation	Zhang [64]
	Human colon cancer cell line	Suppression of nuclear localization of Smad2; decreased expression of TGF-β-targeted gene; inhibition of TGF-β-induced EMT	Hayashida [66]
Neoangiogenesis	HUVEC, rat	Inhibition of bFGF-induced cell proliferation through direct binding to bFGF	Wada [68]
Gene expression	Human colorectal cancer cell line	Upregulation of expression of MRP3, lymphotactin, transgelin and pirin	Yoshikawa [70]
Combination with chemotherapy	Human pancreas cancer cell line	Inhibition of docetaxel-induced NF-κB activation and cIAP-1 expression, and enhancement of apoptosis induced by docetaxel	Zhang [71]
	Human gastric cancer cell line	Inhibition of docetaxel-induced NF-κB activation and survivin expression	Kinoshita [72]

MMP matrix metalloproteinase, *TGF-β* transforming growth factor-β, *EMT* epithelial–mesenchymal transition, *HUVEC* human umbilical vein endothelial cells, *bFGF* basic fibroblast growth factor, *MRP3* multidrug resistance protein 3, *NFκB* nuclear factor-κB, *cIAP-1* cellular inhibitor of apoptosis protein

an EMT assay using TGF-β as the inducer and reported that PSK inhibited EMT.

PSK is known to inhibit angiogenesis [67] and new results on the mechanism have been reported. Wada et al. [68] have shown that PSK administration inhibits basic fibroblast growth factor (bFGF)-induced angiogenesis in rats, and from studies on human umbilical vein endothelial cells, they have speculated that the mechanism is due to direct binding of PSK with bFGF, which results in suppression of bFGF-induced proliferation of endothelial cells. The effect of PSK on vascular endothelial growth factor-induced angiogenesis [69] should also be examined.

Comprehensive gene expression analysis of PSK-treated cancer cells has been attempted. Yoshikawa et al. [70] have used cDNA microarrays to analyze the in vitro effect of PSK on the gene expression profile of the human colorectal adenocarcinoma cell line HCT116, the proliferation of which is inhibited by PSK. After PSK treatment, approximately 450 genes showed altered expression. The expression of genes that were significantly altered was examined further in two cell lines, HCT116 and SW480, using RT-PCR. Multidrug resistance protein 3, lymphotactin, transgelin, and pirin were upregulated in both cell lines. Studies on the relationship between PSK-induced altered gene expression and the biological response are anticipated.

Even when used in conjunction with chemotherapy, PSK directly acts on cancer cells to enhance the effect of combined therapy. Zhang et al. [71] have studied the effect of PSK and low-dose docetaxel on proliferation of the human pancreatic cancer cell line NOR-P1, and have reported the mechanisms as follows. Docetaxel induces apoptosis, but at the same time it also activates NF-κB and induces cellular inhibitor of apoptosis protein (cIAP)-1 expression, to suppress the induction of apoptosis. PSK inhibits the docetaxel-induced NF-κB activation to enhance induction of apoptosis. Kinoshita et al. [72] also have reported that PSK inhibits docetaxel-induced NF-κB activation and survivin expression in the human gastric cancer cell line TMK-1, and enhances antitumor effect of docetaxel through inhibition of survivin expression in tumor tissue in a xenograft model.

Clinical effect of PSK

Clinical trials of PSK were started from around 1972, and the early trials demonstrated the effectiveness of PSK as monotherapy or in combination with chemotherapy, which led to the approval of PSK for clinical use in Japan. In 1980, the criteria for the evaluation of immunotherapy for malignant tumors were published. According to these criteria, immunotherapy should be used in combination with other therapies such as chemotherapy, and the evaluation of its effect should include statistically estimating the overall survival (OS) or disease-free survival (DFS), based on randomized control trials (RCTs) using the base therapy as a control [73]. These criteria apply also to PSK. RCTs were planned and conducted, and submitted for reevaluation, which resulted in reapproval for current indications. In addition, the efficacy of PSK has been verified in multiple meta-analyses.

Gastric cancer (Tables 4, 6)

Anticancer chemotherapy can be classified into two types: systemic chemotherapy for unresectable metastatic or recurrent cancer, and adjuvant chemotherapy for suppressing postoperative relapse. Almost all the clinical results of PSK belong to the latter type. As a study of the former type, Nakao et al. [74] have conducted a clinical trial to determine the efficacy of PSK in 54 patients with unresectable or postoperative recurrent gastric cancer. Patients were treated with intravenous mitomycin C (MMC) and 5-fluorouracil (5-FU) twice weekly for 2 weeks, followed by intravenous MMC/5-FU once weekly plus oral PSK 3 g/day on consecutive days (chemotherapy + PSK group), or MMC/5-FU alone (chemotherapy group). They reported that, although the response rates were not significantly different between the two groups, the survival duration was significantly ($P = 0.03$) prolonged in the chemotherapy + PSK group. Although unresectable, metastatic, and recurrent gastric cancers are not included in the approved indications for PSK at present, this study does suggest a direction for the potential use of PSK.

The results of postoperative adjuvant chemotherapy for gastric cancer have been reviewed by Maehara et al. [75]. In Japan, many controlled trials of postoperative adjuvant chemotherapy compared with surgery alone were conducted from the 1960s, but few studies have reported significant results in favor of postoperative adjuvant chemotherapy. Kondo et al. [76] have conducted a double-blind trial on 144 stage III surgical patients randomized to receive oral PSK 1–3 g/day from 10–15 days after surgery until relapse or distant metastasis (PSK group), or placebo (control group). They reported that PSK treatment significantly prolonged DFS. Their results indicate that PSK, even when used alone, is effective in some patients, although PSK monotherapy is not an approved usage at present. In the study of Maehara et al. [77], 225 patients who underwent histologically curative resection of advanced gastric cancer were treated postoperatively with intravenous MMC once every 3 months for 1 year, together with oral tegafur and PSK from 7 to 10 days after surgery, for long-term (postoperative long-term cancer chemotherapy group; PLCC), or surgery alone. The long-term (15-year) survival rate was 56.9% in the PLCC group

Table 4 RCTs of postoperative adjuvant chemotherapy with PSK for resected gastric cancer

References or trial	Stage	No. of patients	Treatment	Percent survival/years	*P* value	Suggestive data (percent survival/years)
Kondo [76]	III	72	A: PSK	Improvement of DFS	Significant	
		72	B: Placebo			
Maehara [77]	Advanced stage	137	A: MMC + FT + PSK	56.9/15	0.0351	
		118	B: Surgery alone	45.7/15		
Niimoto [79]	[a]	191	A: MMC+FT+PSK	71.7/5	3 groups	
SACG		189	B: MMC+PSK	64.1/5	<0.05	
Chugoku/ Kyushu		199	C: MMC+FT	58.5/5	A versus C <0.01	
Kondo [80]	[a]	145	A: FT+PSK	64.1/8	0.410	A: 86 cases[b], 56.8/8
SACG		159	B: FT	61.0/8		B: 90 cases[b], 43.6/8
Chubu						$P = 0.071$
Kondo [82]	Advanced stage	47	A: CQ+PSK	Not described	0.827	A: 30 cases[c], 43.4/7
TGOG		49	B: CQ			B: 32 cases[c], 32.3/7
						$P = 0.153$
Nakazato [83]	T2, T3	124	A: MMC+5-FU+PSK	73.0/5	0.044	
SIP		129	B: MMC+5-FU	60.0/5		
Hattori [13]	II–III	1,426	A: MMC+FT+PSK	71.6/3	NS	
JFMC01		1,357	B: MMC+FT	69.6/3		
		1,338	C: MMC+FT+OK-432+PSK	69.1/3		
		1,363	D: MMC+FT+OK-432	68.7/3		
Ogawa [84]	I-IV	56	A: MMC+HCFU+PSK	80.2/5	0.811	
		55	B: MMC+HCFU	81.1/5		
JFMC05	T3, T4	215	A: FT+PSK	52.8/5	NS	
		219	B: FT+OK-432	49.3/5		
		217	C: FT	47.0/5		
JFMC11	T1, T2	114	A: CPA+PSK	84.8/5	NS	
		112	B: surgery alone	83.3/5		

DFS disease-free survival, *MMC* mitomycin C, *FT* tegafur, *CQ* carboquone, *T* tumor, *5-FU* 5-fluorouracil, *NS* not significant, *OK-432* picibanil, *HCFU* carmofur, *CPA* cyclophosphamide

[a] Excluding mucosal cancer having no lymph node metastasis

[b] Tumors with serosal invasion

[c] Tumors with serosal invasion with limited lymph node metastasis

and 45.7% in the surgery group, which showed a significant ($P = 0.0351$) survival benefit in the PLCC group (Fig. 4).

Since a subset analysis of multiple clinical trials has shown the effectiveness of postoperative adjuvant chemotherapy compared with surgery alone, studies from the 1980s have compared efficacy between different adjuvant chemotherapies. The effectiveness of PSK as an adjuvant therapy for gastric cancer has been evaluated mainly during this period, by comparing chemotherapy + PSK with chemotherapy alone. To examine the effectiveness of immunostimulators, the Co-operative Study Group of Surgical Adjuvant Chemotherapy for Gastric Cancer (SACG) divided Japan into six blocks in 1978, and each block conducted multicenter RCTs independently in patients with resected gastric cancer, excluding the patients with mucosal cancer having no lymph node metastasis, and compared immunochemotherapy with chemotherapy [78]. In the Chugoku/Kyushu block, 579 patients who underwent curative surgery were given intravenous MMC on the day of and 1 day after surgery, followed 2 weeks later by oral PSK 3 g/day plus tegafur (chemotherapy + PSK group), or PSK alone (PSK group) or tegafur alone (chemotherapy group) for 1 year. Niimoto et al. [79] have reported that the 5-year OS rate in the three groups was 71.7, 64.1, and 58.5%, respectively, and a significant difference ($P < 0.05$) was detected in a three-group comparison. In two-group comparisons, the survival rate was significantly ($P < 0.01$) higher in the chemotherapy + PSK group than in the