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ORIGINAL ARTICLE

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Potassium oxonate, an enzyme inhibitor compounded in S-1, reduces the suppression of antitumor immunity induced by 5-fluorouracil

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Abstract S-1 is an oral formulation combining tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate (Oxo) in a molar ratio of 1:0.4:1. We examined whether Oxo reduces the immunosuppression induced by 5-fluorouracil (5-FU) in the rat. The body weight of rats treated with S-1 (FT + CDHP + Oxo) for seven consecutive days was significantly higher than that of rats treated with a combination of FT plus CDHP (FT + CDHP) for a similar period. The number of peripheral leukocytes was significantly higher in the S-1-treated rats (S-1 group) than that in the FT + CDHP-treated rats (FT + CDHP group). There was no apparent difference between the two treated groups in phenotypic changes of CD3-, CD45-, CD4-, or CD8-positive cells from the spleen or mesenteric lymph nodes. However, the natural killer activities of both spleen cells and mesenteric lymph node cells were significantly higher in the S-1 group than in the FT + CDHP group. Interleukin (IL)-2 production by spleen cells stimulated with concanavalin A was significantly lower in the FT + CDHP group than in the S-1 group. Although IL-2 production by mesenteric lymph node cells in the S-1 group was lower than that in untreated rats, it was higher than that in the FT + CDHP group. These findings suggest that Oxo in S-1 may reduce the suppression of antitumor immunity induced by 5-FU.

Keywords S-1 · 5-fluorouracil · Potassium oxonate · Gastric cancer · Immunosuppression

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Introduction

5-Fluorouracil (5-FU) is widely used to treat solid tumors [1, 2]. S-1 (TS-1®, Taiho Pharmaceutical Co., Ltd., Tokyo, Japan), developed by Shirasaka et al. [3, 4], is a novel oral formulation combining tegafur (FT), a prodrug of 5-FU, with two modulators, 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate (Oxo), in a molar ratio of 1:0.4:1. FT acts as an effector, while both CDHP and Oxo, which lack antitumor activity, act as modulators. Approximately 90% of the administered dose of 5-FU is metabolized by dihydropyrimidine dehydrogenase (DPD) before exerting antitumor activity [5]. Inhibition of DPD activity is therefore likely to potentiate the antitumor activity of 5-FU in vivo. The DPD-inhibitory activity of CDHP is 180-fold higher than that of uracil, which has been confirmed to be an effective DPD-inhibitor in the form of uracil/tegafur (UFT®, Taiho Pharmaceutical Co., Ltd., Tokyo, Japan) in vitro [6]. Accordingly, CDHP leads to the prolonged high concentrations of 5-FU in plasma. We previously reported complete regression of disease after treatment with S-1 in a patient with 5′-deoxy-5-fluorouridine-resistant lung metastasis from gastric cancer. The good response was attributed to the prolonged high concentration of 5-FU in plasma after treatment with S-1 [7]. Oxo, which competitively inhibits orotate phosphoribosyltransferase, decreases the levels of 5-fluorouridine-5′-monophosphate (FUMP) and 5-FU incorporated into RNA by approximately 70% in the small intestine, as compared with only 0–20% in bone marrow and tumors [8]. Consequently, after oral administration high levels of Oxo are found in the digestive tract, where Oxo can

reduce the gastrointestinal toxicity of 5-FU, without negatively affecting its antitumor activity [8, 9].

In a murine model, S-1 had higher antitumor activity than UFT at equitoxic doses [10]. Clinically, a multicenter phase II trial in Japan showed that the overall response to S-1 monotherapy in advanced gastric cancer was 49% (25/51), with a 20% incidence of grades 3 and 4 adverse reactions [11]. These results have led to S-1 being recommended in Japan as standard chemotherapy for patients with advanced or recurrent gastric cancer [12–14].

Most anticancer drugs induce immunosuppression as a side effect [15]. However, compromised immune status can negatively affect response to chemotherapy as well as outcome. This experimental study evaluated whether Oxo can decrease 5-FU-induced immunosuppression. We compared the effects of S-1 on antitumor immunity with those of FT plus CDHP (FT + CDHP) in the rat. To our knowledge, this is the first study to examine the effects of Oxo on antitumor immunity.

Materials and methods

Animals

Male Donryu rats (6–8 weeks old) purchased from Japan SLC (Hamamatsu, Japan) were used in all experiments. The rats were housed in an air-conditioned animal room, maintained at a room temperature of 21–23°C and a relative humidity of 50–60%. Artificial illumination was provided on a 12-h light/dark cycle (lights on between 8:00 a.m. and 8:00 p.m.). The rats were given commercial rodent chow and water ad libitum. Body weights were measured during the course of all experiments.

Drugs

FT + CDHP and S-1 (FT + CDHP + Oxo) were generously provided by Dr. T. Shirasaka (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan) for this study. All other chemicals used were commercially available products. The doses of both FT + CDHP and S-1 are expressed as the dose of FT. Both drugs were dissolved in 0.5% (w/v) hydroxypropyl methylcellulose (HPMC) solution.

Treatments

The rats were divided into three groups (FT + CDHP group, S-1 group, and control group). In the FT + CDHP group, rats were given FT + CDHP dissolved in 0.5% HPMC in an oral dose of 20 mg/kg once daily for seven consecutive days. Rats in the S-1 group similarly received an equivalent oral dose of S-1. In the control group, rats were given 0.5% HPMC alone. All rats were sacrificed on day 8 by an overdose of ether anesthesia. Blood samples were collected by cardiac puncture. The spleen and mesenteric lymph nodes were removed aseptically.

Preparation of spleen cells and mesenteric lymph node cells

Spleen cells and mesenteric lymph node cells were prepared by forcing the spleen or mesenteric lymph nodes through a 150-gauge stainless steel mesh. Spleen cells were additionally treated with red blood cell lysis solution (0.15 M NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$). The cells were washed twice with RPMI 1640 (Nikken, Kyoto, Japan) and finally suspended in RPMI 1640 containing 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol (complete medium). Cell viabilities were determined by Trypan blue exclusion tests. The cell suspensions were adjusted to 4×10^6 cells/ml and assayed in vitro.

Immunophenotypic analysis

Surface phenotypes of spleen cells and mesenteric lymph node cells were identified by immunofluorescence tests with monoclonal antibodies. The monoclonal antibodies used included FITC-IF4 (anti-rat CD3), FITC-MRC OX-33 (anti-rat CD45), FITC-MRC OX-40 (anti-rat CD4), and PE-MRC OX-8 (anti-rat CD8) (PharMingen, San Diego, CA). The percentages of fluorescence-positive cells were determined by fluorescence-activated cell-sorter (FACS) analysis with a FACS Calibur (Becton Dickinson, Mountain View, CA).

Natural killer activity

Target cells of natural killer (NK) cells, YAC-1, were labeled with 100 μCi (per 2×10^6 cells) of $Na_2^{51}CrO_4$ (NEN, Tokyo, Japan) for 60 min at 37°C in complete medium and washed twice with the medium. Labeled targets (1×10^4 cells per well) were incubated in a total volume of 200 μl with effector cells in complete medium in 96-well round-bottom microtiter plates. The plates were centrifuged before incubation and after 4 h of incubation, the supernatants were harvested and counted in a γ counter. Cytotoxicity was calculated as the percentage of releasable counts after subtraction of the spontaneous release.

Production of interleukin-2

Spleen cells and mesenteric lymph node cells were incubated with 0.5 μg/ml of concanavalin A (ConA; Pharmacia, Bucks, UK) on 24-well plates in a humidified atmosphere of 5% CO_2 in air at 37°C. Supernatants harvested after 24-h incubation were tested for interleukin (IL)-2, using ELISA kits (Genzyme, Cambridge, MA).

Statistical analysis

Values are expressed as means ± SD. Student's *t*-test was used for statistical analysis. A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Body weight changes of rats treated with S-1 and FT + CDHP

Changes in the mean body weight ratio of the rats are shown in Fig. 1a. On days 6 and 8, mean body weight ratios of the S-1-treated rats (0.96 ± 0.03 and 0.93 ± 0.05, respectively) were significantly higher than those of the FT + CDHP-treated rats (0.86 ± 0.04 and 0.73 ± 0.06, respectively) ($P < 0.05$). All of the rats treated with FT + CDHP had severe diarrhea and lost body weights dramatically. In contrast, none of the S-1-treated rats had diarrhea, and body weights decreased only slightly. Dermatitis was observed in seven of the ten rats treated with FT + CDHP, as compared with only two of the ten rats treated with S-1. Enteritis was more severe in the FT + CDHP group than in the S-1 group.

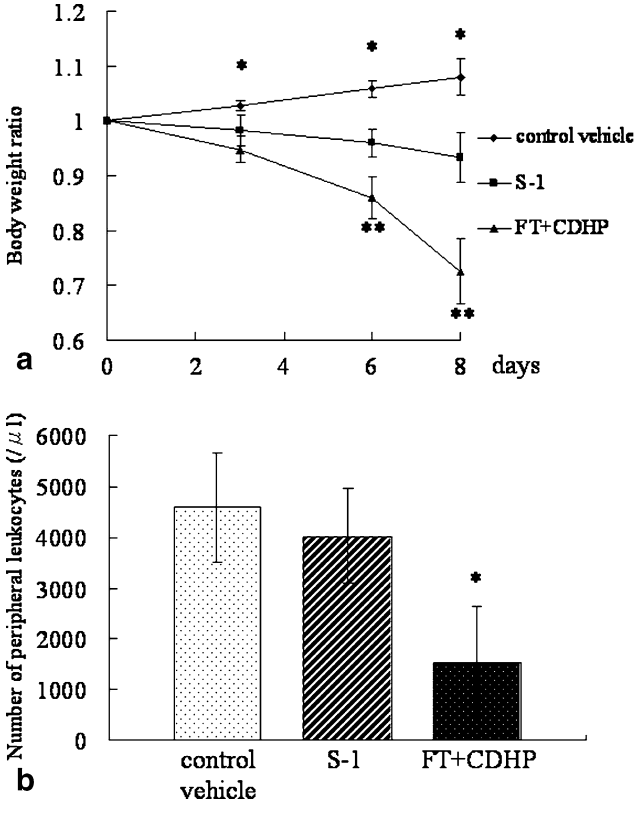

Fig. 1a Effect of daily administration of S-1 or FT + CDHP on the body weight change of rats. The *graph* shows the mean body weight ratio and standard deviation (*bars*) of ten rats treated with vehicle (control), FT + CDHP, or S-1. Significant differences between vehicle-treated rats and FT + CDHP-treated rats or S-1-treated rats were observed after day 3 ($P < 0.05$). Mean body weight of S-1-treated rats was significantly higher than that of FT + CDHP-treated rats after day 6 ($P < 0.05$). *Body weight ratio: body weight/body weight on day 0*. *Bars*, SD. *$P < 0.05$ vs S-1 and FT + CDHP, **$P < 0.05$ vs S-1. b Effects of daily administration of S-1 and FT + CDHP on the number of peripheral leukocytes. All rats treated with vehicle (*control*), S-1, or FT + CDHP were sacrificed on day 8 by an overdose of ether. Blood samples were collected by cardiac puncture. The number of peripheral leukocytes in the S-1 group was significantly higher than that in the FT + CDHP group ($P < 0.05$), whereas there was no significant difference in the number of peripheral leukocytes between the S-1 group and control group. *Bars*, SD. *$P < 0.05$ vs S-1 and control vehicle

Effects of S-1 and FT + CDHP on number of peripheral leukocytes

Changes in the number of leukocytes in peripheral blood are shown in Fig. 1b. Daily treatment with FT + CDHP significantly decreased the number of peripheral leukocytes (1,540 ± 1,106/μl) as compared with the number of leukocytes in S-1-treated rats and control (4,020 ± 934/μl and 4,600 ± 1,070/μl, respectively) ($P < 0.05$). The number of peripheral leukocytes was similar in S-1-treated rats and control.

Immunophenotypes of spleen cells and mesenteric lymph node cells

Phenotypic changes in spleen cells and mesenteric lymph node cells were analyzed by flow cytometry. As compared with control, there were no phenotypic changes in CD3 or CD45 or in CD4 or CD8 in spleen cells or mesenteric lymph node cells in either the S-1 group or FT + CDHP group (data not shown).

Effects of S-1 and FT + CDHP on natural killer cell activity

In the experiments described above, S-1 or FT + CDHP treatment did not change the phenotype of spleen cells or mesenteric lymph node cells of rats. We therefore investigated the NK cytotoxic activities of these cells in rats treated with S-1 or FT + CDHP (Fig. 2). NK activity in the S-1 group (25.6 ± 4.2%) was significantly higher than that in the FT + CDHP group (19.4 ± 3.9%; E:T, 40; $P = 0.04$) in spleen cells. Similarly, in the mesenteric lymph node cells, the NK activity of the S-1 group (9.6 ± 3.2%) was significantly higher than that of the FT + CDHP group (4.8 ± 2.3%; E:T, 40; $P = 0.03$). The differences between the S-1 group and the vehicle group were not significant.

Effects of S-1 and FT + CDHP on IL-2 production

As shown in Fig. 3, IL-2 production by spleen cells in the FT + CDHP group (1,955 ± 784 pg/ml) was significantly lower than that in the control group

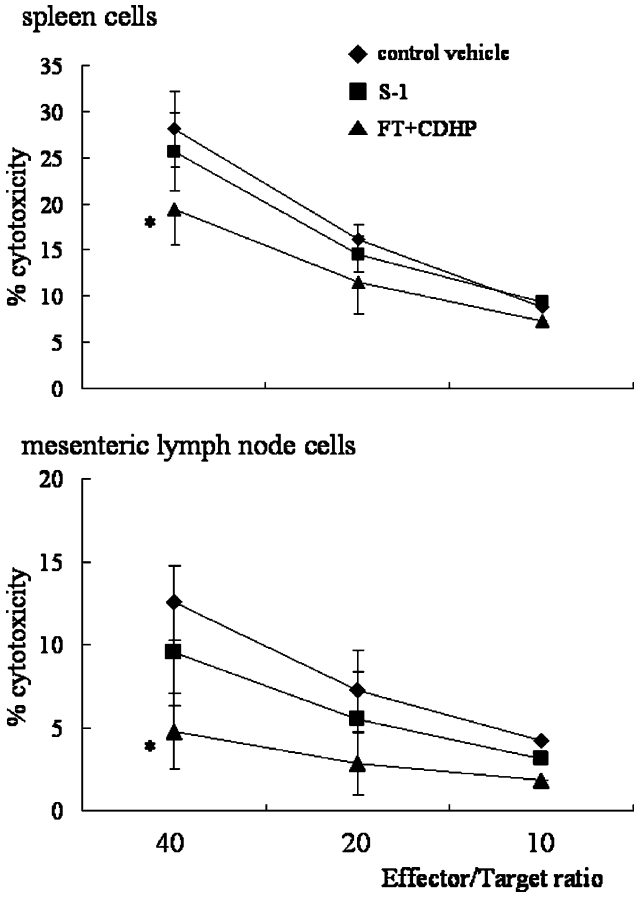

Fig. 2 Natural killer (NK) activities of spleen cells and mesenteric lymph node cells against YAC-1 tumor cells. Rats treated with vehicle (*control*), S-1, or FT + CDHP daily from day 1 to day 7 were sacrificed on day 8, and spleen cells and mesenteric lymph node cells were aseptically obtained from five rats per group. These cells were used as effector cells against YAC-1 tumor targets in a 4-h ^{51}Cr release assay. NK activities against YAC-1 tumor cells in the spleen and mesenteric lymph nodes were significantly higher in the S-1 group than in the FT + CDHP group (E:T, 40; $P < 0.05$). *Bars*, SD. *$P < 0.05$ vs S-1 and control vehicle

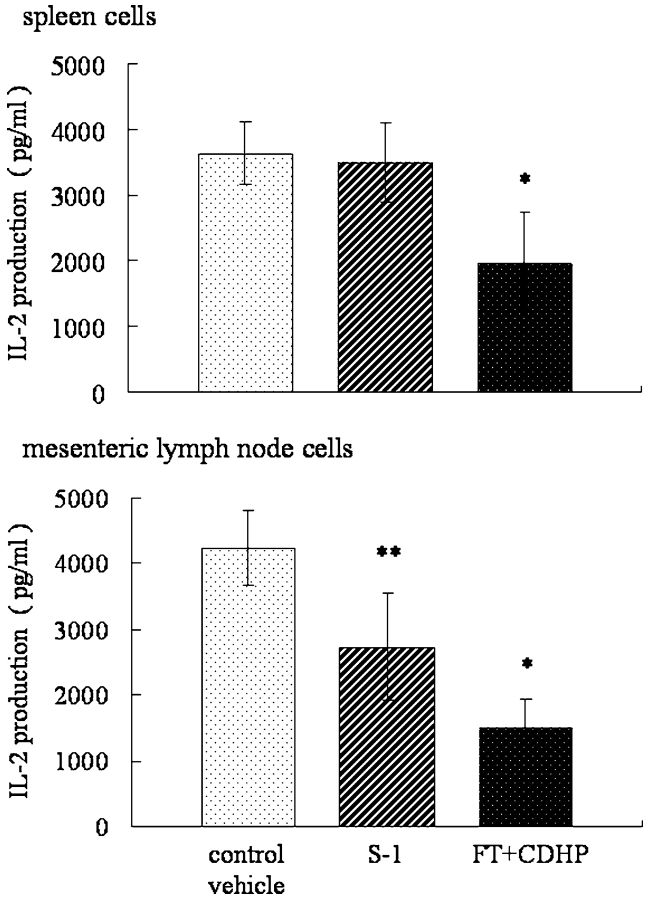

Fig. 3 IL-2 production of spleen cells and mesenteric lymph node cells after incubation with concanavalin A (*ConA*, 0.5 μg/ml). Spleen cells and mesenteric lymph node cells were isolated on day 8, after treatment with FT + CDHP or S-1 from days 1 to 7. These cells were incubated with ConA (0.5 μg/ml) on 24-well plates in a humidified atmosphere of 5% CO_2 in air at 37°C. Supernatants harvested after 24-h incubation were tested for IL-2 using ELISA kits. IL-2 production by both the spleen cells and mesenteric lymph node cells in the S-1 group was significantly higher than that in the FT + CDHP group ($P < 0.05$). *Bars*, SD. *$P < 0.05$ vs S-1 and control vehicle, **$P < 0.05$ vs control vehicle

(3,634 ± 482 pg/ml; $P = 0.005$) and the S-1 group (3,486 ± 596 pg/ml; $P = 0.01$). IL-2 production by spleen cells was similar in the S-1 group and the control group. On the other hand, mesenteric lymph node cells from both of the FT + CDHP-treated rats and S-1-treated rats released lower levels of IL-2 (1,508 ± 427 and 2,729 ± 818 pg/ml, respectively) than control (4,247 ± 573 pg/ml; $P < 0.001$ and $P = 0.01$, respectively). However, IL-2 production was significantly higher in the S-1 group than in the FT + CDHP group ($P = 0.03$).

Discussion

5-FU was first described by Heidelberger in 1957 [16]. The cytotoxic effects of 5-FU involve the inactivation of thymidylate synthase (TS) activity caused by 5-fluorodeoxyuridine-5′-monophosphate (FdUMP), a phosphorylated metabolite of 5-FU [17, 18]. Inhibition of DNA synthesis by 5-FU injures immune cells of the host, causing immunosuppression [19, 20]. Clinically, it is therefore important that immunocompetence is maintained in patients receiving 5-FU.

In this experimental study, we compared the effects of Oxo on antitumor immunity in rats given FT + CDHP and those given FT + CDHP + Oxo (S-1). NK activity is generally considered a representative parameter of immune response in patients with cancer [21–24]. In our study, NK activities in both spleen cells and mesenteric lymph node cells were significantly decreased by treatment with FT + CDHP; however, in the S-1 group, NK activity was not significantly lower than that in the control group, given vehicle alone. Our results suggest that Oxo can inhibit the anticancer-drug-induced decrease in NK activity, which has an important

role in antitumor immunity, without diminishing anticancer activity.

To determine whether Oxo affects cytokine production by these cells, IL-2 production was measured after stimulation with a suboptimal dose of ConA. Although IL-2 production was strongly inhibited in the FT + CDHP group, IL-2 production in the S-1 group was similar to that in the control group for spleen cells. For mesenteric lymph node cells, the decrease in IL-2 production as compared with control was smaller in the S-1 group than in the FT + CDHP group. These findings suggest that Oxo can act to maintain IL-2 production by lymphocytes stimulated by tumor antigens in cancer-bearing rats. In addition, the maintenance of IL-2 production by Oxo may act to preserve antitumor immunity, without disturbing the induction of cytotoxic T cells in vivo. Moreover, since IL-2 promotes the activation of NK cells [25, 26], the maintenance of IL-2 production by Oxo may be linked to the maintenance of NK activity. Oxo seems to maintain immune response by augmenting lymphocyte function rather than altering lymphocyte subsets because immunophenotypic changes in these cells were not observed after treatment.

Yoshisue et al. showed that Oxo inhibits decreased free TS activity in the gastrointestinal tract of rats, suggesting that Oxo-induced suppression of 5-FU phosphorylation helps to lessen damage to gastrointestinal tissue [27]. Korenaga et al. found that treatment with FT + CDHP impairs the barrier function of the gut, thereby increasing the permeability of the intestinal mucosa and causing diarrhea. The inclusion of Oxo may reduce this damage [28]. Previous studies have suggested that Oxo protects immune response by maintaining TS activity in the gastrointestinal tract and preserving gut barrier function. These effects inhibit damage to the host immune system and contribute to the suppression of decreased antitumor immunity. Mori et al. suggested that reduced intestinal toxicity due to Oxo was one factor responsible for the prolonged survival and prevention of cachexia associated with S-1 in a murine model of peritoneal dissemination [29]. Clinically, decreased gastrointestinal toxicity due to Oxo helps to maintain and improve the quality of life (QOL) of patients receiving S-1, thus improving compliance with chemotherapy and augmenting host immune response, adversely affected by 5-FU. Ajani [30] and Ajani and Takiuchi [31] emphasized the importance of QOL, convenience, and cost-containment in patients with gastric cancer who receive chemotherapy and found that oral anticancer drugs meet these requirements. The inclusion of Oxo in S-1 lessens gastrointestinal toxicity and immunosuppression, thereby maintaining the QOL of patients. S-1 thus more closely approximates ideal conditions for a new oral anticancer drug.

In conclusion, our results show that Oxo, acting as a modulator in S-1, inhibits the 5-FU-induced decreases in NK activity and IL-2 production, thereby preventing a reduction in host antitumor immunity.

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