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Oral Administration of a Kampo (Japanese Herbal) Medicine *Juzen-taiho-to* Inhibits Liver Metastasis of Colon 26-L5 Carcinoma Cells

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We have investigated the inhibitory effect of oral administration of *Juzen-taiho-to*, a Kampo Japanese herbal medicine, on liver metastasis by the inoculation of a liver-metastatic variant (L5) of murine colon 26 carcinoma cells into the portal vein. Oral administration of *Juzen-taiho-to* for 7 days before tumor inoculation resulted in dose-dependent inhibition of liver tumor colonies and significant enhancement of survival rate as compared with the untreated control, without side effects. We also found that liver metastasis of L5 cells was enhanced in BALB/c mice pretreated with anti-asialo GM1 serum or 2-chloroadenosine, and in BALB/c *nu/nu* mice, compared to normal mice. This indicates that NK cells, macrophages, and T-cells play important roles in the prevention of metastasis of tumor cells. *Juzen-taiho-to* significantly inhibited the experimental liver metastasis of colon 26-L5 cells in mice pretreated with anti-asialo GM_1 serum and untreated normal mice, whereas it did not inhibit metastasis in 2-chloroadenosine-pretreated mice or T-cell-deficient nude mice. Oral administration of *Juzen-taiho-to* activated peritoneal exudate macrophages (PEM) to become cytostatic against the tumor cells. These results show that oral administration of *Juzen-taiho-to* inhibited liver metastasis of colon 26-L5 cells, possibly through a mechanism mediated by the activation of macrophages and/or T-cells in the host immune system. Thus, *Juzen-taiho-to* may be efficacious for the prevention of cancer metastasis.

Key words: *Juzen-taiho-to* — Liver metastasis — Colon 26-L5 — Macrophage

Despite the advances in diagnostic techniques for the early detection of colon cancer and the significant improvement in surgical procedures, the mortality rate of colon cancer has been increasing year after year,[1–3] and metastasis is a frequent cause of death by cancer. The liver is the most common target of hematogenous metastasis in gastrointestinal tract cancer, especially colon cancer, and the prognosis for cases with liver metastasis is extremely poor.[4, 5] If occult micrometastases, established at the time of surgery, could be inhibited, then the prognosis of patients with colon carcinoma would improve. Suitable experimental metastasis models of colon carcinoma are necessary to develop novel therapies for colon carcinoma. Murine colon 26 carcinoma cells have been utilized in an experimental model of metastasis in BALB/c mice.[6] We have established a liver-metastatic variant (colon 26-L5) of the colon 26 carcinoma by an *in vivo* selection method.[7] Colon 26-L5 cells predominantly metastasize in the liver after inoculation via the portal vein of BALB/c mice. This model has provided a means for evaluating the efficacy of treatments for liver metastasis of cancer, especially for occult micrometastases.[8]

Juzen-taiho-to, a Kampo Japanese herbal medicine, was first described in Daipinghuimin-hejijufang (1151) of the Song dynasty (960–1279) in China. It was introduced into Japan in the Kamakura dynasty (1192–1333) and since then has been used as a cure for consumption, general debility, deficiency and impairment of Yin and Yang, vital energy or blood in the viscera or bowels, and lack of appetite. It is currently administered to patients weakened by long illness, fatigue, loss of appetite, night sweats, circulatory problems, and anemia. It is also used for cancer patients. Several studies have shown that *Juzen-taiho-to* is biologically active, and it has such effects as enhancements of phagocytosis,[9] cytokine induction,[10, 11] antibody production,[12] and spleen cell mitogenic activity.[13] Other studies have demonstrated an anti-tumor effect in combination with surgical excision,[14] augmentation of anti-tumor activity in combination with or without other drugs,[15, 16] and protection from the deleterious effects of anti-cancer drugs,[17] and radiation-induced immunosuppression and bone marrow toxicity.[18, 19] We have reported that *Juzen-taiho-to* effectively prevented weakly malignant tumors from growing progressively upon coimplantation with a gelatin sponge, and may act to induce antioxidative substances, in addition to augmenting the host-mediated

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immune responses.[20] However, to our knowledge, the effect of *Juzen-taiho-to* on tumor metastasis has not been reported.

In the present study, we examined the effect of oral administration of *Juzen-taiho-to* on the experimental liver metastasis of colon 26-L5 cells, as well as the mechanism of the antimetastatic activity.

MATERIALS AND METHODS

Preparation of *Juzen-taiho-to* *Juzen-taiho-to* (TJ-48), obtained from Tsumura & Co., Ltd., Tokyo, is composed of ten crude drugs (Table I), of which the quality is controlled by the Japanese Pharmacopeia XIII. *Juzen-taiho-to* was prepared as follows. A mixture of Astragali radix (3.0 g), Cinnamomi cortex (3.0 g), Rehmanniae radix (3.0 g), Paeoniae radix (3.0 g), Cnidii rhizoma (3.0 g), Atractylodis Lanceae rhizoma (3.0 g), Angelicae radix (3.0 g), Ginseng radix (3.0 g), Hoelen (3.0 g), and Glycyrrhizae radix (1.5 g) was added to 285 ml of water and extracted at 100°C for 1 h. The extracted solution was filtered and the filtrate was spray-dried to obtain the dry extract powder (2.3 g). The blended powder was dissolved in distilled water before oral administration. To control the quality of *Juzen-taiho-to* preparation, the origin of each peak of *Juzen-taiho-to* in the HPLC profiles was identified by comparison of the retention times and UV spectra of each crude drug with those of chemically defined standard compounds (data not shown).

Other chemicals Cisplatin (*cis*-diamminedichloroplatinum II, CDDP) was provided by Nippon Kayaku Co., Ltd., Tokyo. Rabbit anti-asialo GM_1 antiserum was purchased from Wako Pure Chemical Industries, Ltd., Tokyo. 2-Chloroadenosine was purchased from Research Biochemicals Incorporated, Natick, MA.

Animals Specific pathogen-free female BALB/c and BALB/c *nu*/*nu* mice, 6 weeks old, were purchased from Japan SLC, Hamamatsu. The mice were maintained in the Laboratory for Animal Experiments, Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University, under laminar air-flow conditions.

Tumor cells The liver metastatic cell line of the colon 26 carcinoma (colon 26-L5) was obtained by the *in vivo* selection method of Fidler.[7, 21] Colon 26-L5 cells were maintained as monolayer cultures in RPMI-1640 supplemented with 7.5% fetal bovine serum and L-glutamine.

Assay for experimental liver metastasis of tumor cells Log-phase cell cultures of colon 26-L5 cells were harvested with 1 m*M* EDTA in phosphate-buffered saline (PBS), washed three times with serum-free RPMI, and resuspended at appropriate concentrations in PBS. BALB/c mice under ether anesthesia underwent laparotomy by upper median incision, then the duodenal loop was exposed, and an injection of colon 26-L5 ($1–2\times10^4$/200 μl) cells was given into the portal vein through a 29-gauge needle attached to a 1-ml syringe. A sterile absorbable cotton sponge was placed over the injection site as the needle was withdrawn to prevent bleeding and peritoneal dissem-

Table I. The Botanical Origins and Harvesting Seasons of Crude Drugs of *Juzen-taiho-to*

Crude drug	Botanical origin (Family name)	Harvesting time	Representative defined compounds	Ratio
Astragali Radix	*Astragalus membranaceus* Bunge or *A. mongholicus* Bunge (Leguminosae)	autumn	7, 3′-dihydroxy-4′-methoxyisoflavone its 7-*O*-β-D-glucoside	3.0
Cinnamomi Cortex	*Cinnamomum cassia* Mlume or other species of the same genus (Lauraceae)	mainly autumn	cinnamic acid cinnamaldehyde	3.0
Rehmanniae Radix	*Rehmannia glutinosa* Liboschitz var. *purpurea* Makino or *A. glutinosa* Liboschitz (Scrophulariaceae)	autumn	acteoside	3.0
Paeoniae Radix	*Paeonia lactiflora* Pallas or allied plants (Paeoniaceae)	autumn	paeoniflorin benzoic acid	3.0
Cnidii Rhizoma	*Cnidium officinale* Makino (Umbelliferae)	summer	ferulic acid, senkyunolide I	3.0
Atractylodis lanceae Rhizoma	*Atractylodes lancea* De Candolle or *A. chinensis* Koidzumi (Compositae)	autumn		3.0
Angelicae Radix	*Angelica acutiloba* Kitagawa or allied plants (Umbelliferae)	autumn		3.0
Ginseng Radix	*Panax ginseng* C.A. Meyer (Araliaceae)	autumn	ginsenoside Rb1	3.0
Hoelen	The sclerotium of *Poria cocos* Wolf (Polyporaceae)	autumn to winter		3.0
Glycyrrhizae Radix	*Glycyrrhiza uralensis* Fischier, *G. grabra* Linne, or other species of the same genus (Leguminisae)	autumn	liquilitin	1.5

Medicine was prepared by blending the crude drugs in the ratios is indicated above.

ination of the tumor cells. The mice were killed 19 days after tumor inoculation and the number of metastatic colonies in each liver was macroscopically counted. The liver weight was recorded to evaluate the tumor metastasis as previously described.[22, 23] The survival period of the tumor-bearing mice was also determined by allowing them to live until they succumbed to the tumor burden. *Juzen-taiho-to* was orally administered to mice at appropriate doses (4 to 40 mg/mouse) for 7 days before tumor inoculation.

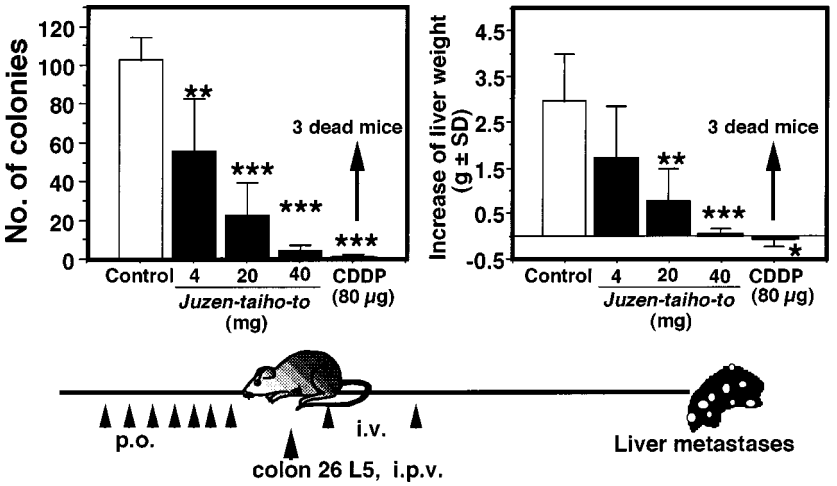

Fig. 1. Effect of oral administration of *Juzen-taiho-to* on experimental liver metastasis produced by the intraportal injection of colon 26-L5 carcinoma cells. Five BALB/c mice per group were inoculated intraportally with colon 26-L5 cells (2×10^4). *Juzen-taiho-to* at the indicated doses was orally administered for 7 days before tumor inoculation. CDDP was injected intravenously on days 1 and 8 after tumor inoculation. Nineteen days after tumor inoculation, mice were killed, the number of liver colonies was manually counted and the liver was weighed. $*$ $P<0.05$, $**$ $P<0.01$, $***$ $P<0.001$ as compared to untreated controls by Student's two-tailed t test.

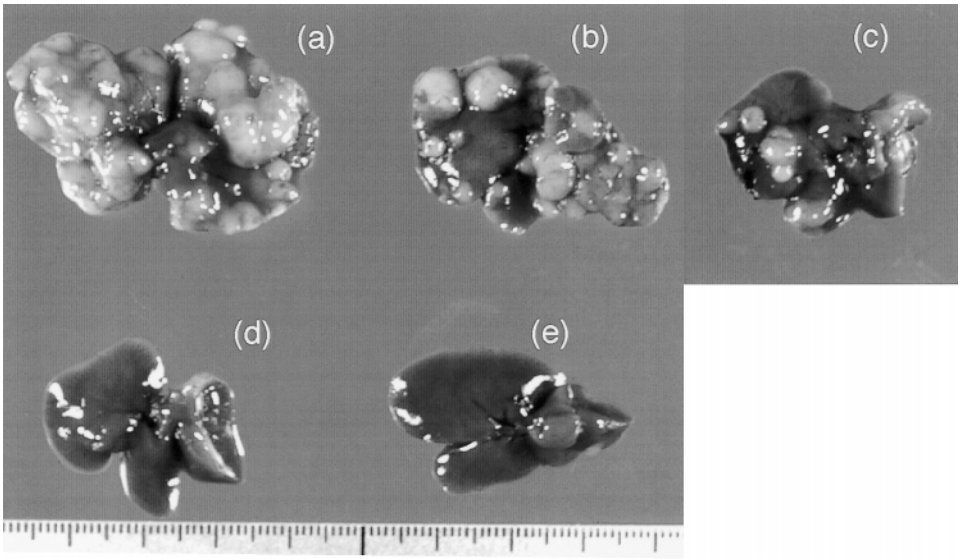

Fig. 2. Macroscopic observation of liver metastasis by colon 26-L5 cells 19 days after tumor inoculation. Five BALB/c mice per group were injected intraportally with colon 26-L5 cells (2×10^4) with or without *Juzen-taiho-to* or CDDP. Nineteen days after tumor inoculation the mice were killed. a, control; b, c or d, 4, 20 or 40 mg *Juzen-taiho-to*, respectively; e, 80 μg CDDP.

Assay for cytostasis Cytostatic activity against the tumor cells was assessed by the WST-1 assay. BALB/c mice were orally administered *Juzen-taiho-to* (40 mg/day) for 7 days and injected i.p. with 1 ml of 3% thioglycolate immediately after the last administration. Peritoneal exudate macrophages (PEM) were collected by peritoneal lavage 4 days after the injection. Colon 26-L5 target cells (5×10^3) were incubated with PEM monolayers in 96-well plates at a PEM:target cell ratio of 10:1. After a 48-h incubation, 10% WST solution (WST-1 Cell Counting Kit; Wako Pure Chemicals, Osaka) was added to each well and the plates were incubated for 2 h more. The absorbance of the culture was measured at 450 nm in an immuno-reader (Immuno Mini NJ-2300, Nippon Inter Med K.K., Tokyo). The cytostatic activity caused by PEM was calculated as follows:

$$\text{Cytostatic activity} = 1 - (\text{OD of target cells with PEM} - \text{OD of PEM}) / \text{OD of target cells}$$

Statistical analysis The statistical significance of differences between the groups was determined by using Student's two-tailed *t* test or the Mann-Whitney U test.

RESULTS

Inhibition of experimental liver metastasis by *Juzen-taiho-to* We first examined the effect of oral administration of *Juzen-taiho-to* on liver metastasis caused by the injection of colon 26-L5 carcinoma cells into the portal vein. The number of tumor colonies in the liver and the liver weight were measured on day 19 after tumor inoculation. Figs. 1 and 2 show that the oral administration of *Juzen-taiho-to* for 7 days before tumor inoculation significantly reduced the number of tumor colonies in the liver and attenuated the increase of the liver weight in a dose-dependent manner (from 4 to 40 mg/day). Intravenous administration of CDDP (80 μg/day) on days 1 and 8 after tumor inoculation inhibited liver metastasis. A marked loss of body weight was observed after the administration of CDDP and 3 of the 6 mice died. The administration of *Juzen-taiho-to* did not have any apparent side effect. These results clearly indicate that the oral administration

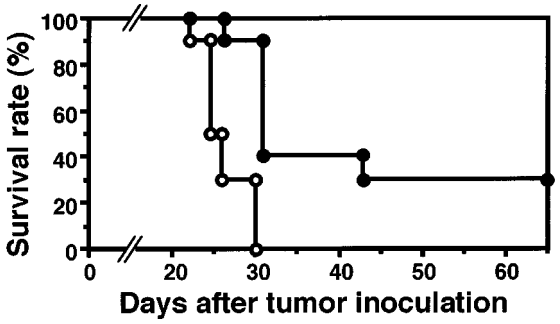

Fig. 3. Effect of *Juzen-taiho-to* on the survival of mice after the inoculation of colon 26-L5 cells into the portal vein. Ten BALB/c mice per group were orally given *Juzen-taiho-to* (40 mg/day) (●) or the vehicle (○) for 7 days before the intraportal inoculation of colon 26-L5 cells (10^4). Survival was monitored as a function of time. $P<0.05$, *Juzen-taiho-to* vs. untreated control (Mann-Whitney U test).

Fig. 4. Effect of anti-asialo GM_1 serum on *Juzen-taiho-to*-mediated inhibition of experimental liver metastasis produced by the intraportal injection of colon 26-L5 cells. Five BALB/c mice per group were orally given *Juzen-taiho-to* (40 mg/day) or the vehicle for 7 days before tumor inoculation. Colon 26-L5 cells (10^4) were intraportally injected into groups of control mice or mice pretreated 24 h earlier with anti-asialo GM_1 serum (20 μl/mouse). Mice were killed 13 days after tumor inoculation. The number of tumor colonies in the liver was manually counted and the liver was weighed. * $P<0.05$, ** $P<0.001$ as compared with an untreated control by Student's two-tailed *t* test.

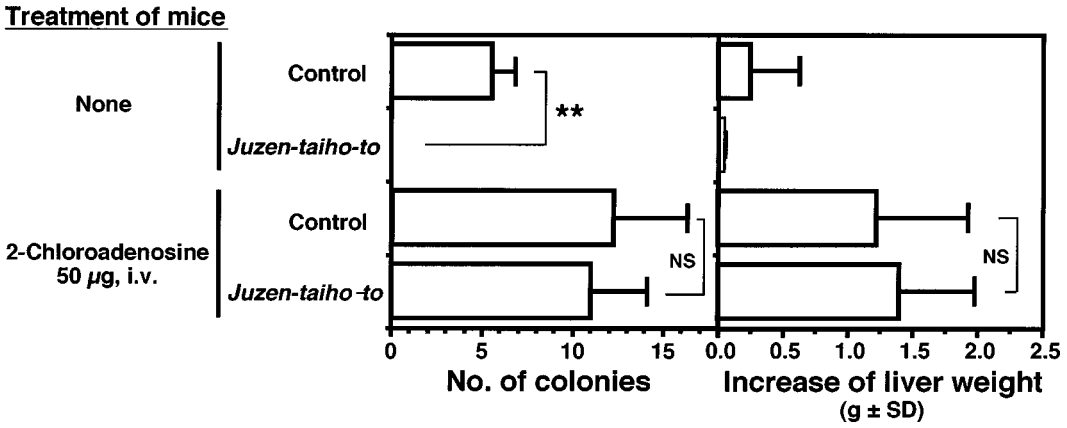

Fig. 5. Effect of 2-chloroadenosine on *Juzen-taiho-to*-mediated inhibition of experimental liver metastasis produced by the intraportal injection of colon 26-L5 cells. Five BALB/c mice per group were orally given *Juzen-taiho-to* (40 mg/day) or the vehicle for 7 days after tumor inoculation. Colon 26-L5 cells (10^4) were intraportally injected into groups of control mice or mice pretreated 24 h earlier with 2-chloroadenosine (50 μg/mouse). Mice were killed 14 days after tumor inoculation. The number of tumor colonies in the liver was manually counted and the liver was weighed. ** $P<0.01$, NS, not significant by Student's two-tailed *t* test.

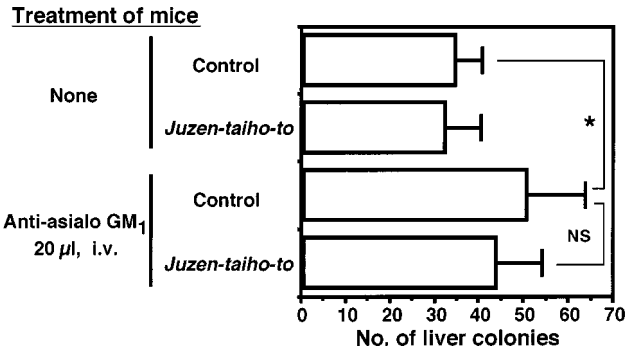

Fig. 6. Effect of *Juzen-taiho-to* on liver metastasis produced by the intraportal injection of colon 26-L5 cells in BALB/c nude mice or nude mice pretreated with anti-asialo GM_1 serum. Five BALB/c nude mice per group were orally given *Juzen-taiho-to* (40 mg/day) for 7 days after tumor inoculation. Colon 26-L5 cells (2×10^4) were intraportally injected into groups of control nude mice or mice pretreated 24 h earlier with anti-asialo GM_1 serum (20 μl/mouse). Mice were killed 18 days after tumor inoculation and the number of tumor colonies in the liver was manually counted. * $P<0.05$, NS, not significant by Student's two-tailed *t* test.

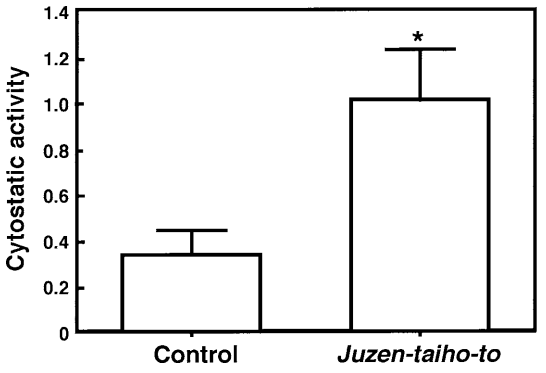

Fig. 7. Cytostatic activity of peritoneal exudate macrophages (PEM) from BALB/c mice treated orally with *Juzen-taiho-to*. BALB/c mice were orally given *Juzen-taiho-to* (40 mg/day) for 7 days and then immediately injected i.p. with 1 ml of 3% thioglycolate. PEM were obtained by peritoneal lavage 4 days after the injection. PEM (5×10^4) were cultured with colon 26-L5 cells (5×10^3) for 48 h and cytostasis was assessed by using the WST-1 assay. * $P<0.01$ as compared with the untreated control by Student's two-tailed *t* test.

of *Juzen-taiho-to* is effective in preventing the experimental liver metastasis caused by colon 26-L5 cells.

Effect of *Juzen-taiho-to* on the survival of mice inoculated with colon 26-L5 cells We also examined the effect of *Juzen-taiho-to* on the survival rate of mice given an intraportal injection of colon 26-L5 cells (Fig. 3). *Juzen-taiho-to* (40 mg/day/mouse) was administered according to the same schedule as in Fig. 1. In this experiment, all the control mice succumbed to the tumor burden within 30 days after tumor inoculation. Mice that received treatment with *Juzen-taiho-to* showed a significant prolongation of survival rate as compared with the control group ($P<0.05$ by Mann-Whitney U test).

Effect of anti-asialo GM_1 serum and 2-chloroadenosine on *Juzen-taiho-to*-mediated inhibition of liver metastasis Since natural killer (NK) cells or macrophages in the

circulation play an important role in the inhibition of tumor metastasis,[24, 25] we next investigated whether the oral administration of *Juzen-taiho-to* can stimulate NK cells or macrophages to induce the inhibition of tumor metastasis. Anti-asialo GM_1 serum can selectively eliminate NK cells[26, 27] and 2-chloroadenosine can eliminate macrophages.[27, 28] Figs. 4 and 5 show that treatment with anti-asialo GM_1 serum or 2-chloroadenosine 24 h before tumor inoculation (i.e., immediately after the last administration of *Juzen-taiho-to*) enhanced the frequency of liver metastasis compared to that of untreated normal mice. Oral administration of *Juzen-taiho-to* for 7 days before the tumor inoculation led to a significant reduction of the number of metastatic colonies and liver weight even in the NK-depleted mice, as well as in normal mice. *Juzen-taiho-to* did not inhibit liver metastasis in the macrophage-deficient mice. We also investigated the effect of *Juzen-taiho-to* on liver metastasis in T-cell-deficient nude mice (Fig. 6). When BALB/c *nu*/*nu* mice and NK-depleted *nu*/*nu* mice (pretreated with anti-asialo GM_1 serum) were orally administered *Juzen-taiho-to* for 7 days before tumor inoculation, no reduction of liver metastasis of colon 26-L5 cells was observed. These results suggest that the inhibition of liver metastasis by *Juzen-taiho-to* is partly mediated by the function of macrophages and/or T-cells.

In vitro* cytostatic activity against tumor cells by PEM of mice given *Juzen-taiho-to We examined the cytostatic activity of PEM obtained from BALB/c mice administered *Juzen-taiho-to* (40 mg/day/mouse) for 7 days. Fig. 7 shows that the oral administration of *Juzen-taiho-to* activated PEM to become cytostatic against the tumor cells.

DISCUSSION

Herbal prescriptions, including Kampo medicines, have been recognized as potentially valid by scientific medical systems and their usage has been increasing. Since the prescriptions are prepared from combinations of many crude drugs, they may have an effect that differs from the combined effect of the individual constituent drugs. To ensure an acceptable efficacy and quality, it is necessary to control the quality of the constituent crude drugs in the prescriptions, because their quality varies with their origin and the time of harvest. In Japan, the quality of crude drugs is controlled by the Japanese Pharmacopeia XIII, which regulates the botanical origin, foreign matter content, loss by drying, total ash, acid-insoluble ash, extract content, essential oil content, and microscopic appearance. We have conducted comparative HPLC analysis of *Juzen-taiho-to* and its constituent crude drugs by using chemically defined compounds (Table I) as standard references to obtain proper prescriptions with constant quality and efficacy. The origin of each peak of *Juzen-taiho-to* was identified by chemical pattern analysis, so-called fingerprinting (data not shown). This analytical method could be used to obtain consistent lots of prescriptions and stable efficacy, even though the active principles in *Juzen-taiho-to* remain unknown. We used a quality-controlled preparation of *Juzen-taiho-to* in the following experiments.

We previously reported that the oral administration of *Juzen-taiho-to* caused significant inhibition of the progressive growth of QR-32 regressor tumors after coimplantation with a gelatin sponge, and prolonged the survival of tumor-bearing mice.[20] *Juzen-taiho-to* inhibited the growth of a progressor tumor which had acquired a more malignant phenotype, when it was orally administered for 7 days after s.c. inoculation of the tumor.[20] To extend our study to the inhibition of malignant progression by *Juzen-taiho-to*, we examined the effect of oral administration of *Juzen-taiho-to* on liver metastasis of colon carcinoma cells *in vivo* and the role of the immune system. Oral administration of *Juzen-taiho-to* before tumor inoculation resulted in the dose-dependent inhibition of liver metastasis and a significant enhancement of survival rate compared to the untreated control (Figs. 1–3). CDDP significantly inhibited liver metastasis at 80 μg/mouse,[16, 17] but it produced severe adverse effects such as decrease of body weight and death. *Juzen-taiho-to* did not produce apparent side effects, nor did it directly affect the tumor cells *in vitro* (data not shown). *Juzen-taiho-to* may be a biological response modifier that inhibits micrometastasis and differs from chemotherapeutic agents.

Since metastasizing tumor cells interact with host cells such as lymphocytes, NK cells, and monocytes, which are important in the destruction of tumor cells,[24, 25] we investigated whether *Juzen-taiho-to* can stimulate immune cells to induce the inhibition of tumor metastasis. Figs. 4–6 show that liver metastasis was enhanced in mice pretreated with anti-asialo GM_1 serum or 2-chloroadenosine, and in T-cell-deficient nude mice, compared to untreated normal mice, which indicates that NK cells, macrophages, and T-cells have an important role in the prevention of the metastatic spread of tumor cells. *Juzen-taiho-to* significantly inhibited the experimental liver metastasis of colon 26-L5 cells in mice pretreated with anti-asialo GM_1 serum as well as untreated normal mice (Fig. 4), whereas it did not inhibit the metastasis in 2-chloroadenosine-pretreated mice or T-cell-deficient nude mice (Figs. 5 and 6). Since *Juzen-taiho-to* was inactive when the contributions of macrophages and T-cells were removed in our system, its inhibitory mechanism is likely to be related to the activation of these cells. We also found that the oral administration of *Juzen-taiho-to* caused PEM to become cytostatic against tumor cells *in vitro* (Fig. 7). Although the exact mechanism responsible for the inhibition of liver metastasis by *Juzen-taiho-to* is unknown, the inhibitory effect produced by *Juzen-taiho-to* is partly associated with the activation of macrophages. Further investigation will be

needed to identify precisely the mechanisms involved, though it is difficult to do *in vitro* experiments using this mixture of ten drugs.

In conclusion, our results show that oral administration of *Juzen-taiho-to* inhibited liver metastasis of colon 26-L5 carcinoma cells and enhanced the survival rate, possibly through the activation of macrophages and T-cells. Thus, *Juzen-taiho-to* may be therapeutically applicable for the prevention of cancer metastasis.

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