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

Yasuharu Ohnishi, Hideki Fujii, Yoshihiro Hayakawa, Rieko Sakukawa, Takeshi Yamaura, Takashi Sakamoto, Kazuhiro Tsukada, Masao Fujimaki, Shinyu Nunome, Yasuhiro Komatsu and Ikuo Saiki

1Department of Pathogenic Biochemistry, Research Institute for Wakan-yaku, 2Second Department of Surgery, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01 and 3Kampo and Pharmacognosy Laboratory, Tsumura Central Laboratories, Tsumura & Co., 3586 Yoshiwara Ami-machi, Inashiki-gun, Ibaraki 300-11

We have investigated the inhibitory effect of oral administration of \textit{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 \textit{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 \textit{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. \textit{Juzen-taiho-to} significantly inhibited the experimental liver metastasis of colon 26-L5 cells in mice pretreated with anti-asialo GM1 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 \textit{Juzen-taiho-to} activated peritoneal exudate macrophages (PEM) to become cytostatic against the tumor cells. These results show that oral administration of \textit{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, \textit{Juzen-taiho-to} may be efficacious for the prevention of cancer metastasis.

Key words: \textit{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, 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. 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. We have established a liver-metastatic variant (colon 26-L5) of the colon 26 carcinoma by an \textit{in vivo} selection method. 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.

\textit{Juzen-taiho-to}, a Kampo Japanese herbal medicine, was first described in Daipinghuimin-heijijufang (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 \textit{Juzen-taiho-to} is biologically active, and it has such effects as enhancements of phagocytosis, cytokine induction, antibody production, and spleen cell mitogenic activity. Other studies have demonstrated an anti-tumor effect in combination with surgical excision, augmentation of anti-tumor activity in combination with or without other drugs, and protection from the deleterious effects of anti-cancer drugs and radiation-induced immunosuppression and bone marrow toxicity. We have reported that \textit{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...
immune responses. 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 Pharmacopoeia 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), Paeniae 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 (3.0 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.

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 | Astragali membranaceus Bunge or A. mongholicus Bunge (Leguminosae) | autumn | 7,3′-dihydroxy-4′-methoxysofavone its 7-O-β-D-glucoside | 3.0   |
| Cinnamomi Cortex | Cinnamomum cassia Mume or other species of the same genus (Lauraceae) | mainly autumn | cinnamic acid | 3.0   |
| Rehmanniae Radix | Rehmannia glutinosa Liboschitz var. purpurea Makino or A. glutinosa Liboschitz (Scrophulariaceae) | autumn | acteoside | 3.0   |
| Paeniae Radix | Paenia lactiflora Pallas or allied plants (Paoniacaeae) | autumn | paoniflorin | 3.0   |
| Cnidii Rhizoma | Cnidium officinale Makino (Umbelliferae) | summer | ferulic acid, senkyunolide I | 3.0   |
| Atractylodis lanceae | Atractylodes lancea De Candolle or A. chinensis Koidzumi (Compositae) | autumn | cinnamaldehyde | 3.0   |
| Angelicae Radix | Angelica acutiloba Kitagawa or allied plants (Ummbelliferae) | autumn | cinnamic acid | 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 Fischer, G. glabra Linne, or other species of the same genus (Leguminosae) | autumn | liquitinin | 1.5   |

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

Inhibition of Liver Metastasis by Juzen-taiho-to
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.\textsuperscript{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. \textit{Juzen-taiho-to} was orally administered to mice at appropriate doses (4 to 40 mg/mouse) for 7 days before tumor inoculation.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{fig1}
\caption{Effect of oral administration of \textit{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\textsuperscript{4}). \textit{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.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{fig2}
\caption{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\textsuperscript{4}) with or without \textit{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 \textit{Juzen-taiho-to}, respectively; e, 80 µg CDDP.}
\end{figure}
Inhibition of Liver Metastasis by *Juzen-taiho-to*

**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 

\( \times 10^3 \) 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:

\[
\text{Cytostatic activity} = 1 - \frac{\text{OD of target cells with PEM} - \text{OD of PEM}}{\text{OD of target cells}}
\]

**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 (10⁶). 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 GM₁ 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 (10⁶) were intraportally injected into groups of control mice or mice pretreated 24 h earlier with anti-asialo GM₁ 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. * * * 0.05, ** * * 0.001 as compared with an untreated control by Student’s two-tailed *t* test.

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**Treatment of mice**

|                | Control | *Juzen-taiho-to* |
|----------------|---------|------------------|
| None           |         |                  |
| Anti-asialo GM₁ |         |                  |
| 20 µl i.v.     |         |                  |
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 peritoneal cavity of BALB/c mice mediated the antitumor effect of Juzen-taiho-to, we next examined the involvement of these cells in the in vivo antitumor effect of Juzen-taiho-to. As shown in Fig. 6, Juzen-taiho-to administration resulted in reduced metastatic liver colony number in the anti-asialo GM$_1$ serum pretreated mice compared with the group without pretreatment. These findings support our hypothesis that the intraperitoneal macrophage population is involved in the anti-liver metastatic activity of Juzen-taiho-to.

**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$^7$) 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$^6$) 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$^6$) were cultured with colon 26-L5 cells (5×10$^4$) 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.
circulation play an important role in the inhibition of tumor metastasis.\textsuperscript{24, 25} we next investigated whether the oral administration of \textit{Juzen-taiho-to} can stimulate NK cells or macrophages to induce the inhibition of tumor metastasis. Anti-asialo GM\textsubscript{1} serum can selectively eliminate NK cells\textsuperscript{26, 27} and 2-chloroadenosine can eliminate macrophages.\textsuperscript{27, 28} Figs. 4 and 5 show that treatment with anti-asialo GM\textsubscript{1} serum or 2-chloroadenosine 24 h before tumor inoculation (i.e., immediately after the last administration of \textit{Juzen-taiho-to}) enhanced the frequency of liver metastasis compared to that of untreated normal mice. Oral administration of \textit{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. \textit{Juzen-taiho-to} did not inhibit liver metastasis in the macrophage-deficient mice. We also investigated the effect of \textit{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\textsubscript{1} serum) were orally administered \textit{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 \textit{Juzen-taiho-to} is partly mediated by the function of macrophages and/or T-cells.

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

\section*{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 Pharmacopoeia 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 \textit{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 \textit{Juzen-taiho-to} was identified by chemical pattern analysis, so-called finger-printing (data not shown). This analytical method could be used to obtain consistent lots of prescriptions and stable efficacy, even though the active principles in \textit{Juzen-taiho-to} remain unknown. We used a quality-controlled preparation of \textit{Juzen-taiho-to} in the following experiments.

We previously reported that the oral administration of \textit{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.\textsuperscript{29} \textit{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.\textsuperscript{30} To extend our study to the inhibition of malignant progression by \textit{Juzen-taiho-to}, we examined the effect of oral administration of \textit{Juzen-taiho-to} on liver metastasis of colon carcinoma cells \textit{in vivo} and the role of the immune system. Oral administration of \textit{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 \mu g/mouse,\textsuperscript{16, 17} but it produced severe adverse effects such as decrease of body weight and death. \textit{Juzen-taiho-to} did not produce apparent side effects, nor did it directly affect the tumor cells \textit{in vitro} (data not shown). \textit{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,\textsuperscript{24, 25} we investigated whether \textit{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\textsubscript{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. \textit{Juzen-taiho-to} significantly inhibited the experimental liver metastasis of colon 26-L5 cells in mice pretreated with anti-asialo GM\textsubscript{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 \textit{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 \textit{Juzen-taiho-to} caused PEM to become cytostatic against tumor cells \textit{in vitro} (Fig. 7). Although the exact mechanism responsible for the inhibition of liver metastasis by \textit{Juzen-taiho-to} is unknown, the inhibitory effect produced by \textit{Juzen-taiho-to} is partly associated with the activation of macrophages. Further investigation will be
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Inhibition of Liver Metastasis by Juzen-taiho-to

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