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Inhibitory Effect of a Traditional Chinese Medicine, *Juzen-taiho-to*, on Progressive Growth of Weakly Malignant Clone Cells Derived from Murine Fibrosarcoma

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We have investigated the inhibitory effect of oral administration of *Juzen-taiho-to*, a Kampo (Chinese herbal) medicine, on progressive growth of a mouse fibrosarcoma. Spontaneously regressive QR-32 tumor cells were able to grow progressively *in vivo* when coimplanted s.c. with a foreign body, gelatin sponge, whereas QR-32 cells alone gradually grew for over 15 days after inoculation and thereafter regressed for up to 25 days. Oral administration of *Juzen-taiho-to* (40 mg/day/mouse) for 7 days after inoculation of QR-32 cells with gelatin sponge resulted in significant inhibition of tumor growth and prolongation of the survival of the tumor-bearing mice. This growth-inhibitory effect of *Juzen-taiho-to* observed on day 25 was dose-dependent over the dose range from 4 to 40 mg/day. Treatment with *Juzen-taiho-to* for 7 days before tumor inoculation with gelatin sponge also significantly suppressed tumor growth examined on day 25, as did the administration of bismuth subnitrate, which is well known to induce metallothionein, an antioxidant. On the other hand, inoculation of progressed tumor cells (QRsP) resulted in growth without gelatin sponge, leading to death in syngeneic mice. Administration of *Juzen-taiho-to* for 7 days after inoculation of QRsP cells resulted in a decrease of the tumor growth and prolongation of the survival of mice, but the effect was less than that on the growth of QR-32 regressor tumor after coimplantation with gelatin sponge. These results suggest that the inhibitory effect of *Juzen-taiho-to* is partly associated with prevention of gelatin sponge-elicited progressive growth, probably mediated by endogenous factors including antioxidant substances, in addition to the augmentation of host-mediated antitumor activity.

Key words: Progressive growth — Tumor progression — *Juzen-taiho-to*

Tumor progression is the process by which tumor cells acquire a more malignant phenotype, such as enhancement of ability to proliferate, to invade and to metastasize, and is affected by various factors.[1,2] However, there have been few studies on the mechanisms, facilitating factors, and inhibitors of progression, because of the lack of a suitable experimental model. We have previously reported that a clone (QR-32 cells) derived from 3-methylcholanthrene-induced fibrosarcoma undergoes spontaneous regression at the inoculated site in normal syngeneic C57BL/6 mice unless extremely large numbers of the cells are inoculated,[3,4] and that the coimplantation of QR-32 cells with a foreign body such as gelatin sponge permits the progressive growth of the tumors that develop *in vivo*. Inoculation of the resultant tumor without gelatin sponge into fresh mice resulted in lethal growth.[5] We have examined the mechanism responsible for the malignant tumor progression.

Juzen-taiho-to (TJ-48), one of the traditional Chinese medicines, is a mixture composed of ten herbs, as shown in Table I. It has been traditionally used to treat patients with anemia, anorexia or fatigue.[6] Recent studies have shown that *Juzen-taiho-to* has various biological activities: enhancement of phagocytosis,[7] cytokine induction,[8,9] antibody production,[10] induction of mitogenic activity of spleen cells,[11] augmentation of anti-tumor activities in combination with or without other drugs,[12,13] and protection from the deleterious effects of anti-cancer drugs[14] as well as radiation-induced immunosuppression and bone marrow toxicity.[15,16] However, the effect of *Juzen-taiho-to* alone on tumor progression or metastasis has not been reported.

We examined the effect of oral administration of *Juzen-taiho-to* on the progressive growth of QR-32 regressor tumor after coimplantation with gelatin sponge in a murine experimental model.

MATERIALS AND METHODS

Animals Specific pathogen-free female C57BL/6 mice, 6–8 weeks old, were purchased from Japan SLC, Inc., Hamamatsu. They were maintained in the Laboratory for Animal Experiments, Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University.

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Table I. Composition of *Juzen-taiho-to*

Herbs	Ratio
Astragali radix	3.0
Cinnamomi cortex	3.0
Rehmanniae radix	3.0
Paeoniae radix	3.0
Cnidii rhizoma	3.0
Atractylodis lanceae rhizoma	3.0
Angelicae radix	3.0
Ginseng radix	3.0
Hoelen	3.0
Glycyrrhizae radix	1.5

Medicine was prepared by blending herbs at the ratio indicated above.

Tumors The origin and characteristics of the tumor cells used in this experiment have been described previously.[3, 17] Briefly, BMT-11 is a transplantable mouse fibrosarcoma induced by 3-methylcholanthrene in a C57BL/6 mouse. We first isolated culture lines of BMT-11 and its clone, BMT-11 cl-9. After exposure of the tumorigenic BMT-11 cl-9 cells to quercetin, and cloning, we obtained QR-32 clone cells which spontaneously regress in normal syngeneic mice. QR-32 cells exhibit regression in mice after challenge with 2×10^5 cells (four times greater than the minimum take dose of the BMT-11 cl-9). Subcutaneous coimplantation of QR-32 cells with gelatin sponge caused progressive growth at inoculated sites, and then the resultant tumors (QRsP) were able to grow in mice even in the absence of gelatin sponge. QR-32 and QRsP cells were maintained as monolayer cultures in Eagle's minimum essential medium supplemented with 7.5% fetal calf scrum, sodium pyruvate, non-essential amino acids and L-glutamine.

Drugs *Juzen-taiho-to* (TJ-48) was kindly provided by Tsumura Co., Ltd., Tokyo. *Juzen-taiho-to* consists of spray-dried hot-water extracts of a mixture of ten medicinal plants at the ratios listed in Table I. The blended powder was dissolved in distilled water, and then administered orally to mice at appropriate doses. For example, 40 mg/day/mouse in this study is approximately 16 times the clinical dose of 7.5 g/day/adult. Bismuth subnitrate was purchased from Sanko Seiyaku Kogyo Co., Ltd., Tokyo.

Assay for *in vivo* growth of QR-32 cells after their coimplantation with gelatin sponge We used the weakly malignant clonal QR-32 cells to define tumor progression as the conversion of tumor cells to a more malignant phenotype that exhibits such properties as acquired tumorigenicity, invasive and metastatic potential and ultimately the ability to kill the host more rapidly.[5] Sterile gelatin sponge (Spongel, Yamanouchi Pharm. Co., Ltd., Tokyo) was cut into $10\times5\times3$ mm sections. Mice were anesthetized with ether and after their backs had been swabbed with 70% alcohol, an approximately 10-mm incision was made in the skin of each mouse on the right flank of the pelvic region. A pocket reaching up to the thorax was made under the skin with the tip of a sterilized scissors. One section of gelatin sponge was inserted under the skin away from the wound, and the wound was closed with sterile clips. QR-32 cells (1×10^5/0.1 ml) were then injected into the pre-inserted gelatin sponge. A tumor developed at the site of gelatin sponge implantation, but not at the wound site. In another experiment, the resultant tumor after the coimplantation with gelatin sponge was cultured, and named QRsP tumor. QRsP tumor cells were injected s.c. without gelatin sponge into mice, causing tumor development. To test the tumorigenicity of these cells, the growth of inoculated tumors was observed as a function of time, and tumor size was assessed by averaging the diameters of the short and long axes of tumor masses measured with vernier calipers. The survival periods of the tumor-bearing mice were also determined by allowing them to live until they succumbed to the tumor burden.

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

RESULTS

Inhibition by *Juzen-taiho-to* of the tumor growth after coimplantation of QR-32 cells with gelatin sponge We first examined the effect of *Juzen-taiho-to* on the growth of QR-32 tumors coimplanted with gelatin sponge (Fig. 1). Subcutaneous injection of 1×10^5 QR-32 cells alone resulted in spontaneous regression in normal mice within 25 days, as reported previously.[4] In contrast, coimplantation of QR-32 cells with gelatin sponge caused progressive growth of the tumor. Oral administration of *Juzen-taiho-to* at a dose of 40 mg/day/mouse for 7 days after tumor inoculation resulted in significant inhibition of tumor growth as compared with the control ($P<0.01$). The results clearly indicate that oral administration of *Juzen-taiho-to* was effective for preventing the gelatin sponge-elicited progressive growth of QR-32 cells.

Effects of *Juzen-taiho-to* on the survival of mice coimplanted with QR-32 cells and gelatin sponge *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 65 days after tumor inoculation (Fig. 2). Mice that had received treatment with *Juzen-taiho-to* had a marked prolongation of survival ($P<0.01$ by the Mann-Whitney U-test) as compared with the control group. All the mice that had been inoculated with QR-32 cells alone were

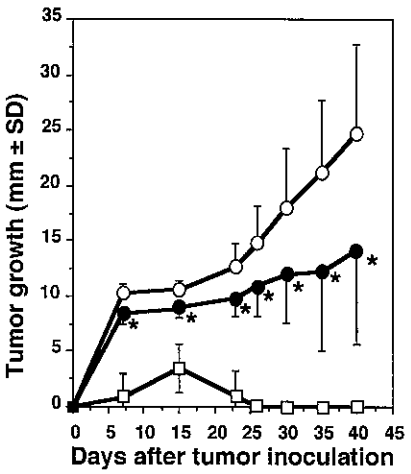

Fig. 1. Effect of *Juzen-taiho-to* on the growth of QR-32 fibrosarcoma after coimplantation with gelatin sponge. Five to ten C57BL/6 mice per group were inoculated s.c. with QR-32 cells (10^5) with or without gelatin sponge ($10\times5\times3$ mm size). *Juzen-taiho-to* (40 mg/day/mouse) was administered orally for 7 days after tumor inoculation. Tumor growth was measured as a function of time after implantation and calculated as average diameter (mm) of the long and short axes. (□), QR-32 alone; (○), QR-32+gelatin sponge; (●), QR-32+gelatin sponge+*Juzen-taiho-to*. * $P<0.01$ as compared with the control (QR-32+gelatin sponge) by Student's two-tailed t test.

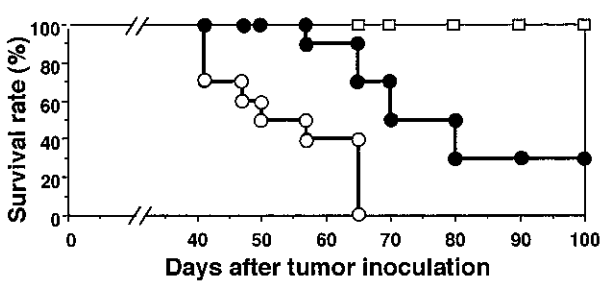

Fig. 2. Effects of *Juzen-taiho-to* on the survival of mice coimplanted with QR-32 cells and gelatin sponge. Treatment with *Juzen-taiho-to* (40 mg/day/mouse) was performed according to the same protocol as in Fig. 1. Animal survival was monitored as a function of time. (□), QR-32 alone; (○), QR-32+gelatin sponge; (●), QR-32+gelatin sponge+*Juzen-taiho-to*. $P<0.01$; QR-32+gelatin sponge+*Juzen-taiho-to* vs. QR-32+gelatin sponge by the Mann-Whitney U-test.

alive without tumor mass at the inoculated sites on day 100 after tumor inoculation. Metastasis to distal organs or regional lymph nodes was not apparent in dead mice. Oral administration of *Juzen-taiho-to* at the same dose to

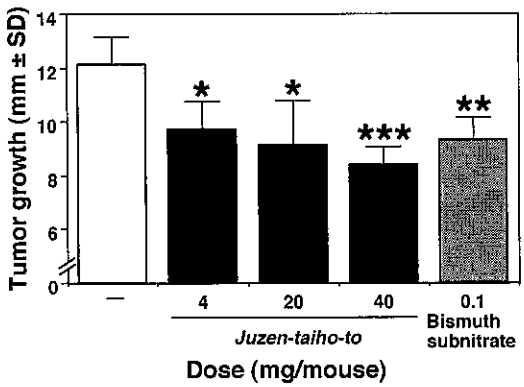

Fig. 3. Dose-dependent effects of *Juzen-taiho-to* on the growth of QR-32 cells coimplanted with gelatin sponge. Five C57BL/6 mice per group were inoculated s.c. with QR-32 cells (10^5) with or without gelatin sponge ($10\times5\times3$ mm size, □). *Juzen-taiho-to* (■) or bismuth subnitrate (▩) at the indicated doses was orally administered for 7 days after tumor inoculation. Tumor size was measured on day 25 when mice that received s.c. injection of QR-32 cells alone showed spontaneous regression, and calculated as average diameter (mm) of the long and short axes. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ as compared with the control by Student's two-tailed t test.

normal mice showed no discernible side effect. Therefore, tumor burden is thought to be the major cause of death.

Dose-dependent effects of *Juzen-taiho-to* on the growth of QR-32 cells coimplanted with gelatin sponge As shown in Fig. 3, gelatin sponge-induced tumor growth was inhibited by treatment with *Juzen-taiho-to* in a dose-dependent manner over the dose range from 4 to 40 mg/day ($P<0.05$–0.001), at the time (day 25) when mice that had received s.c. injection of QR-32 cells alone showed spontaneous regression. Also oral administration of bismuth subnitrate (0.1 mg/day), which induces metallothionein in the tumor tissue and reduces malignant progression,[18] resulted in a significant inhibition of tumor growth.

Effect of timing of *Juzen-taiho-to* administration on the growth of QR-32 cells after coimplantation with gelatin sponge Oral administration of *Juzen-taiho-to* (40 mg/day) for 7 days just before or after tumor inoculation significantly suppressed tumor growth on day 25. On the other hand, treatment with *Juzen-taiho-to* from day 8 to day 14 after tumor inoculation did not significantly affect the tumor growth (Fig. 4).

Inhibition of the growth of QRsP progressor tumor by *Juzen-taiho-to* Since the resultant tumor cells (QRsP) after the coimplantation of QR-32 cells with gelatin sponge were able to acquire the ability for progressive growth even in the absence of gelatin sponge,[5] we next

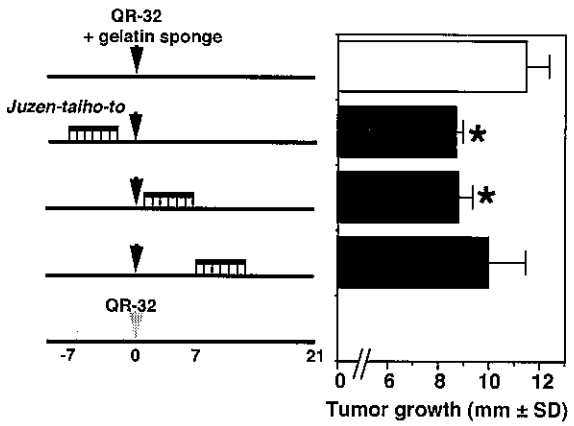

Fig. 4. Effect of administration timing of *Juzen-taiho-to* on the growth of QR-32 cells after coimplantation with gelatin sponge. Five C57BL/6 mice per group were inoculated s.c. with QR-32 cells (10^5) with or without gelatin sponge (↓). *Juzen-taiho-to* (40 mg/mouse) was given orally for 7 days at the indicated administration timing (▭). Tumor size was calculated as average diameter (mm) of the long and short axes on day 25 after tumor inoculation. * $P<0.01$ as compared with the control by Student's two-tailed t test.

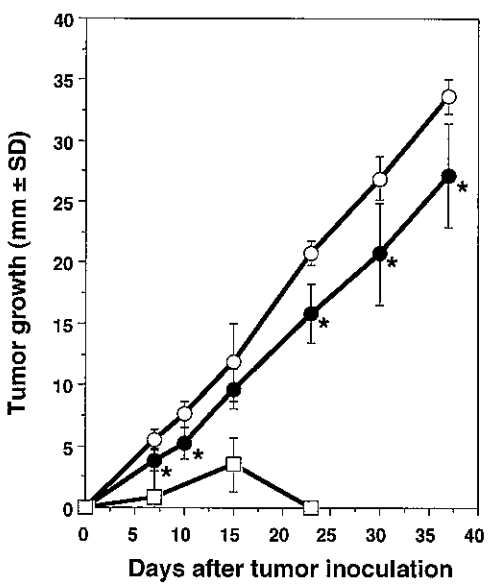

Fig. 5. Effect of *Juzen-taiho-to* on the *in vivo* growth of QRsP progressor tumor. Five C57BL/6 mice per group were injected s.c. with 1×10^5 QRsP tumor cells which had been obtained from tumors that grew progressively after coimplantation with gelatin sponge. *Juzen-taiho-to* (40 mg/day/mouse) was administered orally for 7 days after tumor inoculation. Tumor size was measured as a function of time after tumor inoculation and calculated as average diameter (mm) of the long and short axes. (□), QR-32 alone; (○), QRsP alone; (●), QRsP+*Juzen-taiho-to*. * $P<0.01$ as compared with the control by Student's two-tailed t test.

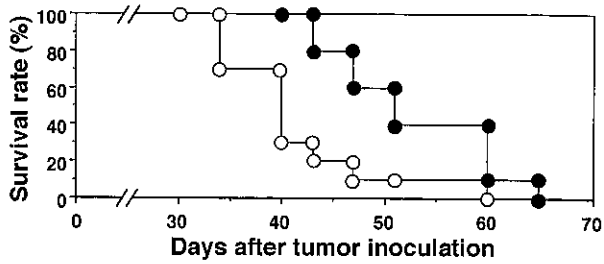

Fig. 6. Effect of *Juzen-taiho-to* on the survival of mice inoculated s.c. with QRsP progressor cells. Treatment with *Juzen-taiho-to* (40 mg/day/mouse) was performed according to the same administration schedule as in Fig. 5. Animal survival was monitored as a function of time. (○), QRsP alone; (●), QRsP+*Juzen-taiho-to*. $P<0.01$; QRsP+*Juzen-taiho-to* vs. QRsP alone by the Mann-Whitney U-test.

examined the effect of oral administration of *Juzen-taiho-to* on the *in vivo* growth of QRsP progressor tumor (Fig. 5). Oral administration of *Juzen-taiho-to* at a dose of 40 mg/day/mouse for 7 days after inoculation of QRsP cells (1×10^5) resulted in a significant reduction of tumor growth as compared with the control ($P<0.01$), but the degree of inhibitory effect was not as marked as that on the growth of QR-32 regressor tumor after coimplantation with gelatin sponge.

Effect of *Juzen-taiho-to* on the survival of mice inoculated s.c. with QRsP cells When QRsP progressor tumor cells were inoculated s.c. into mice, all the control mice succumbed to the tumor burden within 60 days (Fig. 6). Mice that had received treatment with *Juzen-taiho-to* showed significantly enhanced survival as compared with the control ($P<0.01$ by the Mann-Whitney U-test), but nevertheless, 9 out of 10 mice succumbed within 60 days.

DISCUSSION

We have previously reported that QR-32 cells derived from tumorigenic BMT-11 cl-9 cells were not able to grow progressively in normal syngeneic mice because of a decrease in the production of PGE_2,[4] which is considered to promote the *in vivo* growth of tumor cells by suppressing the anti-tumor immune defences of the host.[19–21] In contrast, when QR-32 regressor tumor was coimplanted s.c. with a foreign body such as gelatin sponge, the tumor cells irreversibly acquired the ability to grow progressively at the inoculated sites even without gelatin sponge.[5] We have also shown that such progressive growth of tumors was provoked through the enhancement of PGE_2 production in the tumors, or by oxygen radicals and inflammatory cytokines produced by host

cells reactive to the gelatin sponge.[5, 22, 23] This seems analogous to the fact that malignant progression of tumors followed by metastasis is clinically observed to be elicited by various factors and circumstances including stress, anti-cancer drugs, inflammation, etc. In our experimental progression model, the progressive growth after s.c. coimplantation of QR-32 cells with gelatin sponge was prevented by endogenous induction of antioxidative enzymes or scavengers such as manganese superoxide-dismutase (Mn-SOD) or metallothionein at the tumor sites by orally administered bismuth subnitrate and lipophilic vitamin C.[24]

The present study demonstrated that oral administration of *Juzen-taiho-to*, one of the traditional Chinese medicines, caused significant inhibition of the progressive growth of QR-32 regressor tumors after coimplantation with gelatin sponge (Figs. 1, 3 and 4) and prolonged the survival of tumor-bearing mice (Fig. 2). These results indicate that *Juzen-taiho-to* may be effective for preventing weakly malignant tumors from growing progressively upon coimplantation with gelatin sponge. Such progressive growth, however, may not necessarily be equivalent to malignant progression, because even cells which do not acquire a more malignant phenotype can show transient proliferation depending on the host circumstances and implantation conditions. As shown in Fig. 5, the resultant progressive tumor (QRsP) showed gelatin sponge-independent growth after re-inoculation, as also demonstrated previously.[5] This phenomenon could be regarded as progression.

On the other hand, oral administration of *Juzen-taiho-to* for 7 days after inoculation of QRsP cells resulted in a significant reduction of the tumor growth and enhancement of survival rate in tumor-bearing mice as compared with the control (Figs. 5 and 6), but such inhibitory effects were not as marked as those in the case of QR-32 regressor tumors after coimplantation with gelatin sponge. Since *Juzen-taiho-to* has been reported to possess anti-tumor effects based on the activation of macrophages,[7] cytokine induction,[8, 9] augmentation of NK cell activity,[25] etc., the inhibitory mechanisms of the progressive growth of QR-32 regressor cells and the growth of the resultant QRsP progressor cells may be in part associated with the induction of host-mediated immune surveillance by *Juzen-taiho-to*. However, oral administration of bismuth subnitrate, which induces metallothionein as a scavenger of oxygen radicals in the tumor tissue,[18] resulted in a significant inhibition of gelatin sponge-elicited progressive growth (Fig. 4). These results suggest that *Juzen-taiho-to* may act to induce antioxidants and to reduce PGE_2 production[4] during the progressive process, in addition to augmenting the host-mediated immune responses. Further study will be needed to examine whether or not *Juzen-taiho-to* is actually able to prevent malignant progression, i.e., to see whether tumors obtained from the *Juzen-taiho-to*-treated group grow progressively after re-inoculation without gelatin sponge.

Since several investigations have shown the inhibitory effects of ginsenosides (triterpenoid saponins) of Ginseng Radix, a constituent of *Juzen-taiho-to*, on carcinogenesis, tumor growth and metastasis,[26, 27] we speculate that ginsenosides may be partly responsible for inhibiting the gelatin sponge-induced progressive growth of QR-32 regressor cells and the growth of the resultant QRsP progressor cells. Kampo medicines, including *Juzen-taiho-to*, usually consist of several medicinal plants and contain various active components which will vary according to the harvesting place and time. Therefore, it would be useful to establish methods for pattern analysis of the components contained in such medicines, e.g., by using liquid chromatography and combined mass spectrometry, in order to minimize chemical and biological differences in batches of the medicine, although this is likely to be extremely difficult.

In conclusion, a traditional Chinese medicine, *Juzen-taiho-to*, may provide a therapeutic benefit in preventing progressive growth and subsequent tumor metastasis, although further investigation will be needed to examine in detail the mechanisms responsible for the inhibitory effect of oral administration of *Juzen-taiho-to* on progressive tumor growth *in vivo*.

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