Mao-to, a Kampo medicine, inhibits motility of highly metastatic osteosarcoma cells

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We used a cancer cell motility assay system for the mouse sera (MS) obtained from mice orally given Kampo medicines to find their ability to inhibit metastasis. The motility of the highly metastatic osteosarcoma cell, FBJ-LL, was significantly reduced by MS obtained from mice given Mao-to (TJ-27), but not influenced by those obtained from mice given either Juzen-taiho-to (TJ-48) or Hochu-ekki-to (TJ-41). A reduction in motility was also caused by MS with the addition of Mao-to (TJ-27), suggesting that the reduction can be caused by original compound(s) in Mao-to (TJ-27). Mao-to (TJ-27) caused 80% reduction in FBJ-LL cell motility at a concentration of 100 μg/ml without cytotoxicity, and completely suppressed proliferation of FBJ-LL cells at 1000 μg/ml. Accordingly, Mao-to (TJ-27) may be an inhibitor of metastasis at a low concentration. Mao-to (OMRC-K) inhibited the motility as well as Mao-to (TJ-27). Thus Mao-to generally has an inhibitory effect on the motility. Ephedrae herba and Cinnamomi cortex, which are the ingredients of Mao-to, inhibited cell motility, indicating that these herbs play a critical role in inhibition of cancer cell motility. These results raise the possibility that Mao-to is a candidate for a novel inhibitor of metastasis.

Key words Kampo medicine, Mao-to, metastasis, cell motility, Ephedrae herba, Cinnamomi cortex. Abbreviations MS, mouse serum; FBJ, Finkel-Biskis-Jenkins; FCS, fetal calf serum.

Introduction

Kampo medicines have been used as an adjunctive therapy following surgery, radiation therapy, or chemotherapy of cancer in Japan. The Kampo medicines play a crucial role in recovery from surgery and control of the side effects of radiation therapy and chemotherapy.1,2)

Recently the Kampo medicines have been reported to suppress cancer metastasis in the basic research. Juzen-taiho-to and Shimotsuki-to have been reported to prevent liver metastasis by murine colon carcinoma cells through activation of macrophages and T cells.5,6) Shichimotsuki-koka-to suppresses pulmonary metastasis of B16 melanoma cells by elevating nitric oxide production from macrophages.6) These Kampo medicines share four medical plants (Rehmanniae radix, Paeoniae radix, Cnidii rhizome and Angelicae radix) and suppress cancer metastasis through activation of the immune system. Sho-saiko-to has been reported to inhibit metastasis of malignant melanoma primarily developed in ret-transgenic mice, mediating regulation of the balance between expression levels of matrix metalloproteinase and a tissue inhibitor of the matrix metalloproteinase.9) These reports raise the possibility that Kampo medicines act as novel inhibitors of metastasis.

Metastasis involves numerous different biological processes including dissociation of cancer cells from primary tumor, invasion into surrounding stroma, intravasation, extravasation and generation of secondary tumors. Acceleration of motility of cancer cells is associated with an enhancement of their metastatic ability.10,11) We, therefore, investigated the effect of several Kampo medicines on cancer cell motility to determine if they were candidates of inhibitors of metastasis.

We used in the present study a novel cancer cell motility assay system for MS obtained from mice given the Kampo medicine. Because Kampo medicines are generally administered orally, components of the medicines can be metabolized to active compounds in the gut and tissue cells.12) The serum is assumed to contain both original compounds and metabolites of the Kampo medicine administered.

We chose three Kampo medicines, Juzen-taiho-to, Hochu-ekki-to, and Mao-to, and analyzed their effect on cancer cell motility. Juzen-taiho-to and Hochu-ekki-to are classified as "Hozai", which is a group of the formulations with protective and tonic effects, and have been used as an adjunct cancer therapy. Mao-to is, on the contrary, a representative of "Shazai", which is another group of the formulations with eliminative and purgative effects. Mao-to has generally indications for acute febrile conditions such as fever, chills, lumbago, headache, acute influenza and arthritis, and has never been used for chronic consumptive diseases like malignant diseases. However, it was realized in the present studies that Mao-to can inhibit cancer cell motility without cytotoxicity at low concentrations. On the other hand, Juzen-taiho-to and Hochu-ekki-to had no effect on cancer cell motility. Our results suggest that Mao-to is

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effective as an anti-metastasis medicine.

**Materials and Methods**

**Materials.** Preparations of *Mao-to* (TJ-27), *Jazen-taiho-to* (TJ-48), and *Hochu-ekki-to* (TJ-41) were kindly provided by Tsumura Co. (Tokyo, Japan). The ingredients of these Kampo medicines are shown in Table 1-1, 1-2, and 1-3. Ephedra herba and Glycyrrhizae radix were purchased from UCHIDA WAKANYAKU Co. Ltd. (Tokyo, Japan). Armeniacae semen was purchased from Tsumura Co. (Tokyo, Japan). Cinnamomi cortex was purchased from TochimotoTenkaido, Co. (Osaka, Japan). *Mao-to* (OMRC-K) was prepared in our laboratory as follows. A total (15.5 g) of Armeniacae semen (5.0 g), Ephedrae herba (5.0 g), Cinnamomum cortex (4.0 g) and Glycyrrhizae radix (1.5 g) and 600 ml of distilled water were mixed and boiled until its quantity was halved. The extracted solution was filtered, and the filtrate was lyophilized. Single extracts of Armeniacae semen (5.0 g), Ephedrae herba (5.0 g), Cinnamomi cortex (4.0 g) and Glycyrrhizae radix (1.5 g) were prepared as follows. Each herb and 600 ml of distilled water were mixed and boiled until its quantity was halved. The extracted solution was filtered, and the filtrate was lyophilized. The yields of extracts of *Mao-to* (OMRC-K) and each herb are shown in Table 2.

**Cell line.** Highly metastatic FBJ-LL cells were obtained from the Finkl-Biskis-Jenkins (FBJ) virus-induced osteosarcoma of Balb/c mouse and metastasized into the liver and lung as described by Yamagata et al.13 We showed previously that cell motility is induced by fetal calf serum (FCS).19 FBJ-LL cells were cultured in RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% FCS (SIGMA, St. Louis, MO, USA) at 37°C under a humidified atmosphere of 95% air and 5% CO2.

**MS and motility assay.** We used Balb/c mice to obtain MS, because FBJ-LL cells were established from osteosarcoma of Balb/c mouse. Whole blood was collected from an

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**Table 1-1** The ingredients and botanical origins of *Mao-to* formula

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Botanical origin</th>
<th>Representative compounds</th>
<th>Weight ratio</th>
</tr>
</thead>
<tbody>
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<td>Armeniacae semen</td>
<td><em>Prunus armeniaca</em> L. var. <em>ansa MAXIM</em></td>
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<td>Cinnamomi cortex</td>
<td><em>Cinnamomum cassia</em> <em>BLUME</em></td>
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<tr>
<td>Glycyrrhizae radix</td>
<td><em>Glycyrrhiza uralensis</em> <em>FISHER</em></td>
<td>Glycyrrhizin</td>
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</table>

**Table 1-2** The ingredients and botanical origins of *Jazen-taiho-to* formula

<table>
<thead>
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<td>Angelicae radix</td>
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<td>Cudii rhizoma</td>
<td><em>Cnidium officinale</em> <em>MAKINO</em></td>
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<td>Rehmanniae radix</td>
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<tr>
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<td>Astragali radix</td>
<td><em>Astragalus membranaceus</em> <em>BUNGE</em></td>
<td>Formonoetin</td>
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<tr>
<td>Cinnamomi cortex</td>
<td><em>Cinnamomum cassia</em> <em>BLUME</em></td>
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**Table 1-3** The ingredients and botanical origins of *Hochu-ekki-to* formula

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<td><em>Panax ginseng</em> <em>C. A. MEYER</em></td>
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<td>Glycyrrhizin</td>
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<td>Auranti nobilis pericarpium</td>
<td><em>Citrus unshiu</em> <em>MARKOVICH</em></td>
<td>d-Limonene</td>
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<td>Zingiberis rhizoma</td>
<td><em>Zingiber officinale</em> <em>ROSACE</em></td>
<td>Zingiberol</td>
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<td>Zizyphi fructus</td>
<td><em>Zizyphus jujuba</em> Miller var. <em>inermis</em> <em>REHDER</em></td>
<td>Zizyphus saponin</td>
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<td><em>Cimicifuga simplex</em> <em>WORMSK</em></td>
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abdominal aorta of a specific pathogen-free female Balb/c mouse (8 weeks old) to prepared MS. FBJ-LL cells (5 x 10⁴ cells) were suspended in 100 μl RPMI 1640 medium containing varying concentrations of MS into each of the upper wells of the Transwell (Corning Costar Corporation, Cambridge, MA). To the lower wells 600 μl RPMI 1640 medium containing respective concentrations of MS was added. At 24 h of incubation, cells migrating to the lower wells were counted.

Preparation of MS after oral administration of the *Kampo* medicine. A dose (20 mg) of *Mao-to* (TJ-27), *Juzen-taiho-to* (TJ-48) or *Hochu-ekki-to* (TJ-41) was dissolved in 500 μl of sterile distilled water, and it was administered to each of two given Balb/c mice orally twice a day for 3 days. The control mouse received 500 μl of sterile distilled water. Whole blood was collected from each group of mice on the following 4th day morning. The MS was used for the Transwell assay at a concentration of 0.5%. All animal experiments were performed in accordance with the guidelines for the welfare of animals in experiments in The Kitasato Institute.

Preparation of mixtures of MS and the *Kampo* medicine. Each of *Mao-to* (TJ-27), *Juzen-taiho-to* (TJ-48), *Mao-to* (OMRC-K), Ephedra herba extract, Armeniaca semen extract, Cinnamomi cortex extract and Glycyrrhiza radix extract was mixed with RPMI 1640 medium to the concentration of 10 mg/ml at 37 °C for 30 min. Each solution was centrifuged at 1200 x g for 15 min to remove insoluble materials, and then the supernatant was sterilized by filtration with a disposable syringe filter unit, DISMIC-25cs (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The solution was diluted with RPMI 1640 medium containing 0.5% MS to test the *Kampo* medicines as described in the results. These media were used in the Transwell assay and analyses of cell viability and proliferation.

Analyses of cell viability and proliferation. Cell viability was analyzed as follows. FBJ-LL cells were suspended at 5 x 10⁴ cells in 100 μl RPMI 1640 medium containing 0.5% MS with or without the *Kampo* medicine as indicated in the results in each well of a 96-well plate (IWAKI, Tokyo, Japan) and incubated at 37°C for 24 h. To each well was added 10 μl of Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). After incubation at 37°C for 2 h, the absorbance (450 nm) of formazan generated in each well was measured with a model 680 microplate reader (BIO-RAD laboratories, Inc., CA, USA). Cell growth was analyzed as follows. FBJ-LL cells were suspended at 1 x 10⁴ cells in 100 μl RPMI 1640 medium containing 10% FCS with or without the *Kampo* medicine in each well of the 96-well plate and incubated at 37°C for up to 72 h as indicated in the results. To each well was added 10 μl of Cell Counting Kit-8. After incubation at 37°C for 2 h, the absorbance (450 nm) of formazan generated in each well was measured with the model 680 microplate reader.

Statistical analysis. All data represent mean ± S.D. The data were analyzed by ANOVA. Subsequently the significant differences among groups were determined by Scheffe’s test and the significant difference between control and treatment groups was determined by Dunnett’s test. Values of p<0.05 and p<0.01 were considered significant.

Results

Cell motility assay using MS. To establish the condition of cell motility assay, we examined FBJ-LL cell motility induced by MS. The number of cells migrating into the lower well of the Transwell increased with increase of the MS concentration up to 1% (Fig. 1). The concentration of MS adequate for the assay was estimated at 0.5%, since 0.5% MS-induced motility was significantly higher than that at 0% MS, almost the same as that at 1% MS.

Effect of *Kampo* medicines on FBJ-LL cell motility. *Mao-to* (TJ-27), *Juzen-taiho-to* (TJ-48), *Hochu-ekki-to* (TJ-41), or water as a control, was administered orally to two mice per group, and the whole blood was collected in 4 days. Mouse sera were prepared from the whole blood. These sera were used for the motility assay system. The number of migrated cells was significantly reduced by sera obtained from mice given *Mao-to* (TJ-27) (Fig. 2). There was no significant difference in the number of migrated cells between control serum and sera obtained from mice after administration of *Juzen-taiho-to* (TJ-48) or *Hochu-
ekki-to (TJ-41). These results indicate that the serum obtained from mice given Mao-to (TJ-27) have an inhibitory effect on cancer cell motility.

**Effect of Mao-to (TJ-27) on the motility and viability of FBJ-LL cells.** We examined the motility of FBJ-LL cells in the presence of MS with various concentrations of Mao-to (TJ-27) to clarify the ability of the original compounds of Mao-to (TJ-27) in inhibition of the motility. Juzen-taiho-to (TJ-48) was used as a negative control. The motility was decreased dependently on the concentration of Mao-to (TJ-27), suggesting that original compound(s) in Mao-to (TJ-27) inhibit the cell motility (Fig. 3A). The motility was unchanged by addition of 0.1 to 100 μg/ml of Juzen-taiho-to (TJ-48). A high concentration, 1000 μg/ml, of Juzen-taiho-to (TJ-48) suppressed the motility.

Furthermore, we analyzed the cytotoxicity of Mao-to (TJ-27) and Juzen-taiho-to (TJ-48) under the same medium for the motility assay. The viability of FBJ-LL cells was examined in the presence of MS with various concentrations of Mao-to (TJ-27) or Juzen-taiho-to (TJ-48). Each of the two Kampo medicines had no effect on the cell viability at concentrations of 10 and 100 μg/ml (Fig. 3B). These results indicate that Mao-to (TJ-27) inhibits the cell motility at low concentrations (~100 μg/ml) without cytotoxicity. The viability of cells was decreased significantly by 1 mg/ml of Mao-to (TJ-27) and Juzen-taiho-to (TJ-48) (Fig. 3B), both of which caused a remarkable reduction in the cell motility at 1 mg/ml (Fig. 3A). Therefore, the reduction of cell motility by 1 mg/ml of both Kampo medicines may be caused by their cytotoxicity.

**Effect of Mao-to (TJ-27) on proliferation of FBJ-LL cells.** Mao-to (TJ-27) had no effect on FBJ-LL cell growth at concentrations of 10 and 100 μg/ml and completely inhibited the cell growth at a concentration of 1 mg/ml (Fig. 4A). Juzen-taiho-to (TJ-48) had no effect on FBJ-LL cell growth at concentrations of 10 and 100 μg/ml and significantly
inhibited the cell growth at a concentration of 1 mg/ml (Fig. 4B). At a high concentration (1 mg/ml), Mao-to (TJ-27) showed a stronger inhibition on the cell growth than Juzen-taiho-to (TJ-48).

Comparison of effect on the cell motility among Mao-to (TJ-27), Mao-to (OMRC-K) and the reconstituted Mao-to. Mao-to (OMRC-K) was prepared by extraction of Mao-to formula composed of four herbal medicines (Table 1-1). Each herb was extracted with water to make the reconstituted Mao-to. The yield of each herb extract and the ratio of these extracts in the reconstituted Mao-to are shown in Table 2. The reconstituted Mao-to was made as follows. Extracts of Ephedrae herba, Armeniacae semen, Cinnamomi cortex and Glycyrrhizae radix were mixed in the proportion of 0.409 : 0.265 : 0.124 : 0.202, in accordance with the extraction yields. The inhibitory effect of these three kinds of Mao-to on FBJ-LL cell motility was investigated; both Mao-to (OMRC-K) and the reconstituted Mao-to greatly inhibited the motility as well as Mao-to (TJ-27) (Fig. 5).

Effect of ingredients of the Mao-to on the motility of FBJ-LL cells. Effect of each ingredient of the Mao-to on
the motility of FBJ-LL cells was investigated. We examined the motility of FBJ-LL cells in the presence of MS with 100 µg/ml Miao-to (OMRC-K), or each extract of 40.9 µg/ml Ephedra herba, 26.5 µg/ml Armeniaceae semen, 12.4 µg/ml Cinnamomi cortex and 20.2 µg/ml Glycyrrhiza radix according to the yields of these extracts (Table 2). The inhibitory effect of Ephedra herba on the motility was as large as that of Miao-to (OMRC-K), and Cinnamomi cortex significantly inhibited the cancer cell motility, whereas Armeniace semen and Glycyrrhiza radix had no apparent effect (Fig. 6A). A high concentration of each herb extract, 40 µg/ml, of Ephedra herba, Armeniaceae semen and Cinnamomi cortex significantly inhibited the motility of FBJ-LL cells (Fig. 6B). Especially, Cinnamomi cortex showed almost the same effect of Ephedra herb at 40 µg/ml.

Growth inhibiting effect of Miao-to (OMRC-K) and its ingredients on FBJ-LL cells. Effect of the Miao-to and its ingredients on the growth of FBJ-LL cells was investigated at the cytotoxic level of Miao-to. Ephedra herba (409 µg/ml) suppressed proliferation of FBJ-LL cells as well as the Miao-to (1 mg/ml). However, other herbs had no effect on the growth (Fig. 7). These results suggest that Ephedra herba plays a critical role in Miao-to in suppression of cancer cell growth.

Discussion

We have found for the first time that Miao-to (TJ-27) can suppress migration of cancer cells. The MS with the addition of Miao-to (TJ-27) has an inhibitory effect on cancer cell motility as well as MS obtained from mice orally given Miao-to (TJ-27), suggesting that original compound(s) in Miao-to (TJ-27) play a crucial role in inhibition of the motility. There was found the same inhibitory effect in Miao-to (OMRC-K) (Fig. 5). Therefore, it is suggested that Miao-to generally has the inhibitory effect on motility.

Miao-to suppressed cancer cell motility at low concentrations without effect on cancer cell proliferation (Fig. 3A and 4A). Accordingly, Miao-to may inhibit cancer metastasis without cytotoxicity. In contrast, Juzen-taiho-to had essentially no effect on either cancer cell motility or proliferation.
These results indicate that Mao-to has a direct effect on cancer cell migration, and Juzen-taiho-to acts differently. In fact, Juzen-taiho-to has been reported apparently to suppress cancer metastasis through activation of immune system.\(^6\)

Mao-to to achieve inhibition of cancer metastasis may be required to be administered for a long term, although it is usually considered to be applicable to short term prescriptions because of the side effects of Ephedra herba such as gastrointestinal disturbance, palpitation, hypertension and oliguria. However, differences in the period of administration and the target symptom of the formulae containing Ephedra herba can be allowed by changing the content of the herb in the therapy of diseases with Oriental medicines. The formulae, Mao-to, Dai-seiryu-to, and Epip-i-jiutsu-to, containing daily doses of 5 to 6 g of Ephedra herba are used for the acute stages of infection diseases like influenza, arthritis with fever, lumbago and headache.\(^{15,16}\) On the other hand, the formulae containing daily doses of 1 to 1.5 g of Ephedra herba such as Goshaku-san and Bofu-tsusho-san are used for lifestyle-related diseases for a long time.\(^{17}\) We have shown in the present experiments that both Mao-to and Ephedra herba extracts can effectively suppress the cancer cell motility at low concentrations with no effect on the cell viability. Therefore it is proposed that Mao-to can be used as an inhibitor of metastasis of cancer with reduction in daily doses, and that the formulae of low contents of Ephedra herba are preferable.

Cinnamomi cortex contributes weakly to the inhibitory effects of Mao-to on cancer cell motility (Fig. 6A). However the herb inhibited cancer cell motility as well as Ephedra herba at the concentration of 40 μg/ml (Fig. 6B). The content of Cinnamomi cortex in Mao-to is insufficient to inhibit the cancer cell motility, because the amount of the herb extract is one third that of Ephedra herba extract (Table 2). Thus, the inhibitory effects of Mao-to may be elevated by increasing the content of Cinnamomi cortex. Or it is possible that the side effects are removed from Mao-to with keeping its inhibitory effect on cancer cell motility by decreasing the content of Ephedra herba and increasing the content of Cinnamomi cortex.

Glycyrrhizae radix had no effect on cancer cell motility (Fig. 6B). But, the herb is an important ingredient in Mao-to because it attenuates the side effect of Ephedra herba and reinforces main effects of the prescription.

The molecular mechanisms for inhibitory effects of Ephedra herba and Cinnamomi cortex on the motility have not been clarified yet. We are currently analyzing the mechanism for suppression of migration by these ingredients.

Cinnamic acid, which is one of the principal components in Cinnamomi cortex, has been reported to reduce invasive capacity of metastatic melanoma cells associated with modulation of expression of genes implicated in tumor metastasis (MMP-2 and TIMP-2).\(^{18}\) Therefore, Cinnamic acid may participate in the inhibitory effects of Cinnamomi cortex.

In conclusion, our findings clearly indicate that Mao-to has a strong inhibitory effect on cancer cell motility. We are now investigating the effect of Mao-to on metastasis in vivo.

Acknowledgements

We are grateful to Dr. Takato Yoshida (Photon Medical Research Center Hamamatsu University School of Medicine) and Dr. Nakaaki Ohsawa (Aino Institute for Aging Research) for critical discussions, and to Tsunuma Co. for kindly providing the Kampo medicines. We thank Miss Manami Fujimoto for her assistance of this research.

References

Mao-to inhibits cancer cell motility


**Japanese abstract**

漢方薬の癌転移抑制剤としての可能性を探るために、癌細胞の転移能と高い相関性を示す細胞機能を用い、漢方薬を投与したマウスの血清を用い、高転移性癌細胞の運動能を指標としたアッセイ系を確立した。本アッセイ系により、ツムラより提供された全大補湯（TJ-48）、補中益気湯（TJ-41）及び麻黄湯（TJ-27）を調べた結果、麻黄湯（TJ-27）に強い運動能抑制活性が見出された。当研究所薬剤部から提供された麻黄湯（OMRC-K）を経してエキス剤とし、麻黄湯（TJ-27）と比較したところ、同様の運動能抑制活性が認められたことから、麻黄湯の癌細胞運動能抑制作用は生薬の産地に関係なく存在することが示唆された。さらに、麻黄湯は癌細胞の増殖に影響を与えない低濃度で運動能を抑制することが明らかになった。麻黄湯の各構成生薬（麻黄、杏仁、桂枝）を経してエキス剤とし、運動能に対する効果を解析した結果、麻黄及び桂枝に抑制活性が見られた。以上の結果は麻黄湯に癌転移抑制剤候補としての可能性があることを示している。

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