Suppressive Effect of Shichimotsu-koka-to (Kampo Medicine) on Pulmonary Metastasis of B16 Melanoma Cells

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Shichimotsu-koka-to (SKT) is a Kampo (traditional Japanese herbal) medicine, which is used in Japan to treat hypertension and atherosclerosis. We investigated the inhibitory effect of SKT on experimental pulmonary metastasis of B16 melanoma cells. The intake of SKT at a dose of 430 mg/kg for 6 weeks from 2 weeks before tumor inoculation significantly reduced the number of metastatic surface nodules in the lung and extended the life span. When the duration of SKT intake was examined, survival time was not affected by preintake before B16 melanoma cell inoculation and was slightly extended by postintake after B16 melanoma cell inoculation, although the life span was prolonged by intake throughout the experiment. To address the mechanism underlying the antimetastatic effect of SKT, we studied whether SKT modulated macrophage function, which is involved in killing tumor cells. The intake of SKT for 6 weeks dose dependently increased nitric oxide (NO) production by macrophages following stimulation with lipopolysaccharide. The elevated NO was found to serve as a cytotoxic mediator against B16 melanoma cells in co-culture with macrophages. On the contrary, B16 melanoma-conditioned medium reduced NO production by macrophages. However, SKT treatment reversed the reduction in NO production by the conditioned medium significantly. These findings may suggest that macrophage function-modulating activity by SKT appears to underlie its antimetastatic activity, which leads to a decrease in the number of lung metastatic surface nodules and the extension of life span.

Key words Shichimotsu-koka-to; Kampo medicine; metastasis; nitric oxide; macrophage

The lung, as well as the liver, is one of the organs most frequently involved in metastatic deposits from primary tumors.1–5 Tumor cell metastasis is a complex, multistep process that involves cell separation from the primary tumor, entry into the vascular and lymphatic systems, transport to and arrest within the microcirculation of distant organs, and extravasation.4,5 The emergence of metastasis in organs distant from the primary tumor is the most devastating aspect of cancer. From this point of view, various inhibitors, such as inhibitors of angiogenesis and matrix metalloproteinase, are presently being developed as novel therapeutic drugs.

Several Kampo medicines, such as Keishi-ka-kei-to, Juzen-taiho-to, Shimotsu-to, Unsei-in, Hochu-ekki-to, and Shosaiko-to have been so far reported to exhibit an antimetastatic effect.6–9 Among them, Juzen-taiho-to reduces liver metastasis by colon 26-L5 carcinoma cells as well as pulmonary metastasis by B16-BL6 melanoma cells, and its antimetastatic effect appears likely to be mediated by the activation of macrophages and/or T cells in the host immune system.7 Keishi-ka-kei-to is also able to inhibit the pulmonary metastasis of B16 melanoma cells and its effect is in part due to the stimulation of CD8+ T cells.9 Shosaiko-to suppresses pulmonary metastasis induced by Lewis lung carcinoma cells, and the enhanced number of peritoneal macrophages and elevated binding of C3 cleavage products to macrophages after Shosaiko-to treatment may be related to its antimetastatic effects.9 However, there have been few studies of Kampo medicines in the metastatic setting.

Shichimotsu-koka-to (SKT), which is used to treat hypertension, especially essential and renal hypertension, and atherosclerosis, consists of seven medicinal plants (Paeoniae Radix, Angelicae Radix, Astragali Radix, Rehmanniae Radix, Cnidii Rhizoma, Phellodendri Cortex, and Uncariae Uncus et Ramulus), some of which have been studied to clarify their pharmacological actions. SKT shares four medicinal plants (Rehmanniae radix, Paeoniae radix, Cnidii rhizoma, Angelicae radix) with Shimotsu-to and Unsei-in, which exhibit antimetastatic effects against colon26-L5 carcinoma cells-induced liver metastasis. In addition, SKT shares five medicinal plants (Astragali Radix, Rehmanniae Radix, Paeoniae Radix, Cnidii Rhizoma, Angelicae Radix) with Juzen-taiho-to, which also has antimetastatic effects. However, in addition to its antimetastatic effect, the pharmacological and biological actions of SKT have not been thoroughly investigated to date. A few reports found that SKT decreases systolic blood pressure and attenuates glomerular sclerotic lesions in the kidney after 4 weeks of treatment in salt-induced hypertensive Dahl strain rats,10 and shows radical scavenging activity by increasing superoxide dismutase activity and decreasing xanthine oxidase activity in the brain.11 SKT is able to quench superoxide anion as determined by electron-spin resonance analysis in vitro. Considering that reactive oxygen species play an aggravating role in the stimulation of metastasis, SKT is expected to suppress tumor metastasis. Pharmacological and biochemical studies indicate that immunomodulating activity is found in many Kampo medicines, which rarely produce side effects even after long-term administration.

Taken together, it is worth studying the antimetastatic effect of Kampo medicines. Therefore, in the present study, we examined the antimetastatic effect of SKT using a mouse pulmonary metastasis model.

MATERIALS AND METHODS

Animals Male C57BL/6 mice were purchased from Charles River (Hino, Japan). They were housed in a temperature-controlled room (at 23±2°C) with lighting from 06:00 to 18:00 under specific pathogen-free conditions. The humidity was automatically maintained at 50±10%. They received
a commercial diet (CE-2; Nippon Crea Co. Ltd., Shizuoka, Japan) and water ad libitum. All mice were 8 weeks of age when tumor cells were injected intravenously.

**Tumor Cells** B16 (C57BL/6 mice, melanoma) cells were obtained from Health Science Research Resources Bank (Sennan, Osaka, Japan). B16 melanoma cells were cultured in MEM-Eagle’s salt medium (nonessential amino acids, Irvine Scientific Co., Santa Ana, CA, U.S.A.) supplemented with 10% fetal calf serum (FCS) with penicillin 100 U/ml and streptomycin 100 μg/ml (Life Technologies Inc., Grand Island, NY, U.S.A.).

**Preparation of SKT** SKT (human daily dose) was prepared as follows. Rehmanniae Radix 3.0 g, Angelicae Radix 3.0 g, Cnidii Rhizoma 3.0 g, Paeoniae Radix 3.0 g, Phellodendri Cortex 2.0 g, Uncariae Uncus et Ramulus 4.0 g, and Astragali Radix 3.0 g were added to 500 ml of water, decocted for 1 h, and concentrated to 250 ml. This decoction was lyophilized to give 5.1 g of extract. In this extract, paoniflorin, berberine, ferulic acid, and rhynchophylline were contained as the main components (1.06, 0.50, 0.04, and 0.03, respectively). All of these extracts were kindly provided by Teikoku Pharmaceutical Co., Ltd. (Tokushima, Japan).

**Pulmonary Metastasis Model** Male C57BL/6 mice were injected intravenously with 2×10^5 or 1×10^5 cells/mouse of cultured B16 melanoma cells. Mice injected with 2×10^5 cells were killed 28 d after injection of B16 melanoma cells and the lung was removed. The number of metastatic colonies in the lung were macroscopically counted and their weights were measured. On the other hand, mice injected with 1×10^5 cells were allowed to live until they succumbed to the metastatic tumor burden in the lung. Various concentrations of SKT were dissolved in distilled water and mice received them ad libitum as described in the figure legend.

**Preparation of Conditioned Medium** B16 melanoma cells (3×10^5 cells/ml) were maintained in MEM-Eagle’s salt medium supplemented with 10% FCS for 24 h. Then B16 melanoma-conditioned medium was prepared by incubation of B16 melanoma cells in serum-free MEM-Eagle’s salt medium for 12 h. The conditioned medium was centrifuged at 1000 rpm for 5 min to remove cellular components and stored at −80 °C until use. Control conditioned medium was prepared according to this procedure but without cells.

**Statistical Analysis** Data are expressed as mean±S.E. with the number of animals. Statistical significance was determined by non-paired Student’s t-test, or Dunnett’s test using Stat Light software. p values less than 0.05 were considered significant.

**RESULTS**

To evaluate the effect of SKT on pulmonary metastasis, we first examined the effect of SKT on the number of lung nodules and the weight of the lung in pulmonary metastasis model mice injected with B16 cells. The intake of SKT and B16 cells injection were scheduled as described in the legend to Fig. 1. At 28 d after B16 cells injection, mice were killed and lung weight and the number of lung surface nodules were measured (Figs. 1A, B). The weight of the lung increased significantly in the control group, whereas the intake of SKT, especially at 5 times the human daily dose (430 mg/kg), significantly reduced the lung weight compared with the control group (Fig. 1A). Similarly, about 400 lung nodules were formed in the lungs of control mice, but the intake of SKT at a dose of 430 mg/kg significantly decreased the number of lung nodules to about 200.

We examined the effect of daily intake of SKT dissolved in drinking water and the duration of the intake of SKT on the life span of C57BL/6 mice (Fig. 2). Mice were given SKT at a dose of 430 mg/kg according to three different protocols. In the first group, mice were given SKT for 2 weeks starting 2 weeks before B16 melanoma cells injection (Fig. 2A). In the second group, mice were given SKT from 5 d after B16 melanoma cells injection until all mice succumbed.
LPS stimulation infiltrated into B16 melanoma cell culture. We then assayed whether NO generated by macrophages following LPS stimulation of peritoneal macrophages was harvested as described in Materials and Methods. Following the stimulation of macrophages with LPS 10 μg/ml for 20 h, the amount of NO generated in culture medium was determined (Fig. 3). Although antimetastatic activity shown by SKT was most effective at 5 times the human daily dose, SKT dose dependently increased NO concentration in B16 melanoma cell culture, and decreased the viability of B16 melanoma cells. LPS stimulation increased NO concentration in B16 melanoma cell culture, and the NO concentration in B16 melanoma cell culture was much higher in the co-culture with macrophages prepared from mice treated with SKT 170, 430, and 850 mg/kg, respectively. Each column represents mean±S.E. of 4 wells. Statistical significance was determined by Dunnett’s test using Stat Light software.

It is known that tumor cells effectively suppress macrophage functions to escape the host immunosurveillance system. NO production by macrophages was reduced by co-culture with B16 melanoma cells, although data were not shown. We therefore tested the effect of B16 melanoma-conditioned medium on NO production and the inhibitory effect of SKT. Macrophages were prepared from C57BL/6 mice that received water containing various doses of SKT for 6 weeks and incubated in 75% B16 melanoma-conditioned medium for 20 h in the presence or absence of LPS. B16 melanoma-conditioned medium markedly decreased NO pro-
**DISCUSSION**

In the present study, we evaluated the effect of the Kampo medicine SKT on pulmonary metastasis of B16 melanoma cells and found that the long-term intake of SKT reduced the number of pulmonary metastatic nodules and extended the life span significantly. The most effective dose of SKT was 430 mg/kg, which corresponds to 5 times the human daily dose, and intake throughout the experimental period prolonged the life span significantly.

The process of metastasis involves a series of sequential steps in which malignant cells are released from the primary tumor, disseminate to distant sites via lymphatic and/or circulatory systems, arrest within the microcirculation of distant organs, extravasate, and proliferate in target organs. The metastasis model used in the present study represents only the process following transport of tumor cells by the circulatory systems, arrest within the microcirculation of distant organs, extravasate, and proliferate in target organs. The metastasis model used in the present study represents only the process following transport of tumor cells by the circulatory systems, arrest within the microcirculation of distant organs, extravasate, and proliferate in target organs. The metastasis model used in the present study represents only the process following transport of tumor cells by the circulatory systems, arrest within the microcirculation of distant organs, extravasate, and proliferate in target organs.

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If the enhanced NO production by macrophages is involved in the antimitastatic effect of SKT, long-term intake should be necessary to reduce metastasis and extend the life span. NO has been identified as a major regulator of vascular tone and as a major defense molecule of mononuclear phagocytes against parasites, bacteria, and tumor cells. **Recent accumulating evidence indicates that the role of NO in metastasis appears to depend on the cells that produce NO and the subtypes of NO synthase (NOS). For example, NO, which is produced by endothelial NOS, appears likely to promote metastasis by maintaining vasodilator tone in the blood vessels in and around the melanoma.**

We considered whether it is possible that the antimitastatic effect of SKT resulted from another mechanism. SKT is known to show radical-scavenging activity by increasing superoxide dismutase activity and decreasing xanthine oxidase activity in the brain, and to quench superoxide anion as determined by electron-spin resonance analysis in vitro. Reactive oxygen species (ROS), which are generated by tumor or endothelial cells, are known to play important roles in tumor invasion and metastasis. Expression of P-selectin increases with the exposure of pancreatic tumor cells to ROS, and expression of VLA-4 on B16 melanoma cells also increases...
in response to hydrogen peroxide,\textsuperscript{30}) although these cell adhesion molecules are involved in the recruitment of metastatic tumor cells to a target organ. Scavenging of ROS leads to a reduction in metastasis, evidenced by the fact that catalase or superoxide dismutase effectively suppresses metastasis in an experimental metastasis model.\textsuperscript{31—33}) SKT may effectively prevent metastasis by scavenging ROS, although SKT is not likely to prevent tumor adhesion to endothelial cells as described above.

In the present study, the antimetastatic activity was highest at 5 times the human daily dose of SKT (430 mg/kg), whereas NO production by macrophages was enhanced dose dependently by SKT treatment. This indicates that the antimetastatic activity results from various effects of SKT, including the enhancement of NO production by macrophages, reversal of NO production suppressed by B16 melanoma cells, radical scavenging etc.

In conclusion, SKT shows antimitastatic activity against pulmonary metastasis of B16 melanoma cells, and that activity is at least in part due to stimulation of NO production by macrophages and the reversal of NO production by macrophages suppressed by B16 melanoma cells. The detailed mechanism underlying the antimitastatic effect of SKT is currently under investigation. An antimitastatic agent with the ability to enhance macrophage function has never been developed, so SKT should be a potential candidate for a new antimitastatic agent or an auxiliary to other antimitastatic agents.

REFERENCES