Antiproliferative Effects of the Traditional Chinese Medicine Shimotsu-To, Its Component Cnidium Rhizome and Derived Compounds on Primary Cultures of Mouse Aorta Smooth Muscle Cells

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ABSTRACT — Antiproliferative effects of the Japanese-Sino medicine Shimotsu-to (a combined prescription of cnidium rhizome, angelica root, peony root and rehmannia root) were investigated in the primary culture of smooth muscle cells (SMC) of mouse aorta. Fetal bovine serum (10%)-induced proliferation of primary cultured SMC was inhibited by Shimotsu-to at 4, 20, 100 or 500 µg/ml. The inhibitory effect was selective on SMC and due to cnidium rhizome or angelica root. The IC50 values of senkyunolide H, senkyunolide A, ligustilide and butylidenephthalide derived from cnidium were below 0.1, 1.52, 1.68 and 3.25 µg/ml, respectively. These results indicate that the antiproliferative effect of Shimotsu-to may depend on these cnidium-derived phthalides.

Keywords: Shimotsu-to, Senkyunolide (H and A), Ligustilide

Shimotsu-to, a Japanese-Sino medicine, consists of four crude extracts in the same weight ratio: cnidium rhizome, angelica root, peony root and rehmannia root. Shimotsu-to has been used clinically for improving “Oketsu” (stagnant blood) in traditional Chinese medicine. The “Oketsu” syndrome includes “female” diseases such as blood coagulation and fibrinolysis, atherosclerosis and chronic inflammation (1). The drugs for improvement of “Oketsu” syndrome including Shimotsu-to have been investigated from the aspect of blood diseases (2, 3), but not that of blood vessels. Recently, an abnormal proliferation of vascular smooth muscle cells (SMC) has been recognized to trigger the formation of atherosclerotic plaque (4). Therefore, the aim of the present study was to investigate the effects of Shimotsu-to and its component crude extracts on the proliferation of aortic SMC in primary culture. We further clarified the active antiproliferative compounds that were derived from principle crude extracts in Shimotsu-to.

Mouse aortic SMC in primary culture were prepared by the procedure of Chamley et al. (5) with the following modification (6). The thoracic aorta of a ddY strain male mouse (6–7-week-old, Japan Shizuoka Laboratory Center, Hamamatsu) was isolated in Hank’s solution (136.8 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 1.3 mM CaCl2, 0.4 mM KH2PO4, 0.3 mM Na2HPO4, 4.5 mM NaHCO3 and 2.8 mM glucose, pH 7.3). The aorta was incubated with collagenase (1 mg/ml) and elastase (3.3 unit/ml) (Sigma, St. Louis, MO, U.S.A.) in Hank’s solution for 30 min at 37°C, gently removed of adventitia, then incubated with collagenase and elastase for a further 60 min at 37°C. Isolated cells were collected by centrifugation at 300 × g for 5 min at 4°C and dispersed by pipetting in 2 ml Dulbecco’s modified eagle medium (DMEM, Nissui, Tokyo) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Nissui, Tokyo) and streptomycin sulfate (0.1 mg/ml, Meiji Seiyaku, Tokyo) and penicillin G potassium (160 unit/ml, Banyu Seiyaku, Tokyo). The cells at a density of 2.1 × 10^4 cells/cm² were plated into a glass dish (3.5-cm diameter) and preincubated in 10% FBS-DMEM for 3 days at 37°C under a humidified atmosphere of 5% CO2 and 95% air. The cells were then incubated in 10% FBS-DMEM in the presence or absence of drugs for consecutive days. In the assay of senkyunolide H, SMC at a density of 1.6 × 10^4 cells/cm² were plated and cultured in a 24-well plate (Corning, Corning, NY, U.S.A.) with 10% FBS-DMEM for 6 days in the pres-
ence or absence of it. The culture medium was changed every other day. For the assay of cell proliferation, the cells were detached in 0.25% trypsin (Sigma) and 0.2% EDTA in Ca$^{2+}$, Mg$^{2+}$-free Hank’s solution, and the cell number was counted with a hemocytometer. Viability of the cells was confirmed by the absence of uptake of trypan blue (7). The primary cultured cells exhibited at confluency a hill and valley pattern (8). Shimotsu-to (Tsumura, Tokyo) was the hot water-extract of a combined prescription of cnidium rhizome, angelica root, peony root and rehmannia root at the dry weight ratio of 1:1:1:1. The extracts with 70% methanol from cnidium rhizome, angelica root, peony root (provided by Dr. S. Natori, National Institute of Hygienic Sciences, Tokyo) and rehmannia root (Mikuni Shoten, Osaka) were also used. The chemical structures of senkyunolide H (Tsumura), senkyunolide A, ligustilide, butylidenephthalide, neocnidilide, butylphthalide, cnidilide, ligustilidiol, ferulic acid (provided by Dr. Y. Kano, Hokkaido Institute of Pharmaceutical Sciences) and tetramethylpyrazine (Aldrich, Milwaukee, WI, U.S.A.) are shown in Fig. 2a (9-11). The chemical structure of ligustidiol has not yet been determined. All compounds except for senkyunolide H, ferulic acid and tetramethylpyrazine were provided by Dr. M. Harada (National Institute of Hygienic Sciences). The compounds except for senkyunolide H were administered to the culture medium from the third day in culture. Treatment with senkyunolide H in the culture medium was started on the day the cells were plated. All compounds were dissolved in ethanol and diluted with 10% FBS-DMEM. Final concentrations of ethanol in the culture medium were lower than 0.1%. The data are expressed as the means ± S.E.M. Statistical analyses were performed by 2-way ANOVA (Tukey’s test, Fig. 1), the unpaired t-test or the Welch test.

Shimotsu-to (500 μg/ml) significantly decreased the 10% FBS-stimulated increase in the number of SMC in a time-dependent manner from the ninth to the 17th day in culture (Fig. 1a). The % inhibition of the increase in SMC cell number induced by 10% FBS was 53% at the 17th day in culture. The inhibitory effect of the Shimotsu-to (4, 20, 100 and 500 μg/ml) was concentration-dependent at the 11th day in culture (Fig. 1b). To investigate the constituent crude extracts that contribute to the inhibitory effect of Shimotsu-to, the effects of 70% methanol-extracts from four respective crude extracts were compared at the 11th day. The extracts (50 μg/ml) of cnidium rhizome or angelica root significantly inhibited the 10% FBS-induced cell proliferation, respectively (Table 1). Inhibitory effects of steam-distillate containing essential oil and the hot water-extract without the distillate of cnidium rhizome were also compared. The steam-distillate (50 μg/ml) significantly inhibited the 10% FBS-induced SMC proliferation by 58.1 ± 3.5% at the ninth day, but the remaining hot water-extract (50 μg/ml) did not affect it. These results show that the inhibitory effect of the 70%

Fig. 1. The time-dependent (a) and concentration-dependent (b) inhibitory effects of Shimotsu-to on 10% FBS-induced proliferation of primary cultured SMC of mouse aorta. a: The SMC were cultured in the presence (●) or absence (○) of 500 μg/ml Shimotsu-to for 14 consecutive days. Each value represents the mean ± S.E.M. (n = 3). **P < 0.01: Significantly different from the control without Shimotsu-to by 2-way ANOVA (Tukey’s test). b: The SMC were cultured for 8 consecutive days in 10% FBS-DMEM in the presence or absence of various concentrations of Shimotsu-to. % Inhibition for the SMC number increased by 10% FBS was calculated from the equation: ((without - with)/without Shimotsu-to) × 100. Each value represents the mean ± S.E.M. of % inhibition (n = 6).
methanol-extract of cnidium was due to essential oils in cnidium rhizome. The 70% methanol-extracts (50 μg/ml) of peony root and rehmannia root had no effect (Table 1). These results suggest that essential oils in cnidium rhizome and angelica root may contribute to the inhibitory effect of Shimotsu-to on SMC proliferation.

We further investigated the inhibitory effects of cnidium-derived compounds on the 10% FBS-induced cell proliferation (Fig. 2b). Senkyunolide H (0.1 and 1 μg/ml), senkyunolide A, ligustilide (0.2, 1, and 5 μg/ml), butylidenephthalide (1 and 5 μg/ml) and neocnidilide (5, 10 and 20 μg/ml) inhibited the SMC proliferation in a concentration-dependent manner during the 6-day treatment. The 50% inhibitory concentrations (95% confidence limits) were below 0.1, 1.52 (1.21–1.92), 1.68 (1.27–2.23), 3.25 (1.97–5.36) and 6.22 (4.49–8.62) μg/ml, respectively. From the ninth to the 11th day, the SMC showed no significant increase ((5.0 ± 4.5) x 10⁴ cells) by senkyunolide A (2 μg/ml). When senkyunolide A was removed from the culture medium at the ninth day, the number of SMC was increased by (19.6 ± 3.4) x 10⁴ cells at the 11th day. The increased cell number was similar to the value ((19.2 ± 4.3) x 10⁴ cells) in the control without drug at the 11th day. The increased rate of SMC proliferation was constant from the ninth to the 17th day in the control (Fig. 1). These results indicated that the inhibitory effect of senkyunolide A was completely recovered,
demonstrating that the effect was reversible. In the presence of 2,ug/ml butylidenephthalide at the ninth day, 2.6 ± 0.5% of the total number of SMC were stained by trypan blue, being not different from those in the absence of butylidenephthalide (2.9 ± 0.5%). These results suggest that the cnidium-derived phthalides used do not induce any toxic effect nor damage SMC. Ligustidiol, butylphthalide and cnidilide inhibited the SMC proliferation by less than 50%, showing that they had weak effects. Ligustilidiol, ferulic acid and tetramethylpyrazine had no effect on the SMC proliferation.

Water extracts of cnidium rhizome have been reported to significantly inhibit blood coagulation and accelerate fibrinolysis, but the concentrations of the extracts and its derived phthalides are not clear (2). It is, therefore, difficult to compare the inhibitory potencies of cnidium-derived phthalides against SMC proliferation with their potencies against blood coagulation and fibrinolysis.

The antiproliferative effects of cnidium-derived phthalides on the SMC were compared with those on subcultured endothelial cells (EC, 8 passage) of rat aorta prepared by the previously reported method (12). When EC (1.6 × 10^4 cells/cm^2) plated on type-I-collagen-coated wells were treated with 1 ug/ml senkyunolide H in control medium for 6 days, the number of EC increased by 5% FBS was (13.6 ± 0.4) × 10^4 cells, and was not significantly different from the control without drug ((13.1 ± 0.1) × 10^4 cells) (n = 3, P = 0.05). These results demonstrate that the cnidium-derived phthalides selectively inhibited SMC.

Senkyunolide H, Senkyunolide A, ligustilide, butylidenephthalide and neoecnidilide were contained in the amount of 0.005%, 0.16%, 0.36%, 4.5 × 10^-4% and 0.015% in the essential oil of cnidium rhizome, respectively, in the essential oil of angelica root (13). The results suggest that these phthalides contribute partly to the antiproliferation of SMC caused by the extracts of cnidium rhizome and angelica root.

We have previously reported a mechanism for the inhibitory effect of heparin on DNA synthesis in primary cultured aortic SMC (6). Heparin does not inhibit the competence phase, but does inhibit the progression phase in the phenotype of proliferation of aortic SMC. However, the inhibitory mechanisms of these phthalides for DNA synthesis remain unknown.

In conclusion, senkyunolide H, senkyunolide A, ligustilide and butylidenephthalide are active principles in the cnidium rhizome-derived compounds used that inhibit the aortic SMC proliferation. The inhibitory potencies were in the order of senkyunolide H > senkyunolide A = ligustilide > butylidenephthalide. The anti-proliferative effect of Shimotsu-to may depend on these cnidium-derived phthalides.

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<p>| Table 1. Inhibitory effects of 70% methanol-extracts of crude drugs composing Shimotsu-to on 10% FBS-induced proliferation of aortic smooth muscle cells in primary culture |</p>
<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration (µg/ml)</th>
<th>% Inhibition</th>
</tr>
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<tbody>
<tr>
<td>Cnidium</td>
<td>50</td>
<td>33.1 ± 6.6**</td>
</tr>
<tr>
<td>Angelica</td>
<td>50</td>
<td>32.9 ± 6.0**</td>
</tr>
<tr>
<td>Peony</td>
<td>50</td>
<td>6.7 ± 5.0</td>
</tr>
<tr>
<td>Rehmannia</td>
<td>50</td>
<td>5.2 ± 5.4</td>
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% Inhibition was calculated as described in Fig. 1b. Values are the means ± S.E.M. of 6-9 experiments. **P < 0.01: significantly different from the control (without extract) by the unpaired t-test.
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