The Herbal Medicine Sho-saiko-to Inhibits Proliferation of Cancer Cell Lines by Inducing Apoptosis and Arrest at the G_0/G_1 Phase

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ABSTRACT

Water-soluble ingredients of the herbal medicine sho-saiko-to dose-dependently inhibited the proliferation of a human hepatocellular carcinoma cell line (KIM-1) and a cholangiocarcinoma cell line (KMC-1). Fifty % effective doses on day 3 of exposure to sho-saiko-to were 353.5 ± 32.4 μg/ml for KIM-1 and 236.3 ± 26.5 μg/ml for KMC-1. However, almost no suppressive effects were detected in normal human peripheral blood lymphocytes or normal rat hepatocytes. Sho-saiko-to suppressed the proliferation of the carcinoma cell lines significantly more strongly than did each of its major ingredients, i.e., saikosaponin a, c, and d, ginsenoside Rb1 and Rg1, glycyr rhizizin, baikalin, baicalein, and wogonin, or another herbal medicine, juzen-taiho-to (P < 0.05 or 0.005). Because such ingredients are barely soluble in water, there could be synergistic or additive effects of the ingredients in sho-saiko-to. Morphological, DNA, and cell cycle analyses revealed two possible modes of action of sho-saiko-to to suppress the proliferation of carcinoma cells; (a) it induces apoptosis in the early period of exposure and (b) it induces arrest at the G_0/G_1 phase in the late period of exposure.

INTRODUCTION

Sho-saiko-to consists of seven crude ingredients extracted from herbs, i.e., Bupleuri radix, Pinelliae tuber, Scutellariae radix, Zizyphi fructus, Ginseng radix, Glycyrrhizae radix, and Zingiberis rhizoma. Sho-saiko-to is the most popular herbal medicine in Japan and has been used in the treatment of various chronic liver diseases. Recent in vivo and in vitro studies (1–8) reported its chemopreventive effects and its function as a biological response modifier. Animal studies on rats showed that this drug possesses cancer-preventive effects (7, 8). A clinical study also reported that sho-saiko-to administration suppressed the development of HCC in patients with liver cirrhosis (6).

In 1965 Kerr (17) identified the death of hepatocytes that was different from necrosis, and later Kerr et al. (18) reported “apoptosis” by explaining “apoptosis, or programmed cell death, is a process in which cells die in a controlled manner, in response to specific stimuli, following an intrinsic program.” Since then, various inducers of apoptosis and the mechanisms of apoptosis have been reported (19, 20).

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Preparation of Culture Medium with Sho-saiko-to. Dulbecco’s modified Eagle’s medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 5% fetal bovine serum (Whittaker Bioproducts, Inc., Walkersville, MD), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Chagrin Falls, OH) was used for cell culture as the basal medium. Sho-saiko-to powder (TJ-9; Tsumura & Co., Tokyo, Japan) was dissolved in basal medium to the concentration of 10 mg/ml and was mixed at 37°C for 30 min. This solution was centrifuged (1200 × g, 15 min) to remove insoluble ingredients, and then the supernatant was sequentially passed through 0.45-μm and 0.22-μm filters for sterilization. The solution was diluted with the aforementioned basal medium and prepared at six concentrations (20, 80, 400, 1000, 2000, and 10,000 μg/ml). To compare the effects on the cell lines, we also prepared culture media with a control drug, juzen-taiho-to (TJ-48; Tsumura & Co.), using the same procedures. Juzen-taiho-to is another herbal medicine manufactured by Tsumura & Co. and contains various crude ingredients different from those of sho-saiko-to. In addition, each of the ingredients of sho-saiko-to, i.e., saikosaponin a, c, and d, ginsenoside Rb1 and Rg1, glycyr rhizizin, baikalin, baicalein, and wogonin (Wako Pure Chemical Industries, Japan), was dissolved in dimethylsulfoxide (Wako) to 0.1% or lower concentration, and one of these prepared ingredients was added to the culture. Osmotic pressures and pH values of cultures either with or without sho-saiko-to, except the 10,000 μg/ml group, were within physiological ranges.

Cell Lines. The HCC cell line (KIM-1) and the cholangiocarcinoma cell line (KMC-1) were originally established in our laboratory (25, 26). In this study, we used these two lines and two control cell groups, i.e., normal hepatocytes isolated from male Wistar rats using the collagenase perfusion method (27) and normal human PBL isolated from healthy adults and separated from whole blood by the density centrifugation method (28) using Ficoll-Paque (Pharmacia, Uppsala, Sweden).

Effects of Sho-saiko-to on Cell Proliferation. The numbers of KIM-1 and KMC-1 cells in 6-well plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) were counted to reach confluence in about 11 days. The seeded cells were cultured for 1 day in basal medium. On the next day, the culture medium was replaced with medium containing one of six different concentrations of sho-saiko-to or medium alone as a control. Exchange of culture medium and prepared ingredients was added to the culture. Osmotic pressures and pH values of cultures either with or without sho-saiko-to, except the 10,000 μg/ml group, were within physiological ranges.

Material and Methods

Preparation of Culture Medium with Sho-saiko-to. Dulbecco’s modified Eagle’s medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 5% fetal bovine serum (Whittaker Bioproducts, Inc., Walkersville, MD), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Chagrin Falls, OH) was used for cell culture as the basal medium. Sho-saiko-to powder (TJ-9; Tsumura & Co., Tokyo, Japan) was dissolved in basal medium to the concentration of 10 mg/ml and was mixed at 37°C for 30 min. This solution was centrifuged (1200 × g, 15 min) to remove insoluble ingredients, and then the supernatant was sequentially passed through 0.45-μm and 0.22-μm filters for sterilization. The solution was diluted with the aforementioned basal medium and prepared at six concentrations (20, 80, 400, 1000, 2000, and 10,000 μg/ml). To compare the effects on the cell lines, we also prepared culture media with a control drug, juzen-taiho-to (TJ-48; Tsumura & Co.), using the same procedures. Juzen-taiho-to is another herbal medicine manufactured by Tsumura & Co. and contains various crude ingredients different from those of sho-saiko-to. In addition, each of the ingredients of sho-saiko-to, i.e., saikosaponin a, c, and d, ginsenoside Rb1 and Rg1, glycyr rhizizin, baikalin, baicalein, and wogonin (Wako Pure Chemical Industries, Japan), was dissolved in dimethylsulfoxide (Wako) to 0.1% or lower concentration, and one of these prepared ingredients was added to the culture. Osmotic pressures and pH values of cultures either with or without sho-saiko-to, except the 10,000 μg/ml group, were within physiological ranges.

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The abbreviations used are: HCC, hepatocellular carcinoma; PBL, peripheral blood lymphocytes; EDso, 50% effective dose; BrdU, 5-bromo-2’-deoxyuridine; PBS, phosphate-buffered saline.

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4 Herbal medicines from Tsumura & Co. have been approved as ethical drugs by the Ministry of Health and Welfare of Japan.
Cells incubated in basal medium were analyzed in the same way as controls. Cells treated with 20 μM aphidicolin (Wako), a DNA polymerase inhibitor, were also analyzed as an example of reversible growth arrest at the G1-S border.

**Analysis of DNA.** DNA analysis of KIM-1 and KMC-1 cells was performed on day 2 of culture with sho-saiko-to, using the method of Smith et al. (32). Floating cells and attached cells, which were collected using a scaper, were washed twice together with cold PBS. After centrifugation the pellet, suspended in solution (10 μg/ml Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.5% sodium dodecyl sulfate), was incubated with RNase (20 μg/ml; Sigma) for 60 min at 37°C and then with proteinase K (100 μg/ml; Sigma) for 3 h at 50°C and was extracted with phenol/chloroform, and DNA was collected by ethanol precipitation. Ten μg of the collected DNA were electrophoresed in a 1.6% agarose gel and visualized by UV illumination after staining with 0.5 μg/ml ethidium bromide (Wako).

**RESULTS**

**Effects of Sho-saiko-to on Cell Proliferation.** Sho-saiko-to showed dose-dependent inhibitory effects on the proliferation of both KIM-1 and KMC-1 cells. ED50 values (day 3) were 353.5 ± 32.4 μg/ml (mean ± SE) for KIM-1 and 236.3 ± 26.5 μg/ml for KMC-1. In the cultures of lymphocytes, this drug showed no effects at concentrations less than 2000 μg/ml, and the number of lymphocytes slightly decreased at 2000 μg/ml or higher concentrations. In cultures of normal rat hepatocytes, the proliferation of cells was slightly accelerated by a low dose (20 μg/ml) and slightly suppressed by a concentration of 10,000 μg/ml (Fig. 1).

During the 11-day period, proliferation of KIM-1 and KMC-1 cells was completely inhibited by sho-saiko-to at concentrations higher than the day 3 ED50 value. However, the cell number did not markedly decrease in cultures with 2000 μg/ml or lower concentration of sho-saiko-to, even on day 11, and the same cell number was maintained throughout the period (Fig. 2). Colorimetric assays showed almost the same results (data not shown).

**Comparisons between Total Water-soluble Ingredients of Sho-saiko-to and Each of Its Effective Ingredients and between Sho-saiko-to and Juzen-taiho-to.** The ED50 values on day 3 of culture with juzen-taiho-to were 1625.3 ± 101.2 μg/ml (mean ± SD) for.
In the present study, using water-soluble ingredients of sho-saiko-to, we were able to demonstrate that (a) sho-saiko-to directly inhibits the proliferation of HCC cells and cholangiocarcinoma cells, (b) sho-saiko-to does not affect normal PBL, and (c) at concentrations as low as 20 μg/ml sho-saiko-to slightly promotes cell proliferation of normal rat hepatocytes. The promotion of normal hepatocyte regeneration has been also reported in both in vitro and in vivo studies (33, 34).

Recent studies have reported antitumor effects of the flavonoids quercetin, genistin, and baicalein, which are extracts from plants (9–11). Sho-saiko-to contains various flavonoids, such as baicalein. In the present study, we confirmed much stronger antiproliferative effects of sho-saiko-to, compared with the effect of each ingredient, such as the flavonoids. In addition, it should be noted that we used only water-soluble ingredients of sho-saiko-to in this experiment. In general, the flavonoids are barely soluble in water and also are unstable in water. In other words, a water solution of sho-saiko-to does not contain high levels of flavonoids, which can produce significant antitumor effects. The efficacy of sho-saiko-to cannot be explained by the presence of flavonoids in the drug.
APOPTOSIS OF CANCER CELLS BY AN HERBAL MEDICINE

Fig. 4. Morphological features of KMC-1 cells 48 h after the addition of 400 μg/ml sho-saiko-to, showing apoptotic cells (A and B) and development of insoluble shells (C). Apoptotic cells (arrows in A and B) are characterized by cellular shrinkage and condensed chromatin fragments. A, hematoxylin and cosin, × 200; B, high-power view of A, × 1000; C, phase contrast, × 200.

Fig. 5. Flow cytometric analyses of KIM-1 and KMC-1 cells 48 h after culture with 2000 μg/ml sho-saiko-to, using a fluorescein isothiocyanate-anti-BrdU/propidium iodide double-staining technique. Accumulations of cells in two cell cycle regions, D1 and D3 or A0, are distinct.

only by the presence of flavonoids. Therefore, it is presumed that there could be synergistic or additive effects of various ingredients. In addition, there could be unknown substances present in sho-saiko-to that also inhibit tumor cell proliferation.

In comparison with another herbal medicine, juzen-taiho-to, sho-saiko-to produced significantly stronger inhibitory effects on tumor cells. This result strongly suggests the presence of special substances which suppress tumor cell growth in sho-saiko-to, and at the same time this result argues against the presence of killing effects attributable to other factors, e.g., osmotic pressure or pH, besides the ingredients of this drug. We presume that sho-saiko-to produces specific inhibitory effects on the proliferation of tumor cell lines.
APOPTOSIS OF CANCER CELLS BY AN HERBAL MEDICINE

The ED$_{50}$ values of this drug were 353.5 ± 32.4 µg/ml for KIM-1 and 236.3 ± 26.5 µg/ml for KMC-1. These values seem to be higher than those of other antineoplastic agents, but we consider these levels to be clinically applicable. Because this drug consists of seven different extracts from herbs together with undefined ingredients and, in addition, each ingredient is presumed to be metabolized through a different route, it is rather difficult to determine the blood levels of all ingredients of sho-saiko-to. However, we can use the level of a measurable ingredient, glycyrrhizin, as an index. The maximum level of glycyrrhizin after administration of 7.5 g of this drug (usual daily dose) is approximately 1.2 µg/ml, while the level of this ingredient in 300 µg/ml sho-saiko-to (ED$_{50}$ value in this in vitro study) is approximately 1.5 µg/ml. Therefore, the ED$_{50}$ value of sho-saiko-to in this study is considered to be useful in clinical situations.

Since Kerr et al. (17, 18) reported apoptosis and described it, i.e., “apoptosis, or programmed cell death, is a process in which cells die in a controlled manner, in response to specific stimuli, following an intrinsic program,” apoptosis has been studied in various fields (19, 20) and apoptosis-inducing substances besides physiological phenomenon have been reported, e.g., cyproterone acetate (21), dimethyltrrosamine (22), transforming growth factor β (23), and cycloheximide (24), in hepatocytes and HCC cells.

This study provided some supportive evidence for the presumption that sho-saiko-to induces apoptosis, i.e., shrinkage of cells, chromatin condensation, nuclear fragmentation, development of insoluble shells that are resistant to treatment with various detergents and 6 M guanidine hydrochloride (30), and DNA fragmentation in a ladder pattern, which is a characteristic biochemical marker of apoptosis.

Regarding the relationship between cell cycle and apoptosis, Afanas'ev et al. (35) reported that apoptosis is manifested as the appearance of a cell cycle region with DNA content lower than 2C on histograms. Telford et al. (36) suggested that this state is the A$_0$ region, and Tone (37) suggested that this state is the D$_3$ region. In this experiment, we also confirmed the increase of cells in this cell cycle region (A$_0$ or D$_3$) in the early culture period. Holtzer et al. (38) suggested that premature cells die by passing through ordinary cell cycles and then a specific cell cycle. Tone (37) reported, based upon the findings of Holtzer et al. (38), that those cells which are programmed to die have a last S phase which is different from the ordinary S phase, and then the cells leave the ordinary cell cycle in the next G$_2$ phase. In the last S phase, the DNA content of the cells is between 2C and 4C, but the cells do not incorporate BrdU (D$_1$ phase). Tone (37) proposed that this final S phase is identical to the quantal cell cycle proposed by Holtzer et al. (38). In the present study, we also confirmed increases of cells in the D$_1$ region as the concentration of sho-saiko-to increased. This point indicates that cell cycle analysis using the BrdU/propidium iodide double-staining method is useful to determine apoptosis in the early period.

Many recent studies report that the flavonoids possess inhibitory effects on topoisomerase II activity, e.g., genistein (an isoflavone) inhibits MOLT-4 cell proliferation by suppressing topoisomerase II (9, 39). In addition, induction of apoptosis through topoisomerase II inhibition has been reported (40, 41), and it has been shown that the effect of the drugs on programmed cell death is dependent upon new protein and RNA synthesis, indicating that topoisomerase II has a role

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in the very first stages of the process. Because sho-saiko-to contains various flavonoids, it is possible that this drug induces apoptosis by inhibiting topoisomerase II. Regarding the relationship between topoisomerase II inhibitors and the cell cycle, topoisomerase II poisons were reported to cause G2 arrest, and its extent and duration have been shown to depend on the dose, duration, and phase of drug exposure (42). In the present study, the number of KMC-1 cells in G2/M phase increased on day 2 of culture with sho-saiko-to. However, with both cell lines the number of cells in G0/G1 phase increased chronologically, finally resulting in arrest at G0/G1. This might be attributed to the presence of glycyrrhizin, which was reported to interfere with the cell cycle in the G0/G1 phase (12), or possibly other unknown substances present in sho-saiko-to. The increase of cells in the G0/G1 and D1 phases may indicate the possibility that sho-saiko-to contains inhibitors for enzymes involved in DNA synthesis.

In conclusion, we recognized that the effects of this drug are rather difficult to explain by the effect of one ingredient. We presume that sho-saiko-to possesses at least two different mechanisms of action to inhibit tumor cell proliferation; one is the effect of the flavonoids, which induce apoptosis in actively proliferating tumor cells, and the other is the effect of glycyrrhizic acid, which induces arrest at the G0/G1 phase and decreases DNA synthesis. We presume that there are synergistic or additive effects of various ingredients in sho-saiko-to, and there might be other unknown substances in sho-saiko-to which possess antitumor effects.

Lastly, it would be informative to investigate the general applicability of the drug effects on other cancer cell lines. We recently examined in vitro the direct effects of sho-saiko-to on the growth of 11 human cancer cell lines established from the hepatobiliary system. Dose-dependent growth-inhibitory effects of the drug were demonstrated in all cancer cell lines (43); the concentration of sho-saiko-to required for half-maximal cytostatic activity was approximately 200–1000 μg/ml in eight cell lines. Cell cycle analyses showed accumulation of the treated cells in the G0/G1 phase in all cell lines studied (data not shown). However, significant induction of apoptosis was observed in only a limited number of cell lines.6 Although some cancer cell lines were found to be less susceptible to the apoptosis-inducing effect of sho-saiko-to, this does not diminish the possible relevance of apoptosis for the antitumor activity of the drug; recent accumulating evidence suggests that defects in the process of apoptosis may be closely associated with carcinogenesis and that many cancer cells have defective machinery for self-destruction (44). It is suggested that susceptibility to apoptosis-inducing effects of chemotherapeutic drugs may depend on the intrinsic ability of tumor cells to respond by apoptosis (19). This point may support the specificity of the apoptosis-inducing effect of sho-saiko-to, because the presence of cell lines resistant to apoptosis induction by the drug indicates the possibility that the drug triggers apoptosis by stimulating specific mechanisms in the process that may be defective in some sets of cancer cells. Apoptosis induction, i.e., cell death, means irreversible growth arrest. On the other hand, the action of growth inhibition observed in the remaining viable cells treated with sho-saiko-to appears to be reversible. Therefore, we presume also that multiple mechanisms are involved in the growth-inhibitory effects of sho-saiko-to. Additional studies are currently underway to elucidate these multiple mechanisms for the growth inhibition produced by sho-saiko-to.

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REFERENCES


6 H. Yano, A. Mizoguchi, K. Fukuda, and M. Kojito, unpublished observations.


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