The Herbal Medicine Sho-saiko-to Inhibits Proliferation of Cancer Cell Lines by Inducing Apoptosis and Arrest at the G0/G1 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 percent 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, glycyrrhizin, baicalin, 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 G0/G1 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).

Some investigators have reported antitumor effects of plant extracts from sho-saiko-to, i.e., glycyrrhizin (12) and baicalein (13), have been reported for cholangiocarcinoma cells. In addition, each of the ingredients of sho-saiko-to, i.e., saikosaponin a, c, and d, ginsenoside Rb1 and Rg1, glycyrrhizin, baicalin, and baicalein, 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 G0/G1 phase in the late period of exposure.

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). Apoptosis is more physiological than another type of cell death, 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). Apoptosis is more physiological than another type of cell death, necrosis, and apoptosis inducers for cancer cells are ideal for cancer chemotherapy. For hepatocytes and HCC, such drugs as cyproterone acetate (21), dimethylnitosamine (22), transforming growth factor β (23), and cycloheximide (24) have been reported as apoptosis inducers. However, to our knowledge, no apoptosis inducer has been reported for cholangiocarcinoma cells.

In the present study, we observed that (a) the complete water-soluble ingredients of sho-saiko-to dose-dependently suppressed cell proliferation of a HCC cell line and a cholangiocarcinoma cell line and (b) the complete water-soluble ingredients of sho-saiko-to suppressed cell proliferation much more strongly than did each of the ingredients glycyrrhizin, baicalin, and baicalein. Morphological analysis, DNA analysis, and cell cycle analysis demonstrated that there are at least two modes of the suppressive effects shown by sho-saiko-to; one is the induction of apoptosis at early times of exposure and the other is the induction of arrest at G0/G1 phase in the cell cycle at later times of exposure.

MATERIALS 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 x 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, glycyrrhizin, baicalin, and 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. Osmostic 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 adjusted 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 determination of the number of living cells by the trypan blue dye exclusion method were performed daily until day 5.
test were repeated every 2 days thereafter until day 11 of culture. The ED50 value was determined on day 3 of culture.

PBL and normal rat hepatocytes were seeded on 6-well plates at a density of 5 × 10^5 cells/well and were cultured for 3 days in medium with or without sho-saiko-to. The number of living cells was counted using the trypan blue dye exclusion test on day 3 of culture, and the numbers were compared with those of KIM-1 and KMC-1 cells.

**Colorimetric Assay.** To confirm the reliability of data obtained in the aforementioned experiments, we conducted colorimetric assays using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell growth assay kit (Chemicon, Temecula, CA). KIM-1 and KMC-1 cells (10^4 cells/well) were each seeded on 96-well plates (Falcon) and cultured in basal medium for 1 day. On the next day, the culture medium was exchanged with medium with or without sho-saiko-to. On days 3 and 5, 160 µM/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to the cultures, cells were maintained for 3 h at 37°C, supernatants were gently removed, 100 µl/well of (31), with modification. Briefly, cells were labeled with 10 µM BrdU at 37°C for 30 min, washed twice with PBS, pH 7.6, and fixed in 70% cold ethanol at 4°C overnight. Then, cells were washed with PBS, subjected to double-strand DNA denaturation treatment with 1.5 M HCl at room temperature for 20 min, neutralized with 0.1 M Na2B407 and 0.1% NaBH4, washed with 0.5% Tween 20/PBS, incubated for 20 min at room temperature with 20 µl anti-BrdU antibody (Becton Dickinson Immunocytometry Systems USA), washed twice with PBS, incubated for 20 min at room temperature with 20 µl fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (DAKOPATTS, Denmark), and washed twice with PBS. DNA was counterstained with 5 µg/ml propidium iodide for at least 20 min before flow cytometric analysis.

To determine the reversibility of growth inhibition by the drug, the kinetics of entry into DNA synthesis were analyzed after releasing the cells from the treatment. After treatment with sho-saiko-to (400 and 1000 µg/ml) for 96 h, the cells in monolayer culture were washed 3 times with PBS and the culture was continued for an additional 48 h with basal medium containing 100 µM BrdU. BrdU incorporation into the nuclei of cultured cells was identified under a microscope by immunohistochemical detection using a cell proliferation kit (Amersham, UK), according to the manufacturer’s protocol. Because these cells continue to cycle within 48 h during logarithmic growth phase, judging from their doubling time, cells which did not incorporate BrdU within 48 h after the release of the drug treatment were tentatively assessed as an irreversibly growth-arrested cell population.

**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...
Effects of Sho-sai-to on the Cell Cycle of KIM-1 and KMC-1 Cell Lines. From day 2 of culture with sho-sai-to we observed the accumulation of cells in two cell cycle regions (Fig. 5), (a) a cell cycle region in which cells have between 2C and 4C DNA content but do not incorporate BrdU (D1 region) and (b) a cell cycle region below the G0/G1 phase, indicating apoptosis-associated chromatin degradation (D3 or Ao phase). In KMC-1 cells, the number of cells in G2/M phase increased on day 2, and then the number of cells in G0/G1 phase increased chronologically. On day 6, 46.5% of cells in control culture (without the drug) were in G0/G1 phase, whereas cells in this phase accounted for 68.8% in cultures with 400 µg/ml sho-sai-to and 79.5% in cultures with 2000 µg/ml concentrations of this drug. This indicates the occurrence of arrest in the G0/G1 phase (Fig. 6). On the other hand, KIM-1 cells in G2/M phase on day 2 did not increase in number, but from day 3 cell numbers in G0/G1 phase increased dose-dependently, as observed for KMC-1 cells (data not shown).

Effects on DNA Synthesis. In the KMC-1 cell line, the BrdU incorporation rate decreased time and dose dependently. On the other hand, in the KIM-1 cell line, equivalent decreases in incorporation occurred in cultures with 2000 µg/ml, but no significant difference was observed in cultures with 400 µg/ml (Fig. 7).

We further investigated whether the growth inhibition induced by sho-sai-to is reversible. After treatment with sho-sai-to (400 and 1000 µg/ml) for 96 h, the kinetics of entry into DNA synthesis were analyzed as described in “Materials and Methods.” The results showed no significant difference among control, aphidicolin treatment, and sho-sai-to treatment (data not shown). This indicates that the action of growth inhibition in the remaining viable cells treated with 400 µg/ml or 1000 µg/ml sho-sai-to is reversible. Dying cells were not counted in this study because they detached from the cell monolayer. Because there is no specific marker for the Go phase and reentry of Go-phase cells into the cell cycle may be possible, we tentatively used the term “G0/G1 phase” to define the cell population having 2C DNA content in our flow cytometric analyses.

Effects of Sho-sai-to on the DNA of KIM-1 and KMC-1 Cell Lines. In DNA analysis, a ladder of fragmented DNA of 180–190 base pairs was detected in both cell lines on day 2 of culture with 400 µg/ml or higher concentrations of sho-sai-to (Fig. 8). DNA fragmentation in a ladder pattern indicates internucleosomal chromatin cleavage, which is characteristic of apoptosis.

DISCUSSION

In the present study, using water-soluble ingredients of sho-sai-to, we were able to demonstrate that (a) sho-sai-to directly inhibits the proliferation of HCC cells and cholangiocarcinoma cells, (b) sho-sai-to does not affect normal PBL, and (c) at concentrations as low as 20 µg/ml sho-sai-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, genistein, and baicalein, which are extracts from plants (9–11). Sho-sai-to contains various flavonoids, such as baicalein. In the present study, we confirmed much stronger antiproliferative effects of sho-sai-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-sai-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-sai-to does not contain high levels of flavonoids, which can produce significant antitumor effects. The efficacy of sho-sai-to cannot be explained...
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.
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), dimethylin-

trosoamine (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, Afa


Fig. 6. Time course of the change in the cell cycle of KMC-1 cells cultured with 400 or 2000 µg/ml sho-saiko-to or medium alone (control). The cells in the G$_0$/G$_1$ phase increased dose and time dependently. Data represent the percentage of cells in G$_0$/G$_1$ phase.

Fig. 7. Effect of sho-saiko-to on DNA synthesis of KIM-1 cells (A) and KMC-1 cells (B), determined as BrdU incorporation. Cells were cultured with 400 µg/ml (A) or 2000 µg/ml (B) sho-saiko-to or medium alone as control (C).

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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 gyrrcyrhizin, 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 gyrrcyrhizin, 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. 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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