In vitro cytotoxicities of Shofu-san and its ingredient Bardanae Fructus (Goboshi) extract on human cell lines, HepG2 cells and Chang liver cells

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Abstract

In vitro cytotoxicities of Kampo medicine extracts on cultured-human cell lines were examined by using MTT method. Among twenty-six Kampo medicine preparations, Shofu-san and Unsei-in showed highly significant cytotoxicities against a human hepatoma cell line, HepG2, at a concentration of 250 μg/ml (60.0±2.8 %, $p<0,01$ and 41.1±3.6 %, $p<0.05$, respectively). To see the active component(s) of Shofu-san, we examined the cytotoxicities of extracts of thirteen ingredients of the Kampo preparation on HepG2 cells and Chang liver cells originated from normal tissue. Among the ingredients, only the extract of Bardanae Fructus (Goboshi) at 100 μg/ml showed significantly higher cytotoxicity against HepG2 cells than against Chang liver cells. The IC_{50} value of the Goboshi extract for HepG2 cells was approximately seven-times lower than that for Chang liver cells (25.4±5.4 μg/ml for HepG2 cells ; 174 ±10 μg/ml for Chang liver cells). The cytotoxicity of the Goboshi extract increased with prolongation of the exposure time to HepG2 cells, but the effect of exposure time was not clearly observed on Chang liver cells. These results suggest that the Goboshi extract contains a cytotoxic component(s) which acts strongly against a human hepatoma derived cell line, HepG2 cells.

Key words cytotoxicity, Shofu-san, Bardanae Fructus (Goboshi), HepG2 cells, Chang liver cells, MTT assay.

Abbreviations DMEM, Dulbecco's Modified Eagle's Medium ; HBSS(-), Hanks' balanced salt solution, Ca^{2+}, Mg^{2+} free ; O.D., optical density ; PBS, phosphate buffered saline ; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Introduction

Many antitumor agents clinically used have severe adverse reactions. Considerable efforts to find new drugs, including combinations of anticancer agents with so-called "biological-response modifiers" have been carried out.

Kampo medicines (Chinese traditional medicines) *per se*, having mild adverse reactions, have beneficial effects in enhancing the activity of anticancer agents and reducing the side effects of anticancer agents or radiation. Ikekawa *et al.*[1] have reported Sairei-to and Inchin-gorei-san significantly augmented the anticancer activity of cisplatin, and the combined treatment restored the toxic effects of cisplatin. Similarly, many investigators[2-5] have reported combined effects of Kampo medicines with anticancer agents.

Recently, Mizoguchi *et al.*[6,7] have reported that Sho-saiko-to and its ingredients have some direct inhibitory effects on the growth and function of hepatocellular carcinoma, without influence on normal human peripheral blood lymphocytes and normal rat hepatocytes. Yasui *et al.*[8] have reported the growth inhibitory effects of Sho-saiko-to and its ingredient drugs on HeLa cell and human ovarian cancer cell

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lines. And, Okada *et al.*[9] have reported cytotoxicities of Rhei Rhizoma (Daio) and Scutellariae Radix (Ogon) using MTT assay against a human hepatoma cell line, HepG2. Thus, Kampo medicines have been suggested to be useful for cancer treatment, although the cytotoxicity data do not always reflect the anticancer effect *in vivo*.

In this report, we tested *in vitro* cytotoxicities of twenty six Kampo medicines and found Shofu-san and its ingredient Goboshi showed high cytotoxicity against HepG2 cella, compared with Chang liver cells.

Materials and Methods

Kampo medicines and crude drug extracts : Commercially available granule preparations of twenty-six Kampo medicines tested are listed in Table I. These Kampo prescriptions were purchased from Tsumura Co., LTD., Tokyo, Japan, except for Ninjin-yoei-to (Kanebo Co. LTD., Tokyo, Japan). A simple extract of Shofu-san was kindly supplied from Tsumura, and simple extracts of its ingredients, *i.e.*, Gypsum (Sekko), Rehmanniae Radix (Jio), Angelicae Radix (Toki), Atractylodis Lanceae Rhizoma (Sojutsu), Ledebouriellae Radix (Bofu), Akebiae Caulis (Mokutsu), Anemarrhenae Rhizoma (Chimo), Glycyrrhizae Radix (Kanzo), Sophorae Radix (Kujin), Schizonepeta Herba (Keigai), Bardanae Fructus (Goboshi), Oleum Sesami (Goma) and Cicadae Periostracum (Zentai), were purchased from Hachiro Seiyaku Co., LTD., Nagoya, Japan.

Each Kampo medicine extract or crude drug extract was prepared in sterilized distilled water or Hanks' balanced salt solution, Ca^{2+}, Mg^{2+} free (HBSS(-)), treated with boiling water bath for 15 min, then centrifuged at 350×g for 15 min to remove insoluble materials, and the supernatant was filtered through 0.22 μm membrane filter (NIHON MILLIPORE LTD., Yonezawa, Japan) for sterilization, and diluted with HBSS(-) or phosphate buffered saline (PBS). The samples were freshly prepared before use.

Chemicals : 3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co., St Louis, MO, U.S.A..

All other chemicals used here were special pure grade.

Cell cultures : HepG2 cells were obtained from the Faculty of Biosciences, Fukui Prefectural University (Dr. Toshio Kaido), and Chang liver cells were maintained at the Faculty of Pharmaceutical Sciences, Hokuriku University. Cells were routinely cultured in Dulbecco's Modified Eagle's Medium (Nissui Pharmaceutical Co., LTD., Tokyo, Japan) supplemented with 10 % fetal bovine serum, L-glutamine (4 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5 % CO_2, 95 % air.

In vitro cytotoxicity assay : Cytotoxicity was determined by using MTT assay (Mosmann, T.[10]; Carmichael *et al.*[11]) with minor modifications. Briefly, the cell suspension was plated (180 μl ; 5~6×10³ cells/well) in a 96 well-microculture plate (flat bottom ; Falcon 3027) (Becton Dickinson and Company, New Jersey, U.S.A.). After 24 hr culture, 20 μl of varying concentrations of each sample solution was added to each well and cultured for 3 days. Finally, 20 μl of MTT solution (2 mg/ml in PBS) was added to each well and further incubated for 4 hr. After the incubation, the supernatant was discarded by aspirating, then, 200 μl of dimethyl sulfoxide (Wako Pure Chemical Industries, Tokyo, Japan) was added to dissolve MTT formazan. The optical density (O.D.) was measured with a microplate reader MPR-A4i (TOSOH Co., Tokyo, Japan) at 540/620 nm. The mean value of O.D. of 5~6 wells was used for calculating the % cytotoxicity. The equation is as follows : % cytotoxicity = (1 O.D. treated well/O.D. control well) ×100

The concentration required to inhibit 50 % of the cell-growth (IC_{50}) was determined.

Statistics : Student's *t* test was used to evaluate the significant difference between experimental groups.

Results

In vitro cytotoxicities of Kampo medicines against HepG2 cells

Table I summarizes the results of *in vitro* cytotoxicities of twenty-six Kampo medicines against HepG2 cells. Shofu-san and Unsei-in showed significantly higher cytotoxicity compared with other

Table I Cytotoxicity of Kampo medicine extracts on HepG2 cells.

Kampo medicine extracts	% Cytotoxicity±S.E.
Kakkon-to	3.8±0.8
Jumi-haidoku-to	5.6±7.6
Hachimi-jio-gan	0.8±5.6
Dai saiko-to	7.9±6.5
Sho-saiko-to	1.4±4.9
Saiko-keishi-to	3.7±7.5
Keishi-ka-jutsubu-to	-1.3±6.5
Sho-seiryu-to	6.4±4.6
Shofu-san	60.0±2.8*
Toki-shakuyaku-san	4.6±7.2
Kami-shoyo-san	0.3±5.3
Keishi-bukuryo-gan	1.9±7.1
Shinbu-to	1.8±3.9
Byakko-ka-ninjin-to	2.1±6.0
Chorei-to	15.7±3.5
Hochu-ekki-to	10.6±4.9
Choto-san	0.1±7.0
Juzen-taiho-to	5.5±4.2
Juncho-to	2.8±1.7
Unsei-in	41.1±3.6**
Saiboku-to	1.1±6.9
Dai-kenchu-to	6.4±6.8
Gosha-jinki-gan	-1.5±5.2
Ninjin-yoei-to	10.1±7.2
Seishin-renshi-to	4.7±7.1
Sairei-to	7.0±5.9

Cytotoxicity against HepG2 cells was determined as described in Materials and Methods. Each extract of Kampo medicine was examined at a final concentration of 250 μg/ml. Values represent the mean±S.E. (n=3~5). *, ** ; Significant differences from others at $p<0.01$ and 0.05, respectively.

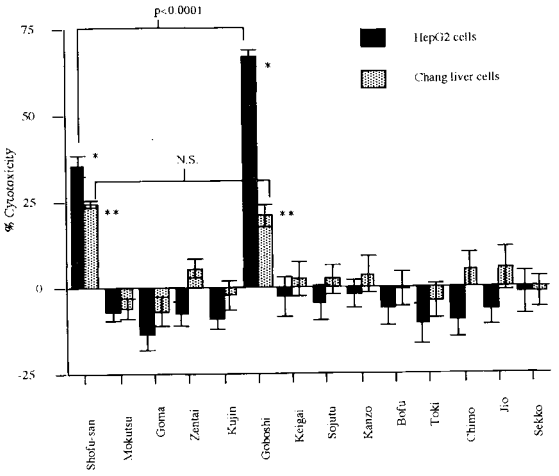

Fig. 1 Cytotoxicities of extracts of ingredients of Shofu-san on HepG2 cells and Chang liver cells. Each extract was examined at a final concentration of 100 μg/ml. Values represent the mean±S.E. (n=8). *, ** ; Significant differences from others at $p<0.0001$ and 0.05, respectively. N.S. ; Not significant.

Table II IC_{50} values of Shofu-san and Goboshi extract against HepG2 cells and Chang liver cells.

	HepG2 cells	Chang liver cells
	IC_{50} (μg/ml)	
Shofuo san	172 ± 7	>250
Goboshi	25.4±5.4	174±10

Values represent the mean±S.E. (n=4~8).

Kampo medicine preparations at a final concentration of 250 μg/ml (60.0±2.8 %, $p<0.01$ and 41.1±3.6 %, $p<0.05$, respectively).

Cytotoxicities of ingredients of Shofu-san, on HepG2 cells and Chang liver cells

Because cytotoxicity of Shofu-san was the most potent among Kampo medicines tested on HepG2 cells, we examined the effects of its ingredients on the malignant cell line, HepG2, and the non-malignant cell line, Chang liver cell. Figure 1 shows the cytotoxicities of 100 μg/ml of simple extracts, not containing excipients, of Shofu-san and its ingredient crude drugs. Among thirteen ingredients, the Bardanae Fructus (Goboshi) extract showed significantly potent cytotoxicity especially against HepG2 cells (against HepG2 cells ; 66.7±2.3 %, $p<0.0001$, and against Chang liver cells ; 20.6 ± 3.2 %, $p<0.05$, respectively), while there was not so large a difference between the cytotoxicities of the Shofu-san extract on these cells (against HepG2 cells ; 35.2±3.0 %, $p<0.0001$, and against Chang liver cells ; 24.2±1.0 %, $p<0.05$), Moreover, it should be noted that the cytotoxicities of the Goboshi extract or the Shofu-san extract differed against cell types. That is, the toxicity against Chang liver cells was the same degree in the Goboshi extract and the Shofu-san extract (20.6± 3.2 % vs 24.2±1.0 % ; not significant). On the other hand, the toxicity against HepG2 cells was much higher in the Goboshi extract than in the Shofu-san extract (66.7±2.3 % vs 35.2±3.0 % ; $p<0.0001$). The cytotoxicities of the Shofu-san and Goboshi extracts were dose-dependent on both HepG2 cells and Chang

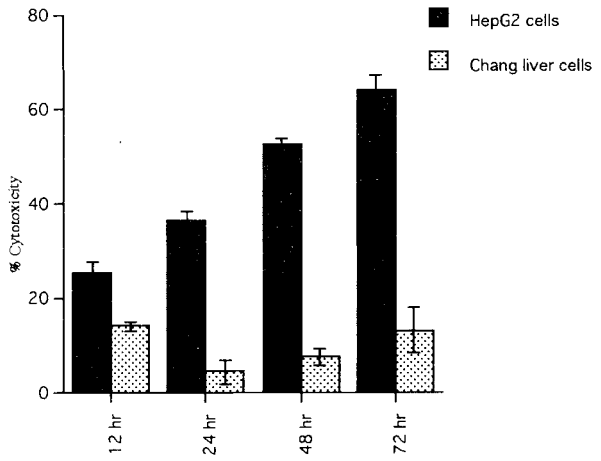

Fig. 2 Cytotoxicities of Goboshi extract in varying exposure times on HepG2 cells and Chang liver cells. Cells were incubated with Goboshi extract at a concentration of 100 μg/ml for 12, 24 and 48 hr, then exchanged with fresh medium without the extract and further incubated. The MTT assay was done 72 hr after addition of the extract. Values represent the mean±S.E. (n=8).

liver cells, and the IC_{50} values are shown in Table II. The cytotoxicity of the Shofu-san extract on HepG2 cells was higher than that for Chang liver cells ; especially, the Goboshi ingredient showed about seven-times higher cytotoxicity on HepG2 cells than on Chang liver cells. When treated with cytotoxic concentrations of Goboshi extract (>50 μg/ml), HepG2 cells appeared to be somewhat of a round-shape and were small size cells, compared with the untreated cells, by phase-contrast microscopic observation three days after the treatment.

Effects of exposure time of Goboshi extract on cultured cells

As shown in Fig. 2, the cytotoxicity of the Goboshi extract on HepG2 cells was increased according to the exposure times, but that of Chang liver cells was hardly changed with the exposure times. This suggest the modes of cytotoxicities of the Goboshi extract on these cells are different.

Discussion

Shofu-san showed the most potent cytotoxicity on the human hepatoma cell line, HepG2, among twenty-six Kampo medicines tested in this study, and Goboshi was revealed to be the active ingredient of the Kampo preparation. Shofu-san has been traditionally used for skin diseases, and Goboshi has been used as antidote, diuretic, anti-inflammatory agent, antimicrobial agent, and antifungal agent. Therefore, the cytotoxic activity of Shofu-san or Goboshi against HepG2 cells may be related to its antimicrobial and/or antifungal activities, though we have no evidence about this.

Sato[12] reported, using an *in vitro* screening test, the selective toxicity of Goboshi on a human cancer cell line (JTC-26), derived from cervical cancer. He has also described that Oren, Obaku and Chimo showed selective toxicity on JTC-26 cells. In this study, Chimo didn't show any cytotoxicities on HepG2 cells and Chang liver cells (Fig. 1), Mizoguchi[6] reported the direct effect of Sho-saiko to on the growth of human cancer cell lines *in vitro* at much high concentrations (>400 μg/ml). However, Sho-saiko to did not show cytotoxicity on HepG2 cells (Table I) at 250 μg/ml of the extract in this study. This may be due to different sensitivities of cultured cell lines against the active component(s). In this study, we used Chang liver cells as the normal control cells of hepatoma HepG2 cells, although Moore *et al.*[13] reported the cells developed the characteristics of an undifferentiated neoplasm. To confirm whether Goboshi has a selective cytotoxicity on hepatoma cells or shows wide spectrum of anticancer activity, further studies on other cell lines are needed.

Goboshi is prepared from fruits of *Arctium lappa* L. and contains arctiin, gobosterin and fatty oils as chemical constituents.[14] Although the active component (s) of the Goboshi extract is unknown now, the extract of the crude drug should contain some constituents, which act time-dependently on the hepatoma cells. Then, further studies to clarify the active constituents are needed. Either way, Shofu-san or Goboshi may be useful for cancer chemotherapy or a "biological response modifier", because of their strong action on human hepatoma-derived cells.

和文抄録

漢方エキス製剤（細粒剤）のヒト由来細胞株に対する *in vitro* における細胞障害性を MTT 法を用いて検討し

た。26種類の漢方エキス製剤のうち，250 μg/ml の濃度では消風散と温清飲が他の漢方エキス製剤と比較して，ヒト肝細胞癌由来の HepG2 細胞に対して有意に強い細胞障害性を示した（消風散；60.0±2.8 %，$p<0.01$，温清飲；41.1±3.6 %，$p<0.05$）。最も強い細胞障害性を示した消風散の活性成分を調べるため，その構成生薬エキスの細胞障害性を HepG2 細胞と正常細胞由来の Chang liver 細胞で比較検討してみた。その結果，13種の生薬エキス剤のうち，100 μg/ml の濃度で強い細胞障害性を示したのは牛蒡子エキスのみで，このものは Chang liver 細胞に対するよりも HepG2 細胞に対してより強い細胞障害性を示した。また，牛蒡子エキスの Chang liver 細胞に対する IC_{50} 値は HepG2 細胞に対する IC_{50} 値の約7倍であった（Chang liver 細胞；174 ± 10 μg / ml，HepG2 細胞；25.4±5.4 μg/ml）。さらに，牛蒡子エキスの HepG2 細胞に対する細胞障害性は，明らかに作用時間に比例して増強することが認められたが，Chang liver 細胞に対してはそのような明らかな作用時間との関係は認められなかった。これらの結果は，牛蒡子エキスがヒト肝細胞癌由来の HepG2 細胞に対して強く作用する細胞障害性成分を有することを示唆している。

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