Antitumor activity of Bardanae Fructus (Goboshi) extract

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Abstract

We have previously indicated that a strong cytotoxicity of Shofu-san (Xiao-Feng-San, 消風散) against human hepatoma HepG2 cells was based on the action of Bardanae Fructus (Goboshi), an ingredient of the Kampo formulation. Goboshi extract showed about 10-fold stronger cytotoxicity against HepG2 cells than against Chang liver cells. The cytotoxicity of Goboshi extract became extremely strong in Chang liver cells, but not in HepG2 cells, by pretreatment with L-buthionine-(S, R)-sulfoximine, an inhibitor of glutathione (GSH) biosynthesis. The cytotoxicity of Goboshi extract on HepG2 cells was weakened by L-cysteine. The contents of nonprotein sulfhydryl (SH)-substances and GSH in Chang liver cells were about 2-fold higher than in HepG2 cells. There was no significant difference between glutathione S-transferase (GST) activities of Chang liver cells and that of HepG2 cells. Murine sarcoma S180 cells contained a similar or less amount of SH-substances than HepG2 cells, and Goboshi extract showed significant antitumor activity against S180-bearing mice by oral administration. These results suggest that Goboshi extract contains some cytotoxic or antitumor components, which may be detoxified by intracellular SH-substances.

Key words Bardanae Fructus (Goboshi), cytotoxicity, antitumor activity, glutathione, glutathione S-transferase, sulfhydryl substances.

Abbreviations BSO, L-buthionine-(S, R)-sulfoximine ; GSH, glutathione ; GST, glutathione S-transferase ; L-Cys, L-cysteine ; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ; SH, sulfhydryl ; PBS, phosphate buffered saline.

Introduction

We have previously reported that a Kampo formulation Shofu-san (消風散) and its ingredient Bardanae Fructus (Goboshi) showed strong *in vitro* cytotoxicities on a human hepatoma derived cell line HepG2 cells.[1] Interestingly, the cytotoxicities of Shofu-san extract and Goboshi extract against Chang liver cells, derived from human normal hepatocyte, were significantly lower than that of HepG2 cells. We hypothesized that this difference in susceptibility between these cell lines was due to a different strength of counteraction against the cytotoxic component(s) of Goboshi extract.

This study deals with (a) the relation between the *in vitro* cytotoxicity of Goboshi extract and contents of sulfhydryl (SH)-substances in the cells and (b) *in vivo* antitumor activity of Goboshi extract on murine ascitic sarcoma S180.

Materials and Methods

Chemicals : Dimethylsulfoxide, 5,5'-dithiobis (2-nitrobenzoic acid), 1-chloro-2,4-dinitrobenzene and L-cysteine (L-Cys) were obtained from Wako Pure Chemical Industries, Tokyo, Japan. L-Buthionine-(S, R)-sulfoximine (BSO), 3-(4,5-dimethylthiazol-2-yl)-

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2,5-diphenyltetrazolium bromide (MTT) and glutathione (GSH, reduced form) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Trypsin (1 : 250) was purchased from Difco Laboratories, Michigan, U.S.A. Goboshi extract was obtained from Hachiro Seiyaku Co., LTD., Nagoya, Japan. All other chemicals were of reagent grade.

Agents used for cytotoxicity assay were dissolved in distilled water or culture medium, then filtered through 0.22 μm membrane filter (NIHON MILLIPORE LTD., Yonezawa, Japan), and diluted with culture medium to various concentrations. The drug solutions were prepared immediately before each experiment.

Cell culture : HepG2 cells and Chang liver 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.

Cytotoxicity assay : Cytotoxicity was determined by using MTT assay [2] and calculated using the following equation :

% cytotoxicity = [1 - (optical density of treated well/optical density of control well)] × 100

The IC_{50} values were determined from full survival curves.

Assay of GSH : Cells, cultured in plastic culture bottles (Falcon 3084, Becton Dickinson and Company, New Jersey, U.S.A.) with or without BSO, were detached from the plastic culture bottles by treatment with trypsin solution (0.25 % trypsin/0.2 % ethylenediamine tetraacetic acid in phosphate buffered saline (PBS), pH 7.4, Ca^+, Mg^+ free), and washed once with PBS. Murine sarcoma S180 cells, which were serially maintained by intraperitoneal passage in female ddY mice (Nippon SLC, Hamamatsu), were harvested from the abdominal cavity about 7 days after the tumor inoculation and washed twice with PBS. Cells were suspended in 50 mM potassium phosphate buffer, pH 7.4, containing 1.15 % KCl and sonicated using TOMY ultrasonic disrupter UD-201, centrifuged at 10,000×g for 10 min, and the supernatant was used as microsomal fraction. Contents of nonprotein SH-substances in the microsomal fraction of the cells were colorimetrically measured using 5,5'-dithiobis (2-nitrobenzoic acid) by a method reported by Palamanda and Kehrer.[3] GSH (reduced form) contents were determined by subtracting the values for nonprotein SH-substances in the cells treated with 50 μM BSO for 24 hr from that in untreated cells.

Assay of GST activity : Total GST activity was determined according to the method of Habig *et al.*[4] The activities in the 105,000 × g supernatant were measured in 100 mM potassium phosphate buffer (pH 6.5) containing 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene.

Protein determination : Protein contents were measured by the method of Bradford using Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, U.S.A.) with bovine serum albumin (Sigma Chemical) as a standard.

Antitumor experiment : Mice (6 weeks old) were intraperitoneally inoculated with S180 cells (1×10^6 cells/mouse), and were orally given 2.5, 5 or 10 mg/kg body weight of Goboshi extract once a day for 10 days from 24 hr after the tumor cell inoculation.

Statistical significance was determined by unpaired two-tailed Student's t-test, with p values <0.05 considered to be significant.

Results

Cytotoxicity of Goboshi extract and effects of BSO and L-Cys

As shown in Table I, cytotoxicity of Goboshi extract on HepG2 cells was markedly higher than that

Table I Effects of BSO treatment on the cytotoxicity of Goboshi extract.

BSO (μM)	Chang liver cells IC_{50} (μg/ml)	HepG2 cells IC_{50} (μg/ml)
0	174±12	12±1
12.5	59±4 *	18±3
25	16±6 *	15±2
50	5.0±0.8*	9.7±1.1

Ten hr after seeding, cells were cultured in the presence of the indicated concentrations of BSO for 14 hr and then incubated with various concentrations of Goboshi extract for 3 days.
*Significant difference from control at $p<0.0005$. Values are represent the mean±S.E. of three to six independent experiments done in quadruplet.

on Chang liver cells. After treatment with BSO (12.5-50 μM) for 14 hr, the effect of Goboshi extract was hardly changed in HepG2 cells, but in Chang liver cells it became strong. On the other hand, by addition of L-Cys to the culture medium, the cytotoxicity of Goboshi extract on HepG2 cells was diminished in a concentration-dependent manner (Fig. 1), without direct interaction of Goboshi extract with L-Cys in the media (data not shown). The effect of L-Cys was almost canceled by co-culture with 50 μM BSO (Fig. 1).

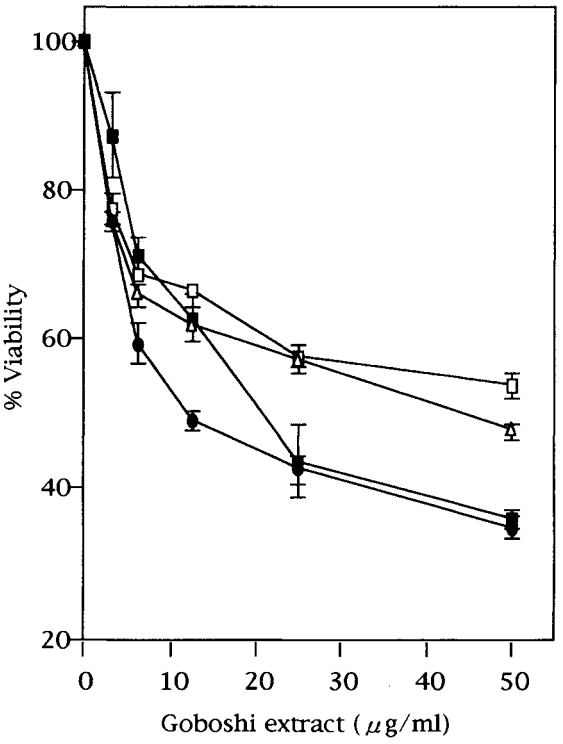

Fig. 1 Effect of L-Cys on the cytotoxicity of Goboshi extract against HepG2 cells. Cells were incubated in the absence (●) or presence of 0.5 (△) and 1.0 mM L-Cys (□) without or with 50 μM BSO (■) for 24 hr, then further incubated with varying concentrations of Goboshi extract for 72 hr. Values represent the mean±S.E. of three independent experiments done in quadruplet.

GST activity and contents of SH-substances in cells

While total GST activity in HepG2 cells was only slightly lower than that in Chang liver cells (Table II), both contents of nonprotein SH-substances and GSH, of which biosynthesis was inhibited by treatment with 50 μM BSO for 24 hr, were 2-fold less in HepG2 cells than in Chang liver cells (Table II).

In vivo antitumor experiment

The antitumor effect of Goboshi extract was examined on murine sarcoma S180 cells. The content of nonprotein SH-substances in S180 cells was 46.5±1.2 nmol/mg protein, which was slightly lower than that of HepG2 cells, and the IC_{50} value of Goboshi extract against the cells was 11.8±5.2 μg/ml.

When orally administered once a day for 10 days,

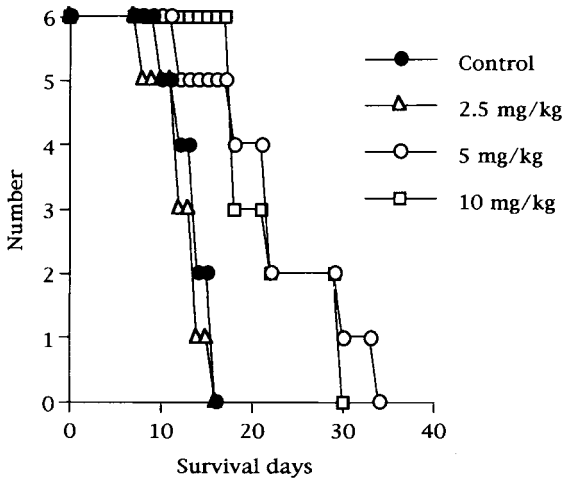

Fig. 2 Antitumor effect of Goboshi extract on S180-bearing mice. Twenty-four hr after tumor inoculation (1×10^6 S180 cells/mouse), the indicated doses of Goboshi extract were orally administered to 6 mice in a group once a day for 10 days.

Table II GSH/GST related biochemical analyses in Chang liver cells and HepG2 cells.

	Chang liver cells	HepG2 cells
GST activity (nmol/min/mg protein)	32.7±4.3	26.9±3.9
SH substance (nmol/mg protein)	98.0±5.0	55.0±4.5*
GSH content (nmol/mg protein)	57.8±6.2	25.0±2.7**

See details in Materials and Methods.
*,**Significant differences from Chang liver cells at $p<0.005$ and $p<0.01$, respectively. Values are represent the mean±S.E. of three to four independent experiments done in triplicate.

high dosages of Goboshi extract (5 and 10 mg/kg) markedly prolonged the life span of S180-bearing mice (Fig. 2).

Discussion

This study suggests that Goboshi extract contains some cytotoxic or antitumor components. These components may be detoxified by SH-substances, such as GSH and L-Cys which is known as for a precursor of GSH synthesis and enhances its synthesis,[5 7] in the cells. It is generally accepted that GSH and GSTs play important roles in the detoxication of various electrophilic chemicals. GSH can conjugate chemicals, and increases in cellular GSH levels and GSTs expression are frequently associated with resistance of tumor to alkylating agents.[8 10] Fujiwara *et al.* reported that significantly increased intracellular GSH levels were found in cisplatin-resistant tumor cells and manipulation of GSH levels with BSO produced a partial sensitization.[11] It has been also reported that the antitumor activities of some alkylating agents can be increased by GSH depletion.[12]

Here, we indicated that human hepatoma HepG2 cells were more sensitive to Goboshi extract than Chang liver cells, which contained high amounts of nonprotein SH-substances and GSH than HepG2 cells, while there was no significant difference between GST activities of Chang liver cells and HepG2 cells. The cytotoxicity of Goboshi extract against Chang liver cells significantly increased after treatment with BSO, an inhibitor of GSH biosynthesis,[13] in contrast the cytotoxicity against HepG2 cells decreased by L-Cys. These results support that components contained in Goboshi extract are detoxified by intracellular SH-substances.

Moreover, Goboshi extract showed antitumor activity against S180-bearing mice by oral administration. These results may imply Goboshi extract could be effective in adjuvant chemotherapy for human cancer. The details for the active components of Goboshi extract are now being studied.

和文抄録

以前に我々は，漢方方剤である消風散エキスがヒト肝癌細胞由来のHepG2細胞に対して強い細胞障害作用を示し，これはその構成生薬の一つである牛蒡子（Bardanae Fructus）に基づくことを示した。牛蒡子エキスは，HepG2細胞に対して，ヒト正常肝細胞由来のChang liver細胞に対するよりもおよそ10倍強い細胞障害作用を示した。Chang liver細胞に対する牛蒡子エキスの細胞毒性は，GSH合成阻害剤であるブチオニンスルフォキシミン（BSO）で処理することにより，用量依存的に著しく増大した。しかしながら，HepG2細胞に対してはこのようなBSO処理の効果は認められなかった。牛蒡子エキスのHepG2細胞に対する細胞障害作用は，L-システイン（L-Cys）で処理することにより有意に減弱した。Chang liver細胞には，HepG2細胞と比較して約2倍の非蛋白性SH物質やグルタチオン（GSH）が含まれていた。両細胞間のGST活性には統計学的に有意な差はなかった。マウス肉種S180は，HepG2細胞と同等かやや少ないSH物質を含んでおり，S180担癌マウスに対して牛蒡子エキスを経口投与することにより，有意な治療効果が認められた。これらの結果は，牛蒡子エキスには何らかの細胞障害性又は抗腫瘍性の成分を含有しており，これらの成分は，細胞内のSH物質によって解毒される性質のものであることを示唆している。

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