***In Vitro* Study of the Chemopreventive Effects of Chinese Herbs against Hepatocarcinogenesis**

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Summary The chemopreventive effects of seven Chinese herbs against hepatocarcinogenesis were evaluated *in vitro*. The water extracts from *Scutellaria barbata* and *Hedyotis diffusa* among the seven herbs as well as *Curcuma zedoaria* (positive control), strongly inhibited cell growth of four human hepatoma cell lines; HuH-7 (p53 mutant), PLC/PRF/5 (p53 mutant), HepG2 (p53 wild-type) and Hep3B (p53 null). These two extracts also induced apoptotic cell death of these cell lines, as evaluated by DNA fragmentation and chromatin condensation. It is suggested that these Chinese herb extracts might contain active components which are able to induce apoptosis independent of the p53-induced process.

Key Words: Chinese herb, chemoprevention, human hepatoma cell line, growth inhibition

Introduction

Apoptosis is a physiologically important form of cell death in response to cytotoxic agents. Apoptosis has been demonstrated to be responsible for anticancer effects in hepatoma in both animal models and clinical research [1–4]. A number of compounds, such as acyclic retinoid [5, 6], transforming growth factor- β [7], cycloheximide [8], and cyproterone acetate [9], have been reported to induce apoptosis on human hepatoma cell lines.

Chinese herbs are widely used as traditional medicine to treat many diseases in Asia. Recently *in vitro* and *in vivo* studies on chemopreventive effects of Chinese herbs and their functions have been reported [10–15]. For instance, the traditional Chinese medicine Xiao-Chai-Hu-Tang contains baicalin, baicalin, and saikosaponin-a, which have been shown to inhibit cell proliferation and induce apoptosis [10, 14], and *Salvia miltiorrhiza*, which con-

tains primaquinone and tanshinone, has antitumor activities [13, 15].

In the present study, we screened seven Chinese herbs, which are commonly used to treat liver cancer patients, for growth inhibitory effects and the ability to induce apoptosis in human hepatoma cell lines.

Materials and Methods

Preparation of water extracts and ultrafiltrated fractions from Chinese herbs. Chinese herbs (Table 1) were gifts from Yi Zheng Hospital of Traditional Chinese Medicine (Yi Zheng, China). Tested Chinese herbs are *Gynostemma pentaphyllum*, *Hedyotis diffusa* (HD), *Picrorrhiza kurrooa* (PK), *Polistes mandarius*, *Salvia yunnanensis*, *Scutellaria barbata* (SB), and *Sparganium simplex*. *Curcuma zedoaria* (CZ), which has been well-investigated, was used as a positive control [16–19]. Each herb (10 g) was soaked in 100 ml of distilled water for 15 min at room temperature and boiled for 15 min. The solution was then filtered, and the resulting filtrate was lyophilized. The water extract was divided into three fractions according to molecular weight, *i.e.*, Fraction I (<5 kDa), Fraction II (5–100 kDa), and Fraction III

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Table 1. The composition of water extract and fractions of Chinese herbs.

Chinese herb	Water extract (g/100 g)	Ultrafiltrated fraction (g/100 g water extract)		
		Fraction I (<5 kDa)	Fraction II (5–100 kDa)	Fraction III (>100 kDa)
1 <i>Curcuma zedoaria</i> (rhizome, stalk)	5.6	63.1	24.3	12.6
2 <i>Picrorrhiza kurroa</i> (rhizome, stalk)	21.3			
3 <i>Sparganium simplex</i> (rhizome, stalk)	4.8			
4 <i>Salvia yunnanensis</i> (rhizome)	21.0			
5 <i>Scutellaria barbata</i> (whole herb)	10.9	75.6	9.5	14.9
6 <i>Gynostemma pentaphyllum</i> (whole herb)	4.3			
7 <i>Hedyotis diffusa</i> (whole herb)	4.6	29.7	64.8	5.5
8 <i>Polistes mandarius</i> (nest)	7.0			

(>100 kDa), by centrifugation using 5- and 100-kDa molecular weight cut-off ultrafiltration membranes (Vivaspin 2, Vivascience Ltd., Göttingen, Germany).

Cell culture. Human tumor cell lines (HuH-7, HepG2, PLC/PRF/5, and Hep3B) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University, Japan. The cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, deactivated), 0.3 mg/ml glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and 2.0 mg/ml NaHCO₃ (as a growth medium) in a humidified atmosphere containing 5% CO₂ at 37°C.

WST-1 dye reduction assay. Chinese herb extracts were dissolved in FBS-free medium as the test medium. Cells were seeded into 96-well plates at 2×10^3 cells/100 µl of growth medium. After 24 h, cells were washed with phosphate buffered saline (PBS) and cultured in test media for 0–72 h. After incubation, viable cell number was counted by WST-1 dye-reduction assay using a Cell Counting Kit (Dotite, Kumamoto, Japan).

DNA fragmentation. Cells were seeded at 2×10^5 cells/10 ml of growth medium into culture dishes 100 mm in diameter. After 24 h, cells were washed with PBS and cultured in test media with/without 100 µM caspase inhibitor (Z-Val-Ala-Asp-FMK, MBL, Co., Ltd., Nagoya, Japan) for 72 h. After incubation, cells were collected, and resuspended in 100 µl of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100, pH 7.4) for 10 min at 4°C. The lysate was centrifuged at $10,000 \times g$ for 5 min. The supernatant was collected, incubated at 37°C for 1 h with RNase A (final conc. 0.1 µg/ml), and then treated with proteinase K (final conc. 0.1 µg/ml) at 37°C for 1 h. After the reaction, DNA was precipitated by addition of 120 µl of 2-propanol

and 20 µl of 5 M NaCl at –20°C overnight. The precipitate was resuspended in Tris-EDTA buffer, and electrophoresed in a 2% agarose gel. The fragmented DNA was detected by ethidium bromide staining.

Statistical analysis. Statistical comparison among several groups was performed using one-way ANOVA, followed by the Newman-Keuls test for multiple comparisons among groups. Student's *t*-test was used for comparisons between two groups. Difference was considered significant at $p < 0.05$.

Results

The compositions of water extracts from the eight Chinese herbs are shown in Table 1. Figure 1 shows cell viability of HuH-7 after incubation for 72 h in the presence of 100 µg/ml Chinese herb extracts in FBS-free medium. Seven of the water extracts, excluding that from PK, reduced the cell viability in comparison with controls (Fig. 1). CZ is commonly used to treat liver cancer patients. The chemopreventive components in CZ are identified [16–19]. Hence, we used the extract of CZ as a positive control in this study. The extracts from HD and SB, like CZ extract, induced the cell growth inhibition. We selected the extracts from SB and HD for further studies.

The water extracts from HD, SB, and CZ exhibited dose- and time-dependent growth inhibitory effects on HuH-7 (data not shown), and inhibited the growth of another three human hepatoma cell lines: PLC/PRF/5, HepG2 and Hep3B (Fig. 2). IC₅₀ values of the water extracts from HD, SB, and CZ at 72 h on HuH-7 were 80.8, 47.1, and 64.0 µg/ml, respectively.

To clarify whether these Chinese herb extracts induce apoptosis of HuH-7 cells, DNA fragmenta-

Fig. 1. Growth inhibitory effects of various Chinese herb water extracts on HuH-7. Cells were incubated for 72 h in the absence or presence of 100 μ g/ml Chinese herb extracts: C, Control; 1, *Curcuma zedoaria*; 2, *Picrorrhiza kurroa*; 3, *Sparganium simplex*; 4, *Salvia yunnanensis*; 5, *Scutellaria barbata*; 6, *Gynostemma pentaphyllum*; 7, *Hedyotis diffusa*; 8, *Polistes mandarius*. Chinese herb extracts were dissolved in FBS-free medium. Results are mean $\pm$ SE values of 8 wells. Symbols represent statistical significance: ** p <0.01, *** p <0.001.

Fig. 2. Growth inhibitory effects of various Chinese herb water extracts on human hepatoma cell lines. Cells were incubated for 72 h in the presence or absence of 100 μ g/ml Chinese herb extracts: 1, Control; 2, *Curcuma zedoaria*; 3, *Scutellaria barbata*; 4, *Hedyotis diffusa*. Results are mean $\pm$ SE values of 8 wells. ** p <0.01 and *** p <0.001 compared with control.

tion and nuclear morphology by Hoechst 33258 staining were examined. The nuclei of the cells treated with these Chinese herb extracts showed nuclear shrinkage and condensation (data not shown). DNA fragmentation was also observed in HuH-7 cells treated with these Chinese herb extracts for 72 h (Fig. 3). These fragmentations were inhibited by the caspase inhibitor (Fig. 3).

The water extracts of HD and SB were fractionated according to molecular size by ultrafiltration into: Fraction I (<5 kDa), Fraction II (5–100 kDa) and Fraction III (>100 kDa). Fraction II of the HD extract comprised 64.8% of the total extract, and the growth inhibitory effect of Fraction II was the same as that of the total water extract. Fraction I of SB

Fig. 3. DNA ladder formation of various Chinese herb water extracts on HuH-7. Cells were incubated for 72 h in the presence or absence of 100 μ g/ml Chinese herb water extracts: lanes 1 and 5, Control; lanes 2 and 6, *Curcuma zedoaria*; lanes 3 and 7, *Scutellaria barbata*; lanes 4 and 8, *Hedyotis diffusa* in the absence (lanes 1–4) or presence (lanes 5–8) of the caspase inhibitor. Chinese herb extracts were dissolved in FBS-free medium. DNA was subjected to 2% agarose gel electrophoresis. Lane M provides a molecular marker showing 200 bps ladder.

Fig. 4. Growth inhibitory effects of ultrafiltrated fractions from *Hedyotis diffusa* (A) and *Scutellaria barbata* (B) water extracts on HuH-7. Cells were incubated for 72 h in the presence or absence of 100 μ g/ml Chinese herb water extracts: 1, Control; 2, total extract; 3, <5 kDa; 4, 5–100 kDa; 5, >100 kDa. Results are mean $\pm$ SE values of 8 wells. Values not sharing a common superscript are significantly difference at p <0.05.

water extract comprised 75.6% of the total water extract, and the growth inhibitory effect of Fraction I was stronger than those of Fractions II and III (Fig. 4).

Discussion

In the present study, the chemopreventive effects of Chinese herbs against hepatocarcinogenesis were investigated.

Chinese herbs are commonly used as traditional

medicine to treat many diseases in Asia, including China and Japan. The Chinese herbs investigated in the present study are used for the treatment of liver cancer. Some of the herbal compounds with antitumor activities not only modify the host immune response but also have been shown to trigger apoptotic cell death [14, 15]. In general, Chinese herbs are usually decocted and the decoctions are given to patients. Accordingly, the chemopreventive effects of water extracts from eight Chinese herbs against hepatocarcinogenesis *in vitro* were tested.

There have been many reports about the anticancer effects of CZ both *in vitro* and *in vivo* [16–19]. CZ has been suggested to contain chemopreventive compounds, such as curcuminoids, sesquiterpenoids, *etc.* [17, 19]. Accordingly, we used the water extract from CZ as a positive control. In this study, the water extract from CZ showed a strong growth inhibitory effect on HuH-7 cells (Fig. 1). The water extracts from HD and SB showed growth inhibitory effects similar to that of CZ. Therefore, we selected these two Chinese herb water extracts for further study, and confirmed that they inhibited growth of HuH-7 cells in a dose- and time-dependent manner (data not shown).

We further investigated whether the inhibition of cell growth induced by these Chinese herb water extracts involves apoptosis. Analysis of DNA fragmentation (Fig. 3) and chromatin condensation (data not shown) indicated that the cell growth inhibition by these three extracts would be at least partially mediated by apoptosis. Moreover, these fragmentations were inhibited by caspase inhibitor. Caspases are known to play crucial roles in the apoptotic pathway. Thus, it is conceivable that the extracts from HD and SB might contain chemopreventive compounds that induce apoptosis of human tumor cells *via* the activation of caspases.

Chemopreventive compounds occurring naturally in many Chinese herbs include polyphenols, aromatic isothiocyanates, flavonoids, terpenoids, retinoids, tocopherols, sterols, and others. When SB and HD extracts were fractioned by molecular size, the chemopreventive compounds from HD were found mainly in Fraction II (5–100 kDa) and those from SB mainly in Fraction I (<5 kDa) (Fig. 5). Further study is warranted to identify these compounds and to clarify their growth inhibitory mechanisms.

Chinese herbs are commonly used in traditional formulas. For example, Bu-Zhong-Yi-Qi-Tang comprises 10 herbs, and its major key compounds

include astrogaloside IV, saikosaponin-a and glycyrrhizin [21]. Kao *et al.* [21] reported that major individual compounds, including those occurring in Bu-Zhong-Yi-Qi-Tang, failed to suppress cell growth of Hep3B, although Bu-Zhong-Yi-Qi-Tang itself and mixtures of its major compounds showed cell growth inhibition. The implication was that the compounds in Bu-Zhong-Yi-Qi-Tang interacted synergistically or additively. Accordingly, those Chinese herbs that did not show growth inhibition in this study might show growth inhibition when they are administered in a traditional formula.

The p53 protein plays a crucial role in suppressing tumorigenicity by inducing cell cycle arrest and/or apoptosis in response to DNA damage [22, 23]. The p53 tumor suppressor gene is the most frequent target for genetic alterations in human cancer, and mutant p53 protein loses its tumor suppressor function. Ectopic expression of mutant p53 in p53-null cell lines can increase tumorigenic potential and drug resistance [22]. Since HuH-7 (mutated p53), PLC/PRF/5 (mutated p53), Hep3B (deleted p53), and HepG2 (wild-type p53) were growth inhibited by our three extracts, the p53 pathway is probably not involved with their growth inhibitory effects.

In conclusion, we identified two Chinese herbs, HD and SB, with growth inhibitory effects on human hepatoma cell lines. Future studies may provide information on novel anticancer compounds contained in these extracts, suggest new drug targets, and identify apoptotic pathways useful in development of novel regimens for cancer therapy. Moreover, clinical application and *in vivo* study in animals (*i.e.* in mice genetically prone to develop spontaneous hepatomas) warrant further exploration.

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