Bioactivity of High-molecular-weight Constituents of *Cistanche tubulosa*

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Summary

Cistanche tubulosa (Schrenk) R. Wight is traditionally eaten in Hotan in Xinjiang Uygur Autonomous Region, an area known for longevity and oasis in the Takla-Makan Desert. In this study, we investigated whether high-molecula-weight constituents of *C. tubulosa* have the same effects on human cell lines as we previously reported for *C. salsa*. As per results, *C. tubulosa* dialysate (CTD) prepared using a 3,500-Da molecular weight cut-off dialysis membrane enhanced IgM production in B-cell line BALL-1 and IgG production in B-cell line HMy-2, induced cell proliferation in BALL-1 and T-cell line Jurkat, and oppositely inhibited cell proliferation in B-cell line Namalwa. Using gel filtration chromatography, we found that, similar to *C. salsa*, the two activities (i.e., enhancement of IgM production and growth inhibition) were concentrated in different fractions. These results suggest that *C. tubulosa* contains high-molecular-weight constituents having immunomodulatory and direct anti-cancer effects, which seem to contribute to the longevity.

Keywords: *Cistanche tubulosa*, high-molecular-weight constituents, immunomodulatory effect, anti-cancer effect.

Introduction

Cistanche is a worldwide genus of holoparasitic desert plants in the family Orobanchaceae. The main species are *C. salsa* (C.A. Meyer) G. Beck and *C. deserticola* Y.C. Ma. The stems of *Cistanche* species have been commonly used traditionally for treating impotence, sterility lumbago, body weakness, and tonic. Beside its common uses, interestingly, the people of the Hotan in Xinjiang Uygur Autonomous Region of China, an area known for longevity and oasis in the Takla-Makan Desert, have traditionally eaten *Cistanche tubulosa* (Schrenk) R. Wight. The daily consumption is believed to the key of their longevity. In Japan, *C. salsa* produced in China has been used as medicinal tonic; however, production of this plant is decreasing. Therefore, *C. tubulosa* is now being used as a substitute for *C. salsa*. The most characteristic constituents of *Cistanche* species are low-molecular-weight constituents phenylethanoids such as acteoside and echinacoside. However, it was reported that their chemical compositions were different among *Cistanche* species [1]. We have already reported the effects of *C. salsa* as a biological response modifier. First, we demonstrated that *C. salsa* extract enhanced the production of IgM and IgG in human lymph node lymphocytes [2]. Then, we showed that *C. salsa* dialysate (CSD) prepared using a 3,500-Da molecular weight (MW) cut-off dialysis membrane enhanced IgM production in human B-cell line BALL-1 with added effect of cell proliferation [3]. CSD also had an opposite effect whereby it induced growth inhibition accompanied by apoptosis in human Burkitt's lymphoma cell line Namalwa [4]. Furthermore, we have shown the possible presence of two types of immunomodulatory fractions in CSD: a high-MW active constituent (HCS) enhancing IgM production and a middle-MW active constituent (MCS) inhibiting cell proliferation. These constituents seem to be polysaccharides because they are water soluble and thermostable [5]. A subsequent study showed that, similar to B-1a cells, BALL-1 cells express CD5 antigen and respond to lipopolysaccharide. In the study, we confirmed that protein-bound polysaccharide-K (PSK) has the same effects as CSD on BALL-1 cells [6]. On the other hand, Hattori et al. have already reported the anti-cancer effect of PSK by using Namalwa cells as haematological malignant cells [7]. PSK, prepared from the basidiomycete *Coriolus versicolor*, is composed of polysaccharide and peptide portions, and has been orally administered to patients with cancer as a therapeutic biological response modifier.

Many studies have been conducted to determine the immunomodulatory effect of PSK on cellular immunity and its anti-cancer effects on various types of tumour cells [8]. We had first demonstrated the direct effect of PSK on a B cell line, thus indicating its direct effect on humoral immunity [6]. Considering the results, we then investigated the effect of CSD on various types of cell lines with PSK as a reference and showed that CSD has a direct anti-cancer effect similar to that of PSK. Briefly, the same fraction from CSD inhibited cell proliferation in Namalwa and mouse melanoma cell line B16 [9].

We have so far reported that *C. salsa* has immunomodulatory and direct anti-cancer effects in its high-MW constituents, which differ from the main characteristic constituents (i.e., phenylethanoids). Therefore, in this study, we investigated whether high-MW constituents of *C. tubulosa* have the same effects as those of *C. salsa*.

Materials and methods

Extract preparation

C. tubulosa produced in China was purchased from Donasis Herb Labo. Co., Japan. *C. tubulosa* dialysate (CTD) was prepared by using a similar method as in our previous study with *C. salsa* [3]. Dried *C. tubulosa* (1 g) was cut into small pieces and treated with 20 ml of purified water at 50°C for 30 min. The extract obtained was centrifuged at 2,200 × g for 20 min. The supernatant was filtered through sterilised gauze and passed through two types of filters (0.45 μm and 0.22 μm). The resulting extract was filtered at 4°C through a dialysis membrane with a 3,500-Da MW cut-off (Spectrum Medical Industries, Inc., USA) to remove the low-MW fraction. After dialysis, the extract was centrifuged, filtered (0.22 μm), and then freeze-dried. Approximately 15–20 mg of dry product was obtained from 1 g of *C. tubulosa*.

Gel filtration column chromatography

CTD was dissolved in 10 mM phosphate buffer (pH 8.0) at 5 mg/ml. The prepared solution (0.8 ml) was filtered (0.22 μm) before application and eluted with the same solution through a column containing TOYOPEARL HW-65S (1.0 × 98 cm; Tosoh Corp., Japan), after which 1 ml (30 drops) fractions were collected.

Active fractions on HW-65S were collected, concentrated by freeze-drying and then eluted through a column containing TOYOPEARL HW-75F (1.0 × 95.5 cm; Tosoh Corp.). To determine the approximate MW of the fractionated constituents, dextrans of known MW— that is, 2,000 kDa (D1), 500 kDa (D2), 70 kDa (D3) and 6 kDa (D4)—obtained from *Leuconostoc* spp. (Sigma-Aldrich Japan K.K., Japan) and cyanocobalamin (VB12, MW = 1355.4; Wako Pure Chemical Industries, Ltd., Japan) were used. The total sugar and protein contents of each fraction were determined by using the phenol–sulphuric acid method and the Lowry method with a PIERCE bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., USA), respectively. Glucose and bovine serum albumin were used as the respective standards. Further, the absorbance of each fraction at 320 nm (A320) was measured. The elution peak of VB_{12} was determined by its absorbance value at 260 nm, and the peaks of the dextrans were determined by using the phenol–sulphuric acid method.

Cell culture

B-cell line BALL-1 (supplied by RIKEN CELL BANK, Japan), Namalwa, HMy-2 and T-cell line Jurkat were cultured in RPMI-1640 medium containing 10% foetal bovine serum (BioSource International, Inc., USA). Cells were plated at 5×10^4 cells/ml in the medium containing various concentrations of CTD or 2–10% of the solution of each fraction under humidified 5% CO_2:95% air atmosphere at 37°C. The acquired cells were counted using a cell counter.

Enzyme-linked immunosorbent assay (ELISA)

The amount of antibodies was measured by ELISA as described in our previous study [2]. The amounts of IgM (BALL-1 and Namalwa) and IgG (HMy-2) were measured. Goat anti-human and horseradish peroxidase (HRP)-conjugated goat anti-human antibodies against IgM and IgG were used. All antibodies were purchased from BioSource International.

Statistical analysis

All data are expressed as the mean ± SD. Statistical significance was analysed by using the Dunnet test, with $P < 0.01$ considered statistically significant.

Results and discussion

Table 1 shows the reactivity of four cell lines with *C. tubulosa* dialysate (CTD).

Table 1. Reactivity of cell lines with CTD.

Cell line	CTD (μg/ml)	Cell number ($\times 10^{-5}$cells)	Immunoglobulin production (ng/ml)
BALL-1	0	7.27 ± 0.08	933 ± 50
	1	7.48 ± 0.11	985 ± 6
	5	7.41 ± 0.21	1,095 ± 14***
	10	8.07 ± 0.11***	1,259 ± 33***
	20	8.54 ± 0.14***	1,427 ± 31***
HMy-2	0	9.95 ± 0.02	58 ± 2
	1	10.0 ± 0.08	60 ± 2
	5	10.0 ± 0.05	77 ± 5***
	10	9.96 ± 0.08	93 ± 5***
	20	10.0 ± 0.14	113 ± 5***
Jurkat	0	11.6 ± 0.35	—
	1	11.7 ± 0.19	—
	5	12.9 ± 0.32***	—
	10	13.9 ± 0.12***	—
	20	14.3 ± 0.22***	—
Namalwa	0	24.2 ± 0.36	339 ± 10
	1	22.4 ± 0.74**	351 ± 14
	5	12.2 ± 0.40***	204 ± 11***
	10	10.9 ± 0.25***	214 ± 6***
	20	11.6 ± 0.36***	203 ± 6***

BALL-1 and HMy-2 cells were cultured in the presence of various concentrations of CTD for 4 days. Jurkat and Namalwa cells were cultured for 5 days. Asterisks indicate values that are significantly different from those of the control group: $^{**}P < 0.01$; $^{***}P < 0.001$.

CTD enhanced IgM production in BALL-1 and IgG production in HMy-2 at 5–20 μg/ml. Furthermore, it induced cell proliferation in BALL-1 at 10–20 μg/ml and Jurkat at 5–20 μg/ml. In contrast, it inhibited cell proliferation in Namalwa at the lower concentration with decreased antibody production. These responses are the same as that of *C. salsa* in our previous study [9]. These results show that *C. tubulosa* has immunomodulatory and anti-cancer effects *via* its high-MW constituents.

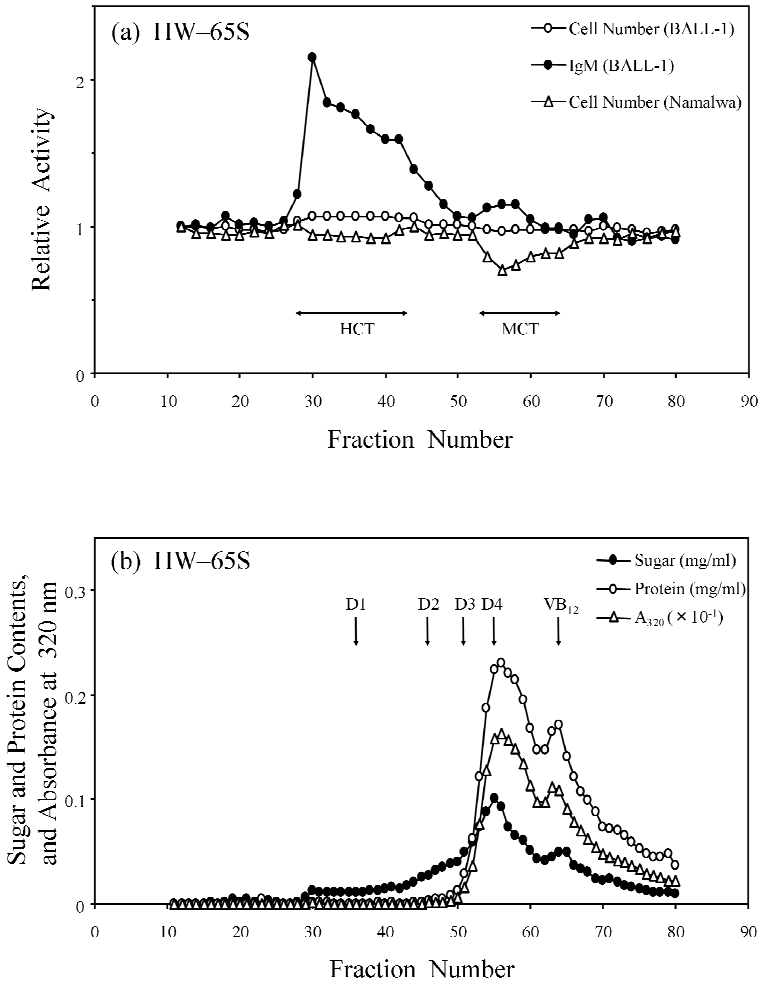

Figure 1. Gel filtration column chromatography of CTD on HW-65S. The activities (a) and chemical properties (b) of the fractions were assessed. BALL-1 and Namalwa cells were treated with 5% and 2%, respectively, of each eluted fraction. The relative activity is the proportional value in each fraction against that in fraction no. 22. The arrows indicate the elution peak of each compound.

Figure 1 shows the gel filtration column chromatograms of CTD on HW-65S. Both CTD activities were concentrated in different fractions, similar to *C. salsa*. The first, concentrated in fraction no. 30, was a high-MW active constituent (HCT) that enhanced IgM production, whereas the second, concentrated in fraction no. 56, was a middle-MW active constituent (MCT) that inhibited cell proliferation. The peak obtained for the HCT was sharp; in our previous study, the peak of the HCS, corresponding to HCT, was not sharp on HW-55F, which is used for lower-MW fractions. This suggested that pure constituents are not easily obtained from the HCS, because there seem to be various constituents with slightly different MWs [5]. In this study, HCT seemed to be eluted in void volume, indicating macromolecule constituents and compositional purity. Therefore, active fractions for BALL-1 cells on HW-65S (HCT: fraction no. 28–43) were collected, concentrated by freeze-drying and then eluted through HW-75F. Sugar and protein in the concentrated sample before application were 90 μg/ml and 10 μg/ml, respectively.

Figure 2 shows the column chromatograms of HCT on HW-75F. Although dextran marker D1 (2,000 kDa) was eluted earlier than D2 (500 kDa), the peaks of D1 and D2 were at the same fraction. Active constituents were eluted in the fractions around D1 and D2. These results suggest that the HCT is a macromolecule polysaccharide including a peptide portion. The MW of the HCT was estimated as approximately 2,000 kDa or more. Although the HCT is a macromolecule constituent, which cannot be absorbed in itself, it might be digested at its peptide portion, or naturally or artificially destroyed to lower molecules similar to the HCS from *C. salsa*.

These findings suggest that high-MW constituents of *C. tubulosa* have the same effects as those of *C. salsa*, and possibly contribute to the longevity in Hotan.

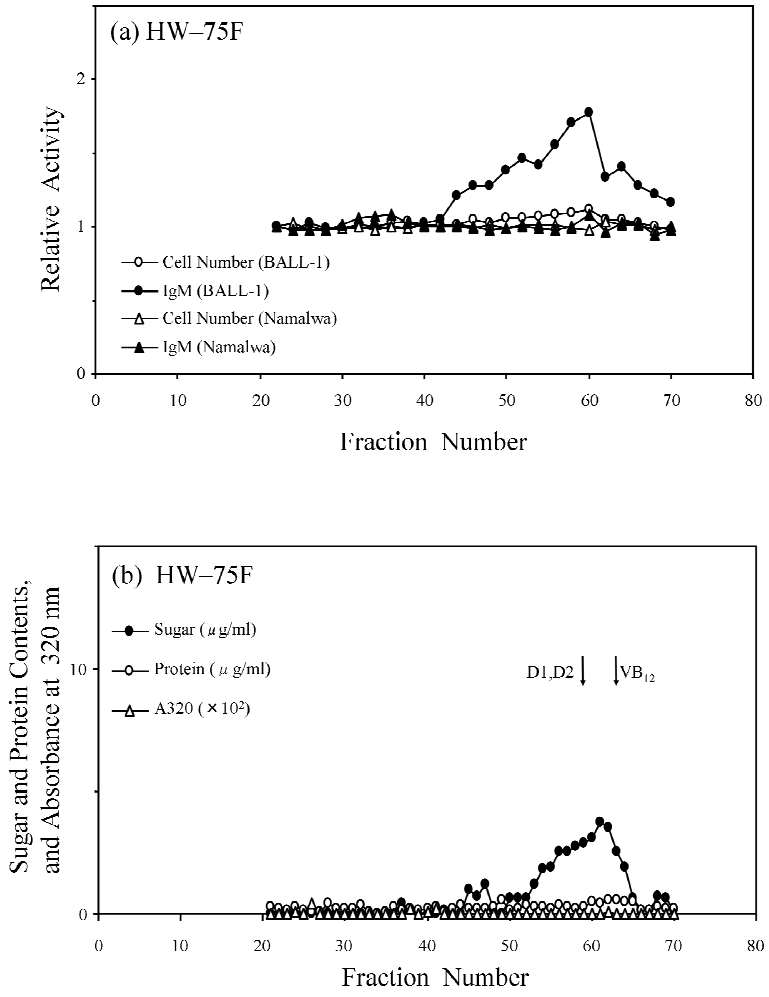

Figure 2. Gel filtration column chromatography of HCT on HW-75F. The activities (a) and chemical properties (b) of the fractions were assessed. BALL-1 and Namalwa cells were treated with 10% of each eluted fraction The relative activity is the proportional value in each fraction against that in fraction no.22. The arrows indicate the elution peak of each compound.

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