J. Microbiol. Biotechnol. (2008), **18**(3), 512–519

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Apoptosis of Human Hepatocarcinoma (HepG2) and Neuroblastoma (SK-N-SH) Cells Induced by Polysaccharides-Peptide Complexes Produced by Submerged Mycelial Culture of an Entomopathogenic Fungus *Cordyceps sphecocephala*

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Received: September 15, 2007 / Accepted: November 5, 2007

Three different polysaccharide-peptide complexes (PPC, named as Fr-I, Fr-II, and Fr-III) were produced by submerged mycelial culture of an entomopathogenic fungus *Cordyceps sphecocephala*, and their anticancer activities were investigated in human hepatocarcinoma (HepG2) and neuroblastoma (SK-N-SH) cells. The highest inhibitory effects of PPC on both HepG2 and SK-N-SH cells were achieved with Fr-I, whereas Fr-III with low molecular mass showed lower inhibition effects. Interestingly, the inhibitory effects of the three fractions were increased after protease digestion, suggesting that the inhibitory effects resulted mainly from the carbohydrate moiety, at least in the case of Fr-II and Fr-III, of PPC. The results of DNA fragmentation in PPC-induced apoptotic cells were confirmed by both DNA ladder assay and comet assay. Our investigation also showed that PPC-induced apoptosis of both cancer cells was associated with intracellular events including DNA fragmentation, activation of caspase-3, and modulation of Bcl-2 and Bax. We conclude that PPC has potential as a novel therapeutic agent for the treatment of both HepG2 and SK-N-SH cancer cells without any cytotoxicity against normal cells.

Keywords: Anticancer activity, apoptosis, comet assay, *Cordyceps sphecocephala*, HepG2, SK-N-SH

Apoptosis or programmed cell death is a normal physiological process serving to eliminate unwanted cells and maintain homeostasis in healthy tissue. Tumor growth is regulated by the balance between cell proliferation and apoptosis. Dysregulated cell proliferation and suppressed cell death together provide the underlying platform for neoplastic progression [8]. It has been suggested that the loss of apoptotic control in favor of cell proliferation is responsible for prostate cancer initiation and progression [42]. In turn, one essential strategy for cancer therapy is to target the lesions that suppress apoptosis in the tumor cells. It has been found that many cancer chemotherapy drugs exert anticancer effects on malignant cells by inducing apoptosis [12].

A vast variety of naturally occurring substances have been shown to protect against experimental carcinogenesis. The recent scientific evaluation of macrofungi such as mushrooms and entomopathogenic fungi has confirmed the efficacy of extracts of either fruiting bodies or mycelia of these candidates in the inhibition of various cancer cells [27, 34, 44, 49].

Cordyceps sphecocephala is a rare entomopathogenic fungal species and it cannot meet the increasing demand for tonic and medicine uses. Therefore, to provide opportunities for increasing its economic benefit and practical use, the submerged culture of some isolates derived from the genus *Cordyceps* have been developed [16, 33]. It is generally accepted that the medicinal merits of cultured mycelia are equally as effective as those of *Cordyceps* species found in nature [18]. In addition, submerged culture has potential advantages for higher mycelial production in a compact space and for a shorter incubation time, with a lesser chance of contamination [4, 15, 21]. Previous studies on extracellular metabolites from various *Cordyceps* species have demonstrated many interesting biological activities, including antitumor activity [3, 31], immunopotentiation [29], hypoglycemic activity [13, 14], and hypocholesterolemic effect [18].

In this study, we demonstrated that the extracellular polysaccharide-peptide complexes (PPC) obtained by submerged mycelial culture of *C. sphecocephala* strongly inhibited the growth of human hepatocarcinoma (HepG2)

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and human neuroblastoma (SK-N-SH) cells by promoting cell apoptosis involving the expression of apoptosis-related proteins. To the best of our knowledge, this is the first report describing the biological activity of PPC obtained by submerged culture of *C. sphecocephala*.

MATERIALS AND METHODS

Cell Line and Culture

Human hepatocarcinoma (HepG2) and human neuroblastoma (SK-N-SH) cells were obtained from the Korean Cell Line Bank of Seoul National University (Seoul, Korea). The cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere with 5% CO_2, and the medium was changed every 2 days. After confluence, the cells were subcultured following trypsinization.

Preparation of PPC

Cordyceps sphecocephala J-201, a newly isolated fungus from dead bees in the mountainous district of the Kyunggi province in Korea, was transferred monthly to fresh nutrient agar medium. A culture of the fungus was maintained on potato dextrose agar (PDA) slants stored at 4°C and subcultured every four weeks. The submerged culture of *C. sphecocephala* for the production of crude PPC was performed in a 5-l stirred-tank bioreactor (Ko-BioTech Co., Incheon, Korea) for 10 days with a six-bladed disc turbine impeller under the following culture conditions (g/l): sucrose 40, yeast extract 6, polypeptone 2, KH_2PO_4 0.46, K_2HPO_4 1, and $MgSO_4 \cdot 7H_2O$ 0.5; temperature, 25°C; aeration rate, 2 vvm; agitation speed, 150 rpm; initial pH, 5.0; working volume, 3 l. Culture broths were centrifuged at 10,000 ×*g* for 20 min, and the resulting supernatant was mixed with four volumes of absolute ethanol, stirred vigorously, and left overnight at 4°C. The precipitated crude PPC were centrifuged at 10,000 ×*g* for 20 min, and the supernatant was discarded. The precipitate of crude PPC was lyophilized. If necessary, crude PPC were purified using gel filtration in a Sepharose CL-6B column, as previously described (Fig. 1) [30].

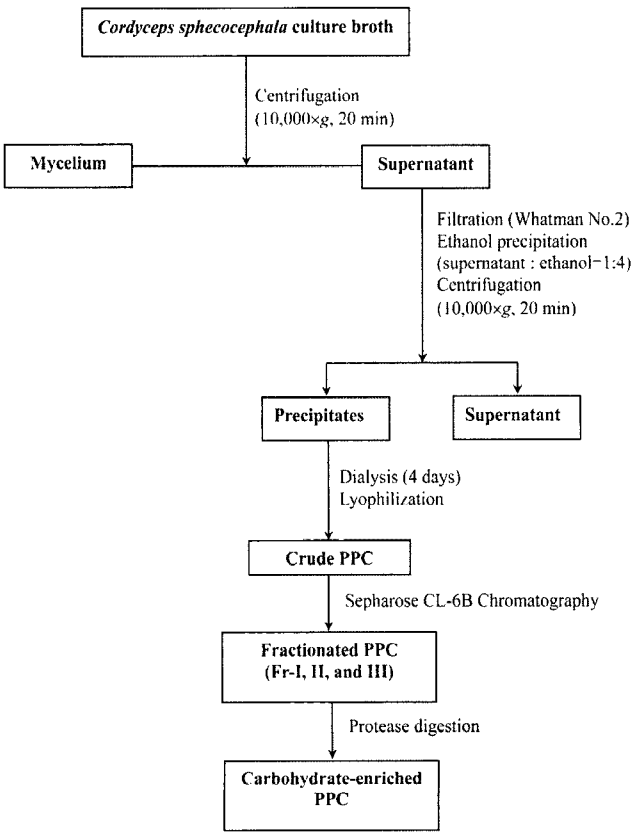

Fig. 1. Preparation of polysaccharide-peptide complexes (PPC) by submerged mycelial culture of *Cordyceps sphecocephala*.

Cell Viability Assay

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay based on the reduction of MTT into formazan dye by active mitochondria [28]. Briefly, The cells were placed in 96-well culture dishes (Nunclon, Roskilde, Denmark) at a density of 5×10^4 cells/ml in MEM culture medium containing 10% FBS at 37°C, 5% CO_2. After 24 h, the cells were washed and placed in culture medium with different concentrations of PPC (0.1, 0.5, and 1.0 mg/ml) for 48 h. Thereafter, 20 μl of MTT solution (5 mg MTT/ml in PBS) was added to each well of a microtiter plate and incubated for 4 h. After washing, the formazan dye precipitates, the amount of which is proportional to the number of live cells, were dissolved in 100 μl of DMSO. The absorbance was read at 540 nm using a microtiter plate reader (Thermo Electron, Vantaa, Finland). The inhibition rate of cell growth was calculated by the following formula: mean value of {(control group-treated group)/control group}×100%. Triplicate wells were analyzed per each concentration.

Protease Digestion of PPC

The three fractions of PPC (40 mg) were separately dissolved in 40 ml of 50 mM Tris-HCl buffer, pH 7.9, containing 10 mM $CaCl_2$, and then 10 mg of protease (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added in each fraction. The reaction mixture was incubated at 37°C for 48 h. The reaction was terminated by boiling for 5 min. The mixture was then dialyzed against deionized water for 4 days and the nondialyzable portion was lyophilized [11, 46].

DNA Ladder Assay

DNA extraction and agarose gel electrophoresis were performed by the method described by Kumar *et al.* [19]. After 24 h of treatment, the DNA was extracted from the cell lysate as follows. Both attached and floating cells were collected, washed with PBS, and centrifuged at 2,000 ×*g* for 5 min to collect the cell pellet, which was resuspended in 0.5 ml of lysis buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.5% SDS, 0.5 mg/ml proteinase K), transferred to a microfuge tube, and incubated for 1 h at 50°C. To this, 4 μl of RNase A (Sigma Chemical. Co., St. Louis, MO, U.S.A.) was added and the tubes were incubated at 37°C for 1 h. To each tube, 0.5 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed, and centrifuged at 13,000 ×*g* for 20 min to separate the DNA-containing upper aqueous phase. Phenol-chloroform extraction was repeated twice, followed by chloroform extraction alone. To the resulting aqueous phase, two volumes of ice-cold absolute ethanol and 1/10th volume of 3 M sodium acetate were added and the

mixture was incubated for 30 min on ice to precipitate DNA. DNA was pelleted by centrifuging at 13,000 ×*g* for 20 min at 4°C, the supernatant was aspirated, and the pellet was washed with 1 ml of 70% ethanol. After repeating the above centrifugation step and removing the last traces of the supernatant fraction, the pellets were air-dried and resuspended with 50 µl of Tris-EDTA (TE) buffer. Equal quantities of DNA were electrophoresed in 1.5% agarose gel containing 0.5 mg/l of ethidium bromide. After electrophoresis, the gel was photographed under UV light.

Single-Cell Gel Electrophoresis (Comet Assay)

For the standard comet assay, three slides were prepared from cells obtained from each group treated at a different concentration [41]. Briefly, about 4×10^6 cells were mixed with 80 µl of 0.7% low-melting agarose in PBS at 37°C in a microtube, and then spread over a window microscopic slide specially designed for this assay locally, precoated with 150 µl of 0.5% normal-melting agarose in PBS. Slides were placed immediately in cold lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, and 1% Triton X-100, 4°C) for a minimum of 1 h. After lysis, the slides were drained and placed in a horizontal gel electrophoresis tank surrounded by ice and filled with fresh cold electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) to a level of approximately 0.25 cm above the slides. Slides were kept in the high pH buffer for 20 min to allow DNA unwinding. Electrophoresis was then carried out for 20 min at 25 V and 300 mA. The slides were then drained and flooded slowly with 3 changes of neutralization buffer (0.4 M Tris, pH 7.5) for 5 min each and then stained with 30 ml of ethidium bromide (20 mg/l) and covered with cover slips. To prevent additional DNA damage from visible light, all the steps described above were conducted under a dimmed light. A total of 50 randomly selected cells per slide were analyzed. Image analysis was performed with a fluorescence microscope (Zeiss Axiovert L410, Germany) attached to a digital camera (Olympus, Tokyo, Japan) equipped with an excitation filter of 549 nm, a barrier filter of 590 nm, and a 100-W mercury lamp. The images were analyzed by the Kinetic Imaging Komet 5.0. The percentage of DNA in the comet tail (designated as "DNA damages") was automatically calculated. At least three slides per one experimental condition, with 50 randomly selected cells per slide, were analyzed.

Western Blot Analysis

HepG2 cells were treated with PPC at concentrations of 0, 0.1, 0.5, and 1 mg/ml for 24 h. At indicated times, the cells were washed with PBS and then lysed (7 M urea, 2 M thiourea, 4% CHAPS, 1 mM PMSF, 20 mM DTT, and 2% IPG buffer). The cell lysates were centrifuged at 10,000 ×*g* for 30 min at 4°C, and the protein content of the cytosolic fraction (supernatant) was measured by using the Bradford method with protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, U.S.A.) [1]. Protein was diluted in 2× sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol] and heated for 5 min at 95°C before SDS-PAGE gel analysis (12 and 15%). Subsequently, they were transferred to microporous polyvinylidene difluoride (PVDF) membrane and incubated overnight with 5% blocking reagent (Amersham Biosciences, Freiburg, Germany) in Tris-buffered salt (TBS) containing 0.1% Tween-20 at 4°C. The membrane was rinsed in four changes of TBS with Tween-20 (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.0), and incubated twice for 5 min and twice for 10 min in fresh washing buffer. It was then incubated for 2 h with blocking solution containing 1:500 dilution of primary antibody (rabbit anti-Bax, mouse anti-Bcl-2, and goat anti-caspase-3; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After four washes, the membrane was incubated again for 2 h in horseradish peroxidase-conjugated anti-mouse IgG, anti-goat IgG, and anti-rabbit IgG secondary antibody (1:1,000; Santa Cruz Biotechnology) and developed using enhanced chemiluminescence (ECL Western blot analysis system kit; Amersham Biosciences). The Western blot was analyzed by scanning with a UMAX PowerLook 1120 (Maxium Technologies, Inc., Dallas, TX, U.S.A.) and digitalizing using image analysis software (KODAK 1D; Eastman Kodak Co., New York, NY, U.S.A.).

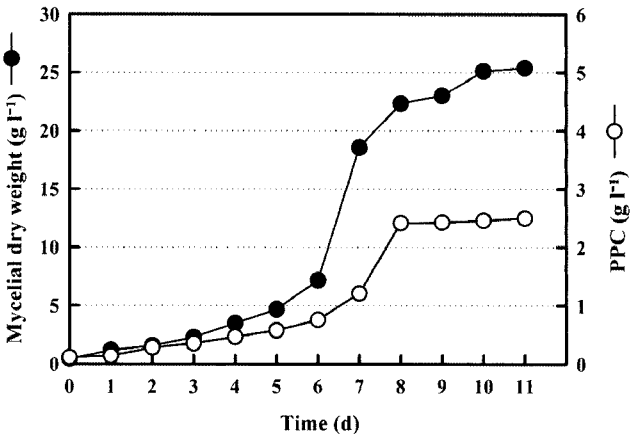

Fig. 2. Typical time profiles for the production of mycelial biomass and polysaccharide-peptide complexes (PPC) in *Cordyceps sphecocephala* in a 5-l stirred-tank bioreactor.

RESULTS

Preparation of the EPS

Fig. 2 shows the typical time profiles of mycelial growth and PPC production achieved in a 5-l stirred-tank bioreactor. The maximum mycelial concentration and PPC production reached 25.4 and 2.5 g/l at day 11, respectively. The crude PPC were fractionated by gel filtration in a Sepharose

Table 1. The chemical characteristics of three different polysaccharide-peptide complexes (Fr-I, II, and III) produced by submerged mycelial culture of *Cordyceps sphecocephala*.

	Fr-I	Fr-II	Fr-III
Protein content[a] (%)	88.3	15.6	46.9
Carbohydrate content[b] (%)	11.7	84.4	53.1
Mannose (%)	56.1	62.1	62.2
Galactose (%)	37.5	30.3	25.9
Glucose (%)	5.4	6.3	18.9
Molecular mass ($\times10^3$ Da)	1,381	27	2.2

[a]Content denotes the weight percentage of each fraction to total amounts of polysaccharide-peptide complexes.
[b]Carbohydrate compositions refer to the weight ratios of each carbohydrate to total carbohydrates except for the trace amounts of other components.

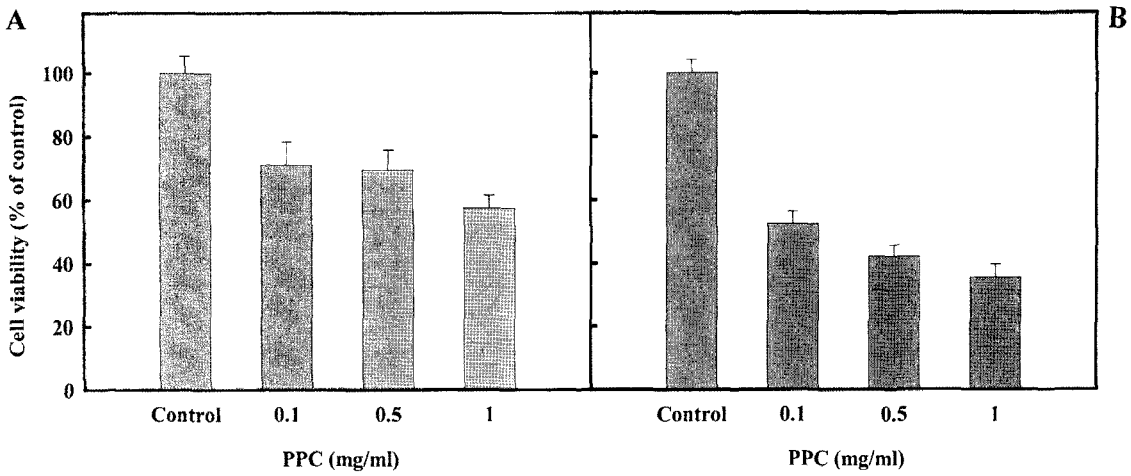

Fig. 3. Inhibitory effects of polysaccharide-peptide complexes (PPC) on the growth of HepG2 (**A**) and SK-N-SH (**B**) cells. All data are expressed as mean±SE.

CL-6B column, by which three different fractions were obtained. Fr-I consisted of mainly proteins (88.3%), whereas Fr-II was composed of mainly carbohydrate (84.4%). In contrast, Fr-III had a similar ratio of polysaccharides and peptides (Table 1).

Inhibitory Effects of PPC on the Growth of Cancer Cells

In order to investigate the inhibitory effects of PPC on the growth of human hepatocarcinoma (HepG2) and human neuroblastoma (SK-N-SH) cell lines, cultures of both cells were treated with crude PPC at various concentrations for 48 h. As shown in Fig. 3, the results demonstrated that crude PPC had significant cytotoxicity on both HepG2 and SK-N-SH cells. After 48 h treatment with crude PPC at a concentration of 1 mg/ml, the inhibitory effects of HepG2 and SK-N-SH cells were 42.6±4.3 and 64.7±1.3%, respectively.

Table 2. Inhibitory effects of three different polysaccharide-peptide complexes (Fr-I, Fr-II, and Fr-III) on the growth of HepG2 and SK-N-SH cancer cells.

Treatment conc. (mg/ml)		Inhibition rate[a] (%)	
		HepG2	SK-N-SH
Fr-I	0.1	53.8±1.5[b]	65.7±4.5
	0.5	60.3±2.0	73.6±2.7
	1	66.4±1.7	75.3±1.9
Fr-II	0.1	35.4±6.5	53.7±4.5
	0.5	48.7±5.9	64.1±3.3
	1	59.7±4.7	70.6±1.4
Fr-III	0.1	16.9±7.1	18.5±4.8
	0.5	27.8±4.4	32.4±5.7
	1	30.0±5.4	33.0±5.1

[a]Inhibition rate was calculated as follows: [1-{(A_{540} of treated cells)/(A_{540} of control cells)}]×100 in MTT assay.
[b]Values are means±SE.

Table 2 shows inhibitory effects of each fraction of PPC on the growth of both cells. The highest inhibitory effects of HepG2 and SK-N-SH cells were achieved with Fr-I, whereas Fr-III with low molecular mass showed lower inhibition rates. To further investigate whether the carbohydrate or peptide moiety played a more important role in the inhibitory effects, each fraction was hydrolyzed with a serine protease. Table 3 shows inhibitory effects of protease-digested fractions of PPC on the growth of both HepG2 and SK-N-SH cells. Interestingly, the inhibitory effects were increased significantly after protease digestion. The maximum enhancements by protease treatments in HepG2 (53.3%) and SK-N-SH (25.8%) cells were achieved by Fr-III.

Effect of PPC on DNA Fragmentation in HepG2 and SK-N-SH Cells

To determine the mode of growth inhibition in HepG2 and SK-N-SH cells induced by PPC, we investigated whether PPC induced internucleosomal degradation of DNA, a characteristic of apoptosis. In the present study, DNA ladders appeared in PPC-treated HepG2 and SK-N-SH cells after exposure to concentrations of 0.1, 0.5, and 1 mg/ml

Table 3. Inhibitory effects of protease-digested polysaccharide-peptide complexes (Fr-I, Fr-II, and Fr-III) on the growth of HepG2 and SK-N-SH cancer cells.[a]

Digested sample	Inhibition rate[b] (%)	
	HepG2	SK-N-SH
Fr-I	84.7±2.1[c]	87.7±0.9
Fr-II	78.8±3.7	83.6±1.2
Fr-III	46.0±3.5	41.5±1.8

[a]Treated concentration was 1 mg/ml.
[b]Inhibition rate was calculated as follows: [1-{(A_{540} of treated cells)/(A_{540} of control cells)}]×100 in MTT assay.
[c]Values are means±SE.

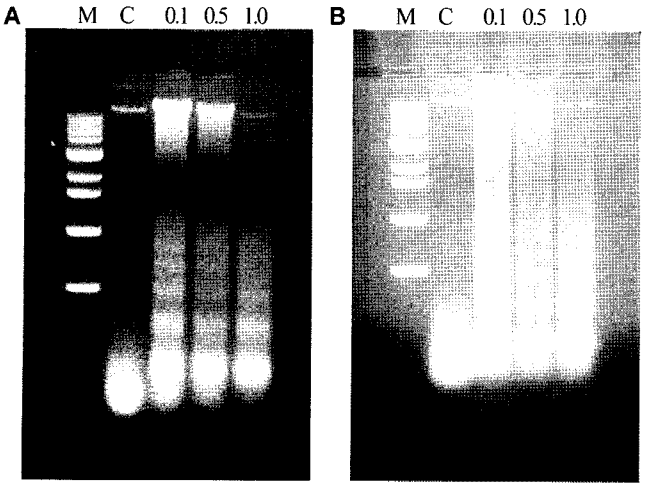

Fig. 4. Effect of polysaccharide-peptide complexes on DNA-fragmentation in HepG2 (**A**) and SK-N-SH (**B**) cells treated with polysaccharide-peptide complexes (PPC) at concentrations of 0, 0.1, 0.5, and 1 mg/ml for 24 h.
M, 1 kb DNA ladder; C, control cells without treatment of PPC.

for 24 h. The control cells of HepG2 and SK-N-SH did not show any DNA fragmentation (Fig. 4).

To further confirm the PPC-mediated apoptosis, we detected DNA damage (50–300 kb fragments) using the comet assay, for HepG2 and SK-N-SH cells treated with crude PPC at concentrations of 0, 0.1, 0.5, and 1 mg/ml for 1 h. The percentage of DNA released to the comet tail was used as the most sensitive parameter to quantify the DNA damage (Table 4). Taken together, it can be concluded that PPC induced apoptosis of both HepG2 and SK-N-SH cells, in a dose-dependent manner.

Effects PPC on Apoptosis-related Gene Expression

To understand the molecular mechanisms by which PPC induced apoptosis, we examined the expression pattern of various apoptosis-related proteins. HepG2 cells were cultured in media with crude PPC at concentrations of 0, 0.1, 0.5, and 1 mg/ml for 24 h. Total protein was isolated and the levels of Bcl-2, Bax, and procaspase-3 were measured by Western blotting. As shown in Fig. 5, the level of the pro-apoptotic molecule, Bax, was increased, whereas the level of the anti-apoptotic molecule, Bcl-2, was decreased in a dose-dependent manner after PPC treatments. In addition, PPC-treated HepG2 cells showed a low procaspase-3 intensity of 32 kDa protein band, implying caspase-3 activation.

Table 4. The results of comet assay for detecting the levels of DNA damage in HepG2 and SK-N-SH cancer cells induced by the PPC for 1 h.

Treatment conc. (mg/ml)	DNA damage[a] (%)	
	HepG2	SK-N-SH
0	1.2±0.9[b]	1.3±0.5
0.1	8.7±2.0	12.8±3.8
0.5	16.5±4.3	22.2±3.8
1.0	24.6±4.5	32.8±4.4

[a]DNA damage was noted as the ratio of comet tail DNA to sum of tail head and tail DNA taken as an average from 100 comets.
[b]Values are means±SE.

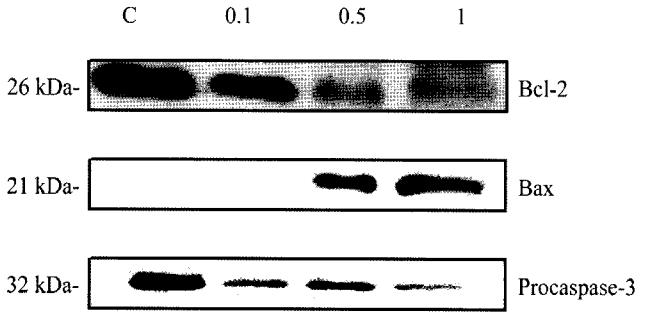

Fig. 5. Changes in the expression of apoptosis-related proteins in HepG2 cells in response to crude polysaccharide-peptide complexes (PPC) at concentrations of 0, 0.1, 0.5, and 1 mg/ml for 24 h.
Cell extracts were subjected to Western blotting to determine the cellular levels of Bcl-2, Bax, and procaspase-3, as described in Materials and Methods.

Discussion

In the present investigation, we found that *C. sphecocephala* PPC markedly reduced the cell viability of HepG2 and SK-N-SH cancer cells in a dose-dependent manner. The suppression of cell proliferation induced by PPC may be due to the induction of apoptosis. Thus, the inhibitory activity of *C. sphecocephala* PPC provides evidence for the *in vitro* cytotoxicity. Interestingly, the inhibitory effects significantly increased after protease digestion, suggesting that the inhibitory effects resulted mainly from the carbohydrate moiety, at least in the case of Fr-II and Fr-III of PPC. This result is consistent with the finding of Yamada *et al.* [45], who pointed out the involvement of the carbohydrate moiety from *Angelica acutiloba* in executing anticomplementary activity. However, there are many controversial results. For example, Liu *et al.* [23] demonstrated that antitumor activity of a protein-rich fraction from *Tricholoma* sp. was in part due to its protein composition. Matsunaga *et al.* [25] also reported that the protein moiety of PSK, a protein-bound polysaccharide obtained from the *Coriolus versicolor* strain CM-101, plays an important role in the exertion of the anticancer activity. More recently, Surenjav *et al.* [37] also demonstrated that protein-bound polysaccharides from the fruiting body of *Lentinus edodes* were favorable to enhancing the anticancer activity.

Although we still have not identified the most active ingredients responsible for the cytotoxic and apoptotic

effects against the two cancer cell lines, our qualitative chemical analysis indicated that galactomannans with higher galactan (or galactooligosaccharides) moiety are probably linked to superior anticancer activities. It has been postulated that macrofungal polysaccharides containing glucose and mannose may have some antitumor action, because a polysaccharide receptor demonstrating high specificity for glucose and mannose has been found on the human macrophage [39, 49]. Moreover, the high molecular mass of Fr-I seems to be related to the higher anticancer activity. Mizuno *et al.* [26] indicated that high molecular mass glucans appear to be more effective for cancer cell apoptosis than those of low molecular mass ranging from 500 to 2,000 kDa.

Cumulated studies showed that polysaccharides or polysaccharides-peptide complexes obtained from macrofungi are able to suppress tumors, either indirectly by enhancing the host's immune systems or directly by inducing apoptosis in tumor cells [27, 44, 50].

For example, polysaccharides like β(1→3) glucans bind to receptors on macrophages and trigger the activation process [39]. Wang *et al.* [43] demonstrated that PPC from cultured mycelia of the *Tricholoma mongolicum* mushroom can activate macrophages, thereby triggering immunoenhancing and anticancer activities. It is believed that structural features such as β(1→3) linkage in the main chain of the glucan and additional β(1→6) branch points are needed for anticancer action [44]. The contributions of the factors related to the antitumor activity of the polysaccharide are, in order; water solubility>chain conformation>molecular weight [37, 38].

Apoptotic cells often produce a unique ladder composed of nucleotide fragments at an interval of 180–200 base pairs, which can be visualized by DNA-agarose gel electrophoresis. In general, cytotoxic drugs induce a massive breakage of DNA into oligonucleosome fragments. The degradation of DNA down into oligonucleosomal fragments is a late event of apoptosis [5, 7, 22]. Thus, the PPC of *C. sphecocephala* induces DNA damage in HepG2 and SK-N-SH cells, thereby causing apoptosis.

The problem with the DNA ladder assay is its low sensitivity. Even with enhancement of the fragmented DNA, ladder formation is observed only when the extent of oligonucleosomal cleavage is prominent, which is usually in the later phase of apoptosis [10]. To overcome this difficulty, a new technique called the comet assay, or single-cell electrophoresis, for detection of apoptosis has been described. This assay can detect various forms of DNA strand breakage, dependent on the pH of electrophoresis, and is generally accepted as specific for cell apoptosis [47]. In combination with the DNA ladder assay, we successfully confirmed PPC-induced apoptosis in both cell lines using the comet assay.

Accumulating evidence suggests that apoptosis is a tightly regulated process that involves changes in the expression of distinct genes. One of the major genes that regulates apoptosis is the protooncogene Bcl-2. The death-promoting molecule, Bax, counteracts the anti-apoptotic effects of Bcl-2 by forming a heterodimer with Bcl-2 [17]. The ratio of Bcl-2 to Bax, rather than the levels of the individual proteins, is considered to be critical in determining the survival/death of cells [9, 32]. We showed that the ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax was substantially decreased by PPC, which may induce apoptotic response in HepG2 cells. Both Bax and its mRNA levels were shown to be upregulated in cells induced to undergo apoptosis by various stimuli [48]. Moreover, Bax overexpression has been demonstrated to accelerate apoptotic cell death [6, 38].

In cells undergoing apoptosis, there is an activation of proteases known as caspases, which have an obligatory cysteine residue within the active site and cleaves peptides adjacent to an aspartic acid residue [40]. Caspase cascade has been extensively demonstrated to be involved in apoptotic signal transduction and execution [2, 36]. Human caspases 1–10 have been described, and activation of the caspase cascade is involved in chemical-induced apoptosis [24], including degradation of DNA repair enzyme poly(ADP-ribose) polymerase [20] and DNA-dependent protein kinase as well as cleavage of chromatin at internucleosomal sites mediated by caspase-activated DNase [35]. Generally, caspases are present as inactive proenzymes, most of which are activated by proteolytic cleavage. Caspase-3 may then cleave vital cellular proteins or activate additional caspases by proteolytic cleavage. In the present study, PPC-treated HepG2 cells showed a low procaspase-3 intensity of 32 kDa protein band.

In conclusion, it was demonstrated that the PPC produced by submerged mycelial culture of *C. sphecocephala* strongly inhibited the growth of both HepG2 and SK-N-SH cancer cells without any cytotoxicity against normal cells (Chang liver cells CCL13, data not shown). In this study, the anticancer activity of PPC was studied against only two cancer cell lines, and it deserves further investigation for possible activity against other cancer cell lines. The inhibitory effect of PPC on the growth of both cancer cell lines was mainly due to the induction of apoptosis, as evidenced by DNA fragmentation and expression of apoptosis-related proteins.

Acknowledgments

This work was supported by Daegu University Research Grant 2007. Oh, J. Y. and Yu, M. B. contributed equally to this work.

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