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SHORT COMMUNICATION

Antitumor Activity of Water Extract of a Mushroom, *Inonotus obliquus*, against HT-29 Human Colon Cancer Cells

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In the current study, it was demonstrated that the hot water extract of *I. obliquus* (IOWE) exerts inhibitory activity against the proliferation of human colon cancer cells (HT-29). The inhibitory effect of IOWE on the growth of HT-29 cancer cells was evaluated by treating cells with IOWE at concentrations of 0.25, 0.5 and 1.0 mg/mL for 24 or 48 h. The IOWE inhibited cell growth in a dose-dependent manner, and this inhibition was accompanied by apoptotic cell death. The maximum inhibitory effect (56%) was observed when IOWE was treated at a concentration of 1.0 mg/mL for 48 h. The apoptotic effect of IOWE on HT-29 cells was also confirmed by flow cytometric analysis. In addition, the apoptotic cell percentage was closely associated with down-regulation of Bcl-2 and up-regulation of Bax and caspase-3. The results suggest that IOWE would be useful as an antitumor agent via the induction of apoptosis and inhibition of the growth of cancer cells through up-regulation of the expression of proapoptotic proteins and down-regulation of antiapoptotic proteins.

Keywords: antitumor activity; *Inonotus obliquus*; apoptosis; Bcl-2; Bax; caspase-3; HT-29.

INTRODUCTION

Recently, the demand for more effective and safer therapeutic agents for the chemoprevention of human cancer has increased. Natural products produced by plants and their synthetic derivatives are expected to play an important role in the development of innovative agents to inhibit the onset of cancer (Taji *et al.*, 2007). Macrofungi such as mushrooms and enthomopathogenic fungi are good sources of natural medicines that exert antitumor activity. Polysaccharides are the best known and most potent mushroom-derived substances because they inhibit the growth of many types of tumors (Wasser, 2002; Moradali *et al.*, 2007). Natural antitumor polysaccharides isolated from mushrooms include acidic and neutral compounds with different types of glycosidic linkages, as well as some that are bound to protein or peptide residues such as polysaccharide–protein complexes (Wasser, 2002; Chen and Chang, 2004; Moradali *et al.*, 2007).

Inonotus obliquus (Pers.: Fr.) Pil. [= *Fuscoporia obliqua* (Pers.: Fr.) Aoshima], known as kabanoanatake (in Japan) and chaga or tchaga (in Korea and Russia), is a white-rot fungus that belongs to the family *Hymenochaetaceae Donk* (Taji *et al.*, 2007). This fungus grows on birch trees in cold northern climates. Since the sixteenth century, this fungus has been used as a folk medicine due to its antitumor properties against several types of cancer cells (Kahlos *et al.*, 1986; Saar, 1991). In some cases, the therapeutic efficiency of *I. obliquus* has been demonstrated clinically. For example, a decoction of fungal sclerotia did not show toxic effects and has been used in the treatment of cancers and digestive system diseases (Park *et al.*, 2005; Nakata *et al.*, 2007). Recently, in addition to polysaccharides, many polyphenolic compounds such as triterpenoids, steroids and ergosterol peroxides from *I. obliquus* have been found to possess biological activities, including antioxidant, antibacterial, hepatoprotective, platelet aggregation inhibitory and antitumor effects (Kahlos *et al.*, 1986; Shin *et al.*, 2001; Park *et al.*, 2004; Cui *et al.*, 2005; Hyun *et al.*, 2006; Mazurkiewicz, 2006; Lee *et al.*, 2007; Nakajima *et al.*, 2007; Shi *et al.*, 2008; Youn *et al.*, 2008). However, the molecular mechanisms by which these antitumor effects occur are not well understood.

This study was conducted to elucidate the antitumor activity of IOWE against HT-29 cancer cells by the MTT assay and flow cytometric analysis. Furthermore, a possible mechanism for IOWE-induced apoptosis was suggested based on analysis of the levels of apoptosis-related molecules such as Bcl-2, Bax and caspase-3. To the best of our knowledge, this is the first report to describe the apoptotic effects of IOWE on HT-29 colon cancer cells.

MATERIALS AND METHODS

Preparation of *Inonotus obliquus* extracts. *I. obliquus* was kindly provided by the Kim Young Pyo Mushroom Co. (Kyungsan, Korea). The fruiting body was extracted

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in water under optimal conditions. Briefly, the fruiting bodies were dried at 50 °C and then ground in a blender, after which the crude powder was used for the extraction. The powder was extracted by heating in water at 90 °C for 3 h or in 70% ethanol at a room temperature for 9 h. After cooling to room temperature, the extracts were filtered. The filtrates were then evaporated at 60 °C in a rotary vacuum evaporator and subsequently freeze-dried to give a powder. The dried hot water extract (IOWE) and 70% ethanol extract (IOEE) were then stored at –80 °C until further use.

Compositional analysis. The sugar composition was analysed by gas chromatography (Varian Co., Model: Star 3600CX, Lexington, MA) using a fused silica capillary column (Na form, 300 mm × 0.25 mm, Supelco Inc., Bellefonte, PA) and a flame ionization detector. The total protein was determined by the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as the standard.

Cell culture. HT-29 human colon carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were seeded in 100 mm petri dishes at a density of 2×10^6 and then maintained in RPMI1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 units/mL of penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere under 5% CO_2. The medium was changed every 2 days during the experimental period. The cells were treated with either IOWE or IOEE and incubated for 24 and 48 h at 37 °C.

Cell viability analysis. Cell viability was assessed by MTT {3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)} assay, which is based on the reduction of a MTT into formazan dye by active mitochondria (Mosmann, 1983). Briefly, the cells were placed in 96-well culture dishes (Nunclon, Roskilde, Denmark) at a density of 5×10^4 cells/well in RPMI1640 culture medium that contained 10% FBS and then incubated at 37 °C under 5% CO_2. After 24 h, the cells were washed and placed in culture medium with different concentrations of either IOWE or IOEE (0.25, 0.5 and 1.0 mg/mL) for 48 h. Next, 20 μL of MTT solution (5 mg MTT/mL in PBS) was added to each well of a microtiter plate and the samples were then incubated for 4 h at 37 °C. After washing, the formazan dye precipitates, which are proportional to the number of live cells, were dissolved in 100 μL of DMSO. The absorbance at 540 nm was then read using a microtiter plate reader (Thermo Electron, Vantaa, Finland). The rate of cell growth inhibition was calculated using the following formula: mean value of {(control group – treated group)/control group} × 100%. The effects of each concentration were analysed in triplicate.

Western blot analysis. The HT-29 cells were treated with IOWE at concentrations of 0.25, 0.5 and 1.0 mg/mL for 48 h. At the 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). Next, the cell lysates were centrifuged at 10 000 × **g** for 30 min at 4 °C, after which the protein content of the cytosolic fraction (supernatant) was measured using the Bradford method (Bradford, 1976) with protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA). Next, the protein was diluted in 2 × sample buffer (50 mM Tris-HCl of pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 5% β-mercaptoethanol) and heated for 5 min at 95 °C prior to SDS-PAGE gel analysis (12% and 15%). The proteins were then transferred to a 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. Next, the membrane was rinsed four times with TBS containing Tween-20 (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.0), after which it was incubated twice for 5 min and twice for 10 min in fresh washing buffer. The membrane was then incubated for 2 h with blocking solution containing a 1:500 dilution of primary antibody (rabbit anti Bax, mouse anti Bcl-2 and goat anti caspase-3, Santa Cruz Biotechnology, Santa Cruz, CA, USA). 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 (1:1000, Santa Cruz Biotechnology) and then developed using enhanced chemiluminescence (ECL Western Blot Analysis System Kit, Amersham Biosciences). The western blot was analysed by scanning with a UMAX PowerLook 1120 (Maxium Technologies, Inc., Dallas, TX, USA) and digitalized using image analysis software (Kodak 1D, Eastman Kodak Co., New York, NY, USA).

Determination of apoptosis. The cells were plated at a density of 5×10^4 cells/well in 6-well plates and allowed to attach for 24 h. The cells were then treated with different concentrations of either IOWE or IOEE (0.25, 0.5 and 1.0 mg/mL) for 48 h. For flow cytometry analysis, the cells were harvested by trypsin treatment and then washed with cold PBS (pH 7.4). After fixing in 80% ethanol for 30 min, the cells were washed twice and then resuspended in PBS (pH 7.4) containing 50 μg/mL of propidium iodide and 25 μg/mL ribonuclease A for DNA staining. Next, the cells were then analysed using a FACS Calibur (Becton Dickson, USA). At least 10 000 events were evaluated. All histograms were analysed using Cell Quest (Becton Dickson, USA) to determine the percentage of nuclei with a hypodiploid content indicative of apoptosis (Hockenbery *et al.*, 1990).

RESULTS

Effects of IOWE on HT-29 cell viability

A compositional analysis revealed that *Inonotus obliquus* extracts (IOWE and IOEE) consisted of carbohydrates (93.5%, 96.4%) and proteins (6.5%, 3.6%). Both IOWE and IOEE primarily consisted of glucose, galactose, xylose and mannose, but their compositions differed, especially the levels of galactose and mannose. To evaluate the effects of IOWE or IOEE on the growth and survival of HT-29 cells, the cells were exposed to various concentrations (0–1.0 mg/mL) of either IOWE or IOEE for 48 h and their viability was then evaluated by an MTT assay. As shown in Fig. 1, treatment with both IOWE and IOEE led to decreases in cell viability in a dose-dependent manner. Furthermore, the decrease

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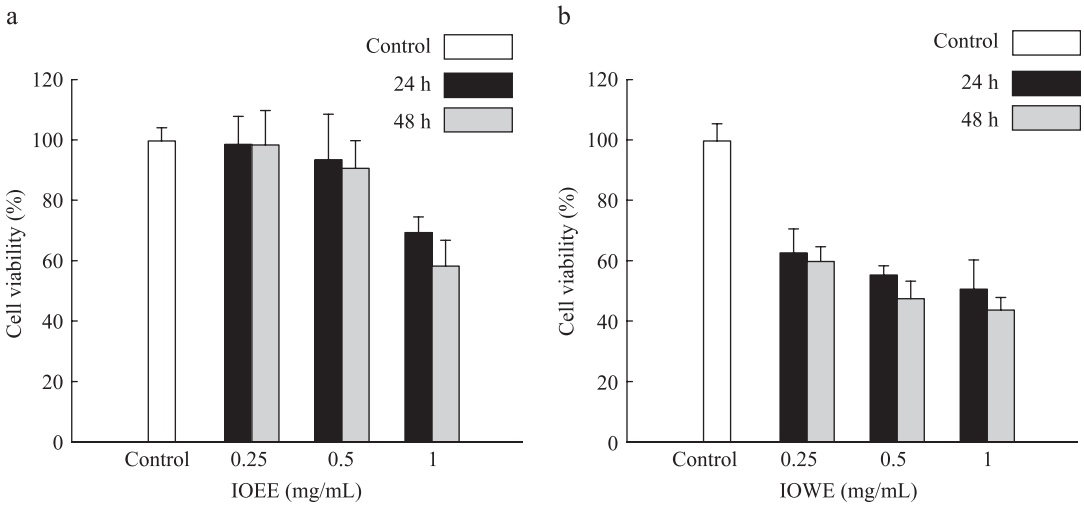

Figure 1. Inhibitory effects of hot water extract (a) and ethanol extract (b) of *Inonotus obliquus* on the growth of HT-29 cells. All data are expressed as the mean ± SE.

induced by IOWE (1.0 mg/mL) was significantly greater than the decrease induced by IOEE (66.01% vs 41.45%). Therefore, further experiments were conducted using IOWE.

Apoptotic HT-29 cell death by IOWE

To determine if growth inhibition is related to cell cycle arrest or apoptosis, the cell cycle distribution was quantified using flow cytometry. Exponentially growing HT-29 cells were treated with IOWE for 48 h and then subjected to cell cycle analysis. As shown in Fig. 2, only 1.3% of the untreated control cells were in the apoptotic phase, whereas significantly greater proportions of the cells that were treated with 0.25, 0.5 and 1.0 mg/mL IOWE were in the apoptotic phase (6.91%, 12.22% and 24.52%, respectively). These findings indicate that treatment with IOWE led to a decrease in the population of HT-29 cells in the G0/G1 phase in a dose-dependent manner.

Effects of IOWE on the expression of apoptosis-related molecules

The regulation of apoptosis is a complex process that involves a variety of Bcl-2 and Bax families. To determine the role that the Bcl-2 families played in IOWE-mediated apoptosis, its effects were investigated on the protein levels of an antiapoptotic member, Bcl-2 and a proapoptotic member, Bax, by western blot analysis. As shown in Fig. 3, IOWE led to a dose-dependent increase in the level of Bax and a decrease in the levels of Bcl-2. IOWE also led to a significant decrease in the level of procaspase-3 in IOWE-treated HT-29 cells,

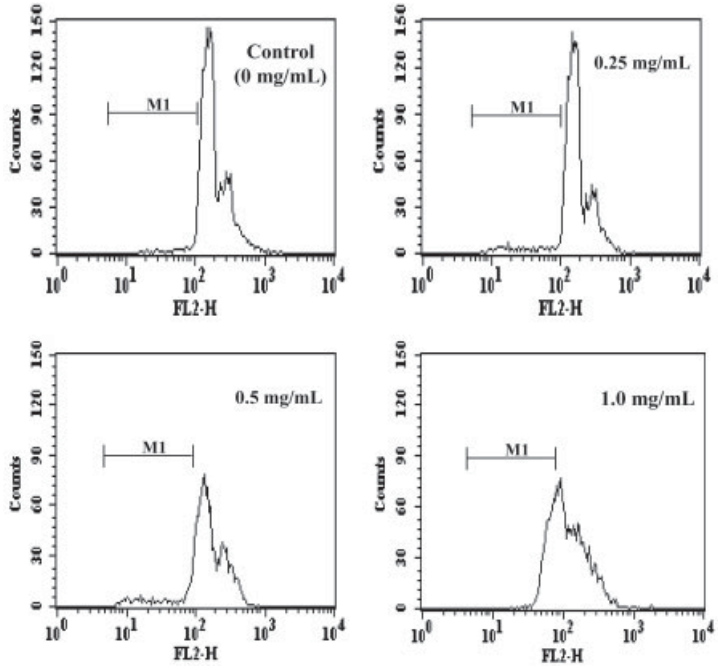

Figure 2. Hot water extract of *Inonotus obliquus* induces apoptotic death of HT-29 cells. The number of apoptotic cells was measured by flow cytometry. The region to the left of the G0/G1 peak, designated M1, was defined as cells undergoing apoptosis-associated DNA degradation.

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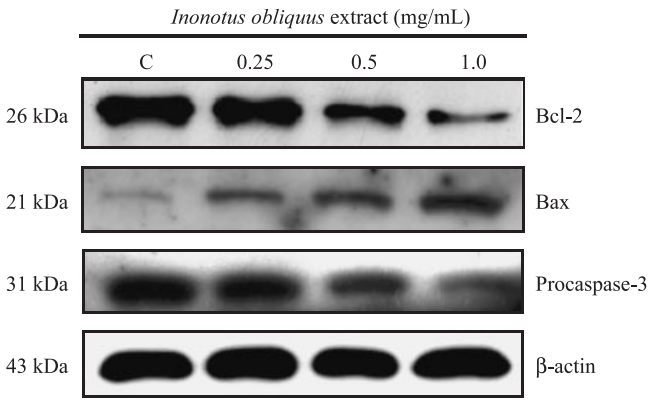

Figure 3. Changes in the expression of apoptosis-related proteins in HT-29 cells in response to hot water extract of *Inonotus obliquus* at concentrations of 0, 0.25, 0.5 and 1 mg/mL for 48 h. Cell extracts were subjected to western blotting to determine the cellular levels of Bcl-2, Bax and procaspase-3, as described in the Materials and Methods.

which suggests that the levels of caspase-3 (an active proapoptotic molecule) increased in a dose-dependent manner.

DISCUSSION

This is the first study to report an antitumor effect of IOWE. IOWE inhibited the growth of HT-29 human colon cancer cells. The prevention of cell growth was exerted through the induction of cancer cell death by apoptosis. Several studies have reported the anticancer activity of the mushroom *I. obliquus*. For example, sclerotia extracts of *I. obliquus* are known to inhibit the growth and protein synthesis of tumor cells. Additionally, Mizuno (1992, 1999) reported that polysaccharides from fungal sclerotia, which are known to include hetero-polysaccharide and homoglycan, showed strong antitumor effects, while polysaccharides from cultured mycelia did not. However, Kim *et al.* (2005, 2006) reported that the polysaccharides extracted from cultivated mycelia (termed endo-polysaccharides) also have anticancer activity. Unlike polysaccharides derived from the fruiting body of *I. obliquus*, mycelia-derived polysaccharides are mannose-rich and have a high protein content (e.g. 22–59%).

The chemical compositions, configurations and physical properties of olysaccharides with antitumor activity differ greatly. Although it is difficult to correlate polysaccharide structure to antitumor activity, some relationships can be inferred. Our IOWE was primarily composed of glucose, galactose and xylose, whereas IOEE was primarily composed of glucose, xylose and mannose. Similarly, Kim *et al.* (2006) reported that water-soluble polysaccharides from the sclerotia of *I. obliquus* were hetero-polysaccharides of xylogalactoglucan composed primarily of glucose, galactose and xylose, with low contents of arabinose and fucose. The presence of protein has been reported to be important for biological activities (Mizuno *et al.*, 1992), and is especially important for the biological activities of hetero-polysaccharides. However, Kim *et al.* (2005, 2006) demonstrated that proteins combined with endo-polysaccharides were unlikely to affect the biological activity.

It is well known that mushroom polysaccharides primarily exert their antitumor activity via activation of the immune response of the host organism (immunoenhancing activity) (Kim *et al.*, 2006). This indicates that mushroom polysaccharides do not directly kill tumor cells. Rather, they help the host to adapt to various biological stresses and exert a nonspecific action on the host, supporting some or all of the major systems. Because mushroom polysaccharides cause no harm and place no additional stress on the body, they are regarded as biological response modifiers. Immunoenhancing activity has been observed in many mushroom polysaccharides (Zhang *et al.*, 2007). However, as found in this study, direct tumor inhibition activity has also been documented in many mushroom polysaccharides (Wang *et al.*, 2002). Although the antiproliferative effect of polysaccharides towards tumor cell lines *in vitro* remains unclear, some studies have indicated that incubation of polysaccharides together with tumor cells can lead to changes in the expression of signals within the tumor cells. Such changes could lead to cell cycle arrest and generate apoptosis, which would explain the *in vitro* antiproliferative effect of polysaccharides (Wang *et al.*, 2002; Chen and Chang, 2004; Li *et al.*, 2004). It is believed that the induction of cell cycle arrest and apoptosis is a good strategy for treating cancer. Therefore, many studies have been conducted to identify compounds produced by mushrooms that induce cell cycle arrest and apoptosis (Hu *et al.*, 2002; Yang *et al.*, 2006). In the present study, there was a concomitant reduction in the number of cells in the G1/G0 phase in response to IOWE treatment, which suggests that IOWE induced the apoptotic phase (Pavletich, 1999).

Recently, mitochondria have been proposed as a novel potential target for the induction of apoptosis in chemotherapy (Jang *et al.*, 1997; Duriez and Shah, 1997). The antiapoptotic function of Bcl-2 may be ascribed to its ability to control several key steps in cell death signaling. For example, Bcl-2 can form ion channels in biological membranes. This formation of ion channels may control apoptosis by influencing the permeability of intracellular membranes, and thus the mitochondrial contents released into the cytoplasm (Pfeiffer *et al.*, 1995; Kim *et al.*, 2003). Accordingly, the formation of ion channels could potentially result in the activation of caspase-3, thereby leading to the induction of apoptosis. However, the overexpression of Bcl-2 protein may rescue cells from apoptosis by maintaining membrane integrity and preventing the release of mitochondrial contents (Jang *et al.*, 1997; Frémont, 2000). In many apoptotic processes, mitochondria play an important role in coordinating caspase activation by releasing proapoptotic factors, such as cytochrome c. This results in the opening of permeability transition pores of mitochondria, which is an irreversible step toward apoptosis (Shimizu *et al.*, 2000; Cheng *et al.*, 2008). The release of proapoptotic factors from the mitochondria is controlled by Bcl-2 and Bax, which are members of the mitochondrial membrane-associated Bcl-2 family of proteins that exert opposing effects on cell life and death (Green and Reed, 1998). Bcl-2 is an integral membrane protein and an antiapoptotic factor. Bax is a proapoptotic factor that translocates from the cytosol to the outer mitochondrial membrane, where it can form heterodimers with Bcl-2 protein to create pores and

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mediate the release of cytochrome c (Park *et al.*, 2007). In an attempt to identify the target molecule and underlying signaling pathway of the IOWE, the study focused on the activation of caspase-3, which is known to play a pivotal role in the formation of apoptotic bodies. The results of this study suggest that inhibition of cell proliferation or the induction of cell death in HT-29 cells by IOWE might be mediated by the induction of G0/G1 phase arrest and the subsequent apoptotic process that is accompanied by caspase-3 activation. Caspase-3 is a downstream effector caspase that is a key protease responsible for the cleavage and inactivation of poly(ADP-ribose) polymerase (PARP), which is believed to be involved in DNA repair and the surveillance of genome integrity in response to environmental stress (Duriez and Shah, 1997). In addition, it is well known that caspase-3 plays a pivotal role in the final execution step of apoptosis induced by various conditions, such as treatment with anticancer drugs (Kim *et al.*, 2002).

In conclusion, the results demonstrated that IOWE significantly inhibits the growth of HT-29 cells and induces apoptosis via the modulation of apoptosis-related proteins.

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