Effects of polysaccharides derived from *Orostachys japonicus* on induction of cell cycle arrest and apoptotic cell death in human colon cancer cells

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Crude *Orostachys japonicus* polysaccharide extract (OJP) was prepared by hot steam extraction. Polysaccharides (OJPI) were separated from OJP by gel filtration chromatography and phenol-sulfuric acid assay. The average molecular weight of the OJPI was 30-50 kDa. The anti-proliferative effect of OJPI on HT-29 human colon cancer cells was investigated via morphology study, cell viability assay, apoptosis assay, cell cycle analysis, and cDNA microarray. OJPI inhibited proliferation and growth of HT29 cells and also stimulated apoptosis in a dose- and time-dependent manner. In cell cycle analysis, treatment with OJPI resulted in a marked increase of cells in the G0 (sub G1) and G2/M phases. To screen for genes involved in the induction of cell cycle arrest and apoptosis, the gene expression profiles of HT-29 cells treated with OJPI were examined by cDNA microarray, revealing that a number of genes were up- or down-regulated by OJPI. Whereas several genes involved in anti-apoptosis, cell proliferation and growth, and cell cycle regulation were down-regulated, expression levels of several genes involved in apoptosis, tumor suppression, and other signal transduction events were up-regulated. These results suggest that OJPI inhibits the growth of HT-29 human colon cancer cells by various apoptosis-aiding activities as well as apoptosis itself. Therefore, OJPI deserve further development as an effective agent exhibiting anticancer activity. [BMB reports 2010; 43(11): 750-755]

INTRODUCTION

*Orostachys japonicus*, a perennial herbaceous plant belonging to the family Crassulaceae, is traditionally used as an anti-inflammatory agent, anti-febrile, hemostatic agent, antidote, and anti-cancer agent (1). Previous studies on *O. japonicus* have revealed the presence of flavonoids, fatty acid esters, triterpenoid, a sterol mixture, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, and gallic acid (2-4). More than 10 million people are diagnosed with cancer every year; it is estimated that there will be 15 million new cases of cancer worldwide every year by 2020 (5). Chemotherapy is considered the most effective method of cancer treatment, but most chemotherapeutic drugs severely affect normal cells (6, 7). Therefore, developing safe and powerful anticancer agents from traditional herbs has become very attractive. Apoptosis plays an important role in homeostasis and tissue development, and imbalances between cell proliferation and apoptotic cell death may result in serious diseases such as cancer. Apoptotic cell death induces a series of morphological changes, such as nuclear condensation and blebs on the cell surface, which lead to the formation of apoptotic bodies that are then phagocytized by macrophages. As programmed cell death does not affect neighboring cells, apoptosis is regarded as the preferred way to manage cancer (8, 9). In this study, we found that polysaccharides from *O. japonicus* (OJPI) showed remarkable anti-proliferative activity in HT-29 human colon cancer cells via apoptosis and various apoptosis-aiding activities.

RESULTS AND DISCUSSION

Extraction and purification of OJPI from *O. japonicus*

Crude polysaccharides (OJPI) were extracted from *O. japonicus* (OJP) and constituted approximately 2% of the whole mass. Fine OJPI were serially purified from OJP by gel filtration chromatography. Although composition analysis of the polysaccharides has not yet been conducted, the average molecular weight of OJPI has been reported to be between 30-50 kDa (10).

Inhibition of cell proliferation and induction of apoptosis by OJPI

The effect of OJPI treatment on proliferation of HT-29 human colon cancer cells was measured by MTS assay. OJPI exhibited a dose-dependent, anti-proliferative effect on HT-29 cells (Fig.
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Fig. 1. Proliferative inhibition of HT-29 cells by OJPI. (A) Amounts of HT-29 cells with disrupted or intact cell membranes were measured. (B) The cells (5 \times 10^5 cells/ml) were treated with graded concentrations of OJPI for 24 or 48 h. Results are represented as percentage of control. *Significantly different from control at P < 0.05.

1). The survival rate of cells treated with 2 mg/ml of OJPI for 24 h decreased dramatically to 28.43% of that of control. Moreover, treatment with 2 mg/ml of OJPI for 48 h inhibited proliferation of cells by approximately 24.92% compared to that of control. Morphological analysis of HT-29 cells treated with OJPI revealed striking changes (Fig. 1). HT-29 cells became shrunken, disintegrated, rounded, and detached from the culture dish. The ability of OJPI treatment to induce apoptosis in HT-29 cells was determined by annexin V-FITC assay to establish a relationship between anti-proliferation and apoptosis. Treatment of HT-29 cells with OJPI (2 mg/ml) for 24 h increased apoptotic death by approximately 3.9-fold and suppressed cell survival rate in culture by 60.37% (Fig. 2). As shown in Fig. 2, the early apoptotic death rate (19.03%, lower right region) of HT-29 cells treated with OJPI (2 mg/ml) was higher than that of control (4.93%) while the late apoptotic or necrotic death rate (20.60%, upper right region) was higher (5.30%). After treatment with OJPI (2 mg/ml) for 48 h, the amount of apoptosis induced was the same as that induced by the 24 h treatment. Specifically, the apoptosis-inducing activities of OJPI caused an increase in the early apoptotic death rate, resulting in poor survival of cells after treatment. Overall, these results show that OJPI may not only have inhibited growth of HT-29 cells but also induced apoptosis. In a previous study, we reported the anti-cancer activity of polysaccharides from Salicornia herbacea in HT-29 cells (8). Other investigators have also demonstrated that polysaccharides exhibit these suppressive effects on several cancer cells (11-13).

Fig. 2. Flow cytometry analysis of apoptotic death of HT-29 cells. The cells were treated with different concentrations of OJPI for 24 h (A) or 48 h (B). Dot plots display the apoptotic death of HT-29 cells treated with 2 mg/ml of OJPI. Annexin+/PI- (LL), viable cells; Annexin+/-PI- (LR), cells undergoing apoptosis; Annexin+/PI+ (UR), cells that are in end-stage apoptosis or are already dead. LL, lower left; LR, lower right; UR, Upper right.

Since many anti-cancer drugs act by inducing apoptosis (14, 15), O. japonicus extract shows promise as a novel anti-cancer agent.

Cell cycle arrest by OJPI
Uncontrolled cell proliferation is a characteristic of cancer (16), and OJPI was shown to inhibit HT-29 cell proliferation. To probe OJPI-mediated cell growth inhibition, we examined the cell cycle by flow cytometry. The effects of OJPI on the cell cycle progression of HT-29 cells are shown in Fig. 3. After 24 h of growth, the G2/M phase cell population of the control was 20.37%. Meanwhile, the G2/M phase cell population of the OJPI-treated cells increased in a dose-dependent manner. Treatment with OJPI at 2 mg/ml resulted in the highest percentage of cells (38.63%) in G2/M phase of the cell cycle. As the G2/M phase population increased, the G1 phase cell pop-

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The results were represented as percentage of total treated cells. Data values are expressed as mean ± SE (n = 3). *Significantly different from control at P < 0.05.

**Fig. 3.** Flow cytometry analysis of cell cycle distribution of HT-29 cells. The cells were treated with different concentrations of OJPI for 24 (A) or 48 h (B). Histograms display cell cycle distribution of HT-29 cells treated with 2 mg/ml of OJPI. Histograms display sub G1, G1, S and G2/M phase of HT-29 cells.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OJPI 0.15</th>
<th>OJPI 2 mg/ml</th>
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<tr>
<td>24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.10±0.66</td>
<td>4.94±0.47</td>
<td>16.23±2.57</td>
</tr>
<tr>
<td>2</td>
<td>38.43±2.47</td>
<td>35.29±1.99</td>
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</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.10±0.66</td>
<td>7.50±0.89</td>
<td>14.90±1.85</td>
</tr>
<tr>
<td>2</td>
<td>38.43±2.47</td>
<td>40.57±1.65</td>
<td>20.70±2.10</td>
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</table>

The cell cycle distribution of HT-29 cells treated with OJPI showed significant changes in the G2/M phase. The G2/M phase population decreased, whereas the S phase cell population showed slight changes within 24 h. G2/M arrest was also observed after 48 h of treatment. These results indicate that OJPI acted on the G2-M transition checkpoint of the cell cycle.

**Gene expression changes induced by OJPI**

A cDNA microarray gene expression profile was used to characterize the anti-proliferative response of cultured HT-29 cells in the presence of OJPI. Of the 978 genes examined, 25 (3.2%) were induced or repressed by at least two-fold. Among the numerous genes affected by OJPI treatment, 12 were considered to be fairly significant (Table 1). Eight genes showed at least two-fold up-regulation, whereas four showed more than two-fold down-regulation. The up-regulated genes included those involved in apoptosis (BAD, FADD, CASP3, 8, 9, and 10) and tumor suppression (TP53BP2 and STAT1) while the down-regulated genes included those involved in cell proliferation and growth (PTK6), anti-apoptosis (BCL2), cell cycle regulation (RFC5), and cancer development (CTSH). Among the numerous genes up-regulated by OJPI treatment, Bcl-2 antagonist causing cell death (BAD), a pro-apoptotic protein of the Bcl-2 family, has been identified as an integrator of several anti-apoptotic signaling pathways in cancer cells (17). The fas-associated death domain (FADD), as a cytoplasmic adaptor protein, plays a significant role in death receptor-mediated cell death (18). Inhibition of apoptosis is an important mechanism for the growth of cancer cells. Caspase protein plays an important role in apoptosis, and most stimuli induce apoptosis through caspase protein cascade activation reactions (19). Tumor suppressor protein p53 binding protein (TP53BP2) also interacts with Bcl-2, an inhibitor of apoptosis, and may be involved in cell cycle progression and cell death. Furthermore, TP53BP2 seems to function in the signal transduction pathway by stimulating p53-mediated transcriptional activation (20). Cytokines can induce apoptosis of cancer cells by activating diverse networks of transcription factors. Cytokines bind to receptors expressed on most nucleated cells and activate a signaling pathway involving the phosphorylation of the cytosolic protein signal transducer and activator of transcription (STAT1) (21). These data collectively imply that OJPI may interact with HT-29 cells to promote apoptosis. Among the genes down-regulated by OJPI treatment, protein tyrosine kinase 6 (PTK6) is an intracellular tyrosine kinase expressed in cancers (22). B-cell CLL/lymphoma 2 (Bcl-2) genes seem to suppress apoptotic function, as they are known to inhibit apoptotic cell death (23). Replication factor C (RFC) is required for DNA replication...
and DNA damage checkpoint control (24). Cathepsin H (CTSH) is involved in lysosomal protein degradation, proenzyme activation, antigen processing, and hormone maturation and is secreted by tumor cells (25). These data suggest that gene down-regulation may be implicated in the induction of HT-29 cell apoptosis, and that OJPI may modulate signal transduction through the apoptosis pathway.

In conclusion, the results of this study suggest that OJPI from O. japonicus has anti-proliferative activity stemming from both an apoptotic effect as well as a cell cycle control effect. We also confirmed substantial changes in the expression patterns of cell survival and apoptosis genes in human colon cancer cells caused by OJPI. Although further studies are needed to elucidate the detailed relationships between cell cycle arrest and apoptosis, this study can serve as a useful foundation for the study and development of new anti-proliferative substances based on these extracts for the treatment of human colon cancer.

MATERIALS AND METHODS

Materials

O. japonicus was provided by Geobugiwason Ltd. (Miryang, Korea). The samples were dried, sliced, and extracted by boiling in water for 3 h under high pressure. OJP was collected by filtration through filter paper (Whatman No. 1). OJPI were purified using a protocol described by Klarzynski et al. (26), with slight modifications. The OJPI were fractionated by filtration using a Sephadex G-50 column (200 × 15 mm) at a flow rate of 3.5 ml/min. The average molecular mass of OJPI was measured by gel filtration chromatography with phenol/sulfuric acid monitoring (27). The OJPI was freeze-dried using a Freeze Dryer (Clean-Vac 24T, Biotron, Korea) to obtain a powder and stored at −4°C.

Table 1. Significant genes up- and down-regulated by OJPI treatment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Fold of expression</th>
</tr>
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<tbody>
<tr>
<td>BAD</td>
<td>BCL2-antagonist of cell death</td>
<td>+3</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated via death domain</td>
<td>+3</td>
</tr>
<tr>
<td>CASP3</td>
<td>Caspase 3</td>
<td>+3</td>
</tr>
<tr>
<td>CASP8</td>
<td>Caspase 8</td>
<td>+3</td>
</tr>
<tr>
<td>CASP9</td>
<td>Caspase 9</td>
<td>+3</td>
</tr>
<tr>
<td>CASP10</td>
<td>Caspase 10</td>
<td>+2</td>
</tr>
<tr>
<td>TP53BP2</td>
<td>Tumor protein p53 binding protein</td>
<td>+3</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal transduction and activator of transcription 1</td>
<td>+2</td>
</tr>
<tr>
<td>PTK6</td>
<td>Protein tyrosine kinase 6</td>
<td>−3</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
<td>−2</td>
</tr>
<tr>
<td>RFC5</td>
<td>Replication factor C5</td>
<td>−2</td>
</tr>
<tr>
<td>CTSH</td>
<td>Cathepsin H</td>
<td>−2</td>
</tr>
</tbody>
</table>

Cell cultures

The HT-29 cell line (human colon cancer cells) was purchased from the Korean Cell Line Bank. Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) with 5,000 units penicillin/ml and 5,000 μg streptomycin/ml in 0.85% saline (Gibco) in a humidified incubator containing 5% CO2 at 37°C.

Proliferation assay

Inhibition of cell proliferation by OJPI was measured by the MTS method using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). MTS assays were performed as directed by the manufacturer. HT-29 cells were plated in 96-well plates (5 × 104 cells/ml). After 24 h of incubation, the cells were treated with different concentrations (0.15, 0.3, 1, 2 mg/ml) of OJPI for 24 and 48 h. Culture medium was aspirated and 20 μl of MTS reagent in culture medium was added to each well. Cells were incubated for an additional 4 h at 37°C. Proliferation levels were detected by optical density (O.D.) at 490 nm using a Fluorescence Multi-Detection Reader (Synergy HT, Biotex, USA).

Morphological observations

Cells used in this study were observed constantly under an inverted microscope. HT-29 cells were placed in 24-well plates (5 × 104 cells/ml). Photographs were taken after cells were incubated with different concentrations (0.15, 0.3, 1, 2 mg/ml) of OJPI for 24 and 48 h.

Apoptosis assay

The apoptotic status of HT-29 cells was evaluated by measuring the exposure of phosphatidylserine on cell membranes using annexin V-fluorescein isothiocyanate (annexin V-FITC) and propidium iodide (PI) staining (28). A BD Pharmingen Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) was used for the apoptosis assay. HT-29 cells were placed in a 24-well plate (1 × 105 cells/ml), and after 24 h of incubation, the cells were treated with graded concentrations of OJPI for 24 or 48 h and then harvested. After centrifugation, the cell pellets were washed twice with cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, pH 7.4) and suspended in 100 μl of 1 × binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). The cells were then incubated with 5 μl of annexin V-FITC and 10 μl of PI at room temperature for 15 min in the dark. After incubation, 400 μl of 1 × binding buffer was added to each tube. The cells were immediately analyzed by FACSCalibur flow cytometry (Becton Dickinson, USA) (29).

Cell cycle analysis

Cell cycle phase was assayed by DNA fragment staining using PI. A Cell Cycle Phase Determination Kit (Cayman Chemical,

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Ann Arbor, MI, USA was used according to the manufacturer’s protocol. Cells (1 × 10⁶) were plated in each well of a 12-well plate for 24 h, followed by treatment with different concentrations (0.15, 0.3, 1, 2 mg/ml) of OJPI for 24 or 48 h. After treatment, the cells were harvested and centrifuged. Cell pellets were suspended to a density of 1 × 10⁶ cells/ml in assay buffer after washing twice with assay buffer. The cells were fixed and permeabilized by adding 1 ml of a fixative (100% ethanol) to each tube for more than 2 h. After centrifugation, the fixatives were decanted and cell pellets were suspended in 0.5 ml of staining solution containing 200 μl of DNase-free RNase and 200 μl of PI, followed by incubation for 30 min at room temperature in the dark. The cells were analyzed immediately by FACSCalibur flow cytometry.

Total RNA isolation
Cells were placed in a petri dish (1 × 10⁶ cells/ml) for 24 h and then treated with 2 mg/ml of OJPI for 24 h. Total RNA was isolated using an EasySpin [DNA-free] Total RNA Extraction Kit (iNtRON Biotechnology, Korea) as described by the manufacturer. The purity and concentration of the total RNA were checked by spectrophotometry.

cDNA microarray and analysis
Fluorescent cDNA probes were synthesized from each RNA sample by reverse transcription with Cy3- or Cy5-dUTP using the Micromax™ Direct cDNA Microarray System (PerkinElmer Life Sciences, Inc.). After purification using a Microcon YM-100 Column (Millipore, USA), fluorescent probes were hybridized to the Gene-Plorer TwinChip Cancer-1K (Digital Geneomics, Inc.) in 25% formamide hybridization solution at 55°C for 18 h. After washing (1 × SSC/0.1% SDS for 5 min at 55°C, 0.1 × SSC/0.1% SDS for 5 min at 25°C, and 0.1 × SSC for 5 min at 25°C), the DNA chips were scanned using a ScanArray Express scanner (PerkinElmer, Inc.) at two wavelengths to detect emissions from both Cy3 (red) and Cy5 (green). The overall intensities were normalized using a correction coefficient obtained from the ratios of four housekeeping genes.

Statistical analysis
All experiments were performed in triplicate. Data are expressed as means ± standard error. Significant differences (P < 0.05) between the means of the control and OJPI-treated cells were analyzed by Student’s t-test.

Acknowledgements
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REFERENCES