Prev. Nutr. Food Sci. 2016;21(1):31-37
http://dx.doi.org/10.3746/pnf.2016.21.1.31
pISSN 2287-1098 · eISSN 2287-8602

Extracts of *Opuntia humifusa* Fruits Inhibit the Growth of AGS Human Gastric Adenocarcinoma Cells.

Sahng-Wook Hahm[1,4], Jieun Park[2], Kun-Young Park[3], Yong-Suk Son[4], and Hyungchul Han[1]

[1]Department of Animal Sciences, Colorado State University, Fort Collins, CO 80523, USA
[2]Integrative Research Support Center, The Catholic University of Korea, Seoul 06591, Korea
[3]Department of Food Science and Nutrition, Pusan National University, Busan 46241, Korea
[4]Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Korea

ABSTRACT: *Opuntia humifusa* (OHF) has been used as a nutraceutical source for the prevention of chronic diseases. In the present study, the inhibitory effects of ethyl acetate extracts of OHF on the proliferation of AGS human gastric cancer cells and the mode of action were investigated. To elucidate the antiproliferative mechanisms of OHF in cancer cells, the expression of genes related to apoptosis and cell cycle arrest were determined with real-time PCR and western blot. The cytotoxic effect of OHF on AGS cells was observed in a dose-dependent manner. Exposure to OHF (100 μg/mL) significantly induced ($P<0.05$) the G1 phase cell cycle arrest. Additionally, the apoptotic cell population was greater ($P<0.05$) in OHF (200 μg/mL) treated AGS cells when compared to the control. The expression of genes associated with cell cycle progression (Cdk4, Cdk2, and cyclin E) was significantly downregulated ($P<0.05$) by the OHF treatment. Moreover, the expression of Bax and caspase-3 in OHF treated cells was higher ($P<0.05$) than in the control. These findings suggest that OHF induces the G1 phase cell cycle arrest and activation of mitochondria-mediated apoptosis pathway in AGS human gastric cancer cells.

Keywords: anticancer activities, gastric cancer cell, *Opuntia humifusa*

INTRODUCTION

The genus *Opuntia* is a member of the Cactaceae family and is widely distributed throughout the world (1). *Opuntia humifusa* (OHF) is one of the cold-hardy cactus species and can be grown during the winter in areas where temperatures reach −20°C (2,3). The therapeutic properties of the OHF cactus parts have been researched for the anti-diabetic effect of the stem (4), the anti-inflammatory activity of the fruit (5), and the prevention of osteoporosis of the seeds (6). Previously, we found that OHF extracts have potent antiproliferative effects on HeLa cervical carcinoma cells (7) and U87MG glioblastoma cells (8). These studies suggested that extracts of OHF might serve as a cell cycle arrest inducer to inhibit the growth of cancer cells. Additionally, it was reported that taxifolin and dihydrokaempferol were the main flavonoids in the extracts of OHF fruits (7). These phytochemicals have been considered as important contributors for the prevention of chronic diseases (9,10).

Gastric cancer is the second most common cause of cancer-related death in the world (11). The case-fatality ratio of gastric cancer is higher than those of colon and breast cancers (12). Despite widely accepted treatment options for cancer, including radiotherapy and chemotherapy, they have limited efficacy. Thus, the discovery of extracts or compounds from natural products is still necessary as an alternative for cancer treatment. Moreover, elucidating the critical events related to carcinogenesis provides an opportunity for inhibiting cancer development via dietary intervention, particularly with functional foods (13).

In spite of several studies related to the nutraceutical effects of OHF, the anticancer effects of OHF on human gastric cancer cells have not been evaluated. Therefore, the aim of the present study was to investigate the antiproliferative activities of OHF and the molecular mechanisms in the AGS human gastric adenocarcinoma cells.

Received 30 October 2015; Accepted 5 January 2016; Published online 31 March 2016
Correspondence to Hyungchul Han, Tel: +1-970-988-3679, E-mail: hyungchul.han@colostate.edu

MATERIALS AND METHODS

Extraction of the OHF fruit

OHF fruits were collected from a plantation located in the city of Goyang, Korea. Each fruit (seed-free) was freeze-dried using a freeze drier (EYELA, Tokyo, Japan) at −70°C and grounded using a cyclone mill (UDY Co., Fort Collins, CO, USA). The powder from OHF fruits was extracted using methanol 3 times at room temperature. Methanol extracts were subsequently filtered through Whatman No. 2 filter paper. Methanol-extracted samples were concentrated using a rotary evaporator (Buchi, Flawil, Switzerland) at 40°C. Methanol extracts were suspended in water and then partitioned in hexane to produce a hexane-soluble layer. The aqueous layer was then partitioned using ethyl acetate to yield ethyl acetate-soluble (OHF) and water soluble residues. All extracts were stored at −20°C.

Cell culture

Human gastric cancer cell line AGS was purchased from the Korean Cell Line Bank. Cells were maintained in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (Gemini, West Sacramento, CA, USA), 1% penicillin/streptomycin (Caisson Labs, North Logan, UT, USA). Cells were grown at 37°C and 5% CO_2 in humidified air.

Assay for cytotoxicity

Cell cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,3 diphenyltetrazolium bromide (MTT) assay (Roche, Mannheim, Germany). This assay is used to indirectly determine cytotoxicity by measuring the activity of mitochondrial enzymes. MTT, a yellow tetrazolium salt, is metabolized by mitochondrial dehydrogenases to form blue formazan (methyltriazole). Briefly, cells were plated in 96-well plates at an initial density of 1×10^4 cells per well. After incubation for 24 h at 37°C, cells were treated with 100 μg/mL and 200 μg/mL concentrations of OHF and incubated for 24 h. Solubilized formazan products were quantified using an enzyme-linked immunosorbent assay reader (Bio-Tek, Winooski, VT, USA) at 550 nm with a reference wavelength of 690 nm.

Cell cycle analysis

AGS human gastric cancer cells were seeded into 6-well plates at 3×10^5 cells per well. After 24 h of exposure to OHF (100 μg/mL or 200 μg/mL), cells were harvested by trypsinization and combined with floating cells collected from the media. Cells were then fixed in 70% ethanol and resuspended in 200 μL of 100 μg/mL propidium iodide (PI) (Sigma-Aldrich Co., St. Louis, MO, USA) in phosphate buffered saline. The cell cycle stage was determined by flow cytometry using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with CellQuestPro software (Becton Dickinson).

Determination of the apoptotic cell population

AGS human gastric cancer cells (3×10^5 cells/well) were plated in 6-well plates. Cells were treated with OHF (100 μg/mL or 200 μg/mL) and incubated for 24 h. Upon completion of treatment with OHF, the cells were collected by trypsinization and combined with floating cells collected from the media. Cells were double stained with 50 μg PI (Sigma-Aldrich Co.) and FITC Annexin V (BD Pharmingen, San Jose, CA, USA) at a dilution of 1:100, and cell death was determined by flow cytometry. The percentage of cells undergoing apoptosis was determined using a FACSCalibur (Becton Dickinson) equipped with CellQuestPro software (Becton Dickinson).

RNA isolation and quantitative real-time PCR

The total RNA from AGS human gastric cancer cells was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Isolated RNA was further purified by using the RNeasy Mini Kit (Quiagen, Valencia, CA, USA) with RNase-free DNase (Quiagen) and quantified by the use of a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Synthesis of cDNA was performed using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Real-time PCR was conducted using the CFX-96 (BioRad) with 96-well plates. The Ct values were obtained from the BioRad CFX-Manager software (BioRad) and adjusted for GAPDH Ct values for each sample to determine ΔCt and relative expression of the target mRNA. Data were presented as $2^{-\Delta\Delta Ct}$ (14). The primers used in this study included the following: p53 (NM_001126115.1), forward: 5'-GGC CAT CTA CAA GCA GTC ACA GCA C-3' and reverse: 5'-CTC ATT CAG CTC TCG GAA CAT CTC G-3'; p21 (NM_001291549.1), forward: 5'-CCC GTG AGC GAT GGA ACT-3' and reverse: 5'-CGA GGC ACA AGG GTA CAA GA-3'; Cdk4 (NM_000075.3), forward: 5'-GAG GGG GCC TCT CTA GCT T-3' and reverse: 5'-CAC GGG TGT AAG TGC CAT CT-3'; Cyclin D1 (NM_053056.2), forward: 5'-ACG GAC TAC AGG GGA GTT TTG-3' and reverse: 5'-GAA ATC GTG CGG GGT CAT TG-3'; Cdk2 (NM_052827.3), forward: 5'-CAC TGA GAC TGA GGG TGT GC-3' and reverse: 5'-GGA CTC CAA AAG CTC TGG CT-3'; Cyclin E1 (NM_001238.2), forward: 5'-CAT CAT GCC GAG GGA GCG-3' and reverse: 5'-TTT GCC CAG CTC AGT ACA GG-3'; Bax (NM_001291428.1), forward: 5'-CCC TTT TGC TTC AGG GTT TCA T-3' and reverse: 5'-ACA GGG ACA TCA GTC GCT TC-3'; Bcl-2 (EU_287875.1), forward: 5'-CTC GTC GCT ACC GTC GTG ACT TGG-3' and reverse: 5'-CAG ATG CCG GTT CAG GTA CTC AGT C-3'; Caspase-3 (NM_004346.3), forward: 5'-CAA

ACT TTT TCA GAG GGG ATC G-3' and reverse: 5'-GCA TAC TGT TTC AGC ATG GCA-3'; GAPDH (NM_001289745.1), forward: 5'-GAA GAC GGG CGG AGA GAA AC-3' and reverse: 5'-AAA TGA GCC CCA GCC TTC TC-3'.

Western blot analysis

Whole cells were lysed in a lysis buffer [20 mM Tris-HCl (pH 8.0), 1% nonyl phenoxypolyethoxylethanol-40, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 50 mM NaF, and 1 mM Na_3VO_4] supplemented with a protease inhibitors cocktail (Roche Applied Science, Indianapolis, IN, USA). After incubation on ice, lysed tissues were centrifuged, and equal amounts of protein were dissolved in 4× sodium dodecyl sulfate (SDS) sample buffer. Samples were separated in 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) and blocked with 5% nonfat dried milk. The membrane was incubated with the primary antibody (1:1,000 dilution) at 4°C overnight. Antibodies against p53, p21, Cdk4, Cyclin D1, Cdk2, Cyclin E1, Bax, Bcl-2, Caspase-3, and β-actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The membrane was washed with Tris-buffered saline with Tween 20, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.). Proteins were detected using the chemiluminescence (Western ECL substrate, BioRad). Membrane images were recorded using a ChemiDoc XRS system (BioRad). Protein bands were quantitated by densitometry using the UN-SCAN-IT gel software (Silk Scientific, Orem, UT, USA).

Statistical analysis

Data are presented as the mean±SE. Differences between the mean values for individual groups were assessed with a one-way analysis of variance with Duncan's multiple-range test (SAS version 9.3, SAS Institute Inc., Cary, NC, USA). Differences were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

OHF inhibit cell proliferation on AGS human gastric cancer cells

The inhibitory effects of OHF on AGS human gastric cancer cells proliferation were evaluated using the MTT

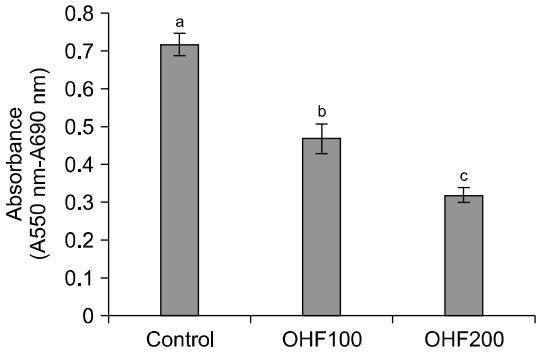

Fig. 1. Inhibitory effect of the *Opuntia humifusa* (OHF) on the growth of AGS human gastric cancer cells. The AGS cell proliferation was significantly suppressed by OHF in a dose-dependent manner. The cells were treated with OHF at 100 μg/mL (OHF100) or 200 μg/mL (OHF200) and incubated for 24 h. Data are presented with the mean±SE. Mean values with different letters (a-c) above the bars are significantly different ($P<0.05$) according to Duncan's multiple-range test.

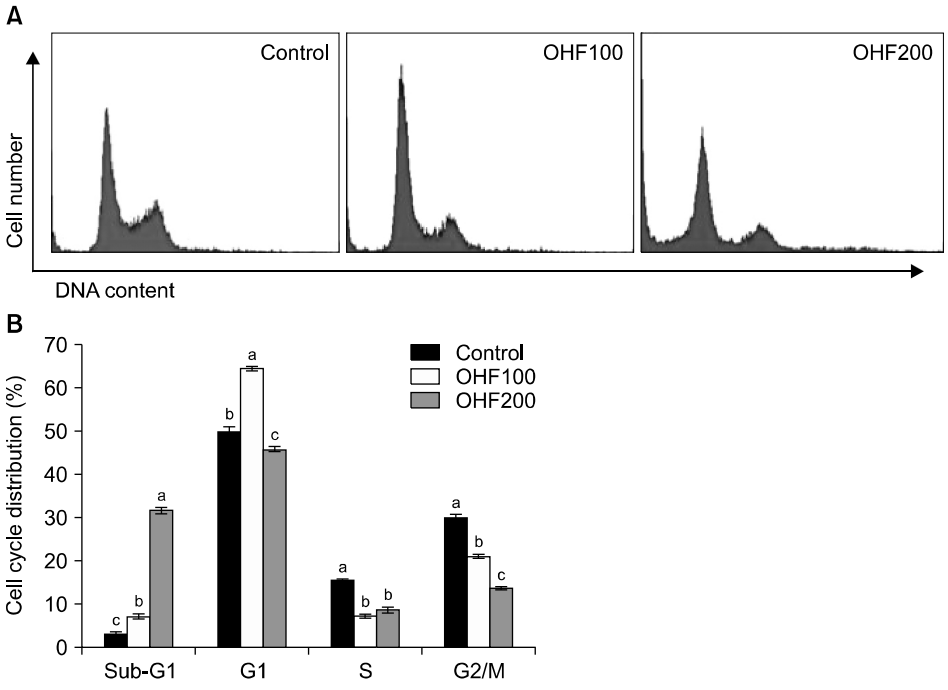

Fig. 2. Effect of *Opuntia humifusa* (OHF) on the cell cycle in AGS human gastric cancer cells. (A) Flow cytometric histograms are representative of triplicate experiments. (B) The percentage of cells in each phase of cell cycle was evaluated using a flow cytometer. Data are presented with the mean±SE. Mean values with different letters (a-c) above the bars are significantly different ($P<0.05$) according to Duncan's multiple-range test. The cells were treated with OHF at 100 μg/mL (OHF100) or 200 μg/mL (OHF200) and incubated for 24 h.

assay. The AGS cell proliferation was significantly suppressed ($P<0.05$) by the OHF treatment in a dose-dependent manner (Fig. 1). Treatment with OHF100 and OHF200 decreased the proliferation of AGS cells by 34.7% and 55.6%, respectively. These results indicated that OHF had cytotoxic effects against AGS cells. Similar to this finding, the inhibitory effects of the extracts of OHF fruits were observed in U87MG glioblastoma cells and HeLa cervical cancer cells (7,8). In addition, OHF fruits contained the flavonoids taxifolin and dihydrokaempferol (7). These compounds have been shown to possess antiproliferative effects on colon cancer cells (15, 16). Therefore, flavonoids in OHF may induce the inhibitory action on AGS cells growth.

Based on the results of the MTT assay, we investigated the effects of OHF on cell cycle progression. It is known that inhibition of the cell cycle is a target for the management of cancer development (17). As shown in Fig. 2, OHF100 significantly increased ($P<0.05$) the number of cells in the G1 phase. This result indicated that OHF have the potential for induction of G1 phase cell cycle arrest in AGS cells. Additionally, an increment of sub-G1 cells was observed ($P<0.05$) in cells treated with OHF 200 (Fig. 2). To further characterize the cell death induced by OHF in AGS cells, cells were analyzed by PI/FITC Annexin V staining with flow cytometry. Exposure to OHF resulted in significant apoptosis (Fig. 3). The treatment of OHF100 and OHF200 increased ($P<0.05$) the early apoptotic cell populations significantly by 14.2% and 31.1%, respectively, compared with the control value of 5.6%. Moreover, significant increases of late apoptotic cells were observed ($P<0.05$) in OHF treated cells. Cancer has been associated with dysregulated apoptotic processes, leading to the inhibition of cell death (13). Thus, apoptosis induction of OHF in AGS cells is also an important event for inhibition of cancer cell proliferation.

Effect of OHF on cell cycle- and apoptosis-related gene expression

To further analyze the cell cycle arrest associated molecular mechanisms of OHF, real-time PCR (Table 1) and

Table 1. Altered mRNA expression of genes involved in cell cycle regulation and apoptosis in AGS human gastric cancer cells treated with or without *Opuntia humifusa*

Gene	Control	OHF100	OHF200
p53	1.05±0.28^{b}	1.32±0.34^{b}	2.52±0.25^{a}
p21	1.14±0.25^{c}	2.03±0.20^{b}	3.30±0.29^{a}
Cdk4	1.04±0.13^{a}	0.57±0.26^{b}	0.36±0.24^{b}
Cyclin D1	1.23±0.32^{a}	1.09±0.19^{a}	0.29±0.28^{b}
Cdk2	1.12±0.24^{a}	0.59±0.21^{b}	0.55±0.23^{b}
Cyclin E	1.14±0.25^{a}	0.56±0.22^{b}	0.49±0.29^{b}
Bax	1.08±0.13^{c}	4.49±0.19^{b}	5.57±0.20^{a}
Bcl-2	1.44±0.18^{a}	1.21±0.25^{a}	0.32±0.22^{b}
Caspase-3	1.20±0.10^{b}	1.69±0.17^{a}	1.84±0.14^{a}

Data are presented as the mean±SE. Values with different letters (a-c) in the same row are significantly different ($P<0.05$) according to Duncan's multiple-range test. The cells were treated with OHF at 100 μg/mL (OHF100) or 200 μg/mL (OHF200) and incubated for 24 h.

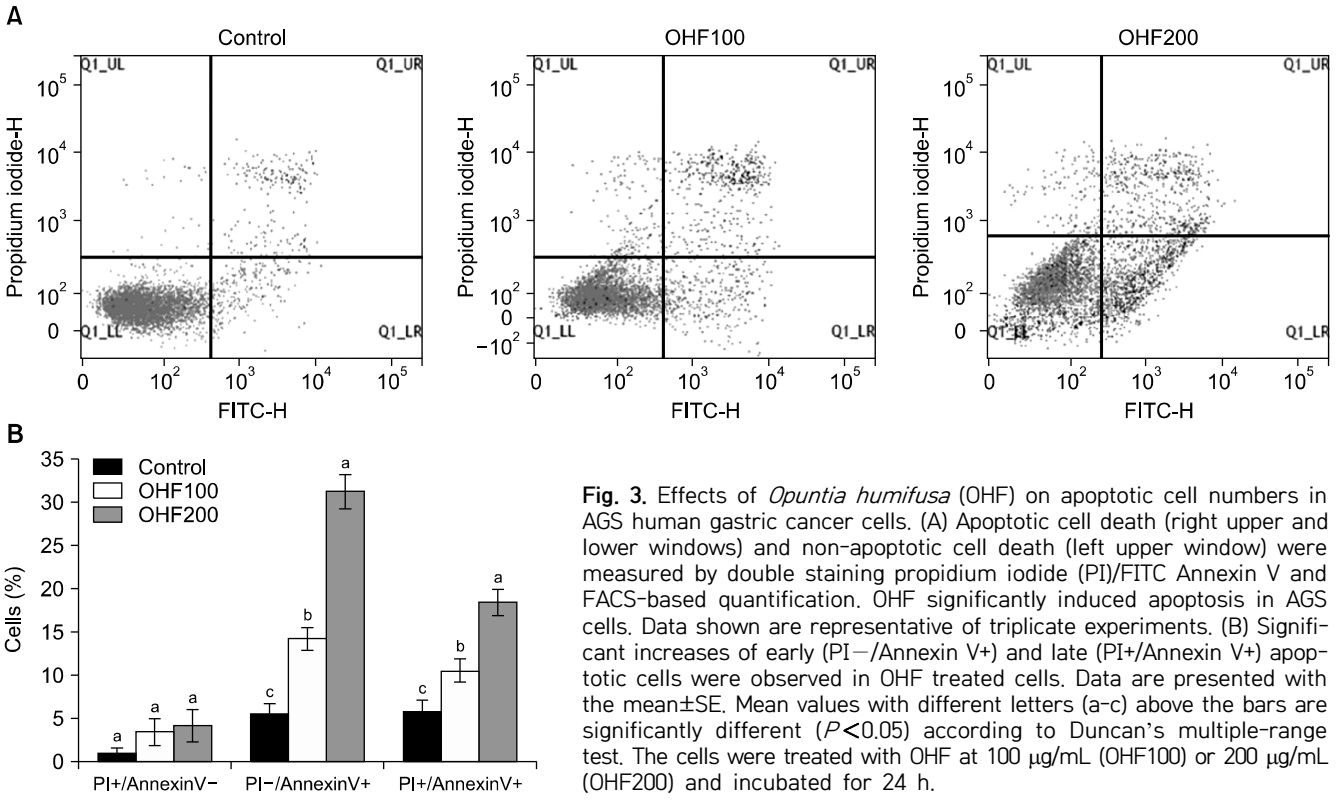

Fig. 3. Effects of *Opuntia humifusa* (OHF) on apoptotic cell numbers in AGS human gastric cancer cells. (A) Apoptotic cell death (right upper and lower windows) and non-apoptotic cell death (left upper window) were measured by double staining propidium iodide (PI)/FITC Annexin V and FACS-based quantification. OHF significantly induced apoptosis in AGS cells. Data shown are representative of triplicate experiments. (B) Significant increases of early (PI−/Annexin V+) and late (PI+/Annexin V+) apoptotic cells were observed in OHF treated cells. Data are presented with the mean±SE. Mean values with different letters (a-c) above the bars are significantly different ($P<0.05$) according to Duncan's multiple-range test. The cells were treated with OHF at 100 μg/mL (OHF100) or 200 μg/mL (OHF200) and incubated for 24 h.

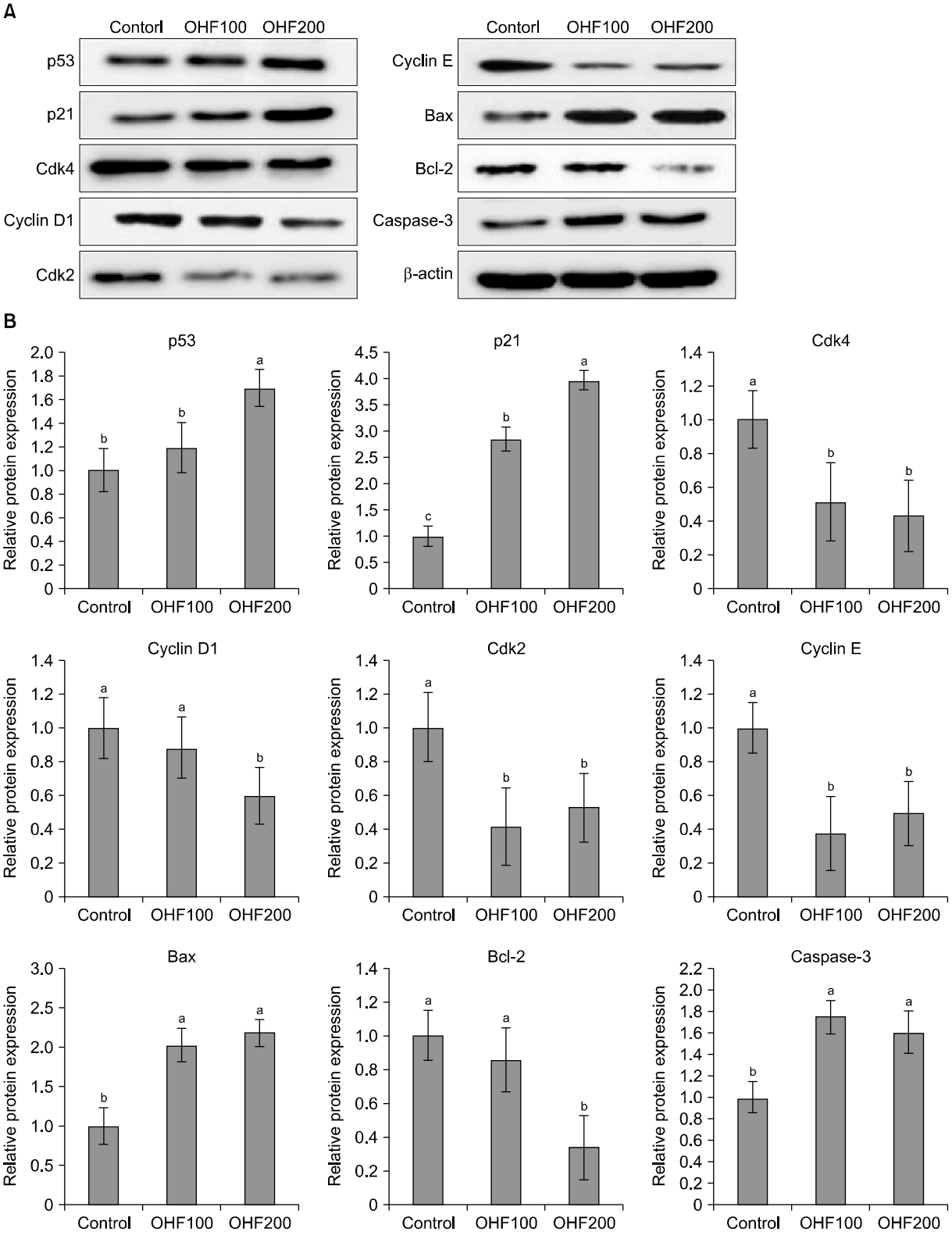

Fig. 4. Effects of *Opuntia humifusa* (OHF) on the apoptosis and cell cycle regulatory proteins in AGS human gastric cancer cells. (A) Representative blot showing protein expression following OHF treatment. (B) Band intensity was measured by densitometric analysis and expressed as fold change of the control. Data are presented with the mean±SE. Mean values with different letters (a-c) above the bars are significantly different ($P<0.05$) according to Duncan's multiple-range test. The cells were treated with OHF at 100 μg/mL (OHF100) or 200 μg/mL (OHF200) and incubated for 24 h.

western blot analysis (Fig. 4) for p21, cyclins, and Cdks were performed. The mRNA and protein levels of p21 in AGS cells were upregulated ($P<0.05$) by the OHF treatment, compared to the control, whereas the expressions of Cdk4 and Cdk2 were downregulated ($P<0.05$). Additionally, OHF200 significantly decreased ($P<0.05$) the

expression levels of cyclin D1 and cyclin E in AGS cells. Cyclin D1 related to the cell cycle progression through the G1 phase by interactions with Cdk2 and Cdk4 (18). When cyclin D1 is overexpressed at the G1 phase, cell proliferation is accelerated (19). The overexpression of cyclin D has been observed in various tumor cells (20, 21). Thus, inhibition of cell cycle progression is an important incident for anticancer activity. The cell cycle inhibitory proteins negatively regulate the cell cycle and discontinue the cell cycle process to the next phase (22). The induction of the G1 phase cell cycle arrest is correlated with the upregulation of Cdk inhibitor, p21 is associated with the inhibition of Cdk2 and Cdk4 (23). The effective approach to inhibit cyclin D1 activity is by reducing its associated kinases Cdk4 (24). Therefore, OHF arbitrated the G1 phase cell cycle arrest by disrupting the expression of cyclin D1 and Cdk4 via the upregulation of p21. It has also been reported that extracts of OHF fruits induced the increment of p21 protein expression in human cervical carcinoma cells (7).

To elucidate the mechanisms underlying the apoptosis by OHF, the expression of p53, Bax, Bcl-2, and caspase-3 was measured by real-time PCR and western blot. As shown in Table 1 and Fig. 4, the mRNA and protein expression levels of Bax were significantly increased ($P<0.05$) in OHF treated groups when compared to the control AGS cells. Moreover, OHF200 inhibited ($P<0.05$) Bcl-2 expression, which caused a dose-dependent increase of the Bax/Bcl-2 ratio. With OHF treatment, the expression of caspase-3 was elevated ($P<0.05$) when compared to the control. Furthermore, OHF200 treatment increased ($P<0.05$) the expression level of p53.

The anti-apoptotic protein Bcl-2 is expressed on the outer mitochondrial membrane surface and prevents the release of cytochrome c into the cytosol (25,26). In contrast, Bax permeabilizes the outer membrane of the mitochondria and admits the release of cytochrome c, which triggers the activation of caspases to induce apoptosis of the cell (27). The activation of caspase-3 is an important downstream event in the apoptotic process (28). The increase in the Bax/Bcl-2 ratio also leads to the activation of caspase-3 (29). Thus, the balance modulation between Bax and Bcl-2 is decisive for the induction of apoptosis. In the present study, apoptosis induction by the OHF treatment was related to upregulation of Bax and downregulation of Bcl-2, leading to an increase of caspase-3 expression in AGS cells. The expression level of p53 was also increased in OHF treated AGS cells. p53 also advances apoptosis through the mitochondrial pathways (30). The p53-dependent apoptosis leads to mitochondrial apoptotic changes via activation of the Bax and caspases cascade (31). Furthermore, p53 activates the p21 protein to instigate a the G1 phase cell cycle arrest (32). In the current study, the different p53 expression level was observed in the 2 doses of OHF treatment. Therefore, the distinct increased levels of p53 expression were considered to be supportive for the induction of cell cycle arrest and apoptosis in OHF treated AGS cells.

In conclusion, the present study demonstrated that OHF inhibits the growth of AGS cells by causing cell cycle arrest and the induction of apoptosis. OHF induced cell cycle arrest through the p21-mediated G1 phase arrest. Moreover, the activation of the mitochondria-mediated apoptosis pathway is the main events in the process of apoptosis incidence by OHF treatment. OHF was implicated the regulation of Bax and Bcl-2, followed by the significant increment of caspases-3 expression. This research provides a new insight into understanding the inhibition mechanisms of AGS cell proliferation by OHF. Further studies may be needed to identify the phytochemical constituents in OHF.

ACKNOWLEDGEMENTS

This work was carried out with the support of a Korea University Grant, Republic of Korea.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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