Alpinia japonica extract induces apoptosis of hepatocellular carcinoma cells through G0/G1 cell cycle arrest and activation of JNK

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ABSTRACT

Hepatic cancer was the third most prevalent cause of cancer-related death worldwide in 2018, and its incidence is increasing. While therapeutic agents for hepatic cancer have improved, these agents can cause serious side effects, including damage to healthy tissues. To overcome this limitation, more than 3,000 plants have been used globally as common alternatives for cancer treatment. The anti-cancer activity of Alpinia japonica, one of the traditional herbal medicines (Korean name: Kkot-yang-ha), was investigated. Water extract of A. japonica (AJ) decreased the cell viability of hepatic cancer cells. AJ extract showed greater than 70% loss of mitochondrial potential in HepG2 cells as demonstrated by JC-1 staining. Apoptosis was induced by treatment with AJ extract as shown through FACS analysis, and G0/G1 phase arrest of 76.66% HepG2 cells was confirmed through cell cycle analysis and quantitative RT-PCR. Improper regulation of ERK1/2 might contribute to cell death, and JNK activation is necessary for apoptosis induced by stress stimuli. AJ extract stimulated the phosphorylation of JNK and ERK1/2, mitogen-activated protein kinases (MAPKs), in HepG2 cells. AJ extract has anticancer activity by inhibiting cell cycle progression, leading to apoptosis of hepatic cancer cells. This extract could potentially be used as a therapeutic agent for hepatic cancer.

Introduction

Hepatocellular carcinoma (HCC), a primary liver cancer, is the most common malignancy and the third leading cause of cancer-related death (1). An estimated 905,700 people were diagnosed with and 830,200 people died from liver cancer globally (2). Because of the absence of specific symptoms in the early stages and the lack of early diagnostic markers, most HCC patients are diagnosed in an advanced stage after radical resection is not a feasible option. These patients have a poor prognosis (3). Because HCC is a serious global problem, more effective and novel prevention, diagnosis, and therapeutic strategies are needed.

Plant extracts and their bioactive compounds have been used for medicinal purposes from ancient times. Traditional medicinal plants usually have low or no toxicity, and their chemotherapeutic activities are being investigated for clinical application. Numerous natural compounds activate or inhibit mechanisms associated with disease prevention or pathogenesis. These diseases include cancer, infection, and other diseases associated with inflammation (4-8).

Vinca alkaloids, taxol and podophyllotoxins, and their derivatives are the most important examples of plant-derived anticancer drugs (9, 10). In particular, the Vinca alkaloids vinblastine and vincristine isolated from Catharanthus roseus, commonly known as periwinkle, possess strong anticancer activities (11).

The genus Alpinia has been widely used in traditional herbal medicine and cultivated in many diverse regions (12, 13). A. japonica is commonly used to treat abdominal crymodynia, lung problems, rheumatism and menoxenia in southeast China, Japan, and Korea (14). Approximately 200 compounds with significant bioactive potential have been identified from species of the genus Alpinia (15). Some recently isolated sesquiterpenoids from rhizomes of these plants inhibit NO production (16), and alpinone 3-acetate from A. japonica has shown anti-inflammatory potential (17).

In this study, the anticancer activity of A. japonica was tested on hepatocellular carcinoma cells. To evaluate the cytotoxic effect on hepatic cancer cells, MTT assay was performed on cells treated with AJ extract. Cell cycle progression and apoptosis of AJ extract-treated cancer cells were tested by flow cytometry. Additionally, expression of mRNAs and proteins related to cell apoptosis and the mechanism of AJ action in cancer cells were assayed.

Materials and Methods

Cells and cultivation

The hepatocellular cancer cells, HepG2, Huh7 and PLC/PRF5 were obtained from KCLB (Korean Cell Line Bank, Korea). All cells were cultured in DMEM medium (Welgene, Korea) with 10% fetal bovine serum (FBS, Gibco, MA, USA) and 1% penicillin/streptomycin (Corning, NY, USA) at 37 °C in 5% CO2.

Preparation of A. japonica water extract

Leaves of A. japonica were acquired from Jeju Island. The extracts were produced using 3 L of distilled water containing 300 g of the sample at 80 °C for 8 hours.
concentrated at 40 °C using a rotary evaporator, and freeze-dried. Then, 10 mg of extract powder was dissolved in 1 mL dimethyl sulfoxide (DMSO) for the experiments.

**Cell viability assay**

Cell viability was measured according to the previously reported method (18, 19). For this, 10^4 hepatocellular cancer cells per well were added to a 96-well plate and incubated for 24 hours. The indicated concentrations of AJ extract (3.125-100 μg/mL) were added, and the cells were further incubated for 48 hours. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, MO, USA) was added and incubated for 3 hours at 37 °C. Cells were suspended in DMSO for 10 minutes. Absorbance was calculated from optical density (OD_{540}) values.

**Annexin V/PI analysis by Flow Cytometry**

An eBioscience Annexin V apoptosis detection kit APC (Thermo Fisher Scientific, MA, USA) was used. AJ extract (5, 25 and 50 μg/mL) was added and incubated for 24 hours. Cells were harvested by trypsinization, re-suspended in a 1x binding buffer, and stained with 2.5 μL of annexin V with 2.5 μL of propidium iodide solution for 15 minutes at room temperature. FACS analysis was then carried out according to the manufacturer’s instructions, using the BD Accuri C6 Plus System (BD Bioscience, CA, USA). More than 30,000 counts were recorded in each analysis (18, 20).

**JC-1 staining analysis by fluorescence microscope**

HepG2 cells were added to the wells of a 24-well plate at a density of 10^5 cells per well and incubated for 24 hours. AJ extract (5, 25 and 50 μg/mL) was added and incubated for 24 hours. Cells were stained with 5 μM of JC-1 (Biotium, CA, USA) for 30 minutes at 37 ℃. Photographic images were acquired under an inverted fluorescence microscope (EVOS FL Cell Imaging System, Thermo Fisher, MA, USA) (18, 21).

**Quantitative reverse transcriptase PCR (qRT-PCR) analysis**

HepG2 cells in DMEM were added to the wells of a 24-well plate at a density of 10^5 cells per well and incubated for 24 hours. AJ extract (5, 25 and 50 μg/mL) was added and further incubated for 24 hours. qRT-PCR was performed following the manufacturer’s method with slight modification (22). A reverse transcriptase (NanoHelix, Korea) reaction was prepared using 0.8 μg of RNA to obtain cDNA. Primer sequences used in this study are listed in Table 1.

**Western blot analysis**

Protein was extracted with RIPA buffer and quantified with the Bradford reagent (Sigma, MO, USA). Protein samples were separated by 10-15% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF, Bio-Rad, CA, USA) membranes. Antibodies for Bax, Bcl-2, p-p53, caspase-9, caspase-3, JNK, p-JNK, p38, GAPDH (Santa Cruz Biotechnology, USA), p-p38, ERK1/2, p-ERK1/2 and p53 (Cell Signaling Technology, TX, USA) were used. The membranes were developed using enhanced ECL (Bio-Rad, CA, USA) on a UVITEC imaging system (UVITEC Cambridge, UK) (18, 19).

**Cell cycle analysis**

The cell cycle distribution was determined by flow cytometry with propidium iodide (PI, Sigma-Aldrich, MO, USA) staining. HepG2 cells were incubated with vehicle control (0.45% DMSO) or AJ extract (5, 25 and 50 μg/mL) for 24 hours. Cells were harvested, fixed with 10% neutral buffered formalin (NBF), and stained with PI. Stained cells were analyzed with the BD Accuri C6 Plus System (BD Bioscience, CA, USA) (23).

**Statistical analysis**

All data are expressed as mean ± S.D. Significant differences among the groups were determined using Student’s t-test or one-way ANOVA, p < 0.01 was considered significant.

**Results**

**AJ extract reduced cell viability of hepatic cancer cells.**

The effects of AJ extract on viability of hepatic cancer cell lines HepG2, Huh7, and PLC/PRF5 were investigated. AJ extract reduced cell viability in a dose-dependent manner (Fig. 1), with IC_{50} values of 67 and 57.4 μg/mL in HepG2 and Huh7 cells, respectively, cells at 24 hours. AJ

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>Bax</td>
<td>TTTGCTTCAGGGTTCATCCAG</td>
<td>CTCCATGTTCAGTCATCCAG</td>
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<tr>
<td>Bcl-2</td>
<td>TCCCTCGCTGCACAATAC</td>
<td>ACGACCAGATGGCATAGA</td>
</tr>
<tr>
<td>p53</td>
<td>AGGCCCTGGAACTCAAGGAT</td>
<td>TTATGGGCGAGTGAAGCTG</td>
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<td>CDK1</td>
<td>CAGGTCGAAGTGAGCCATG</td>
<td>ACCTGGAATTCGATAAGC</td>
</tr>
<tr>
<td>CDK2</td>
<td>TACTGCGTTCCATCCGAG</td>
<td>GTACGTCGCTTCCGATTTT</td>
</tr>
<tr>
<td>CDK4</td>
<td>CTGAGAATGGCTACCTCTCG</td>
<td>CGAACTGTGCTGATGGGAAG</td>
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<tr>
<td>CDK6</td>
<td>CCAGAAGTCTGCTCCAGTCC</td>
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<td>CCNB1</td>
<td>TAAGGCAGACATCACAATGG</td>
<td>GCTTCCTTCATGAGCAT</td>
</tr>
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<td>CCND1</td>
<td>CTGTGCTGGCAAGTGGGAAACC</td>
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</tr>
<tr>
<td>CCNE1</td>
<td>ACACCATGAAGGAGGACG</td>
<td>CACAGACTGCTATTGCCC</td>
</tr>
<tr>
<td>CDFKN1B</td>
<td>AAGGGTTTGGAGGCGGCTG</td>
<td>GATCCAAATGCACTGGAGAC</td>
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<tr>
<td>GAPDH</td>
<td>GTGAAGGTGGAGGATVACC</td>
<td>TGAGGTCATGAGGGAGTC</td>
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</table>

**Table 1. Primer sequences used in this study.**
the G₀/G₁ phase was increased by 76.66% at 50 μg/mL of AJ treatment compared with control cells, 67.97% (Fig. 4A and Table 2). Also, the fraction of cells in the G₂/M phase was decreased by 7.87% at 50 μg/mL of AJ treatment compared with control cells, 15.66%. Regulation of extract also reduced cell viability with IC₅₀ values of 77.3 μg/mL in PLC/PRF5 cells. These results showed that AJ extract had anticancer activities in hepatic cancer cells.

AJ extract induced apoptotic cell death in HepG2 cells.

To investigate how AJ extract inhibits the cell growth of hepatic cancer cells, JC-1 staining was used to detect cellular mitochondrial membrane potential. AJ extract induced the loss of membrane potential in a dose-dependent manner as demonstrated by fluorescence microscopic analysis (Fig. 2A). Apoptotic HepG2 cells were significantly increased (26.93%) by AJ extract compared to control solvent-treated cells (3.88%) as determined by flow cytometric analysis (Fig. 2B).

AJ extract induced the expression of apoptosis-related genes and proteins.

To validate the expression of apoptosis-related genes and proteins, qRT-PCR and western blot analysis were performed. The pro-apoptotic gene Bax sustained its expression, but expression of Bcl-2, an anti-apoptotic gene, was decreased by approximately 27.8 % in AJ-treated (50 μg/mL) cells. The expression ratio of Bax/Bcl-2 was increased 3.76-fold at 50 μg/mL of AJ treatment (Fig. 3A). mRNA expression of p53 was increased by 65.2% at 50 μg/mL of AJ treatment (Fig. 3B). Expression of Bax and phosphorylated p53 protein increased in a dose-dependent manner as indicated western blot analysis, while Bcl-2 was decreased as the concentration of AJ extract increased (Fig. 3C, D). Caspase-9 and caspase-3 are included in the caspase family and are mediators of apoptosis (24). Caspase-9 and its cleaved form, and caspase-3 also increased in response to treatment with AJ (Fig. 3E).

Cell cycle was arrested in G₀/G₁ by AJ treatment.

Cell cycle distribution was analyzed in HepG2 cells by flow cytometry and qRT-PCR. The fraction of cells in the G₀/G₁ phase was increased by 76.66% at 50 μg/mL of AJ treatment compared with control cells, 67.97% (Fig. 4A and Table 2). Also, the fraction of cells in the G₂/M phase was decreased by 7.87% at 50 μg/mL of AJ treatment compared with control cells, 15.66%. Regulation of
mRNA expression of genes related to cell cycle progression by AJ treatment in HepG2 cells was further analyzed. mRNA expression of CDK4 and CDK6 was increased. CDKN1B, p27 and one of the Cyclin-dependent kinase inhibitors (CKI) was increased in a dose-dependent manner. However, CDK1 (7.22 ± 3.65%), CDK2 (5.53 ± 2.60%), CCNB1 (28.45 ± 13.72%), CCND1 (45.29 ± 12.31%), CCNA2 (56.35 ± 0.13%) and CCNE1 (64.10 ± 4.01%) were decreased in HepG2 cells with 50 μg/mL of AJ treatment. (Fig. 4B-D). These results indicate that HepG2 cells were arrested in the G₀/G₁ phase of the cell cycle by AJ treatment.

Table 2. AJ treatment arrests cell cycle distribution of HepG2 cells at G₀/G₁.

<table>
<thead>
<tr>
<th>AJ Concentration (μg/mL)</th>
<th>% of the Cell Population</th>
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<tbody>
<tr>
<td></td>
<td>G₀/G₁ fraction</td>
</tr>
<tr>
<td>0</td>
<td>64.97</td>
</tr>
<tr>
<td>5</td>
<td>59.64</td>
</tr>
<tr>
<td>25</td>
<td>67.93</td>
</tr>
<tr>
<td>50</td>
<td>76.66</td>
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</table>

JNK and ERK1/2 were activated by AJ treatment.

To investigate the anticancer mechanism of AJ, MAPK pathways of JNK, p38, ERK1/2 and their phosphorylated forms were analyzed by western blot. AJ extract significantly increased the phosphorylation of JNK in HepG2 cells in a dose-dependent manner. AJ extract significantly increased p-ERK1/2 at 5 μg/mL, but this gradually decreased with increasing AJ extract dosage. AJ extract did not alter the activation of p38 in HepG2 cells (Fig. 5). To investigate whether JNK activated by AJ extract was involved in the apoptosis of hepatic cancer cells, combination effects of AJ extract were evaluated with a JNK inhibitor (SP600125) by cell viability assay and western blot analysis. HepG2 cells were treated with 50 μg/mL AJ extract alone or in combination with 10 μM of SP600125 for 24 hours. Only SP600125-treated HepG2 cells showed no changes in p-JNK; however, p-JNK was decreased when treated with SP600125 and AJ extract together (Fig. 6A and B). In the presence of SP600125, the cell viability of HepG2 cells was not changed, but HepG2 cells treated with SP600125 and AJ extract together showed increased
the viability (Fig. 6C). These results suggest that activation of JNK is important for inducing apoptosis in HepG2.

Together, these results indicate that JNK activation contributes to AJ-induced apoptosis in HepG2 cells.

Discussion

HCC is the most common and serious primary liver malignancy with a high mortality and incidence worldwide (25). Nutraceutical intervention with plant compounds might be an effective way to treat HCC and improve patients’ quality of life (26, 27). Plant-derived bioactive components, including paclitaxel (from Taxus brevifolia), camptothecin (from Camptotheca acuminata), podophyllotoxin (from Podophyllum emodi), and vinblastine (from Catharanthus roseus), have been developed as potential anticancer agents (28, 29). The genus Alpinia is widely used as an herbal medicine with antioxidant and antimicrobial activity (30, 31). The anticancer activity results for the genus Alpinia have been already validated in lung and breast cancer cells (32, 33). In the present report, we demonstrated that AJ strongly inhibited the growth of several hepatic cancer cell lines in a dose-dependent manner.

Mitochondria plays an essential role in apoptosis since apoptosis signals damage mitochondria and triggers mitochondrial membrane permeabilization (34). Our JC-1 staining and Annexin V and PI analysis, demonstrate that AJ extract changes the mitochondrial membrane potential and induces the apoptosis of HepG2 cells.

AJ extract induced mRNA expression and up-regulation of the p53 by phosphorylation in HepG2 cells. Consistent with an increase in disruption of mitochondrial membrane potential and the ratio of Bax/Bcl-2, AJ extract showed dose-dependent caspase-9 activation and caspase-3 accumulation which suggest that AJ extract induces apoptosis in cancer cells.

Regulation of the cell cycle is key in controlling tumor progression (35). Abnormal regulation of the cell cycle could disrupt tumor progression and cause cell death (36). DNA content as demonstrated by FACS analysis showed that G0/G1 HepG2 cells were increased after AJ treatment. Thus, AJ extract treatment resulted in G0/G1 cell cycle arrest. Cyclins and cyclin-dependent kinases (CDKs), such as CDK4 and CDK6, regulate cell cycle's progression (37). AJ extract induced mRNA expression of CDKN1B (p27), a CDK inhibitor of G1 cell cycle progression. mRNA expression of CCND1, CDK1, CDK2, CCNB1, and CCNE1 was reduced by AJ treatment. CDK and cyclin mRNA are generally decreased, and CKIs are increased with cell cycle arrest (38). Dysregulation of the cell cycle check-
point proteins, such as CDK4 and CDK6, is a hallmark of apoptosis. The mRNA expression of CDK4/6 is generally decreased in apoptosis, but in this study, it was increased by treatment with AJ extract. AJ extract may cause cell cycle arrest in G$_1$/G$_0$ through decreased cyclin D1 expression and increased expression of CDKN1B.

MAPKs are key regulators of cell growth and survival in physiological and pathological processes. ERKs regulate proliferation and survival signals in normal and cancer cells, so dysregulation of ERK1/2 can induce cell death (39). Phosphorylation of ERK1/2 was increased at 5 μg/ml of AJ treatment, but gradually decreased to a similar level to the untreated control as the treatment concentration increased. However, JNKs generally induce pro-apoptotic effects. In lymphoid cells, JNK pathway activation by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) caused apoptosis activation (40). AJ extract showed significantly increased activation of JNK. This suggests that AJ extract stimulates activation of JNK to induce the apoptosis of HepG2 cells. The p38 pathway was unaffected by AJ treatment.

In conclusion, the anticancer activity and mechanism of AJ extract action in hepatic cancer cells were investigated in this study. However, further detailed studies of the anticancer effects of individual components of AJ need to be conducted. In vivo experiments will be performed to confirm the effect of AJ extract.

This study showed that AJ extract has anticancer activity in HepG2 cells by inducing apoptosis. AJ treatment induced apoptosis in HepG2 cells through Bcl-2 protein family members, mitochondrial dysregulation, and activation of caspases. AJ treatment decreased mRNA expression of cyclin D1 and increased CDKN1B. Together with previous results, AJ treatment was shown to arrest the cell cycle progression in G$_1$/G$_0$ as demonstrated through FACS analysis. AJ also activated JNK, decreasing viability of HepG2 cells.

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Interest conflict
The authors have no conflicts of interest to declare.

Author’s contribution
The authors confirm these contributions to the paper: study conception and design: J.G. Kim and K.Y. Kim; literature search: J.G. Kim, W. Kim and K.Y. Kim; experimental studies, data acquisition: J.G. Kim; data analysis: J.G. Kim, W. Kim and K.Y. Kim; manuscript preparation: J.G. Kim; manuscript editing and review: J.G. Kim, W. Kim and K.Y. Kim. All authors reviewed the results and approved the final version of the manuscript.

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