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Je-Chun-Jun induced apoptosis of human cervical carcinoma HeLa cells[1]

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ABSTRACT

AIM: To study the mechanism of Je-Chun-Jun (JCJ)-inducing the apoptosis of the human cervical carcinoma, HeLa cells. **METHODS:** The cell viability was assessed using MTT assay. The optical density was measured at 570 nm. The caspase activity was measured using 50 mmol/L of fluorogenic substrate, AC-DEVD-AMC (caspase-3), AC-VEID-AMC (caspase-8) or AC-LEHD-AFC (caspase-9). To confirm the expression of proteins, Western blotting was performed. To detect the characteristic of apoptosis chromatin condensation, HeLa cells were stained with Hoechst 33258 in the presence of JCJ. For the cell cycle analysis, HeLa cells were incubated with Propidium iodide (PI) solution. Fluorescence intensity of cell cycle was measured using flow cytometry system. **RESULTS:** The loss of viability occurred following the exposure of 10 g/L JCJ. Cells treated with 10 g/L JCJ for 3 d exhibited the apoptotic morphology (brightly blue-fluorescent condensed nuclei by Hoechst 33258-staining) and the reduction of cell volume. Cells incubated with JCJ for 48 h were arrested at the G_1 phase of cell cycle and their G_1 checkpoint related gene products such as cyclin D_1 were transiently decreased. We showed that JCJ induced the p38 MAPK activation in HeLa cells. The p38 MAPK inhibitor, SB203580 protected Hela cells from the JCJ-induced death as well as intervened the JCJ-induced accumulation of cells at the G_1 phase. In contrast, MEK1 (-ERK upstream) inhibitor, PD98059 had no effect on HeLa cells. **CONCLUSION:** JCJ induced cell cycle arrest and apoptosis of HeLa cells through p38 MAPK pathway.

INTRODUCTION

As part of our continuing efforts in the search for biologically active anti-cancer agents from medicinal resources, the anti-cancer effect of Je-Chun-Jun (JCJ) was investigated. JCJ, a traditional herb formulation, has been successfully used for the treatment of diseases in women in Korea, China, and Japan[1]. However, its underlying therapeutic mechanism is not clear. In the present study, to determine the therapeutic potential of JCJ in cervical carcinoma, we examined the JCJ-induced cytotoxic effect as well as the signaling pathways in human cervical carcinoma cells-HeLa cells.

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Cervical carcinoma is one of the prevalent cancers in females. It has been demonstrated that human cervical carcinoma cells appear to retain functional apoptotic machinery because they respond to a wide spectrum of stimuli by undergoing apoptotic death[2-4]. In this study, we characterized in detail the role of JCJ in HeLa cells and found that JCJ-triggered cell death depended on p38 MAPK (mitogen activated protein kinase). The mechanism of JCJ-induced apoptosis included the regulatory effect on the cell cycle in human cervical carcinoma cells.

MATERIAIS AND METHODS

Preparation of JCJ extracts Je-Chun-Jun (JCJ) extracts were prepared by decocting the prescription of dried herbs with boiling water (75.315 g/L) for 4 h. The extracts were freezer-dried for 4 d, then dissolved with distilled water and filtered and stored at 4 ºC until used. These dried herbs were obtained from Oriental Medicine Hospital, Wonkwang University (Iksan, South Korea). A voucher specimen (No 99-02-0026) was deposited at the Herbarium at the College of Dental Medicine, Wonkwang University. The yield (w/w) of aqueous form from starting crude materials was about 10 %.

The composition of JCJ were as follows:
Dang-Gui: Radix Angelicae gigantis (11.25 g)
Suk-Jee-Hwang: Rhizoma Rehmanniae (7.5 g)
U-Sool: Radix Achyranthis (7.5 g)
O-yak: Radix Linderae (3.75 g)
Yook-Kae: Cortex Cinnamomi (3.75 g)
Do-In: Semen Persicae (2.1 g)
Total: 35.85 g

Cell culture Human cervical carcinoma HeLa cells were maintained in D-MEM supplemented with 10 % FBS, benzylpenicillin (100 kU/L), and streptomycin (100 mg/L) in a humidified 5 % CO_2 incubator.

MTT assay The viability of cells was assessed in the MTT assay as described previously[5]. Briefly, MTT was added to cells at a final concentration of 0.5 g/L. Cells were incubated at 37 ºC for 5 h, the the medium was aspirated, and the formazan product was solubilized with dimethylsulfoxide. The absorbance at 630 nm (background absorbance) was subtracted from absorbance at 570 nm.

Morphological detection of apoptosis Cells were fixed for 5 min in 3 % paraformaldehyde in phosphate-buffered saline. After air-drying, cells were stained for 10 min in Hoechst 33258 (10 g/L), mounted in 50 % glycerol containing 20 mmol/L citric acid and 50 mmol/L orthophosphate, and stored at -20 ºC before analysis. Nuclear morphology was evaluated using a Zeiss IM 35 fluorescent.

Cell cycle analysis For cell cycle analysis, 1×10^6 HeLa cells were washed twice with phosphate-buffered saline (PBS) and then incubated with PI (Propidium iodide) solution (0.5 mL of 3.4 mmol/L sodium citrate, 10 mmol/L NaCl, 0.1 % NP-40, and 50 mg/L of PI) for 30 min. Fluorescence intensity was measured using flow cytometry (the PAS system: PARTEC, Germany). For each sample, 10 000 cells were analyzed using program M cycle software (Coulter).

The assessment of the caspase activity by fluorogenic substrate assay Cell lysates were prepared by lysing the cells in a buffer containing 1 % Nonidet P-40, NaCl 200 mmol/L, Tris/HCl 20 mmol/L, pH 7.4, leupeptin 10 mg/L, aprotinin (trypsin inhibitor 0.27 kU/L) and 100 mm PMSF. Caspase protease activity was determined by incubating the lysate (25 mg of total protein) with 50 mmol/L of fluorogenic substrate, AC-DEVD-AMC (caspase-3), AC-VEID-AFC (caspase-8) or AC-LEHD-AFC (caspase-9) in the buffer (HEPES 10 mmol/L, pH 7.4, containing mannitol 220 mmol/L, sucrose 68 mmol/L, NaCl 2 mmol/L, KH_2PO_4 2.5 mmol/L, EGTA 0.5 mmol/L , $MgCl_2$ 2 mmol/L, pyruvate 5 mmol/L, PMSF 0.1 mmol/L, and dithiothreitol 1 mmol/L). The caspase activity was assessed by measuring fluorescent 7-amino-4-methylcoumarin released for 1 h at a 2-min intervals by a spectrofluorometer.

Western blot analysis Western blot analysis was performed as described[6]. Briefly, the whole-cell lysates were prepared by lysing cells with the buffer containing 1 % Nonidet P-40, HEPES 50 mmol/L (pH 7.5), NaCl 100 mmol/L, EDTA 2 mmol/L, pyrophosphate 1 mmol/L, sodium orthovanadate 10 mmol/L, phenylmethylsulfonyl fluoride 1 mmol/L, and sodium fluoride 100 mmol/L. Equal amounts of the lysates were subjected to sodium dodecyl sulfate-10 % polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore) in transfer buffer (Tris 25 mmol/L, glycine 192 mmol/L, methanol 20 % [v/v]). The membranes were rinsed in Tris-buffered saline (TBS: Tris 10 mmol/L [pH 7.4], NaCl 150 mmol/L) and blocked with TBS-5 % bovine serum albumin (BSA) overnight at room temperature. Anti-Bcl-2 and anti-Bax antibodies were used at the dilution 1:1000 in TBS-5 % BSA. The antibody-antigen complexes were detected with the

aid of horseradish peroxidase-conjugated protein A or horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) and chemiluminescent substrate development kit (Kirkegaard & Perry Laboratories).

Statistical analysis The statistical significance of the differences were evaluated by applying analysis of variance (ANOVA) and two-tailed Student's *t*-tests.

RESULTS

JCJ induced G_1 arrest and apoptosis of HeLa cells JCJ decreased the cell viability in a dose-dependent manner (Fig 1A). Treated with JCJ at 10 g/L, JCJ decreased the cell viability in a time-dependent manner (Fig 1B). The number of apoptotic cells was significantly increased after the treatment of JCJ for 72 h. Within the incubation time (48 h), we could not detect any sign of apoptosis, although Fig 1B showed the cell viability was decreased 48 h after the JCJ treatment. However, MTT assay has been used as an assay for proliferation like the method of thymidine incorporation. This suggests the existence of JCJ also contributes to the anti-proliferative potential in HeLa cells. The treatment of JCJ for 2 d increased the G_1 fraction of HeLa cells (middle panel). The arrest of cell cycle (G_1 phase) which was obtained 48 h later was less pronounced but the increase of sub G_1 fraction cells became much more

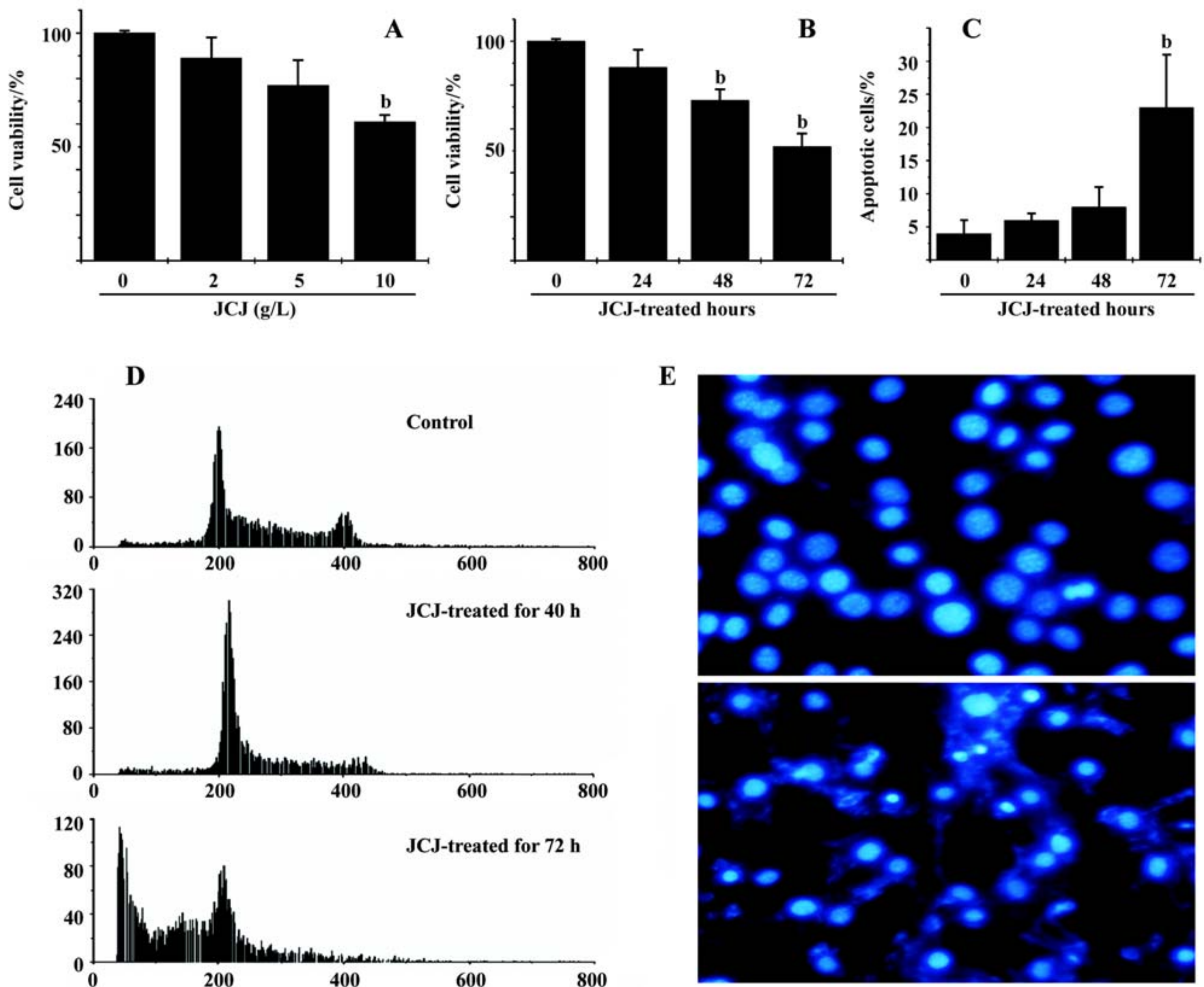

Fig 1. Je-Chun-Jun (JCJ) induced apoptosis in HeLa cells. (A) A dose-dependent effect of JCJ on cell viability. (B) A time-dependent effect of JCJ 10 g/L on cell viability. (C) Apoptosis assay of cells incubated with JCJ 10 g/L by Hoechst staining. *n*=4 experiments. Mean±SEM. $^bP<0.05$ *vs* control. (D) Cell cycle analysis using PAS. Cells were incubated with JCJ 10 g/L for 48 or 72 h. (E) Morphology of apoptotic cells. Upper panel: control; Lower panel: JCJ 10 g/L for 72 h. Hoechst 33258 stain. ×400.

apparent at later time point: 72 h (Fig 1D, lower panel). Fig 1E also showed that JCJ induced apoptotic pattern within 72-h incubation (Hoechst staining). Our data suggest that JCJ-induced accumulation of cells in G_1 stage may lead to apoptosis in later stage (eg, 72 h) in HeLa human cervical carcinoma cells.

JCJ activated caspase-3, -6, -8, and -9 in HeLa cells Cells undergo apoptosis through the activation of the proteolytic caspase cascades in diverse biological systems. The members of the caspase family are synthesized as proforms that are proteolytically cleaved and thus activated during apoptosis[6]. In various cells undergoing apoptosis, caspase-3 constitutes the major pool of the activated caspases regardless of the initial apoptotic stimuli[7]. However, other caspases –such as caspase-1, -2 and -8 are also activated during the execution phase of apoptosis[8,9]. Therefore, the fluorescence intensity of the caspase protease cleavage product AMC was monitored at various concentrations of JCJ (0, 2, 5, and 10 g/L). JCJ activated caspase-3 in HeLa cells. In addition, JCJ activated caspase-6, -8 and -9 but not caspase-1. Our data showed that caspase-3, -6, -8, and -9-like cysteine protease activities were involved in JCJ-induced apoptosis (Fig 2).

JCJ induced cell cycle arrest in HeLa cells JCJ regulated the expressions of cell cycle-associated proteins in HeLa human cervical carcinoma cells. The DNA flow cytometric analysis indicated that JCJ induced the G_1 phase arrest of HeLa cells (Fig 1C). Treated with JCJ 10 g/L, the transient down-regulations of cyclin D_1, D_2, and D_3 and cyclin E were detected (Fig 3).

JCJ regulated the expressions of Bcl-2 and Bax in HeLa cells JCJ (10 g/L) had no effect on the expression of Bcl-2 protein. JCJ, on the other hand, increased the expression of pro-apoptotic protein-Bax (Fig 4). JCJ increased the ratio of Bax/Bcl-2 in human cervical carcinoma cells. The results suggested that JCJ might induce apoptosis by increasing the expression of Bax. Within 48 h of the treatment with JCJ, the expression of Bax was not increased. After 48 h of incubation with JCJ, the increase of Bax was detected. This suggested that the expression of Bax might contribute to the JCJ-induced apoptosis but not to the cell cycle arrest.

JCJ induced apoptosis as well as cell cycle arrest in HeLa cells through p38 pathway To assess the role of MAPK in the JCJ-induced apoptosis, we examined the effect of the cell permeable MEK inhibitor PD098059 and the p38 MAP kinase specific inhibitor SB203580. Prior to the exposure to JCJ, HeLa cells were treated with 10 mmol/L PD098059 or 10 mmol/L SB203580 for 30 min and the JCJ-induced death was

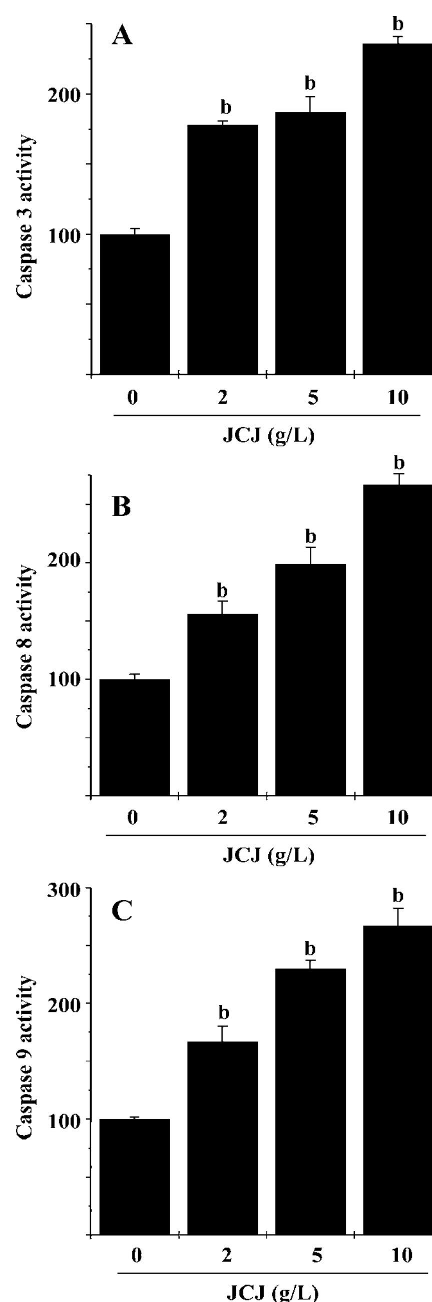

Fig 2. Je-Chun-Jun (JCJ) induced caspase-3, -8, or -9 activation in HeLa cells. HeLa cells were exposed to various concentrations of JCJ for 72 h. Cell extracts (50 μg) were then incubated in the presence of fluorescent caspase-3 substrate, DEVD-AFC (100 μmol/L) (A), caspase-8 substrate, VEID-AFC (100 μmol/L) (B), or caspase-9 substrate, LEHD-AFC (100 μmol/L) (C) for 1 h at 37 °C. All of the caspase activity was measured fluorometrically after substrate cleavage with excitation at 400 nm and emission at 505 nm. Data are expressed as the percent of controls that were non-treated. $^bP<0.05$ *vs* control.

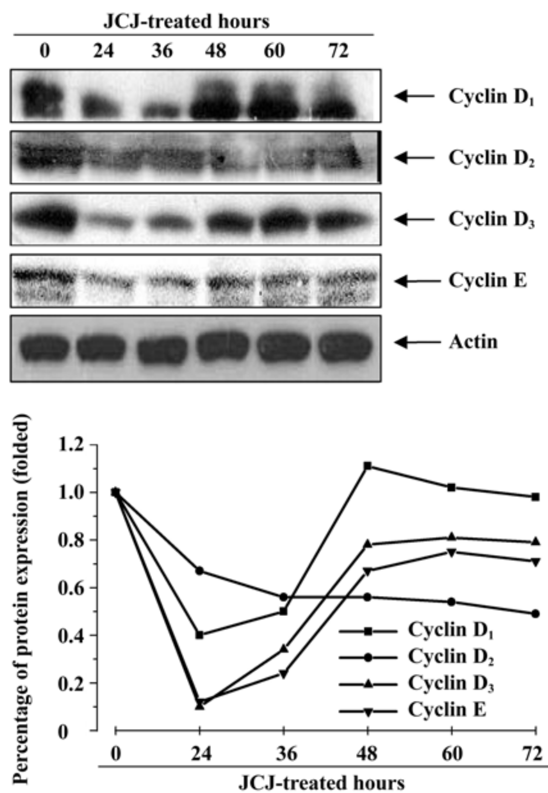

Fig 3. Je-Chun-Jun (JCJ) regulated cell cycle-associated proteins in HeLa cells. Cells were subjected to 10 g/L JCJ for 0, 24, 36, 48, 60, or 72 h. The cell lysates were prepared and separated on SDS-PAGE and transferred onto a nitrocellulose membrane. Upper: Western blot analysis; Lower: The percentage of cell cycle-associated protein expression.

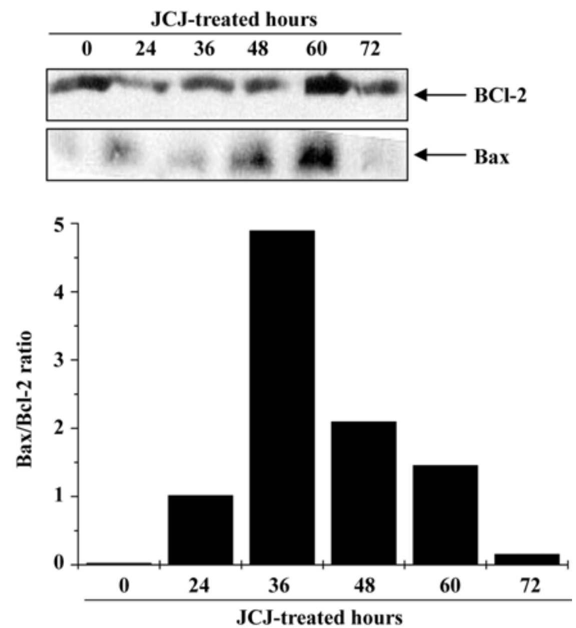

Fig 4. JCJ regulated the expressions of Bcl-2 and Bax in HeLa cells. Cells were subjected to 10 g/L JCJ for 0, 24, 36, 48, 60, or 72 h. The cell lysates were prepared and separated on SDS-PAGE and transferred onto a nitrocellulose membrane. Upper: Western blot analysis; Lower: the ratio of Bcl-2/Bax expression.

measured in the MTT assay. PD98059 had no effect on the JCJ-induced apoptosis. This indicated that ERK was not involved in the JCJ-induced apoptosis in HeLa cells (Fig 5A). SB203580, on the other hand, inhibited the JCJ-induced apoptosis. This indicated that p38 MAP kinase might be involved in the JCJ-induced apoptosis in HeLa cells. Fig 5B showed the effect of SB203580 on the JCJ-induced activation of caspase-3. Treated with JCJ for 72 h, the p38 inhibitor, SB203580 partially but significantly reduced the JCJ induced caspase-3 activity. Furthermore, SB203580 intervened the JCJ-induced cell cycle arrest (Fig 5C). The data showed that p38 MAP kinase may be involved in the JCJ-induced G_0/G_1 arrest as well as apoptosis in HeLa cells.

JCJ activated p38 MAPK in HeLa cells Previously, the p38 MAPK pathway has been reported to be involved in the apoptosis pathway in various cell types[13]. Here we observed that JCJ stimulated the expression of phospho-p38 MAPK within 10 min. After a 120-min incubation with JCJ, the expression of phospho-p38 MAPK was returned to normal (Fig 6).

DISCUSSION

Cervical carcinoma is the most prevalent malignancy in the female reproductive system. Chemotherapy with cytotoxic agents has thus been suggested as a growth inhibitor of cancer cells. Agents capable of inducing apoptosis, inhibiting cell proliferation, or modulating signal transduction are currently used for the treatment of cancer. A combination of multiple chemo-preventive agents or agents with multiple targets is considered to be more effective than a single agent[14]. Among crude ingredients of JCJ, *Semen Persicae* was reported to have anti-cancer effect on cancer cells[15]. The anti-proliferative effects of *Persicae Semen* appear to be attributable to its induction of apoptotic cell death, as *Persicae Semen* induced nuclear morphology changes and internucleosomal DNA fragmentation. Another ingredient of JCJ, *Cinnamomi Cortex*, a component of Keishi-Ka-Kei-To (KKKT: a traditional Chinese herbal medicine), also played an important role on KKKT-induced inhibitory metastasis in mice implanted with melanoma cells[16].

Here, we showed that JCJ induced apoptosis in

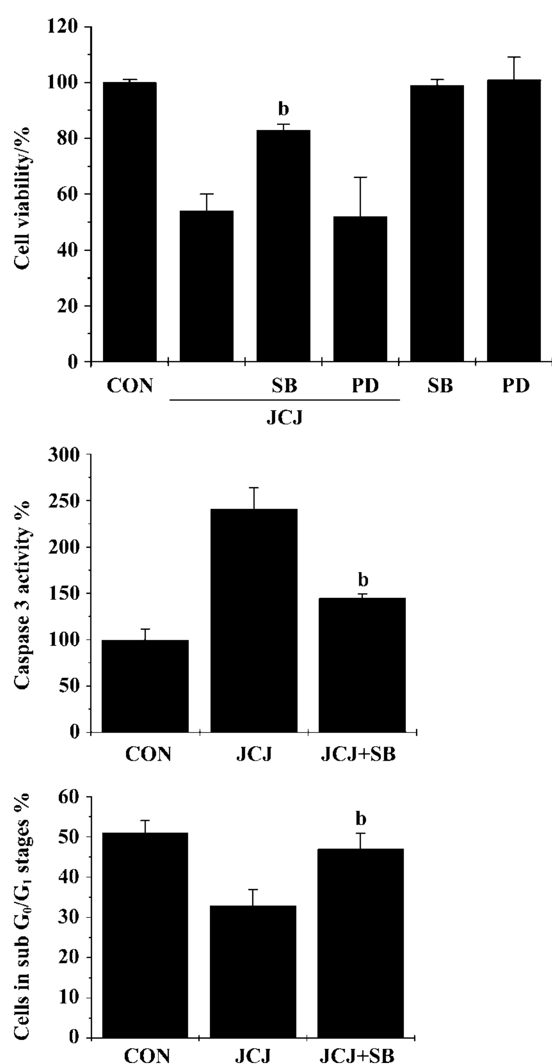

Fig 5. SB 203580, p38MAPK inhibitor, reduced Je-Chun-Jun (JCJ)-induced cell death in HeLa cells. (A) Cells were treated with 10 g/L JCJ and subsequently incubated with SB203580 10 μmol/L or PD98059 10 μmol/L for 72 h. (B) Cells were treated with 10 g/L JCJ in the presence or absence of SB203580 10 μmol/L for 72 h. Cell extracts (50 mg) were then incubated in the presence of fluorescent caspase-3 substrate DEVD-AFC (100 μmol/L). (C) Cells were treated with 10 g/L JCJ in the presence or absence of SB203580 10 μmol/L for 48 h. The cells were stained with propidium iodide (PI) and the portion of G_0/G_1 stages was assayed with PAS analyzer. *n*=3 experiments. Mean±SEM. ^bP<0.05 *vs* control.

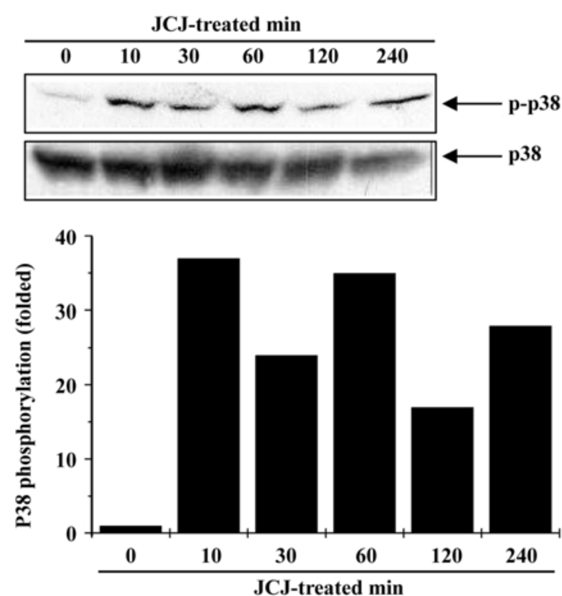

Fig 6. Je-Chun-Jun (JCJ) 10 g/L phosphorylased p38 MAPK in HeLa cells. The cell lysates were prepared and separated on SDS-PAGE and transferred onto a nitrocellulose membrane. Upper: Western blot analysis; Lower: The percentage of p38 expression.

human cervical carcinoma, HeLa cells. In addition, our data suggested that p38 MAPK (Mitogen-Activated Protein Kinase) may be involved in the JCJ-induced cell death. MAP kinases, especially p38 MAPK has been reported to play important roles in the pathways that regulate both cell cycle and apoptosis[17,18]. The observation that p38 MAP kinase facilitates apoptosis through regulation of cell cycle is consistent with these reports[18].

This study showed that JCJ inhibits cell proliferation by transiently suppressing the cell cycle progression genes, cyclin D and E, leading to the arrest at the G_1 phase followed by apoptosis. Cell cycle progression is regulated by a highly complex network of proteins that are fluctuated throughout the cell cycle. Examples of such proteins are cyclins D, E, and A[19]. In the presence of mitogens, the level of D-type cyclins, including cyclins $D_{1,2}$ and $_3$, gradually rises to the mid-to-late G_1 phase. Another early consequence of the DNA replication-, the entry into S phase is the increase of cyclin E levels resulting in the formation of active cyclin E/CDK2 complexes. At this stage, cells become irreversibly committed to entering the S phase and no longer depend on further growth factor stimulation[20-22]. Based on these reports, the transient regulation of cyclin D and E shows the regulatory effect on DNA replication and the entry into S phase in human cervical carcinoma cells, HeLa.

Caspases, a family of cysteine proteases-, that is homologous to the Caenorhabditis elegans death gene ced-3, are common and critical components of the cell death pathways. Among these caspases, caspase-3-like proteases mediate the initiation and/or execution stages of programmed cell death[23,24]. We detected the activity of caspases-3, 8 and 9 in the cells treated with JCJ. However, we could not detect caspase-1 activity

in response to JCJ using another fluorescent peptide substrate (data not shown). This suggests that JCJ induces apoptotic cell death through caspase-3, -8 and -9 activation. Furthermore, treatment with the caspase inhibitors-, such as the pan caspase inhibitor Z-VAD-FMK, or the caspase-3 inhibitor CHO-DEVD, abrogated the ability of JCJ-inducing the apoptosis of HeLa cells (data not shown).

In addition, we showed here that JCJ induces apoptosis by regulating the expression of Bcl-2 and Bax. Considering that the expression of Bax was significantly detected 60 hrs after JCJ treatment when apoptosis could be detected clearly, Bax expression may contribute to the JCJ-induced apoptosis, rather than to the cell cycle arrest in HeLa cells. The anti-apoptotic function of Bcl-2 is inhibited by the pro-apoptotic proteins, Bax. The JCJ-induced regulation of the expressions of Bax and Bcl-2 may contribute to the apoptotic actions of JCJ in HeLa cells. Consistent with these results, several lines of evidence have suggested that the ratio of Bcl-2 and Bax can be the regulator of apoptosis[25,26].

In summary, the inhibition of p38 MAPK significantly reduced the JCJ-induced apoptosis of Hela human cervical carcinoma cells. Furthermore, the treatment of SB203580 had a regulatory effect on the JCJ-induced cell cycle arrest. These findings are of potentially clinical importance. Our results suggest that when administered to cervical carcinoma patients, JCJ may induce the cell cycle arrest and subsequent apoptosis of cancer cells through the p38 MAPK pathway.

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