Effects of *Citrus Reticulata* on the Cell Detachment and Apoptosis in Human Gastric Cancer SNU-668 Cells

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The purpose of this study was to examine the effects of *Citrus Reticulata*(CR) on the Cell Detachment and Apoptosis in Human Gastric Cancer SNU-668 Cells. The effect of CR on apoptosis was investigated through MTT assay, DAPI staining, and TUNEL assay. We also performed RT-PCR for apoptotic genes including BCL-2, BAX, and caspase-3, the caspase-3 activity assay, and western blotting for pro-CASP-3. Then, to detect that adhesion of cell to ECM was reduced by CR, we investigated mRNA expression of CDH1 and PTK2 using RT-PCR, and their protein expressions using western blotting, and immunocytochemistry in SNU-668 cells. In this study, the results showed that treatment of CR induced time and dose-dependent cell death in SNU-668 cells. Downregulated mRNA expression of BCL-2, and upregulated *m*RNA expressions of BAX and CASP-3 indicated that the cell death was due to apoptosis. Protein expression of inactivated CASP-3, and caspase-3 activity assay also showed that apoptosis was induced in CR-treated cells.

Key words : *Citrus Reticulata*(CR), Cell Detachment, Apoptosis, BCL-2, BAX, caspase-3

Introduction

Apoptosis is known as programmed cell death which occurs in several pathological situations. It has been recognized that induction of apoptosis is a highly desirable mode as a chemopreventive strategy for cancer control[14]. Anoikis is that the detachment of cells to extracellular matrix (ECM) induces cell death, which induces changes in the expression and/or activity of proteins involved in the control of apoptosis[7]. Accordingly, induction of anoikis is also valuable as strategy for cancer control[11].

Gastric cancer is one of the most common cancers in the world[18,27]. Gastric cancer inherently resists to many current anticancer drugs, and the 5-year survival rate of gastric cancer is very low[22,24]. Recently, the development of new anticancer drug is key issue for cancer chemotherapy because of the reality that cancer cells which are resistant to chemotherapy will eventually cause the mortality. Many researchers tried to find the therapeutic key step of gastric cancer and to develop new anticancer compounds. Herb medicines as substitute cancer remedies have attracted a great deal of interest because of their low toxicity and costs.

Citrus Reticulatae Viride Pericarpium (CR, the immaturepeels of *Citrus reticulata* Blanco) has traditionally been used to promote the flow of liver Qi and to alleviate the pain in chest, breast, hypochondriac region, or hernia-like pain. It breaks up and reduces Qi-accumulations such as food-stagnation with symptoms as pain and distention. And it is used to dry dampness and to transform phlegm[4]. Related with this study, CR has been thought to possess anti-proliferating effects on several cancer cells[9,15,25]. However, the molecular mechanism of CR was little investigated in respect of apoptosis. Thus, in the present study, the cell attachment and apoptotic mechanism by CR was evaluated in SNU-668 cells.

Previously, it was investigated whether CR induced apoptotic cell death in human gastric cancer cell line, SNU-668. The effect of CR on apoptosis was investigated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 6-diamidineo-2-phenylindole(DAPI) staining, and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. We also performed reverse transcription-polymerase chain reaction (RT-PCR) for apoptotic genes including B-cell CLL/lymphoma 2 (BCL-2),

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BCL-2-associated X protein (BAX) and caspase-3 (CASP-3), the caspase-3 activity assay, and western blotting for pro-CASP-3. Then, to detect that adhesion of cell to ECM was reduced by CR, we investigated mRNA expression of E-cadherin (CDH1) and protein tyrosin kinase 2 (PTK2) using RT-PCR, and their protein expressions using western blotting, and immunocytochemistry in SNU-668 cells.

Materials and Methods

1. Preparation of Extract

Citrus reticulata Blanco (Rutaceae) was obtained from Semyung University Oriental Medicine Hospital. The sample was authenticated by college of Oriental medicine, Semyung University, where the voucher specimen was preserved. Methanol extracts of CR (yield: 19.7% of dry wt.) were obtained by 48 h maceration at room temperature. The methanol extract was filtered through a 0.45 μm filter (Osmonics, Minnetonka, MN), lyophilized, and kept at 4°C. The dried extract was re-solublized in saline before use.

2. Cell Culture

The SNU-668 cell line was purchased from Korean Cell Line Bank (KCLB, Seoul). SNU-668 cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY). Cells were maintained in a humidified incubator at 37℃ in an atmosphere of 5% CO_2 and 95% air, and the medium was changed every 2 days.

3. MTT assay

Cytotoxicity was measured by MTT assay (Sigma, St. Louis, MO) as previously described[13]. In order to detect the cytotoxicity of CR, SNU-668 cells were treated with CR at concentrations of 1, 10, 50 and 100 μg/ml at 6, 12, 24, 36 and 48 h. The control group was treated with the same amount of vehicle. SNU-668 cells with or without CR extract were washed and treated with MTT labeling reagent. After the cells were incubated in the dark for 4 h, absorbance at a test wavelength of 595 nm with a reference wavelength of 690 nm were measured using a microtiter plate reader (Bio-Tek, Winooski, VT). The optical density (O.D.) was calculated as the difference between the absorbance from reference wavelength and from test wavelength. Percent viability was calculated as (O.D. of drug-treated sample / O.D. of none treated sample) ×100.

4. DAPI staining

Apoptotic cells induced by CR were determined by DAPI staining (Sigma, St. Louis, MO) according to the manufacturer's protocol[28]. SNU-668 cells were cultured on four-chamber slides (Nalge Nunc International, Naporvile, IL). After 50 μg/ml CR treatment, the cells were fixed by incubation in 4% paraformaldehyde for 30 min, and were incubated for 30 min in the dark, including 1 μg/ml DAPI solution. Then, treated cells were observed through a fluorescence microscope (Zeiss, Oberköchen).

5. TUNEL assay

For in situ detection of apoptosis cells, TUNEL assay was performed using Roche in site cell death detection kit (Roche, Indianapolis, IN). After treatment with CR (50 μg/ml) for 24 h, the cells were fixed in acetic acid for 5 min at 20℃. The fixed cells were incubated with TUNEL-reaction mixture of enzyme solution (terminal deoxynucleotidyl transferase) and label solution (nucleotide mixture) for 1 h at 37℃, and were then washed at room temperature. A converted-POD (antibody conjugated with peroxidase) was added and the cells were incubated for 30 min. The DNA fragments were stained using 3, 3'-diaminobenzidine (Sigma, St. Louis, MO) as the substrate for the peroxidase. In addition, the percentage of TUNEL positive cells was calculated as (drug-treated sample / none treated sample) ×100.

6. RT-PCR analysis

Total RNA was isolated from SNU-668 cells with RNAzol^{TM}B (TEL-TEST, Friendswood, TX, USA) according to the manufacturer's instruction. RT-PCR was performed for apoptotic gene (*BCL-2*, *BAX*, and *CASP-3*), and for cell adhesion gene (*CDH1* and *PTK2*). The *CYCLOPHILIN* was used as an internal control (Table 1). The RT-PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Table 1. Sequences of the primers used in RT-PCR analysis

Primer name	Primer sequence (sense / anti-sense)	Fragment length (bp)	Annealing temperature (°C)
BCL-2	5'-tccgtgcctgactttagcaagctg-3'	333	50
	5'-ggaatcccaaccagagatctcaa-3'		
BAX	5'-aacatggagctgcagaggatgatt-3'	249	56
	5'-ctggtcttggatccagccagcccaacag-3'		
CASP-3	5'-cttggtagatcggccatctgaaac-3'	405	57
	5'-ggtcccgtacaggtgtgcttcgac-3'		
CDH1	5'-ccaactggaccattcagtacaac-3'	719	55
	5'-catgtcagccagcttcttgaacg-3'		
PTK2	5'-agccttatgacgaaatgctg-3'	263	58
	5'-ttggtttgtaccatgtgctg-3'		
CYCLOPHILIN	5'-accccaccgtgttcttcgac-3'	300	56
	5'-catttgccatggacaagatg-3'		

7. Capase-3 activity assay

Caspase-3 activity was measured using an enzyme linked immunosorbent assay (ELISA) kit from Sigma. SNU-668 cells were lysed with lysis buffer after treatment with CR (1, 10, 50 and 100 μg/ml) for 24 h. Caspase-3 was used as positive control for comparison. All mixtures were incubated overnight in humidified environment at 37℃. P-nitroaniline (p-NA) released from the substrate was measured at 405 nm.

8. Western blotting

Protein was isolated using Pro-prep® protein extraction solution (Intron Biotech, Seoul, Korea). Protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA). A sample of the protein (20 μg) was separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred ontoa nitrocellulose membrane (Schleicher & Schuell, Postfach, Germany). The membrane was incubated with primary anti-bodies (Santa Cruz, CA, USA) overnight at 4℃. Horseradish peroxidase-conjugated anti-mouse anti-body for pro-CASP-3, and CDH1, and anti-rabbit anti-body for PTK2 (Serotec, Oxford, UK) were used as secondary anti-bodies. Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

9. Imunocytochemistry

CDH1, and PTK2 proteins in cell were detected by using a standard avidin-biotin peroxidase complex technique (De Nigris et al., 2003). Cells were grown on glass coverslip for 24 h with 50 μg/ml of CR, and fixed with the 4% paraformaldehyde at room temperature for 10 min. After quenching the endogenous peroxidase activity, cells were incubated overnight with CDH1, and PTK2 anti-body (Santa Cruz Biotechnology, Diego, CA). Then, the cells were stained by an avidin biotinylated horseradish-peroxidase procedure using commercially available kit (ABC Elite,Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen.

10. FACS analysis

FACS analysis was performed as previously described[16]. Briefly, after treatment with 50 μg/ml CR, cells were collected, and resuspended in ice-cold phosphatebuffer saline (PBS) with 0.5% bovine-serum-albumin (BSA, Usb, Cleveland, OH, USA). To this suspension, CDH1, and PTK2 anti-bodies were added. After an incubation for 30 min at 4℃, cells were washed with PBS and added FITC-conjugated anti-rabbit anti-body and anti-mouse anti-body (Jackson Immunoresearch, West Grove, PA, USA), respectively. After incubation for 20 min at 4℃, unbound anti-bodies were washed off with PBS, and cells were analyzed usingFACScan (Becton Dickinson, San Jose, CA, USA). To negative control, primaryanti-bodies were not added, and only FITC-conjugated secondary anti-bodies were added.

11. Statistical analysis

Results were expressed as mean ± SEM. The data were analyzed by one-way ANOVA followed by Dunnett's post-hoc analysis using SPSS. Differences were considered significant at $P < 0.05$.

Results

1. Effect of CR on cytotoxicity of SNU-668 cells

Time and dosage effect of CR extract on the cytotoxicity of SNU-668 cells are shown in Fig. 1. CR extract treatment displayed significantly time and dose-dependent decrease of cell viability. Viability of cell treated with CR at concentrations of 1, 10, 50 and 100 g/ml for 24 h was 100 4%, 71 1%, 60 11% and 51 6% of control value, respectively (Fig. 1). This result indicated that CR extract induced cell death in SNU-668 cells with time and dose-dependent manner.

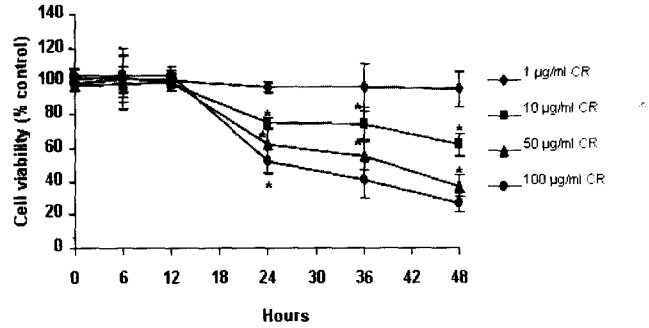

Fig. 1. Cytotoxicity of *Citrus reticulata*. The effect of *Citrus reticulata* on viability of human gastriccancer cells, SNU-668 according to time and concentrations was evaluated by MTT assay. Results are presented as mean ± SEM. Theexperiments were done triplicates. * represents P < 0.05 compared with the control.

2. Effect of CR on morphological changes

DAPI staining and TUNEL reaction were performed to detect whether the death of SNU-668 cells was caused by apoptosis. SNU-668 cells treated with CR (50 μg/ml) for 24 h revealed apoptotic cellular bodies through phase-contrast microscopy (Fig. 2B), whereas apoptotic cells were not observed in the untreated cells (Fig. 2A). DAPI staining showed the occurrence of nuclear condensation, DNA fragmentation and perinuclear apoptotic bodies upon CR treatment (Fig. 2D), while the untreated cells were not shown apoptotic features (Fig. 2C). As shown in Fig. 2F, analyzing

through TUNEL assay was ascertained that DNA strand breaks occur, and it indicated the induction of apoptosis by CR in SNU-668 cells. TUNEL-positive cells were stained with dark brown. In Fig. 2E, the untreated control was not revealed TUNEL-positive cells. The percentages of TUNEL positive cells at control and 50 g/ml CR were 11.8 ± 8.4% and 57.5 ± 6.5%, respectively. CR (50 g/ml) significantly increased TUNEL positive cells compared with untreated control. This result suggested that treatment of 50 g/ml CR induced apoptosis in SNU-668 cells.

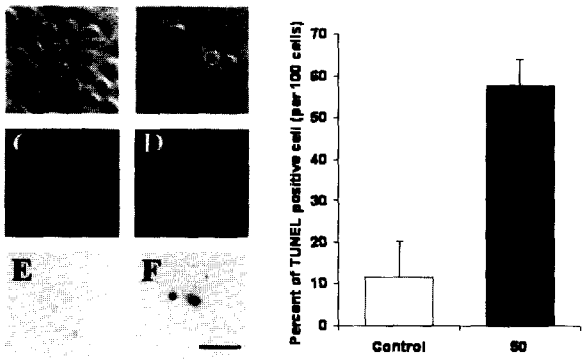

Fig. 2. Effect of *Citrus reticulata* on apoptotic bodies and TUNEL positive cells in SNU-668 cells. Cells were cultured without *Citrus reticulata* (A, C, and E) or with 50 μg/ml *Citrus reticulata* (B, D,and F). Morphology (top): phase-contrast microscopy showed cell shrinkage, irregularity in shape and cellular detachment in *Citrus reticulata*-treated cultures (B). DAPI staining (middle): SNU-668 cells stained with DAPI. Condensed nuclei were revealed by DAPI staining (D). TUNEL assay (bottom): SNU-668 cells stained using TUNEL method. Condensed and marginated chromatin showed to be stained dark brown (F). The number of TUNEL positive cells increased significantly in 50 g/ml of *Citrus reticulata* compared to control. * represents P < 0.05 compared to the control. The experiments were done triplicate. Scale bar, 100 μm.

3. Effect of CR on mRNA expression of apoptosis and adhesion related genes

The effect of CR was examined on mRNA expressions of anti-apoptotic genes (*BCL-2*), pro-apoptotic gene (*BAX* and *CASP-3*), and cell adhesion gene (*CDH1* and *PTK2*). In cells treated with CR, mRNA expressions of BAX and CASP-3 were increased compared with none-treated cells, while the expression of BCL-2 was decreased (Fig. 3B). In cell adhesion genes, mRNA expressions of CDH1 and PTK2 were diminished by treatment of CR (Fig. 3C). As a consequence, CR increased mRNA expressions of apoptotic genes, decreased those of cell adhesion genes.

4. Effect of CR on caspase-3 activity

Caspase-3 activity by various concentrations of CR was shown in Fig. 4. Caspase-3 activity was measured using DEVD peptide-nitroanilide (*p*NA). Caspase-3 activity was increased by CR with a dose-dependent manner (Fig. 4). This result suggested that CR induced apoptosis via caspase-3 activation in SNU-668 cells.

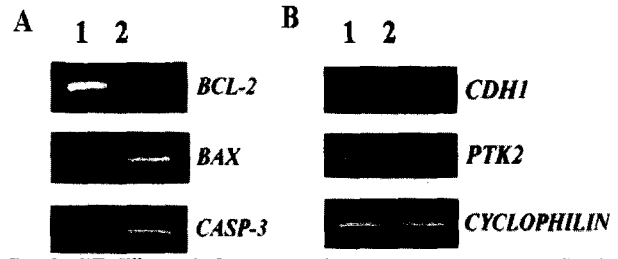

Fig. 3. RT Effect of *Citrus reticulata* on apoptosis and adhesion related genes. (A) Apoptotic genes (BCL-2, BAX and CASP-3) and (B) cell adhesion genes (CDH1 and PTK2) were reverse-transcribed and amplifiedby RT-PCR. CYCLOPHILIN was used as the internal control. Independent experiment was performed three times. 1, Control; 2, 50 μg/ml *Citrus reticulata*-treated cells.

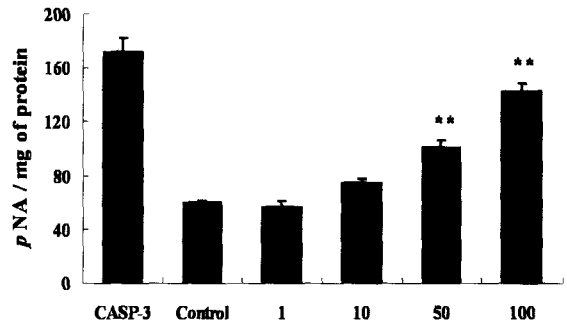

Fig. 4. Effect of *Citrus reticulata* on caspase-3 activity. The rate of DEVD-*p*NA cleavage was measured at 405 nm. *Citrus reticulata* was treated at 1, 10, 50 and 100 μg/ml. Caspae-3 was used as a positive control. The experiments were done triplicate. ** represents P < 0.01 compared with the control.

5. Effect of CR on apoptosis and adhesion related protein expression

Protein expressions of Pro-CASP-3, CDH1, and PTK2 were detected by western blot. The expressions of cell adhesion proteins (*CDH1* and *PTK2*) were decreased by treatment of CR (50 μg/ml)(Fig. 5A). CASP-3, apoptosis-related proteins in mitochondria, was detected as pro-CASP3, whose expression was attenuated by treatment of CR (Fig. 5B). These protein expressions indicated that anoikis was induced by CR in SNU-668 cells.

6. Effect of CR on expression of cell adhesion proteins by immunocytochemistry

In more study for protein expressions, CDH1 and PTK2 were detected in SNU-668 cells treated with CR using immunocytochemistry. CDH1 and PTK2 were observed on ECM stained with dark brown (Fig. 6). As shown in Fig. 6, CR decreased the expression of CDH1 and PTK2 compared with untreated control.

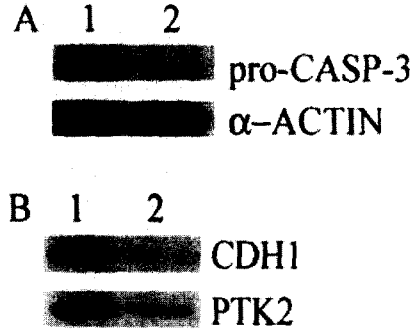

Fig. 5. Effect of *Citrus reticulata* on expressions of apoptosis and adhesion related proteins. The expressions of apoptotic protein, Pro-CASP-3 (A), and cell adhesion proteins, CDH1 and PTK2 (B), were assessed by western blotting analysis. α-ACTIN was used as a protein loading control. Western blots were representative of three experiments. 1, Control; 2, 50 μg/ml *Citrus reticulata*-treated cells.

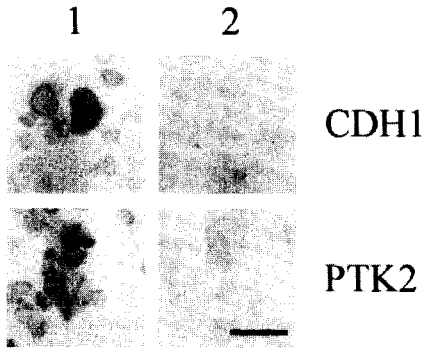

Fig. 6. Effect of *Citrus reticulata* on adhesion proteins by immunocytochemistry. The extracellular matrix for CDH1, and PTK2 anti-bodies was stained with dark brown. 1, Control; 2, 50 μg/ml *Citrus reticulata*-treated cells. Independent experiment was performed three times. Scale bar, 100 μm.

7. Effect of CR on expression of cell adhesion proteins by FACS analysis

CDH1 and PTK2 were detected in SNU-668 cells treated with CR using FACS analysis. The treatment of CR (50 μg/ml) shifted the fluorescence histogram of CDH1 to the left compared with untreated control (Fig. 7).

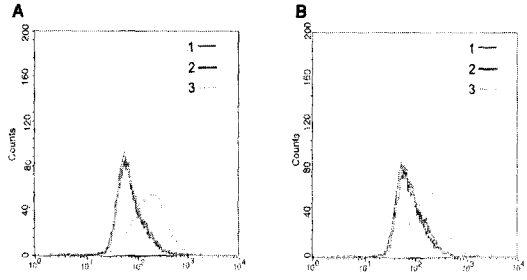

Fig. 7. Effect of *Citrus reticulata* on the population of adhesion proteins. CDH1 (A) and PTK2 (B) detected by FITC-conjugated secondary anti-body were analyzedusing FACScan. 1, Negative control; 2, 50 μg/ml *Citrus reticulata*-treated cells; 3, None-treated cells. Independent experiment was performed three times.

Discussion

In this study, the results showed that treatment of CR induced time and dose-dependent cell death in SNU-668 cells. Downregulated *m*RNA expression of BCL-2, and upregulated mRNA expressions of BAX and CASP-3 indicated that the cell death was due to apoptosis. Protein expression of inactivated CASP-3, and caspase-3 activity assay also showed that apoptosis was induced in CR-treated cells.

Anoikis was that the loss of cell-ECM adhesion leads to changes in the expression of proteins involved directly in the control of apoptosis[7,8]. According to prior studies, several components of mitochondrial apoptotic pathways have been shown to be regulated by anoikis, as well as, to participate in anoikis. Particularly, in the process of anoikis, BCL-2, an anti-apoptotic member of BCL-2 family, was downregulated[6,26], whereas BAX, a pro-apoptotic member, was upregulated; in the end, those were disrupted mitochondrial membrane potential[10,21]. In particular, CASP-3 was believed as the most common executor of apoptosis[12], and cell detachment was prerequisite for activation of caspase-3[19,20]. Considering these previous reports, it was hypothesized that CR-induced apoptosis would be involved with anoikis. Accordingly, we investigated changes of cell adhesion genes, and proteins by CR.

Cadherins, and integrins included in cell adhesion molecule superfamilies[17]. Cadherins are localizedin specialized cell-cell adhesion sites termed as adherence junctions. At these sites cadherins establish linkages with the actin containing cytoskeleton[2]. CDH1 among them mediates cell contact, and acts as an important suppressor of epithelial tumor cell invasiveness and metastasis[1]. Integrins, transmembrane glycoproteins, play a part as receptors for extracellular matrix protein, transduced a variety of survival signals[3]. PTK2, which becomes activated upon integrin ligand binding, is an important member of focal adhesion complexes that functions as a key mediator to transmit the signals from the extracellular matrix to cytoplasm[28]. This study showed that mRNAs and proteins of CDH1, and PTK2 were reduced in CR-treated cells. Taken together, These results suggests that CR induces the detachment of cell to extracellular matrix and subsequently apoptosis in SNU-668 cells.

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