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Hesperidin Induces Apoptosis in SNU-668, Human Gastric Cancer Cells

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Abstract

Hesperidin, known as a flavonoid constituent of citrus, has been known to reduce the proliferation of several cancer cells. We investigated whether hesperidin-induced cell death on SNU-668, human gastric cancer cells. The cytotoxicity of hesperidin on SNU-668 cells was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay at the concentration of 1, 10, 50, and 100 μM. Cell viability by hesperidin was 53.18 ± 2.85% of control value at 100 μM. The cell death by hesperidin showed apoptotic features, which were confirmed using a combination of 4, 6-diamidino-2-phenylindole (DAPI) staining and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. In the apoptosis-regulating genes, treatment of hesperidin decreased mRNA expression of B-cell CLL/lymphoma 2 (*BCL2*), whereas expression of BCL2-associated X protein (*BAX*) was increased. The mRNA expression and the activity of caspase3 (CASP3), a major apoptotic factor, was significantly increased by hesperidin treatment. These results suggest that hesperidin could induce apoptosis through CASP3 activation on SNU-668, human gastric cancer cells.

Keywords: Hesperidin, Apoptosis, Caspase3, Human gastric cancer cell

Gastric cancer remains the fourth most common cancer and the second most common cause of cancer-related death worldwide, killing more than 700,000 people every year. Despite the overall decline in incidence, it is predicted that due to the ageing of the world's population its incidence may in reality increase, especially in developing countries, to represent in some countries a major public health problem during the next decades[1].

Searching new compounds in foods or plant medicines showing anti-cancer effects is one of realistic and promising approaches to prevent human cancer incidence[2]. A variety of compounds have undergone clinical trials against gastric cancer based upon this strategy[3]. Flavonoids exist extensively in all parts of plants including fruits, vegetables, nuts, leaves, flowers, and bark, and their biological functions such as apoptosis-inducing activity, free radical scavenging activity, and antitumorigenic activities have been identified[4-7]. Hesperidin belongs to the class of flavonoids called flavanones, and is found largely in citrus. It was reported that hesperidin has several health-beneficial effects inhibiting skin tumorgenesis[8], and carcinogenesis of bladder[9]. Additionally, hesperidin suppressed cell proliferation in azoxymethane-induced rat colon carcinogenesis[10]. Hesperidin has been thought to possess anti-proliferating effects on several cancer cells including colon cancer were well known, however it is not clearly understood how hesperidin prevents cancer. In this study, the activity of hesperidin on apoptosis inducing effects was investigated in human gastric cancer cell line, SNU-668.

Hesperidin-induced Cytotoxicity and Morphological Change in SNU-668 Cells

The viabilities of cells exposed to hesperidin at concentrations of 11, 10, 50, and 100 μM were 87.58 ± 4.60, 84.89 ± 6.29, 73.45 ± 2.26, and 53.18 ± 2.85% of control value, respectively. Hesperidin showed cytotoxic effect as a concentration-dependent manner on SNU-668 cells (Figure 1A). Additionally, SNU-668 cells treated with hesperidin (100 μM) for 24 hr revealed apoptotic cellular bodies through phase-contrast microscopy (Figure 1B, upper panel). DAPI staining showed the occurrence of nuclear condensation, DNA fragmentation and perinuclear apoptotic bodies upon hesperidin treatment (Figure 1B, middle panel). As shown in Figure 1B (lower panel), analyzing through TUNEL assay was ascertained that DNA strand breaks occur, and it indicated the induction of apoptosis by hesperidin in SNU-668 cells.

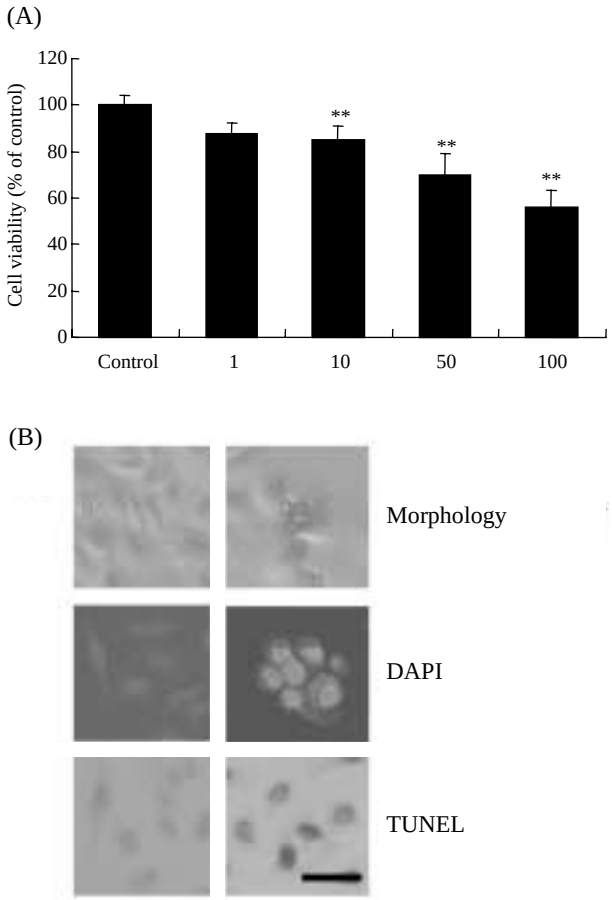

Figure 1. Characterization of hesperidin-induced cell death in SNU-668 human gastric cancer cells. (A) Cells were treated with various concentrations of hesperidin for 24 hr prior to the determination of cellular viability by MTT assay. Results are presented as the mean ± SEM (**$P < 0.01$ vs. control group). (B) Cells were cultured with or without hesperidin (100 μM). Morphology (top): phase-contrast microscopy shows cell shrinkage, irregularity in shape and cellular detachment in hesperidin-treated cells. DAPI staining (middle): condensed nuclei are observed. TUNEL assay (bottom): condensed and marginated chromatin is labeled dark brown. Scale bar, 100 μm. Three independent experiments were performed.

Effect of Hesperidin on Apoptotic Gene Expression and Caspase3 Activity

The effect of hesperidin on the expression of apoptotic gene (*BAX*, *BCL2* and *CASP3*) in SNU-668 cells was examined by RT-PCR. *BCL2* gene expression was decreased, whereas *BAX* and *CASP3* gene expression was increased after hesperidin treatment (Figure 2A). Caspase3 activity of SNU-668 cells treated with hesperidin was measured by level of DEVD-pNA. From the activity assay, the quantity of DEVD-pNA cleavage was elevated in the cells treated with hesperidin, compared with the untreated cells.

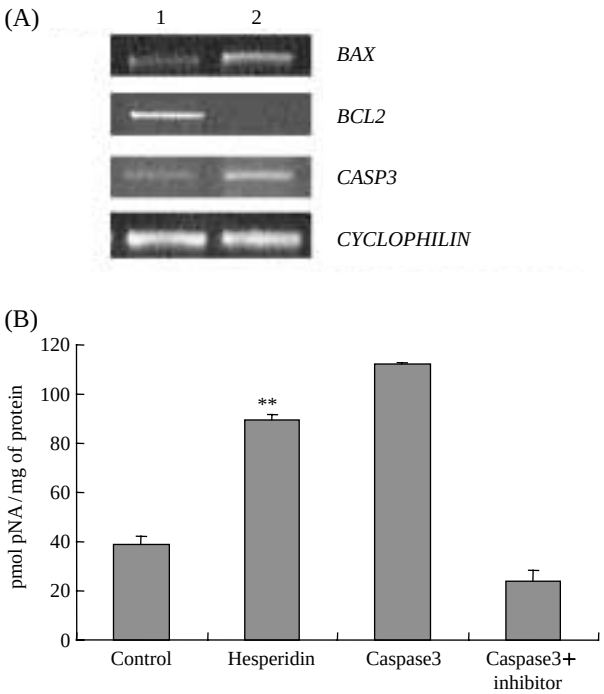

Figure 2. Activation of caspase3 (*CASP3*) by hesperidin treat-ment. (A) The mRNA expression of *BAX*, *BCL2*, and *CASP3* after hesperidin treatment (100 μM, 24 hr), were examined using RT-PCR analysis. As an internal control, *CYCLOPHILIN* mRNA was also reverse-transcribed and amplified. 1, Control; 2, Hesperidin. (B) Caspase3 (*CASP3*) activity was examined in hesperidin-treated SNU-668 cells. The rate of cleavage of the *CASP3* substrate, acetyl-Asp-Glu-Val-Asp p-Nitroanilide (Ac-DEVD-pNA), was measured at 405 nm. Hesperidin was used at 100 μM. *CASP3* was used as a positive control. Results are presented as the mean ± SEM (**$P < 0.01$ vs. control group). Three independent experiments were performed.

Discussion

Hesperidin treatment showed significant dose-dependent cytotoxicity in SNU-668 cells. We investigated that hesperidin induced cell death via apoptotic pathway in SNU-668 cells. Apoptosis is known as programmed cell death, which occurs in several pathological situations in multicultural organism, and it is a form of common mechanism for cell replacement, tissue remodeling, and removal of damaged cell[11]. Besides, apoptosis is characterized by morphological changes including progressive cell shrinkage with condensation and fragmentation of nuclear chromatin and membrane blebbing[12]. After SNU-668 cells were treated with hesperidin for 24 hr, the mor-

phologic observations revealed apoptotic changes including apoptotic morphology of cellular bodies, and the chromatin condensation with DAPI staining. In addition, it is known that DNA strand breaks occur during the process of apoptosis, and the nicks in DNA molecules can be quantitatively and qualitatively detected the apoptosis status of cells through TUNEL assay[13]. In present study, typical apoptotic characteristic TUNEL staining was observed in hesperidin-treated cells.

In a number of studies, it has been documented that the progress of apoptosis is regulated by the expression of several genes. One of these genes is a member of the *BCL2* family[14]. The *BCL2* family can be classified into two functionally distinct groups: anti-apoptotic genes and pro-apoptotic genes. *BCL2*, an anti-apoptotic gene, is known for regulating the apoptotic pathways and protecting cell death, while *BAX*, a pro-apoptotic gene of the family, is expressed abundantly and selectively during apoptosis, promoting cell death[15]. Our data showed that hesperidin regulated the expression of apoptosis-regulating genes. *BCL2* gene expression was decreased and *BAX* gene expression was increased after hesperidin treatment. We focused our attention on *CASP3*, one of the most commonly involved mediators in the execution of apoptosis in various cell types[16,17] in which it cleaves most caspase-related substrates that are induced in apoptosis regulation[18]. In our data, it is shown that the *CASP3* gene expression was increased after hesperidin-treatment and the cleaving activity of *CASP3* was increased as well.

In conclusion, the induction of apoptosis by hesperidin treatment in gastric cancer cells may be related with modulation of *BCL2* family resulting in the activation of *CASP3* expression. These results suggest the possible usage of hesperidin on gastric cancer patient as an alternative herb therapy.

Methods

Materials

Hesperidin, MTT, DAPI, DAB, and caspase3 assay kit were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). An in situ cell death detection TUNEL kit was obtained from Roche (Indianapolis, IN, USA).

Cell Culture

The SNU-668 cell line was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. Cultures were maintained in a humidified incubator at 37˚C in an atmosphere of 5% CO_2, 95% air, and media were changed every 2 days.

Cell Viability Assay

In order to determine the effects of hesperidin in SNU-668 cells, cell viability test was performed using the MTT assay kit as per the manufacturer's protocol. After cells were cultured in 96-well plates, study groups were treated with hesperidin at concentrations of 1, 10, 50, and 100 μM for 24 hr. The control group was added with saline of equal volume. The MTT labeling reagent was added to each well, and the plates were incubated for 4 hr. The cells were then incubated in solubilization solution for 12 hr, and the absorbance was measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 540 nm with a reference wavelength of 650 nm.

4, 6-Diamidino-2-Phenylindole (DAPI) Staining

In order to determine whether hesperidin induced apoptosis, DAPI staining was performed as described previously[19]. Cells treated with hesperidin (100 μM) for 24 hr were fixed in 4% paraformaldehyde and incubated in 1 μg/mL DAPI solution for 30 min in the dark. The cells were then examined on a fluorescence microscope (Zeiss, Oberköchen, Germany).

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) Assay

For in situ detection of apoptotic cells, TUNEL assay was performed using ApoTag® peroxidase in situ apoptosis detection kit. After a 24 hr exposure to 100 μM hesperidin, cells were fixed in acetic acid at −20 ˚C. Fixed cells were incubated with TUNEL reaction mixture (terminal deoxynucleotidyl transferase and nucleotide mixture) for 1 hr at 37˚C, followed by the addition of peroxidase-conjugated detection antibody. The DNA fragments were stained using DAB as the substrate for the peroxidase.

Reverse Transcription-polymerase Chain Reaction

Total RNA was isolated from SNU-668 cells using RNAzolB reagent (Tel-Test, Friendswood, TX, USA) according to the manufacturer's protocol. PCR was performed using the following primers: for *BAX*, 5′-AAC ATG GAG CTG CAG AGG ATG ATT-3′ and 5′-CTG GTC TTG GAT CCA GCC AGC CCA ACA

G-3′; for *BCL2*, 5′-CCT CTG TTT GAT TTC TCC TGG CTG T-3′ and 5′-TGG AAA GCG AAT CTA TGT TTA CAG G-3′; and for *CASP3*, 5′-ATC TCG GTC TGG TAC AGA TGT CGA T-3′ and 5′-TGA ATT TCG CCA AGA ATA ATACCA-3′. Primer for *CYCLOPHILIN*, 5′-ACC CCA CCG TGT TCT TCG AC-3′ and 5′-CAT TTG CCA TGG ACA AGA TG-3′, were used as an internal standard. RT-PCR products were resolved on 1.5% agarose gel and visualized by staining with ethidium bromide. The amplified fragment sizes were 476 bp (*BAX*), 249 bp (*BCL2*), 442 bp (*CASP3*), and 300 bp (*CYCLOPHILIN*).

Caspase3 Activity Assay

Caspase3 enzyme activity was measured using an assay kit according to the manufacturer's protocol. In brief, after treatment with hesperidin (100 μM, 24 hr), cells were lysed with chilled cell lysis buffer. Caspase3 substrate (Ac-DVED-pNA) was added to cell lysates and the mixtures were incubated overnight in a humidified environment at 37°C. Control lysates were preincubated with the caspase3 inhibitor, Ac-DEVD-CHO, to determine on-specific background substrate breakdown. The concentration of pNA released from the caspase3 substrate was measured as absorbance values at 405 nm and calculated from a calibration curve of pNA standards.

Statistical Analysis

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

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