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Genistein induces apoptosis in human hepatocellular carcinomas via interaction of endoplasmic reticulum stress and mitochondrial insult

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ABSTRACT

Hepatocellular carcinoma is a very common malignancy and is chemoresistant to currently available chemotherapeutic agents. Endoplasmic reticulum (ER) stress-induced apoptotic pathway is suggested to be less affected by the resistance mechanisms, becoming a potential target of chemotherapeutic strategy. The anticancer effects and expression of GADD153, a transcription factor induced by ER stress, were examined in hepatocellular carcinoma Hep3B cells. The correlation between these two parameters was constructed under flavonoid stimulation with a correlation coefficient (r) of 0.8. The data also showed that genistein (isoflavone) was the most effective one. Genistein induced the activation of several ER stress-relevant regulators, including m-calpain, GADD153, GRP78 and caspase-12. Furthermore, genistein-induced effect was inhibited in cells transfected with antisense GADD153 cDNA, indicating a functional role of GADD153. Notably, genistein induced the activation of caspase-2, whereas did not cause the DNA damage. It also triggered the production of ROS. The antioxidant trolox significantly reduced ROS accumulation, but did not modify genistein-induced apoptotic cell death. The long-term exposure (48 h) of cells to genistein caused Mcl-1 down-regulation and Bad cleavage; furthermore, cyclosporin A (an inhibitor of mitochondrial permeability transition pore) almost completely abolished genistein-induced loss of mitochondrial membrane potential, and induced a 30% reverse of apoptosis caused by long-term treatment (48 h) of genistein, suggesting the involvement of mitochondrial stress in the late phase of genistein-induced effect. Taken together, it is suggested that genistein induces the anticancer effect through a mechanism initiated by ER stress and facilitated by mitochondrial insult in Hep3B cells.

1. Introduction

Flavonoids, chemical structures of a common phenylchromanone skeleton with one or more hydroxyl substituents, are part of a family of naturally occurring compounds and represent one of the most widespread classes of component in fruits, vegetables and medical herbs [1]. The flavonoids exert a wide spectrum of pharmacological activities, with one of the most elucidated effects being the anticancer activities. The flavonoids are well known to inhibit cell growth and

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induce apoptosis in numerous types of cancer cells [2,3]. Increasing evidence suggests that the anticancer effects of flavonoids result from various mechanisms, including the regulation of cell cycle progression [3], inhibition of kinase and protease activities [4,5], suppression of the secretion of matrix metalloproteinases [6] and inhibition of the induction of activator protein-1 activity [7].

Human hepatocellular carcinoma (HCC) is a very common malignancy and is highly chemoresistant to currently available chemotherapeutic agents [8]. In a large screening test, we found that numerous flavonoids could prevail over the resistant capacity and display effective anti-proliferative activities in HCC. In this study, 16 flavonoids of several classes, including flavones, flavonols, flavanones, flavanols, and isoflavones, were used to examine their anticancer effects in HCC. Moreover, the anticancer mechanisms were investigated based on three predominant apoptosis pathways, such as mitochondria-mediated intrinsic pathway, death receptor-induced extrinsic pathway and the apoptotic signaling evoked by endoplasmic reticulum (ER) stress [9–11]. Caspases are intracellular cysteine protease responsible for and associated with these three apoptosis pathways. The intrinsic apoptotic pathway is a mitochondria-involved signaling cascade in which caspase-9 is the predominant initiator caspase. In the presence of ATP, the association of procaspase-9 with cytochrome c and the adaptor molecule apoptotic protease-activating factor 1 (Apaf-1) and oligomerization of this complex cause the activation of caspase-9 [9,10]. With contrast, the extrinsic apoptotic pathway is mediated by death receptors, such as the receptors for Fas ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and caspase-8 is a major initiator caspase in this pathway [9,10,12]. Furthermore, it has been suggested that there is cross-talk between the intrinsic and extrinsic apoptosis pathways [12,13]. Recent studies identify the ER as a third subcellular compartment implicated in apoptotic execution [11]. The ER, which is the site for folding and assembly of proteins, lipid biosynthesis, vesicular traffic, and cellular calcium storage, is sensitive to alterations in homeostasis. Several stimuli, such as the expression of misfolded proteins, glucose deprivation, misrepresented glycosylation, and perturbation in calcium homeostasis, can disrupt ER homeostasis and, subsequently, induce ER stress [11,14]. Notably, it has been suggested that ER stress and the associated activation of NF-κB, ATF-6 and mitogen-activated protein kinases (MAPKs) may contribute to hepatocarcinogenesis [15,16]. However, recently the trigger of ER stress as an anticancer strategy has been suggested in a variety of tumor types including HCC [17,18]. Furthermore, increasing evidence suggests that the ER stress precedes the mitochondria event in HCC responsive to apoptotic stimuli [18,19]. To date, there are few studies delineating flavonoid-mediated anticancer mechanisms in HCC based on the aforementioned three apoptosis pathways. In this study, several biological assays and techniques were used and the apoptosis pathways were examined to identify the anticancer mechanisms of flavonoids. To our knowledge, this is the first report that the intrinsic, extrinsic and ER stress-mediated apoptosis pathways are investigated in flavonoid-induced apoptosis in human hepatocellular carcinoma cells.

2. Materials and methods

2.1. Materials

FBS and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). Antibodies to Bid, caspase 7, 8 and 9 were from Cell Signaling (Beverly, MA, USA). Anti-Bcl-xL antibody was from Upstate. Antibodies to other Bcl-2 family member proteins and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Transduction Lab (Lexington, KY, USA). Antibodies to caspase 3 and GADD153 were from IMGENEX (San Diego, CA, USA) and Affinity BioReagents (Golden, CO, USA), respectively. Antibody to caspase-12 was from Abcam Limited (Cambridgeshire, UK). 2′,7′-Dichlorofluorescin diacetate (DCF-DA) was from Molecular Probes (Eugene, Oregon, USA). RPMI-1640 medium, phenylmethylsulphonylfluoride, leupeptin, aprotinin, glycerophosphate, NaF, sodium orthovanadate, sulforhodamine B (SRB), Hoechst 33342, etoposide (VP-16) and all of the flavonoids were obtained from Sigma Chemical (St. Louis, MO, USA). The purities of the flavonoids are also obtained: quercetin, ≥98%; (−)-epigallocatechin-3-gallate (EGCG), ≥95%; diosmin, ≥95%; genistin, ≥99%; genistein, ≥98%; epicatechin, ≥98%; catechin, ≥98%; hesperidin, 80%; hesperetin, ≥95%; naringin, ≥90%; naringenin, 95%; rutin, ≥95%; myricetin, ≥85%; gossypin, ≥90%; phloridzin, ≥99%; daidzein, ≥98%.

2.2. Cell cultures

Hep3B and HepG2 cells (American Type Culture Collection) were cultured in RPMI-1640 medium supplemented with 10% FBS (v/v) and penicillin (100 U/ml)/streptomycin (100 μg/ml). Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

2.3. SRB assay method

Cells were seeded in 96-well plates in medium with 5% FBS. After 24 h, cells were fixed with 10% trichloroacetic acid (TCA) to represent cell population at the time of flavonoid addition (T_0). After additional incubation of vehicle (0.1% DMSO) or flavonoid for 48 h, cells were fixed with 10% TCA and SRB at 0.4% (w/v) in 1% acetic acid was added to stain cells. Unbound SRB was washed out by 1% acetic acid and SRB bound cells were solubilized with 10 mM Trizma base. The absorbance was read at a wavelength of 515 nm. Using the following absorbance measurements, such as time zero (T_0), control growth (C), and cell growth in the presence of flavonoid (T_x), the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition was calculated as: $[(T_x - T_0)/(C - T_0)] \times 100$ for concentrations for which $T_x \geq T_0$. Growth inhibition of 50% (IC_{50}) is determined at the drug concentration which results in 50% reduction of total protein increase in control cells during the compound incubation.

2.4. Immunoprecipitation assay

After treatment with vehicle (0.1% DMSO) or compound, cells were washed twice with ice-cold PBS, lysed in 700 μl ml of lysis

buffer containing 20 mM Tris, pH 7.5, 1 mM $MgCl_2$, 125 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulphonylfluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 mM β-glycerophosphate, 50 mM NaF, and 100 μM sodium orthovanadate, and centrifuged. The supernatant was immunoprecipitated with respective antibody against the indicated protein in the presence of A/G-agarose beads overnight. The beads were washed four times with lysis buffer for immunoblotting.

2.5. Western blotting analysis

After the indicated exposure time of cells to vehicle (0.1% DMSO) or compound, cells were washed twice with ice-cold PBS and reaction was terminated by the addition of 100 μl ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100). For Western blot analysis, the amount of proteins (40 μg) were separated by electrophoresis in a 10 or 15% polyacrylamide gel and transferred to a nitrocellulose membrane. After an overnight incubation at 4 °C in PBS/5% nonfat milk, the membrane was washed with PBS/0.1% Tween 20 for 1 h and immuno-reacted with the indicated antibody for 2 h at room temperature. After four washings with PBS/0.1% Tween 20, the anti-mouse or anti-rabbit IgG (dilute 1:2000) was applied to the membranes for 1 h at room temperature. The membranes were washed with PBS/0.1% Tween 20 for 1 h and the detection of signal was performed with an enhanced chemiluminescence detection kit (Amersham).

2.6. In situ labeling of apoptotic cells

In situ detection of apoptotic cells was performed using Hoechst 33342 staining and the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) apoptosis detection methods. After a 48-h treatment with or without genistein (100 μM), the cells were washed twice with PBS, stained with Hoechst 33342 (1 μg/ml) for 15 min at 37 °C, and fixed for 15 min with 4% paraformaldehyde. They were examined under a confocal laser microscopic system (Leica TCS SP2). The TUNEL method identifies apoptotic cells using TdT to transfer biotin-dUTP to the free 3′-OH of cleaved DNA. The biotin-labeled cleavage sites were then visualized by reaction with fluorescein conjugated avidin (avidin-FITC). Cells were treated with or without genistein. Then, the cells were washed, fixed and stained for apoptotic detection according to the protocol provided by the suppliers (Promega, Madison, WI, USA). The photomicrographs were obtained with a fluorescence microscope (Nikon).

2.7. Immunofluorescence and confocal microscopic examination

After the treatment, the cells were washed twice with PBS and followed by methanol permeabilization for 5 min. The cells were stained with primary anti-GADD153 antibody at room temperature for 1 h. Then, the cells were washed and stained with secondary antibody of FITC-conjugated anti-mouse immunoglobulin. The immunofluorescence was analyzed by a confocal laser microscopic system (Leica TCS SP2).

2.8. FACScan flow cytometric assay

After the treatment of cells with vehicle (0.1% DMSO) or compound for the indicated time courses, the cells were harvested by trypsinization, fixed with 70% (v/v) alcohol at 4 °C for 30 min and washed with PBS. After centrifugation, cells were incubated in 0.1 ml of phosphate-citric acid buffer (0.2 M $NaHPO_4$, 0.1 M citric acid, pH 7.8) for 30 min at room temperature. Then, the cells were centrifuged and resuspended with 0.5 ml propidium iodide solution containing Triton X-100 (0.1%, v/v), RNase (100 μg/ml) and propidium iodide (80 μg/ml). DNA content was analyzed with the FACScan and CellQuest software (Becton Dickinson, Mountain View, CA).

2.9. Transfection of GADD153 antisense

The morpholino GADD153 antisense oligonucleotides (5′-TGCAGTTGGATCAGTCTGGAAAAGC-3′, Gene Tools, LLC, Corvallis, OR, USA) or control oligonucleotides (5′-CCTCTTACCTCAGTTACAATTTATA-3′) were used for the inhibition of GADD153 expression. Cells were cultured in 6-well plates with serum-containing medium. After a 24-h incubation, the medium was refreshed with complete medium (10% serum) containing Endo-Porter reagent (Gene Tools, LLC, Corvallis, OR, USA). The cells were incubated at 37 °C for 16 h. After the treatment, the SRB assays and cytofluorometric analysis were performed as described previously.

2.10. Measurement of reactive oxygen species (ROS)

Cells were incubated in the absence or presence of the indicated agents for 1, 6 or 24 h. Thirty minutes before the termination of incubation period, DCF-DA (final concentration of 10 μM) was added to the cells and incubated for the last 30 min at 37 °C. Then, the cells were harvested for the detection of ROS accumulation using FACScan flow cytometric analysis.

2.11. Comet assay for monitor the integrity of chromosomal DNA

Compound-treated cells (2×10^5, 30 min) were pelleted and resuspended in ice-cold PBS. The resuspended cells were mixed with 1.5% low melting point agarose. This mixture was loaded onto a fully frosted slide that had been pre-coated with 0.7% agarose and a coverslip was then applied to the slide. The slides were submerged in pre-chilled lysis solution (1% Triton X-100, 2.5 M NaCl, and 10 mM EDTA, pH 10.5) for 1 h at 4 °C. After soaking with pre-chilled unwinding and electrophoresis buffer (0.3 N NaOH and 1 mM EDTA) for 20 min, the slides were subjected to electrophoresis for 15 min at 0.5 V/cm (20 mA). After electrophoresis, slides were stained with 1× Sybr Gold (Molecular Probes) and nuclei images were visualized and captured at 400× magnifications with an Axioplan 2 fluorescence microscope (Zeiss) equipped with a CCD camera (Optronics). Over hundreds of cells were scored to calculate the overall percentage of comet tail-positive cells.

2.12. *Measurement of the change of mitochondrial membrane potential ($\Delta\psi_m$)*

Cells were treated with or without the indicated agent. Thirty minutes before the termination of incubation, a rhodamine 123 solution (final concentration of 5 μM) was added to the cells and incubated for the last 30 min at 37 °C. The cells were finally harvested and the accumulation of rhodamine 123 was determined using FACScan flow cytometric analysis.

2.13. *Data analysis*

Data are presented as the mean ± S.E.M. for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by Bonferroni t-test and P-values less than 0.05 were considered significant.

3. Results

3.1. *Identification of anti-proliferative effect of flavonoids and the correlation with ER stress*

The anti-proliferative effect of 16 flavonoids of several classes, including flavones, flavonols, flavanones, flavanols, and isoflavones, was examined by SRB assays. The data showed that quercetin (flavonol), (−)-epigallocatechin-3-gallate (EGCG, flavanol) and genistein (isoflavone) were more effective than the other flavonoids to inhibit the proliferation of Hep3B cells with IC_{50} values of 49.2, 82.2 and 21.4 μM, respectively (Fig. 1A). We next examined the expression of GADD153, a transcription factor induced by ER stress [11,18], in cells responsive to flavonoids. As demonstrated in Fig. 1A, quercetin, EGCG and genistein induced a dramatic increase of GADD153 level. The correlation between anti-proliferative effect and GADD153

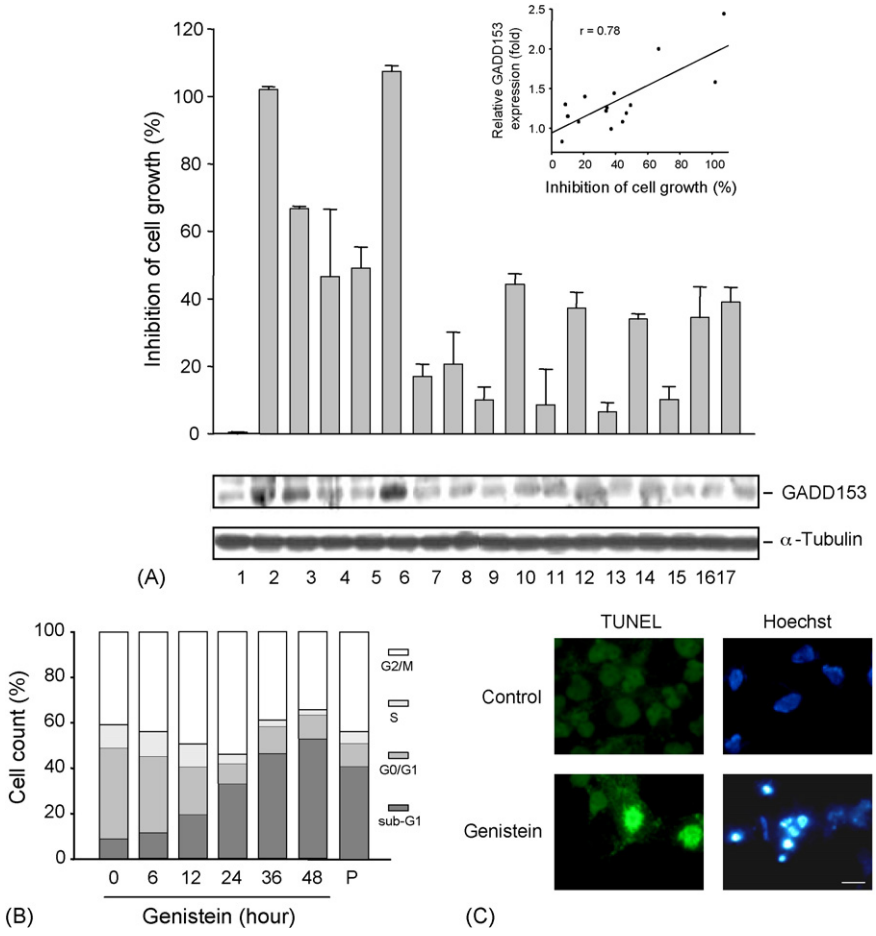

Fig. 1 – Identification of flavonoid-induced anticancer effect in Hep3B cells. (A) The flavonoid (100 μM) was added to cells for 48 h. Then, cells were fixed and stained with SRB. After a series of washing, bound SRB was subsequently solubilized and the absorbance was read at a wavelength of 515 nm. Data are expressed as mean ± S.E.M. of four determinations (each in triplicate). The expression of GADD153 was detected by Western blot. The correlation between inhibition of cell growth and GADD153 protein level was also constructed. Lane 1, control; 2, quercetin; 3, EGCG; 4, diosmin; 5, genistin; 6, genistein; 7, epicatechin; 8, catechin; 9, hesperidin; 10, hesperetin; 11, naringin; 12, naringenin; 13, rutin; 14, myricetin; 15, gossypin; 16, phloridzin; 17, daidzein. (B) Cells were treated with genistein (100 μM) for the indicated time course. Then, cells were fixed and stained with PI to analyze DNA content by FACScan flow cytometry. "P" represents the positive control of etoposide (3 μM). (C) Cells were treated with or without genistein (100 μM) for 48 h. The apoptosis was detected by TUNEL- and Hoechst 33342 reaction technique as described in Section 2. Scale bar, 10 μm.

level was constructed with a correlation coefficient (*r*) of 0.78. The similar results were obtained in HepG2 cells (data not shown). The data provided a rationale for further investigation of ER stress in flavonoid-induced anticancer effect in HCC. Since genistein displayed the most effective anti-proliferative effect, the following study was done to elucidate genistein-induced anticancer mechanisms in Hep3B cells, a representative HCC which contains copies of hepatitis B virus (HBV) genomes in their chromosomes and actively secrets HBsAg [20,21].

Genistein induced a time-dependent arrest of G2/M phase and a subsequent increase of hypodiploid phase (apoptosis) of the cell cycle (Fig. 1B); the apoptosis was also identified by TUNEL-reaction technique (Fig. 1C). Furthermore, genistein induced an increase of Ca^{2+} mobilization (an increase of 169 nM by Fluo-3 AM loading assays) and an early cleavage of *m*-calpain, followed by an increase of protein expression of GADD153 and GRP78 and caspase-12 activation (Fig. 2). Furthermore, the immunofluorescence and confocal microscopic examination showed that genistein induced a time-dependent increase of nuclear translocation of GADD153 (Fig. 3). The disturbance of Ca^{2+} homeostasis always involves in ER stress-induced effect [4]. Furthermore, there are several lines of evidence that caspase-12, GRP78 and GADD153 are specific markers of ER stress [11,14,18]. The data in this study suggest that genistein may induce ER stress in Hep3B cells.

3.2. *Effect of genistein on intrinsic and extrinsic apoptosis pathway*

Caspases are cysteine proteases that are activated through several apoptotic pathways. The activation of different initiator and executor caspases is widely exploited to characterize the involvement of distinct apoptotic signaling pathways. The Western blot showed that both caspase-8 and -9 were present as un-cleaved procaspase types although the procaspase-9 was moderately increased by genistein (Fig. 4A and B). Furthermore, in our unshown data, the exposure of genistein (100 μM) to cells for 24 h did not alter the protein levels of several death receptors (Fas, DR4 and DR5) and their ligands (FasL and Apo-2L/TRAIL). These data indicated that both intrinsic and extrinsic apoptosis pathways did not contribute to genistein-induced effect in Hep3B cells.

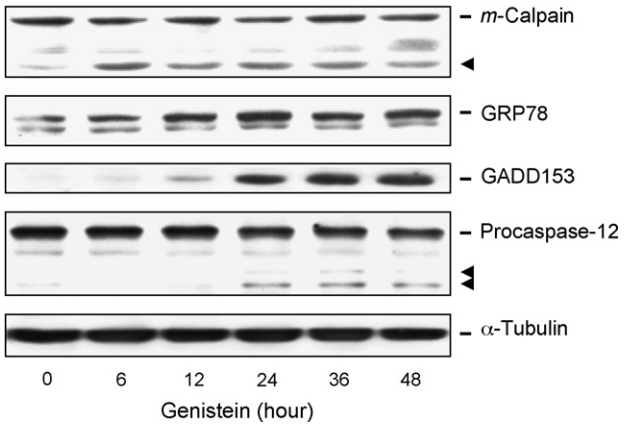

Fig. 2 – Effect of genistein on ER stress-related protein expression. Cells were treated with or without genistein (100 μM) for the indicated time course. Then, the cells were harvested and lysed for the detection of protein expressions by Western blot analysis. For Western blotting, the amount of proteins (40 μg) was separated by electrophoresis in a 10 or 15% polyacrylamide gel, transferred to a nitrocellulose membrane and immunoreacted with the indicated antibody. Arrowhead, the cleaved form of the indicated protein.

The expression of several effector caspases was also detected. The data demonstrated that genistein did not affect the protein levels of procaspase-6, whereas induced the activation of caspase-3 and -7, and the cleavage of their substrate PARP after a 24-h treatment (Fig. 4C). Since genistein induced an up-regulation of procaspase-9, the association of procaspase-9 and cytochrome *c* with Apaf-1 was examined by immuno-precipitation assays. Interestingly, the association of cytochrome *c* in the apoptosome was significantly increased by genistein, although the absence of cleaved caspase-9 (35 kDa) was confirmed once again (Fig. 4D). To explain the inability of caspase-9 activation, the expression of inhibitors of apoptosis proteins (IAPs) including XIAP, CIAP-1 and CIAP-2 was determined. The data showed that the protein level of XIAP was dramatically increased by genistein, although it was not the predominant IAP family member in Hep3B cells (Fig. 4E). In an analysis of the composition and assembly kinetics of Apaf-1 apoptosomes, Hill and the colleagues suggest that XIAP is able to rapidly recruit to the apoptosome [22]. By using immuno-precipitation analysis, our data showed that genistein induced a significant recruitment of XIAP to the apoptosome (Fig. 4F), suggesting that Apaf-1-mediated processing of procaspase-9 was blocked by XIAP.

3.3. *Effect of genistein on DNA damage reaction*

In this study, the Western blot analysis showed that genistein induced a time-dependent activation of caspase-2 (Fig. 5A). There are several lines of evidence that the caspase-2 can be activated in cells responsive to DNA damage. Moreover, it has been suggested that genistein can inhibit topoisomerase II and induce DNA damage by stabilizing topoisomerase II-DNA cleavage complex [23]. However, the comet assay showed that the exposure (1 h) to etoposide, a topoisomerase II inhibitor, but not genistein induced significant DNA strand breaks (Fig. 5B). The data suggest that genistein has little direct effect on topoisomerase II activity and DNA damage although it induces apoptotic cell death in a long-term treatment. The data also indicate that genistein-induced caspase-2 activation does not result from the inhibition of topoisomerase II activity in Hep3B cells.

3.4. *Effect of genistein on mitochondria-related regulators*

Changes in the expression of Bcl-2 family of proteins, including the anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1) and the pro-apoptotic proteins (Bax, Bak, and Bad) after the exposure of cells to genistein were examined by immunoblotting. The levels of Bcl-2, Bcl-xL, Bax and Bak were not altered, whereas the expression of Bad was up-regulated by genistein in a time-dependent manner. Furthermore, after a long-term

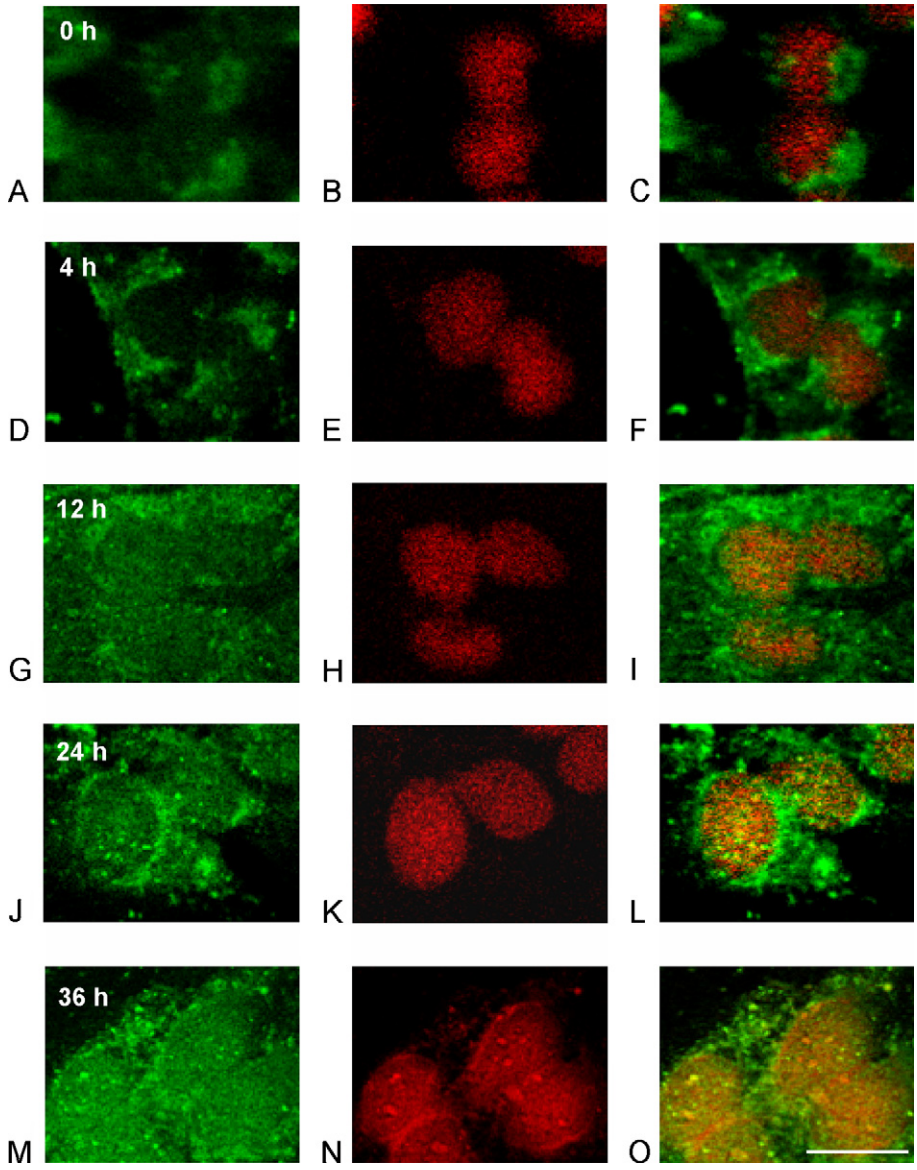

Fig. 3 – Effect of genistein on GADD153 nuclear translocation. Cells were incubated in the absence (A–C) or presence of genistein (100 μM) for 4 (D–F), 12 (G–I), 24 (J–L) or 36 h (M–O). The cells were fixed and stained with primary antibody to GADD153. Then, FITC-labeled secondary antibodies were used (green fluorescence) and the protein was detected by a confocal laser microscopic system. The nuclei were apparent by PI staining (red fluorescence). Areas of colocalization between GADD153 expression and nuclei in the merged panels (C, F, I, L and O) are yellow. Scale bar, 20 μm.

(48 h) treatment of genistein, the Bad was cleaved into a 15 kDa fragment and the Mcl-1 expression was down-regulated (Fig. 6A). The data indicated that an interaction with mitochondria might partially explain the apoptotic cell death during a long-term exposure to genistein. Furthermore, the ROS production and change of $\Delta\psi_m$ were also examined to monitor the mitochondrial function. The data showed that genistein induced a significant increase of ROS production and loss of $\Delta\psi_m$ (Fig. 6B). Notably, although trolox (a water-soluble Vitamin E analog) and cyclosporin A (a mitochondrial permeability transition pore inhibitor) significantly inhibited ROS production and loss of $\Delta\psi_m$ induced by genistein (Fig. 6B), only cyclosporin A displayed a partial inhibition (30%) of genistein-induced increase of sub-G1 phase (apoptosis) of the cell cycle (Fig. 6C).

3.5. *Identification of functional role of GADD153*

The aforementioned data showed that genistein significantly induced the ER stress, leading to the up-regulation and nuclear translocation of CHOP/GADD153. To clearly identify the functional role of this transcription factor, the control or a plasmid containing antisense GADD153 cDNA was transiently transfected into the cells and several cellular functions were assessed. As demonstrated in Table 1, the antisense-treated cells caused a 44% reduction of genistein (100 μM)-induced

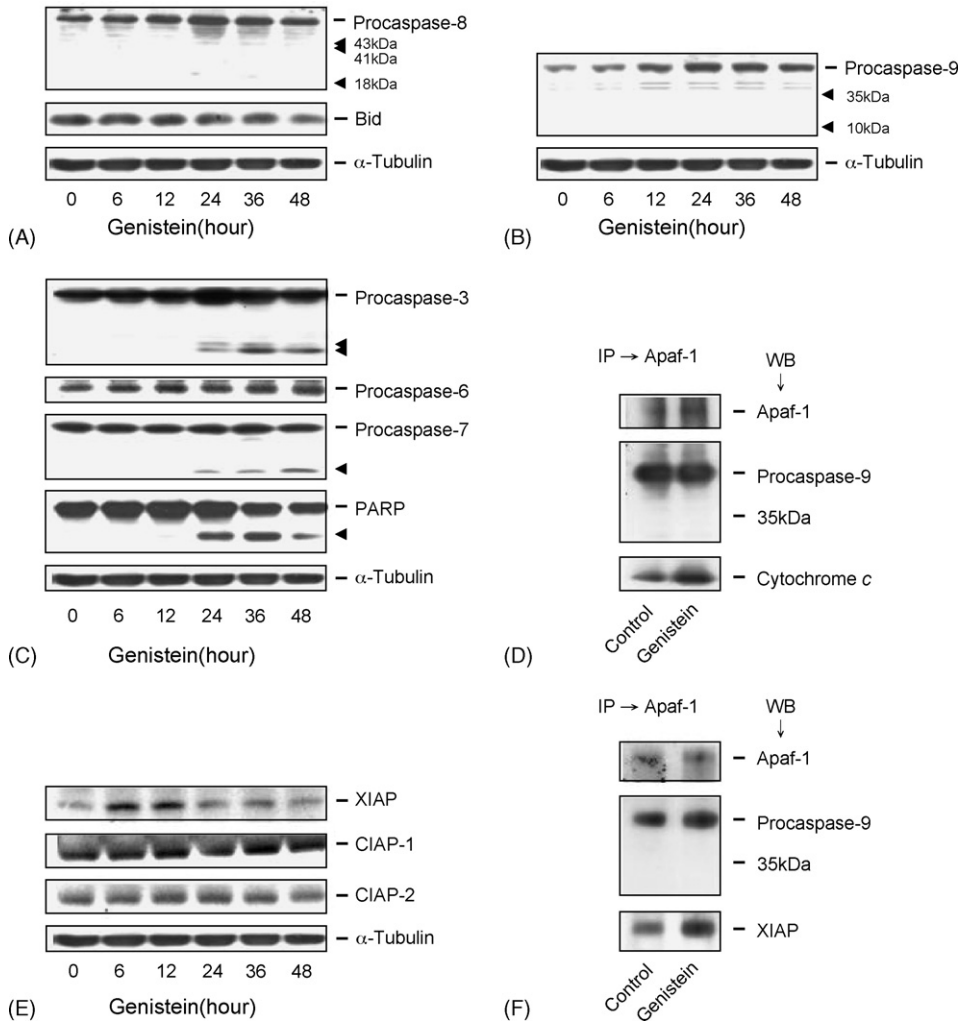

Fig. 4 – Effect of genistein on the expression of caspases and IAPs, and Apaf-1-associated proteins. Hep3B cells were treated with genistein (100 μM) for the indicated time course (A–C and E) or for 24 h (D and F). Then, the cells were harvested and lysed for the detection of protein expression by Western blot analysis (A–C and E). For Western blotting, the amount of proteins (40 μg) was separated by electrophoresis in a 10 or 15% polyacrylamide gel, transferred to a nitrocellulose membrane and immuno-reacted with the indicated antibody. The arrowhead indicates the cleavaged form of caspase. To detect the proteins associated with Apaf-1 (D and F), the immunoprecipitation analysis was performed as described in the Materials and methods section. The Western blot analysis of immunoprecipitated protein complexes with the indicated antibodies shows the expression of Apaf-1, procaspase-9, cytochrome c and XIAP. IP: immunoprecipitation, WB: Western blotting.

GADD153 expression; furthermore, the inhibition of cell growth and apoptotic cell death caused by genistein were also blocked in a moderate level (Table 1). The data indicate that genistein-induced effect is, at least partly, contributed to the induction of GADD153 expression.

4. Discussion

Most currently used anticancer agents are effective against proliferating cells. Apoptotic signaling mechanisms induced by numerous such agents are impaired in tumor cells, causing therapy resistance. The apoptotic pathway induced by ER stress may be less affected by chemotherapy-induced resistance mechanisms, suggesting that induction of ER stress response of tumor cells may be a potential therapeutic strategy [24,25]. Two signaling mechanisms are involved in ER stress-mediated apoptosis: the accumulation of unfolded or misfolded protein and the Ca^{2+} signaling The latter always explains cytotoxic agent-induced apoptosis in tumor cells [4]. In this study, our data revealed that genistein induced Ca^{2+} mobilization (an increase of 169 nM by Fluo-3 AM loading assays) and activation of *m*-calpain (Ca^{2+}-dependent intracellular cysteine proteases) indicating that alterations in Ca^{2+} homeostasis are implicated in genistein-induced effect. We further investigated specific markers of ER stress, such as caspase-12, GRP78 and GADD153. Caspase-12 that locates at the cytoplasmic side of ER is proteolytically activated following ER stress and *m*-calpain

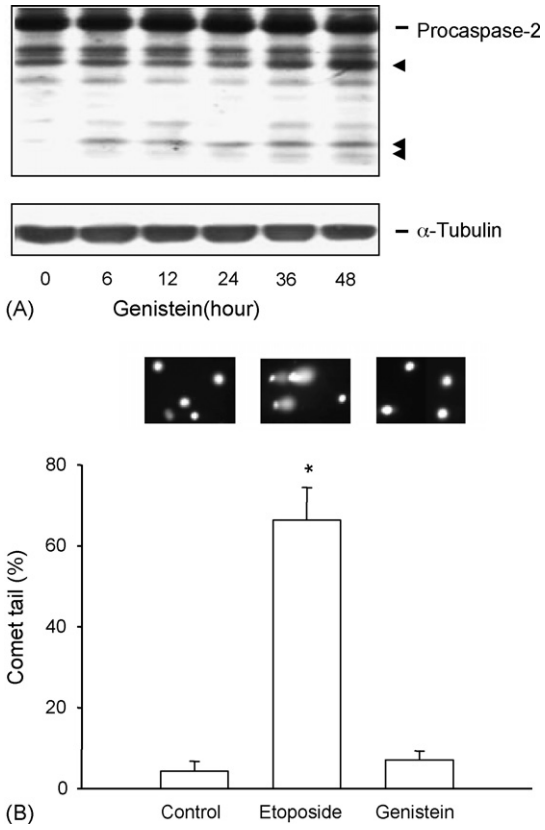

Fig. 5 – Effect of genistein on the expression of caspase-2 and DNA damage. (A) Hep3B cells were treated with genistein (100 μM) for the indicated time course. Then, the cells were harvested and lysed for the detection of protein expression by Western blot analysis. For Western blotting, the amount of proteins (40 μg) was separated by electrophoresis in a 15% polyacrylamide gel, transferred to a nitrocellulose membrane and immuno-reacted with capase-2 antibody. The arrowhead indicates the cleavaged form of caspase. (B) The comet assay was employed to examine the integrity of chromosome DNA upon treatment of genistein (100 μM for 1 h). Etoposide (50 μM) was included as positive controls. One hundred of cells were scored to calculate the overall percentage of comet tail-positive cells. Data are expressed as mean ± S.E.M. of three independent experiments.
*** $P < 0.01$ compared with control.**

Table 1 – Determination of the functional role of GADD153 in genistein-induced effect

Events	Nonsense	Antisense
GADD153 protein level (%)	100	56.0
Inhibition of cell growth (%)	90.5 ± 1.4	28.6 ± 4.4**
Increase of hypodiploid cells (apoptosis) (%)	14.8 ± 1.8	4.0 ± 2.8*

Hep3B cells were exposed to nonsense or GADD153 antisense oligonucleotides for 16 h, and the cells were treated with or without genistein (100 μM) for 48 h. Then, the cells were harvested for the detection of GADD153 expression by Western blot analysis, cell cycle progression by FASCcan flow cytometric analysis of PI staining or cell growth inhibition by SRB assays. Data are expressed as mean ± S.E.M. of three independent experiments.
* $P < 0.05$ compared with nonsense condition.
** $P < 0.001$ compared with nonsense condition.

activation [26]. GRP78 is a central regulator of ER function in the coordination of ER protein processing with mRNA translation during ER stress [27]. GADD153 is expressed at very low levels in growing cells. However, it is highly expressed in response to several cellular stresses. The agents, such as thapsigargin which depletes ER calcium stores and tunicamycin which inhibits protein glycosylation, are inducers of GADD153 [28], suggesting that induction of GADD153 is highly responsive to ER stress. The data showed that genistein induced the up-regulation and/or activation of these marker proteins, indicating the occurrence of ER stress in Hep3B cells. Furthermore, an increase of nuclear translocation of GADD153 was induced by genistein, indicating a functional role of this transcription factor. There is increasing evidence that GADD153 contributes to apoptosis of cancer cells induced by numerous chemotherapeutic drugs, such as etoposide and cisplatin [29,30]. In this study, the data showed that the anti-proliferative activity and apoptosis induced by genistein were reduced in cells transfected with antisense GADD153 cDNA, suggesting a crucial role of GADD153. Notably, it has been suggested that GADD153 gene is highly inducible by genotoxic agents [31]. Furthermore, genistein has been examined to induce a DNA damage pathway that activates p53 and Chk2 [32]. Accordingly, the comet assay was used in this study to measure DNA strand breakage in individual cells and to examine the relation between DNA damage and genistein. However, there was limited comet signal being detected in cells treated with genistein, revealing that genistein did not cause DNA damage in Hep3B cells.

The initiation of apoptosis signaling following treatment with numerous cytotoxic agents leads to activation of the mitochondrial (intrinsic) apoptosis pathway. Furthermore, apoptosis mediated by death receptor (extrinsic) pathways contributes to direct cytotoxicity and/or augmentation of sensitivity of tumor cells towards cytotoxic insult. Both pathways converge into a signaling cascade of caspase activation. In this study, the initiator caspase-8 and caspase-9 were not activated, indicating that neither extrinsic pathways nor intrinsic pathways contributed to genistein-induced apoptotic signaling cascades. Interestingly, genistein induced a moderate up-regulation of procaspase-9 and an association of cytochrome *c* in Apaf-1, while did not cause the cleavage of procaspase-9. To investigate this phenomenon, the expression of IAPs was detected since IAPs could inhibit apoptosis through direct inhibition of caspases and, in particular, XIAP could recruit to and inhibit Apaf-1-mediated activation of procaspase-9 [33]. The Western blotting and immuno-precipitation analysis showed that genistein induced a significant increase of XIAP level and its recruitment to Apaf-1, suggesting that XIAP might play a central role on the inhibition of procaspase-9 processing.

Caspase-2, a unique caspase with characteristics of both initiator and effector caspases, is one of the best conserved caspases across species. The activation of caspase-2 appears to be necessary for apoptotic onset triggered by several stimuli, including DNA damage and ER stress [34,35]. Our data support the assumption that genistein induces the activation of caspase-2 relevant to ER stress other than DNA damage.

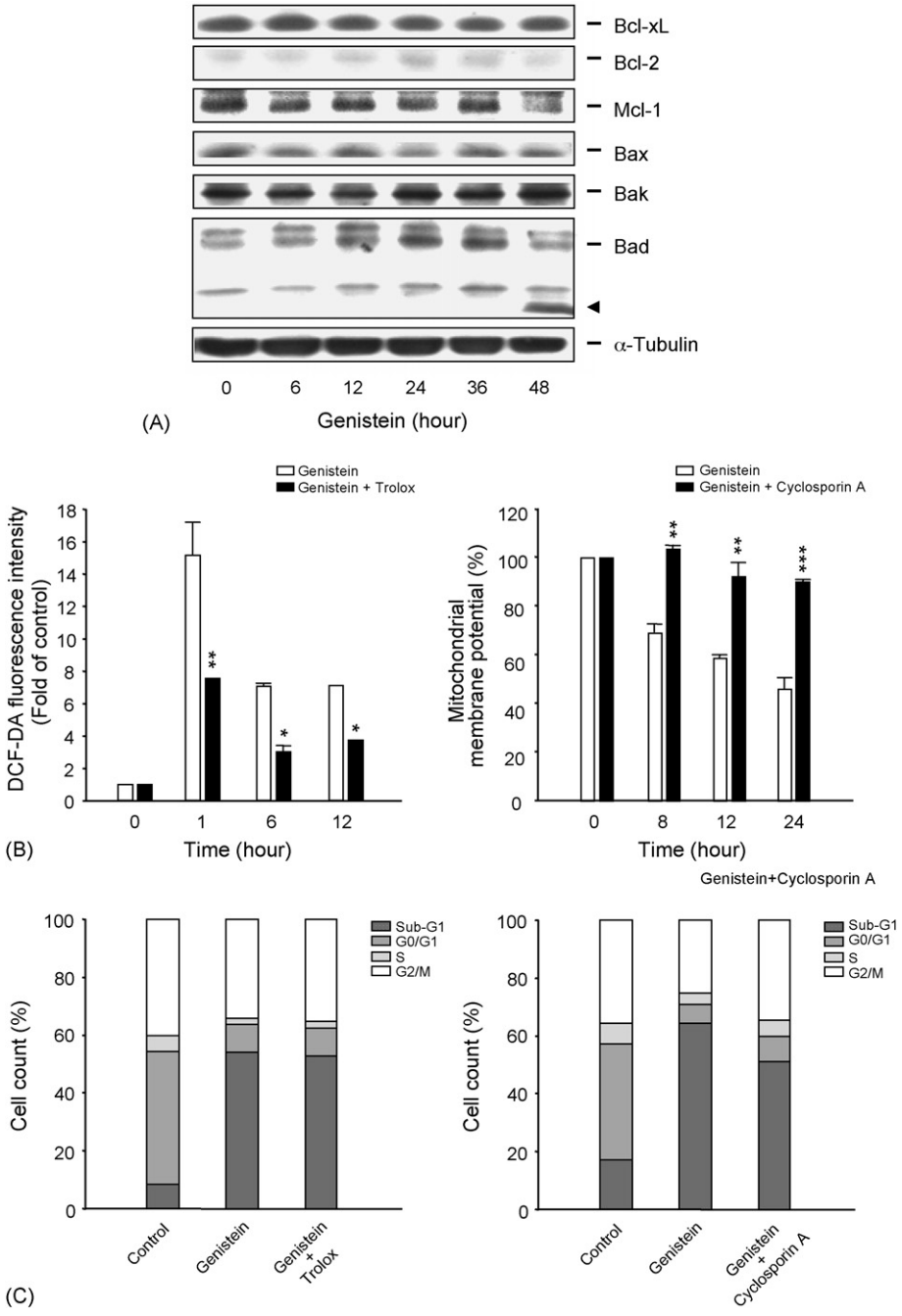

Fig. 6 – Effect of genistein on expressions of Bcl-2 family member proteins, ROS production and $\Delta\psi_m$. (A) Hep3B cells were treated with genistein (100 μM) for the indicated time course. Then, the cells were harvested and lysed for the detection of protein expression by Western blot analysis. The arrowhead indicates the cleavaged form of Bad. (B) Cells were incubated in the indicated agent (genistein, 100 μM; trolox, 300 μM; cyclosporin A, 5 μM) for several time courses. Thirty minutes before the termination of the incubation period, cells were incubated with DCF-DA or rhodamine 123 for the last 30 min at 37 °C. The cells were washed and harvested for the detection of ROS accumulation and $\Delta\psi_m$, respectively, using FACScan flow cytometric analysis. Data are expressed as mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with genistein alone. (C) Cells were treated with the indicated agent for 48 h. Then, the cells were fixed and stained with PI to analyze DNA content by FACScan flow cytometry. Data are representative of three independent experiments.

Recently, the compensatory caspase activation has been investigated during several apoptotic signaling cascades. It has been suggested that the elimination of caspase-9 (e.g., caspase-$9^{-/-}$) in hepatocyte apoptosis caused by the Fas agonistic antibody Jo2 induces a compensatory activation of caspase-2 [36]. The compensatory effect may also explain the deficiency of caspase-9 activity while induction of caspase-2 activation in genistein-mediated apoptosis in Hep3B cells.

ROS is well recognized to act as secondary messengers in diverse intracellular signaling cascades. Increasing evidence suggests that ROS can also induce apoptotic cell death and can act as a target for anticancer strategy. In this study, the data showed that genistein induced a significant increase of ROS production and loss of $\Delta\psi_m$. However, trolox (a water-soluble Vitamin E analog) did not modify genistein-induced apoptotic effect. Similar results of ROS-independent anticancer effects are also apparent in other reports that 2-methoxyestradiol and paclitaxel induce apoptosis in hepatoma cells through caspase-involved but ROS-independent pathways, although an increase of ROS production occurs [37]. However, two reasons should be addressed to keep the possibility of oxidative stress in this study. The first, although trolox significantly decreased the ROS production, it still remained 50% of ROS that might have a role in apoptosis. The second, There is evidence that combined ROS production and Ca^{2+} release is required for ER stress-induced apoptosis [38]. In this study, the single treatment to diminish ROS production (by trolox) could not work because of the lack of calcium blockade. We ever tried to do combined treatment by trolox and BAPTA-AM (a chelator of intracellular Ca^{2+}). However, the combined treatment, by itself, induced cytotoxic effect in Hep3B cells. Interestingly, a long-term (48 h) exposure to genistein caused a significant down-regulation of Mcl-1 protein level; furthermore, the expression of Bad was up-regulated and, subsequently, was cleaved into a 15 kDa fragment. Recently, there are several lines of evidence that Bad is cleaved into a 15-kDa truncated protein, which is a more potent inducer of apoptosis than the wild-type protein [39]. It is likely that the truncated Bad may facilitate genistein-induced apoptosis. In addition, cyclosporin A (a mitochondrial permeability transition pore inhibitor) displayed a partial inhibition of genistein-induced apoptotic cell death. Taken together, the data suggest that an interaction with mitochondria might partially explain the apoptotic cell death during a long-term exposure to genistein.

There are several lines of evidence that inhibition of tyrosine kinase augments ER stress-mediated apoptosis in numerous types of cancer cells [40]. Furthermore, it has been suggested that the activation of phosphatidylinositol (PI) 3-kinase/Akt and MEK/ERK plays a crucial role in cell survival by resisting ER stress-induced cell death [41]. Genistein is able to indirectly attenuate the activity of PI 3-kinase/Akt and MEK/ERK through the inhibition of tyrosine kinase [42,43]. In our unshown data, the PI 3-kinase/Akt- and MEK/ERK-involved signals were inhibited in genistein (100 μM)-treated cells, indicating that the inhibition of tyrosine kinase may also play a role in the apoptotic cell death.

In summary, it is suggested that genistein is an effective isoflavonoid that induces apoptotic signaling in a sequential manner in Hep3B cells. It causes ER stress, which is characterized by the increase of calcium mobilization, cleavage of *m*-calpain, up-regulation of GRP78 and GADD153 expression, and activation of caspase-12. Genistein also induces the activation of executor caspase-3 and caspase-7. Moreover, the interaction with mitochondrial stress to down-regulate Mcl-1 level and to generate truncated Bad may facilitate genistein-mediated apoptosis in Hep3B cells.

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