

Genistein Induces Receptor and Mitochondrial Pathways and Increases Apoptosis During Bcl-2 Knockdown in Human Malignant Neuroblastoma SK-N-DZ Cells

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The potent antiapoptotic molecule Bcl-2 is markedly upregulated in a majority of cancers, including neuroblastoma. Genistein is an isoflavone with antitumor properties. The present study sought to elucidate the molecular mechanism of genistein-induced apoptosis and also to examine the effect of genistein in increasing apoptosis during Bcl-2 knockdown in human malignant neuroblastoma SK-N-DZ cells. The cells were transfected with Bcl-2 siRNA plasmid vector, treated with 10 µM genistein, or the combination, and subjected to TUNEL staining and FACS analysis. Semiguantitative and real-time RT-PCR experiments were performed for examining expression of Fas ligand (FasL), tumor necrosis factor- α (TNF- α), Fasassociated death domain (FADD), and TNFR-1-associated death domain (TRADD). The cell lysates were analyzed by Western blotting for levels of molecules involved in both receptor- and mitochondria-mediated apoptotic pathways. Treatment with the combination of Bcl-2 siRNA and genistein resulted in more than 80% inhibition of cell proliferation. TUNEL staining and FACS analysis demonstrated apoptosis in 70% of cells after treatment with the combination of both agents. Apoptosis was associated with increases in Bax:Bcl-2 ratio, mitochondrial release of cytochrome c, and activation of caspases through the mitochondria-mediated apoptotic pathway. Genistein triggered the receptor-mediated apoptotic pathway through upregulation of TNF- α , FasL, TRADD, and FADD and activation of caspase-8. Combination of Bcl-2 siRNA and genistein triggered a marked increase in cleavage of DFF45 and PARP that resulted in enhanced apoptosis. Our study demonstrates that Bcl-2 knockdown during genistein treatment effectively induced apoptosis in neuroblastoma cells. Therefore, this strategy could serve as a potential therapeutic regimen to inhibit the growth of human malignant neuroblastoma. © 2009 Wiley-Liss, Inc.

Key words: apoptosis; Bcl-2; genistein; neuroblastoma; SK-N-DZ

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Neuroblastomas are extracranial malignant tumors that affect mainly children, and these are the most common solid cancers in infancy, with a dismal prognosis (Park et al., 2008). About 50% of neuroblastomas occur in children younger than 2 years old, and 75% occur in children less than 4 years old (Resnick and Schaeffer, 2000).

Dysregulation of apoptotic pathways plays a significant role in the progression of neuroblastomas and also in responses to therapeutic interventions (Fulda and Debatin, 2003; Goldsmith and Hogarty, 2005). Several molecular alterations associated with transformation and tumor progression may also be implicated in the regulation of cell death pathways and development of drug resistance (Zunino et al., 1997). Highly invasive tumor cells are protected from apoptosis through upregulation of the Bcl-2 (B-cell lymphoma 2) family of antiapoptotic proteins that stabilize the mitochondrial permeability transition pore and inhibit the release of cytochrome c from mitochondria. The different signals that converge on mitochondria result in the release of cytochrome c into the cytosol, triggering the activation of intrinsic caspase cascade for apoptosis (Martinez-Caballero et al., 2005; George et al., 2007). The use of a potent proapoptotic molecule, which also induces the extrinsic caspase pathway, can yield a synergistic effect for the induction of apoptosis in neuroblastomas.

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Genistein (4',5,7-trihydroxyisoflavone) is an isoflavone with profound antitumor properties (Farina et al., 2006; Banerjee et al., 2008). In higher concentrations, genistein inhibits tyrosine kinases, the enzymes involved in phosphorylation of intracellular proteins that trigger a cascade of events leading to changes in gene expression (Akiyama et al., 1987). Dietary genistein inhibits metastasis of human prostate cancer in mice (Lakshman et al., 2008). Genistein and other dietary compounds that prevent cancer may enhance the efficacy of cancer therapeutics by modifying the activity of key cell proliferation and survival pathways, such as those controlled by Akt, nuclear factor- κ B (NF- κ B), and cyclooxygenase-2 (Sarkar and Li, 2006). The strategies for treatment of cancer using a combination of agents with distinct molecular mechanisms are currently considered highly promising.

Tumor necrosis factor- α (TNF- α) and the type II transmembrane protein Fas ligand (FasL) play significant roles in induction of apoptosis in both physiological and pathological conditions (Wajant, 2006). Binding of TNF- α and FasL (CD95L) to their receptors, Fas (CD95) and TNF receptor-1 (TNFR-1), respectively, triggers the formation of a death-inducing signaling complex (DISC) involving recruitment of their adaptor proteins Fas-associated death domain (FADD) and TNFR-1-associated death domain (TRADD), respectively. In addition to its carboxyl-terminal death domain, FADD contains an amino-terminal death effector domain (DED) that binds to DEDs present on caspase-8, leading to activation of caspase-8 (Nagata, 1997). The active caspase-8 in turn cleaves Bid to produce truncated Bid (tBid) that translocates to mitochondria and induces conformational changes in Bak to form oligomer channels in the mitochondrial membrane for cytochrome c release into the cytosol. Cytosolic cytochrome c then initiates formation of an apoptosome along with apoptotic protease-activating factor-1 (Apaf-1) in the presence of adenosine nucleotides (Liu et al., 1996; Zou et al., 1997). The apoptosome processes procaspase-9 to generate active caspase-9 that triggers activation of caspase-3 for cleavage of key substrates leading to apoptosis.

The introduction of siRNA through a plasmid vector is an effective tool with which to down-regulate a specific mRNA and thus the protein level of a target gene (George and Tsutsumi, 2007). Upregulation of the antiapoptotic molecule Bcl-2 in tumor cells confers a survival advantage (Metrailler-Ruchonnet et al., 2007). The aim of our present investigation was to elucidate the molecular mechanism of genistein-induced apoptosis and also to study the effect of genistein in increasing apoptosis during Bcl-2 knockdown in human malignant neuroblastoma SK-N-DZ cells.

MATERIALS AND METHODS

Cell Culture Conditions

Human neuroblastoma SK-N-DZ cell line was procured from American Type Culture Collection (ATCC, Manassas, VA). We have selected the SK-N-DZ cell line for testing efficacy of our therapeutic strategy because it is a malignant and aggressive cell line among human neuroblastoma cell lines. The cells were propagated in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% fetal bovine serum (FBS) and antibiotics in a fully humidified incubator containing 5% CO₂ in air at 37°C. Genistein (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide to a concentration of 100 mM. Genistein was further diluted to a final concentration of 10 μ M in serum-free medium and used for all cell culture experiments.

Construction of Bcl-2 siRNA cDNA Expression Vector

The Bcl-2 siRNA cDNA was constructed into a mammalian expression vector, pRNAT-CMV3.2/Neo (GenScript, Piscataway, NJ), between the BamHI and the XhoI sites. We prepared three siRNA sequences, and the most effective one was selected based on percentage of Bcl-2 knockdown at mRNA and protein levels. The selected siRNA sequence to target human Bcl-2 mRNA began at nucleotide 974 (BC027258): 5'-GGA TGC CTT TGT GGA ACT GTAtt-3' (sense) and 3'-TAC AGT TCC ACA AAG GCA TCC-5' (antisense). The scrambled siRNA sequences were 5'-GTG TTA AGG TGC TGT TCG ACAtt-3' (sense) and 3'-TGT CGA ACA GCA CCT TAA CAC-5' (antisense). The loop selected was 5'-TTG ATA TCCG-3'. The linear siRNA construct with the sense and antisense strand, loop, termination signal, and BamHI and XhoI restriction sites was annealed with the complimentary strand and ligated into the siRNA expression vector (pRNAT-CMV3.2/Neo) between the BamHI and the XhoI sites. In this vector, the powerful cytomegalovirus (CMV) promoter drives the expression of siRNA, and the SV40 promoter drives the expression of the neomycin resistance gene. This vector also carries coral green fluorescence protein (cGFP) as a marker for tracking the transfection efficiency in cell cultures. The siRNA sequence was confirmed by DNA sequencing. The plasmid vector carrying the Bcl-2 siRNA cDNA cassettes was transformed into JM109 competent cells (Promega, Madison, WI) and the positive colonies were screened using the Qiagen miniprep plasmid DNA purification kit (Qiagen, Valencia, CA). The highly expressing colony was selected and propagated in LB broth containing neomycin and purified using maxiprep plasmid DNA purification kit (Qiagen).

Treatment of SK-N-DZ Cells With Bcl-2 siRNA Plasmid Vector and Genistein

About 80% confluent cultures were transfected with mammalian expression vector carrying Bcl-2 siRNA cDNA or treated with a final concentration of 10 μ M genistein or both agents in combination in serum-free medium. A dose of 10 μ M genistein was selected based on a dose-response study for cell viability as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983) as well as by TUNEL staining. This dose of genistein was found to be optimal to induce apoptosis in SK-N-DZ cells. The plasmid vectors were transfected using Fugene HD (Roche Diagnostics, Indianapolis, IN) with a ra-

Transcript	GeneBank No.	Primer sequence	Primer position	Primer length (bases)	Product size (bp)
Bcl-2	BC027258	5'-TGTGGATGACTGAGTACC-3'	913F	18	117
		5'-AGGAGAAATCAAACAGAGG-3'	1029R	19	
TNF-α	NM_000594	5'-TTCCTCAGCCTCTTCTCC-3'	263F	18	195
	_	5'-AGGGTTTGCTACAACATGG-3'	457R	19	
FasL	U11821	5'-AGCAAATAGGCCACCCCAGTCC-3'	450F	22	241
		5'-TGGCTCAGGGGCAGGTTGTTG-3'	690R	21	
TRADD	NM_003789	5'-CCACCTGCCCAGACTTTTC-3'	669F	19	102
		5'-CCATTTGAGACCCACAGAGC-3'	770R	20	
FADD	NM_003824	5'-GGAGTCACTGAGAATCTGG-3'	723F	19	120
		5'-TGCTGAACCTCTTGTACC-3'	842R	18	
GAPDH	NM_002046	5'-CCACCCATGGCAAATTCC-3'	254F	18	304
		5'-CAGGAGGCATTGCTGATGAT-3'	557R	20	

TABLE I. Description of the Primers Used for Semiquantitative and Real-Time RT-PCR

tio of 3:1 (3 μ l Fugene and 1 μ g DNA). Transfection efficiency was examined with an inverted fluorescent microscope (Olympus IX71, Tokyo, Japan) for the expression of cGFP. The cells were also transfected with the vector carrying the scrambled siRNA cDNA sequence for Bcl-2. After 24 hr, the medium was replaced with fresh medium containing 5% FBS, and the cells were incubated for another 24 hr.

RT-PCR and Western Blotting for Bcl-2

Semiquantitative RT-PCR and Western blotting were performed to examine the down regulation of Bcl-2 at mRNA and protein levels, respectively, after transfection with the plasmid vector carrying Bcl-2 siRNA cDNA and treatment with genistein. The primer sequences used in RT-PCR experiments are shown in Table I. The primers were transcribed with 300 ng of isolated total RNA using a single-step RT-PCR kit (Invitrogen, Carlsbad, CA) on a PCR cycler (Eppendorf, Westbury, NY) with annealing at 56°C. Western blotting was carried out for Bcl-2 protein levels with Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), as we describe below. Both RT-PCR and Western blotting images were quantified using Gel-Pro analyzer software (Media Cybernetics, Silver Spring, MD).

TUNEL Staining

We employed TUNEL staining to detect the apoptotic cells after treatments with Bcl-2 siRNA or/and genistein. TUNEL staining was performed using a fluorogenic TUNEL kit (Millipore, Bedford, MA). Briefly, the cells were fixed to the slides, washed in PBS, and permeabilized with 0.2% Triton X-100 for 5 min. The cells were washed, equilibrated with equilibration buffer (supplied in the kit), and incubated with 50 µl recombinant TdT-fluorescein-12-dUTP cocktail for 1 hr at 37°C in a fully humidified chamber. The reaction was terminated, and the slides were washed thrice in PBS and mounted with antifade agent (Biomeda, Foster City, CA). Slides were dried in the dark, examined under a fluorescent microscope (Olympus, Tokyo, Japan), and photographed. The staining areas were examined in control and treated cells in 10 uniform fields, and the intensity of fluorescent staining was measured using Image-Pro Discovery software (Media Cybernetics). The data are presented as percentage of apoptotic cells based on the scoring index (square micrometers).

FACS Analysis for Apoptotic DNA Fragmentation

FACS was performed to determine the percentage of apoptotic cells after transfection with Bcl-2 siRNA, treatment with genistein, or both agents together. Cells were harvested using TrypLE (Invitrogen) and washed twice with PBS. The cells were then fixed with 70% ethanol for 15 min on ice. After removing ethanol, the cells were dispersed in 1 ml propidium iodide (50 μ g/ml) staining solution (Biosure, Grass Valley, CA) on ice and incubated for 30 min in dark. The cells were sorted on a FACS machine (FACS Calibur; BD, Franklin Lakes, NJ) and analyzed based on the red fluorescence at 488 nm. Data were presented as FACS histograms and percentage of apoptotic and healthy cells.

Semiquantitative and Real-Time RT-PCR for Death Factors and Death Domains

Semiquantitative and real-time RT-PCR experiments were conducted for levels of mRNA expression of TNF- α , FasL, TRADD, and FADD in the cells after the treatments. Total cellular RNA was isolated using the Aurum kit (Bio-Rad, Hercules, CA). The quality of the isolated RNA was validated with UV spectrometry. Gene-specific primers were designed using Beacon Designer software (Premier Biosoft, Palo Alto, CA). The details of all the primers used are shown in Table I. The primers were transcribed with 300 ng isolated total RNA using a single-step RT-PCR kit (Invitrogen) on a PCR cycler (Eppendorf, Westbury, NY) with the following reaction conditions: cDNA synthesis at 50°C for 30 min, reverse transcriptase inactivation at 94°C for 2 min, PCR amplification (denature at 94°C for 20 sec, annealing at 56°C for 30 sec, chain extension at 72°C for 45 sec) for 35 cycles, and a final chain extension at 72°C for 10 min. The amplified products were separated on 1.5% agarose gels, stained with ethidium bromide (1 µg/ml), and visualized using a transilluminating UV chamber (Alpha Innotech, San Leandro, CA). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene.

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Furthermore, we used the primer sequences (Table I) for real-time quantitative RT-PCR analysis for mRNA expression of TNF- α , FasL, TRADD, and FADD. Real-time RT-PCR experiments were carried out using 100 ng total isolated RNA and one-step RT-PCR kit containing SYBR green (Bio-Rad) on a real-time PCR machine (iCycler iQ5; Bio-Rad) with the following reaction conditions: cDNA synthesis 10 min at 50°C, reverse transcriptase inactivation at 95°C for 5 min, PCR cycling and detection at 95°C for 10 sec, and data collection at 56°C for 30 sec.

Western Blotting for Proteins Involved in Receptor and Mitochondrial Pathways of Apoptosis

After the treatments, cells were washed twice with icecold PBS and scraped with freshly prepared RIPA buffer containing protease inhibitors [50 mM Tris-HCl, pH 7.4, containing 1% Nonidet P-40, 150 mM NaCl, 1 mM sodium orthovanadate (activated), 1 mM sodium fluoride, 1 mM PMSF, 1 mM EDTA, 5 µg/ml aprotinin, and 5 µg/ml pepstatin]. Cells were centrifuged at 5,000 rpm for 10 min at 4°C in an Eppendorf microfuge. The supernatant was discarded, and the cell pellet was suspended in 100-300 µl RIPA buffer and sonicated (Kontes, Vineland, NJ). Cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were collected. The protein concentration in the supernatant was determined using the Coomassie Plus protein assay reagents (Pierce Biotechnology, Rockford, IL), and the samples were stored at -20°C until use. Mitochondria were isolated from the total cell lysate using the mitochondria isolation kit (Pierce Biotechnology, Rockford, IL) for cultured cells. Cytochrome c levels were determined in both mitochondrial and cytosolic fractions. The nuclear fraction was extracted from the cultured cells using the nuclear extraction kit (Panomics, Fremont, CA), and the nuclear protein poly-(ADP-ribose) polymerase (PARP) was determined in the nuclear fraction.

The proteins were resolved on 4-20% SDS-polyacrylamide gradient gels (Bio-Rad) and electroblotted to activated polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking the nonspecific binding sites with 5% no-fat milk, the membranes were incubated overnight on a rocker at 4°C with specific antibodies for various protein molecules. The primary IgG antibodies for caspase-8, caspase-3, Bax, tBid, TNFR-1, Fas (CD95), FasL (CD95L), TRADD, FADD, and cytochrome c oxidase 4 (COX-4) were purchased from Santa Cruz Biotechnology; Apaf-1, Bcl-xL, caspase-7, PARP, and TNF-a were procured from Cell Signaling Technology (Danvers, MA); caspase-9 and cytochrome c were purchased from BD Biosciences (San Jose, CA); and DNA fragmentation factor-40 (DFF40) was obtained from Millipore. After incubation with primary antibodies, the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary IgG antibodies (Biomeda, Foster City, CA) for 2 hr at room temperature. The membranes were washed again, treated with enhanced chemiluminescence reagent (Amersham, United Kingdom), exposed to BioMax XAR autoradiography film (Kodak, New Haven, CT), and developed. The membranes were reprobed using Western reprobe buffer (Gbiosciences, St. Louis, MO) for GAPDH content using a monoclonal primary IgG antibody (Novus Biologicals, Littleton, CO) to demonstrate uniform loading of cytosolic protein in each lane. Also, COX-4 was used as loading control for the mitochondrial fraction. Autoradiograms were imaged and quantified in Gel-Pro analyzer software (Media Cybernetics). Quantitative data were compiled and presented only for molecules with marked changes.

Statistical Analysis

Arithmetic mean and standard deviation (SD) were calculated using all quantitative data obtained from cell culture experiments (n = 6). The results were statistically evaluated using one-way analysis of variance (ANOVA). The least significant difference method was used to compare the mean values of control or scrambled siRNA-transfected samples with those of Bcl-2 siRNA-transfected or genistein-treated samples. The individual Bcl-2 siRNA or genistein mean values were also compared with the combination treatment mean values. The difference between two values was considered statistically significant at P < 0.05.

RESULTS

Cognate siRNA Down Regulated Bcl-2 at mRNA and Protein Levels

We examined the effects of treatments on expression of Bcl-2 and cell viability in in SK-N-DZ cells (Fig. 1). Transfection of cells with a plasmid encoding Bcl-2 shRNA results in marked down-regulation of Bcl-2 at mRNA (Fig. 1A) and protein (Fig. 1B) levels. Expression of GAPDH was used as an internal control in experiments for examining mRNA and protein levels. There was no notable alteration in Bcl-2 at mRNA and protein levels after transfection with a plasmid vector carrying the scrambled Bcl-2 siRNA cDNA. Treatment of cells with genistein alone resulted in significant down-regulation of Bcl-2 at mRNA and protein levels, and treatment with the combination of both agents resulted in marked and almost complete knockdown of Bcl-2 (Fig. 1C). Transfection of cells with Bcl-2 siRNA, treatment with genistein, and the combination of both caused 66%, 27%, and 92% knockdown of Bcl-2 mRNA, respectively, and also 69%, 31%, and 93% knockdown of Bcl-2 protein, respectively (Fig. 1C).

Effect of Bcl-2 siRNA and Genistein on Cell Viability

MTT assay, which is based on activity of enzyme system in mitochondria in viable cells, is a specific measure of cell viability. We subjected the SK-N-DZ cells to MTT assay after Bcl-2 siRNA transfection, genistein treatment, or the combination of both. Results from MTT assay showed significant decreases in cell viability after transfection with Bcl-2 siRNA and treatment with genistein (Fig. 1D). Cell viability was reduced to 16% after combined treatment, indicating the highest effect of the combination on reduction in cell viability. These

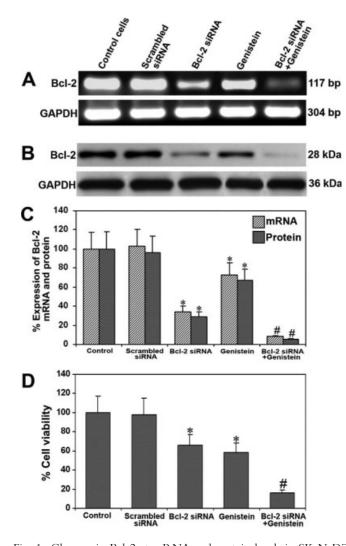


Fig. 1. Changes in Bcl-2 at mRNA and protein levels in SK-N-DZ cells. Treatments (48 hr): control, transfection with a plasmid vector carrying scrambled siRNA cDNA, transfection with a plasmid vector carrying Bcl-2 siRNA cDNA, 10 µM genistein, and combination of Bcl-2 siRNA cDNA and 10 µM genistein. A: Semiquantitative RT-PCR for mRNA levels of Bcl-2. GAPDH mRNA expression was used as an internal control. B: Western blotting for protein levels of Bcl-2. Western blots were reprobed for GAPDH content to demonstrate equal loading of protein in all lanes. C: Evaluation of percentage knockdown of Bcl-2 at mRNA and protein levels. Western blots were quantified using Gel-Pro analyzer software. The data are mean \pm SD of six experiments (*P < 0.001 compared with the control mean values, ${}^{\#}P < 0.001$ compared with Bcl-2 siRNA or genistein mean values). D: MTT assay for cell viability and proliferation. The data are mean \pm SD of six independent experiments in duplicate (*P < 0.001 compared with the control mean values, ${}^{\#}P < 0.001$ compared with Bcl-2 siRNA or genistein mean values).

data reflected loss of mitochondrial integrity and increase in mitochondrial permeability in SK-N-DZ cells after combined treatment, contributing to dramatic decrease in cell viability (Fig. 1D).

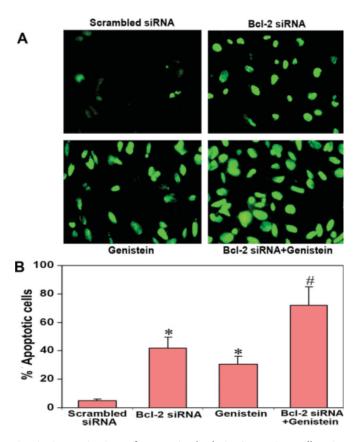


Fig. 2. Determination of apoptotic death in SK-N-DZ cells using the TUNEL staining. Treatments (48 hr): transfection with a plasmid vector carrying scrambled siRNA cDNA, transfection with a plasmid vector carrying Bcl-2 siRNA cDNA, 10 μ M genistein, and combination of Bcl-2 siRNA cDNA and 10 μ M genistein. **A**: TUNEL staining for detection of apoptotic cells. **B**: Quantitation of TUNEL-positive cells in Image-Pro Discovery software. The treatment with combination of Bcl-2 siRNA and genistein caused more apoptotic death than either treatment alone. Data are mean \pm SD of six independent experiments in duplicate (*P < 0.001 compared with the scrambled siRNA mean values, "P < 0.001 compared with Bcl-2 siRNA or genistein mean values). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Combination of Bcl-2 siRNA and Genistein Increased Apoptosis as Indicated by TUNEL Staining

We performed fluorescent TUNEL staining and determined apoptosis after Bcl-2 siRNA transfection, genistein treatment, or the combination of both in SK-N-DZ cells (Fig. 2). TUNEL staining showed apoptotic cells distinctly after transfection with Bcl-2 siRNA or treatment with genistein (Fig. 2A). Bcl-2 siRNA was more effective than genistein at inducing apoptosis. Treatment with the combination of both agents resulted in more than a two-fold increase in number of apoptotic cells compared with either treatment alone. Also, TUNEL staining indicated hardly any apoptosis in untreated control cells (not shown) and cells transfected with scrambled siRNA (Fig. 2A). Quantitation of

TUNEL stained cells revealed 42%, 31%, and 73% apoptosis after treatments with Bcl-2 siRNA, genistein, and the combination, respectively (Fig. 2B). The mean value of the treatment with combination was significantly different (P < 0.001) from that of individual mean values of transfection with Bcl-2 siRNA or treatment with genistein. The cells transfected with scrambled siRNA were considered as the treated control.

Combination of Bcl-2 siRNA and Genistein Increased Apoptosis as Indicated by FACS Analysis

We performed FACS analysis of SK-N-DZ cells for determination of apoptosis after treatments with Bcl-2 siRNA, genistein, and the combination of both agents (Fig. 3). The cells transfected with scrambled siRNA were used as the treated control. The FACS histograms of the cells demonstrated the extent of apoptotic DNA fragmentation after the treatments (Fig. 3A). A marked increase in cell population in the sub-G1 phase (gray area) indicated accumulation of apoptotic cells with DNA fragmentation. The cell population in sub-G1 phase appeared to peak after treatment with combination of Bcl-2 siRNA and genistein. Consequently, the normal cell population in the G0/G1 phase (black area) was remarkably reduced (Fig. 3A). The cell population in the sub-G1 phase was significantly increased (P < 0.001) after transfection with Bcl-2 siRNA or treatment with genistein compared with the cells transfected with Bcl-2 scrambled siRNA (Fig. 3B). The treatments with Bcl-2 siRNA, genistein, and the combination of both agents produced 39%, 31%, and 74% apoptotic cells, respectively. The FACS data well correlated (r = 0.996) with the TUNEL data for apoptosis.

Increases in mRNA Levels of Death Factors and Death Domains

Because TNF- α , FasL, and their respective death domains TRADD and FADD play significant roles in apoptosis, we examined changes in the mRNA expression of TNF- α , FasL, and the related death domains using both semiquantitative and real-time RT-PCR experiments (Fig. 4). Semiquantitative RT-PCR demonstrated conspicuous increases in the mRNA levels of TNF- α , FasL, TRADD, and FADD after treatment with genistein alone or in combination with Bcl-2 siRNA (Fig. 4A). Transfection with scrambled siRNA or Bcl-2 siRNA did not show noticeable alterations in the mRNA levels of any of the molecules studied. The increase of mRNA expression of TNF- α was the most prominent among the three other molecules studied. Furthermore, real-time RT-PCR analysis for the mRNA expression of TNF- α , FasL, TRADD, and FADD showed similar expression patterns (Fig. 4B). The levels of transcription of all the genes examined were signigicantly increased after treatment with genistein alone or a combination of Bcl-2 siRNA and genistein. For both semiquantitative and real-time RT-PCR experiments, the treatment with scrambled siRNA was used as treated control.

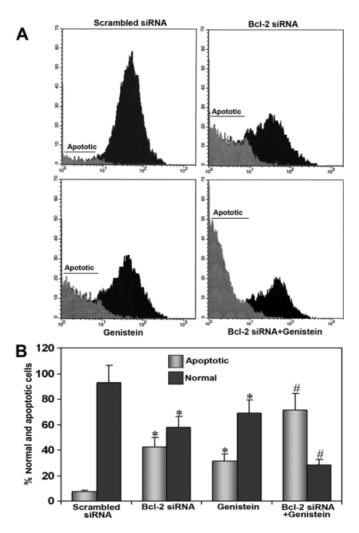


Fig. 3. FACS analysis for determination of DNA fragmentation in apoptotic SK-N-DZ cells. Treatments (48 hr): transfection with a plasmid vector carrying scrambled siRNA cDNA, transfection with a plasmid vector carrying Bcl-2 siRNA cDNA, 10 μ M genistein, and combination of Bcl-2 siRNA cDNA and 10 μ M genistein. A: Representative FACS histograms of SK-N-DZ cells after the treatments. The cells were stained with 50 μ g/ml propidium iodide for 30 min at 4°C in the dark. An increase in cell population in sub-G1 phase (gray area marked as apoptotic) indicated an increase in apoptosis after treatment with Bcl-2 siRNA, genistein, or both together. **B**: Quantitative presentation of FACS data of normal and apoptotic cells. Data are mean \pm SD of six independent experiments (*P <0.001 compared with scrambled siRNA mean values, "P < 0.001 compared with Bcl-2 siRNA or genistein mean values).

Increases in Protein Levels of Death Factors and Death Domains

We used Western blotting to examine and measure the protein levels of TNFR-1, Fas, TNF- α , FasL, TRADD, and FADD after transfection with Bcl-2 siRNA, treatment with genistein, or both in combination (Fig. 5). Western blots showed apparently no altera-

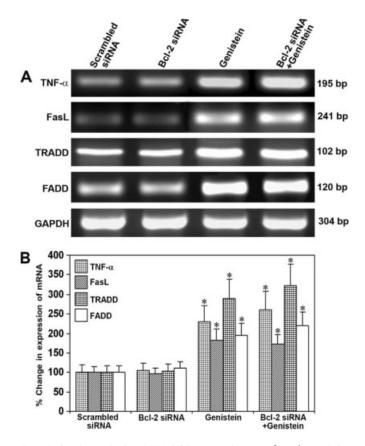


Fig. 4. Semiquantitative RT-PCR experiments for determining changes in the mRNA expression of selective genes in SK-N-DZ cells. Treatments (48 hr): transfection with a plasmid vector carrying scrambled siRNA cDNA, transfection with a plasmid vector carrying Bcl-2 siRNA cDNA, 10 µM genistein, and combination of Bcl-2 siRNA cDNA and 10 µM genistein. **A**: Semiquantitative RT-PCR for examining the mRNA expression of TNF-α, FasL, TRADD, and FADD after the treatments. The mRNA expression of GAPDH was used as a housekeeping gene expression. **B**: Real-time RT-PCR analysis using SYBR green for quantitative evaluation of the mRNA expression of TNF-α, FasL, TRADD, and FADD. The data are mean ± SD of six samples (*P < 0.001 compared with the mean values of scrambled siRNA-transfected samples).

tions in the protein levels of any of these molecules after transfection with scrambled siRNA of Bcl-2 siRNA (Fig. 5A). However, treatment with genistein resulted in a marked upregulation of all the molecules. Treatment with the combination of Bcl-2 siRNA and genistein showed similar results. Reprobing of membranes for GAPDH demonstrated equal loading of protein in each lane. The quantitative data were also obtained from protein levels of death factors and death domains (Fig. 5B). The highest upregulation of TRADD was observed after treatment with genistein alone and also after treatment with combination of Bcl-2 siRNA and genistein (Fig. 5B). Treatment with scrambled siRNA was considered as the treated control.

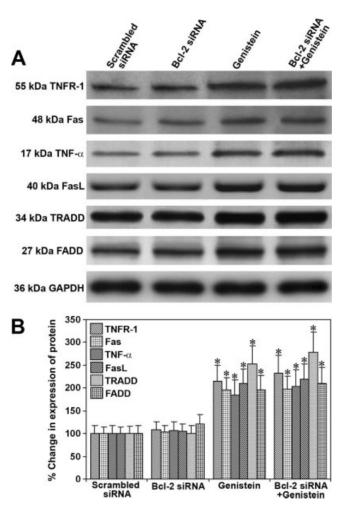


Fig. 5. Western blotting for determining the changes in levels of proteins of receptor pathway of apoptosis in SK-N-DZ cells. Treatments (48 hr): transfection with a plasmid vector carrying scrambled siRNA cDNA, transfection with a plasmid vector carrying Bcl-2 siRNA cDNA, 10 μ M genistein, and combination of Bcl-2 siRNA cDNA and 10 μ M genistein. **A**: Representative Western blots of TNFR-1, Fas, TNF- α , FasL, TRADD, and FADD in the cell lysate of SK-N-DZ cells. Western blots were reprobed for GAPDH content to demonstrate that same amount of protein was loaded in each lane. **B**: Evaluation of percentage changes in the protein levels of TNFR-1, Fas, TNF- α , FasL, TRADD, and FADD. Western blot images were quantified using Gel-Pro analyzer software. Data are mean \pm SD of six independent experiments (*P < 0.001 compared with the mean values of scrambled siRNA-transfected samples).

Combination of Bcl-2 siRNA and Genistein Increased Molecular Events Related to Receptor and Mitochondrial Pathways of Apoptosis

We performed Western blotting to examine and measure the effects of Bcl-2 siRNA and genistein on molecular events (Fig. 6) known to be associated with the receptor and mitochondrial pathways of apoptosis. Treatment of cells with genistein generated active caspase-8 that cleaved Bid to produce tBid for its translocation to mitochondria (Fig. 6A), indicating activation of

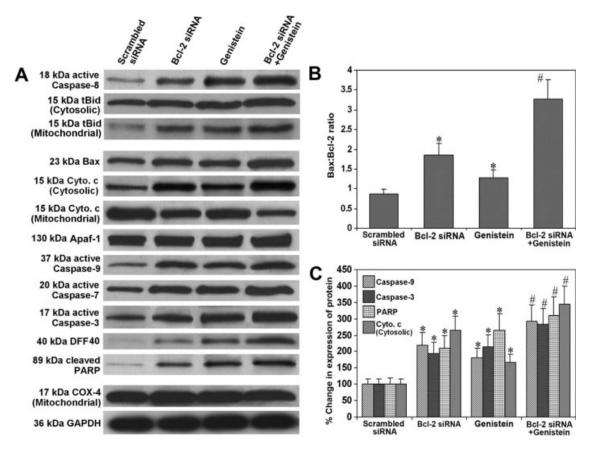


Fig. 6. Western blotting for determining the activation of both receptor and mitochondrial pathways of apoptosis in SK-N-DZ cells. Treatments (48 hr): transfection with a plasmid vector carrying scrambled siRNA cDNA, transfection with a plasmid vector carrying Bcl-2 siRNA cDNA, 10 μ M genistein, and combination of Bcl-2 siRNA cDNA and 10 μ M genistein. **A**: Representative Western blots of specific proteins after the treatments. Mitochondria were isolated from the total cell lysate, and both tBid and cytochrome c levels were determined in the mitochondrial fraction. Cleaved PARP was determined in the nuclear fraction. Western blots were reprobed for

COX-4 and GAPDH to demonstrate that equal amounts of mitochondrial and cytosololic proteins, respectively, were loaded in all lanes. **B**: Determination of changes in Bax:Bcl-2 ratio after the treatments. **C**: Evaluation of percentage changes in active fragments of caspase-9, caspase-3, cleaved PARP, and cytosolic cytochrome c. Western blot images were quantified in Gel-Pro analyzer software. Data are mean \pm SD of six independent experiments (*P < 0.001compared with the mean values of scrambled siRNA-transfected samples, ${}^{\#}P < 0.001$ compared with the mean values of Bcl-2 siRNA- or genistein-treated samples).

molecular components of the receptor pathway to augment the mitochondrial pathway as well. The highest activation of caspase-8 and also production and translocation of tBid to mitochondria occurred after treatment with the combination of Bcl-2 siRNA and genistein. Treatment with the combination of Bcl-2 siRNA and genistein also caused a dramatic increase in Bax (Fig. 6A) and decrease in Bcl-2 (Fig. 1B), resulting in a highly significant increase in the Bax:Bcl-2 ratio (Fig. 6B) to trigger directly the mitochondrial pathway of apoptosis. Treatment with a combination of Bcl-2 siRNA and genistein showed an almost two-fold increase in Bax:Bcl-2 ratio when compared with the effect of either agent alone (Fig. 6B). Cytosolic cytochrome c level was significantly increased because of its release from mitochondria after Bcl-2 siRNA transfection, genistein treatment, or the combination of both (Fig. 6A,C). However, cytosolic Apaf-1 levels remained almost the same following treatments. The active subunits of caspase-9, caspase-7, and caspase-3 were increased prominently after Bcl-2 siRNA transfection and genistein treatment (Fig. 6A,C). Significant increase in activation of caspase-3 (Fig. 6A,C) correlated well with nuclear upregulation of DFF40 (also known as caspase-3-activated DNase, or CAD) and cleavage of 116-kDa PARP (a DNA repair enzyme) to 89-kDa PARP (Fig. 6A), confirming the fulfillment of prerequisites for nuclear DNA fragmentation in the cells. Reprobing and analysis of the Western blots for COX-4 and GAPDH demonstrated equal loading of mitochrondrial and cytosolic proteins, respectively, in all the lanes (Fig. 6A). The transfection of cells with scrambled siRNA did not show alteration in the level of any of the molecules studied and thus was considered as the treated control.

Combination of Bcl-2 siRNA and Genistein Activated Molecular Events for Apoptosis

We propose, based on our experimental data, the following molecular events associated with the induction of both receptor and mitochondrial signaling pathways for apoptosis in neuroblastoma SK-N-DZ cells following treatment with combination of Bcl-2 siRNA and genistein. Genistein upregulates TNF- α and FasL and their corresponding adaptor proteins TRADD and FADD bearing a death-domain-homologous region as well as a death effector domain to which procaspase-8 can bind. This association processes procaspase-8 to generate active caspase-8. Active caspase-8 cleaves Bid to tBid, which translocates to mitochondria and induces conformational changes in Bak to form oligomer channels in the mitochondrial membrane for cytochrome c release. The antiapoptotic Bcl-2 inhibits mitochondrial cytochrome c release through stabilization of mitochondrial membrane. The cognate siRNA knockdowns Bcl-2 and promotes the release of cytochrome c from mitochondria. The association of cytosolic cytochrome c with procaspase-9 and Apaf-1 can process procaspase-9 to active caspse-9 that in turn converts procaspase-3 to active caspase-3. The active caspase-3 cleaves DFF45 to release and translocate DFF40 to nucleus, and it also cleaves PARP, creating the perfect environment for nuclear DNA fragmentation and apoptosis.

DISCUSSION

This study delineates the receptor and mitochondrial events that genistein activated for apoptosis and also shows the efficacy of the combination of Bcl-2 siRNA and genistein for increasing apoptosis in human malignant neuroblastoma SK-N-DZ cells. Inactivate apoptotic pathways play a crucial role in tumorigenesis and have also been implicated in tumor growth in human malignant neuroblastomas (Maris and Matthay, 1999; Fulda and Debatin, 2003). Caspases play an important role as effector molecules in mediation of apoptosis in both receptor and mitochondrial caspase signaling pathways. Because inappropriate caspase activity is potentially detrimental to cell survival, activation of caspases is tightly controlled in the cells (Igney and Krammer, 2002). Proteins of the Bcl-2 family are critical regulators of activation of caspases via mitochondrial pathway, acting as gatekeepers to monitor and integrate death and survival signals and set the threshold for mitochondrial release of cytochrome c (Goldsmith and Hogarty, 2002). Through dramatic upregulation of the potent antiapoptotic molecule Bcl-2, cancer cells including neuroblastomas gain a survival advantage based on apoptosis resistance that promotes tumorigenesis.

Investigating the effects of genistein during silencing of a powerful antiapoptotic molecule is a novel strategy for enhancing induction of apoptosis. In most of the tumor cells, upregulation of antiapoptotic molecules (e.g., Bcl-2) contribute to the resistance to various chemotherapeutic agents and radiation (Sartorius and Krammer, 2002; Wacheck et al., 2003a). Combination of Bcl-2 siRNA and cisplatin resulted in a massive increase in apoptotic death, with almost complete suppression of cell growth in malignant melanoma (Wacheck et al., 2003b). Furthermore, silencing of Bcl-2 using siRNA-sensitized human hepatoblastoma cells to chemotherapeutic agents suggested that silencing of Bcl-2 in combination with chemotherapy would be a potential therapeutic strategy against human hepatoblastoma (Lei et al., 2007). In the present study, we observed that knockdown of this potent antiapoptotic molecule Bcl-2 in malignant neuroblastoma cells and genistein treatment resulted in massive induction of apoptosis, as evidenced from TUNEL and FACS data and significant increases in the active fragments of effector caspases for execution of apoptosis.

Previous studies showed that genistein induced apoptosis due to increases in Bax:Bcl-2 ratio; mitochondrial release of cytochrome c; and activation of caspase-9, caspase-12, and caspase-3 in human malignant neuroblastoma SH-SY5Y cells (Das et al., 2006). Genistein is a known inhibitor of protein tyrosine kinase (PTK) and thus inhibits PTK-mediated signaling mechanisms to attenuate the growth of cancer cells (Akiyama et al., 1987). There is a growing body of experimental evidence showing that inhibition of human cancer cell growth by genistein is mediated via modulation of the genes that control cell cycle and apoptosis. It has been shown that genistein is also a powerful inhibitor of NFκB and Akt signaling pathways, which are highly important for cell survival (Li and Sarkar, 2002). Genistein inhibits the translocation of NF-KB to the nucleus, preventing NF- κ B from binding to its target DNA and thereby inhibiting the transcription of the NF-KB-dependent genes. This process ultimately inhibits cell growth and induces apoptotic death (Banerjee et al., 2008).

A major problem in the treatment of human tumors with chemotherapeutic agents is the development of drug resistance, which is often achieved through upregulation of antiapoptotic genes. Thus, we thought that down-regulation of the most prominent antiapoptotic molecule during treatment with a new anticancer agent would be an appropriate strategy to inhibit cell proliferation and induce apoptosis in malignant neuroblastoma cells. Several recent reports confirm the antiproliferative and anticancer effects of genistein on tumor cells (Sarkar, 2006; Lakshman et al., 2008). Epidemiological evidence suggests that high phytoestrogen diets may reduce the incidence of hormone-responsive cancers, primarily breast cancer and prostate cancer (Adlercreutz, 2002). Genistein has a good therapeutic effect on BRCA1-associated breast cancer (Tominaga et al., 2007). The anticancer effects of genistein also include inhibition of invasion, angiogenesis, cell growth, and proliferation (Ravindranath et al., 2004). Furthermore, genistein acts synergistically with anticancer drugs such as tamoxifen, cisplatin, 1,3-bis-2-chloroethyl-1-nitrosourea (BCNU), dexamethasone, daunorubicin, and tiazofurin (Ravindranath et al.,

2004). An in vitro concentration of genistein more than 5 μ M is regarded as non-physiological, considering the levels of dietary genistein intake and bioavailability (Klein and King, 2007). In the present study, we observed remarkable inhibition of cell proliferation and induction of apoptosis in human malignant neuroblastoma SK-N-DZ cells after treatment with 10 μ M genistein, and the effects of genistein were dramatically elevated during Bcl-2 knockdown in the cells. A concentration of genistein just above the physiological level is important to induce apoptosis selectively in fast-dividing tumor cells without affecting the normal cells. Thus, the combination of Bcl-2 siRNA and a concentration of genistein just above the physiological level would be ideal for killing malignant neuroblastoma cells and inhibiting tumor progression.

In conclusion, our study demonstrates genisteinactivated receptor and mitochondria-mediated molecular events for induction of apoptosis and also shows that treatment with the combination of Bcl-2 siRNA and genistein was a very effective strategy to increase apoptosis in human malignant neuroblastoma SK-N-DZ cells. The efficacy of the combination of Bcl-2 siRNA and genistein can also be explored for controlling the growth of human malignant neuroblastoma in preclinical animal models.

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