Original Article

Flavonoids Activated Caspases for Apoptosis in Human Glioblastoma T98G and U87MG Cells But Not in Human Normal Astrocytes

Arabinda Das, PhD[1]; Naren L. Banik, PhD[1]; and Swapan K. Ray, PhD[2]

BACKGROUND: Human glioblastoma is a deadly brain cancer that continues to defy all current therapeutic strategies. The authors induced apoptosis in human glioblastoma T98G and U87MG cells after treatment with apigenin, (−)-epigallocatechin, (−)-epigallocatechin-3-gallate (EGCG), and genistein, which did not induce apoptosis in human normal astrocytes. **METHODS:** Induction of apoptosis was examined using Wright staining and ApopTag assay. Production of reactive oxygen species (ROS) and increase in intracellular free Ca^{2+} were measured by fluorescent probes. Analysis of mRNA and Western blotting indicated increases in expression and activities of the stress kinases and cysteine proteases for apoptosis. JC-1 showed changes in mitochondrial membrane potential ($\Delta\Psi_m$), and use of specific inhibitors confirmed activation of kinases and proteases in apoptosis. **RESULTS:** Treatment of glioblastoma cells with apigenin, (−)-epigallocatechin, EGCG, or genistein triggered ROS production that induced apoptosis with phosphorylation of p38 mitogen-activated protein kinase (MAPK) and activation of the redox-sensitive *c-Jun* N-terminal kinase 1 pathway. Pretreatment of cells with ascorbic acid attenuated ROS production and p38 MAPK phosphorylation. Increases in intracellular free Ca^{2+} and activation of caspase-4 indicated involvement of endoplasmic reticulum stress in apoptosis. Other events in apoptosis included overexpression of Bax, loss of $\Delta\Psi_m$, mitochondrial release of cytochrome *c* and Smac into the cytosol, down-regulation of baculoviral inhibitor-of-apoptosis repeat-containing proteins, and activation of calpain, caspase-9, and caspase-3. (−)-Epigallocatechin and EGCG also induced caspase-8 activity. Apigenin, (−)-epigallocatechin, EGCG, and genistein did not induce apoptosis in human normal astrocytes. **CONCLUSIONS:** Results strongly suggest that flavonoids are potential therapeutic agents for induction of apoptosis in human glioblastoma cells. ***Cancer*** **2010;116:164–76.** *© 2010 American Cancer Society.*

KEYWORDS: apoptosis, flavonoids, glioblastoma, T98G, U87MG.

Glioblastoma is the most common primary malignant brain tumor, comprising about 20% of all primary brain tumors in adults. It is also called grade IV astrocytoma.[1] Glioblastoma is aggressive and characterized by rapid cell growth and local spread. Chemotherapy alone is not typically used for its treatment, but is often used as part of a multimodality treatment strategy. The major limitations of chemotherapy for treatment of glioblastoma are the inability of many drugs to pass through the blood-brain barrier and their low efficacy for induction of apoptosis. Therefore, the choice of active drugs is limited. Many clinical trials are evaluating new therapeutic agents for treatment of newly diagnosed glioblastoma or recurrent disease.

Flavonoids constitute the largest and most important group of polyphenolic compounds in plants. Flavonoids are present in vegetables, fruit, and beverages of plant origin that have antioxidant, antimutagenic, and antiproliferative properties.[2-5] They may thus have a potential protective role in various chronic diseases, including cancers,[6-8] and explain, at least in part, the well-established associations between high consumption of vegetables and fruit and reduced risk of several neoplasms. Over the past few years, flavonoids have been demonstrated to act on multiple key elements in signal transduction pathways related to cellular proliferation, differentiation, cell-cycle progression, apoptosis, inflammation, angiogenesis, and metastasis; however, these molecular mechanisms of action are not completely characterized, and many

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features remain to be elucidated.[9] It has been shown that flavonoids can trigger apoptosis through modulation of several key elements in cellular signal transduction pathways linked to apoptosis. Earlier studies suggest that flavonoids may exert regulatory activities in cells through actions at different signal transduction pathways such as cyclin-dependent kinases, caspases, Bcl-2 family members, epidermal growth factor/epidermal growth factor receptor, phosphatidylinositol-3-kinase/Akt, mitogen-activated protein kinase (MAPK), and nuclear factor kappa B (NF-κB), which may affect cellular function by modulating genes or phosphorylating proteins.[10] It has been shown repeatedly that antioxidants and their derivatives selectively induce apoptosis in cancer cells but not in normal cells in culture. Apigenin, a flavone abundantly found in fruits and vegetables, exhibits antiproliferative, anti-inflammatory, and antimetastatic activities. Treatment with apigenin was accompanied by an increase in reactive oxygen species (ROS) and phosphorylation of the MAPKs, p38 and ERK.[11] (−)-Epigallocatechin and (−)epigallocatechin-3-gallate (EGCG) are present in green tea at low levels and reduce the proliferation of human breast cancer cells in vitro and decrease breast tumor growth in rodents.[12] EGCG can induce apoptotic changes, including loss of mitochondrial membrane potential ($\Delta\Psi_m$) and activation of *c-Jun* N-terminal kinase 1 (JNK1), caspase-9, and caspase-3.[13] However, comprehensive mechanisms to explain the diverse effects of EGCG in causing apoptosis remain to be explored. Like most flavonoids, genistein exists in nature in its 7-glycoside form, genistin, rather than in its aglycone form. Genistein, a isoflavonoid, is a specific inhibitor of protein tyrosine kinase,[14] and it induces $\Delta\Psi_m$ change, caspase-3 activation, and poly(ADP-ribose)polymerase cleavage. Furthermore, genistein is a powerful inhibitor of NF-κB and Akt signaling pathways, both of which are important for cell survival.[15]

In the present study, we demonstrated that apigenin, (−)-epigallocatechin, EGCG, and genistein induce apoptosis in human glioblastoma T98G and U87MG cells but not in human normal astrocytes. We also observed that these flavonoids induced apoptosis with increase in ROS production, loss of $\Delta\Psi_m$, and activation of kinases and proteases.

MATERIALS AND METHODS

Cell Culture and Treatments

Human glioblastoma T98G and U87MG cells were purchased from the American Type Culture Collection (Manassas, Va). We purchased passage number 1 of the human normal astrocytes of human brain tissue from ScienCell Research Laboratories (Carlsbad, Calif). We grew the human normal astrocytes in our laboratory and used passage number 3 for monitoring flavonoid-induced cell death, ROS production, and caspase activities. Glioblastoma cells were grown in 75-cm^2 flasks containing 10 mL of 1 × RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in a fully humidified incubator containing 5% CO_2 at 37°C, whereas human normal astrocytes were grown in Dulbecco modified Eagle/F12 medium with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pyridoxine, and $NaHCO_3$ (Sigma, St. Louis, Mo), supplemented with 2% Sato's components, 1% penicillin and streptomycin (GIBCO-Invitrogen, Grand Island, NY), and 2% heat-inactivated FBS (Hyclone, Logan, Utah). Before drug treatments, the cells were starved in medium supplemented with 1% FBS for 24 hours. Dose-response studies were conducted to determine the suitable concentration of the drugs used in the experiments. Cells were treated with 50 μM apigenin, 50 μM (−)-epigallocatechin, 50 μM EGCG, and 50 μM genistein for 24 hours for induction of apoptosis. After treatments, cells were used for determination of the mechanisms of apoptosis.

Wright Staining and ApopTag Assay

Cells from each treatment were washed with 1 × phosphate-buffered saline (PBS), pH 7.4, sedimented onto the microscopic slide, and fixed. The morphological (Wright staining) and biochemical (ApopTag assay) features of apoptosis were examined, as we described previously.[16,17] After Wright staining and ApopTag assay, cells were counted under the light microscope to determine percentage of apoptosis.

Fura-2 Assay for Determination of Intracellular Free Ca^{2+}

Level of intracellular free Ca^{2+} was measured using the fluorescence Ca^{2+} indicator fura-2/AM, as we described previously.[16,18] The value of K_d, a cell-specific constant, was determined experimentally to be 0.387 μM for the T98G cells and 0.476 μM for the U87MG cells, using standards of the Calcium Calibration Buffer Kit With Magnesium (Molecular Probes, Eugene, Ore).

Analysis of mRNA Expression

Extraction of total RNA, qualitative reverse transcriptase polymerase chain reaction (RT-PCR), and agarose gel

Table 1. Human Primers Used to Determine Levels of mRNA Expression of Specific Genes

Gene	Primer Sequence	Product Size (bp)
β-Actin	Sense: 5′-TAT CCC TGT ACG CCT CT-3′ Antisense: 5′-AGG TCT TTG CGG ATG T-3′	460
baxα	Sense: 5′-AAG AAG CTG AGC GAG TGT-3′ Antisense: 5′-GGA GGA AGT CCA ATG TC-3′	265
bcl-2α	Sense: 5′-CTT CTC CCG CCG CTA C-3′ Antisense: 5′-CTG GGG CCG TAC AGT TC-3′	306
BIRC2	Sense: 5′-CAG AAA GGA GTC TTG CTC GTG-3′ Antisense: 5′-CCG GTG TTC TGA CAT AGC ATC-3′	536
BIRC3	Sense: 5′-GGG AAC CGA AGG ATA ATG CT-3′ Antisense: 5′-ACT GGC TTG AAC TTG ACG GAT-3′	368
BIRC4	Sense: 5′-AAT GCT GCT TTG GAT GAC CTG-3′ Antisense: 5′-ACC TGT ACT CAG CAG GTA CTG-3′	470
BIRC5	Sense: 5′-GCC CCA CTG AGA ACG-3′ Antisense: 5′-CCA GAG GCC TCA ATC C-3′	302

electrophoresis were performed as we described previously.[18] For real-time quantitative RT-PCR experiments, the cells from all treatments were homogenized, and total RNA samples were extracted according to the RNAwiz protocol (Ambion, Austin, Tex). In each reaction, 1 μg of total RNA was used for cDNA synthesis. First-strand cDNA synthesis was performed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, Calif). Both *bax* and *bcl*-2 mRNA levels were determined by real-time quantitative RT-PCR in ABI 7000 Prism (Applied Biosystems), using the manufacturer's suggested protocol. At the end of the runs, melting curves were obtained to make sure that there were no primer-dimer artifacts. The products were verified by agarose gel electrophoresis analysis as well. The threshold cycle values were calculated with the standard software (Applied Biosystems), and quantitative fold changes in mRNA were determined relative to β-actin in each treatment group. All primers (Table 1) for RT-PCR experiments were designed using Oligo software (National Biosciences, Plymouth, Minn). The level of β-actin gene expression served as an internal control.

Antibodies

Monoclonal antibody against β-actin (Sigma) was used to standardize cytosolic protein loading on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anti-cytochrome *c* oxidase subunit IV (COX4) antibody (Molecular Probes, Eugene, Ore) was used to standardize the mitochondrial protein level. COX4 is a membrane protein in the inner mitochondrial membrane, and it remains in the mitochondria regardless of activation of apoptosis.[16,17] Antibodies against α-spectrin (Affiniti, Exeter, UK), and phospho-p38 MAPK (Promega, Madison, Wis), were also used. All other primary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, Calif) and Calbiochem (Gibbstown, NJ). The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig) G (ICN Biomedicals, Aurora, Ohio) and horseradish peroxidase-conjugated goat antirabbit IgG (ICN Biomedicals).

Western Blotting

Western blotting was performed as we described previously.[16,17] The isolation of cytosolic, mitochondrial, and nuclear fractions was performed by standard procedures.[16,17] Cytochrome *c* in the supernatants and pellets and also caspase-activated DNase in nuclear fractions were analyzed by Western blotting. The autoradiograms were scanned using Photoshop software (Adobe Systems, Seattle, Wash), and optical density of each band was determined using Quantity 1 software (Bio-Rad, Hercules, Calif).

Determination of ROS Production

The fluorescent probe 2′,7″-dichlorofluorescein diacetate was used for assessment of intracellular ROS production. This is a reliable method for measurement of intracellular ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical, and hydroperoxides.[19] Briefly, cells were seeded (1×10^5 cells/well) in 6-well culture plates. The next day, cells were washed twice with Hank's balanced salt solution (GIBCO-Invitrogen) and loaded with 1 mL of 1 × RPMI containing 5 μM of 2′,7″-dichlorofluorescein diacetate and different concentrations of apigenin, (−)-

epigallocatechin, EGCG, or genistein. Cells were then incubated at 37°C for 30 to 1440 minutes, and the fluorescence intensity was measured at 530 nm after excitation at 480 nm in Spectramax Gemini XPS (Molecular Devices, Sunnyvale, Calif). The increase in fluorescence intensity was used to assess the generation of net intracellular ROS.

Change in $\Delta\Psi_m$

Loss of $\Delta\Psi_m$ was measured by using the fluorescent probe JC-1. Control cells and cells treated with apigenin, (−)-epigallocatechin, EGCG, or genistein were incubated in medium containing 5 μg/mL JC-1 during treatment from 0.5 hours to 24 hours (30-1440 minutes).[19] After being stained, the cells were washed twice with PBS. When excited at 488 nm, the fluorescence emission of JC-1 was measured at wavelengths corresponding to its monomer (530 ± 15 nm) and *J* aggregate (>590 nm) forms. Fluorescence was measured in a fluorescent plate reader (Molecular Devices, Sunnyvale, Calif).

Colorimetric Assays for Caspase Activities

Measurements of caspase activities in cells were performed using the commercially available caspase-8, caspase-9, and caspase-3 assay kits (Sigma). The colorimetric assays were based on the hydrolysis of the Ac-IETD *p*-nitroaniline by caspase-8, Ac-LEHD *p*-nitroaniline by caspase-9, and Ac-DEVD *p*-nitroaniline by caspase-3, resulting in the release of the *p*-nitroaniline moiety. Proteolytic reactions were carried out in extraction buffer containing 200 μg of cytosolic protein extract and 40 μM Ac-IETD *p*-nitroaniline, 40 μM Ac-LEHD *p*-nitroaniline, or 40 μM Ac-DEVD *p*-nitroaniline. The reaction mixtures were incubated at room temperature for 2 hours, and the formation of *p*-nitroaniline was measured at 405 nm in a colorimeter. The concentration of the *p*-nitroaniline released from the substrate was calculated from the absorbance values. Experiments were performed in triplicate.

Trypan Blue Dye Exclusion Assay After Pretreatment With Inhibitors

Loss of membrane integrity was determined on the basis of inability of cells to exclude the vital dye trypan blue. For inhibitor studies, cells were cultured as described before and either left untreated or were pretreated (1 hour) with 5 μM ascorbic acid (ASC), 10 μM caspase-8 inhibitor II, 10 μM calpeptin, 10 μM caspase-9 inhibitor I, and 10 μM caspase-3 inhibitor IV. After 24 hours, cells were removed from each treatment, diluted (1:1) with trypan blue (Sigma), and counted (at least 600 cells) for calculation of percentage of residual cell viability.

Statistical Analysis

All results obtained from different treatments were analyzed using StatView software (Abacus Concepts, Berkeley, Calif). Data were expressed as mean + standard error of mean of separate experiments ($n \geq 3$) and compared by 1-way analysis of variance followed by Fisher post hoc test.

RESULTS

Morphological and Biochemical Features of Apoptotic Death

We examined morphological and biochemical features of apoptotic death (Fig. 1). Treatment of T98G and U87MG cells with 50 μM apigenin, 50 μM (−)-epigallocatechin, 50 μM EGCG, or 50 μM genistein increased apoptotic cells, based on morphological (Wright staining) and biochemical (ApopTag assay) features (Fig. 1A and B). Quantification based on ApopTag assay indicated that treatment of cells with a flavonoid significantly increased apoptosis (Fig. 1C).

Flavonoids Induced ROS Production and p38 MAPK Phosphorylation for Apoptosis

We determined ROS production and p38 MAPK phosphorylation after the treatments (Fig. 2). To examine ROS production in apoptosis, we measured fluorescence intensity (as a measure of ROS production) resulting from the oxidation of dichlorofluorescin in T98G and U87MG cells after exposure to a flavonoid (apigenin, [−]-epigallocatechin, EGCG, or genistein). Time-dependently, flavonoid treatment promoted the oxidation of dichlorofluorescin in T98G (Fig. 2A) and U87MG (Fig. 2B) cells, and inclusion of 5 μM ASC pretreatment completely blocked ROS production (data not shown). The results suggested that these flavonoids induced apoptosis by a mechanism that required increase in intracellular ROS levels. Western blotting demonstrated that treatment with flavonoid increased phosphorylation of p38 MAPK in T98G (Fig. 2C) and U87MG (Fig. 2D) cells. Pretreatment with 5 μM ASC (an antioxidant) completely blocked an increase in phosphorylation of p38 MAPK (Fig. 2C and D), indicating involvement of ROS in generation of phosphorylation of p38 MAPK. We did not see any change in p38 MAPK because of treatments.

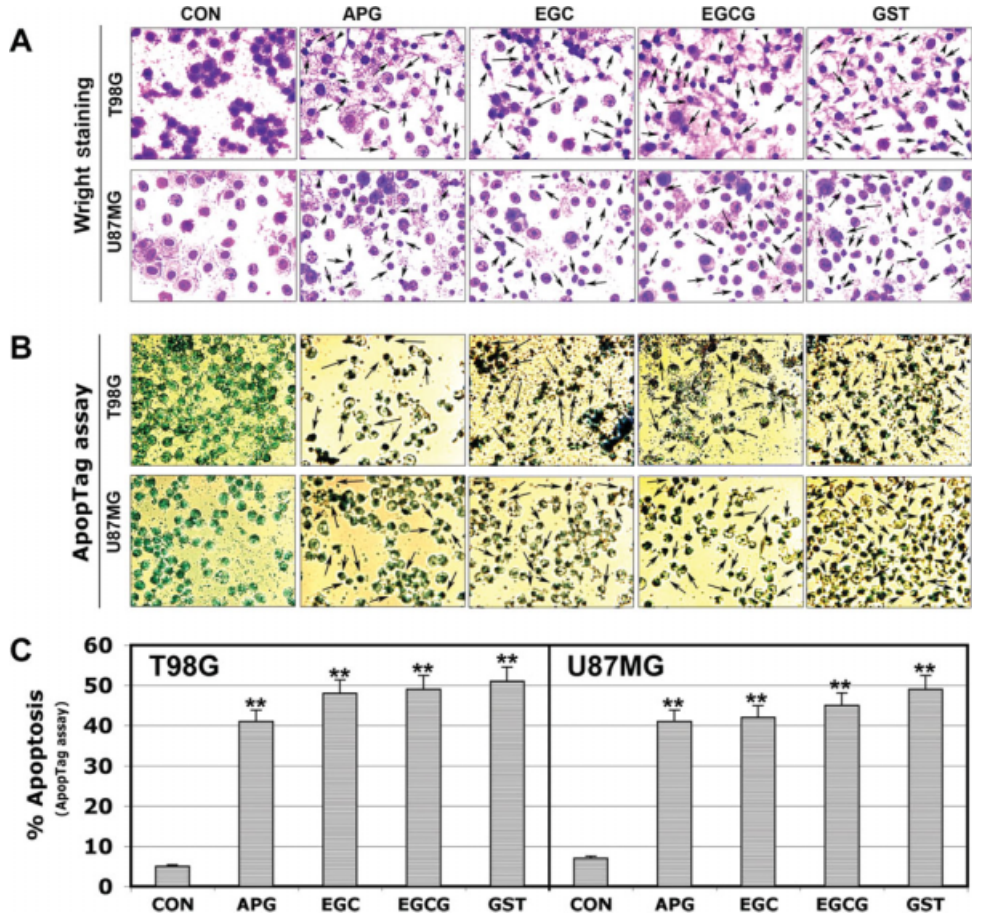


Figure 1. Morphological and biochemical features of apoptosis in T98G and U87MG cells are shown. Treatments (24 hours) were as follows: control (CON), 50 μM apigenin (APG), 50 μM (−)-epigallocatechin (EGC), 50 μM (−)epigallocatechin-3-gallate (EGCG), and 50 μM genistein (GST). (A) Wright staining is shown. (B) ApopTag assay is shown. (C) A bar diagram shows percentage of apoptosis based on ApopTag assay. Arrows indicate apoptotic cells. $^{**}P < .01$, significant difference between control and flavonoid (APG, EGC, EGCG, or GST) treatment.

Treatment with 4-(4-fluoropheny)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580), the specific inhibitor of phosphorylation of p38 MAPK, has previously been reported to show no inhibitory action on p42 of 44 MAPK and JNK1.[20] We found that pretreatment of cells with 5 μM SB203580 almost completely blocked apoptotic death (data not shown). Thus, ROS was required for promotion of phosphorylation of p38 MAPK for apoptosis after exposure to flavonoids.

Flavonoids Activated JNK1 and Suppressed Expression of Antiapoptotic and Inflammatory Proteins

The active form of JNK1 is phosphorylated JNK1, which regulates the transcription of several genes for cell death. Our Western blotting revealed an increase in 46-kD phosphorylated JNK1 after treatment with a flavonoid (Fig. 3). The involvement of activated JNK1 pathway in cell death process was confirmed in our experiments using cell-permeable JNK inhibitor I. Pretreatment of cells with 10 μM JNK inhibitor I provided protection from flavonoid (apigenin, [−]-epigallocatechin, EGCG, or genistein)-mediated cell death (data not shown), indicating involvement of JNK1 pathway in cell death. Our Western blotting also showed decreases in 60-kD phosphorylation of Akt at Ser-473 and 26-kD Bcl-2 (antiapoptotic protein)[21] in glioblastoma cells after treatment with flavonoids (Fig. 3). Almost uniform expression of total 60-kD Akt was detected in treated cells. Thus, our results showed suppression of Akt and Bcl-2 survival pathways during apoptosis. Because NF-κB and cyclooxygenase-2 (COX-2) are involved in the inflammation and potentiation of cell

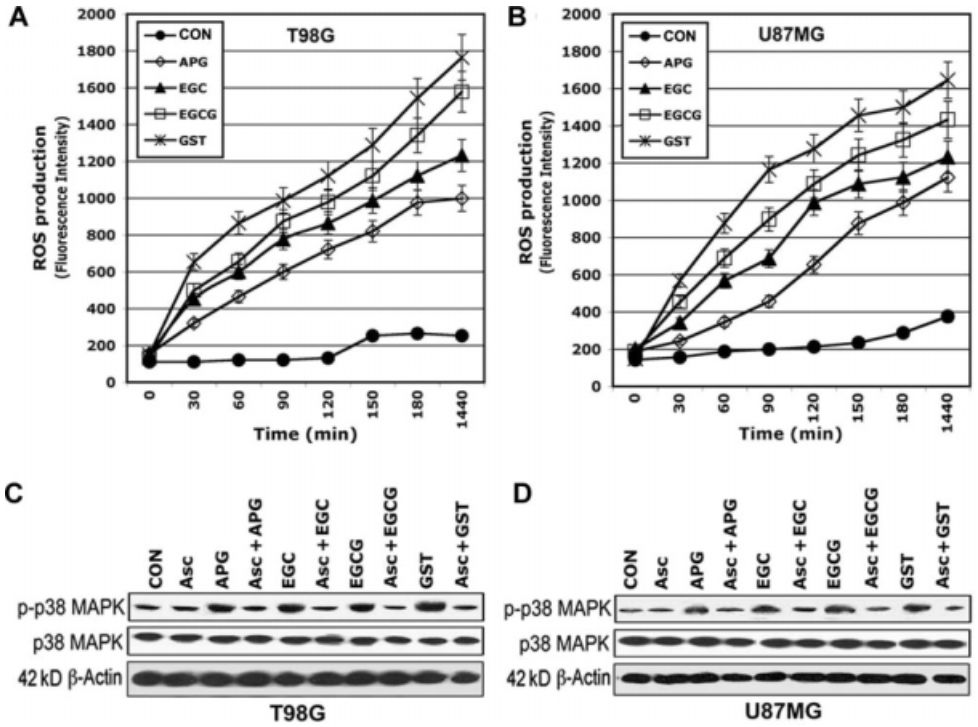


Figure 2. Determination of reactive oxygen species (ROS) production and p38 mitogen-activated protein kinase (MAPK) phosphorylation in T98G and U87MG cells are shown. Treatments (0, 30, 60, 90, 120, 150, 180, and 1440 minutes) in the presence of 5 μM 2,7-dichlorofluorescin diacetate were as follows: control (CON), 50 μM apigenin (APG), 50 μM (−)-epigallocatechin (EGC), 50 μM (−)epigallocatechin-3-gallate (EGCG), and 50 μM genistein (GST). (A) ROS production in T98G cells is shown. (B) ROS production in U87MG cells is shown. Western blotting was used to show levels of phosphorylation of p38 MAPK, p38 MAPK, and β-actin in (C) T98G cells and (D) U87MG cells after the following treatments (24 hours): CON, 10 μM ascorbic acid (Asc), 50 μM APG, 10 μM Asc (1-hour pretreatment) + 50 μM APG, 50 μM EGC, 10 μM Asc (1-hour pretreatment) + 50 μM EGC, 50 μM EGCG, 10 μM Asc (1-hour pretreatment) + 50 μM EGCG, 50 μM GST, and 10 μM Asc (1-hour pretreatment) + 50 μM GST. Pretreatment with Asc prevented phosphorylation of p38 MAPK.

growth in human glioblastoma, we examined expression of NF-κB and COX-2 after exposure to flavonoids. Our results demonstrated down-regulation of 65-kD NF-κB and 70-kD COX-2. β-Actin expression was monitored to ensure that equal amounts of protein were loaded in all lanes.

(−)-Epigallocatechin and EGCG Induced Caspase-8 Activation and Proteolytic Cleavage of Bid

To determine the involvement of the death receptor pathway of apoptosis, we examined activation and activity of caspase-8 in glioblastoma cells after treatments (Fig. 4). Our results showed an increase in the active 18-kD caspase-8 band in T98G and U87MG cells because of treatment with (−)-epigallocatechin and EGCG (Fig. 4A). But we did not observe any increase in the active 18-kD caspase-8 band in cells treated with apigenin and genistein (Fig. 4A). β-Actin expression was monitored to ensure that an equal amount of cytosolic protein was loaded in each lane. Caspase-8 activation induced proteolytic cleavage of Bid to tBid, which could translocate from cytosol to mitochondrial membrane to stimulate efficient oligomerization of Bax and thereby activate the mitochondrial pathway of apoptosis. We examined the level of tBid in the mitochondrial fraction and also monitored the level of COX4 as a mitochondrial internal control (Fig. 4A). We found a dramatic increase in tBid in mitochondria in both T98G and U87MG cells treated with (−)-epigallocatechin and EGCG (Fig. 4A), indicating an increase in caspase-8 activity. Furthermore, colorimetric assay showed a significant increase in total caspase-8 activity in cells treated with (−)-epigallocatechin and EGCG (Fig. 4B).

Apoptosis via Mitochondrial Pathway

Apoptosis may occur with involvement of mitochondria, which release cytochrome *c* and other proapoptotic factors

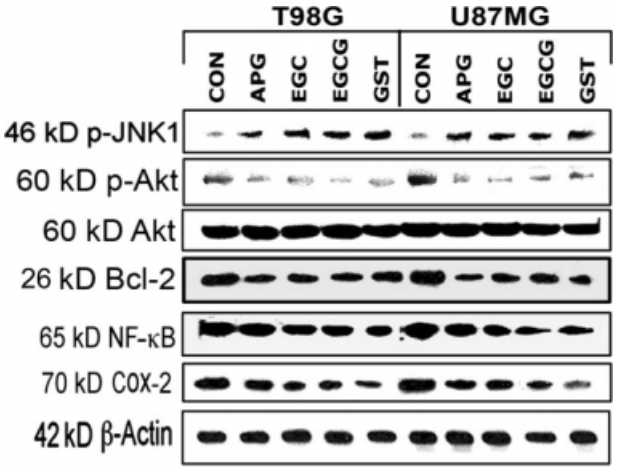


Figure 3. Western blotting is shown for the levels of phosphorylated *c-Jun* N-terminal kinase 1 (JNK1), survival factors (p-Akt and p-Bcl-2), and inflammatory factors (nuclear factor kappa B [NF-κB] and cyclooxygenase-2 [COX-2]) in T98G and U87MG cells. Treatments (24 hours) were as follows: control (CON), 50 μM apigenin (APG), 50 μM (−)-epigallocatechin (EGC), 50 μM (−)epigallocatechin-3-gallate (EGCG), and 50 μM genistein (GST). Protein levels are shown for phosphorylated JNK1, p-Akt, total Akt, Bcl-2, NF-κB, COX-2, and β-actin.

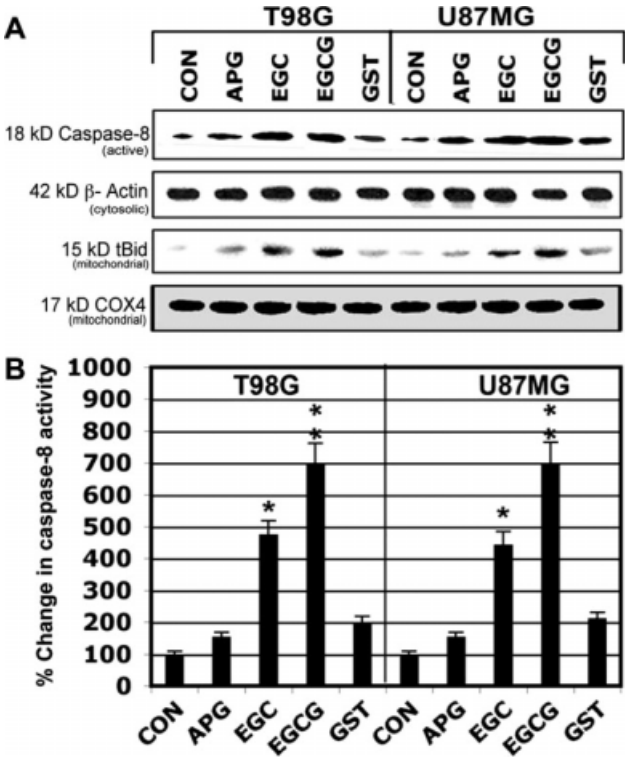
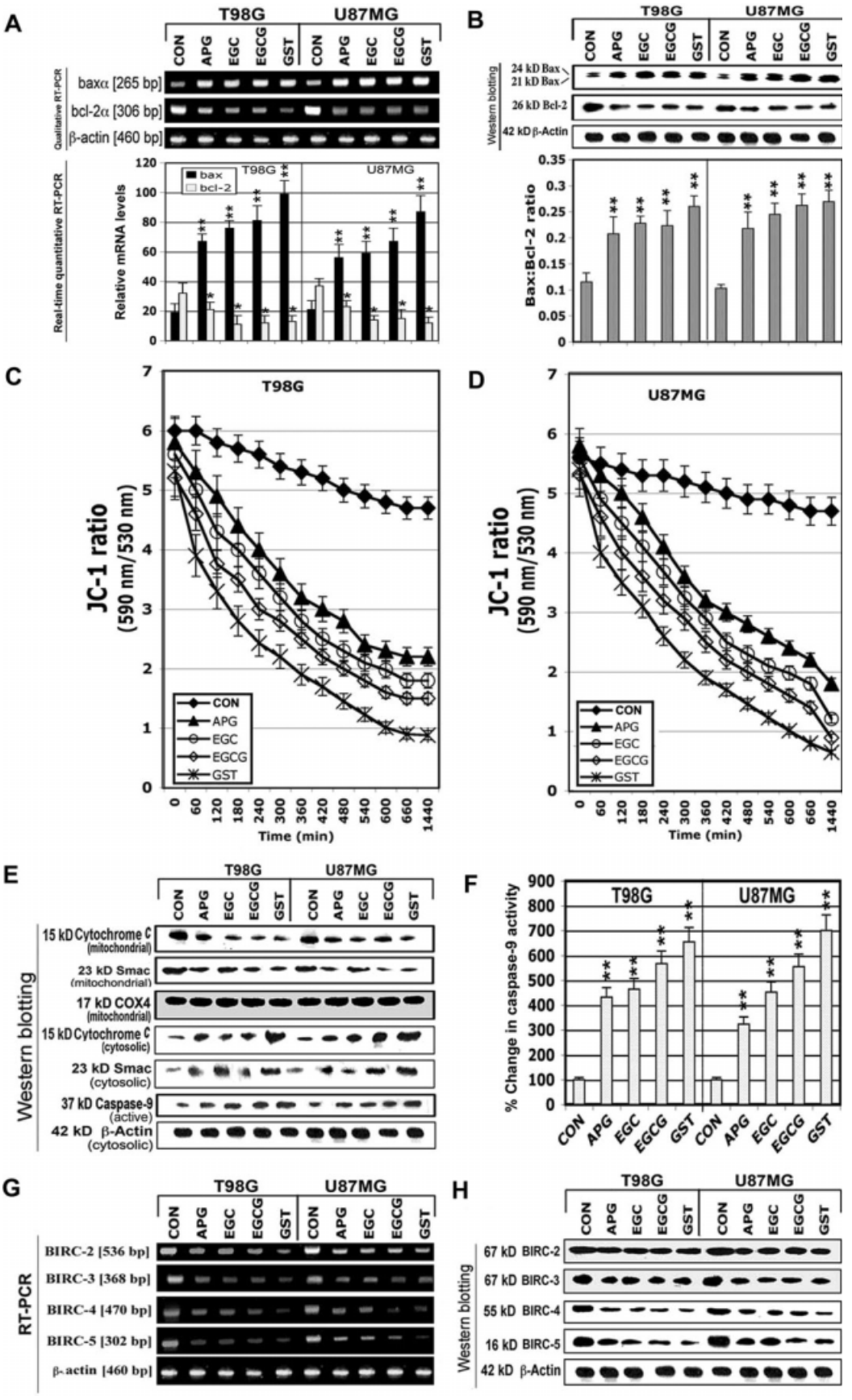


Figure 4. Determination of caspase-8 activation and activity in T98G and U87MG cells is shown. Treatments (24 hours) were as follows: control (CON), 50 μM apigenin (APG), 50 μM (−)-epigallocatechin (EGC), 50 μM (−)epigallocatechin-3-gallate (EGCG), and 50 μM genistein (GST). (A) Western blotting shows levels of caspase-8, β-actin, tBid, and cytochrome *c* oxidase subunit IV (COX4). (B) Colorimetric determination of caspase-8 activity is shown. $^{*}P < .05$, $^{**}P < .01$, significant difference between control and flavonoid (APG, EGC, EGCG, or GST) treatment.

during different forms of cellular stress.[22] We examined the involvement of mitochondrial events in apoptosis in glioblastoma cells after the treatments (Fig. 5). Treatment of cells with apigenin, (−)-epigallocatechin, EGCG, and genistein increased Bax but decreased Bcl-2 expression at mRNA and protein levels (Fig. 5A). Real-time quantitative RT-PCR experiments were carried out to determine the relative mRNA levels of the *bax* and *bcl-2* genes. Expression of β-actin was used to normalize the values. Similar changes in mRNA levels were observed in both qualitative and quantitative RT-PCR (Fig. 5A). On the basis of Western blotting, we measured the Bax:Bcl-2 ratio, which was significantly increased after treatments (Fig. 5B). Mitochondrial damage is often associated with loss of mitochondrial membrane potential ($\Delta\Psi_m$), which is readily measured using JC-1 staining.[13] Control T98G and U87MG cells showed a high JC-1 ratio (590 nm/530 nm). After treatments, the mean red and green fluorescence ratio in the mitochondria dropped slowly in a biphasic way, indicating the collapse of the $\Delta\Psi_m$ during apoptosis in T98G (Fig. 5C) and U87MG (Fig. 5D) cells. This collapse of $\Delta\Psi_m$ was associated with mitochondrial release of 15-kD cytochrome *c* and 23-kD Smac into the cytosol (Fig. 5E) to cause activation of caspases. We used

Figure 5. Components involved in the mitochondrial pathway of apoptosis in T98G and U87MG cells are examined. Treatments (24 hours) were as follows: control (CON), 50 μM apigenin (APG), 50 μM (−)-epigallocatechin (EGC), 50 μM (−)epigallocatechin-3-gallate (EGCG), and 50 μM genistein (GST). (A) mRNA levels of *bax* and *bcl-2* were determined by qualitative reverse transcriptase polymerase chain reaction (RT-PCR) as well as by real-time quantitative RT-PCR. (B) Western blotting for examination of Bax and Bcl-2 expression in protein levels and determination of Bax:Bcl-2 ratio based on Western blotting are shown. JC-1 ratio (590 nm/530 nm) is shown in (C) T98G and (D) U87MG cells after the treatments for different times (30, 60, 120, 180, 240, 300, 360, 420, 480, 540, 600, 660, and 1440 minutes). (E) Western blotting shows levels of cytochrome *c*, Smac, cytochrome *c* oxidase subunit IV (COX4), caspase-9, and β-actin. (F) Determination of caspase-9 activity using a colorimetric assay is shown. (G) Qualitative RT-PCR and (H) Western blotting show mRNA and protein levels of BIRC2-BIRC5 and β-actin. $^{**}P < .01$, significant difference between control and flavonoid (APG, EGC, EGCG, or GST) treatment.


A
T98G
U87MG
CON
APG
EGC
EGCG
GST
Qualitative RT-PCR
baxα [265 bp]
bcl-2α [306 bp]
β-actin [460 bp]
Real-time quantitative RT-PCR
Relative mRNA levels
bax
bcl-2
B
Western blotting
24 kD Bax
21 kD Bax
26 kD Bcl-2
42 kD β-Actin
Bax:Bcl-2 ratio
C
T98G
JC-1 ratio
(590 nm/530 nm)
Time (min)
D
U87MG
E
Western blotting
15 kD Cytochrome C (mitochondrial)
23 kD Smac (mitochondrial)
17 kD COX4 (mitochondrial)
15 kD Cytochrome C (cytosolic)
23 kD Smac (cytosolic)
37 kD Caspase-9 (active)
42 kD β-Actin (cytosolic)
F
% Change in caspase-9 activity
G
RT-PCR
BIRC-2 [536 bp]
BIRC-3 [368 bp]
BIRC-4 [470 bp]
BIRC-5 [302 bp]
β-actin [460 bp]
H
Western blotting
67 kD BIRC-2
67 kD BIRC-3
55 kD BIRC-4
16 kD BIRC-5
42 kD β-Actin

COX4 as a loading control for mitochondrial protein. We observed an increase in active 37-kD caspase-9 fragment in cells after treatments (Fig. 5E). β-Actin expression was used to ensure that an equal amount of cytosolic protein was loaded in each lane. A colorimetric assay confirmed a significant increase in total caspase-9 activity for apoptosis in cells after treatments (Fig. 5F). In response to apoptotic stimuli, Smac is released from mitochondria into the cytosol to block inhibitory function of the baculoviral inhibitor-of-apoptosis repeat-containing (BIRC) proteins so as to promote caspase-9 activation.[17] We examined the expression of BIRC molecules at mRNA (Fig. 5G) and protein (Fig. 5H) levels to understand their roles in apoptosis in T98G and U87MG cells after the treatments. The decreases in BIRC2-BIRC5 at mRNA and protein levels correlated well with the increases in cytosolic Smac. Together, these results suggested that loss of $\Delta\Psi_m$, mitochondrial release of cytochrome *c* and Smac into the cytosol, and subsequent activation of caspase-9 played key roles in apoptosis in glioblastoma cells.

Increase in Intracellular Free Ca^{2+} and Activation of Caspase-4, Calpain, and Caspase-3 for Apoptosis

We explored the contribution of intracellular free Ca^{2+}, capsase-4, calpain, and caspse-3 to apoptosis in glioblastoma cells (Fig. 6). Fura-2 assay showed significant increases of intracellular free Ca^{2+} in T98G and U87MG cells after treatments with the flavonoids (Fig. 6A). These results suggested a role for intracellular free Ca^{2+} in apoptosis. Human caspase-4 is localized to endoplasmic reticulum (ER) membrane and activated during ER stress.[23] We found involvement of ER stress and activation of caspase-4 during apoptosis when cells were treated with the flavonoids (Fig. 6B). The Ca^{2+}-dependent protease calpain was overexpressed and activated, leading to degradation of calpastatin, the endogenous inhibitor of calpain, in glioblastoma cells after exposure to the flavonoids (Fig. 6B). The final executioner caspase-3 was also overexpressed and activated for mediation of apoptosis (Fig. 6B). Degradation of 270-kD α-spectrin to 145-kD spectrin breakdown product and 120-kD spectrin breakdown product has been attributed to proteolytic activities of calpain and caspase-3, respectively.[16-19] Cells treated with flavonoids produced 145-kD spectrin breakdown product and 120-kD spectrin breakdown product (Fig. 6B), indicating increased activities of calpain and caspase-3, respectively. Increased activity of caspase-3 also cleaved inhibitor of caspase-activated DNase to release and translocate caspase-activated DNase to the nucleus (Fig. 6B) to cause degradation of nuclear DNA. Almost uniform expression of β-actin served as a loading control for cytosolic proteins. We stained 1 set of gel with Coomassie Blue to ensure loading of equal amounts of nuclear proteins in all lanes (Fig. 6B). A colorimetric assay indicated very significant increases in total caspase-3 activity in glioblastoma cells after exposure to flavonoids (Fig. 6C).

Prevention of Cell Death by Pretreatment With Different Inhibitors

Pretreatment of glioblastoma cells for 1 hour with Asc and caspase-3 inhibitor IV inhibited cell death caused by treatment with flavonoids (Fig. 7). In contrast, caspase-9 inhibitor I showed a partial inhibitory effect. Pretreatment of cells with calpeptin (calpain inhibitor) blocked cell death in apigenin and genistein treatments, whereas caspase-8 inhibitor II blocked cell death in (−)-epigallocatechin and EGCG treatments. Blocking of >60% cell death occurred by calpeptin in cells treated with (−)-epigallocatechin and EGCG, and by caspase-8 inhibitor II in cells treated with apigenin and genistein (Fig. 7). These results indicated the roles of ROS production and also calpain and caspase activities in cell death.

Human Normal Astrocytes Remained Resistant to Flavonoids

We examined the effects of flavonoids on human normal astrocytes (Fig. 8). Residual cell viability was determined by trypan blue dye exclusion test under the light microscope after treatment of human normal astrocytes with the flavonoids for 24 hours. Compared with control cells, cells treated with flavonoids did not show any significant change in viability (Fig. 8A). These results demonstrated that apigenin, (−)-epigallocatechin, EGCG, and genistein were cytotoxic to glioblastoma T98G and U87MG cells but not to human normal astrocytes. Time-dependently, flavonoid treatment did not promote considerable oxidation of dichlorofluorescin in human normal astrocytes (Fig. 8B). The results suggested that flavonoids did not sufficiently increase intracellular ROS levels in human normal astrocytes to induce cell death. Colorimetric assays showed insignificant changes in activities of caspase-8, caspase-9, and caspase-3 in human normal astrocytes after treatment with flavonoids (Fig. 8C). These results indicated that human normal astrocytes did not commit apoptosis following exposure to flavonoids.

DISCUSSION

Flavonoids have emerged as potential therapeutic agents for treatment of cancers, especially because of their ability to induce apoptosis. In this investigation, we demonstrated that flavonoids (apigenin, [−]-epigallocatechin, EGCG, and genistein) induced apoptosis in human glioblastoma T98G and U87MG cells (Figs. 1-7) without affecting the human normal astrocytes (Fig. 8). We explored the molecular mechanisms of induction of apoptosis in glioblastoma T98G and U87MG cells after exposure to these flavonoids.

Diverse chemotherapeutic agents can induce apoptosis in cancer cells via death receptor and mitochondrial pathways because of increased ROS generation.[24] Phosphorylation of p38 MAPK induces apoptosis, whereas phosphorylation of p42 of 44 MAPK exerts cytoprotective effects.[22,23] The addition of ASC completely blocked the phosphorylation of p38 MAPK, suggesting an involvement of ROS in phosphorylation of p38 MAPK (Fig. 2). Thus, production of ROS provided a signal for selective phosphorylation of p38 MAPK and induction of apoptosis in glioblastoma cells after treatment with flavonoids.

Our data showed activation or phosphorylation of JNK1 in flavonoid-treated glioblastoma cells, which correlated with production of ROS, suggesting an essential role of phosphorylated JNK1 in induction of apoptosis (Fig. 3). We also observed that flavonoids down-regulated expression of antiapoptotic protein Bcl-2 and activation of the key antiapoptotic kinase Akt (Fig. 3). NF-κB and COX-2 are known to be involved in the inflammatory responses. Dysregulation of the NF-κB and COX-2 pathways plays an important role in the development of various cancers.[24,25] Our results demonstrated that flavonoids suppressed expression of both NF-κB and COX-2 in glioblastoma cells (Fig. 3).

Cleavage of Bid to tBid as a result of caspase-8 activation promotes Bax-mediated mitochondrial release of cytochrome *c*. We found caspase-8 activation and also its

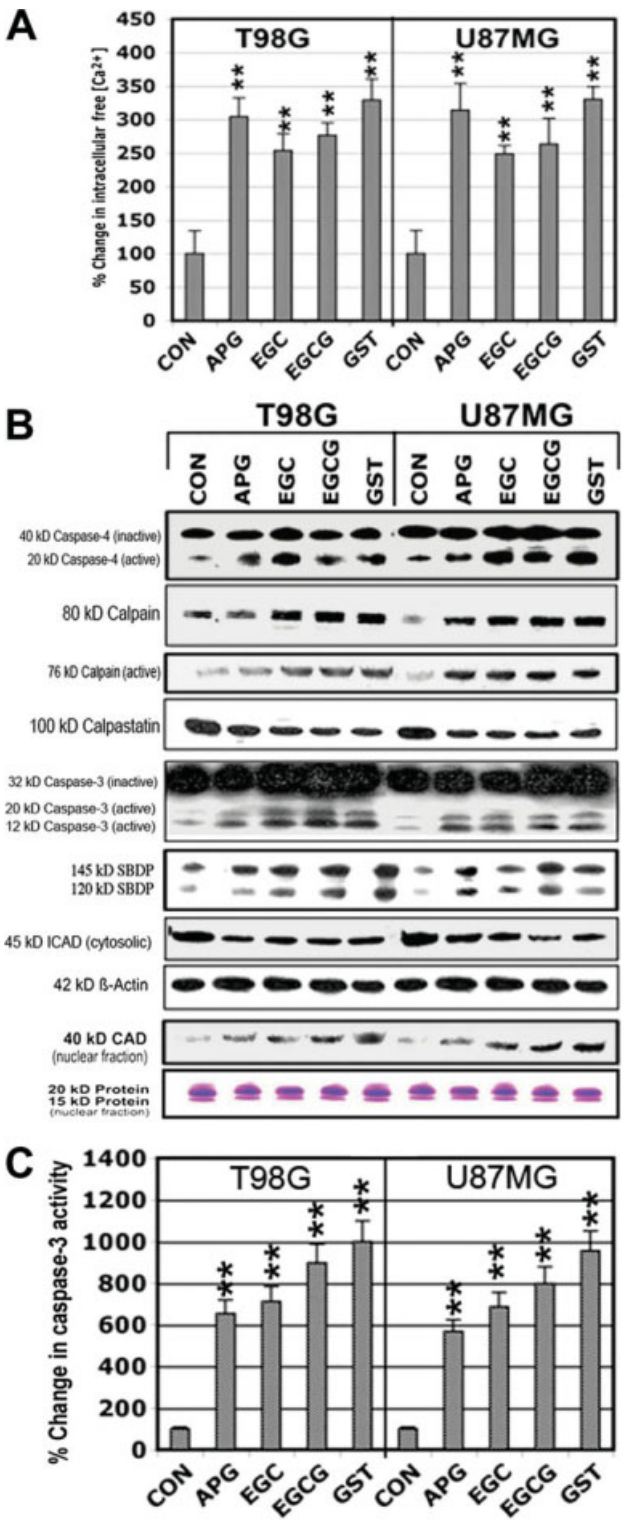


Figure 6. Increase in intracellular free Ca^{2+} and activation of caspase-4, calpain, and caspase-3 in T98G and U87MG cells are examined. Treatments (24 hours) were as follows: control (CON), 50 μM apigenin (APG), 50 μM (−)-epigallocatechin (EGC), 50 μM (−)epigallocatechin-3-gallate (EGCG), and 50 μM genistein (GST). (A) Determination of intracellular free Ca^{2+} is shown. (B) Western blotting shows levels of caspase-4, inactive and active calpain, calpastatin, spectrin breakdown product (SBDP), inactive and active caspase-3, inhibitor of caspase-activated DNase (ICAD), caspase-activated DNase (CAD), and β-actin. (C) Determination of caspase-3 activity by a colorimetric assay is shown. $^{**}P < .01$, significant difference between control and flavonoid (APG, EGC, EGCG, or GST) treatment.

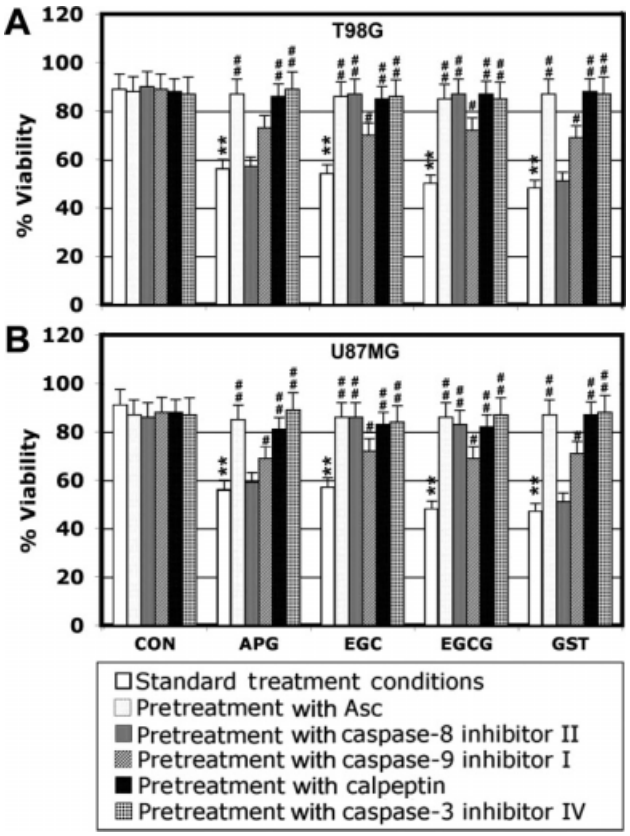


Figure 7. Pretreatment with selective inhibitors prevented flavonoid-mediated cell death in T98G and U87MG cells. Treatments (24 hours) were as follows: control (CON), 50 μM apigenin (APG), 50 μM (−)-epigallocatechin (EGC), 50 μM (−)epigallocatechin-3-gallate (EGCG), and 50 μM genistein (GST) without any inhibitor and with 1-hour pretreatment with 5 μM ascorbic acid (Asc), 10 μM caspase-8 inhibitor II, 10 μM caspase-9 inhibitor I, 10 μM calpeptin (calpain-specific inhibitor), or 10 μM caspase-3 inhibitor IV. Determination of percentage of viability is shown in (A) T98G cells and (B) U87MG cells. **P < .01, significant difference between control and flavonoid (APG, EGC, EGCG, or GST) treatment. #*P* < .05, ##*P* < .01, significant difference between flavonoid (APG, EGC, EGCG, or GST) treatment and inhibitor pretreatment (1 hour) + flavonoid (APG, EGC, EGCG, or GST) treatment.

activity in the cleavage of Bid to tBid in T98G and U87MG cells treated with (−)-epigallocatechin and EGCG but not with apigenin and genistein (Fig. 4). Translocation of tBid from cytosol to mitochondrial membrane could stimulate more efficient oligomerization of Bax for inducing the mitochondrial pathway of apoptosis. The relative levels of Bax (proapoptotic) and Bcl-2 (antiapoptotic) in mitochondria determine the fate of cells. Heterodimerization of Bax with Bcl-2 prevents apoptosis, whereas Bax homodimerization triggers an apoptotic process with the mitochondrial release of cytochrome *c* and Smac into the cytosol. After flavonoid treatments, an

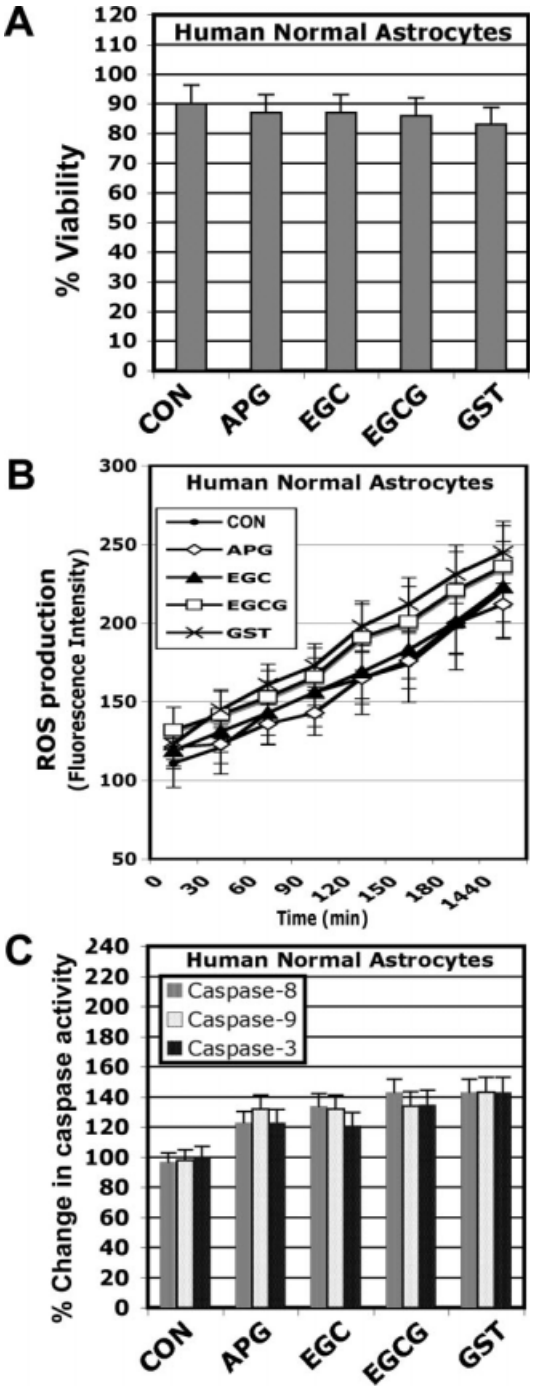


Figure 8. Effects of flavonoids on human normal astrocytes are shown. Treatments (24 hours) were as follows: control (CON), 50 μM apigenin (APG), 50 μM (−)-epigallocatechin (EGC), 50 μM (−)epigallocatechin-3-gallate (EGCG), and 50 μM genistein (GST). (A) Percentage of cell viability was determined by trypan blue dye exclusion test. (B) Reactive oxygen species (ROS) production was determined in human normal astrocytes at different time intervals (0, 30, 60, 90, 120, 150, 180, and 1440 minutes). (C) Colorimetric assays determined caspase-8, caspase-9, and caspase-3 activities.

increase in Bax:Bcl-2 ratio and decrease in $\Delta\Psi_m$ triggered accumulation of cytosolic cytochrome *c*, increasing activation and activity of caspase-9 (Fig. 5). Because cytosolic Smac could suppress survival effects of BIRC proteins, we examined alteration in the levels of BIRC proteins in T98G and U87MG cells after treatment with flavonoids and found that some of these survival proteins (BIRC2-BIRC5) were down-regulated for promoting apoptosis (Fig. 5).

Our findings support a direct relationship among increases in intracellular free Ca^{2+}, ER stress (caspase-4 activation), calpain activation, and caspase-3 activation for apoptosis in glioblastoma cells after exposure to flavonoids (Fig. 6). Increased calpain activity cleaved calpastatin and also generated 145-kD spectrin breakdown product, whereas increased caspase-3 activity generated 120-kD spectrin breakdown product and cleaved inhibitor of caspase-activated DNase to release and translocate caspase-activated DNase to the nucleus (Fig. 6). We used specific inhibitors to confirm that glioblastoma cells committed cell death because of ROS production and activation of caspase-8, caspase-9, calpain, and caspase-3 (Fig. 7). On the basis of our results, we suggest that apigenin, (−)-epigallocatechin, EGCG, and genistein activate multiple pathways for induction of apoptosis in glioblastoma cells, but not in human normal astrocytes. It should be noted that flavonoids do not require a p53-dependent pathway for mediation of apoptosis, because they are capable of inducing apoptosis in both T98G (mutant p53) and U87MG (wild-type p53) cells.

In conclusion, our results demonstrated that flavonoids (apigenin, [−]-epigallocatechin, EGCG, and genistein) induced apoptosis in T98G and U87MG cells via multiple mechanisms, including increase in ROS production, activation of kinases, down-regulation of survival pathways and inflammatory factors, and activation of death receptor and mitochondrial pathways. Our data suggested the following relative potency of these flavonoids (at 50 μM): apigenin < (−)-epigallocatechin < EGCG < genistein for induction of apoptosis in T98G and U87MG cells. Finally, these flavonoids induced apoptosis in human glioblastoma cells but not in normal astrocytes, indicating their selective action for controlling glioblastoma. This approach of using flavonoids for induction of apoptosis specifically in glioblastoma cells is particularly appealing, because currently a major obstacle in cancer therapy is extensive drug toxicity to the normal cells.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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