Genistein Induces Apoptosis in T Lymphoma Cells via Mitochondrial Damage

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Abstract: The soy isoflavone genistein has been identified as having antiproliferative and apoptotic effects on various malignant cell types derived from solid tumors. Because little information regarding the effect of genistein on hematopoietic malignancies is available, we undertook this study of T-cell lymphomas. We tested the effect of genistein on murine T-cell lines derived from thymic lymphomas induced by an oncogenic murine leukemia virus. When T lymphoma cells were treated with genistein concentrations of 15 μM and greater, it was observed that the percentage of viable cells was significantly reduced in a dose- and time-dependent manner. The observed cell killing was found to be the result of apoptosis as detected by flow cytometric analysis of cells stained with annexin V and propidium iodide and assays for caspase-3 activation and DNA fragmentation. Cell staining with the mitochondrial specific dye JC-1 and detection of caspase-9 activation revealed that genistein produced mitochondrial depolarization as an early step in the induction of apoptosis. Bongkrekic acid inhibition of mitochondrial depolarization identified the mitochondria permeability transition pore (PTP) as a potential target of genistein activity. These results indicate that the induction of apoptosis by pharmacological concentrations of genistein in T lymphoma cells occurs via mitochondrial damage with the involvement of the PTP.

Introduction

Epidemiological evidence has implicated high-soy diets as playing a role in the observed lower incidence of certain cancers within Asian populations (1,2). It is thought that the anticancer effect of soy is due primarily to the isoflavone genistein (3,4). Studies of the effects of genistein on different types of malignant cells have been carried out in both in vitro and in vivo experiments (5–10). These studies have shown that genistein is able to induce apoptosis in various types of malignant cells. This activity of genistein could be potentially exploited in the treatment of tumors that have become resistant to chemotherapeutic drugs and radiation therapy. Most of the aforementioned studies were performed with malignant cells derived from solid tumors, such as breast and prostate (5,10–12). Because little is known about the effects of genistein on hematopoietic malignancies, this study was undertaken to identify the effects of genistein on T-cell lymphomas.

A major apoptotic pathway, the intrinsic pathway, involves mitochondria as a point of initiation of programmed cell death (13–15). Some of the apoptotic stimuli that function via this pathway include ultraviolet radiation, DNA damage, serum starvation, and various chemotherapeutic agents (15). Damage to the mitochondrial membrane by these reagents results in the release of cytochrome c, a mitochondrial protein involved in energy production, into the cytoplasm (16). The binding of cytoplasmic cytochrome c to the apoptosis activating factor-1 and ATP results in the recruitment and activation of procaspase-9 (17). Activated caspase-9 subsequently results in the activation of caspase-3, which initiates the execution phase of apoptosis.

Mitochondrial damage can occur by different mechanisms, one of which involves the sustained opening of the mitochondrial permeability transition pore (PTP) (13,15,18). The PTP regulates the transient depolarization of the mitochondrial membrane. Its sustained opening leads to mitochondrial depolarization and the influx of solutes, which results in outer membrane damage and cytochrome c release (19). It has been shown that certain stress factors, such as increases in intracellular calcium, generation of reactive oxygen species, and changes in cytosolic pH, induce mitochondrial damage via the PTP (19).

In this study, we have observed that pharmacological concentrations of genistein are able to induce apoptosis in cell lines derived from murine thymic lymphomas in a dose- and time-dependent manner. Investigation into the mechanism of apoptosis by genistein revealed an early involvement of mitochondria via the PTP.

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Materials and Methods

Cell Culture

Dr. Nancy DiFronzo (Children's National Medical Center, Washington, DC) graciously provided T-cell lymphoma lines (92316T, 92284T, and 92290T) that were isolated from thymic lymphomas induced by the MCF247 murine leukemia virus. 92316T and 92284T are clonal cell lines. Cell line 92290T is polyclonal. Cells were maintained in RPMI 1640 supplemented with L-glutamine, 10% fetal bovine serum (FBS), 2 mM sodium pyruvate, 50 μM 2-β-mercaptoethanol, 15 mM HEPES, and 20 units/ml penicillin-streptomycin at 37°C and 5% CO_2 in 75-cm^2 tissue culture flasks. Cells were cultured at 5×10^5 cells per milliliter 24 h prior to the addition of genistein (Toronto Research Chemicals, Toronto, Canada). Cells were washed once with RPMI 1640 and resuspended at 1×10^6 cells per milliliter at the time of genistein exposure. A genistein stock solution was prepared in dimethyl sulfoxide (DMSO) at a concentration of 125 mM. The same volume of DMSO was added to control cells, resulting in a final concentration of DMSO of <0.05%. For bongkrekic acid (BA) studies, cells were treated with 50 μM BA (Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C prior to the addition of genistein.

Single-cell suspensions of lymphocytes were prepared from thymuses of AKR/J mice (Jackson Laboratories, Bar Harbor, ME) by pressing thymic tissue through a wire screen into cold RPMI 1640 containing 2% FBS. Thymic lymphocyte viability at isolation was >90% as determined by trypan blue exclusion.

Flow Cytometric Analysis of Apoptotic Cells and Mitochondrial Depolarization

A quantitative analysis of viable, dead, and apoptotic cell populations was conducted by staining cells with propidium iodide (PI, Sigma-Aldrich) and fluorescein isothiocyanate–conjugated annexin V (annexin V-FITC) (PharMingen, BD Biosciences, San Diego, CA). It has been shown that flow cytometric analysis of cells stained with PI and annexin V-FITC is an effective method to distinguish among live (PI–, annexin V–), apoptotic (PI–, annexin V+), and dead (PI+, annexin V+) cells (20). After genistein treatment at different concentrations for 24 h, 10^5 cells were washed twice with cold phosphate-buffered saline (PBS) and stained with 0.5 μg/ml PI and 0.6 μg/ml annexin V-FITC in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Stained cells were incubated at room temperature (RT) in the dark for 15 min, after which time 400 μl of binding buffer was added. Cells were analyzed by flow cytometry using a Becton Dickson FACScan flow cytometer (Wayne State University and Karmanos Cancer Institute Flow Cytometry Core Facility) within 1 h of staining. Data were collected on 2×10^4 cells using CELLQuest software (Becton Dickinson, BD Biosciences). Gating was estab-

lished on single-color controls. *P* values were calculated by Student's *t*-test.

5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes, Inc., Eugene, OR) was used as an indicator dye for mitochondrial depolarization, and 10^6 cells were treated with 60 μM genistein for 4 h, after which time JC-1 was added to a final concentration of 5 μg/ml for 15 min at RT. Stained cells were pelleted, washed twice in cold PBS, and resuspended in 500 μl PBS. As a positive control, cells were treated with valinomycin (Sigma-Aldrich) at 2 μg/ml final concentration for 1 h at 37°C. Cells were analyzed by two-color flow cytometry. Gating was established with untreated and valinomycin-treated cells.

Caspase-3 Assay

We treated 2×10^7 cells with 0, 15, 30, or 60 μM genistein for different times. Cells were subsequently collected, washed twice with PBS, and lysed in 200 μl 50 mM Tris-HCl, pH 7.5, containing 0.03% Nonidet P-40 and 1 mM dithiothreitol. Cell lysates were centrifuged at 1,200 *g* for 5 min at 4°C to pellet nuclei. Cytosolic fractions were recovered and stored at –70°C. Fifty microliters of the cellular extract was used to assay for caspase-3 activity using the EnzChek caspase-3 assay kit (Molecular Probes, Eugene, OR) in a 96-well plate format. After a 30-min incubation, fluoromethylcoumarin fluorescence, resulting from the cleavage of the Z-DEVD-AMC peptide substrate by caspase-3, was detected with a Spectra MAX Gemini fluorometer (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 350 nm and emission wavelength of 450 nm. An AMC standard curve was prepared in the range of 0.137–17.5 μg of fluorophore. Protein amounts were measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis

We treated 5×10^6 cells with 0, 15, 30, or 60 μM genistein for 24 h, after which time cellular DNA was extracted with the Apoptotic DNA Ladder Kit (Boehringer Mannheim/Roche, Indianapolis, IN). Cells were washed once and resuspended in 200 μl PBS. We added 200 μl lysis buffer (6 M guanidine-HCl, 10 mM Tris-HCl, pH 4.4, 10 mM urea, and 20% Triton X-100) to cells followed with an incubation at RT for 10 min. Samples were mixed with 100 μl isopropanol and centrifuged for 1 min at 8,000 rpm through a glass fiber column. DNA was washed twice and eluted with 200 μl 10 mM Tris-HCl, pH 8.5. We electrophoresed 1.5 μg DNA through a 1.5% agarose gel for 1.5 h at 100 V in 1 × TBE (81 mM Tris base, 81 mM boric acid, and 1.8 mM EDTA). DNA was visualized by staining with 0.5 μg/ml ethidium bromide and exposure to UV light.

Western Blot Analysis

Cells were treated with 0, 15, 30, or 60 µM gensitein for 24 h, after which time total cell or nuclear extracts were prepared as described (21) and measured by the Pierce protein assay. We added 20 µg protein to reducing buffer [62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% sodium dodecylsulfate (SDS), 0.01% bromophenol blue, and 5% β-mercaptoethanol] and boiled for 4 min. Samples were electrophoresed in running buffer of 25 mM Tris, 192 mM glycine, and 1% SDS, pH 8.3, through a 9% SDS-polyacrylamide gel at 120 V for 1.5 h and transferred to polyvinylidene difluoride membrane (BioRad, Hercules, CA) at 350 mA for 2 h at 4°C. Membranes were probed with a rabbit antibody specific for either caspase-9 (9504, Cell Signaling Technology, Beverly, MA) or β-actin (A5441, Sigma-Aldrich) overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit serum (Pierce) for 1 h at RT. Protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ) and visualized on Biomax MR film (Kodak, Rochester, NY).

Results

Genistein Reduces the Viability of Murine T Lymphoma Cells

To determine the effect of genistein on T-cell lymphomas, we examined the viability of cell lines derived from three different murine T-cell lymphomas (92316T, 92284T, and 92290T) after exposure to 15, 30, and 60 µM genistein for 24 h. We observed that for all three cell lines genistein reduced the percentage of viable cells at these concentrations compared with untreated cells (Fig. 1A). A dose-dependent decrease of percent cell viability was detectable for all three cell lines. When normal thymocytes were treated under the same conditions, we detected no decrease of cell viability due to genistein treatment. In this experiment, we also tested concentrations of genistein lower than 15 µM and detected no effect on cell viability (data not shown). Thus, genistein concentrations at 15 µM and greater were sufficient to reduce the viability of thymic tumor cells without producing the same effect on the viability of normal thymic lymphocytes. For a kinetic analysis of the percent viability of these cells after treatment with different concentrations of genistein, we treated 92316T cells over a period of 3 days (Fig. 1B). We observed that the viability of these T lymphoma cells declined over this time period in a dose-dependent manner.

Genistein Induces Apoptosis in T Lymphoma Cells

Because genistein is reported to induce apoptosis in a variety of cell types, we examined the influence of genistein on T lymphoma cells for apoptosis. As an assay for apoptosis, we stained cells with annexin V-FITC and PI after a 24-h exposure to the concentrations of genistein that reduced cell viability (15, 30, and 60 µM). Cells undergoing apoptosis expose phosphatidyl serine residues at their membrane surface, which are detected by annexin V binding. Flow cytometric analysis of 92316T cells stained with annexin V-FITC and PI revealed that the percentage of live cells declined with increasing genistein concentrations (Table 1). The percentage of dead and apoptotic cells within the population increased correspondingly. These results demonstrated that the decrease in cell viability, which resulted from exposure to genistein, could be attributed to the induction of apoptosis.

A

B
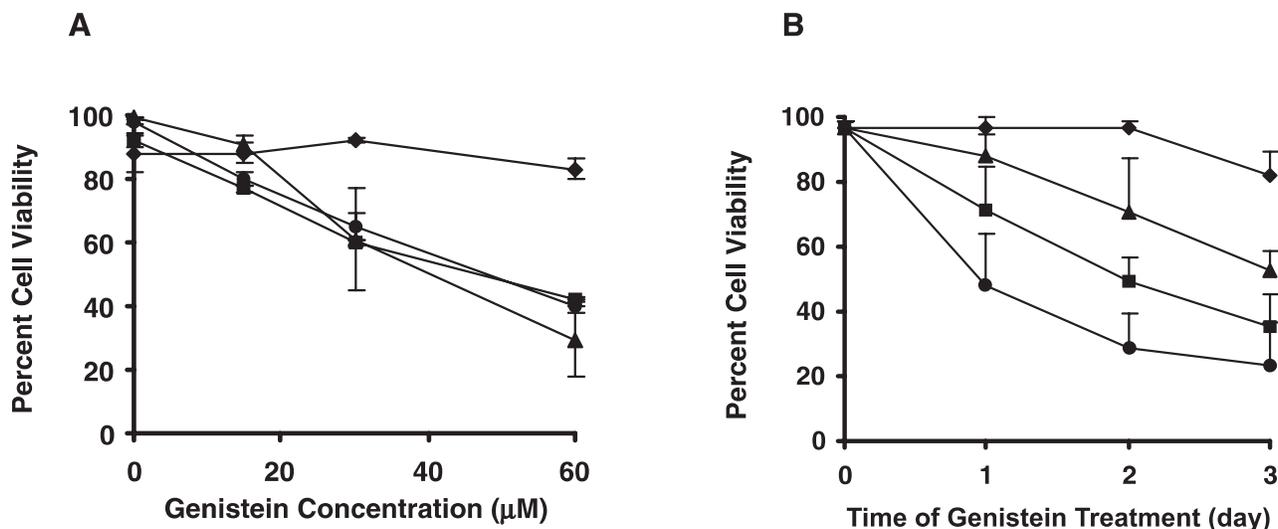
Figure 1. Percent viability of T lymphoma cells treated with genistein. A: Comparison of T lymphoma cell lines and normal thymocytes treated with genistein for 24 h. We treated 10^6 cells per milliliter of 92316T (■), 92284T (◆), and 92290T (●) cells with genistein at concentrations of 0, 15, 30, and 60 µM for 24 h. Normal thymocytes extracted from 9-wk-old AKR/J mice (◆) were treated under the same conditions. B: Kinetic analysis of 92316T cells treated with genistein for 3 days. For 3 days, 10^6 cells per milliliter of 92316T cells were untreated (◆) or treated with 15 µM (▲), 30 µM (■), and 60 µM (●) genistein. Cells were stained with trypan blue and counted with a hemacytometer. The results shown are the mean values and SDs from three independent experiments.

**Table 1.** Annexin-V FITC and PI Staining of T Lymphoma Cells After Genistein Treatment[a]

Genistein Concentration (μM)	% Live	% Dead	% Apoptotic
0	90.0 ± 2.2	6.7 ± 1.6	3.0 ± 0.5
15	84.3 ± 4.7[b]	10.4 ± 3.6[b]	5.2 ± 1.3[b]
30	45.2 ± 8.7[b]	32.2 ± 12.3[b]	18.9 ± 0.5[b]
60	18.3 ± 2.9[b]	63.8 ± 9.5[b]	17.1 ± 6.2[b]

a: 92316T cells were treated with 15, 30, and 60 μM genistein for 24 h. The results shown are the means of the values obtained from three independent experiments.

b: $P < 0.04$ compared with untreated cells as determined by the Student's t-test.

Furthermore, the extent of apoptosis was dose dependent, similar to our observations for cell viability (Fig. 1A). The percentage of dead cells, which also increased with genistein concentration, could be attributed to both apoptotic as well as necrotic cells because genistein can induce necrosis at higher concentrations (22).

As an alternative assay for apoptosis, we examined caspase-3 activation, which occurs as a relatively early event in the induction of apoptosis (15). 92316T lymphoma cells were exposed to concentrations of genistein of 15, 30, and 60 μM, and caspase-3 activity was measured after 4, 6, 8, and 24 h of exposure. Beginning at 6 h of genistein exposure and continuing to 24 h, an increase in caspase-3 activity was observed in cultures treated with genistein compared with untreated cells (Fig. 2). At 6 h, we detected a statistically significant increase in caspase-3 activity for 30 and 60 μM genistein ($P < 0.05$, as calculated by Student's t-test). At later times, statistically significant increases were detectable for all concentrations tested, including 15 μM genistein. After 24 h of exposure to 60 μM genistein concentration, we detected an approximately sevenfold increase in caspase-3 activity compared with untreated cells. Thus, caspase-3 activa-
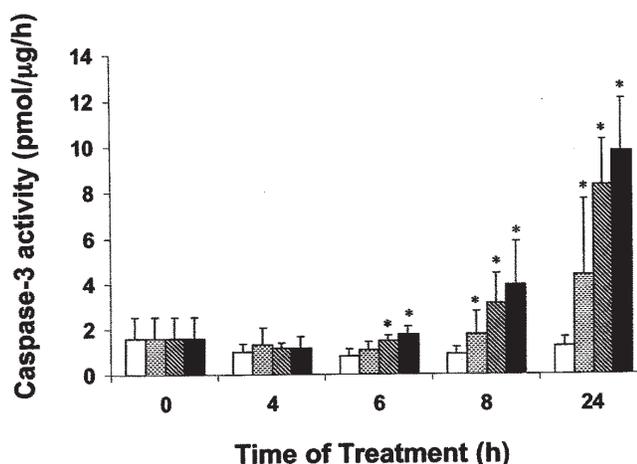
Figure 2. Caspase-3 activation in T lymphoma cells treated with genistein. 92316T cells were treated with the range of genistein concentrations of 0 μM (white bar), 15 μM (horizontal dashes), 30 μM (diagonal stripes), and 60 μM (black bar) and assayed at 0, 4, 6, 8, and 24 h of exposure. The results shown are the mean values and SDs calculated from six independent experiments. *$P < 0.05$ in comparison with untreated cells as calculated by Student's t-test.

tion was dependent on both duration of exposure to and concentration of genistein.

To verify that T lymphoma cells treated with genistein underwent apoptosis, we performed DNA fragmentation analysis of total cellular DNA extracted from cells of all three T-cell lymphoma lines treated with the range of genistein concentration of 15, 30, and 60 μM for 24 h. The production of characteristic 180- to 200-bp DNA fragments resulting from cleavage of cellular DNA is considered to be an end-stage event in the apoptotic pathway (23). Total cellular DNA was stained with ethidium bromide after agarose gel electrophoresis to detect the presence of DNA ladders. Figure 3A shows the results from 92316T cells, which was typical for the three T-cell lymphoma lines. DNA fragmentation was observed beginning at 15 μM genistein concentration and continued to increase with higher concentrations of genistein (Fig. 3A, lanes 3–5). To determine whether DNA fragmentation occurred earlier than 24 h of exposure to genistein, total cellular DNA was extracted from 92316T cells exposed to 60 μM genistein after 0, 4, 8, and 24 h (Fig. 3B). DNA laddering significantly increased beginning at 8 h of exposure to genistein (lane 6) and was greatest at 24 h (lane 8).

Taken together, these results show that genistein decreased cell viability in T lymphoma cell lines in a time- and dose-dependent manner. Furthermore, cell killing by genistein can be attributed to the induction of apoptosis as indicated by phosphatidyl serine inversion, caspase-3 activation, and DNA fragmentation.

Genistein Induces Mitochondrial Depolarization and Caspase-9 Activation

It has been reported that genistein can initiate apoptosis by depolarization of the mitochondrial membrane (24,25). Thus, to determine whether mitochondrial damage occurs as an early event in genistein-induced apoptosis in T lymphoma cells, we examined 92316T cells at 4 h after exposure to genistein with the mitochondrion-specific dye JC-1 (Molecular Probes). We selected this time-point because it is before we detected caspase-3 activation, which occurs downstream of mitochondrial damage in the intrinsic apoptotic pathway (15). JC-1 selectively enters mitochondria where it forms red fluorescent J-aggregates (26,27). Upon damage to the mitochondrial membrane and decrease in the transmembrane potential, JC-1 exists as fluorescent green monomers. Thus, a shift of fluorescence from red to green is an indication of depolarization of the mitochondrial membrane.

By flow cytometric analysis, we observed that 72.5% of cells treated with 60 μM genistein showed a decrease in red fluorescence and an increase in green fluorescence (Fig. 4B). These populations represented only 0.6% of control cells (Fig. 4A). As a positive control, cells were exposed to the potassium ionophore valinomycin, which is known to produce mitochondrial depolarization (28), and resulted in the depolarization of practically all of the cells (Fig. 4C). From this analysis our data indicate that mitochondrial depolarization
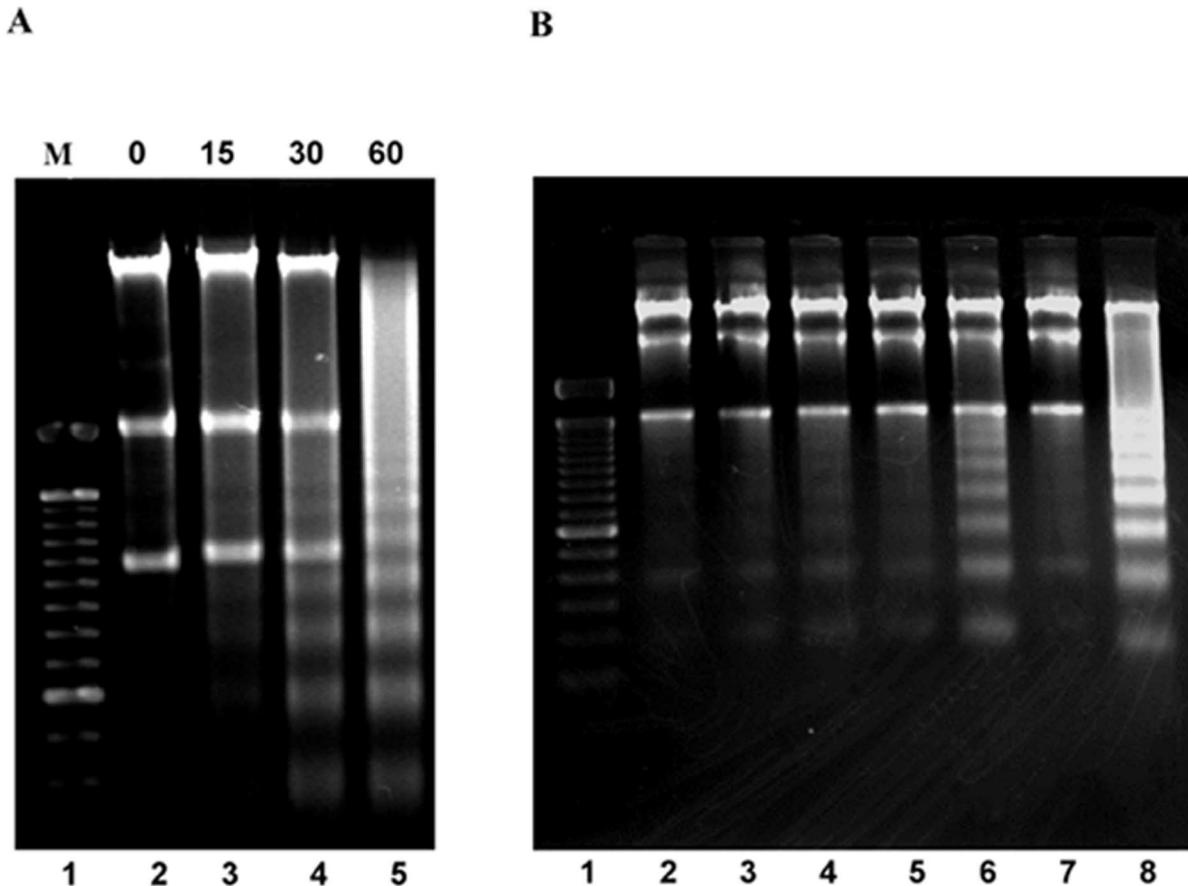
Figure 3. DNA fragmentation in T lymphoma cells treated with genistein. A: 92316T cells were treated with the range of genistein concentrations of 0, 15, 30, and 60 μM for 24 h (lanes 2–5), at which time total cellular DNA was extracted from cells. We electrophoresed 1.5 μg DNA through a 1.5% agarose gel for 1.5 h at 100 V in 1 × TBE. Lane 1 contains 100-bp DNA molecular size markers. Agarose gels were stained with ethidium bromide for visualization by UV light. B: Kinetic analysis of DNA fragmentation. Total cellular DNA was extracted from 92316T cells treated with 60 μM genistein at 0, 4, 8, and 24 h of exposure. Results are shown for cells prior to genistein exposure (lane 2), untreated cells at 4, 8, and 24 h (lanes 3, 5, and 7), and genistein-treated cells at 4, 8, and 24 h (lanes 4, 6, and 8).

occurs as a relatively early step in the induction of apoptosis by genistein.

We next evaluated caspase-9 activation because it typically occurs as a result of mitochondrial damage. We examined total cellular protein extracts from 92316T cells exposed to a range of genistein concentrations (0, 15, 30, and 60 μM) for 24 h by Western blot analysis using an antibody specific for caspase-9 (Fig. 5A). At 15 μM genistein, we detected the appearance of a 37-kDa band, which corresponds to the active form of caspase-9 (29,30). This band increased in intensity with a concomitant decrease in the 49-kDa procaspase band with increasing genistein concentrations (Fig. 5A, lanes 3–5). To determine when caspase-9 activation occurs, we performed a kinetic study by examining protein extracts from cells exposed to 60 μM genistein for 0, 2, 4, 6, 8, 12, and 24 h by Western blotting. Figure 5B shows that the initial cleavage of procaspase-9 to the activated caspase-9 form occurred at 4 h of genistein exposure, which is when we detected mitochondrial depolarization in a significant fraction of the cells. These results are consistent with our observations that mitochondrial damage by genistein occurs relatively early after genistein exposure.

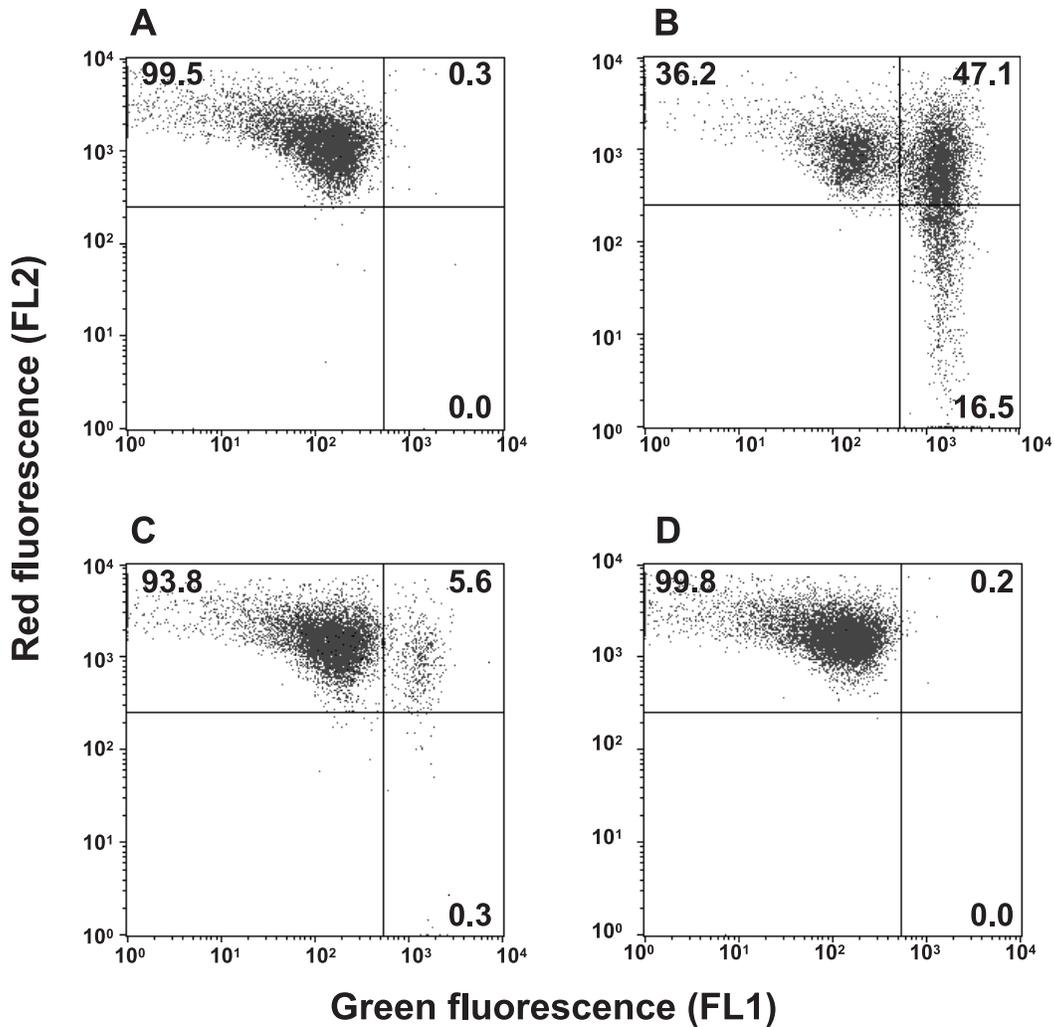## Genistein Induces Mitochondrial Depolarization Via the Permeability Transition Pore

Because it has been reported that genistein is able to induce mitochondrial depolarization in some cell types as a result of association with the membrane PTP (24,25), we tested whether a similar mechanism is involved in T lymphoma cells. 92316T cells were pretreated with BA, which is a specific inhibitor of the mitochondrial PTP (31). BA was added to cells to a final concentration of 50 μM for 1 h prior to a further 4-h incubation with 60 μM genistein (Fig. 6C). Flow cytometric analysis with JC-1 staining to detect mitochondrial depolarization showed that these cells resembled untreated control cells (Fig. 6A) or cells treated with BA alone (Fig. 6D). Flow cytometric analysis of cells treated with genistein with no BA pretreatment is shown in Fig. 6B. These data indicate that under these conditions BA nearly completely inhibited mitochondrial depolarization induced by genistein, suggesting that mitochondrial damage occurs via dysregulation of the PTP. Furthermore, BA was able to reduce cell killing by genistein at 24 h, when 66% of
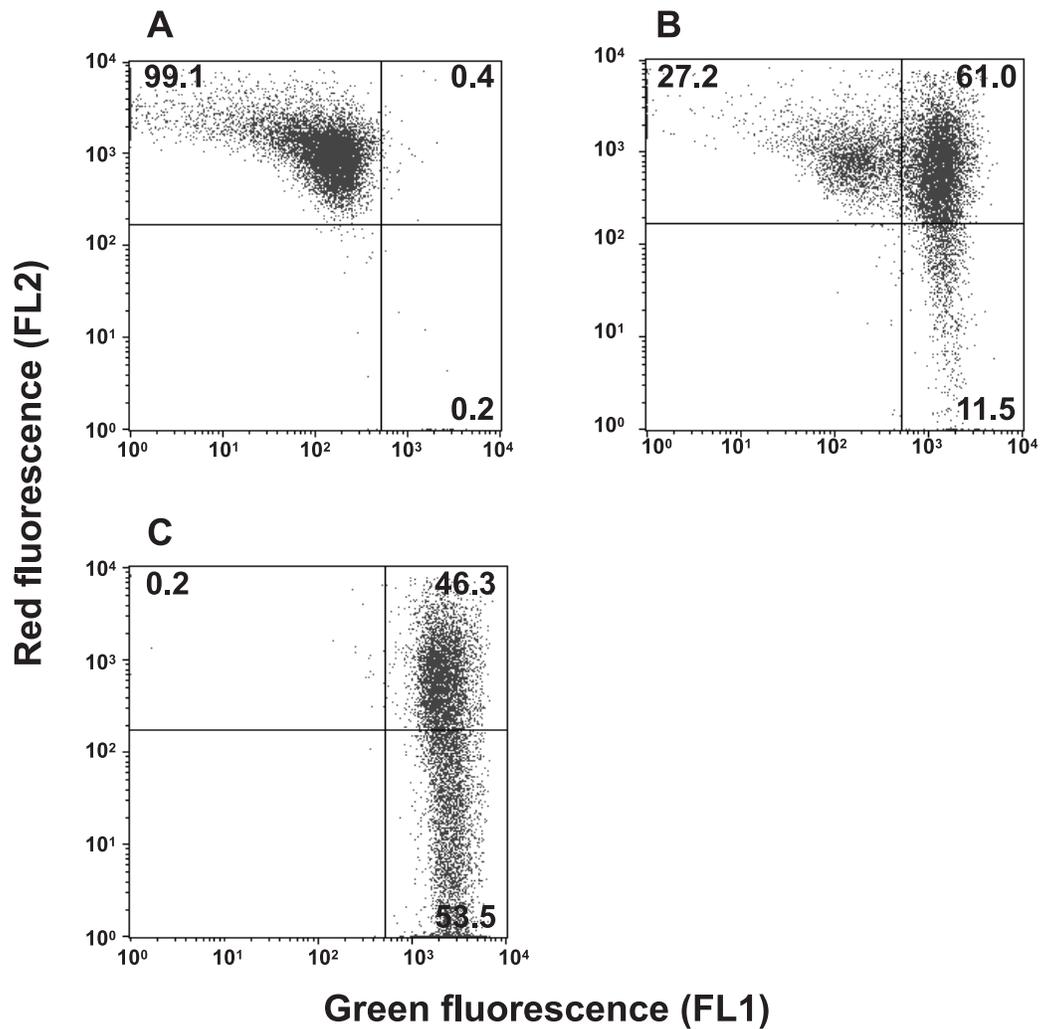
Figure 4. Genistein causes mitochondrial depolarization. We treated 10^6 92316T lymphoma cells for 4 h with DMSO (A) or 60 μM genistein (B) or for 1 h with 2 μg/ml valinomycin (C). JC-1 (5 μg/ml final concentration) was subsequently added to cells for 15 min, after which time two-color flow cytometric analysis was performed. Number in each quadrant indicates the percentage of total cells analyzed. Dot plots are representative data from three independent experiments.
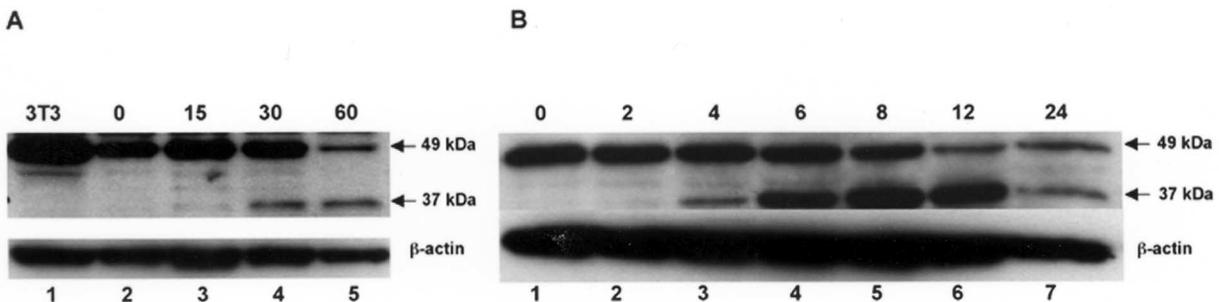
Figure 5. Caspase-9 activation in T lymphoma cells treated with genistein. A: Western blot detection of caspase-9 from cells treated with a range of genistein concentration. Analysis of 20 μg total cell protein from 92316T cells treated with 0, 15, 30, and 60 μM genistein for 24 h (lanes 2–5). Protein extracts were electrophoresed through a 9% SDS-polyacrylamide gel. The upper panel shows the 49- and 37-kDa bands for procaspase-9 and activated caspase-9, respectively, as indicated by arrows. NIH3T3 total cell extract was utilized as a positive control for antibody binding (lane 1). The lower panel displays β-actin staining as a loading control. B: Western blot of time-course analysis of caspase-9 activation in 92316T cells treated with 60 μM genistein. Isolated total cell protein was analyzed at 0, 2, 4, 6, 8, 12, and 24 h of exposure (lanes 1–7). The upper panel shows the 49-kDa procaspase-9 and 37-kDa activated caspase-9 bands. The bottom panel shows β-actin detection as a loading control.

Figure 6. Mitochondrial depolarization occurs via the PTP; 10^6 92316T lymphoma cells were untreated (A), treated with 60 µM genistein for 4 h with no bongkrekic acid (BA) pretreatment (B), pretreated with 50 µM BA for 1 h before the addition of 60 µM genistein for an additional 4 h (C), or treated with 50 µM BA for 5 h (D). Cells were subsequently stained with JC-1 and analyzed by two-color flow cytometry as described for Fig. 4. Number in each quadrant indicates the percentage of total cells analyzed. Dot plots are representative data from three independent experiments.

genistein-treated cells were viable compared with 85% viable cells pretreated with BA as determined by trypan blue exclusion (data not shown).

Discussion

The ability of genistein to induce apoptosis has been reported for several malignant cell types, mainly derived from solid tumors, such as breast and prostate (10,11). Because very little is known about the effects of genistein on hematopoietic malignancies, we undertook this study of T lymphoma cells to determine whether genistein also induces apoptosis in this cell type and, if so, identify some of the cellular processes that are involved in this phenomenon. We

have observed that the treatment of murine T lymphoma cell lines with genistein did indeed induce cell killing via apoptosis, which occurred in a dose- and time-dependent manner. Various kinetic assays for apoptosis revealed increasing caspase-3 activity beginning at 6 h after exposure to genistein. We also detected an increase in DNA laddering beginning at 8 h after genistein exposure, which is consistent with the occurrence of DNA fragmentation as a downstream step from caspase-3 activation (23).

Activation of caspase-3 in the apoptotic cascade may occur through activated caspase-9, which results from mitochondrial depolarization, or as a death receptor–mediated response (15). Our kinetic analysis of caspase-9 activation demonstrated that it occurred before an increase in caspase-3 activity was detectable, thereby suggesting that mitochon-

drial depolarization is involved in the induction of apoptosis by genistein. Flow cytometric results from our staining of genistein-treated cells with the mitochondrion-specific dye JC-1 revealed that as many as 73% of the cells contained depolarized mitochondria at the time that caspase-9 activation was detectable. These data, thus, implicate mitochondrial damage as an early event in the induction of apoptosis by genistein.

One way that mitochondrial depolarization can occur is via the PTP. Sustained opening of the PTP results in depolarization and ultimately in irreversible damage of the mitochondria (15). To determine whether the PTP is involved in mitochondrial depolarization by genistein, we used BA, which specifically blocks the open conformation of the PTP and results in the inhibition of mitochondrial depolarization (31). Our observation that BA was able to prevent the depolarization of mitochondria by genistein supports the idea that genistein functions via the PTP. The effect of genistein on the PTP may be either direct or indirect. An indirect effect may involve either the translocation of the pro-apoptotic protein Bax to the PTP without an effect on total cytosolic Bax levels or an increase in intracellular calcium levels, both of which result in PTP opening and subsequent mitochondrial depolarization (13,32–34). Further studies are required to distinguish between these possible mechanisms for T lymphoma cells.

It has been shown that genistein can have additional effects in a cell, some of which are involved in the induction of apoptosis. These include the inhibition of protein kinases, cell cycle arrest, and NF-κB down-regulation (6,35–38). We have also observed that the treatment of T lymphoma cells with genistein results in the decrease of activated NF-κB levels, which is detectable after 24 h of genistein treatment (39). Interestingly, we have also observed that BA, which inhibited mitochondrial depolarization by genistein, prevented the reduction of NF-κB. The present study demonstrates that mitochondrial damage by genistein occurs much earlier than its effects on NF-κB levels. The results from this study do not exclude the possibility that genistein may have still other effects in T lymphoma cells in the induction of apoptosis.

In conclusion, we have demonstrated that T lymphoma cells exposed to genistein undergo apoptosis and the initiation of programmed cell death is both time and dose dependent. We have determined that the induction of apoptosis by genistein is a result of mitochondrial damage, which produces caspase-9 activation and the subsequent activation of caspase-3. Furthermore, this study shows that mitochondrial damage by genistein occurs as an early event and results from the sustained opening of the mitochondrial PTP. These results suggest that the soy isoflavone genistein may be effective in the treatment of human T-cell lymphoma and leukemia. Studies are currently under way to determine whether a similar mechanism is involved in the induction of apoptosis by genistein in malignant human T cells.

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