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Genistein Induces Apoptosis of RPE-J Cells by Opening Mitochondrial PTP

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Although previous studies demonstrated that genistein-induced apoptosis of various cell types including RPE-J cells, the involvement of mitochondrial events in such types of apoptosis has not been demonstrated to date. In this investigation of genistein-induced apoptosis of RPE-J cells, genistein induced the reduction of the mitochondrial membrane potential and the release of cytochrome c to cytosol. A mitochondrial permeability transition pore (PTP) blocker bongkrekic acid prevented the reduction of the mitochondrial membrane potential and cytochrome c release, and consequently abolished caspase-3 activation, nuclear condensation, and DNA fragmentation. On the other hand, zVAD-fmk did not inhibit the mitochondrial event such as the reduction of the mitochondrial membrane potential and cytochrome c release although it prevented caspase-3 activation, nuclear condensation, and DNA fragmentation. Taken together, genistein induces apoptosis of RPE-J cells by opening the mitochondrial PTP, and the mitochondrial event in this type of apoptosis is caused independently of caspase.

Key Words: **genistein; apoptosis; RPE-J cell; mitochondria; caspase; PTP.**

Apoptosis is an evolutionarily conserved, innate process by which cells systemically inactivate, disassemble, and degrade their own structural and functional components to complete their own demise. It can be activated intracellularly through a genetically defined developmental program or extracellularly by endogenous proteins, cytokines and hormones, as well as drugs, xenobiotic compounds, radiation, oxidative stress, and hypoxia. Cells undergoing apoptosis usually develop characteristic changes, including nuclear condensation, and degradation of DNA into oligonucleosomal fragments (1, 2). Apoptotic cell death is thought to result ultimately from the proteolytic actions of caspase (3).

Genistein is a specific natural tyrosine kinase (TK) inhibitor (4). It has been documented to produce cell cycle arrest and apoptosis in a variety of cells (5–9). Although several possible explanations regarding the mechanisms of genistein-induced apoptosis have been put forth, the mechanism is still controversial (10–14).

Recently, it was demonstrated that alterations in mitochondrial function in general and induction of the mitochondrial permeability transition play a key part in the regulation of apoptosis (15, 16). Mitochondrial intermembrane space was proposed to contain several potentially apoptogenic factors, including cytochrome c, procaspases 2, 3, and 9, and AIF, which are liberated through the outer membrane in order to participate in the degradation phase of apoptosis (17–23). To date, however, there has been no report demonstrating the mitochondrial involvement in genistein-induced apoptosis.

The present study was undertaken to investigate whether mitochondria are involved in genistein-induced apoptosis of RPE-J cells. As will be shown, genistein induces apoptosis of RPE-J cells by opening mitochondrial permeability transition pore (PTP), and the mitochondrial event in this type of apoptosis is caused independently of caspase.

MATERIALS AND METHODS

Reagents

The following reagents were obtained commercially.

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0006-291X/00 $35.00

Antibodies. Rabbit polyclonal anti-horse cytochrome c and anti-human caspase-3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); Texas Red-conjugated goat anti-rabbit IgG antibody was from Molecular probes (Eugene, OR).

Culture media and serum. Dulbecco's modified Eagle's medium (DMEM) and FCS were from Gibco (Gaithersburg, MD).

Caspase inhibitor and permeability transition pore (PTP) blockers. z-VAD-fmk was from Kamiya Biomedical Co. (Seattle, WA); bongkrekic acid (BKA) was from Calbiochem (San Diego, CA); cyclosporin A (CsA) was from Sigma (St. Louis, MO); aristolochic acid (ArA) was from BiomoL (Plymouth Meeting, PA).

Other reagents. Dimethyl sulfoxide (DMSO), Hoechst 33342, RNase A, proteinase K, aprotinin, leupeptin, PMSF were from Sigma (St. Louis, MO); TUNEL reaction mixture was from Roche Molecular Biochemicals (Mannheim, Germany); ECL Western blotting detection reagents were from Amersham International (Buckinghamshire, UK).

Cell Culture

RPE-J cells were purchased from the American Tissue Culture Collection (Rockville, MD). Cells were maintained at 33°C with 10% CO_2 in air atmosphere in DMEM with 4.5 g/L glucose, 2 mM L-glutamine, and 0.1 mM non-essential amino acids supplemented with 4% FCS. To maintain reproducibility, cells past passage 20 were not used for experiments and early passage cells thawed to renew the culture.

Genistein Treatment and the Effect of Caspase Inhibitor or PTP Blockers

Twenty four hours after RPE-J cells were subcultured, the original medium was removed. Cells were washed with PBS and then incubated in the same fresh medium. Genistein from a stock solution (25 mM) was added to the medium to obtain 50 μM dilutions of the drug. In order to study the effect of caspase inhibitor, cells were preincubated for 60 min with zVAD-fmk (50 μM) before addition of genistein. To study the effect of PTP blockers, cells were preincubated for 5 min with BKA (100 μM), CsA (10 μM), or CsA (10 μM) plus ArA (50 μM) before addition of genistein. The concentration of DMSO or ethanol used in this study as a vehicle had no effect on RPE-J cell viability in our preliminary studies.

Assay of Mitochondrial Membrane Potential

Changes in mitochondrial membrane potential 24 h after treatment with genistein were determined by staining cells with the indicator dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1). JC-1 was added directly to the cell culture medium (1 μM final concentration) and incubated for 15 min. The medium was then replaced with PBS, and cells were quantitated for J-aggregated fluorescence intensity in a modular fluorimetric system (Spex Edison, NJ) using excitation and emission filters of 492 and 590 nm, respectively.

Western Blot Analysis

2×10^6 cells were washed twice with ice-cold PBS, resuspended in 200 μl ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris–Cl (pH 7.6), 0.5% TritonX-100, 2 mM PMSF, 2 μl/ml aprotinin, and 2 μl/ml leupeptin] and incubated at 4°C for 30 min. The lysates were centrifuged at 14,000 rpm for 15 min at 4°C and SDS and Na-DOC (final concentration 0.2%, respectively) were added. Protein concentrations of cell lysates were determined by the method of Bradford (Bio-Rad protein assay) and equivalent amounts were loaded onto 15% SDS–PAGE. The gels were transferred to a nitrocellulose membrane (Amersham) and reacted with caspase-3. Immunostaining with the antibody was performed using ECL western blotting reagents and detected by LAS-1000PLUS (Fujifilm, Japan).

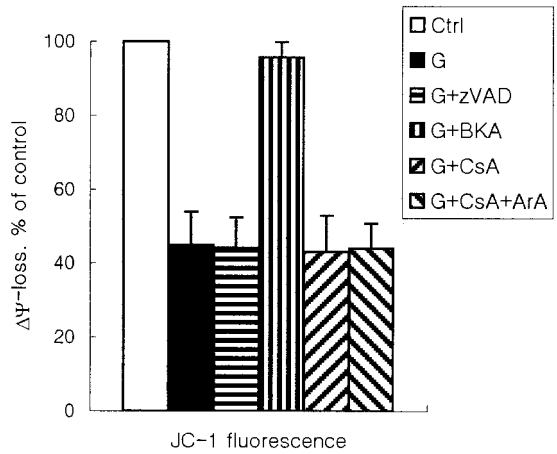

FIG. 1. Loss of mitochondrial membrane potential ($\Delta\Psi$m) at 24 h after exposure of RPE-J cells to 50 μM genistein (G). $\Delta\Psi$m was quantitated by measurement of J aggregate (JC-1) fluorescence in cells treated with 50 mM genistein, 50 μM genistein plus zVAD-fmk (50 μM), 50 μM genistein plus BKA (100 μM), 50 μM genistein plus CsA (10 μM), and 50 μM genistein plus CsA (10 μM) and ArA (50 μM). The percent of control is calculated $\Delta\Psi$m of treated cells/$\Delta\Psi$m of untreated control cells. Four independent assays were performed and data shown are the mean ± SD of the means obtained from triplicates of each experiment.

Immunocytochemistry and Nuclear Staining

Twenty four hours after incubation of 50 μM genistein, cells were harvested and cell suspension was centrifuged onto a clean, fat-free glass slide with a cytocentrifuge. Cytocentrifuged samples were fixed for 10 min in 4% paraformaldehyde. Cells were incubated with anti-cytochrome C antibody for 1 h, washed three times each for 5 min, and then incubated with Texas Red-conjugated secondary antibody for 1 h at room temperature. Cells were costained with 2 μg/ml Hoechst 33342 for 30 min at 37°C to observe the nuclear morphology.

TUNEL Quantitation to Measure DNA Fragmentation

Cytocentrifuged cells were fixed for 30 min in 4% paraformaldehyde, incubated in permeabilisation solution for 2 min on ice, and labeled in TUNEL reaction mixture for 60 min at 37°C.

Cell Counts

Samples were observed under an epifluorescence microscope. For each time point, the number of cells that had lost the punctate staining pattern for cytochrome c, showed condensed or fragmented nuclei with Hoechst staining, or demonstrated positive TUNEL reaction was determined by a blinded observer from a random sampling of 250–300 cells per experiment. Four independent experiments were conducted.

RESULTS

Mitochondrial membrane potential ($\Delta\Psi$m) was quantitated by measuring J aggregate (JC-1) fluores-

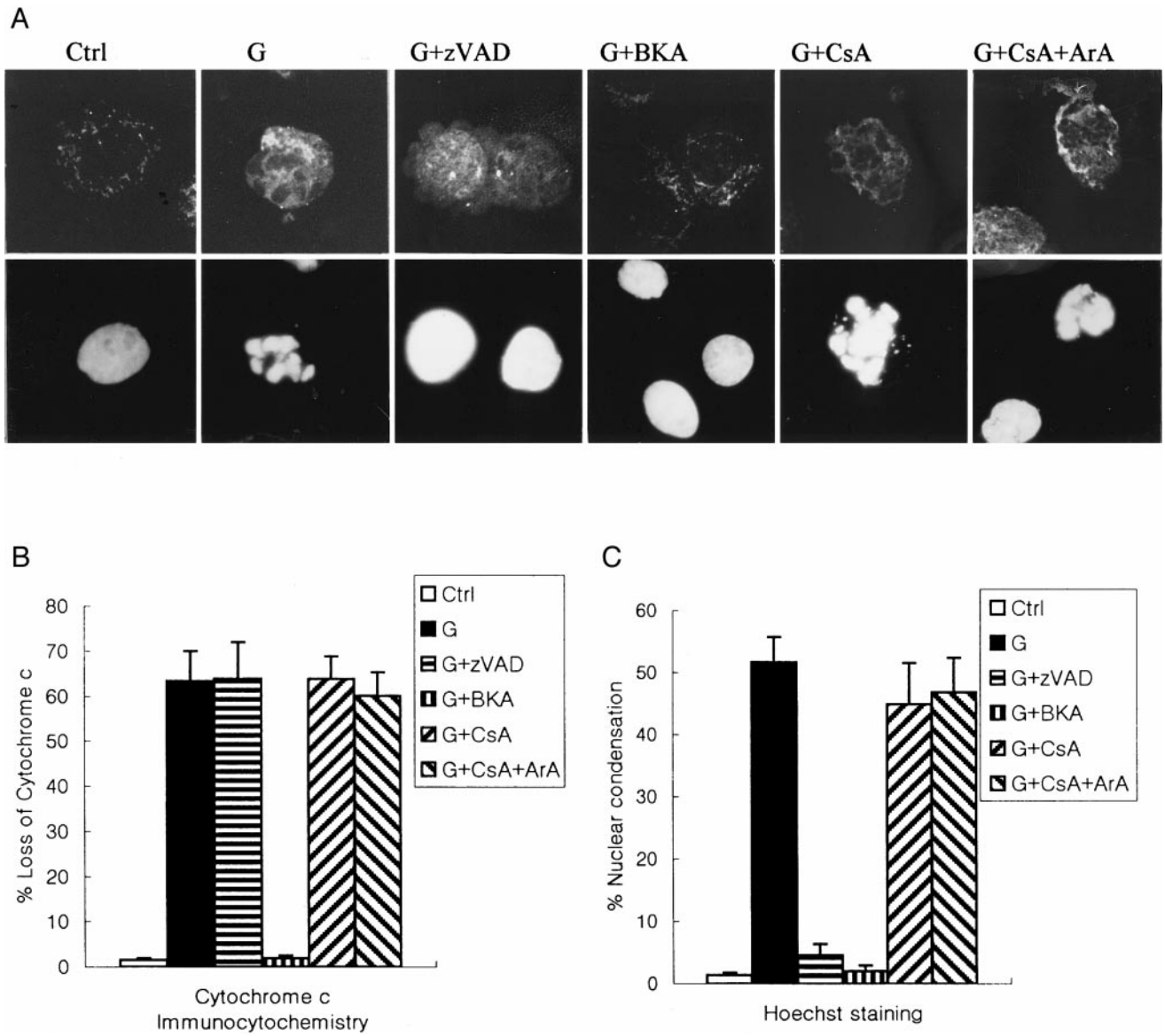

FIG. 2. Subcellular distribution of cytochrome c and nuclear morphology 24 h after treatment of 50 μM genistein (G). Cells were fixed, immunostained, and costained with Hoechst. (A) Upper panel, immunofluorescent micrographs showing localization of cytochrome c. Lower panel, Hoechst staining. (B) The number of cells exhibiting diffuse cytochrome c staining. (C) The number of cells having condensed or fragmented nuclei. (B) & (C) The number of cells was determined with 250–300 cells counted for each condition. Four independent assays were performed and data shown are the mean ± SD of the means obtained from triplicates of each experiment.

cence. As demonstrated in Fig. 1, genistein induced reduction of $\Delta\Psi$m. BKA prevented reduction of $\Delta\Psi$m but CsA, CsA plus ArA, or zVAD-fmk did not.

We examined by immunofluorescence the cellular distribution of cytochrome c. Costaining with Hoechst demonstrated the nuclear morphology simultaneously. As shown in Fig. 2A, control cells had a normal round nucleus and a bright, punctate cytoplasmic cytochrome c distribution, clearly excluded from the nuclear space and a consistent mitochondrial location. Over 50% of RPE-J cells treated with genistein for 24 h had condensed or fragmented nuclei. In cells with condensed or fragmented nuclei, the cytochrome c staining was diffuse and uniform throughout the cytoplasm, indicating that cytochrome c had been released from the mitochondria. Although zVAD-fmk prevented nuclear condensation completely, it did not inhibit the cytochrome c release from mitochondria. On the other hand, BKA prevented both nuclear condensation and cytochrome c translocation. CsA, or CsA plus ArA did not block neither nuclear condensation or cytochrome c translocation. The number of cells exhibiting diffuse cytochrome c staining and nuclear condensation was counted and depicted in Figs. 2B and 2C, respectively.

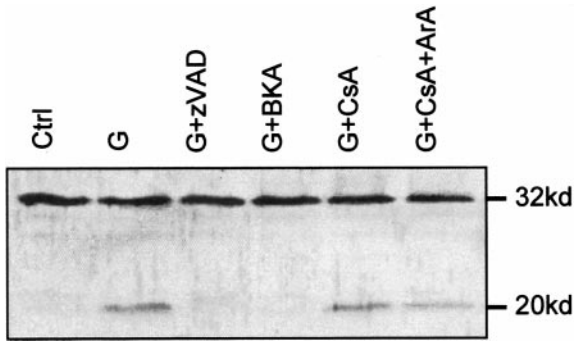

FIG. 3. Caspase-3 activity of RPE-J cells 24 h after 50 μM genistein treatment. Genistein induced caspase-3 degradation and cleavage. Both zVAD-fmk and BKA prevent the degradation and cleavage but CsA or CsA with ArA did not. Intact 32-kd caspase-3 and its 20-kd cleaved products are indicated. Data shown are a representative of four independent experiments.

Western blotting data are shown in Fig. 3. Genistein treatment induced caspase-3 degradation, and produced the processed caspase-3 p20 cleaved product. When cells were treated with zVAD-fmk or BKA, caspase-3 activation was abolished. But CsA only, or CsA plus ArA did not prevent caspase-3 degradation and cleavage.

DNA fragmentation was determined by TUNEL technique. Figure 4 shows that genistein-induced DNA fragmentation, and genistein-induced DNA fragmentation was prevented by zVAD-fmk or BKA but not by CsA, or CsA plus ArA.

DISCUSSION

There have been two possible explanations regarding the apoptosis-inducing mechanism by genistein. The first explanation proposes that the induction of apoptosis is directly linked to TK activity (9, 10). To date the mechanisms of cell cycle inhibition by genistein have been put forth. Genistein might perturb the process of phosphorylation/dephosphorylation of tyrosine residues of cdc2 kinase which may lead to arrest at G_2-M (24–26), and S phase entry and transition from G_2 to mitosis are both regulated by phosphorylation and dephosphorylation of $pp34^{cdc2}$ protein kinase (27, 28). The second explanation suggests that the interaction between genistein and DNA topoisomerase-II may be related to occurrence of apoptosis (11–13). As a matter of fact, most topoisomerase II interacting drugs are able to produce S and G_2/M phase cell cycle arrest, which are sometimes associated with extensive DNA degradation and apoptosis of blocked cells (29–31). However, a recent study demonstrated that topoisomerase II-mediated DNA cleavage is not required for the induction of apoptosis although topoisomerase II served as the enzymatic target of genistein. Therefore, the mechanism, by which genistein induce apoptosis, had remained elusive before this study.

Mitochondria have been demonstrated to be involved in many aspects of the death response. One currently favored hypothesis for the induction of apoptosis is that perturbations of mitochondria allow the release of cytochrome c. Much evidence has been accumulated to suggest that release of cytochrome c from mitochondria is an important step in apoptosis (32). In response to a variety of death-promoting stimuli, cytochrome c is released from its normal position within the intermembrane space of mitochondria, in association with changes in mitochondrial permeability, membrane potential, and ultrastructure (33). Once in the cytosol, cytochrome c binds to Apaf-1 in a dATP/ATP-dependent manner, an event that triggers oligomerization of Apaf-1/cytochrome c in complexes that activate procaspase-9 (34). The ensuing recruitment and activation of caspase-9 results in activation of caspase-3, caspase-6, caspase-7, which function as downstream effectors of the cell death program (35). However, several recent genetic studies demonstrated that T lymphocytes from mice lacking Apaf-1 or caspase-9 underwent apoptosis normally in response to the agents functioning as agonists for Fas receptor signalling. According to these observations two discrete apoptotic signalling pathways were suggested: a “cellular stress” or “mitochondrial pathway;” and a “death ligand” or “death receptor” pathway. Whereas the mitochondrial pathway is dependent on cytochrome c, Apaf-1, and caspase-9. The other pathway is mediated by other signalling proteins such as FADD and caspase-8 (36, 37).

We report here for the first time that genistein induces apoptosis of RPE-J cells through a mitochondrial

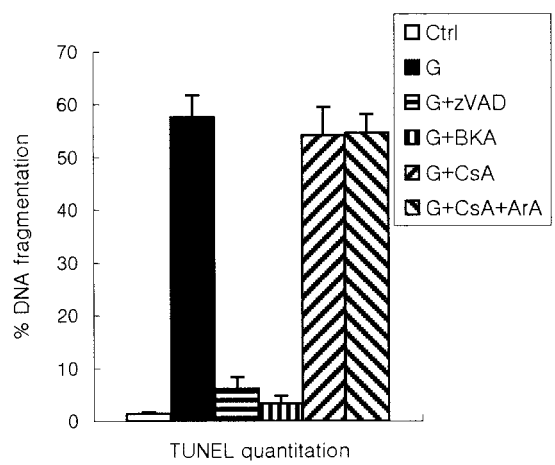

FIG. 4. DNA fragmentation. TUNEL positive cells were counted under an epifluorescence microscope with 250–300 cells counted for each condition. DNA fragmentation was determined as a percentage to total cells. Genistein induced DNA fragmentation, and genistein-induced DNA fragmentation was prevented by zVAD-fmk or BKA but not by CsA, or CsA plus ArA. Four independent assays were performed and data shown are the mean ± SD of the means obtained from triplicates of each experiment.

pathway. The involvement of mitochondria is supported by the data that genistein-induced cytochrome c release and reduction of mitochondrial membrane potential and that a PTP blocker bongkrekic acid prevented caspase-3 activation, nuclear condensation, and DNA fragmentation. Taken together, genistein-induced cytochrome c release, and consequent activation of caspase-3, nuclear condensation, and DNA fragmentation by opening of the mitochondrial PTP.

Opening of the mitochondrial PTP was proposed previously as one possible means of enabling cytochrome c release to the cytosol (15) although cytochrome c release from mitochondria is independent of permeability transition pore, and cytochrome c release was not accompanied by changes in the mitochondrial membrane potential in a certain situation (20, 38, 39). We here demonstrated that BKA prevented cytochrome c translocation and the reduction of the mitochondrial membrane potential in genistein-induced apoptosis. These results suggest that opening of the PTP is involved in the release of mitochondrial factors including cytochromc c during this type of apoptosis (21, 40). However, another PTP blocker CsA did not prevent both the release of cytochrome c to the cytosol and decrease in $\Delta\Psi$m. Theses results suggest that genistein releases cytochrome c and decreases $\Delta\Psi$m dependently of ANT (BKA target) and independently of cyclophilin D (cyclosporin target).

In our previous study of genistein-induced apoptosis of RPE-J cells, we demonstated that the caspase inhibitor zVAD-fmk abolished the activation of caspase-3 and nuclear condensation (9). Therefore, we concluded genistein-induced apoptosis of RPE-J cells is associated with caspase activation. zVAD-fmk, in the present study, was shown to abolish DNA fragmentation besides activation of caspase-3 and nuclear condensation. However, it was not able to inhibit either genistein-induced cytochrome c release or reduction in $\Delta\Psi$m. Theses results suggest that genistein triggers cytochrome c and reduction in $\Delta\Psi$m independently of caspase. Accordingly, caspases are explained to activate downstream of mitochondria in genistein-induced apoptosis of RPE-J cells.

In conclusion, genistein induces apoptosis of RPE-J cells by opening the mitochondrial PTP, and mitochondrial events in this type of apoptosis are caused independently of caspase.

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