Genistein-Induced Apoptosis of p815 Mastocytoma Cell

Ki-Hoon Song, M.D., Ki-Ho Kim, M.D.

Department of Dermatology, Dong-A University College of medicine, Pusan, Korea

***Background* : Numerous studies demonstrated that genistein induced the decrease of proliferation and apoptosis in a variety of cells. However, there is no report about the effect of genistein on proliferation and demise of mast cells.**

***Objective* : This study was conducted to investigate genistein-induced apoptosis of mast cells as it pertains to both its basic drug mechanism and the potential therapeutics of the pathologic conditions accompanying mast cell proliferation.**

***Methods* : p815 murine mastcytoma cell line was used to assess the effects of genistein treatment including viability and proliferation, morphologic study, DNA electrophoresis, the effect of caspase inhibitor, western blotting, and mitochondrial event.**

***Results* : Genistein induced many apoptotic manifestations as evidenced by changes in cell morphology, generation of DNA fragmentation, activation of caspase 3, and DNA hypoploidy. The reduction of mitochondrial membrane potential and the release of cytochrome c to cytosol were also demonstrated. However, reduction of mitochondrial membrane potential and cytochrome c release were not prevented by caspase inhibitors zVAD-fmk and BocD.fmk, or PTP (permeability transition pore) blockers such as bongkrekic acid and cyclosporin A.**

***Conclusions* : This in vitro study suggests that pathologic increases in mast cell number possibly be regulated in vivo by therapeutic strategy enhancing apoptosis by treatment of genistein. (Ann Dermatol 14(2) 88-97, 2002).**

Key Words : Genistein, Apoptosis, Mastocytoma, Caspase

Apoptosis is an evolutionary conserved, innate process by which cells systemically inactivate, disassemble, and degrade their own structural and functional components to complete their own de mise. Cells undergoing apoptosis usually develop characteristic morphological changes, including nuclear condensation and pyknosis, and degradation of DNA into oligonucleosomal fragments[1,2]. 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[1,2].

To date, several genes involved in regulating apoptotic cell death have been identified. Multiple lines of evidence indicate that apoptosis can be triggered by the activation of caspase[3]. Among them caspase-3 has been studied intensively, which is activated proteolytically when cells are signaled to undergo apoptosis[4]. Several substrates of caspase-3 have been demonstrated, including PARP(poly-(ADP-ribose)-polymerase). It is not known yet whether the cleavage of the substrates plays a causal role in apoptosis.

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[5,6]. Their

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Reprint request to : Ki-Hoon Song, M.D., Department of Dermatology, Dong-A University Hospital, Dong-A University College of Medicine.
3Ga-1, Dongdaeshin-Dong, Seo-Gu, Pusan, 602-715, Korea
Tel. (051)240-5430, Fax. (051)253-0787
E-mail > tatabox@hananet.net

intermembrane space was proposed to contain several potentially apoptogenic factors, including cytochrome c, procaspases 2, 3 and 9, and AIF(apoptosis inducing factor), which were liberated through the outer membrane in order to participate in the degradation phase of apoptosis[7].

Although tissue mast cell numbers under normal conditions are relatively constant from individual to individual, mast cells proliferate rapidly and become activated in various pathological conditions and processes including mastocytosis, chronic inflammatory conditions or allergic skin diseases[8]. However, mast cells are rapidly eliminated from inflammatory tissues in recovery from allergic diseases. The number of mast cells in tissue were demonstrated to be regulated in part by apoptosis[9]. Since induction of apoptosis of mast cells in tissue can be the potential therapeutic modality for such various pathological conditions, understanding its mechanism is important. Numerous studies have focused on the targeted induction of apoptosis in order to control the unlimited growth of cells. Moreover, induction of apoptosis of the activated cell may promote therapeutic efficiency. As a matter of fact, anti-inflammatory and anti-allergic effect of a certain drug was demonstrated to be caused by its apoptosis-inducing mechanism[10]. Genistein is a tyrosine kinase inhibitor derived from soybean. Although the pharmacological action of genistein results from its inhibitory effects on tyrosine kinase, its apoptosis inducing activity was also demonstrated. Thus, genistein also has been investigated as a therapeutic potential for proliferative diseases[11-16].

This study was conducted in order to investigate the effect of genistein on mast cell proliferation and demise as it pertains to both its basic drug mechanism and the potential therapeutics of the pathologic conditions accompanying mast cell proliferation. Although the tyrosine kinase inhibitor, genistein, has been reported to inhibit growth and induce apoptosis of various cells, there is no report of its effects on mast cell demise to date. In this study, genistein has been demonstrated to induce apoptosis of p815 mastocytoma cell line.

MATERIALS AND METHODS

1. Materials

(1) Antibodies

The following reagents were obtained commercially: Rabbit polyclonal anti-horse cytochrome c and anti-human caspase-3, and mouse monoclonal anti-mouse PARP antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA,); FITC-conjugated goat anti-rabbit IgG antibody was from Vector (Burlingame, CA).

(2) Culture media

Dulbeccos modified Eagles medium (DMEM) and FCS were from Gibco (Gaitherburg, MD).

(3) Reagents

Genistein, dimethyl sulfoxide (DMSO), Hoechst 33342, RNase A, proteinase K, aprotinin, leupeptin, PMSF, eugenol and propidium iodide were from Sigma (St Louis, MO); TUNEL reaction mixture and photometric enzyme immunoassay kit to detect histone-associated DNA fragments were from Boehringer Mannheim (Mannheim, Germany); ECL western blotting detection reagents were from Amersham International (Buckinghamshire,UK); FluorAce apopain assay kit were from Biorad (Hercules,CA).

(4) Caspase inhibitor and permeability transition pore (PTP) blockers

zVAD-fmk was from Kamiya Biomedical Co. (Seattle,WA). Cyclosporin A (CsA) was from Sigma (St Louis, MO). Aristolochic acid (ArA) was from BiomoL (Plymouth Meeting, PA). BocD.fmk and bongkrekic acid (BKA) were from Calbiochem (San Diego,CA)

2. Cell culture

p815 murine mastcytoma cell line was purchased from the ATCC (Rockville, Maryland). Cells were maintained at 37 ℃ with 5% CO_2 in air atmosphere in DMEM with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 1.0 mM sodium pyruvate supplemented with 10% FBS.

3. Genistein treatment

Twenty four hours after p815 cells were subcultured, the original medium was removed. The cells were washed with PBS and then incubated in the same fresh medium. Genistein from a stock solution was added to the medium to obtain 1, 5, 10, 25, 50 or 100 μM dilutions of the drug. Stock solutions of genistein (25mM) made by dissolving the

drug in DMSO were kept frozen at 20℃ until use. The concentration of DMSO, 0.004 - 0.1 % (vol/vol), used in this study, both as a vehicle for genistein and as a control, had no effect on p815 cell proliferation in our preliminary studies.

4. Genistein treatment and assessment of cell viability and proliferation

Cells treated with various concentrations of genistein were harvested 24 h after treatment, stained with trypan blue and then counted using a hemocytometer. Since viability and proliferation assays demonstrated evident induction of cell death at 50μM genistein, this concentration was utilized for further assessment of apoptosis.

5. Quantification of DNA hypoploidy by flow cytometry

Ice cold 95% ethanol with 0.5% Tween 20 was added into cell suspension to final 70% ethanol. Fixed cells were pelleted, and washed in 1% BSA-PBS solution. Cells were resuspended in 1 ml PBS containing 11 Kunitz U/ml RNase, incubated at 4°… for 30 min, washed once with BSA-PBS, and resuspended in PI solution (50 g/ml). After cells were incubated at 4℃ for 30 min in the dark and washed with PBS, DNA content was measured on a FACScan flow cytometry system (Becton Dickinson, San Jose, CA) and data were analyzed using the Modfit software which allowed a simultaneous estimation of cell-cycle parameters and apoptosis.

6. Morphological assessment of apoptosis

(1) *Light microscopy* Cells were observed and photographed under phase contrast microscope. Cells were then harvested and cell suspension was centrifuged onto a clean, fat-free glass slide with a cytocentrifuge. The samples were stained in 4 g/ml Hoechst 33342 for 30 min at 30℃, fixed for 10 minutes in 4% paraformaldehyde and observed under an epifluorescence microscope.

(2) *Electron microscopy* Cells treated with genistein for 24 h were collected, centrifuged at 200 x g and fixed in 2.5% glutaldehyde for 1.5 h. Cells were then postfixed in 1% osmic acid for 1.5 h and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and morphological changes were examined using a Hitachi H600-3 electron microscope.

7. DNA electrophoresis

2 X10^6 cells were resuspended in 1.5 ml of lysis buffer [10 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), 10 mM NaCl and 0.5% SDS] into which proteinase K (200μg/ml) was added. After samples were incubated overnight at 48℃, 200 μl of ice cold 5 M NaCl was added and the supernatant containing fragmented DNA was collected after centrifugation. The DNA was then precipitated overnight at-20℃ in 50% isopropanol and RNase A-treated for 1 h at 37℃. A loading buffer containing 100 mM EDTA, 0.5% SDS, 40% sucrose, and 0.05% bromophenol blue was added at 1:5(v/v). Separation was achieved in 2% agarose gels in Tris-acetic acid/EDTA buffer (containing 0.5 μg/ml ethidium bromide) using 50 mA for 1.5 h.

8. Quantitation of apoptosis

Cells treated with genistein were harvested at various time points for the following procedures: 1) *Trypan blue stain as above; 2) Hoechst staining for assessing nuclear apoptosis.* Cells were prepared as described in the above light microscopy section. Total cell number was counted under DIC optics and cells undergoing apoptosis were assessed by nuclear morphology under an epifluorescence microscope; 3) *TUNEL quantitation to measure DNA fragmentation.* Cells were fixed for 30 minutes in 4% paraformaldehyde, incubated in permeabilisation solution for 2 min on ice, and labeled in TUNEL reaction mixture for 60 min at 37 ℃. Total cell number, at least 300 cells from each group, was counted under DIC optics and the percentage of TUNEL positive cells was calculated under an epifluorescence.

9. The effect of caspase inhibitor

Cells were preincubated for 1 h with 50 μM zVAD-fmk or BocD.fmk and then, maintaining the inhibitor in the culture medium, genistein was added.

10. Western blot analysis

2×10^6 cells treated with genistein 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, 2mM PMSF, 2 μl/ml aprotinin and 2 μl/ml leupeptin] and incubated at 4 ℃ for 30 min. The lysates were centrifuged at 14,000 rpm for 15 min at 4℃ 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 7.5% SDS/PAGE. The gels were transferred to a PVDF membrane (Amersham) and reacted with each antibody. Immunostaining with antibodies was performed using ECL western blotting reagents and detected by LAS-1000PLUS (Fujifilm, Japan).

11. Assessment of mitochondrial event

(1) *Assay of mitochondrial membrane potential* Cells were preincubated for 60 min with zVAD-fmk (50 μM), or for 5 min with CsA (10 μM), CsA (10 μM) and ArA (50 μM) or BKA (100 μM) before addition of genistein. 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.

(2) *Immunofluorescent staining* Cells were preincubated for 60 min with zVAD-fmk (50 μM), or for 5 min with CsA (10 μM), CsA (10 μM) and ArA (50 μM) or BKA (100 μM) before addition of genistein. Twenty four hours after treatment with genistein, cells were harvested and cytocentrifuged. Samples were incubated with anti-cytochrome C antibody for 1 h, washed 3x each for 5 min, and then incubated with FITC-conjugated secondary antibody for 1 h at room temperature. To observe the nuclear morphology simultaneously, counter staining with Hoechst was conduced as above. Cells were mounted with PBS, and observed and photographed under an epifluorescence microscope.

RESULTS

Genistein-induced inhibition of p815 mastocytoma cell proliferation resulted from G2/M block and subsequent apoptosis (Fig. 1-3).

Genistein produced a dose-dependent decrease in cell proliferation (Fig. 1A) and viability (Fig. 1B). Flow cytometric analysis which allows the simultaneous estimation of cell-cycle parameters and apoptosis, elucidated not only the rates at which genistein altered cell cycle progression but the correlation between cell cycle distribution (Fig. 2). Genistein at 50 μM resulted in an increase in the percentage of G_2/M-phase cells while producing a concomitant fall in the percentage of G_0/G_1 phase cells. Since the microscopic examination of cultures did not reveal any increase in the number of mitotic nuclei, it can be assumed that genistein

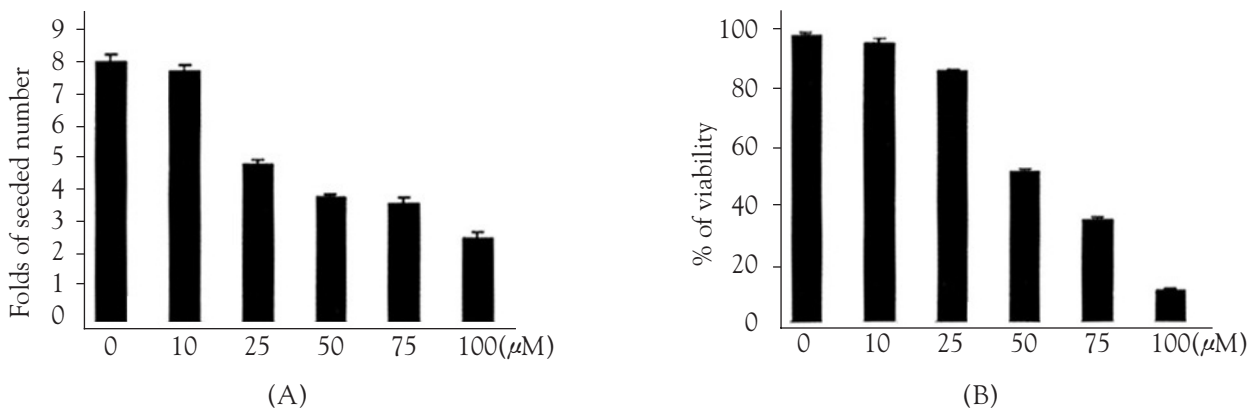


Fig. 1. Effect of different genistein concentrations (0-100 μM) on viability and proliferation of p815 cells as determined by trypan blue dye exclusion. Twenty four hours after cells were treated with genistein, cell numbers were measured by a hemocytometer. Three independent assays were performed and data shown is the mean $\pm$ SD of the means obtained from triplicates of each experiment. (A) Effect of genistein on in vitro proliferation of p815 cells. (B) Effect of genistein on viability of p815 cells.

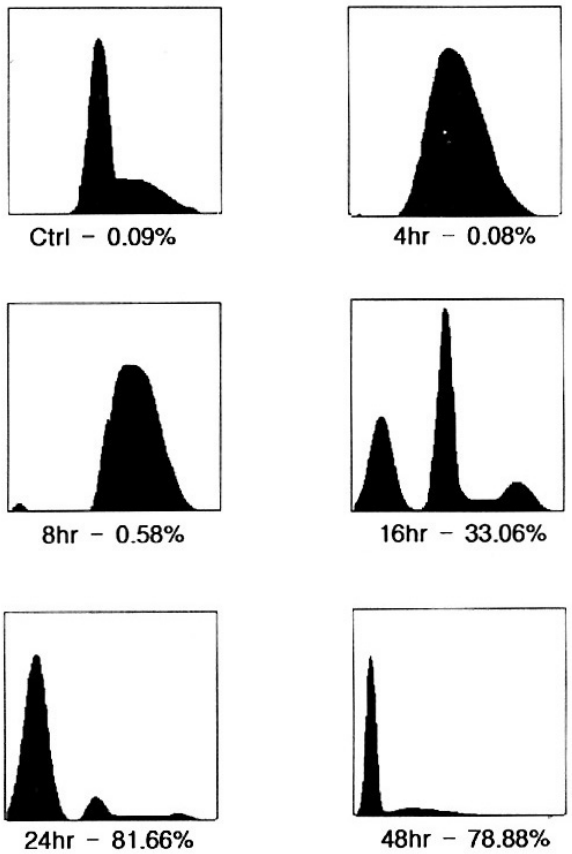


Fig. 2. DNA histograms demonstrating the distribution of DNA content quantitated by flow cytometric analysis of propidium iodide-stained cells. Data shown is a representative of three independent experiments. 50 μM genistein-treated group.

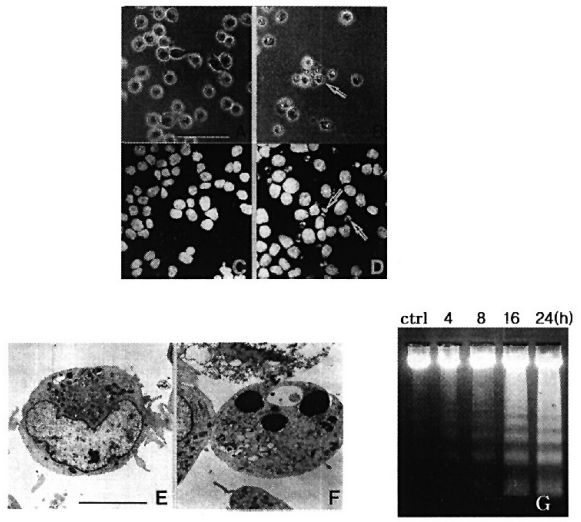
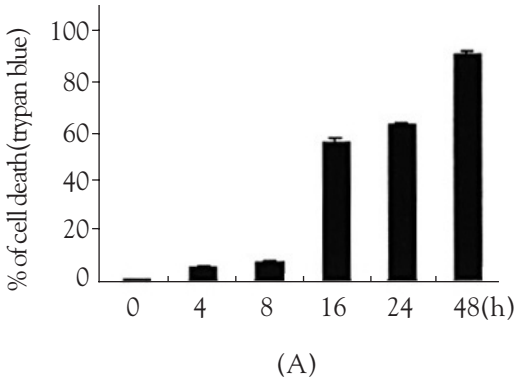
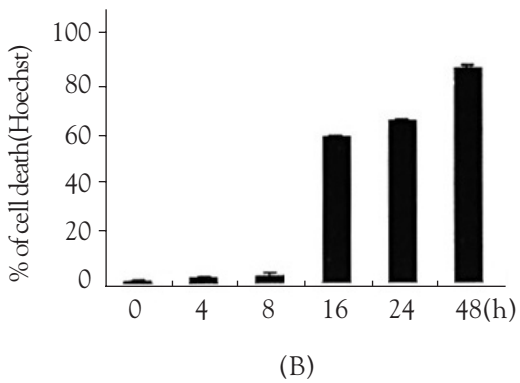


Fig. 3. Demonstration of apoptosis in p815 mastocytoma cells treated with 50 μM genistein. The results presented are representatives of three independent experiments. A scale bar in A indicates 40 μm for A-D. That in E indicates 4 μm for E-F. (A) & (B), Phase contrast micrographs. (A) Control cells. (B) Cells treated with genistein for 24 h show cytoplasmic shrinkage (arrow heads) and bleb formation (arrows). (C) and (D), Immunofluorescent micrographs after Hoechst staining. (C) Control cells showing the typical round nuclei. (D) Cells treated with genistein for 24 h show fragmented nuclei (arrows). (E) & (F) Electron micrographs. (E) A representative control cell exhibiting the typical p815 morphology which includes microvilli and a nucleus containing evenly dispersed nuclear chromatin. (F) A cell treated with genistein for 8 h. A representative early apoptotic cell showing the disappearance of surface folds and condensation of nuclear poles (arrows). (G) DNA electrophoresis. The control group without any treatment, showed no DNA fragmentation. Cells treated for 4, 8, 16, and 24 h clearly showed DNA degradation characteristic of apoptosis with a ladder pattern of DNA fragments.

might produce an arrest at the critical point of the cell cycle that regulates M-phase entry. The increase in the G_2/M phase cell percentage and concomitant fall in the percentage of G_0/G_1 phase cells paralleled an increase in apoptotic portions (0.09% at 0 h, 0.08% at 4h, 0.58% at 8h, 33.06% at 16 h, 81.66% at 24 h and 78.88% at 48h).

Genistein at 50 μM resulted also in several morphological and biochemical changes associated with apoptosis. Phase contrast pictures showed cytoplasmic shrinkage and bleb formation (Fig. 3A and B). Hoechst stain demonstrated that genistein induced a change in nuclear morphology. Compared to the typical round nuclei of the control cells (Fig. 3C), apoptotic p815 cells displayed condensed and fragmented nuclei (Fig. 3D). Transmission electron microscopical observation also supported that genistein induced apoptosis of p815 cells. As shown in figure 3E, control p815 cells exhibited a typical p815 morphology with a corrugated cell surface and a nucleus containing evenly dispersed nuclear chromatin. After genistein treatment, cells showed disappearance of the surface folds and the condensation of nuclear pole (Fig. 3F). DNA fragmentation was evidently demonstrated by DNA electrophoresis. Cells treated with genistein showed DNA degradation characteristic of apoptosis with a ladder pattern of DNA fragments (Fig. 3G).





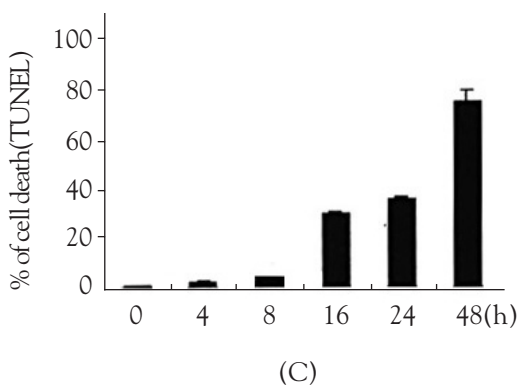
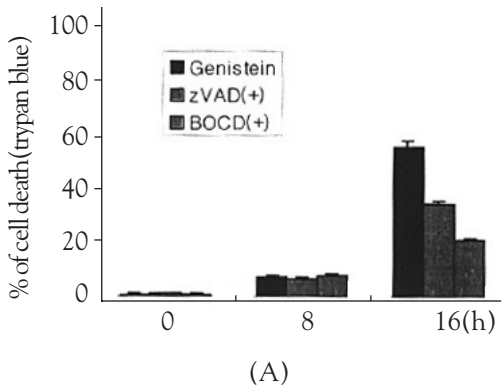


Fig. 4. Time course of 50 μM genistein -induced apoptosis of p815 mastocytoma cells. The amount of apoptosis was quantitated by three different assays for evaluating various aspects of apoptosis. Three independent assays were performed and data shown are the mean $\pm$ SD of the means obtained from triplicates of each experiment. (A) Trypan blue exclusion. (B) Hoechst stain. (C) TUNEL quantitation. (A)-(C) The percent of cell death shown on the y-axis was calculated as the number of apoptotic cells as compared to the total cell.



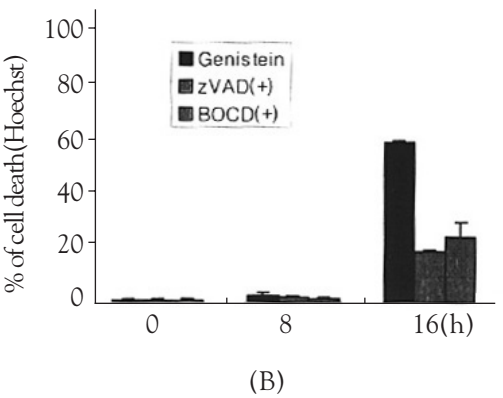


Fig. 5. Effect of caspase blockers on 50 μM genistein -induced apoptosis of p815 mastocytoma cells. The amount of apoptosis was quantitated by two different method. Three independent assays were performed and data shown is the mean $\pm$ SD of the means obtained from triplicates of each experiment. (A) Trypan blue exclusion. (B) Hoechst stain. The percent of cell death shown on the y-axis was calculated as the number of apoptotic cells as compared to the total cell.

Genistein induced apoptosis of p815 mastocytoma cells was caspase-dependent (Fig. 4-6).

The time course quantitation of genistein-induced cell death of p815 cells is depicted in figure 4A-C. Based on trypan blue, Hoechst and TUNEL stains, the percentage of cell death gradually increased in a time-dependent manner compared to control cells (Fig. 4A-C). Both caspase blockers BocD.fmk and zVAD-fmk prevented cell death partially (Fig. 5 A and B). In the next step, caspase and subsequent PARP activation was assayed. As expected, caspase-3 and PARP were degraded and the cleaved subunits was detected in genis-

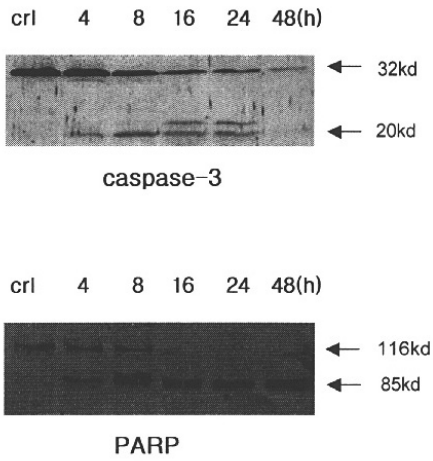


Fig. 6. Western blotting showing caspase-3 and PARP degradation and cleavage.

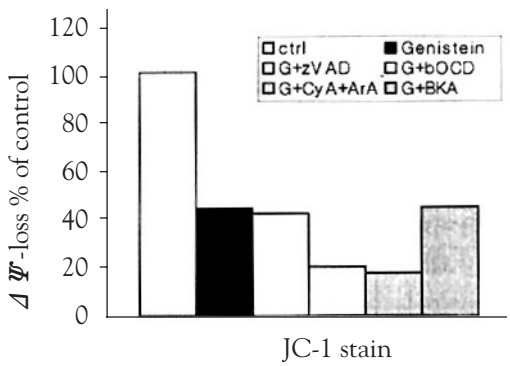


Fig. 7. Effect of caspase inhibitors and PTP blockers on genistein-induced loss of mitochondrial membrane potential ($\Delta\Psi$m) at 24 h after exposure of cells to genistein. $\Delta\Psi$m was quantitated by measurement of J aggregate (JC-1) fluorescence in cells treated without (control) or with 50 μ M genistein, genistein plus zVAD-fmk (50 μM), genistein plus BocD-fmk (50 μM), genistein plus CsA (10 μM) and ArA (50 μM), and genistein plus BKA (100 μM). The percent of control is calculated $\Delta\Psi$m of treated cells/$\Delta\Psi$m of control cells. Data shown is a representative of three independent experiments.

tein-treated cells (Fig. 6).

Mitochondrial events were involved in genistein-induced apoptosis of p815 mastocytoma cells (Fig. 7-8).

Loss of mitochondrial membrane potential ($\Delta\Psi$m) is known common events to many pathways of

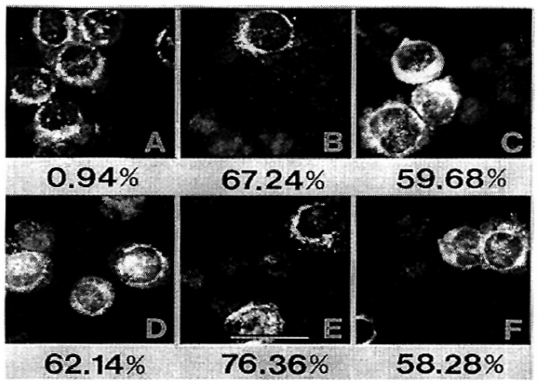


Fig. 8. Immunofluorescent micrographs showing localization of cytochrome c. (A) Control cells. (B) Cells treated with 50 μM genistein. (C) Cells treated with genistein plus zVAD-fmk (50 μM). (D) Cells treated with genistein plus BocD.fmk (50 μM). (E) Cells treated with genistein plus CsA (10 μM) with ArA. (50 μM) (F) Cells treated with genistein plus BKA (100 μM). A scale bar in E indicates 20 μm for each picture

apoptosis induction. To analyze mitochondrial events that may be associated with this type of apoptosis, the potentia$\Delta\Psi$l-sensitive fluorescent probe JC-1 was used to detect loss of $\Delta\Psi$m at 24 h after exposure of cells to genistein. In control cells, JC-1 formed the characteristic J-aggregates in the mitochondria, which was demonstrated under an inverted fluorescent microscope. However, little red fluorescence was observed in genistein-treated cell (morphological data not shown). Change of $\Delta\Psi$m was fluorospectrophotometrically quantitated. As depicted in figure 7, treatment of cells with genistein caused the reduction of membrane potential. Translocation of proapoptogenic molecule cytochrome c was also demonstrated (Fig. 8). PTP blockers such as CsA and BKA as well as both caspase inhibitors zVAD-fmk and BocD.fmk did not significantly block the reduction of $\Delta\Psi$m and translocation of cytochrome c (Fig. 7, 8).

DISCUSSION

Tyrosine kinases (TK) are associated with membrane receptors for several growth factors as well as with a number of oncogene products[17]. In this study, genistein treatment was able to inhibit mast cell growth and additionally it is noteworthy, that apoptotic cell death could also be induced. Al-

though many studies have reported the apoptosis of mast cells in various conditions, this is the first study describing that genistein could induce apoptotic death of mast cells.

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[18]. 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 G2-M[19-21], and S phase entry and transition from G2 to mitosis are both regulated by phosphorylation and dephosphorylation of $p34^{cdc2}$ protein kinase[22, 23]. The second explanation suggests that the interaction between genistein and DNA topoisomerase II may be related to occurrence of apoptosis[24-26]. 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[27-29]. 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. A recent study showed that genistein induced apoptosis by opening mitochondrial PTP[30].

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[31]. 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[32]. 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[33]. 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[34]. 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 signaling. According to these observations two discrete apoptotic signaling 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 signaling proteins such as FADD and caspase-8[35, 36].

We here report for the first time that genistein induces apoptosis of mast cells through a mitochondrial pathway. The involvement of mitochondria is supported by the data that genistein induced cytochrome c release and reduction of mitochondrial membrane potential.

Caspases are typically constitutively present within cells as single-chain inactive proenzymes that are proteolytically processed during apoptosis to form active heterodimeric enzymes with a specific substrate cleavage activity. Caspases are known to cleave a number of proteins and the consequences of this cleavage event was suggested to be responsible for many of the phenotypic changes in the cell undergoing apoptosis[14,15]. This study demonstrates that genistein-induced apoptosis of p815 cells is associated with caspase activation. Caspase inhibitor was shown to abolish DNA fragmentation besides activation of caspase-3 and nuclear condensation. However, a certain type of apoptoisis was prevented only by BocD.fmk but not by zVAD-fmk[37].

Much evidence has accumulated to suggest that release of cytochrome c from mitochondria is an important step in apoptosis[31]. However, BocD.fmk blocked cell death although it failed to prevent the passage of cytochrome c from mitochondria to the cytosol in a certain type of apoptosis[38]. Therefore, it is controversial still that the release of cytochrome c is essential for the induction of all types of apoptosis. Moreover, it is still not clear exactly how cytochrome c release is achieved. Although opening of a permeability transition pore was proposed previously as one possible means of enabling cytochrome c release to the cytosol[5], cytochrome c release has been observed in situations where no loss in mitochondrial transmembrane

potential was observed[38]. In this study, the release of cytochrome c was not prevented by BocD.fmk. These results suggest that genistein triggers cytochrome c release independently of caspase. Besides the release of mitochondrial cytochrome c, other mitochondrial components such as AIF might participate in the induction of the characteristic biochemical and morphological changes associated with apoptosis. However, the investigation of other mitochondrial components was beyond the scope of the present study.

In this study, reduction in mitochondrial transmembrane potential was observed after genistein treatment. By the way, reduction in $\Delta\Psi$m was not prevented by BocD.fmk. These results suggest that genistein triggers reduction in $\Delta\Psi$m independently of caspase. This data, demonstrating a decrease in $\Delta\Psi$m, even in the presence of BocD.fmk, are compatible with the possibility that mast cell death pathway may be mediated in these cells by alterations in mitochondrial function. Whether such alterations constitute commitment for the demise of the cells or whether they function as a potent amplificatory loop remains to be established in genistein-treated mast cells. In this study, two PTP blockers employed did not prevent both the release of cytochrome c to the cytosol and decrease in $\Delta\Psi$m. These results suggest that genistein releases cytochrome c and decrease in $\Delta\Psi$m independently of at least two major components of the PTP, cyclophilin D (cyclosporin target), and the ANT (BKA target).

In conclusion, this study has shown for the first time that genistein has a dose-dependent antiproliferative and apoptotic effect on mast cells in vitro. Although many important issues of this type of mast cell death remain to be elucidated, this in vitro study suggests that pathologic increases in mast cell numbers may possibly be regulated in vivo by therapeutic strategies enhancing apoptosis by treatment of genistein, and provides insight into how mast cell number may be controlled in vivo.

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