Genistein-induced apoptosis of human breast cancer MCF-7 cells involves calpain–caspase and apoptosis signaling kinase 1–p38 mitogen-activated protein kinase activation cascades

Hye-Young Shim[a,*], Jong-Hwa Park[a,*], Hyun-Dong Paik[b], Seung-Yeol Nah[c], Darrick S.H.L. Kim[d] and Ye Sun Han[a]

The molecular mechanisms of genistein-induced apoptosis of human breast cancer MCF-7 cells were investigated. Genistein showed 50% cell growth inhibition at $IC_{50} = 27.5 \pm 0.8$ μmol/l in 24 h incubation under 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay conditions. Genistein is known to express both cell growth activity at nanomolar concentrations and anti-cell growth activity at micromolar concentrations. It was found that genistein at 100 μmol/l concentration effectively induced apoptosis of MCF-7 cells in 24 h. Genistein-induced apoptosis involved activation of calpain, caspase 7 and poly(ADP ribose) polymerase. Dantrolene, an inhibitor of Ca^{2+} release from the endoplasmic reticulum, inhibited genistein-induced activation of calpain and caspase 7, in addition to effectively negating genistein-induced apoptosis. MCF-7 cells treated with genistein also showed increased phosphorylation of p38 mitogen-activated protein kinase, whereas no effect was observed for extracellular signal-regulating kinase 1/2. Phosphorylation of apoptosis signaling kinase 1, an upstream regulator of p38 mitogen-activated protein kinase, was also increased by genistein treatment. Genistein-induced phosphorylation of apoptosis signaling kinase 1 and p38 mitogen-activated protein kinase was diminished by the presence of dantrolene. These results suggest that genistein-induced apoptosis in MCF-7 cells is mediated through calpain–caspase 7 and apoptosis signaling kinase 1–p38 mitogen-activated protein kinase activation cascades that involve Ca^{2+} release from the endoplasmic reticulum. *Anti-Cancer Drugs* 18:649–657 © 2007 Lippincott Williams & Wilkins.

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[a]Department of Advanced Technology Fusion, [b]Division of Animal Life Science and [c]Department of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University, Seoul, South Korea and [d]CurXceL Corporation, Technology Center, Cumberland Ave. West Lafayette, Indiana, USA

Correspondence to Professor Ye Sun Han, PhD, Department of Advanced Technology Fusion, Konkuk University, 1, Hwayang-dong, Gwangjin-gu, Seoul, 143-701, South Korea
Tel: +82 2 2049 6050; fax: +82 2 452 5558; e-mail: yshan@konkuk.ac.kr

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[*]Hye-Young Shim and Jong-Hwa Park contributed equally to the writing of this article.

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Introduction

Genistein is a soy-derived isoflavone, having a heterocyclic diphenolic structure that mimics estrogen, which has shown potent antitumor activity against various human cancer cell lines and xenograft systems of human tumors, suggesting a potentially effective tumor-suppressive agent [1–6]. The mechanism, however, behind its anticancer activity is not yet fully understood. In this report, we attempt to elucidate the mechanisms behind genistein-induced apoptosis using human breast cancer MCF-7 cells.

Genistein has been shown to modulate cell growth through inhibitory and/or stimulatory interactions with estrogen receptors (ERs) [7,8]. Genistein possesses dose-dependent agonistic and antagonistic properties with respect to cell growth; estrogen-like proliferative activities at low (nanomolar) concentrations and antiproliferative activities at higher (micromolar) concentrations [9,10]. Genistein also possesses antiangiogenic and antimetastasic properties and antioxidant activities [11–13]. In addition, genistein has been shown to modulate genes that regulate cell proliferation, cell cycle, apoptosis, oncogenesis, transcription regulation, cancer cell invasion and metastasis [14–17].

Genistein showed different effects against breast cancer cells depending on the concentration and cell types (ER positive and negative cells) [18]. In the absence of endogenous estrogens, low concentrations of genistein (1 nmol/l to 1 μmol/l) stimulated the growth of ER-positive MCF-7 cells, whereas no activity was observed

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against ER-negative breast cancer cells such as MDA-MB 468 and HBL. At high concentrations ($>10\,\mu mol/l$) of genistein, the growth of MCF-7 cells at G_2/M phase was significantly arrested through protein regulation and mRNA expression of cell cycle-related genes [19,20]. A recent report showed that genistein induced the depletion of endoplasmic reticulum Ca^{2+} stores to cause the increase of intracellular Ca^{2+} concentration, resulting in the activation of Ca^{2+}-dependent proapoptotic protease μ-calpain and caspase 12 [21].

Intracellular Ca^{2+} is implicated in the induction of apoptosis and regulation of the apoptotic signaling kinase 1 (ASK1)–p38 mitogen-activated protein kinase (MAPK) cascade [22,23]. ASK1 is a MAPK kinase kinase family member that activates MAPK kinase 3/6–p38 MAPK and stress-activated protein kinase kinase 1 (SEK1)–c-Jun *N*-terminal kinase (JNK) signaling cascades [24–26]. ASK1 has been suggested to be a key element in endoplasmic reticulum stress-induced cell death and hydrogen peroxide-induced apoptosis [27,28]. ASK1 was shown to play a critical role for induction of apoptosis in various cytotoxic stresses such as tumor necrosis factor, Fas and reactive oxygen species (ROS) [27,29].

The family of MAPK plays a central role in the signaling pathways of cell proliferation, differentiation, survival and apoptosis [30,31]. Major enzymes belonging to this family are extracellular signal-regulating kinase 1/2 (Erk1/2 or p44/42 MAPK), JNK and p38 MAPK, which are activated in response to a variety of extracellular stimuli. Erk1/2 is predominantly activated by mitogens through a Ras/Raf/MEK signaling cascade leading to cell growth and survival. JNK and p38 MAPK are preferentially activated by proinflammatory cytokines and oxidative stress, resulting in cell differentiation and apoptosis [32,33]. The activation of JNK and p38 MAPK pathways leads to phosphorylation of various proapoptotic downstream effectors. Herein, we report an investigation on molecular mechanisms involved in genistein-induced apoptosis of MCF-7 cells.

Materials and methods

Cell and cell culture

Human breast cancer MCF-7 cells were obtained from KCLB (Korean Cell Line Bank), and were cultured in Dulbecco's modified Eagle's minimum media (DMEM; Invitrogen, Carlsbad, California, USA) supplemented with 10% FBS (Invitrogen) and 1% penicillin-streptomycin solution (Sigma, St Louis, Missouri, USA) at 37°C in a 5% CO_2 incubator. All experiments were performed on six-well plates, unless noted otherwise. MCF-7 cells were seeded onto six-well plates at a density of 1×10^6 cells per well and incubated for 24 h before the experiment. Cells were washed with phosphate-buffered saline (PBS, pH 7.4), and treated with fresh media containing different concentrations of genistein (Sigma) dissolved in ethanol and dimethylsulfoxide (DMSO) (1:1 v/v). The vehicle control only received ethanol and DMSO (0.05%, 1:1 v/v).

Cell growth inhibition study using the MTT assay

Cell growth inhibition by genistein was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MCF-7 cells cultured overnight on 96-well plates were washed with PBS, media containing different concentrations of genistein were added, incubated for 24 h, was added 10 μl of MTT from a stock solution (5 mg/ml), and incubated for an additional 4 h. Blue formazans were eluted from cells by adding 100 μl of DMSO under gentle shaking and absorbance was determined at 570 nm using an enzyme-linked immunosorbent assay reader (SpectraMAX; Molecular Devices, Sunnyvale, California, USA).

Cell viability

Cell viability was determined by the Trypan blue dye exclusion assay. A cell suspension was prepared using brief trypsinization. One hundred microliters of cell suspension was mixed with 100 μl of 1% Trypan blue solution and left for 5 min at room temperature. The stained (dead) cells and the total cells per square of the cell chamber (four squares per suspension) were counted under a microscope (Olympus, Tokyo, Japan).

Determination of DNA fragmentation

Genistein-treated or nontreated cells were harvested, washed twice with PBS and treated with lysis buffer [500 μl, 20 mmol/l Tris–HCl (pH 8.0), 5 mmol/l ethylenediaminetetraacetic acid, 400 mmol/l NaCl, 1% sodium dodecyl sulfate (SDS) and 10 mg/ml proteinase K] for 1 h at 55°C. Fragmented DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v), precipitated with ethanol and resuspended in Tris-ethylenediaminetetraacetic buffer (pH 8.0) containing 20 μg/ml RNase A. After quantitative analysis of DNA content, an equal amount of DNA was separated in 1.5% agarose gels. DNA in gel was visualized under ultraviolet light after staining with ethidium bromide.

Protein extraction and Western blot analysis

MCF-7 cells harvested from six-well plates were washed twice with ice-cold PBS and treated with lysis buffer [100 μl, 50 mmol/l Tris–HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid, 1 mmol/l NaF, 1% Nonidet P-40, 1 mmol/l phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Sigma)] for 30 min at 4°C. Protein extracts were collected after centrifugation at 14 000 rpm for 10 min. Protein concentration was determined using Bio-Rad protein assay kit (Hercules, California, USA). An equal amount of protein extracts were resolved by SDS–polyacrylamide gel electrophoresis and transferred

to polyvinylidine diflouride membranes (Amersharm, Piscataway, New Jersey, USA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline Tween-20 (TBST) [10 mmol/l Tris–HCl (pH 8.0), 137 mmol/l NaCl, 0.05% Tween-20 (v/v)] for 2 h and probed with primary antibodies. After washes with TBST (2×), membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 2 h. Membranes were washed (3×) and the chemiluminescent signals were visualized with an enhanced chemiluminescence reagent (ECL; Pierce, Rockford, Illinois, USA) as described in the manufacturer's manual. All antibodies used in this experiment were purchased from Cell Signaling (Beverly, Massachusetts, USA). For Western blot and autoradiogram images, band densities were measured using Image acquisition and analysis software version 4.5 (UVP; Upland, California, USA).

Calpain activity assay

Calpain activities were determined using the InnoZyme calpain activity kit (Calbiochem, San Diego, California, USA) with a minor modification. Total cell lysates were prepared as described above. Protein extracts (50 μg) were mixed with 4 nmol calpain substrate (DABCYL)-TPLKSPPPSPR-(EDANS) and incubated for 30 min at room temperature with gentle shaking. Changes in fluorescence were measured at an excitation wavelength of 320 nm and an emission wavelength of 480 nm with a fluorescence microplate reader (Geminin EM; Molecular Devices; Sunnyvale, California, USA).

Determination of reactive oxygen species generation

The generation of intracellular ROS by genistein or hydrogen peroxide was measured based on intracellular peroxide-dependent oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma; St Louis, Missouri, USA) to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) as described previously [34]. Cells were seeded onto 24-well plates at a density of 5×10^4 cells per well and cultured for 24 h. After washing with PBS, fresh medium containing 100 μmol/l genistein or 1 mmol/l H_2O_2 was added and incubated for 12 h. Cells were incubated with 20 μmol/l of DCFH-DA for 30 min at 37°C, rinsed with PBS and 2 ml of PBS was added to each well. The fluorescent intensity was determined using fluorescence microscope (Carl Zeiss, Gaottingen, Germany).

Statistical analysis

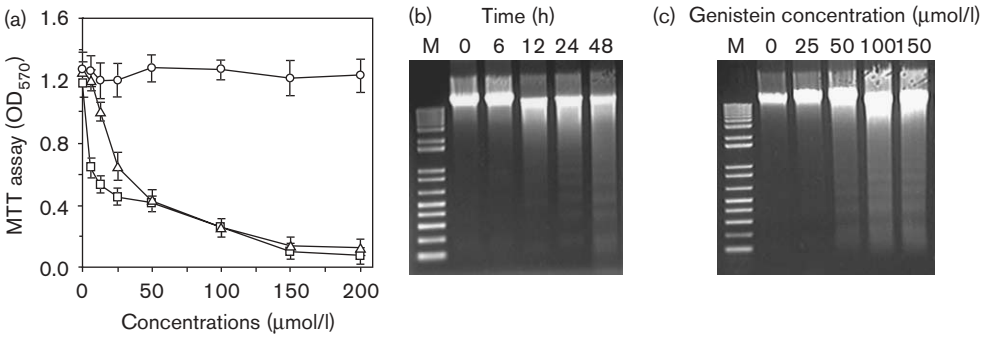
Experiments were performed three times and statistical analysis was conducted using Student's *t*-test for significance between the different values. Data were expressed as means ± SD and $P < 0.05$ was considered significant.

Results

Genistein induces apoptosis in human breast cancer MCF-7 cells

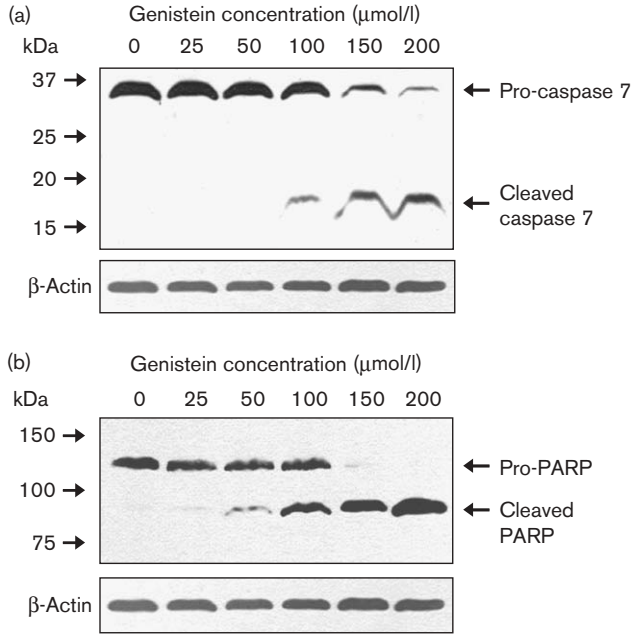
Genistein-induced MCF-7 cell growth inhibition was determined using the MTT assay. Genistein showed 50% cell growth inhibition (IC_{50}) at 27.5 ± 0.8 μmol/l (Fig. 1a) that was higher than that of a positive control etoposide (IC_{50} = 10 ± 0.5 μmol/l). To determine the amount of genistein needed to induce apoptosis in an experimentally reasonable time frame, cells were incubated with 25–150 μmol/l concentrations of genistein for 6, 12, 24 or 48 h and apoptotic DNA fragmentation was analyzed using agarose gel electrophoresis on DNA purified from the cells. At 100 μmol/l genistein treatment, the apoptotic DNA fragmentation was observed after 12 h incubation (Fig. 1b), whereas 48 h incubation was needed for 50 μmol/l genistein treatment to observe the apoptotic DNA fragmentation (Fig. 1c), suggesting that genistein-induced apoptotic DNA fragmentation was dose- and time-dependent. Thus, 100 μmol/l concentration of

Fig. 1



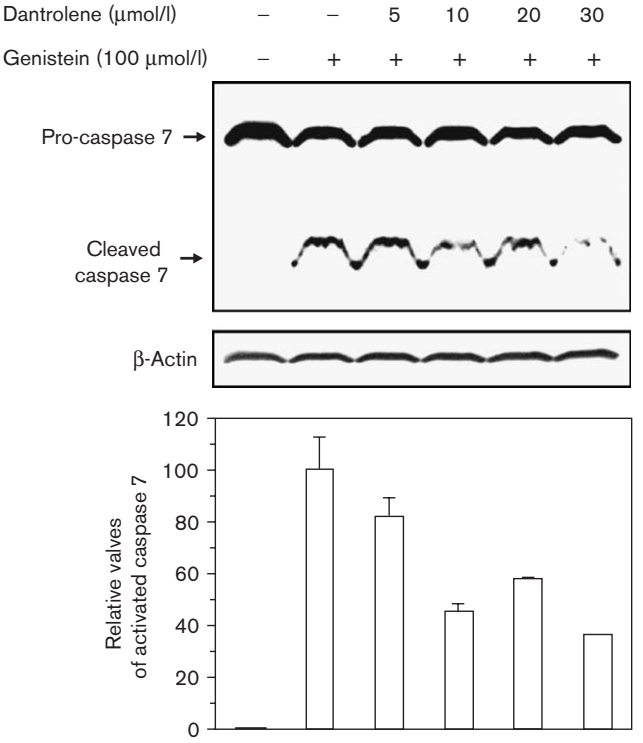
Genistein inhibits MCF-7 cell proliferation and induces apoptosis. (a) MCF-7 cells were treated without and with different concentrations of genistein for 24 h. Then, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was performed as described under Materials and methods (circle, control; triangle, genistein; square, etoposide). Data are mean ± SD from three independent experiments and each experiment was conducted in triplicate. (b and c) DNA fragmentation was analyzed by agarose gel electrophoresis. MCF-7 cells were treated with 100 μmol/l genistein for the indicated time (b) or treated with increasing doses of genistein for 48 h (c). M, 100 bp DNA ladder size marker.

Fig. 2



Genistein induces the activation of caspase 7 and poly(ADP ribose) polymerase (PARP). MCF-7 cells were treated with or without increasing doses of genistein for 24 h. Equal amounts of whole-cell extracts were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, and caspase 7 (a) and PARP (b) were detected by Western blot analysis. β-Actin was used as a control.

Fig. 3



The activation of caspase 7 by genistein is inhibited by dantrolene. MCF-7 cells were treated with the indicated concentrations of dantrolene and 100 μmol/l of genistein for 24 h. Equal amounts of whole-cell extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and caspase 7 was detected by Western blot analysis. β-Actin was used as a control. The bar diagram shows means of relative band intensities from two independent experiments. Maximum intensity was estimated as 100.

genistein was used throughout the molecular mechanism study involved in genistein-induced apoptosis of MCF-7 cells.

The caspase cascade is involved in genistein-induced apoptosis

Activation of caspase 7 in genistein-treated MCF-7 cells was investigated using Western blot analysis (Fig. 2a). Active caspase 7 (~17 kDa) produced from the cleavage of procaspase 7 (~35 kDa) was observed from 100 μmol/l genistein-treated MCF-7 cells. Furthermore, an increase of the poly(ADP ribose) polymerase (PARP) 85-kDa cleavage form, a marker from caspase cascade-activated apoptosis, was observed in genistein-treated cells (Fig. 2b). Apoptotic signals to activate caspase 3 or 7 have been suggested to mediate through a caspase activation cascade controlled by one of two distinct pathways that are associated with either caspase 8 or 9 [35]. Activation of caspase 8 or 9, however, was not observed in genistein-treated MCF-7 cells (data not shown).

Dantrolene inhibits genistein-induced apoptosis

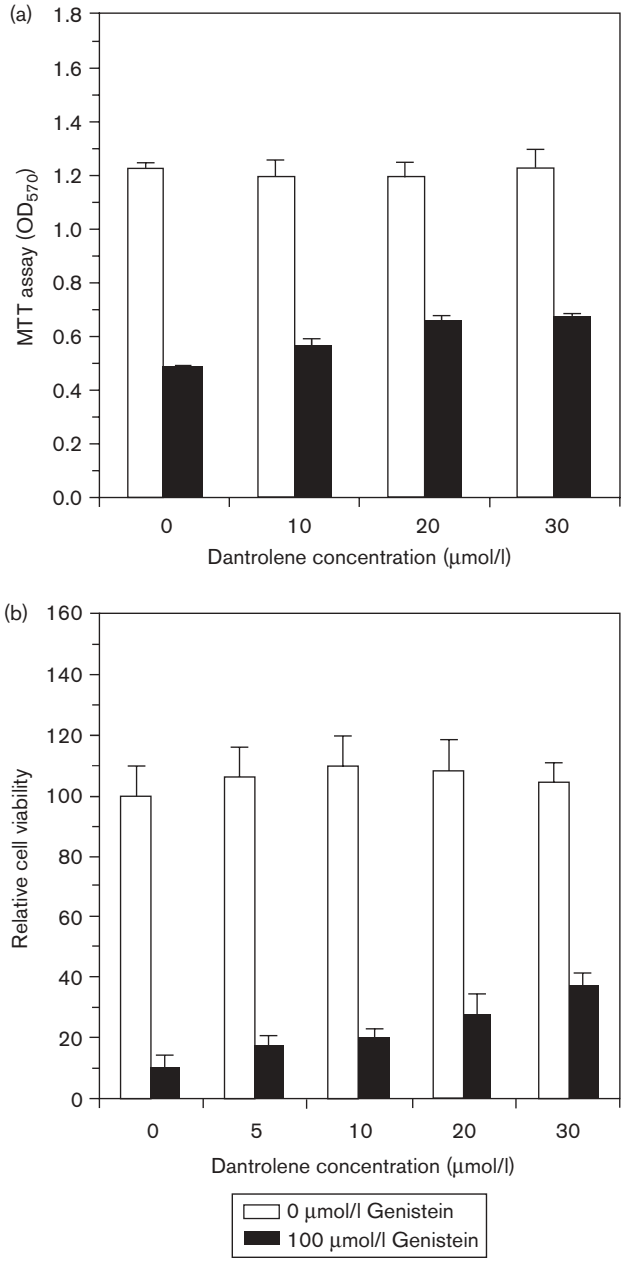
We investigated whether or not genistein-induced caspase 7 activation involves Ca^{2+} disruption of the endoplasmic reticulum. MCF-7 cells were treated with genistein in the presence of Ca^{2+} release inhibitors, dantrolene and thapsigargin. Cells treated with different concentrations of dantrolene for 1 h before a treatment with 100 μmol/l genistein for 24 h showed decrease in genistein-induced caspase 7 activation (Fig. 3). The presence of dantrolene reversed genistein-induced cell growth inhibition by 20–30% as determined using the MTT assay (Fig. 4). Thapsigargin, a Ca^{2+}-ATPase inhibitor, had no effect on genistein-induced apoptosis in MCF-7 cells (data not shown).

Calpain activities were measured using proteins extracted from genistein (100 μmol/l)-treated MCF-7 cells as described. Genistein-treated cells showed increased calpain activity, whereas those treated with 1 nmol calpain inhibitor Z-LLY-FMK showed decreased calpain activity (Fig. 5a). Cells incubated with dantrolene for 1 h before the addition of genistein showed decreased genistein-induced calpain activity (Fig. 5b).

Genistein induces the phosphorylation of p38 mitogen-activated protein kinase

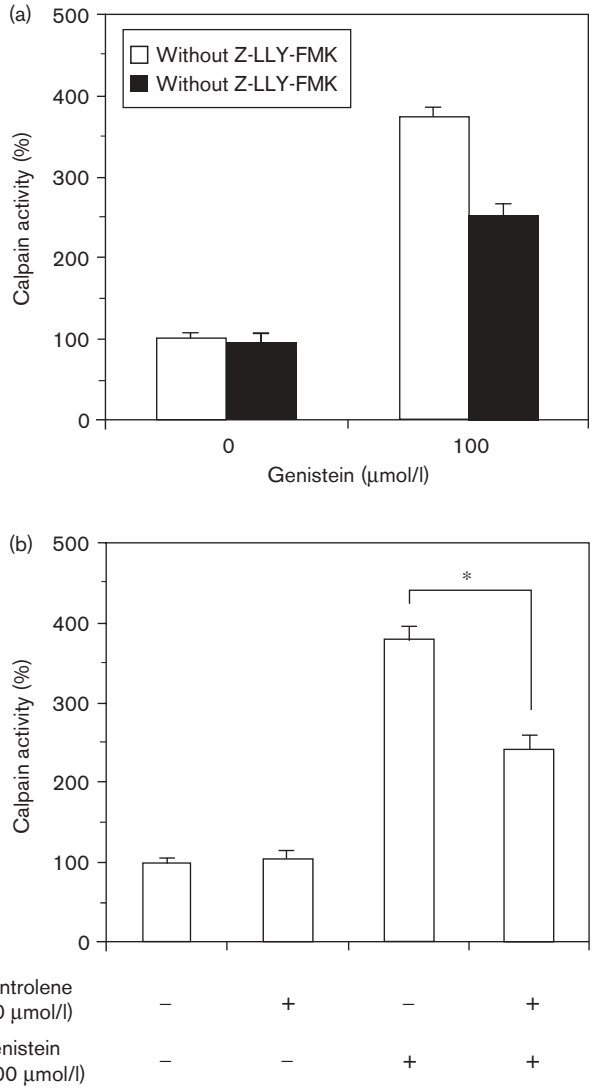
The involvement of MAPK signaling pathways in genistein-induced apoptosis was determined by treating

Fig. 4



Dantrolene treatment with genistein increases the cell proliferation of MCF-7 cells. MCF-7 cells were treated with the indicated concentrations of dantrolene and 100 μmol/l genistein for 24 h. Then, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (a) and cell viability assay (b) were performed. Open and closed bars represent 100 μmol/l genistein nontreated and treated cells, respectively. Data are mean ± SD from three independent experiments and each experiment was conducted in triplicate.

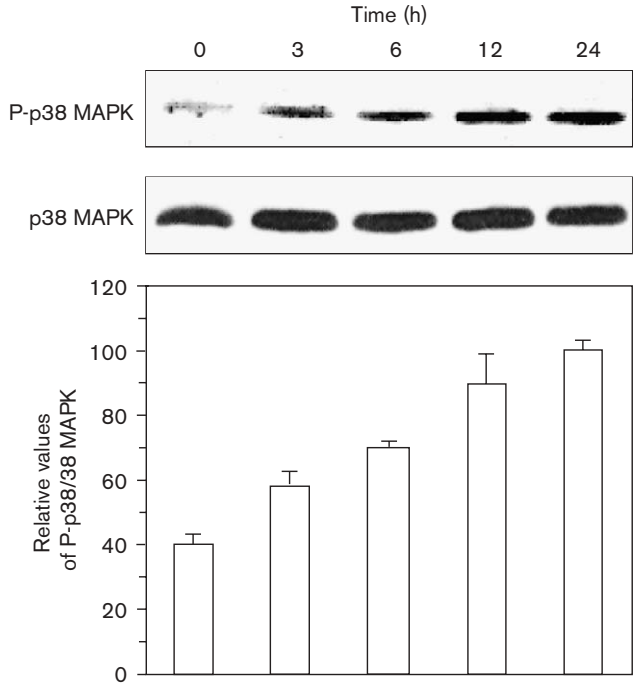
Fig. 5



Genistein increases calpain activity, and it is decreased with dantrolene treatment. (a) MCF-7 cells were treated with 100 μmol/l of genistein for 24 h. Same amounts of cell extracts (50 μg) were incubated with 4 nmol calpain substrate for 30 min, and the increase of fluorescence resulting from the cleavage of calpain substrate by calpain was measured at an excitation and emission wavelength of 320/480 nm. To confirm the fluorescence enhancement caused by calpain activity, a calpain inhibitor (Z-LLY-FMK, 1 nmol) was added to the protein extract–calpain substrate mixture. (b) Nontreated or pretreated MCF-7 cells with 20 μmol/l dantrolene for 1 h were incubated with 100 μmol/l genistein for 24 h and calpain activity was measured as described above. Data are mean ± SD from three independent experiments and each experiment was conducted in triplicate (*$P < 0.05$).

MCF-7 cells with 100 μmol/l genistein for 0, 3, 6, 12 or 24 h in the presence of phosphorylated p38 MAPK (P-p38 MAPK) or Erk1/2 (P-Erk1/2). The results were analyzed by Western blot analysis using antiphospho-p38 MAPK or antiphospho-Erk1/2 antibody. As shown in Fig. 6, genistein-induced activation of p38 MAPK was evident after 3 h treatment, whose intensity increased over 24-h periods. Genistein, however, did not change P-Erk1/2 phosphorylation (data not shown). Cells treated with a specific p38 MAPK inhibitor SB203580 (10 μmol/l; Calbiochem) [34] for 1 h before incubation with

Fig. 6



Genistein enhances the phosphorylation of p38 MAPK. MCF-7 cells were treated with 100 μmol/l genistein for the indicated times. Same amounts of whole-cell extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and phospho-p38 MAPK was detected by Western blot analysis. β-Actin was used as a control. The bar diagram shows means of relative band intensities from two independent experiments. Maximum intensity was estimated as 100.

100 μmol/l of genistein for 6 h showed a significant decrease in genistein-induced phosphorylation of p38 MAPK (Fig. 7a). SB203580 was able to reverse the genistein-induced cell growth inhibition by 60% (Fig. 7b). Cells treated with dantrolene for 1 h before adding 100 μmol/l genistein for 12 h also showed a decrease in genistein-induced P-p38 MAPK (Fig. 7c).

Apoptotic signaling kinase 1 activation is associated with genistein-induced apoptosis of MCF-7 cells

Genistein's potential involvement with the ASK1–p38 MAPK activation cascade was investigated. MCF-7 cells were treated with 100 μmol/l of genistein for 0, 1, 3, 6, 9, 12 and 24 h, and the degree of ASK1 activation was observed by Western blot analysis using antiphospho-ASK1 antibody (Fig. 8a). ASK1 activation was observed 1 h after genistein treatment, with the maximum activation peaking at 9 h. Genistein-induced ASK1 activation that relates to Ca^{2+} release was investigated by treating the cells with 100 μmol/l genistein and dantrolene. Genistein activated ASK1, whereas dantrolene retarded the genistein-induced ASK1 activation (Fig. 8b).

Genistein does not induce reactive oxygen species generation in MCF-7 cells

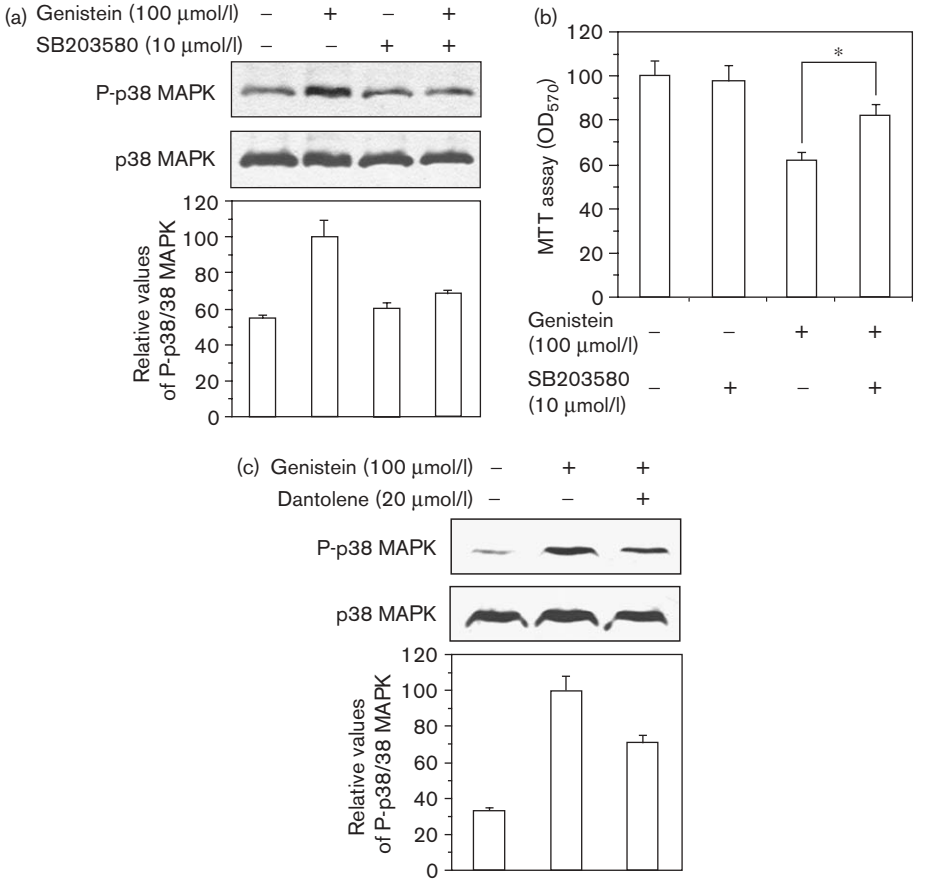
The generation of intracellular ROS by genistein in MCF-7 cells was evaluated using intracellular peroxide-dependent oxidation of DCFH-DA. Cells were treated with H_2O_2 (1 mmol/l) as a positive control and compared with genistein-treated cells. Fluorescent DCF from 1 mmol/l H_2O_2-treated cells was observed as expected, whereas 100 μmol/l genistein-treated cells did not show any DCF fluorescence (data not shown). Cells were also treated with genistein or H_2O_2 in the presence of *N*-acetyl-L-cysteine (NAC) for 1 h to show that the addition of NAC effectively protected cells from H_2O_2-generated ROS, whereas genistein-induced cell growth inhibition was not affected (data not shown).

Discussion

In this report, we investigated molecular mechanisms involved in genistein-induced apoptosis using human breast cancer MCF-7 cells. Genistein was found to inhibit 50% cell growth at $IC_{50} = 27.5 \pm 0.8$ μmol/l using the MTT assay (Fig. 1a). As the MTT assay is a technique to measure the cell viability that does not necessarily reflect apoptosis, the effective amount of genistein to induce apoptosis was determined using 50 and 100 μmol/l genistein, and observing the apoptotic DNA fragmentation over 12, 24 and 48 h of treatment (Fig. 1b and c). At 100 μmol/l of genistein with 24 h incubation was determined to be reasonable dose and condition for our genistein-induced apoptosis study using MCF-7 cells.

Apoptosis mechanisms have been shown to involve dose-dependent activations of caspase 3, 8 and 9, or alternative expression of Bcl-2 family proteins that act in either a proapoptotic or an antiapoptotic manner to regulate the release of cytochrome *c* from mitochondria. Caspase 3 was shown to stimulate caspase 8-mediated cell surface death receptors and caspase 9-mediated perturbation of mitochondria. As MCF-7 cells do not possess caspase 3, however, owing to 47-base pair deletion in the caspase 3 gene [36], caspase 7, a caspase 3 subfamily, has been shown to be involved in the apoptosis of MCF-7 cells [37,38]. In our investigation, activation of caspase 7 and PARP was observed in Western blot analysis of 100 μmol/l genistein-treated MCF-7 cells (Fig. 2a and b). Bcl-2 or Bax protein levels remained constant regardless of genistein treatment, whereas the genistein-induced caspase 7 activation cascade did not involve caspase 8 or 9 (data not shown). These results suggested that genistein-induced apoptosis occurs through the activation of caspase 7, instead of in a proapoptotic or antiapoptotic manner that is regulated by the release of cytochrome *c* from mitochondria. Caspase 3 or 7 could also be activated by caspase 12 activation cascade, which is induced by endoplasmic reticulum stress [39].

Fig. 7

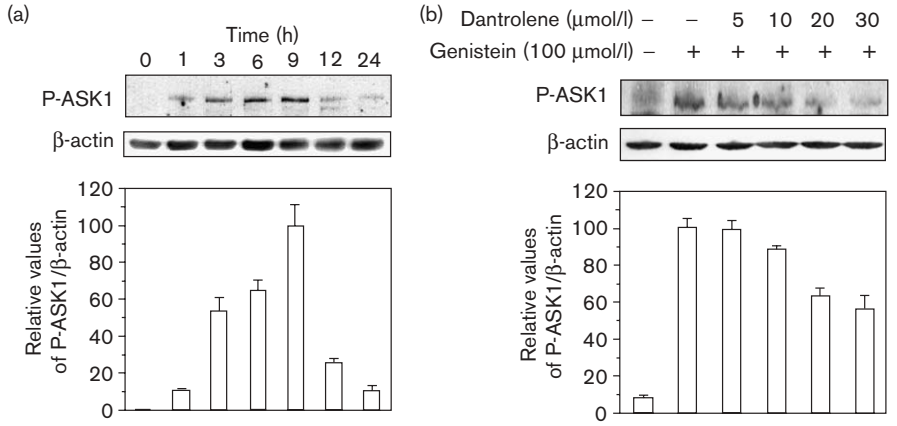


SB203580 and dantrolene reduces the phosphorylation of p38 mitogen-activated protein kinase (MAPK) induced by genistein. (a) MCF-7 cells were treated with either SB203580 or genistein alone and in combination for 6 h. Equal amounts of whole-cell extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and phospho-p38 MAPK was detected by Western blot analysis. (b) MCF-7 cells were treated with either SB203580 or genistein alone and in combination for 6 h. Then, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed. Data are mean ± SD from three independent experiments and each experiment was conducted in triplicate (*$P < 0.05$).
(c) MCF-7 cells were treated with either dantrolene or genistein alone and in combination for 12 h. Phospho-p38 MAPK was detected by Western blot analysis. Non-phospho-p38 MAPKs in (a) and (c) were used as a control. The bar diagrams in (a) and (c) show the mean of relative band intensities from two independent experiments. Maximum intensity was estimated as 100.

Genistein has recently been reported to induce Ca^{2+}-mediated, calpain/caspase 12-dependent apoptosis in MCF-7 cells [21]. Genistein treatment of MCF-7 cells resulted in an increase of intracellular Ca^{2+} concentration through depletion of the endoplasmic reticulum Ca^{2+} stores [21]. Genistein treatment of MCF-7 cells activated calpain (Fig. 5a), whereas dantrolene, a drug that inhibits Ca^{2+} release from its intracellular stores by blocking the ryanodine receptor/calcium release channel [40], inhibited the activation of calpain and genistein-induced caspase 7 (Figs 3 and 5b). Genistein-induced apoptosis was also retarded by the addition of dantrolene (Fig. 4a and b). These results suggest genistein-induced apoptosis in MCF-7 cells is perhaps associated with the calpain–caspase 7 signaling pathway. Although MCF-7 cells have not been known to express the ryanodine receptor, dantrolene was shown to decrease Ca^{2+} presented in cytoplasm (data not shown) and was used to effectively inhibit genistein-induced calpain activity. This inhibition was thought to be mediated by the disruption of Ca^{2+} release induced by genistein treatment. These results suggested that genistein-induced caspase 7 activation in MCF-7 cells involves the activation of calpain, which has been known to be induced by Ca^{2+} depletion of the endoplasmic reticulum. Thus, genistein-induced apoptosis in MCF-7 cells is suggested to be associated with calpain/caspase 7 signaling pathway.

Although genistein treatment did not show any change in phosphorylation of Erk1/2 in MCF-7 cells (data not shown), a gradual increment of P-p38 MAPK in time-dependent manner was observed (Fig. 6). SB203580, a specific inhibitor of p38 MAPK, blocked the genistein-induced activation of p38 MAPK (Fig. 7a). SB203580 treatment was also able to reduce genistein-induced

Fig. 8



The phosphorylation of apoptosis signaling kinase 1 (ASK1) is induced by genistein and dantrolene reduces its phosphorylation by genistein. (a) MCF-7 cells were treated with 100 μmol/l genistein for the indicated times. Equal amounts of whole-cell extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and phospho-ASK1 was detected by Western blot analysis. (b) MCF-7 cells were treated with either dantrolene or genistein alone and in combination for 9 h. Equal amounts of whole-cell extracts were separated by SDS–PAGE and phospho-ASK1 was detected by Western blot analysis. β-Actin was used as a control. The bar diagram shows the mean of relative band intensities from two independent experiments. Maximum intensity was estimated as 100.

apoptosis (Fig. 7b). These results suggested that p38 MAPK activation is critical for genistein to induce apoptosis in MCF-7 cells. Activation of p38 MAPK by genistein is thought to be a distinctive characteristic for MCF-7 cells. Genistein was shown to differently regulate the activation of Erk1/2 or p38 MAPK according to cell types [41,42]. Genistein inhibited prostate epithelial cell proliferation by inactivating Erk1/2, but induced apoptosis in neuronal cells by enhancing activated Erk1/2. The activation of p38 MAPK by genistein was, however, not observed in these cells. p38 MAPK was able to be activated by the increase of intracellular Ca^{2+} [24]. When MCF-7 cells were treated with genistein in the presence of dantrolene, genistein-activated p38 MAPK level was retarded (Fig. 7c). These results suggested that genistein-induced p38 MAPK activation is affected by the increase of intracellular Ca^{2+} released from the endoplasmic reticulum.

ASK1 has been shown to be involved in Ca^{2+}-induced p38 MAPK activation [23]. As shown in Fig. 8a, phosphorylation of ASK1. Genistein treatment was shown to enhance phosphorylation of ASK1. Dantrolene, however, retarded this ASK1 enhancement (Fig. 8b). Thus, the activation of ASK1 involved in the genistein-induced apoptotic process of MCF-7 cells was related to Ca^{2+} release from the endoplasmic reticulum. Although ASK1 has been reported to induce apoptosis in various cells through mitochondria-dependent caspase activation [24–26], caspase 9 activation or changes in Bcl-2 and Bax protein levels were not detected in genistein-treated MCF-7 cells; instead, genistein-induced ASK1 activation that influences p38 MAPK activation was suggested.

As ROS have been suggested to activate ASK1 [27,29], potential involvement of ROS in genistein-induced activation of the ASK1–p38 MAPK cascade was investigated. DCF, a fluorescent compound that is generated from peroxidase-dependent oxidation of DCFH-DA, was not detected in genistein-treated MCF-7 cells. Cell growth inhibition by genistein was not recovered by a treatment with a ROS trapping agent NAC (data not shown). These results demonstrated that genistein does not involve ROS generation to induce the ASK1–p38 MAPK activation cascade, which involves Ca^{2+} release from the endoplasmic reticulum to induce apoptosis.

In summary, we investigated the molecular mechanisms related to genistein-induced apoptosis in MCF-7 cells. We demonstrated that genistein-induced apoptosis was mediated through the activation of the calpain–caspase 7 cascade by Ca^{2+} released from the endoplasmic reticulum. We further demonstrated that genistein also achieved apoptosis of MCF-7 cells by activating ASK1 and p38 MAPK through Ca^{2+} release from the endoplasmic reticulum.

References

1 Adlercreutz CH, Goldin BR, Gorbach SL, Hockerstedt KA, Watanabe S, Hamalainen EK, *et al.* Soybean phytoestrogen intake and cancer risk. *J Nutr* 1995; **125**:S757–S770.
2 Li Y, Che M, Bhagat S, Ellis KL, Kucuk O, Doerge DR, *et al.* Regulation of gene expression and inhibition of experimental prostate cancer bone metastasis by dietary genistein. *Neoplasia* 2004; **6**:354–363.
3 Yu Z, Li W, Liu F. Inhibition of proliferation and induction of apoptosis by genistein in colon cancer HT-29 cells. *Cancer Lett* 2004; **215**:159–166.
4 Hewitt AL, Singletary KW. Soy extract inhibits mammary adenocarcinoma growth in a syngeneic mouse model. *Cancer Lett* 2003; **192**:133–143.
5 Banerjee S, Zhang Y, Ali S, Bhuiyan M, Wang Z, Chiao PJ, *et al.* Molecular evidence for increased antitumor activity of gemcitabine by genistein *in vitro*

and *in vivo* using an orthotopic model of pancreatic cancer. *Cancer Res* 2005; **65**:9064–9072.
6 Mohammad RM, Banerjee S, Li Y, Aboukameel A, Kucuk O, Sarkar FH. Cispain-induced antitumor activity is potentiated by the soy isoflavone genistein in BxPC-3 pancreatic tumor xenografts. *Cancer* 2006; **106**: 1260–1268.
7 Kuiper GG, Carlsson B, Gtandien K, Enmark E, Haggblad J, Nilsson S, *et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor α and β. *Endocrinology* 1997; **138**: 863–870.
8 Middleton E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 2000; **52**:673–751.
9 Kelloff GJ, Crowell JA, Steele VE, Lubet RA, Malone WA, Boone CW, *et al.* Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. *J Nutr* 2000; **130**:467S–471S.
10 Messina MJ. Soy intake and cancer risk: a review of the *in vitro* and *in vivo* data. *Nutr Cancer* 1994; **21**:113–131.
11 Fotsis T, Pepper M, Aktas E, Breit S, Rasku S, Adlercreutz H, *et al.* Genistein, a dietary-derived inhibitor of in vitro angiogenesis. *Proc Natl Acad Sci U S A* 1993; **90**:2690–2694.
12 Sonee M, Sum T, Wang C, Mukherjee SK. The soy isoflavone, genistein, protects human cortical neuronal cells from oxidative stress. *Neurotoxicology* 2004; **25**:885–891.
13 Su SJ, Yeh TM, Chuang WJ, Ho CL, Chang KL, Cheng HL, *et al.* The novel targets for anti-angiogenesis of genistein on human cancer cells. *Biochem Pharmacol* 2005; **69**:307–318.
14 Davis JN, Singh B, Bhuiyan M, Sarkar FH. Genistein-induced upregulation of $p21^{WAF1}$, downregulation of cyclin B, and induction of apoptosis in prostate cancer cells. *Nutr Cancer* 1999; **32**:123–131.
15 Sarkar FH, Li U. Soy isoflavones and cancer prevention. *Cancer Invest* 2003; **21**:744–757.
16 Alhasan SA, Pietrasczkiwicz H, Alonso MD, Ensley J, Sarkar FH. Genistein-induced cell cycle arrest and apoptosis in a head and neck squamous cell carcinoma cell line. *Nutr Cancer* 1999; **34**:12–19.
17 Li Y, Sarkar FH. Down-regulation of invasion and angiogenesis-related genes identified by cDNA microarray analysis of PC3 prostate cancer cells treated with genistein. *Cancer Lett* 2002; **186**:157–164.
18 Dampier K, Hudson EA, Howells LM, Manson MM, Walker RA, Gescher A. Differences between human breast cell lines in susceptibility towards growth inhibition by genistein. *Br J Cancer* 2001; **85**:618–624.
19 Lian F, Bhuiyan M, Li YW, Wall N, Kraut M, Sarkar FH. Genistein-induced G_2-M arrest, $p21^{waf1}$ upregulation, and apoptosis in a non-small-cell lung cancer cell line. *Nutr Cancer* 1998; **31**:184–191.
20 Choi YH, Zhang L, Lee WH, Park KY. Genistein-induced G_2/M arrest is associated with the inhibition of cycline B1 and the induction of p21 in human breast carcinoma cells. *Int J Oncol* 1998; **13**:391–396.
21 Sergeev IN. Genistein induces Ca^{2+}-mediated, calpain/caspase-12 dependent apoptosis in breast cancer cells. *Biochem Biophys Res Commun* 2004; **321**:462–467.
22 Mathiasen IS, Sergeev IN, Bastholm L, Elling F, Norman AW, Jaattela M. Calcium and calpain as key mediators of apoptosis-like cell death induced by vitamin D compounds in breast cancer cell. *J Biol Chem* 2002; **277**:30738–30745.
23 Takeda K, Matsuzawa A, Nishitoh H, Tobiurne K, Kishida S, Ninomiya-Tsuji J, *et al.* Involvement of ASK1 in Ca^{2+}-induced p38 MAP kinase activation. *EMBO Rep* 2004; **5**:161–166.
24 Hatai T, Matsuzawa A, Inoshita S, Mochida Y, Kuroda T, Sakamaki K, *et al.* Exclusion of apoptosis signal-regulating kinase 1 (ASK1)-induced apoptosis by the mitochondria-dependent caspase activation. *J Biol Chem* 2000; **275**:26576–26581.
25 Nishitoh H, Saitoh M, Mochida Y, Takeda K, Nakano H, Rothe M, *et al.* ASK1 is essential for JNK/SAPK activation by TRAF2. *Mol Cell* 1998; **2**: 389–395.
26 Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, *et al.* Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 1998; **17**:2596–2606.
27 Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, *et al.* ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* 2002; **16**:1345–1355.
28 Machino T, Hashimoto S, Maruoka S, Gon Y, Hayashi S, Mizumura K, *et al.* Apoptosis signal-regulating kinase 1-mediated signaling pathway regulates hydrogen peroxide-induced apoptosis in human pulmonary vascular endothelial cells. *Crit Care Med* 2003; **31**:2776–2781.
29 Tobiume K, Matsuzawa A, Takahashi T, Nishitoh H, Morita K, Takeda K, *et al.* ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep* 2001; **2**:222–228.
30 Su B, Karin M. Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr Opin Immunol* 1996; **8**:402–411.
31 Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascade. *Adv Cancer Res* 1998; **74**:49–139.
32 Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature* 2001; **411**:342–348.
33 Chen YR, Meyer CF, Tan TH. Persistent activation of c-Jun *N*-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. *J Biol Chem* 1996; **271**:631–634.
34 Kanno S, Shouji A, Hirata R, Asou K, Ishikawa M. Effects of naringin on cytosine arabinoside (Ara-C)-induced cytotoxicity and apoptosis in P388 cells. *Life Sci* 2004; **75**:353–365.
35 Cryns V, Yuan J. Proteases to die for. *Genes Dev* 1998; **12**:1551–1570.
36 Janicke RU, Sprengart ML, Wati MR, Porter AG. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* 1998; **273**:9357–9360.
37 Hu CA, Tang CA, Wang J. Caspase activation in response to cytotoxic Rena catesbeiana ribonuclease in MCF-7 cells. *FEBS Lett* 2001; **503**: 65–68.
38 Twiddy D, Cohen GM, MacFarlane M, Cain K. Caspase 7 is directly activated by the ~700 kDa apoptosome complex and is released as a stable XIAP-caspase 7 ~200 kDa complex. *J Biol Chem* 2006; **281**: 3876–3888.
39 Hitomi J, Katayama T, Taniguchi M, Honda A, Imaizumi K, Tohyama M. Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase 3 via caspase 12. *Nurosci Lett* 2003; **357**:127–130.
40 Alvarez-Vega M, Baamonde A, Hidalgo A, Menendez L. Effects of the calcium release inhibitor dantrolene and the Ca^{2+}-ATPase inhibitor thapsigargin on spindal nociception in rats. *Pharmacology* 2001; **62**: 145–150.
41 Wang X, Clubbs EA, Bomser JA. Genistein modulates prostate epithelial cell proliferation via estrogen- and extracellular signal-regulated kinase-dependent pathways. *J Nutr Biochem* 2006; **17**:204–210.
42 Linford NJ, Yang Y, Cook DG, Dorsa DM. Neuronal apoptosis resulting from high doses of the isoflavone genistein: role for calcium and p42/44 mitogen-activated protein kinase. *J Pharmacol Exp Ther* 2001; **299**:67–75.