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Caspase-3 status is a determinant of the differential responses to genistein between MDA-MB-231 and MCF-7 breast cancer cells

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Abstract

Genistein, a soy isoflavone with anti-tumor properties, has both estrogenic and non-estrogenic activities. Genistein sensitive/estrogen receptor negative (ER-) MDA-MB-231 cells and genistein resistant/ER+MCF-7 cells are frequently cited as examples of differential responses to genistein due to different ER status. Other factors that may affect genistein response, however, are largely unknown. Based on our finding that MCF-7 is caspase-3 deficient, we examined whether caspase-3 status plays a role in the differential responses between the two cell lines. We demonstrate that reconstitution of caspase-3 significantly sensitizes MCF-7 cells to genistein. Specific knockdown of caspase-3 in MDA-MB-231 cells renders the cells resistant to genistein. We also found that caspases-4 and -10 were downregulated in MCF-7 cells. Reconstitution of caspase-10 in MCF-7 cells, however, resulted in little sensitization. Moreover, we show that caspase-3 downregulation is very common in breast cancer cell lines and tumor tissues. Taken together, our data indicate that caspase-3 is a critical determinant of cellular response to genistein, which may have important implications in studying soy/genistein-mediated anti-tumor activities.

Keywords: Genistein; Caspase-3; Apoptosis; Breast cancer; MCF-7; MDA-MB-231

1. Introduction

A low incidence of breast cancer in Asian women has been associated with a diet high in soy products [1], and many soy-mediated anti-tumor properties have been attributed to genistein, a major soy isoflavone [2]. Identification of the factors that affect genistein-mediated tumor inhibition is of great significance in the implication of soy/genistein in tumor prevention and/or treatment. Available evidence suggests genistein functions by modulating multiple signaling pathways [3], including estrogen receptor (ER) pathway [4], receptor tyrosine kinase (RTK) pathways [5], and NF-KB pathway [6]. Phenotypically, genistein-induced apoptosis and/or cell cycle arrest links the modified cellular activities and tumor growth inhibition [3].

The response of breast cancer cells to genistein vary significantly from cell line to cell line [3,7–9]. *In vitro* and *in vivo* results indicate genistein dose and estrogen dependency of the cancer cells appear to be the most prominent factors [10] associated with genistein's SERM (selective estrogen receptor modulator) property [11]. For ER-α negative/estrogen-independent cells, such as MDA-MB-231 cells, genistein inhibits cell growth at both low and high concentrations [12,13]. Nevertheless, genistein exhibits a concentration dependent bi-phasic effect on ER-α positive/estrogen-dependent cells, such as MCF-7. In these cells, genistein promotes cell growth at a lower concentration (<5 μM), while inhibiting growth at a higher concentration (>10 μM) [14–17]. Among the numerous cell lines tested, MCF-7 and MDA-MB-231 cell lines are the most popular breast cancer cell lines used for genistein studies [12,13,17–19]. Most studies indicate that MDA-MB-231 cells are more sensitive than MCF-7 [4,9,20–22], although one group reported that MCF-7 cells were more sensitive than MDA-MB-231 cells [23]. Currently, the differential responses to genistein between the two cell lines are primarily explained by their ER status [4,18,24–26]. While ER status is critical in genistein responses, the two cell lines are also distinctive in p53 and caspase-3 status, both of which are critical in cell cycle

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and/or apoptosis regulation. MDA-MB-231 cells express mutant p53 and functional caspase-3 [12,23], whereas MCF-7 cells express wild type p53 and are caspase-3 deficient [23,27,28].

Caspase-3 is a key member of the caspase family, a group of cysteine proteases that mediate apoptotic execution [29]. It can be activated by apoptotic signals from both death receptor and intracellular/mitochondrial pathways [30]. Caspase-3 functions as a major effector caspase by cleavage of numerous cell death substrates, to cellular dysfunction and destruction [31]. Involvement of caspase-3 in genistein-induced apoptosis has been detected in previous studies [11,12,32]. Given the functional overlap among the caspase family members and the different status of p53 between MCF-7 and MDA-MB-231 cells lines, however, it is unclear if and how much caspase-3 status contributes to the differential response to genistein. Recently, caspase-3 deficiency and downregulation have been associated with breast carcinogenesis [33], suggesting caspase-3 could be a biomarker in cancer prevention and treatment. This is supported by our previous reports showing that functional caspase-3 is critical for effective chemo- and radio-therapy [34,35]. In this report, we studied the role of caspase-3 in genistein-induced apoptosis by characterization of apoptosis in caspase-3 reconstituted MCF-7 cells and MDA-MB-231 cells with caspase-3 knockdown. We found that caspase-3 status, in addition to ER status, significantly contributes to the differential responses between the two cell lines. While our data clarifies an important issue in genistein responses in breast cancer cell lines, it also underscores the significance of apoptosis, and caspase-3 in particular, in genistein-mediated anti-tumor mechanisms.

2. Materials and methods

2.1. Reagents

DMEM/F12 medium, genistein, 5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma (St. Louis, MO). Antibodies against caspases-3, -4 and -10, and Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against ER-α was from Upstate Biotechnology, Inc. (NY). Antibody against Poly (ADP-ribose) polymerase (PARP) was from BD Biosciences (San Diego, CA). Horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from Amersham/GE Health Care (Arlington Height, IL). Caspase multiplex PCR set-5 and 6 were purchased from BioSources (Carmarillo, CA). Apoptosis ELISA kit was from Roche Diagnostics (Indianapolis, IN).

2.2. Cell lines, cell culture and breast tumor tissues

MCF-7 and MDA-MB-231 cells, and all the other cells lines tested in Fig. 4 were from ATCC. MCF-7/PV and MCF-7/C3 cells are stable MCF-7 cell lines transfected with pBabe/puro vector or caspase-3 encoding vector as described before [35]. MCF-7/C10 cell line was established by transfecting MCF-7 cells with caspase-10 (Mch4) encoding plasmid followed by G418 selection [27]. The cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum (FBS). To prepare for protein lysate, the cells were inoculated into 60 mm dish at 1×10^6/ dish 24 h before genistein treatment and sample collection. The controls for genistein treatment were the cells treated with 0.1% DMSO (solvent for genistein) at the indicated time. Primary breast tumor samples were obtained from patients who had undergone breast surgery at Evanston Northwestern Healthcare Research Institute with Institutional Review Board (IRB) approval.

2.3. MTT assay

The cells were inoculated into 96-well-plate at 1×10^3 cells/well, or 24-well-plate at 3×10^3 cells/well 24 h before the treatment. The cells were then treated with increasing concentrations of genistein at indicated concentrations for 6 days. After removal of the medium, 50 μl of MTT solution (500 μg/ml) was added to each well followed by 4 h incubation. MTT solution was replaced by DMSO to dissolve blue formazan crystals, and absorbance was measured at 562 nm using a microplate reader. Each group contains 8 parallel samples. Survival fractions were calculated as the absorbance in the treated cells over the control (treated with 0.1% DMSO). Statistical differences were analyzed with Student's *t*-test.

2.4. Western blot

Control and drug treated cells or primary breast cancer tissues were lysed with lysis buffer [36] on ice for 30 min. Protein concentration was determined using BCA assay kit (Pierce Biotechnology Inc. IL). Fifty μg of protein lysate was loaded onto each lane of a gel. Proteins were separated with 12% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with a specific primary antibody at a dilution of 1:1000 to 2000, followed by washing and probing with a corresponding secondary antibody. The specific protein band was visualized by autoradiography using an ECL kit.

2.5. Short hairpin RNA (shRNA) transfection

For silencing the expression of caspase-3 in MDA-MB-231 cells, caspase-3 specific and control shRNA (New England Biolab, Ipswich, MA) was transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For the preparation of Western blot lysate, 3×10^5 cells were inoculated into each well of 6-well plates 24 h before transfection. The cells in each well were transfected with 200 pmol of control or caspase-3 specific shRNA. Genistein was added 16 h after transfection. Cell lysates were collected 48 h post transfection. For MTT assay, the cells were inoculated into 24-well plate at 3000 cells/well. shRNA was transfected as above. Genistein at indicated concentrations were added to the transfected cells 16 h post transfection and left for 4 days before MTT assay.

2.6. RNA extraction, reverse transcription (RT), and multiplex PCR

Total RNA of indicated cell lines was isolated using RNeasy Mini Kit (QIAGEN, CA). First strand cDNA synthesis was performed using SuperscriptIII™ First Strand synthesis system (Invitrogen). After reverse transcription (RT) with 3 μg of total RNA, one tenth of the RT product was used as template for PCR reaction. mRNA levels of specific caspases were detected using 2 sets of multiplex PCR kits, which are set 5 and set 6 of the apoptosis series of multiplex PCR from BioSource (Carmarillo, CA). With gene specific primers, set 5 detects caspases-3, -5, -8, -9, Apaf-1, and GAPDH. Set 6 detects caspases-2, -4, -6, -7, -10, and GAPDH. Following the manufacturer's protocol, cDNAs of specific caspases were amplified for 30 cycles. Twenty μl of PCR products were electrophoresed on 2% agarose gel and visualized by Ethidium Bromide staining. The image was acquired and analyzed using UPVII gel documentation system.

2.7. Apoptosis detection by ELISA

Induction of apoptosis was assessed with a cell death detection ELISA kit (Roche Diagnostics) by following the manufacturer's instruction. This test is based on the quantitative determination of cytoplasmic histone-associated DNA fragments in the form of mononucleosomes and oligonucleosomes after induced apoptotic death. In brief, cells in a 24-well plate were treated with genistein at indicated concentrations for 30 h. Both floating and adherent cells were

collected for lysis preparation. Based on the absorbance at 405 nM from three parallel samples, the data were analyzed using Student's *t*-test.

3. Results

3.1. Differential responses to genistein and the status of ER and caspase-3 in MCF-7 and MDA-MB-231 cells

As a basis for further characterization of genistein-induced cellular response in MDA-MB-231 and MCF-7 cells, we first examined cell proliferation/growth inhibition of the two cell lines post genistein using MTT assay. As shown in Fig. 1A, MDA-MB-231 cells were more sensitive to genistein than MCF-7 cells. Analysis of the response patterns indicates genistein inhibits MDA-MB-231 cells in a concentration dependent manner, whereas genistein treatment resulted in a bi-phasic response in MCF-7 cells, which are consistent with previous reports [37]. To demonstrate the expression of ER and caspase-3 in MDA-MB-231 and MCF-7 cells, we then examined the protein levels of ER-α and caspase-3 in the two cell lines. Immunoblot analysis indicated MCF-7 cells express high levels of ER-α and non-detectable caspase-3. In contrast, MDA-MB-231 cells express very low levels of ER-α but high levels of caspase-3. These results suggest that besides ER-α status, caspase-3 status may also contribute to the differential responses between the two cell lines.

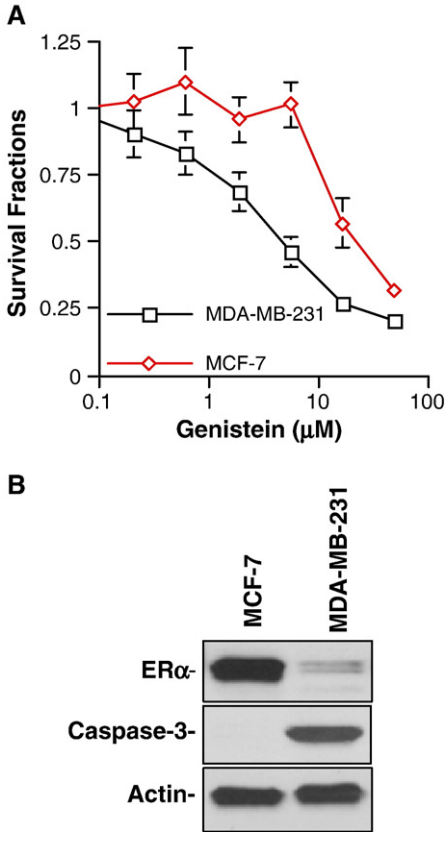

Fig. 1. Different ER-α and caspase-3 status and genistein responses in MCF-7 and MDA-MB-231 cells. (A) MDA-MB-231 cells are more sensitive to genistein than MCF-7 cells. Cells from each line were inoculated into 96-well plates at 1000 cells/well. Genistein was added 24 h later for a 6-day treatment. Cell survival fractions were measured using MTT assay as detailed in Materials and methods. (B) Expression of ER-α and caspase-3 in MCF-7 and MDA-MB-231 cells detected with Western blot.

3.2. Reconstitution of caspase-3 sensitizes MCF-7 cells to genistein

To test whether caspase-3 deficiency contributes to genistein resistance in MCF-7 cells, we studied the effect of genistein on MCF-7/PV and MCF-7/C3 cells, which are MCF-7 cells transfected with control vector and caspase-3, respectively (Fig. 2A) [35]. As shown in Fig. 2B, the survival fractions of genistein treated MCF-7/C3 cells were consistently lower than MCF-7/PV cells, even at lower concentrations, indicating caspase-3 reconstitution significantly sensitizes MCF-7 cells. We also have results suggesting the sensitization was not caused by modified ER-α levels (data not shown). To examine the effect of caspase-3 on genistein-induced apoptosis in MCF-7/C3 cells, we detected the cleavage of PARP, a common proteolytic marker of apoptosis [38], in genistein treated MCF-7/PV and MCF-7/C3 cells (Fig. 2C). A significant increase in PARP cleavage, as indicated by the production of p85 subunit in genistein treated MCF-7/C3 cells, suggests caspase-3 sensitizes MCF-7 cells to genistein by inducing enhanced apoptosis. In MCF-7/C3 cells treated with 25 μM of genistein, we observed increase in both total PARP and the cleaved product (p85). Increase in total PARP is likely caused by increased PARP expression, because we have data showing that phytoestrogens, including genistein, induces PARP expression at higher concentrations in MCF-7 cells (unpublished data). To further demonstrate that caspase-3 reconstitution sensitizes MCF-7 cells to genistein through enhanced apoptosis, we evaluated apoptotic responses in the paired cell lines using an apoptosis ELISA kit from Roche. As shown in Fig. 2D, apoptotic readings in genistein treated MCF-7/C3 cells were significantly higher than those in the control cells under the same conditions. Taken together, results from caspase-3 specific MCF-7 cell lines demonstrate that functional caspase-3 is critical to genistein-induced apoptosis and suggests caspase-3-deficiency in MCF-7 cells contributes to its resistance to genistein.

3.3. Caspase-3 knockdown in MDA-MB-231 cells renders the cells more resistant to genistein

To demonstrate the specific role of caspase-3 in genistein-mediated apoptosis in MDA-MB-231 cells, we examined the effect of caspase-3 knockdown on genistein-mediated growth inhibition in MDA-MB-231 cells. As shown in Fig. 3A, transfection of caspase-3 specific shRNA resulted in significant downregulation of caspase-3. MDA-MB-231 cells with caspase-3 knockdown displayed reduced growth inhibition as compared to the cells transfected with control oligonucleotides (Fig. 3B). Consistently, caspase-3 knockdown in MDA-MB-231 cells led to decreased PARP cleavage (Fig. 3C). Quantitative results from apoptosis ELISA assay also showed that caspase-3 knockdown significantly inhibited genistein-mediated apoptosis in these cells (Fig. 3D). These

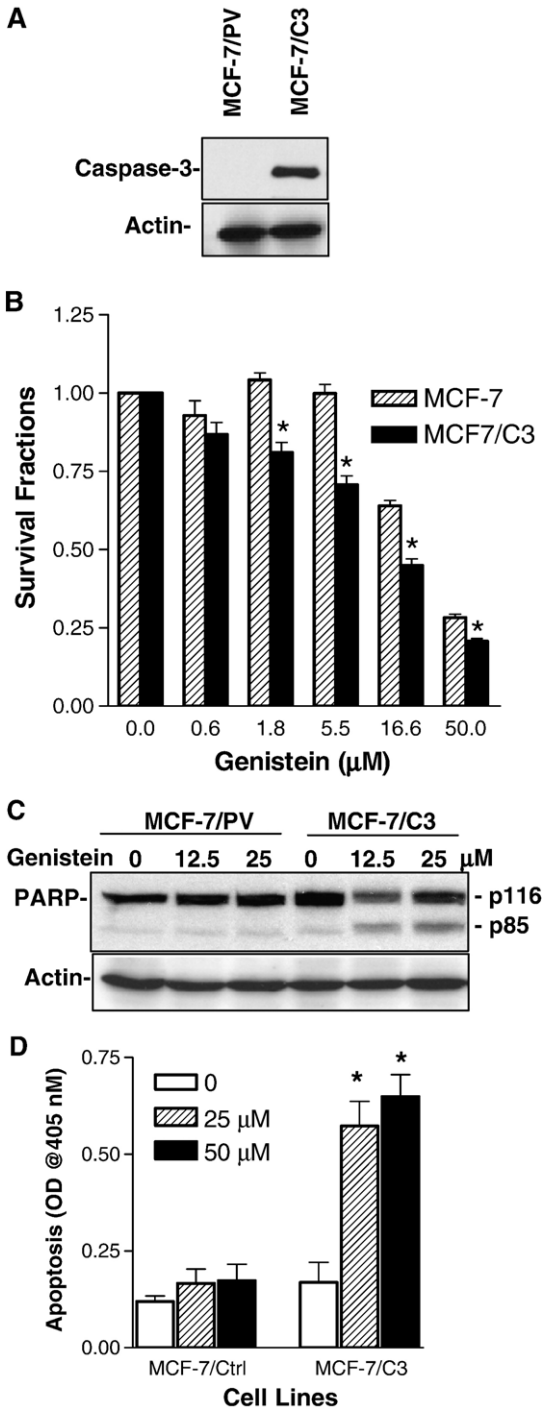

Fig. 2. Caspase-3 reconstitution sensitizes MCF-7 cells to genistein. (A) Caspase-3 status in control (MCF-7/PV) and caspase-3 reconstituted (MCF-7/C3) MCF-7 cells detected with Western blot. (B) Genistein-mediated growth inhibition in MCF-7/PV and MCF-7/C3 cells. Survival fractions of genistein treated cells based on 8 parallel samples were determined using MTT assay. *$p<0.01$ as compared to MCF-7/PV cells under the same conditions. (C) PARP cleavage in genistein-treated MCF-7/PV and MCF-7/C3 cells. Each cell line was treated with genistein at indicated concentrations for 48 h, followed by Western blot detection of PARP. (D) Genistein-induced apoptosis in control (MCF-7/PV) and MCF-7/C3 cells. Cells from each line were inoculated into 24 well plates at 1×10^5/well 24 h, followed by genistein treatment for 30 h. Apoptosis in the treated cells was detected using an apoptosis ELSIA kit from Roche.

results indicate that functional caspase-3 is essential in genistein-induced apoptosis and growth inhibition in MDA-MB231 cells and functional caspase-3 in these cells contributes to their sensitive response to genistein.

3.4. Downregulation of caspase-3 in breast cancer cell lines and tissues

Results from the above experiments suggest that caspase-3 status is associated with cellular response to genistein treatment, and that breast cancer cells with downregulated caspase-3 might be resistant to genistein treatment. To explore the clinical relevance of caspase-3 status in predicting response to genistein treatment, we examined caspase-3 protein levels in 18 breast cancer cell lines and 9 primary breast cancer tissues (Fig. 4). The results indicate that caspase-3 levels varied significantly among different cell lines. As compared to the other cell lines, HS-578T, T47D, MDA-MB-415, ZR-75-1, and SK-BR-3 cells express lower levels of caspase-3 than the other cell lines. Among the primary breast cancer tissues, three of nine expressed very low levels of caspase-3. This suggests that caspase-3 downregulation is common in breast cancers and that breast cancers with caspase-3 downregulation may be resistant to genistein.

3.5. Differential regulation of multiple caspases between MCF-7/and MDA-MB-231 cells

Recent advances suggest that in addition to proteolytic activation, caspase activity is also regulated by other mechanisms, such as DNA methylation and differential transcription [39,40]. We then asked whether the expression of other caspases is also differentially regulated between MCF-7 and MDA-MB-231 cells. To this end, we examined the mRNA levels of caspases-3, -4, -6, -7, -8, -9, -10, and Apaf-1 in control and genistein treated MCF-7 and MDA-MB-231 cells using multiplex PCR (Fig. 5). In this experiment, the band of caspase-3 was detected in MCF-7 cells, which might be the truncate transcript of caspase-3 mRNA that does not yield a mature protein [28]. Analysis of other caspases indicates MDA-MB-231 cells also express higher basal levels of caspases-4 and -10 than MCF-7 cells. This was confirmed by the protein levels of caspases-4 and -10, detected using Western blots (Fig. 5B). Furthermore, genistein treatment also upregulated Apaf-1 and caspase-8 in MDA-MB231 cells, suggesting that not only the basal level, but also the dynamic regulation of certain caspases, were also different between the two cell lines. Specific roles of differentially regulated caspases-4, -8, -10, and Apaf-1 between the two cell lines will be followed in future studies.

3.6. Reconstitution of caspase-10 has little effect on genistein-induced apoptosis in MCF-7 cells

In our previous studies on granzyme B-induced apoptosis [27], we had established an MCF-7 subline that overexpresses caspase-10 (MCF-7/C10) (Fig. 6A). To test whether reconstitution of caspase-10 sensitizes MCF-7 cells to genistein, we

examined genistein-mediated growth inhibition in control (MCF-7/PV) and MCF-7/C10 cells using MTT assay. As shown in Fig. 6B, caspase-10 reconstitution failed to sensitize MCF-7 to genistein to a significant level. Consistently, genistein-induced apoptosis in both cell lines measured with the apoptosis ELISA revealed similar results (Fig. 6C). These data suggest that the role of caspase-10 in genistein-induced apoptosis appears not as significant as caspase-3. Please note that MCF-7/C10 cells remain deficient in caspase-3 and caspase-4. Whether caspase-10-mediated apoptosis in genistein treated cells requires caspase-3 or caspase-4 will be addressed in future studies.

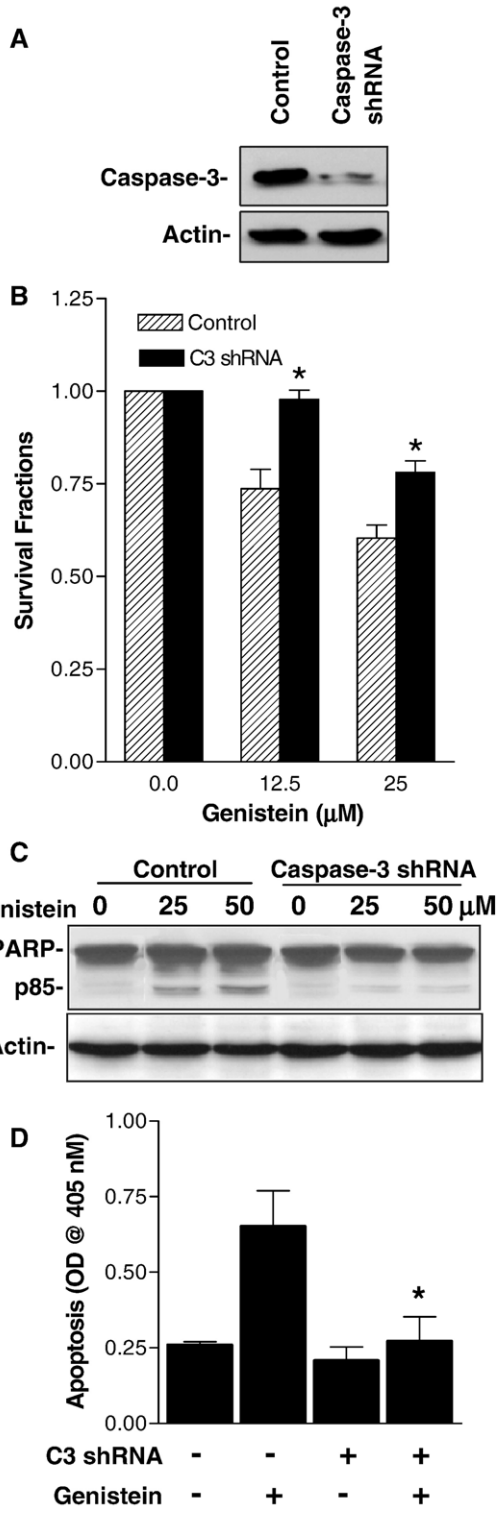

Fig. 3. Caspase-3 knockdown in MDA-MB-231 cells renders the cells resistant to genistein. (A) Caspase-3 levels in control and caspase-3 shRNA transfected cells. MDA-MB-231 cells were transfected with control or caspase-3 specific shRNA as described in Materials and methods. Protein lysate were collected 48 h, followed by caspase-3 detection using Western blot. (B) Genistein-mediated growth inhibition in control and caspase-3 shRNA transfected cells. MDA-MB-231 cells transfected with control or caspase-3 specific shRNA were treated with genistein 16 h post transfection. The survival fractions of the treated (a 4-day treatment) cells based on three parallel samples were determined by MTT assay. *$p<0.01$ as compared to MDA-MB-231 cells transfected with control siRNA under the same conditions. (C) PARP cleavage in genistein treated MDA-MB-231 cells transfected with control or caspase-3 specific shRNA. shRNA transfected cell were treated with genistein at indicated concentrations for 48 h, followed by Western blot detection of PARP. (D) Effect of caspase-3 knockdown on genistein-induced apoptosis in MDA-MB-231 cells. The cells were inoculated in a 24-well plate at 8×10^4/well 16 h, followed by transfection with control (−) or caspase-3 specific shRNA (+) and genistein treatment at 25 μM for 30 h. Apoptosis in the treated cells was detected using an apoptosis ELSIA kit from Roche. *$p<0.01$ as compared to genistein treated MDA-MB-231 cells transfected with control siRNA.

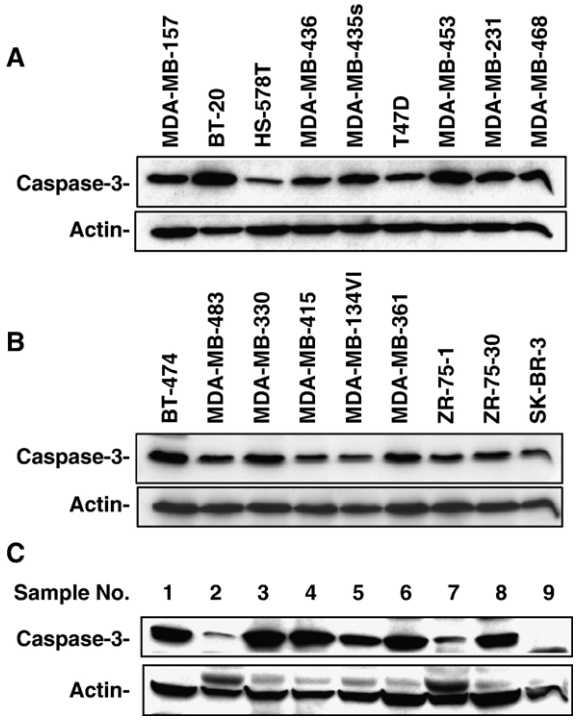

Fig. 4. Expression of caspase-3 in 18 breast cancer cell lines and 9 primary breast cancer tissues. Protein lysates were prepared from non-synchronized cells of the indicated cell lines (A and B) or primary breast cancer tissues (C). Caspase-3 and actin were probed with corresponding specific antibodies using Western blot.

4. Discussion

In this report, we focused on the correlation between caspase-3 status and the differential responses to genistein in

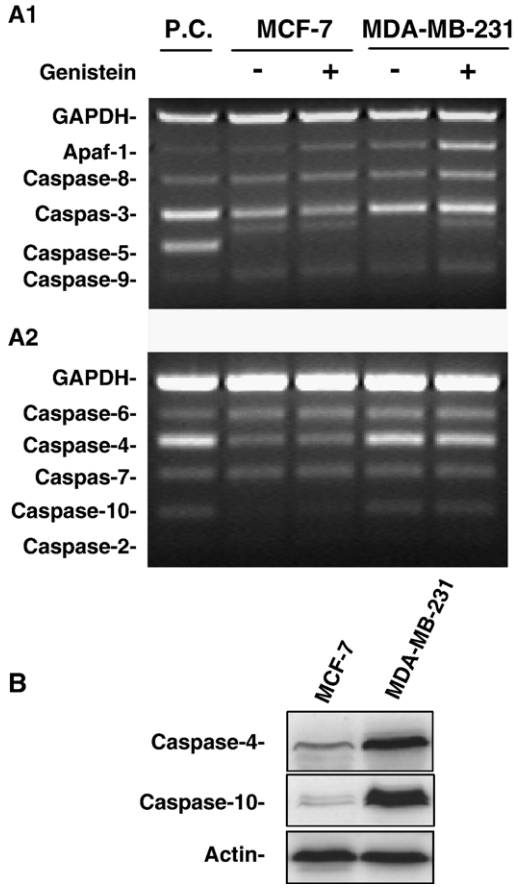

Fig. 5. Differential expression of more apoptotic factors between MFC-7 and MDA-MB-231 cells. (A) mRNA levels of caspases-3, -5, -8, -9, and Apaf-1 (panel A1); and caspases 2, 4, 6, 7, and 10 (panel A2) in genistein treated MCF-7 and MDA-MB-231 cells. Total RNA was extracted from control (−) and genistein (50 μM for 20 h) treated (+) cells. Three μg of total RNA were used for cDNA synthesis. One tenth of the RT product was used for amplification. mRNA levels of the selected caspases were amplified by PCR for 30 cycles using a multi-complex PCR kit (BioSource) containing specific primers of corresponding caspases according to the manufacturer's protocol. P.C., positive control from the kits. (B) Protein levels of caspases-4 and -10 in MCF-7 and MDA-MB-231 cells detected with Western blot.

MCF-7 and MDA-MB-231 cells. This is based on the fact that, other than ER-α status, the cellular factors that affect genistein's sensitivity are largely unknown. Since genistein-mediated non-estrogenic activities are also critical to its inhibitory effect on tumor cell growth, identification of those factors is important for the implication of genistein in clinical oncology. We demonstrate that reconstitution of caspase-3 in MCF-7 cells sensitizes those cells to genistein, and knock-down caspase-3 from MDA-MB-231 cells renders the cells resistant to genistein. These data indicate that the differential responses between the two cell lines are not only determined by ER status but also by caspase-3 status. Caspase-3 deficiency in MCF-7 cells contributes to its resistance to genistein.

The mechanisms of genistein-mediated anti-tumor activities have been extensively studied [3]. Genistein treatment elicits a number of cellular changes that modulate cell/tumor growth and apoptosis. These include modulation of estrogen receptor pathways, inhibition of protein tyrosine kinase and topoisomerase I and II, induction of antioxidant activities, inhibition of angiogenesis, downregulation of Bcl-2, and upregulation of Bax [3]. In theory, any defect in the pathways affected may contribute to genistein resistance. However, due to functional overlap and interactions among these pathways, the factors that have meaningful impact on genistein sensitivities are elusive. As a phytoestrogen with SERM properties, genistein's role in modulation of estrogen signaling has been well established. The possible role of other factors, such as caspase-3, is not adequately recognized.

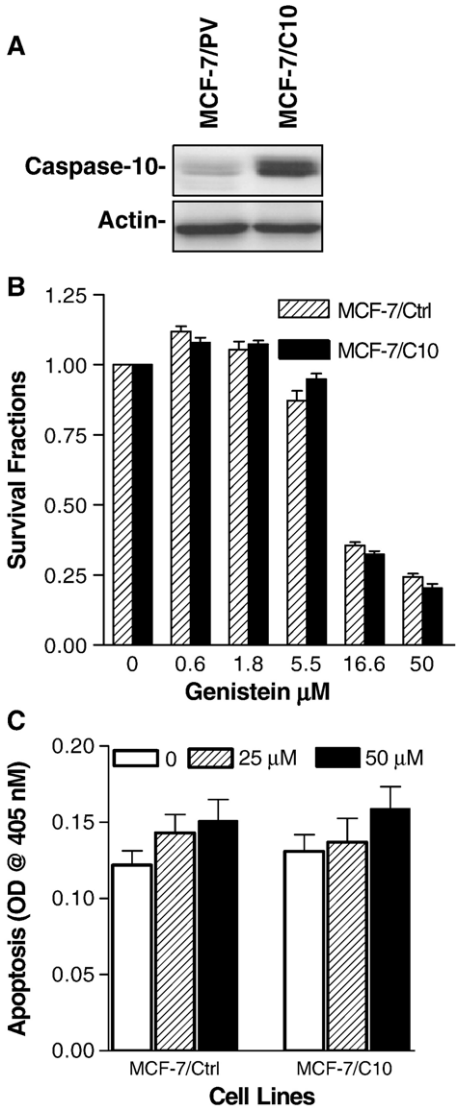

Fig. 6. Genistein-induced apoptosis is not enhanced by caspase-10 overexpression in MCF-7 cells. (A) Caspase-10 status in control (MCF-7/PV) and caspase-10 reconstituted (MCF-7/C10) MCF-7 cells detected with Western blot. (B) Genistein-mediated growth inhibition in control (MCF-7/PV) and MCF-7/C10 cells. After genistein treatment at indicated concentrations for 6 days, the survival fractions of the treated cells based on 8 parallel samples were determined using MTT assay. (C) Genistein-induced apoptosis in control (MCF-7/PV) and MCF-7/C10 cells. Cells from each line were inoculated into 24 well plates at 1×10^5/well 24 h, followed by genistein treatment for 30 h. Apoptosis in the treated cells was detected using an apoptosis ELSIA kit from Roche.

Although p53 status is also different between MCF-7 and MDA-MB-231 cells, sensitive responses to genistein in MDA-MB-231 cells, which express mutant p53, suggests genistein-induced apoptosis may be p53 independent. However, functional regulation of p21/Waf-1, a p53 target gene, appears to be required in genistein-mediated growth inhibition. Involvement of caspase-3 in genistein-induced apoptosis has been reported by several groups [11,12,32] and mainly indicated by cleavage of procaspase-3 and the use of synthetic inhibitors, such as DEVD-CHO. Due to functional overlap among effector caspases (caspases-3, -6 and -7), and since DEVD-CHO may also affect caspases-6 and -7 [41], the specific role of caspase-3 in genistein-induced apoptosis remains undefined. With caspase-3 specific isogenic cell lines (MCF-7/PV and MCF-7/C3), and caspase-3 shRNA-mediated knockdown in MDA-MB-231 cells, our results provide direct evidence supporting the critical role of caspase-3 in genistein-induced apoptosis, and demonstrates a functional correlation between caspase-3 status and the differential responses between the two cell lines.

Caspase-3 is a key caspase that plays a critical role in apoptotic machinery [29]. Activation of caspase-3 has been employed as a marker of apoptosis induced by different mechanisms [42]. Although the cells with deficient caspase-3, such as MCF-7, can undergo apoptosis eventually, the efficacy of the apoptotic responses in these cells is significantly damaged [28,35]. Despite the fact that caspase-3 is classified as an effector caspase, we have shown that caspase-3 functions beyond a typical caspase. Besides its role in the proteolysis of cellular death substrate, caspase-3 activity is required for maximal activation of caspases-6 and -7 [35]. In particular, caspase-3 has feedback action on apical caspases [43,44]. Collectively, the essential role of caspase-3 in apoptotic execution may explain why caspase-3 status is critical to genistein-mediated apoptosis/growth inhibition.

Soy/genistein has potential applications as a chemopreventive or a pharmaceutical agent in anti-cancer studies [8]. The chemopreventive effect of soy/genistein is usually associated with intake of soy meal or low dose isoflavones. The serum concentration of genistein under these conditions is generally lower than 5 μM [45]. In contrast, test of genistein as a pharmaceutical agent often uses concentrations up to 50 μM [46]. In our study, we found that caspase-3 reconstitution sensitized MCF-7 cells to genistein at both low and high concentrations in MTT assay, which measured genistein's effect after 6-day treatment. Since genistein induces both apoptosis and cell cycle arrest, genistein-mediated apoptosis is usually examined at higher concentrations for a shorter treatment. Therefore, our observation may be more relevant to genistein's therapeutic potential.

In this study we also examined caspase-3 expression in nine breast cancer tissues and 18 breast cancer cell lines. The common incidence of caspase-3 deficiency/downregulation in breast cancer underscores the significance of the correlation between caspase-3 status and genistein sensitivity. This is further supported by previous reports showing the frequent detection of caspase-3 downregulation in a larger cohort setting [33]. Although the causes of caspase-3 deficiency/downregulation are not clear, available reports suggest that caspase-3 expression could be affected at multiple levels. Caspase-3 defect in MCF-7 cells is caused by gene mutation [28]. Caspase-3 transcription may be regulated by transcription factor E2F-1 [47]. Based on reports on other caspases [48], DNA methylation could also be a mechanism that contributes to caspase-3 downregulation. These results suggest that caspase-3 deficiency/downregulation is emerging as a clinical problem. Since genistein is also being studied for prevention/intervention of cancers originating from other tissues such as prostate and pancreatic cancers [46,49], identification of caspase-3 status as a determinant of genistein sensitivity may have broader implications.

Results from multiplex PCR screening indicate that other than caspase-3 status, MCF-7 cells also express lower levels of caspases-10 and -4 than MDA-MB-231 cells. We also found that genistein upregulates Apaf-1 and caspase-8 in MDA-MB-231 cells but not in MCF-7 cells. Lower level expression of caspase-10 in MCF-7 cells is consistent with a previous report [27], whereas a lower level expression of caspase-4 in MCF-7 has not been documented. Genistein-induced differential responses in Apaf-1 and caspase-8 appear to be a secondary effector of other factors, such as modified E2F activities [47,50].

With the MCF-7/C10 cell line established in our previous study [27], we found that reconstitution of caspase-10 had little effect on genistein-mediated overall growth inhibition and apoptosis, as indicated by MTT and apoptosis ELISA assays, respectively (Fig. 6). The results suggest that functional impact of caspase deficiency may vary from one caspase to another, which underscores the significance of caspase-3 deficiency. Since MCF-7/C10 cells are also deficient in caspases-3 and -4, the significance of caspase-10 deficiency will be concluded after the tests in cells with normal levels of other caspases. The specific impact of caspase-4 deficiency, and -8 induction, on the differential responses to genistein between the two cell lines will be examined in future studies.

In summary, we examined the functional role of caspase-3 in genistein-induced apoptosis in MCF-7 and MDA-MB-231 cells. Using caspase-3 specific approaches, we demonstrated that caspase-3 status is a critical determinant, in addition to ER status, that contributes to the differential responses between the two cell lines. The clinical relevance of this finding is supported by the common incidences of caspase-3 downregulation in breast cancer tissues and cell lines. Differential expression and responses of caspases-4, -8, -10 and Apaf-1 in genistein treated MCF-7 and MDA-MB-231 cells suggest that genistein sensitivity may be regulated by more factors, which warrants further studies.

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