Genistein induces Gadd45 gene and G_2/M cell cycle arrest in the DU145 human prostate cancer cell line

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Abstract Genistein is the most abundant isoflavone of soybeans and has been shown to cause growth arrest in various human cancer cell lines. However, the precise mechanism for this is still unclear. We report here that the growth arrest and DNA damage-inducible gene 45 (gadd45) gene is induced by genistein via its promoter in a DU145 human prostate cancer cell line. The binding of transcription factor nuclear factor-Y to the CCAAT site of the gadd45 promoter appears to be important for this activation by genistein.

Keywords: Genistein; Cell cycle arrest; Growth arrest and DNA damage-inducible gene 45; Promoter; CCAAT site; Nuclear factor-Y

1. Introduction

Many epidemiological studies have suggested that the dietary intake of soybeans is associated with a low risk for developing certain cancers [1,2]. Genistein (4,5,7-trihydroxyisoflavone) is the most abundant isoflavonic component of soybeans. Others and we have reported that genistein arrests cell cycle progression at the G_2/M or G_1/S phases [3,4]. However, the mechanism responsible for this arrest is not understood.

Several studies have shown that gadd45 causes cell cycle arrest [5–7], and is involved in apoptosis [8] and genomic stabilization [9]. In addition, it is known to be a target gene of the tumor suppressor gene p53. In this report, we demonstrate that genistein induces gadd45 activation via its promoter, through a p53-independent pathway.

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Abbreviations: gadd45, growth arrest and DNA damage-inducible gene 45; NF-Y, nuclear factor-Y; C/EBP, CCAAT/enhancer-binding protein

2. Materials and methods

2.1. Reagents

Genistein was obtained from Fujikko (Tokyo, Japan). It was dissolved in dimethylsulfoxide (DMSO) and diluted to final concentrations in each culture medium used (below).

2.2. Cell culture and cell growth

DU145 cells (a p53-mutant human prostate cancer cell line) were maintained in RPMI1640 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (MP Biomedicals, Irvine, CA, USA). Cells (3×10^4) were seeded and 24 h later treated with various concentrations of genistein. Numbers of cells were counted every 24 h. Data were analyzed using two-tailed Student's *t* tests and differences were considered to be statistically significant from the controls at $P < 0.05$.

2.3. Cell cycle analysis

Unsynchronized cells were exposed to genistein for 24 h and then harvested and fixed in 70% ethanol. After treating with RNase A and propidium iodide, DNA contents were measured using FACSCalibur (Becton Dickinson, Inc., Franklin Lakes, NJ, USA). The ModFit LT V2.0 software package (Verity Software, Topsham, ME, USA) was used to analyse the data.

2.4. Protein assay

Gadd45 protein was analyzed by immunoblotting. Cells were lyzed with 20 mM Tris buffer (pH 8.0) containing 137 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1% (v/v) Triton X-100 [10]. Each protein (75 μg) was separated by electrophoresis on polyacrylamide gels. Transferred PVDF membranes were probed with monoclonal antibodies for gadd45 (Santa Cruz Biotech., Inc., Santa Cruz, CA, USA) and for β-actin (Oncogene Res. Prod., San Diego, CA, USA) as a loading control. Signals were detected using an ECL Western blot analysis system (Amersham Biosciences, Buckinghamshire, UK).

2.5. RNA isolation and Northern blot analysis

The isolation of total RNA was as described [11]. Each aliquot of RNA (10 μg) was separated by electrophoresis on a 1.5% agarose gel, transferred to a membrane (Gene Screen Plus; DuPont New England Nuclear, Boston, MA, USA), and hybridized with an [α-^{32}P]dCTP-labeled *Xba*I/*Hin*dIII fragment from pCMV-45 (kindly provided by Dr. A.J. Fornace, Jr.). Hybridization was carried out at 68 °C in PerfectHyb hybridization solution (Toyobo Co., Ltd., Osaka, Japan). The same membrane was rehybridized with an [α-^{32}P]dCTP-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe

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(Clontech Laboratories, Inc., Palo Alto, CA, USA) as a loading control.

2.6. Construction of plasmid DNA

Constructions of the human gadd45 promoter–luciferase fusion plasmids, pG45-luc, pG45-1325, pG45-817, pG45-234, pG45-81, and pG45-10, were as previously described [11]. A QuikChange site-directed mutagenesis kit (Stratagene Cloning Systems, Co., La Jolla, CA, USA) was used to generate point mutations at the Oct-1 or the CCAAT site of the pG45-234 reporter plasmids. The primer used to mutate the Oct-1 site in the region between −75 and −62 bp of the pG45-234 plasmid was 5′-CCA ATG GCC AAG CTG CAT GCA AGC GAG GCG GAA GGT GGT TGG-3′ (the mutated nucleotides are underlined). The primer used to mutate the CCAAT site in the region between −55 and −46 bp was 5′-GAG GCG GAA GGT GGT TTT ATG AGG GTT GGC AGG ATA ACC C-3′ (the mutated nucleotides are underlined). The vacant control luciferase plasmid pGVB2 was purchased from Nippon Gene (Tokyo, Japan).

2.7. DNA transfection and luciferase assay

After DU145 cells had been cultured for 24 h, 0.5 μg of reporter plasmid DNA was transfected using a CellPhect transfection kit (Amersham Pharmacia Biotechnology, Inc., Piscataway, NJ, USA). Twenty-four hours after transfection, the cells were treated with or without genistein and 24 h later the cells were collected for luciferase assay. This was measured on cell lysates as described [11], and each was normalized for the amount of protein in the cell lysates, measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Data were analyzed using two-tailed Student's *t* tests and differences were considered statistically significant from controls at $P < 0.05$.

2.8. Electrophoretic mobility shift assay (EMSA)

Annealed double-stranded oligonucleotides containing the sequence of the CCAAT site (5′-GCA TAG CCC AAT GGC CAA GC-3′), the mutated CCAAT site (5′-GCA TAG CCT TGT GGC CAA GC-3′; mutated oligonucleotides are underlined), the Oct-1 site (5′-AGC AGG CTG ATT TGC ATA GC-3′), and the mutated Oct-1 site (5′-AGC AGG CTG GCT TGC ATA GC-3′; mutated oligonucleotides are underlined) between positions −234 and −81 bp were labeled with [α-^{32}P]dCTP and used as probes. Competition reactions were performed using a 100- and 200-fold molar excess of unlabeled oligonucleotides. Nuclear extracts (9 μg) treated with or without 75 μM genistein were incubated in a 20 μL reaction volume containing the appropriate buffer and 1 μg of poly(dI–dC). An [α-^{32}P]dCTP-labeled probe was added and the reaction mixture was incubated. In supershift experiments, antibodies against NF-YA, NF-YB, C/EBP, and Oct-1 (Santa Cruz Biotechnology) were preincubated with nuclear extracts before addition of the probe.

3. Results

3.1. Genistein blocks cell growth at the G_2/M phase of DU145 human prostate cancer cells

We first examined the effect of genistein on the growth of DU145 cells. Fig. 1A shows the growth rate of DU145 cells in the presence or absence of various concentrations of genistein. A significant dose-dependent inhibition of cell growth was observed. After 72 h, cell growth was inhibited to 51%, 12%, 10%, and 8% of controls by genistein at 20, 50, 75, and 100 μM, respectively. Next, we examined changes in the distribution of DNA content after 24 h incubation in the presence or absence of genistein (75 μM) using flow cytometry. As shown in Fig. 1B, significant G_2/M cell cycle arrest was observed in cells treated with genistein. Treatment with 75 μM genistein changed the percentages of cells in the G_1, S, and G_2/M phases from 39%, 36%, and 25% to 4%, 15%, and 81%, respectively.

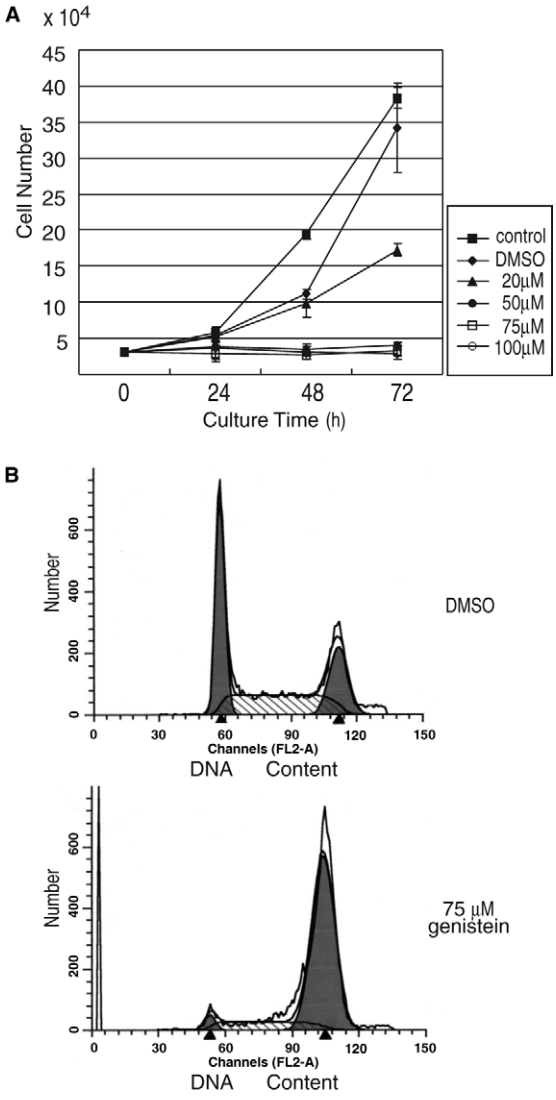

Fig. 1. Effect of genistein on the growth and cell cycle of DU145 cells. (A) One day after inoculation of DU145 cells, genistein dissolved in DMSO was added at 20, 50, 75 or 100 μM, and cell growth was compared with a control culture with equivalent DMSO alone. Numbers of cells were counted every 24 h. The data represent means of triplicate experiments and the bars show SDs. (B) Unsynchronized cells were incubated in the presence or absence (DMSO alone) of 75 μM genistein for 24 h and the DNA content of the cells was determined by flow cytometry.

3.2. Genistein induces production of the Gadd45 protein and mRNA in DU145 cells

As shown in Fig. 2A, we found that treatment with genistein stimulated the production of Gadd45 protein in a dose-dependent manner. We next measured the gadd45 mRNA level in genistein-treated DU145 cells using Northern blot analysis. Twenty-four hours exposure to genistein induced 1.3-, 3.7-, 4.7-, and 4.7-fold of gadd45 mRNA relative to control at 20, 50, 75, or 100 μM, respectively (Fig. 2B). A time course study showed that the gadd45 mRNA was induced by 6 h after treatment with 75 μM genistein (Fig. 2C).

3.3. Genistein stimulates gadd45 promoter activity

Next, we investigated whether genistein could stimulate the activity of the gadd45 gene promoter. As shown in Fig. 3A,

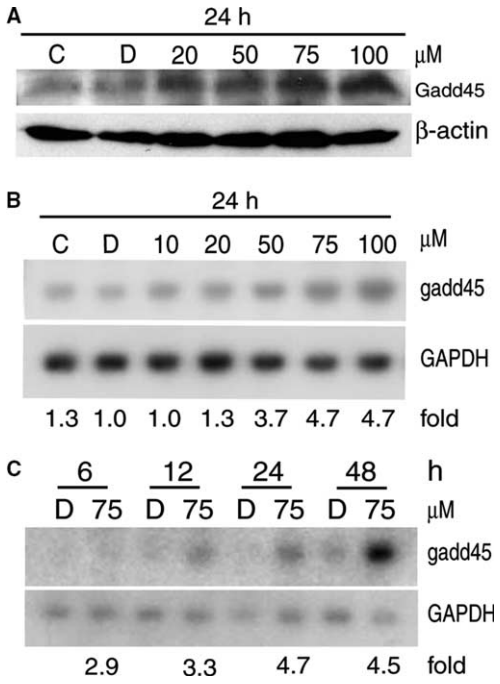

Fig. 2. The gadd45 gene expression was induced by genistein in DU145 cells. (A) Western blot analysis of DU145 cells treated with various concentrations of genistein for 24 h. (B) Northern blot analysis of gadd45 mRNA from DU145 cells treated with various concentrations of genistein for 24 h. The mRNA level of gadd45 was standardized against that of glyceraldehyde-3-phosphate dehydrogenase. (C) Northern blot analysis of gadd45 mRNA from DU145 cells treated with 75 μM genistein for the indicated times.

genistein increased the luciferase activity in a dose-dependent manner. We then determined which regions of the gadd45 promoter were responsive to genistein. For this purpose, we prepared various lengths of 5′-deletion constructs of the gadd45 promoter–luciferase fusion plasmids (Fig. 3B). As shown in Fig. 3C, the response to genistein decreased with deletion of the region between −234 and −81 bp. This suggests that a genistein-responsive element exists within 153 bp relative to the transcriptional start site. This region (from −234 to −81 bp) contains Oct-1 and CCAAT sites, which are conserved among species [12]. Therefore, this region appears to be essential for the activation of gadd45 by genistein. To determine the genistein-responsive site of pG45luc-234 precisely, we prepared two constructs, pG45luc-234mOct-1 and pG45luc-234mCCAAT, which contained point mutations at the Oct-1 or the CCAAT site of pG45luc-234, respectively (Fig. 3B). As shown in Fig. 3D, the response to genistein decreased because of the point mutation at the CCAAT site, while there was little change resulting from the point mutation at the Oct-1 site.

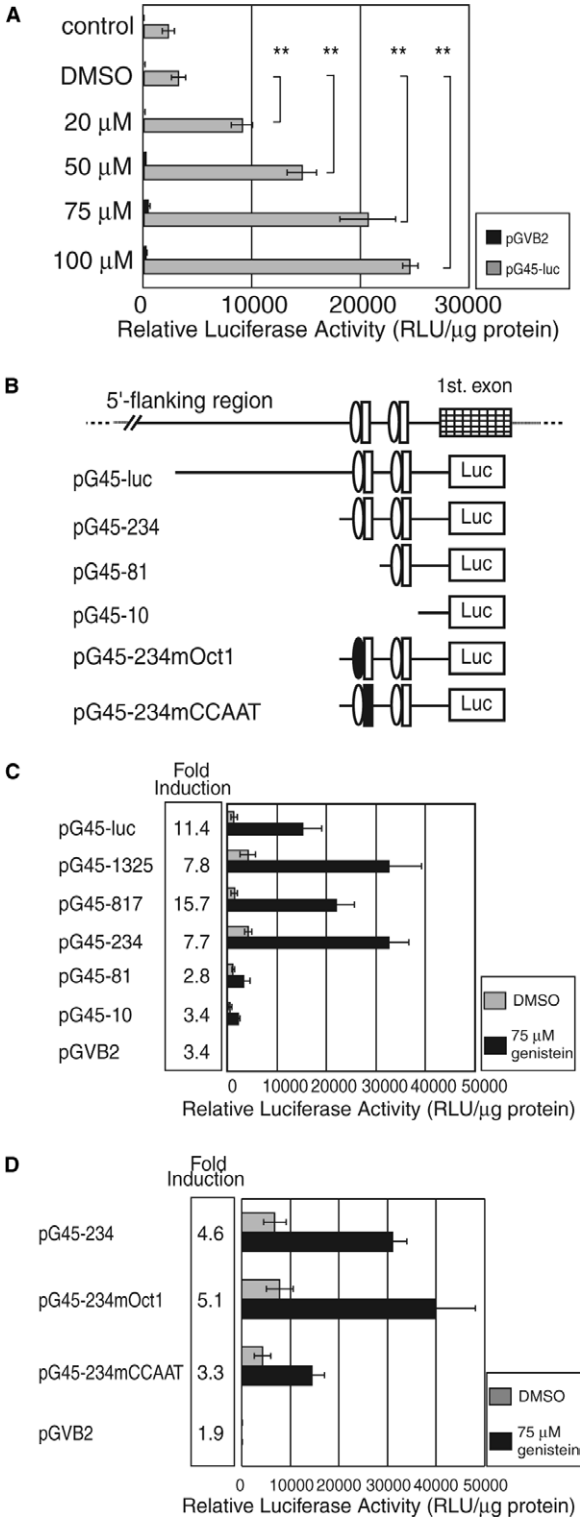

Fig. 3. Activation of gadd45 promoter activity in DU145 cells by treatment with genistein. (A) DU145 cells were transiently transfected with gadd45–luciferase fusion plasmids (pG45-luc) or with vacant control luciferase plasmids (pGVB2), and luciferase activities were measured after incubation with medium containing various concentrations of genistein for 24 h. The data represent means of triplicate experiments and the bars show SDs. $^{**}P < 0.01$. (B) Prepared plasmid constructs are shown. Various 5′-deletion mutants of pG45-luc and pG45-234 reporter plasmids harboring point mutations in the Oct-1 site, closed circle, or the CCAAT site, closed square, were used to identify the genistein-responsive element. (C) Deletion analysis of the gadd45 promoter. DU145 cells were treated in the presence or absence (DMSO alone) of 75 μM genistein. The response to genistein decreased with deletion of the region between −234 and −81 bp. (D) Mutation analysis of the gadd45 promoter. The response to genistein decreased because of the point mutation at the CCAAT site, whereas there was little change caused by the point mutation at the Oct-1 site.

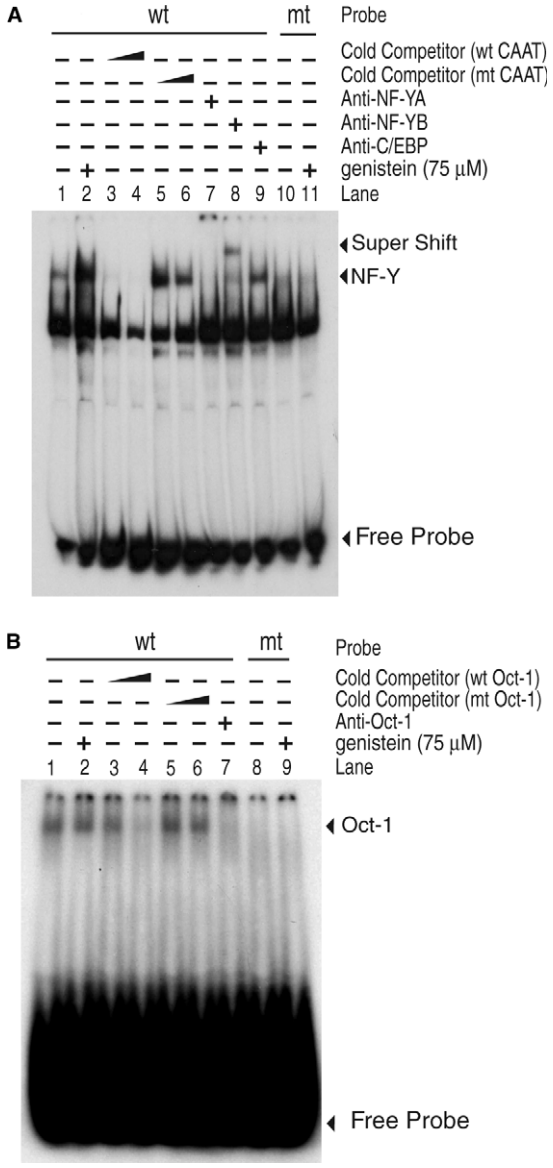

Fig. 4. Binding of nuclear proteins to the Oct-1 site or the CCAAT site of the gadd45 gene. EMSAs were carried out with nuclear extracts prepared from DU145 cells. (A) Specific binding of NF-Y to the CCAAT site. Treatment with 75 μM genistein enhanced the binding of NF-Y to the CCAAT site (lanes 1 and 2). (B) Specific binding of Oct-1 to the Oct-1 site. Treatment with 75 μM genistein did not result in any difference of the binding of nuclear protein Oct-1 to the Oct-1 site (lanes 1 and 2).

3.4. Genistein increases the binding of NF-Y to the CCAAT site

We examined whether genistein changed the binding of transcription factors to the Oct-1 and the CCAAT sites. The Oct-1 and the CCAAT sites of the promoter region of gadd45 are of great significance [7,11,13]. The transcription factors Oct-1 and NF-Y are known to bind mainly to Oct-1 and CCAAT sites, respectively. As shown in Fig. 4A, 75 μM genistein increased the binding of NF-Y to the CCAAT site, but did not increase the amount of NF-Y protein (data not shown). On the other hand, genistein did not produce any change in the binding of transcription factor Oct-1 to the Oct-1 site (Fig. 4B).

4. Discussion

Others and we have shown that genistein causes G_2/M arrest in various cancer cell lines [3,4,14]. Choi et al. [14] reported that the induction of p21/WAF1 and the reduction of cyclin B1 by genistein cause inhibition of the kinase activities of cdk2 and cdc2 in human breast cancer cell lines. However, we did not observe a reduction of cyclin B1 by genistein in DU145 cells, although weak induction of p21 protein was observed (data not shown). In this study, we showed that genistein induces gadd45 gene expression in this p53-mutant human prostate cancer cell line DU145. Similar results were observed in the p53-null human prostate cancer cell line PC3 (data not shown). Gadd45 is a target gene of p53 and in addition a p53-independent pathway has also been reported [7,11,12,15].

Others and we have reported that the Oct-1 and the CCAAT sites of the promoter region are important for induction of the gadd45 gene [7,11,13]. In this study, we found that the genistein-responsive element is the CCAAT site. The results of EMSA (Fig. 4A) suggest that this induction of gadd45 by genistein is caused by the increased binding of NF-Y to the CCAAT site of the promoter region. Shimizu et al. [16] reported that genistein induced the bone sialoprotein (BSP) gene through its promoter and that the CCAAT site of the BSP promoter region was of significance. The CCAAT site of both the BSP and gadd45 genes may thus be important for activation by genistein.

The Gadd45 protein is reported to have roles in cell cycle regulation [5–7], apoptosis [8], and genomic stability [9]. Therefore, finding an agent that transcriptionally upregulates this gene in cancer cells may contribute to new strategies for the prevention of or therapy for cancer, which we have termed 'gene-regulating chemoprevention or chemotherapy' [17,18]. In this sense, genistein, which activates gadd45 gene expression, would represent a gene-regulating chemopreventive agent.

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