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Caffeine Overcomes Genistein-Induced G2/M Cell Cycle Arrest in Breast Cancer Cells

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Although inhibition of tumor cell growth by genistein is mediated by different types of cell cycle arrest, its regulation of genes related to the cell cycle is not clear. In this study, genistein caused a concentration-dependent growth inhibition in the hormone-independent cell line MDA-MB-435S. Flow cytometric analysis showed that genistein induced a concentration-dependent accumulation of cells in the G2/M phase of the cell cycle. Caffeine enhanced the inhibition of cell proliferation induced by genistein. Caffeine alone did not have an appreciable effect on the phases of the cell cycle, but caffeine at 3 mM completely eliminated genistein-induced G2/M cell cycle arrest. cDNA microarrays were used to investigate the mechanism of genistein-induced, caffeine-negated G2/M arrest. We identified 12 genes, which had opposite responses to genistein and caffeine treatments. Among these, 5 genes were upregulated by genistein and downregulated by caffeine; 7 genes were downregulated by genistein and upregulated by caffeine. Reversal by caffeine of genistein-induced G2/M phase arrest in breast cancer cell lines could increase their sensitivity to genistein and enhance genistein-induced inhibition of cell growth. Genes that have opposite responses to genistein and caffeine may be involved in regulation of the G2/M phase of the cell cycle.

INTRODUCTION
Genistein, the major isoflavonoid contained in soybeans, is believed to exert pleiotropic effects including those that are estrogenic or growth promoting and those that are anticarcinogenic (1-3). Epidemiological studies, as well as work performed with animal models (4,5), have suggested that it is responsible for chemopreventive effects on breast, colon, and skin tumors. Genistein potently inhibits cell proliferation and induces cell differentiation or apoptosis (6,7). Several mechanisms for the effects of genistein in tumor cells, such as inhibition of tyrosine kinase and topoisomerases, have been proposed (7).

For several drugs, the anticancer effects are mediated by cell cycle arrest and involve modulation of the cyclins and cyclin-dependent kinases that regulate cell cycle progression (8). Previous studies have suggested that after genistein treatment of breast cancer cells, G2/M cell cycle arrest and alterations in cyclinB1 and cdc2 proteins may be involved in the effects of genistein and other phytoestrogens (9,10). Inhibition of the G2 checkpoint in cells often leads to a marked increase in the sensitivity of these cells to chemotherapeutic agents (11).

The sensitizing action of caffeine, a known inhibitor of the cell cycle at the G2 checkpoint, has raised hopes that for cancer patients, adjunctive therapy with G2 checkpoint inhibitors will increase the therapeutic efficacies of radiation and other genotoxic therapies. The molecular targets of caffeine for radiosensitization/chemosensitization, however, remain unclear.

In this study, we evaluated the effects of genistein and caffeine on cell growth and cell cycle distribution in the hormone-independent cell line MDA-MB-435S. By use of microarrays, we investigated changes of gene expression after exposure of cells to genistein and/or caffeine to understand the molecular mechanisms involved in regulation of the cell cycle.

MATERIALS AND METHODS
Cell Culture
The spindle-shaped MDA-MB-435S cells evolved from the parental line MDA-MB-435, which was derived from cells in the pleural effusion of a 31-year-old female with metastatic, ductal adenocarcinoma of the breast (12). MDA-MB-435 cells have a gene expression pattern compatible with a melanocyte origin (13). Because the MDA-MB-435 and M14 melanoma cell lines are essentially identical with respect to cytogenetic characteristics and gene expression patterns, MDA-MB-435 cells were not considered to be a model of breast cancer (14). Nevertheless, these cells can be induced to express proteins that are specific for breast differentiation and to secrete milk lipids, characteristics of well-established breast cancer cell lines. Thus, MDA-MB-435 is most likely a breast epithelial cell line that has undergone lineage infidelity (15). It is possible that some breast tumors have gene expression profiles similar to melanoma. In our laboratory, MDA-MB-435S cells were routinely maintained in RPMI 1640 medium (containing phenol red) with 10% fetal bovine serum (FBS) and grown at 37°C in a 5% CO₂ atmosphere.
Genistein and caffeine, purchased from Sigma (St. Louis, MO), were dissolved in ethanol and frozen until use. Control cells were exposed to ethanol only; at the concentration used (0.1%), ethanol did not interfere with cell growth.

Cell Growth Assay
Experiments were accomplished in 96-well plates containing RPMI 1640 supplemented with 10% FBS. Cells were seeded at a plating density of 2 × 10^5 cells/well in 200 µl of medium and then cultured for 24 h to allow their adhesion to the plate. At that time, the culture medium was changed to the experimental medium supplemented with genistein and/or caffeine. Following culture with genistein and/or caffeine for 2, 4, or 6 days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added, and optical densities of the plate samples were determined at 490 nm with a scanning multwell spectrophotometer. Data points represented the means of values for 8 wells.

Cell Cycle Analysis
We incubated 5 × 10^5 cells at 37°C overnight in triplicate 10-cm plastic dishes in medium containing 10% FBS and then for 2 days with various concentrations of genistein and/or caffeine. Cells were trypsinized, washed in cold phosphate-buffered saline (PBS; pH 7.4), fixed in 70% ethanol/30% PBS, and stored at 4°C until processing. A portion (1 ml) of the fixed cell suspension containing 1 × 10^6 cells was washed twice in cold PBS. The fixed cells were treated for 30 min at 4°C in the dark with fluorochrome DNA staining solution (1 ml) containing 40 µg of propidium iodide and 0.1 mg of ribonuclease A; the stained cells were analyzed by flow cytometry.

Isolation of Total RNA
Cells were grown in 10-cm dishes (1 × 10^6 cells/dish). At 24 h after plating, cells were treated with 0.1% ethanol, 20 µM of genistein alone, or 20 µM of genistein plus 3 mM of caffeine for 48 h. At this time, cells were harvested for RNA extraction. Total RNA was prepared by use of the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA was measured spectrophotometrically. For quality control, RNA purity was evaluated by the OD260/280 ratio.

Complementary DNA (cDNA) Microarray Analysis
Deoxyribonuclease I-treated total RNA (50 µg) was reverse transcribed to generate cDNA probes labeled with fluorescent dye Cy3 (control samples) or Cy5 (treated samples). The labeled probes were hybridized to a commercial cDNA microarray containing 4,000 immobilized fragments of cancer-related genes (Biostar Genechip Inc., Shanghai, China) that encode for proteins involved in apoptosis, cell cycle progression, and stress response; for tumor suppressors, transcription factors, growth factors, and receptors; and for oncogenes, kinases, phosphatases, and G-proteins. These genes are generally expressed in the eukaryotic cells and not breast epithelial-specific genes. The control genes, including housekeeping genes, were printed in duplicate. The fluorescence intensities of the Cy3- and Cy5-labeled probes bound to each target were measured and scanned separately with a ScanArray 4.00 (Gsi Lumonic, Novi, MI). Data analysis was performed with the supplied GenePix Pro 3.0 software (Axon Instruments). The results were normalized for the labeling and detection efficiencies of the 2 fluorescence dyes (Cy3 is green and Cy5 is red) and then used to detect differential gene expression in the control and treated cell samples. A differentially expressed gene was defined by a ratio of treated:control >2.0 or <0.5. A ratio of 2 and higher (for upregulation) and a ratio of 0.5 and lower (for downregulation) between background-corrected normalized gene expression levels were considered to be significant. Two independent experiments were performed to verify the reproducibility of results.

Northern Blot Hybridization
RNA samples (30 µg) were separated on 1% formaldehyde agarose gels and transferred onto nylon membranes. The RNA was fixed on to the membrane by ultraviolet (UV) cross-linking. The membranes were then hybridized with the different 32P-labeled probes at 2 × 10^6 cpm/ml for 2 days at 42°C. Probes were labeled by use of the Megaprime DNA labeling system (Amersham, Piscataway, NJ) according to the manufacturer’s instructions. Membranes were washed twice in 1× SSC, 0.1% sodium dodecyl sulfate for 1 h at 65°C. All RNA samples were also probed for β-actin messenger RNA (mRNA), and these values were used to normalize changes in expression stimulated by various treatments. After exposure to x-ray film (Kodak; Rochester, NY), the intensities of the bands were measured by densitometry (Analytical Uniscan, Newark, Delaware). Densitometer readings were normalized for β-actin RNA content and expressed as relative values.

Statistical Analyses
Data were expressed as means ± SD. Statistical differences were analyzed by 1-way analysis of variance followed by Tukey’s post hoc test. A value of P < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Genistein Inhibits the Proliferation in MDA-MB-435S Cells
A concentration- and time-dependent inhibition of cell proliferation was observed for MDA-MB-435S cells exposed to genistein (Fig. 1A). Treatment with 5, 10, or 20 µM genistein for 2, 4, or 6 days significantly reduced cell proliferation compared to controls (P < 0.05; n = 8).
FIG. 1. Effects of genistein and caffeine on proliferation of breast cancer cells. A: MDA-MB-435S cells were treated with 0, 5, 10, or 20 µM concentrations of genistein for 2, 4, or 6 days. B: Cells were treated similarly in the absence or presence of caffeine (1 or 3 mM) for 4 days. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed as described in Materials and Methods. Results represent mean ± SD; n = 8. *, Significantly different from control at the same concentration of caffeine (P < 0.05); #, significantly different from control at the same concentration of genistein (P < 0.05).

Caffeine Enhances Genistein-Induced Cell Growth Inhibition

MDA-MB-435S cells were treated with 0, 5, 10, or 20 µM concentrations of genistein with or without caffeine (0, 1, 2, 3 mM) for 4 days. As shown in Fig. 1B, caffeine alone inhibited the proliferation of MDA-MB-435S cells in a concentration-dependent manner. The presence of caffeine also enhanced genistein-induced cell growth inhibition in a concentration-dependent manner.

Genistein Arrests MDA-MB-435S Cells at the G2/M Phase

MDA-MB-435S cells were treated with 0, 5, 10, or 20 µM concentrations of genistein for 2 days. In a concentration-dependent manner, genistein induced an accumulation of cells in the G2/M phase of the cell cycle, with a parallel depletion of the percentage of cells in G0/G1 (Fig. 2A). The increase of the percentage of cells in the G2/M phase of the cell cycle after treatment with 20 µM genistein was 4.4-fold compared to the control (P < 0.05; n = 3).

Gene Expression Pattern of MDA-MB-435S Cells Treated with Genistein or Caffeine

To investigate the mechanism of genistein-induced, caffeine-negated G2/M arrest, the gene expression profiles of MDA-MB-435S cells treated with genistein or caffeine were assessed by
Table 1
List of Genes Upregulated by Genistein and Downregulated by Caffeine in MDA-MB-435S Cells

<table>
<thead>
<tr>
<th>Gene Bank Symbol</th>
<th>Gene Description</th>
<th>Genistein Average Ratio</th>
<th>Caffeine Average Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITPR1</td>
<td>Inositol 1,4,5-triphosphate receptor, type 1</td>
<td>2.10 ± 0.11</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>ASNS</td>
<td>Asparagine synthetase</td>
<td>2.44 ± 0.53</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>PRNP</td>
<td>Prion protein (p27-30)</td>
<td>2.30 ± 0.26</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>PPP2R2B</td>
<td>Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform</td>
<td>2.37 ± 0.48</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>TXNRD1</td>
<td>Thioredoxin reductase 1</td>
<td>4.20 ± 0.92</td>
<td>0.28 ± 0.05</td>
</tr>
</tbody>
</table>

Values are fold change to control. Fold change values represent the average of 2 independent microarray experiments. All genes listed are upregulated at least twofold by genistein and downregulated at least 2.5-fold by caffeine.

Table 2
List of Genes Downregulated by Genistein and Upregulated by Caffeine in MDA-MB-435S Cells

<table>
<thead>
<tr>
<th>Gene Bank Symbol</th>
<th>Gene Description</th>
<th>Genistein Average Ratio</th>
<th>Caffeine Average Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-2</td>
<td>Tyrosinase-related protein 2</td>
<td>0.31 ± 0.01</td>
<td>3.81 ± 0.19</td>
</tr>
<tr>
<td>KIAA0481</td>
<td>KIAA0481 protein</td>
<td>0.42 ± 0.04</td>
<td>2.37 ± 0.18</td>
</tr>
<tr>
<td>CYP4B1</td>
<td>Cytochrome P450, subfamily IVB, polypeptide 1</td>
<td>0.35 ± 0.13</td>
<td>4.25 ± 0.68</td>
</tr>
<tr>
<td>TNFSF4</td>
<td>Tumor necrosis factor (ligand) superfamily, member 4</td>
<td>0.33 ± 0.19</td>
<td>2.54 ± 0.24</td>
</tr>
<tr>
<td>HMP</td>
<td>Myelin proteolipid protein</td>
<td>0.32 ± 0.03</td>
<td>2.45 ± 0.06</td>
</tr>
<tr>
<td>MGC:27353</td>
<td>Clone MGC:27353 IMAGE:4671816</td>
<td>0.34 ± 0.03</td>
<td>3.72 ± 0.54</td>
</tr>
<tr>
<td>SERPINA3</td>
<td>Serine (or cysteine) proteinase inhibitor, clade A, member 3</td>
<td>0.33 ± 0.02</td>
<td>4.20 ± 0.41</td>
</tr>
</tbody>
</table>

Values are fold change to control. Fold change values represent the average of 2 independent microarray experiments. All genes listed are upregulated at least twofold by caffeine and downregulated at least 2.5-fold by genistein.
Genistein could be useful in combination cancer therapy along their sensitivity to several anticancer agents. With this effect, the G2 checkpoint function in human tumor cell lines increases an "epiphenomenon." There is growing evidence that ablation of cell cycle arrest and that the effects on cell cycle arrest may be these observations, it is likely that the antiproliferative mechanism of genistein is related to the mechanism required for cell cycle arrest and that the effects on cell cycle arrest may be an "epiphenomenon." There is growing evidence that ablation of the G2 checkpoint function in human tumor cell lines increases their sensitivity to several anticancer agents. With this effect, genistein could be useful in combination cancer therapy along with radiation or other DNA-damaging agents (23). Thus, our results suggest that caffeine, by reversing the genistein-induced G2/M phase arrest in breast cancer cell lines, could increase their sensitivity to genistein and enhance genistein-induced cell growth inhibition.

Our results show that 1 mM caffeine partly and 3 mM caffeine completely eliminated the genistein-induced G2/M arrest in MDA-MB-435S cells and that genistein or caffeine alone did not have appreciable effects on the S phase of the cell cycle. Thus, the percentages of cells in the G0/G1, S, and G2/M phases following treatment with 3 mM caffeine plus 20 µM genistein were similar to that for control cells and for cells exposed to 3 mM caffeine alone. Caffeine is thought to inhibit at the G2 checkpoint by blocking ataxia-telangiectasia-mutated-Rad3-related (ATR)-dependent phosphorylation of checkpoint kinase 1 (Chk1). Additional cell culture studies have suggested that caffeine overcomes radiation-induced G2 arrest by inhibiting Cdc2 phosphorylation on Tyr-15 (24). Alternatively, it is possible that caffeine exerts this effect by inhibiting the p53- and ATR-independent, p38 kinase-mediated Cdc25B signal transduction pathway or by decreasing the level of Wee1 (25). Further research is needed to identify the specific molecular targets for the effect of caffeine in overcoming the genistein-activated blockade at the G2/M checkpoint.

Although many genes were upregulated or downregulated by genistein or caffeine treatment, only 12 had opposite responses to genistein and caffeine (Tables 1 and 2). Because no gene was found to be commonly regulated by genistein and caffeine, the cell growth inhibition induced by genistein and caffeine may be through different pathways.

Inositol 1,4,5-triphosphate receptor, type 1 (ITPR1), a ligand-gated Ca²⁺ channel protein located in the membrane of the endoplasmic reticulum, has 2 putative phosphorylation sites for cyclin-dependent kinases. Cdc2/CyB phosphorylation positively regulates IP3-gated Ca²⁺ signaling (26). Because ITPR1 was upregulated by genistein and downregulated by caffeine, it is possible that caffeine eliminates the G2/M checkpoint by inhibiting genistein-induced ITPR1 phosphorylation through upregulation of Cdc2/CyB activity, thereby enhancing mitosis.

The presence of redox-sensitive motifs in several cell-cycle regulatory proteins indicates that periodic oscillations in intracellular redox state could be involved in regulation of progression from G0/G1 to S to G2 and M cell cycle phases (27). The thioredoxin reductase 1 gene (TXNRD1) is related to oxidative stress. In HeLa cells, overexpression of wild-type TXNRD1 causes a marked increase in the number of cells at the G2/M phase of the cell cycle, whereas cells that express the mutant TXNRD1 do not demonstrate this effect (28). Our results show that TXNRD1 was upregulated by genistein and downregulated by caffeine, a finding confirmed by northern blots (Fig. 4). The results suggest a role for TXNRD1 in the regulation of the G2/M phase of the cell cycle.
The protein phosphatase type 2A (PP2A) is encoded by PPP2R2B (a tumor suppressor gene). Viral protein R (Vpr) upregulates PP2A and induces G2 arrest in cells from distantly related eukaryotes; mutations in genes coding for a regulatory or catalytic subunit of PP2A reduce Vpr-induced G2 arrest (29), suggesting that PP2A is involved in induction of G2 arrest. Data from our investigation also provide evidence for such a function for PP2A. Genistein unregulated PPP2R2B and induced G2/M arrest. Further, inhibition of PPP2R2B by caffeine overcame genistein-induced G2/M arrest.

In this study, TRP-2 was downregulated by genistein and upregulated by caffeine. When cultured human melanocytes were exposed to UV light, a decrease in the amount of TRP-2 was observed (32). The UV irradiation protocol that was followed blocked melanocytes in the G2-M phase of the cell cycle. Our results, together with others (32), suggests that the TRP-2 gene is downregulated when cell G2/M arrest occurs. Furthermore, caffeine reversed the downregulation of TRP-2 gene induced by genistein. Another 6 genes that showed no direct relationship with cell cycle arrest may still be involved in regulation of the cell cycle.

In summary, the results of this study demonstrate that in breast cancer cells, caffeine overcomes the G2/M cell cycle arrest induced by genistein and enhances inhibition of cell growth induced by genistein. Further, the results link this blockade to the deregulation of cell cycle-related genes and illustrate the potential for use of gene expression profiling as a tool to identify cell cycle mechanisms and pathways.

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