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Genistein Arrests Cell Cycle Progression at G_2-M

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ABSTRACT

Genistein, an isoflavone, is a specific inhibitor of tyrosine kinase and topoisomerase II. However, its effect on cell growth is unknown. Therefore, we examined the effects of genistein on cell growth and cell cycle progression and compared its effects with other flavonoids. Genistein inhibited in a dose-dependent manner the growth of HGC-27 cells derived from human gastric cancer. Flow-cytometric analysis showed that genistein almost completely arrested the cell cycle progression at G_2-M. This effect was reversible when genistein was removed from the culture medium. In contrast, other flavonoids such as flavone, luteolin, and the structurally similar daidzein arrested the cell cycle at G_1. Consistent with the flow-cytometric analysis, microscopic observation showed that genistein did not increase the mitotic index, which supposes that genistein may arrest the cell cycle at G_2 or early M. These results suggest that the G_2-M arrest by genistein is a unique effect among flavonoids.

INTRODUCTION

Flavonoids are found in many plants, including edible fruits and vegetables. Various pharmacological activities of flavonoids have been extensively studied (1–5). Recently, we have reported that the flavonoid quercetin markedly inhibits the growth of human gastric and colon cancer cells by inhibiting cell cycle progression at the G_1-S boundary (6, 7). In the present study, we determined whether other flavonoids show this property. We found that while other flavonoids induce G_1 arrest similar to one caused by quercetin (6, 7), genistein specifically inhibits the cell cycle of HGC-27 cells at G_2-M. This is the first report that a flavonoid causes growth inhibition of malignant tumor cells by G_2-M arrest. Therefore, we postulate that genistein may inhibit tumor cell growth through a mechanism unique among flavonoids.

MATERIALS AND METHODS

Chemicals. Genistein, daidzein, flavone, and luteolin were purchased from Funakoshi Chemicals Co., Ltd., Tokyo, Japan. These flavonoids were dissolved in dimethyl sulfoxide and diluted to their final concentrations in each culture dish. An equivalent volume of dimethyl sulfoxide was added to control dishes and it had no measurable effects on HGC-27 cells. RPMI 1640 was obtained from Nissui Seiyaku Co., Ltd., Tokyo. RNase was purchased from Sigma, St. Louis, MO. Propidium iodide was obtained from Calbiochem Corp., La Jolla, CA. All other chemicals used were of reagent grade.

Cell Culture. Human gastric cancer cell line, HGC-27, established by Akagi and Kimoto (8) was used. Cultures were maintained in RPMI 1640 containing 10% fetal bovine serum and were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Growth Inhibition of Cells. Cells were seeded at a density of 4×10^4 cells/2 ml medium in 35-mm diameter dishes. Flavonoids were added at various concentrations 2 days after the inoculation. At day 3 and 4, the number of viable cells was counted by a trypan blue dye exclusion test.

Analysis of Cell Cycle Progression. Cells were plated at a density of 1×10^5 cells/5 ml medium in 60-mm diameter dishes. Two days after the inoculation drugs were added. Cells were removed at the indicated time from culture dishes by trypsinization and centrifugation. After washing with PBS(−),[1] they were suspended in PBS(−) containing 0.1% Triton X-100 to prepare nuclei. After the suspension was filtered through 50 μm nylon mesh, 0.1% RNase and 50 μg/ml propidium iodide were added. DNA contents in stained nuclei were analyzed with FACStar (Becton Dickinson). The suspension of 1×10^5 cells was analyzed for each DNA histogram. The number of stained nuclei in each phase was measured according to the S-fit program in the FACStar (9). To elucidate the reversibility of the effect of genistein, medium containing genistein was aspirated off 24 h after the addition, and cells were washed with PBS(−), then refed with 5 ml fresh medium.

Mitotic Index. Cells were seeded at a density of 2×10^5 cells/10 ml medium on slides in 100-mm diameter dishes. Sixty μM genistein was added 2 days after inoculation of cells. The slides were stained with Giemsa 1 day after the addition of genistein. Mitotic cells were microscopically examined. The percentage of mitotic cells in 1000 cells was obtained. Data are expressed as means ± SD (n = 3).

RESULTS

Effect of Genistein on Growth of HGC-27 Cells. Fig. 1 shows the growth curve of HGC-27 cells in the various concentrations of genistein. A dose-dependent inhibition of growth was observed between 10 and 60 μM. On day 4, the growth of cells decreased to 72, 30, and 11% of the control level with 10, 30, and 60 μM genistein, respectively. The 50% inhibitory concentration of genistein was 20 μM on day 4.

Effects of Flavonoids on Cell Cycle Progression. To investigate the effect of genistein on cell cycle progression of HGC-27 cells, the DNA content of HGC-27 nuclei was measured by flow-cytometric analysis after 24 h following the addition of genistein. In Fig. 2*A*, DNA histograms show that genistein increased the population of G_2-M cells in a dose-dependent manner, while it decreased the percentage of G_1 cells. At 60 μM genistein, very few G_1 cells were observed. The percentage of S phase cells was also reduced with genistein. Although cells in early S were almost completely abolished at 60 μM, some population of late S still remained. This accumulation in late S might be some spillage from G_2-M, although there is a possibility that genistein might cause a weak block in late S. In Fig.2*B*, S-fit analysis shows that concentrations greater than 25 μM genistein were necessary to alter cell cycle progression with maximal accumulation of G_2-M cells observed at 40 μM or more. G_1 percentage was reduced at 25 μM or more of genistein and completely abolished at 50 μM. The percentage of cells in S phase did not decrease at 30–60 μM; on the contrary there was S phase reduction of cells according to the DNA histogram at the same concentrations in Fig. 2*A*. This discrepancy may be an artifact caused by inappropriate histogram analysis of S-fit program. To elucidate the rate at which genistein altered cell cycle progression, cells were treated with 60 μM genistein and cell cycle progression was determined at multiple time points over the next 48 h. With this concentration, genistein showed a cytostatic effect on the growth of HGC-27 cells as shown in Fig. 1. In Fig. 3, the percentage of G_2-M cells initially increased beginning at 12 h and rapidly increased up to 24 h. Thereafter, G_2-M cells gradually increased by 48 h. The percentage of G_1 cells decreased after the addition of genistein

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[1] The abbreviations used are: PBS(−), phosphate-buffered saline without Ca^{2+} and Mg^{2+}; topo II, topoisomerase II; HSP, heat shock protein.

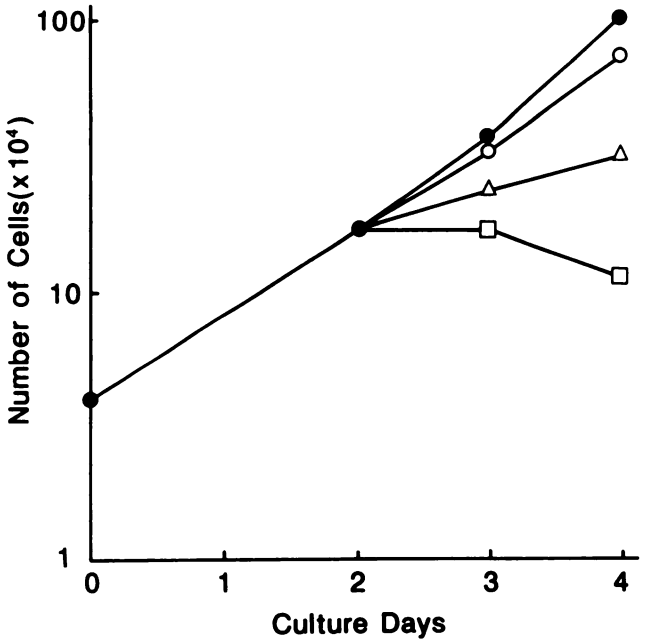

Fig. 1. Effects of genistein on growth of HGC-27 cells. On day 0, 4 × 10^4/2ml of HGC-27 cells were seeded. On day 2, genistein was added at 10 (○), 30 (△), and 60 (□) μM compared with control culture (●). On day 3 and 4, viable cell number by a trypan blue dye exclusion test was determined. Data are means of duplicate experiments.

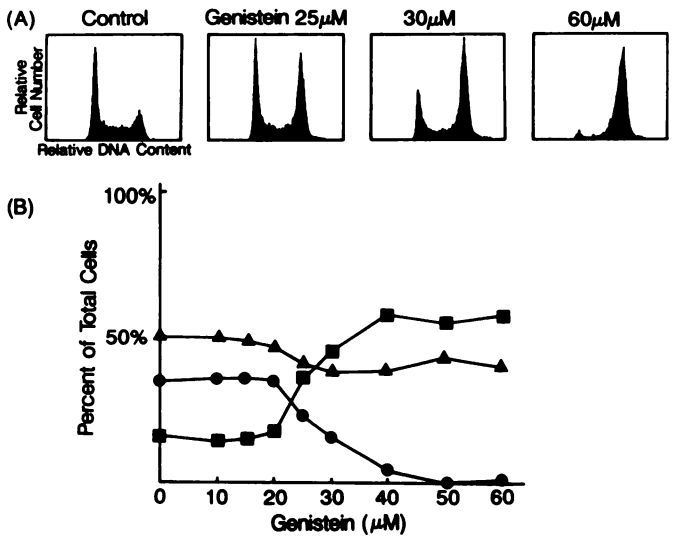

Fig. 2. Effect of genistein on cell cycle progression. On day 0, 1 × 10^5/5ml of HGC-27 cells were seeded, and on day 2 various concentrations of genistein were added. After 24 h from the addition, DNA histograms of cells were obtained by flow-cytometric analysis (*A*). The percentages of G_1 (●), S (▲), and G_2-M (■) are also shown (*B*). Data represent means of duplicate experiments.

and reached its nadir by 24 h. S phase population showed a slight accumulation up to 12 h and thereafter rapidly decreased during the following 12 h. At 48 h, about 74% of the cells accumulated at G_2-M. Fig. 4 shows that the effect of genistein on cell cycle progression (G_2-M arrest) was reversible 24 h after the cells which had been exposed to genistein were washed. These results demonstrated that genistein almost completely, yet reversibly, arrested the cell cycle at G_2-M, and that most of the associated cell population changes were observed within 24 h. The rate of change observed in the cell populations reflects the length of the cell cycle of HGC-27 cells. The result in Fig. 1 showed that the doubling time of HGC-27 cells was about 18 h. Therefore, the accumulation of cells at the G_2-M boundary would be expected to take one cell cycle, approximately 18 h.

To investigate whether G_2-M arrest is commonly induced by other flavonoids, we examined the effects of daidzein, flavone, and luteolin. As shown in Fig. 5, daidzein is very similar to genistein in chemical structure, only missing a single hydroxyl group in position 5. As shown in Fig. 6*A*, the cell cycle progression was inhibited at G_1, but not at G_2-M 24 h following the addition of daidzein at 200 μM, at which HGC-27 cells were in cytostatic state (data not shown). Similarly, G_1 arrest was observed 16 h after the addition of 40 μM flavone or 40 μM luteolin, which are also cytostatic concentrations (Fig. 6*B*).

Mitotic Index. The ratio of mitotic cells in the presence of 60 μM genistein for 24 h was 1.3 ± 0.2% (SD; n = 3; total of 1000 cells) compared with 3.3 ± 1.8% (n = 3, total of 1000 cells) for control. Also we did not observe any swelling of cytoplasm of cells by the treatment of genistein at 60 μM for 24 h. These results, including the flow cytometric analysis, suggest that genistein arrested the cell cycle

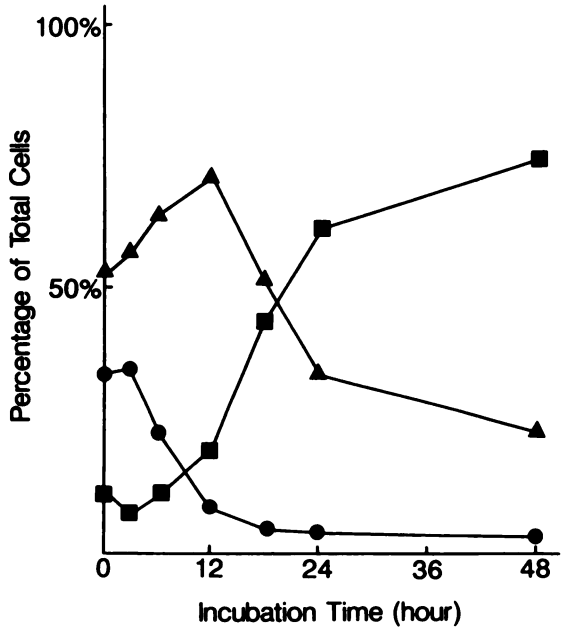

Fig. 3. Kinetic analysis of the effect of genistein on cell cycle progression. Genistein at a concentration of 60 μM was added to the culture 2 days after inoculation of 1 × 10^5 cells of HGC-27 and incubated for 48 h. The percentages of G_1 (●), S (▲), and G_2-M (■) were obtained from DNA histograms by quantitative analysis. Data represent means of duplicate experiments.

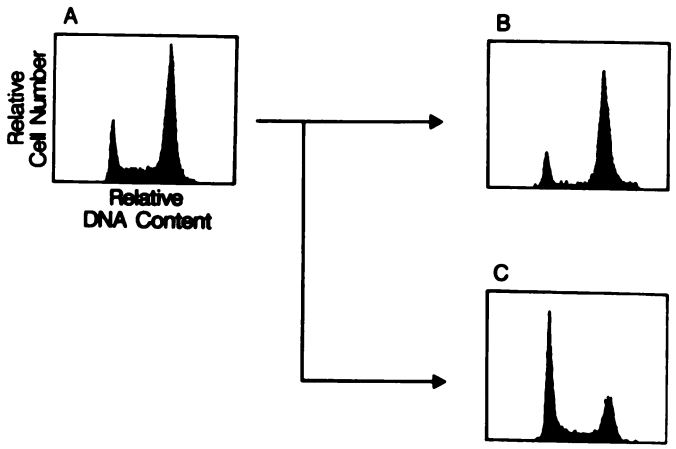

Fig. 4. Reversibility of G_2-M arrest by genistein. Cells were incubated with 60 μM genistein for 24 h (*A*), then the culture was maintained with genistein for an additional 24 h (*B*), or the medium was changed to a fresh one without genistein and was incubated for 24 h (*C*).

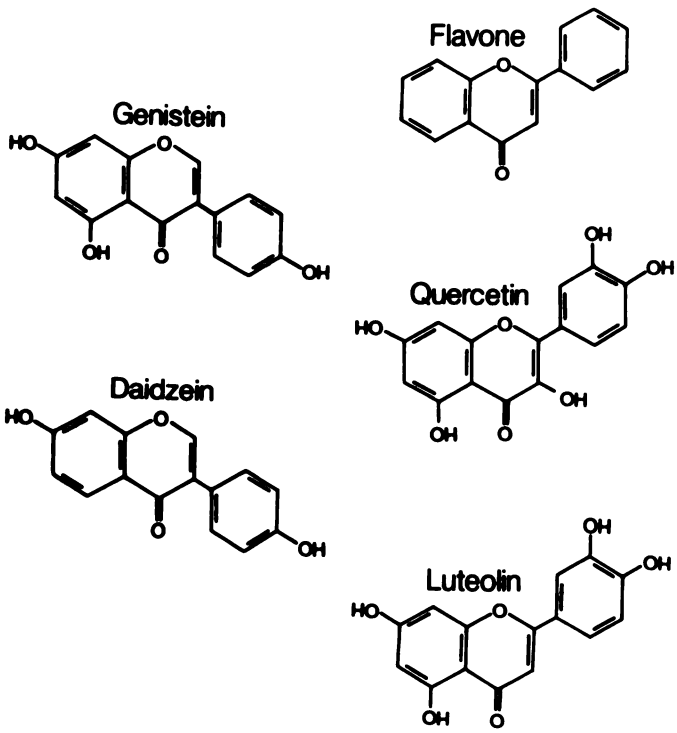

Fig. 5. Structures of genistein, daidzein, and other flavonoids. These flavonoids are classified into three groups; that is, isoflavones (genistein, daidzein), flavones (flavone, luteolin), and flavonols (quercetin). Flavone is the parent compound of quercetin and luteolin.

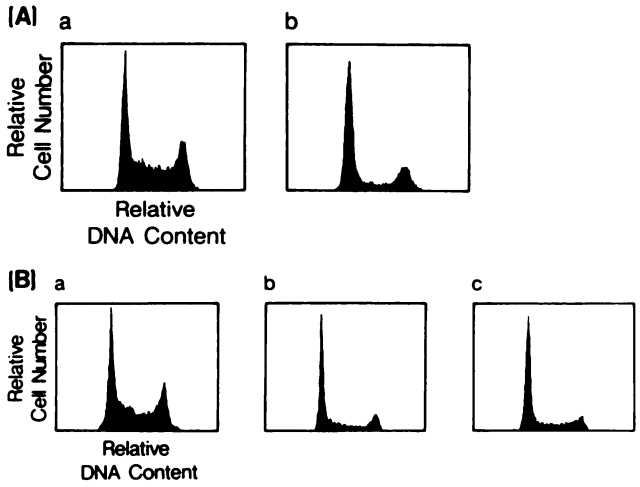

Fig. 6. DNA histogram of HGC-27 cells exposed to flavonoids. (*A*) DNA histogram exposed to daidzein. On the second day after inoculation of 1×10^5/5 ml of HGC-27 cells, 200 μM daidzein or its solvent was added. The cultures were continued for 24 h without daidzein (*a*), with 200 μM daidzein (*b*), and were examined by flow-cytometric analysis. (*B*) DNA histogram exposed to flavone or luteolin. As in (*A*), on the second day, 40 μM flavone, 40 μM luteolin, or their solvent was added. The cultures were continued for 16 h with only solvent (*a*), 40 μM flavone (*b*), or 40 μM luteolin (*c*), and were examined by flow-cytometric analysis.

at G_2 rather than at M because there was no increase of the mitotic index, although there is a possibility that this arrest might be at early M.

DISCUSSION

Flavonoids are plant pigments classified into several groups, such as flavones, flavonols, and isoflavones, according to their structural differences. We previously reported that quercetin (a flavonol) (Fig. 5) reversibly arrests the cell cycle of HGC-27 cells at G_1 (6). In the present study we have shown that genistein (an isoflavone) reversibly arrested the cell cycle progression of HGC-27 cells at G_2-M, whereas daidzein (an isoflavone), flavone, and luteoline (flavones) inhibited it at G_1 as quercetin does. Microscopic observation showed that genistein did not work as a spindle poison, such as vincristine or colcemid.[2] These results suggest that genistein has a unique effect on the cell cycle among closely related flavonoids and might be used as an antiproliferative or anticancer drug.

Biochemical studies of flavonoids have provided insight into the mechanisms of cell cycle inhibition by genistein. There are at least two possible explanations as to how genistein works.

First, genistein is a specific inhibitor of tyrosine-protein kinase. Even though daidzein has a very similar structure by missing only a single hydroxyl group in position 5 (Fig. 5), daidzein shows no inhibitory activities on tyrosine kinase (10). On the contrary, quercetin nonspecifically inhibits tyrosine kinase as well as other kinase activities (11, 12). These results suggest that specific inhibition of tyrosine kinase activity may be responsible for G_2-M arrest by genistein.

Among many kinases, cdc2 kinase is a component of the maturation-promoting factor, which is known to initiate M. Tyrosine residues of cdc2 kinase are phosphorylated from S to G_2. The activity of the maturation-promoting factor is induced by dephosphorylation of tyrosine residues of cdc2 kinase at M initiation (13–15). There is a possibility that genistein might perturb the process of phosphorylation/dephosphorylation of tyrosine residues of cdc2 kinase, which may lead to arrest at G_2-M, although we have not obtained any direct proofs of our hypothesis.

As the second possibility, genistein is reported to inhibit topo II (16–18). Topo II is an essential nuclear enzyme that resolves severe tangling problems of DNA by forming transient breaks on both DNA strands during replication, transcription, and other DNA processes. Topo II inhibitors interfere with the breakage-reunion step of DNA strand passage (19). Topo II inhibitors can be classified into two groups, intercalators into DNA (*e.g.*, Adriamycin, actinomycin D, amsacrine), and non-intercalators into DNA (*e.g.*, VP-16, VM-26, fostriecin). Genistein is found to be a nonintercalative topo II inhibitor, whereas daidzein has no inhibitory effect (20). One of the intercalators, Adriamycin, is well known to induce G_2 arrest and not M arrest with the mitotic selection procedure (21, 22), or with flow cytometric analysis (23). Nonintercalators, VP-16 and VM-26, also evoke G_2 arrest (24, 25), while fostriecin shows S and G_2 arrest (26). These facts suggest that genistein may work as a topo II inhibitor to arrest the cell cycle at G_2.

We have reported that genistein and other flavonoids (quercetin, luteolin, flavone) commonly inhibit the expression of HSPs (27). The role of HSPs on cell cycle regulation is controversial. In our previous study, we showed that cyclopentenone prostaglandins arrest cell cycle at G_1 and induced HSPs specifically at G_1 (28–30). Therefore, induction of HSPs is supposed to be associated with the G_1 block. Furthermore, Iida and Yahara (31) observed the similar phenomenon with the use of yeast and other eukaryotes. In contrast, other groups reported that HSPs are involved in promoting G_1 (32–34). In this study, genistein induced G_2-M arrest while the other flavonoids induced G_1 arrest. These results suggest that G_1 block by the flavonoids may have no relation with the expression of HSPs.

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[2] Unpublished data.

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