Blocking effects of genistein on cell proliferation and possible mechanism in human gastric carcinoma

Hong-Bin Cui, Xiao-Lin Na, Dan-Feng Song, Ying Liu

AIM: To study the blocking effects of genistein on cell proliferation cycle in human gastric carcinoma cells (SGC-7901) and the possible mechanism.

METHODS: MTT assay was applied in the detection of the inhibitory effects of genistein on cell proliferation. Flow cytometry was used to analyze the cell cycle distribution. Immunocytochemical technique and Western blotting were performed to detect the protein expression of cyclin D1, cyclin B1, and p21^{waf1/cip1}.

RESULTS: Genistein significantly inhibited the growth and proliferation of human gastric carcinoma cells (SGC-7901). Seven days after treatment with different concentrations of genistein (2.5, 5.0, 10.0, 20.0 µg/mL), the growth inhibitory rates were 11.2%, 28.8%, 55.3%, 84.7% respectively and cell cycles were arrested at the G(2)/M phase. Genistein decreased cyclin D1 protein expression and enhanced cyclin B1 and p21^{waf1/cip1} protein expression in a concentration-dependent manner.

CONCLUSION: The growth and proliferation of SGC-7901 cells can be inhibited by genistein via blocking the cell cycle, with reduced expression of cyclin D1 and enhanced expression of cyclin B1 and p21^{waf1/cip1} protein in the concentration range of 0-20 µg/mL.

Abstract

AIM: To study the blocking effects of genistein on cell proliferation cycle in human gastric carcinoma cells (SGC-7901) and the possible mechanism.

METHODS: MTT assay was applied in the detection of the inhibitory effects of genistein on cell proliferation. Flow cytometry was used to analyze the cell cycle distribution. Immunocytochemical technique and Western blotting were performed to detect the protein expression of cyclin D1, cyclin B1, and p21^{waf1/cip1}.

RESULTS: Genistein significantly inhibited the growth and proliferation of human gastric carcinoma cells (SGC-7901). Seven days after treatment with different concentrations of genistein (2.5, 5.0, 10.0, 20.0 µg/mL), the growth inhibitory rates were 11.2%, 28.8%, 55.3%, 84.7% respectively and cell cycles were arrested at the G(2)/M phase. Genistein decreased cyclin D1 protein expression and enhanced cyclin B1 and p21^{waf1/cip1} protein expression in a concentration-dependent manner.

CONCLUSION: The growth and proliferation of SGC-7901 cells can be inhibited by genistein via blocking the cell cycle, with reduced expression of cyclin D1 and enhanced expression of cyclin B1 and p21^{waf1/cip1} protein in the concentration range of 0-20 µg/mL.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Key words: Gastric carcinoma; Genistein; Cell proliferation; Cell cycle

Introduction

Genistein is a natural ingredient in soybean. Recently, it has attracted more and more attention in the field of cancer prevention[1-3]. A number of epidemiological and laboratory studies have shown that genistein is a potential cancer chemopreventive agent for sex hormone-dependent cancers, such as breast cancer and prostate cancer[4-9]. However, there are few reports about the effect of genistein on non-sex hormone-dependent cancers, such as gastric cancer[10-12]. Gastric cancer is common in China and supposed to be caused by environmental factors, in which diet is an important modifying agent[10,14].

In this study, human gastric carcinoma cells (SGC-7901) were used as the model in vitro to investigate the effect of genistein on cell proliferation and its possible mechanism.

Materials and Methods

Reagents and cell lines

Genistein (purity >98%) and trypsin were purchased from Sigma. 'H-TdR was purchased from China Atomic Energy Research Academy. SP-9000 kit was the product of Zyme. Monoclonal antibodies to cyclin D1, cyclin B1 and p21^{waf1/cip1} were the products of Santa Cruz and purchased from Zhongshan Co., China. Human gastric carcinoma cells (SGC-7901), provided by the Cancer Research Institute of Beijing, were cultured in RPMI1640 (Gibco) medium supplemented with 10% fetal calf serum, penicillin (100×10^3 U/L) and streptomycin (100 mg/L) at 37 ℃ in a 50 mL/L CO2-atmosphere. Genistein was dissolved in DMSO at the concentration of 20 mg/mL and then diluted to the required concentration with culture medium.

Assessment of cell proliferation

MTT assay was conducted to detect the cell proliferation. SGC-7901 cells were seeded in 96-well plates, each well containing 5×10^3 cells. After 24 h, the culture medium was replaced by media in which genistein concentrations were 0, 2.5, 5.0, 10.0 and 20.0 µg/mL respectively. There were four wells for each concentration. From 1 to 7 d, one of the plates was taken out and 20 μL fresh 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 5g/L PBS) was added to each well. After 4 h incubation, the culture media were discarded, 150 μL of DMSO was added to each well and vibrated to dissolve the deposer. The optical density (A value) was measured at 570 nm with a microplate reader. The inhibitory rate (IR) of genistein on SGC-7901 cells on the 7th d was calculated as follows: IR(%) = (1- treated group A/control group A)×100%.

Flow cytometric analysis

After an exponential growth phase, SGC-7901 cells were treated with different concentrations of genistein (0, 5.0, 10.0 and 20.0 µg/mL) for 24 or 48 h. The cells were collected and stained with propidium iodide (PI), then the DNA content of cells was assessed by flow cytometric analysis using FITC-labeled anti-PI antibody.

Immunocytochemistry

Cultured cells treated with genistein for 24 or 48 h were harvested and fixed in 4% citromint solution, and then embedded in
paraffin. Four micrometer-thick sections were cut and deparaffinized in xylene and dehydrated with graded alcohol. Sections were treated with microwave to retrieve antigens, then incubated overnight at 4 °C with cyclin B1 and cyclin D1 antibodies (1:50 dilution) respectively. Other steps were according to the description of SP kit. Chromogenic reaction was developed with diaminobenzidine (DAB), and restained with methylgreen. All sections were observed under microscope and the number of positive cells per 1000 cells was counted.

**Western blot analysis**
Cultured cells treated with genistein for 48 h were harvested and washed with PBS. The cells were lysed in protein extract solution. Protein concentration was determined by Coomassie light blue methods. One hundred micrograms of cell protein was degenerated by heat, separated on 10% polyacrylamide gel electrophoresis and transferred to nitrocellulose filter membrane at 30 V. The membranes were incubated with blocking solution (containing antibodies against p21WAF1/CIP1) for 2 h at 37 °C and washed twice with PBS, then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Chromogenic reaction was developed with DAB and the bands were recorded and the peak areas of protein were scanned 1 h. Chromogenic reaction was developed with DAB and the restained with methylgreen. All sections were observed under microscope and the number of positive cells per 1000 cells was counted.

**Statistical analysis**
Data analysis was performed using Student’s t test. P<0.05 was considered statistically significant.

## RESULTS

### Inhibitory effect of genistein on SGC-7901 cell growth

MTT assay was conducted to detect the inhibitory effect of genistein on SGC-7901 cells. As shown in Figure 1, cell proliferation slowed down with the increase of genistein concentration and elongation of action time in a dose- and time-dependent manner. On d7, the inhibitory rates of genistein on SGC-7901 cell growth at concentrations of 2.5, 5.0, 10.0 and 20.0 µg/mL were 11.2%, 55.3% and 84.7%, respectively.

![Figure 1](image)

**Figure 1** Inhibitory effect of genistein on growth of SGC-7901 cells. The cells were treated with various concentrations of genistein for 1-7 d, the antiproliferative effect was measured by MTT assay. Results were expressed as mean±SD from 4 wells.

### Changes of cell cycle detected by flow cytometric analysis

As shown in Table 1, the cell cycle of SGC-7901 cells was changed obviously. The number of cells in Go/G1 phase of cell cycle was decreased gradually. The progression of cell cycle was partly arrested at G2/M phase, but the change of S phase was insignificant.

| Genistein (µg/mL) | 24 h | 48 h |
|-------------------|------|------|
|                   | G0/G1 | S    | G2/M | G0/G1 | S    | G2/M |
| 0.0               | 57.90 | 32.52 | 9.57 | 64.13 | 29.75 | 6.12 |
| 5.0               | 50.64b | 30.05 | 19.31b | 56.16b | 29.41 | 14.43b |
| 10.0              | 43.01bd | 30.18b | 27.80bd | 49.85bd | 30.01 | 20.14bd |
| 20.0              | 36.96bd | 30.66b | 32.38bd | 39.26bd | 36.88bd | 23.86bd |

*P<0.01, vs genistein 0.0 µg/mL, 0P<0.01, vs genistein 10.0 µg/mL, 0P<0.01, vs genistein 5.0 µg/mL, 0P<0.05, vs genistein 0.0 µg/mL.

### Expression of cyclin B1 and cyclin D1 in SGC-7901 cells treated with genistein

![Table 2](image)

**Table 2** Expression of cyclin B1 and cyclin D1 in SGC-7901 cells treated with genistein

| Genistein (µg/mL) | 24 h | 48 h |
|-------------------|------|------|
|                   | cyclin B1 | cyclin D1 |
|                   | positive rate (%) | positive rate (%) |
| 0.0               | 36.8 | 91.9 |
| 5.0               | 46.5b | 70.5b |
| 10.0              | 53.4bd | 49.3bd |
| 20.0              | 72.3bd | 25.4bd |

*P<0.01, vs genistein 0.0 µg/mL, 0P<0.01, vs genistein 5.0 µg/mL, 0P<0.01, vs genistein 10.0 µg/mL.

### Expression of p21WAF1/CIP1 protein by Western blotting

The expression of p21WAF1/CIP1 protein is shown in Figure 2 and the peak areas of bands were analyzed with gel digit image instrument (Chemilumager 4000).

![Figure 3](image)

**Figure 3** Calculation of areas of p21WAF1/CIP1 protein by Chemilumager 4000.

### DISCUSSION

MTT chromatometry is a common method to detect cell stock and growth. Ectogenesis of MTT can be reduced by succinic acid.
acid dehydrogenase existing in mitochondria of live cells and forms indissoluble blue-purple crystal mass (formazan) and deposits in cells. The crystal mass is dissolved by DMSO. By detecting the A value with a microplate reader, the quantity of live cells can be gained indirectly. The findings from our research group suggest that genistein could significantly inhibit the proliferation of SGC-7901 cells in a dose- and time-dependent manner. As shown in Figure 1, the inhibitory rates of different genistein concentrations (2.5, 5.0, 10.0 and 20.0 µg/mL) on d 7 are 11.2%, 28.8%, 55.3% and 84.7%, respectively. Genistein is a growth inhibitor of gastric carcinoma cells, the mechanism is unknown. However, we discovered that supplemented with genistein, the number of SGC-7901 cells after incubation in culture media was decreased and the cell cycle was arrested at G1/M phase.

Cyclins are a group of proteins with cell cycle specificity. Up to the present, cyclins A, B, C (B1, C), D (D1, D2, D3), E, F, G and H have been found. Cyclin D1 is synthesized in pre-DNA-synthetic gap (early G1 phase), and plays an important role in G1 to S phase and induces cells into S phase. In general, cyclin D1 is the key regulator of cell cycle progression and the key protein of the signal transduction in G1 phase cell proliferation. If cyclin D1 is over-expressed, the checkpoint of G1/S will be out of control and lose its role in the signaling of proliferation. This further promotes cell cycle progression and cell proliferation, and causes carcinomatous change of cells. Thus cyclin D1 is called the shirking protein of G1/S checkpoint. It has been proved that cyclin D1 is overexpressed in several neoplasms, such as esophageal carcinoma, mammary cancer, pulmonary and gastric carcinoma[13]. Suppressed expression of cyclin D1 in cancer cells would help recover normal cell cycle and control proliferation speed of tumor cells. In this study, we found that genistein showed significant inhibition on the expression of cyclin D1 in SGC-7901 cells, suggesting that genistein might inhibit cell proliferation of gastric carcinoma by decreasing the over-expression of cyclin D1.

Cyclin B1 and cyclin-dependent kinase 1 (CDK1) are two proteins required for cells to traverse from G2 into M. G2 phase cell proliferation. If cyclin B1 protein by Western blotting. Researchers previously believed that p21<sup>waf/cip1</sup> protein was a regulatory factor of cell cycle in G1 phase. But now, more and more evidence indicates the expression of p21<sup>waf/cip1</sup> protein relates with G2/M phase arrest.[16,23] While p21<sup>waf/cip1</sup> binds to a variety of CDKs and cyclins, and exerts inhibitory activity on cyclin/CDK complexes, including cyclin-A/CDK1 and cyclin-B1/CDK1. Therefore p21<sup>waf/cip1</sup> protein has an intimate relationship with G2 and M phases of cell cycle. When SGC-7901 cells are incubated with genistein for 48 h, the expression of p21<sup>waf/cip1</sup> is reduced in a dose-dependent manner. All these demonstrate that the inhibitory effect of genistein on human gastric carcinoma cells relates with genistein-induced expression of p21<sup>waf/cip1</sup> and genistein arrests tumor cells in G1/M phase.

Cell cycle regulation involves many factors and is very complicated[23]. The data from our studies indicate that genistein could arrest cell cycle progression of SGC-7901 cells at G1/M phase. The possible mechanism is that genistein promotes the expression of p21<sup>waf/cip1</sup> and reduces the degradation of cyclin B1 protein in tumor cells. Therefore tumor cells are unable to pass the checkpoint pathway of G2/M and cannot proceed to mitosis. Genistein could also inhibit the expression of cyclin D1 in tumor cells. In a word, neoplasia is a disease of cell over-proliferation and correlates with cell cycle regulation disorder. Genistein inhibits tumor cell growth and proliferation by increasing the expression of cyclin B1 and p21<sup>waf/cip1</sup> and decreasing the expression of cyclin D1 in SGC-7901 cells. This result suggests that the inhibitory effect of genistein on SGC-7901 cell proliferation relates to cell cycle.

REFERENCES

1. Myong H, Hong SP, Yun PY, Lee JH, Kim MJ. Anti-cancer effect of genistein in oral squamous cell carcinoma with respect to angiogenesis and in vitro invasion. Cancer Sci 2003; 94: 215-220
2. Dixon RA, Ferreira D. Genistein. Phytochemistry 2002; 60: 205-211
3. Arliss RM, Biermann CA. Do soy isoflavones lower cholesterol, inhibit atherosclerosis, and play a role in cancer prevention? Holist Nurs Pract 2002; 16: 40-48
4. Jones JL, Daley BJ, Enderson BL, Zhou JR, Karlstad MD. Genistein inhibits tamoxifen effects on cell proliferation and cell cycle arrest in T47D breast cancer cells. Am Surg 2002; 68: 575-577; discussion 577-578
5. Lamartiniere CA, Cotroneo MS, Fritz WA, Wang J, Mentor-Marcel R, Elgavish A. Genistein chemoprevention: timing and mechanisms of action in murine mammary and prostate. J Nutr 2002; 132: 5526-5585
6. Frey RS, Li J. Singletary KW. Effects of genistein on cell proliferation and cell cycle arrest in nonneoplastic human mammary epithelial cells: involvement of Cdk2, p21(waf/cip1), p27(kip1), and Cdc25c expression. Biochem Pharmacol 2001; 61: 979-989
7. Castle EP, Thrasher JB. The role of soy phytoestrogens in prostate cancer. Urol Clin North Am 2002; 29: 71-81, viii-ix
8. Wang J, Eltoum IE, Lamartiniere CA. Dietary genistein suppresses chemically induced prostate cancer in Lobund-Wistar rats. Cancer Lett 2002; 186: 11-18
9. Li Y, Sarkar FH. Gene expression profiles of genistein-treated PC3 prostate cancer cells. J Nutr 2002; 132: 3623-3631
10. Song D, Liu Y, Wang X, Yang Y. Inhibitory effects of genistein on the synthesis of DNA and the protein expression of cyclin D1 in human gastric carcinoma cell-line. Weisheng Yanjiu 2002; 31: 106-108
11. Song D, Na X, Liu Y, Chi X. Study on mechanisms of human gastric carcinoma cells apoptosis induced by genistein. Weisheng Yanjiu 2003; 32: 128-130
12. Piontek M, Hengels KJ, Porschens R, Strohmeyer G. Antiproliferative effect of tyrosine kinase inhibitors in epidermal growth factor-stimulated growth of human gastric cancer cells. Anticancer Res 1993; 13: 2119-2123
13 Liu JR, Li BX, Chen BQ, Han XH, Xue YB, Yang YM, Zheng YM, Liu RH. Effect of cis-9, trans-11-conjugated linoleic acid on cell cycle of gastric adenocarcinoma cell line (SGC-7901). *World J Gastroenterol* 2002; 8: 224-229

14 Wang DX, Fang DC, Liu WW. Study on alteration of multiple genes in intestinal metaplasia, atypical hyperplasia and gastric cancer. *Shijie Huaren Xiaohua Zazhi* 2000; 8: 855-859

15 Barnes DM, Gillett CE. Cyclin D1 in breast cancer. *Breast Cancer Res Treat* 1998; 52: 1-15

16 Cappelletti V, Fioravanti L, Miodini P, Di Fronzo G. Genistein blocks breast cancer cells in the G2/M phase of the cell cycle. *J Cell Biochem* 2000; 79: 594-600

17 Tu SP, Jiang SH, Tan JH, Jiang XH, Qiao MM, Zhang YP, Wu YL, Wu YX. Proliferation inhibition and apoptosis induction by arsenic trioxide on gastric cancer cell SGC-7901. *Shijie Huaren Xiaohua Zazhi* 1999; 7: 18-21

18 Kasahara T, Kuwayama C, Hashiba M, Harada T, Kakinuma C, Miyauchi M, Degawa M. The gene expression of hepatic proteins responsible for DNA repair and cell proliferation in tamoxifen-induced hepatocarcinogenesis. *Cancer Sci* 2003; 94: 582-588

19 Palazon LS, Davies TJ, Gardner RL. Translational inhibition of cyclin B1 and appearance of cyclin D1 very early in the differentiation of mouse trophoblast giant cells. *Mol Hum Reprod* 1998; 4: 1013-1020

20 Shao ZM, Alpaugh ML, Fontana JA, Barsky SH. Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by P21WAF1/CIP1 induction, G2/M arrest, and apoptosis. *J Cell Biochem* 1998; 69: 44-54

21 Stewart ZA, Leach SD, Pietenpol JA. p21(Waf1/Cip1) inhibition of cyclin E/Cdk2 activity prevents endoreduplication after mitotic spindle disruption. *Mol Cell Biol* 1999; 19: 205-215

22 Davis JN, Singh B, Bhuiyan M, Sarkar FH. Genistein-induced upregulation of p21WAF1, downregulation of cyclin B, and induction of apoptosis in prostate cancer cells. *Nutr Cancer* 1998; 32: 123-131

23 Kim MH, Gutierrez AM, Goldfarb RH. Different mechanisms of soy isoflavones in cell cycle regulation and inhibition of invasion. *Anticancer Res* 2002; 22: 3811-3817

*Editorial by Wang XL, Zhang JZ and Zhu LH*