Apoptosis of human primary gastric carcinoma cells induced by genistein

Hai-Bo Zhou, Juan-Juan Chen, Wen-Xia Wang, Jian-Ting Cai, Qin Du

Hai-Bo Zhou, Juan-Juan Chen, Wen-Xia Wang, Jian-Ting Cai, Qin Du, Department of Gastroenterology, Second Affiliated Hospital of Zhejiang University, Hangzhou 310009, Zhejiang Province, China

Correspondence to: Dr. Hai-Bo Zhou, Department of Gastroenterology, Second Affiliated Hospital of Zhejiang University, Hangzhou 310009, Zhejiang Province, China. zhoubaihuzh@163.com

Abstract

AIM: To investigate the apoptosis in primary gastric cancer cells induced by genistein, and the relationship between this apoptosis and expression of bcl-2 and bax.

METHODS: MTT assay was used to determine the cell growth inhibitory rate in vitro. Transmission electron microscope and TUNEL staining were used to quantitatively and qualitatively detect the apoptosis of primary gastric cancer cells before and after genistein treatment. Immunohistochemical staining and RT-PCR were used to detect the expression of apoptosis-associated genes bcl-2 and bax.

RESULTS: Genistein inhibited the growth of primary gastric cancer cells in dose-and time-dependent manner. Genistein induced primary gastric cancer cells to undergo apoptosis with typically apoptotic characteristics. TUNEL assay showed that after the treatment of primary gastric cancer cells with genistein for 24 to 96 h, the apoptotic rates of primary gastric cancer cells increased time-dependently. Immunohistochemical staining showed that after the treatment of primary gastric cancer cells with genistein for 24 to 96 h, the positivity rates of Bcl-2 proteins were apparently reduced with time and the positivity rates of Bax proteins were apparently increased with time. After exposure to genistein at 20 µmol/L for 24, 48, 72 and 96 respectively, the density of bcl-2 mRNA decreased progressively and the density of bax mRNA increased progressively with elongation of time.

CONCLUSION: Genistein is able to induce the apoptosis in primary gastric cancer cells. This apoptosis may be mediated by down-regulating the apoptosis-associated bcl-2 gene and up-regulating the expression of apoptosis-associated bax gene.

Zhou HB, Chen JJ, Wang WX, Cai JT, Du Q. Apoptosis of human primary gastric carcinoma cells induced by genistein. World J Gastroenterol 2004; 10(12): 1822-1825

http://www.wjgnet.com/1007-9327/10/1822.asp

INTRODUCTION

Genistein is a planar molecule with an aromatic A-ring, has a second oxygen atom 11.5 Å from the one in the A ring, a molecular weight similar to those of the steroidal estrogens. It has estrogenic properties in receptor binding assays[5,6], cell culture[5,6], and uterine weight assays[5,6]. Genistein inhibits topoisomerase II[9], platelet-activating factor- and epidermal growth factor-induced expression of c-fos[9], diacylglycerol synthesis[9], and tyrosine kinases[11]. It also inhibits microsomal lipid peroxidation[12] and angiogenesis[13]. Genistein exhibits antioxidant properties[14-16] and was reported to induce differentiation of numerous cell types[17-19]. Moreover, a recent report shows that genistein is a potent cancer chemopreventive agent[20-22]. The anti-tumor activity of genistein might be related to induce the apoptosis of tumor cells but the precise mechanism of antitumor activity is not well understood.

The Bcl-2 family plays a crucial role in the control of apoptosis. The family includes a number of proteins which have homologous amino acid sequences, including anti-apoptotic members such as Bcl-2 and Bcl-xL, as well as pro-apoptotic members like Bax and Bad[23-26]. Overexpression of Bax has the effect of promoting cell death[27-31]. Conversely, Overexpression of antiapoptotic proteins such as Bcl-2 will repress the function of Bax[25-28]. Thus, the ratio of Bcl-2/Bax appears to be a critical determinant of cell apoptosis[32].

In this study, MTT assay was used to determine the cell growth inhibitory rate. Transmission electron microscope and TUNEL staining method were used to quantitatively and qualitatively detect the apoptosis status of primary gastric cancer cells before and after the genistein treatment. Immunohistochemical staining and RT-PCR were used to detect the expression of apoptosis-associated genes bcl-2 and bax.

MATERIALS AND METHODS

Materials

Genistein and MTT were obtained from Sigma Chemical Co, Ltd. In situ cell detection kit, anti-Bcl-2 monoclonal antibody and anti-Bax monoclonal antibody were purchased from Beijing Zhongshan Biotechnology Co, Ltd. Stock solution of genistein was made in dimethylsulfoxide (DMSO) at a concentration of 40 µmol/L. Working dilutions were directly made in the cell culture medium.

Methods

Cell culture Fresh sample from a patient with gastric cancer was obtained in operating room. A single-cell suspension of tumor cells with the concentration of 5x10^6/mL was prepared for seeding. Primary gastric cancer cells were purified after culture.

MTT assay Cells 1x10⁵/well in a 96-well plate after incubation for 24 h were treated with different concentrations of genistein (5, 10, 20, 40 µmol/L) for 24, 48 and 72 h respectively. A 10 µL of 5 g/L of MTT was added to the medium triplicate at each dose and incubated for 4 h at 37 °C. Culture media were discarded follow by addition of 0.2 mL DMSO and vibration for 10 min. The absorbance (A) was measured at 570 nm using a microplate reader. The cell growth inhibitory rate was calculated as follows: [(A of control group - A of experimental group)/(A of control group - A of blank group)] x100%.

Transmission electron microscopy Cells treated with 20 µmol/L genistein were harvested after incubation for 24 h. Subsequently
the cells were fixed in 40 g/L glutaral and immersed with Epon 821, imbedded for 72 h at 60 °C. After that the cells were prepared into ultrathin section (60 nm) and stained with uranyl acetate and lead citrate. Cell morphology was observed by transmission electron microscopy.

**TUNEL assay** Apoptosis of primary gastric cancer cells was evaluated by using an *in situ* cell detection kit. The cells were treated in the presence or absence of 20 µmol/L genistein for 24 to 96 h and fixed in ice-cold 800 mL/L ethanol for up to 24 h, treated with proteinase K and then 3 mL/L H2O2, and labeled with fluorescein dUTP in a humid box for 1 h at 37 °C. Cells were then combined with POD-horseradish peroxidase, colored with DAB and counterstained with methyl green. Controls received the same treatment except the labeling of omission of fluorescein dUTP. Cells were visualized under light microscope. The apoptotic index (AI) was calculated as follows: AI=(Number of apoptotic cells/Total number)×100%.

**Immunohistochemical staining** Immunohistochemical staining was done by an avidin-biotin technique. Primary gastric cancer cells treated in the presence or absence of 20 µmol/L genistein for 24 to 96 h were grown on six-well glass plates and were fixed by acetone. After washed in PBS, the cells were incubated in 3 mL/L H2O2 solution at room temperature for 5 min. The cells were then incubated with anti-Bcl-2 or anti-Bax monoclonal antibody at a 1:300 dilution at 4 °C overnight. After wash of cells in PBS, the second antibody, biotinylated antirat IgG was added and the cells were incubated at room temperature for 1 h. After wash of cells in PBS, ABC compound was added and the cells were then incubated and labeled at room temperature for 10 min. DAB was used as the chromagen. After 10 min, the brown color signifying the presence of antigen bound to antibodies was detected by light microscopy and photographed at ×200. Controls were treated the same as the experimental group except the incubation of the primary antibody instead of second antibody. The positive rate (PR) was calculated as follows: PR=(Number of positive cells/Total number)×100%.

**RT-PCR** The primary gastric cancer cells were treated in the presence or absence of 20 µmol/L genistein for 24 to 96 h and total RNA was extracted. The concentration of RNA was determined by absorption at 260 nm. The primers for Bcl-2, Bax and β-actin were as follows: β-actin (500 bp): 5'- GTGGGG GCGCCGACCA CCA 3', 5'-CTCCTTATGTCACG CACGATTTC 3'; bcl-2 (716 bp): 5'- GGAATATGGCCGC ACCT3' .5'- TACATGGCGCAGATG 3'; bax (508 bp): 5'- CCAGCTCTGAGCATCATG 3', 5'- TATCAGCCCA TTCTCTCCC 3'. Polymerase chain reactions were performed in a 25 µL reaction volume. PCR for Bcl-2 and β-actin was run in the following procedures: at 94 °C for 7 min, 1 circle; at 94 °C for 1 min, at 72 °C for 1 min, 30 cycles; at 72 °C for 7 min, 1 circle. PCR for Bax was run in the following procedure: 94 °C for 1 min, 60 °C for 45 s, 72 °C for 45 s, 35 cycles. Ten µL PCR product was placed onto 15 g/L agarose gel and observed by EB staining using Gel-Pro analyzer.

**Statistical analysis** Data were analyzed by the paired two-tailed Student’s *t* test, and significance was considered when *P*<0.05.

**RESULTS**

**MTT assay** Primary gastric cancer cells were exposed to increasing concentrations (5 µmol/L to 40 µmol/L) of genistein for 24 to 72 h, respectively. Primary gastric cancer cells showed death in a dose- and time-dependent manner. The data are summarized in Table 1.

| Table 1 A value of primary gastric cancer cells treated with different concentrations of genistein | 24 h | 48 h | 72 h |
|---|---|---|---|
| Control | 0.400±0.008 | 0.406±0.007 | 0.404±0.008 |
| 5 µmol/L genistein | 0.361±0.002 | 0.334±0.012 | 0.305±0.004 |
| 10 µmol/L genistein | 0.325±0.004 | 0.313±0.003 | 0.248±0.004 |
| 20 µmol/L genistein | 0.308±0.003 | 0.249±0.002 | 0.206±0.003 |
| 40 µmol/L genistein | 0.265±0.004 | 0.215±0.004 | 0.159±0.002 |

*P*<0.05; *P*<0.01; *P*<0.001 vs control group.

**Morphological changes** After treatment of primary gastric cancer cells with genistein (20 µmol/L) for 24 h, some cells presented apoptotic characteristics including chromatin condensation, appearance of chromatin crescent, nuclear fragmentation that could be seen by transmission electron microscope (Figure 1).

**Expression of Bcl-2 proteins**

Positive staining located in the cytoplasm. After treatment with genistein (20 µmol/L) for 24 to 96 h, Apoptotic index of the cells was apparently increased with the increase of treatment time (*P*<0.05) (Table 2).

**Expression of Bax proteins**

Positive staining located in the cytoplasm. After treatment with genistein (20 µmol/L) for 24 to 96 h, PRs of Bax proteins were apparently increased with increase of treated time (*P*<0.05)
Genistein could reduce Bcl-2 expression and enhance bax mRNA expression. The ratio of Bcl-2/Bax was decreased when primary gastric cancer cells were treated with genistein, which could trigger the apoptosis of primary gastric cancer cells.

The present study demonstrated that genistein was able to induce the apoptosis in primary gastric cancer. This apoptosis may be mediated by down-regulating the expression of apoptosis-associated gene bcl-2 and up-regulating the expression of apoptosis-associated gene bax. Genistein may be used as a potential chemotherapeutic drug in the anti-gastric carcinoma chemotherapy.

**REFERENCES**

1. Shutt DA, Cox RL. Steroid and phyto-oestrogen binding to sheep uterine receptors in vivo. J Endocrinol 1972; 52: 299-311
2. Mathieson RA, Kits WD. Binding of phytoestrogen and estradiol-17beta by cytoplasmic receptors in the pituitary gland and hypothalamus of the ewe. J Endocrinol 1980; 85: 317-325
3. Martin PM, Horowitz KB, Ryan DS, McGuire WL. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. Endocrinology 1978; 103: 1860-1867
4. Makela S, Davis VL, Talley WC, Korkman J, Salo L, Viikho R, Santti R, Korach KS. Dietary estrogens act through estrogen receptor-mediated processes and show no antiestrogenicity in cultured breast cancer cells. Environ Health Perspect 1994; 102: 572-578
5. Thigpen JE, Haseman JK, Saunders H, Locklear J, Caviness G, Grant M, Forysthe D. Dietary factors affecting uterine weights of immature CD-1 mice used in uterotrophic bioassays. Cancer Detect Prev 2002; 26: 381-393
6. Polkowski K, Mazurek AP. Biological properties of genistein. A review of in vitro and in vivo data. Acta Pol Pharm 2000; 57: 135-155
7. Okura A, Arakawa H, Oka H, Yoshinari T, Monden Y. Effect of genistein on topoisomerase activity and on the growth of [Val12] Ha-ras-transformed NIH 3T3 cells. Biochem Biophys Res Commun 1988; 157: 183-189
8. Tripathi YB, Lim RW, Fernandez-Gallardo S, Kanda JC, Gunakara RV, Shukla SD. Involvement of tyrosine kinase and protein kinase C in platelet-activating-factor-induced c-fos gene expression in A421 cells. Biochem J 1992; 286( Pt 2): 527-533
9. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itho N, Shibuya M, Fukami Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 1987; 262: 5592-5595
10. Dean NM, Kanemitsu M, Boynton AL. Effects of the tyrosine kinase inhibitor genistein on DNA synthesis and phospholipid-derided second messenger generation in mouse 10T1/2 fibroblasts and rat liver T51B cells. Biochem Biophys Res Commun 1989; 175: 705-701
11. Jia HC, von Recklinghausen G, Zilliken F. Inhibition of in vitro microuosomal lipid peroxidation by isoflavonoids. Biochim Biophys Acta 1989; 102: 225-229
12. Fotsis T, Pepper M, Adlercreutz H, Fleischman G, Hase T, Montesano R, Schweigerer L, Genestin, a dietary-derived inhibitor of in vitro angiogenesis. Proc Natl Acad Sci U S A 1993; 90: 2690-2694
13. Mitchell JH, Gardner PT, McPhail DB, Morrow PC, Collins AR, Dubois GG. Antioxidant efficacy of phytoestrogens in chemical and biological model systems. Arch Biochem Biophys 1998; 350: 142-148
14. Tikkkanen MJ, Adlercreuz H. Dietary soy-derived isolavones of phytoestrogens. Could they have a role in coronary heart disease prevention? Biochem Pharmacol 2000; 60: 1-5
15. Wei H, Bowen R, Cai O, Barnes S, Wang Y. Antioxidant and anti-promotional effects of the soybean isolate avonoids. Proc Soc Exp Biol Med 1999; 220: 124-130
16. Watanabe T, Kondo K, Ishii M. Induction of in vitro differentiation of mouse erythroleukaemia cells by genistein, an inhibitor of tyrosine protein kinases. Cancer Res 1993; 51: 764-768
17. Miller DR, Lee GM, Maness PF. Increased neurite outgrowth induced by inhibition of protein tyrosine kinase activity in PC12...
pheochromocytoma cells. J Neuroendocrin 1993; 60: 2134-2144
19 Simon HU, Youssefi S, Blaser K. Tyrosine phosphorylation regu-
lates activation and inhibition of apoptosis in human eosinophil
and neutrophils. Int Arch Allergy Immunol 1995; 107: 338-339
20 Huang P, Robertson LE, Wright S, Plunkett W. High molecular
weight DNA fragmentation: a critical event in nucleoside ana-
logue-induced apoptosis in leukemia cells. Clin Cancer Res 1995;
1: 1005-1013
21 Xu LH, Owens LV, Sturge GC, Yang X, Liu ET, Craven RJ, Cance
WG. Attenuation of the expression of the focal adhesion kinase
induces apoptosis in tumor cells. Cell Growth Differ 1996; 7: 413-418
22 Davis JN, Singh B, Bhuiyan M, Sarkar FH. Genistein-induced
upregulation of p21WAF1, downregulation of cyclin B, and in-
duction of apoptosis in prostate cancer cells. Nutr Cancer 1998; 32:
123-131
23 Konopleva M, Konoplev S, Hu W, Zaritskey AY, Afanasiev BV,
Andreeff M. Stromal cells prevent apoptosis of AML cells by up-
regulation of anti-apoptotic proteins. Leukemia 2002; 16: 1713-1724
24 van der Woude CJ, Jansen PL, Tiebosch AT, Beuving A, Homan
M, Kleibeuker JH, Moshage H. Expression of apoptosis-related
proteins in Barrett’s metaplasia-dysplasia-carcinoma sequence:
A switch to a more resistant phenotype. Hum Pathol 2002; 33:
696-692
25 Panaretakis T, Pokrovskaja K, Shoshan MC, Grander D. Activ-
ation of Bak, Bax and BH3-only proteins in the apoptotic response
to doxorubicin. J Biol Chem 2002; 277: 44317-44326
26 Bellissillo B, Villamar N, Lopez-Guillermo A, Marce S, Bosch F,
Campo E, Montserrat E, Colomer D. Spontaneous and drug-in-
duced apoptosis is mediated by conformational changes of Bax
and Bak in B-cell chronic lymphocytic leukemia. Blood 2002; 100:
1810-1816
27 Matter-Reissmann UB, Forte P, Schneider MK, Filgueira L,
Groscurth P, Seebach JD. Xenogeneic human NK cytotoxicity
against porcine endothelial cells is perforin/ granzyme B depen-
dent and not inhibited by Bd-2 overexpression. Xenotransplantation
2002; 9: 325-337
28 Lanzi C, Cassinelli G, Cucurru G, Supino R, Zuco V, Ferlini C,
Scambia G, Zunino F. Cell cycle checkpoint efficiency and cellular
response to paclitaxel in prostate cancer cells. Prostate 2001;
48: 254-264
29 Mertens HJ, Heineman MJ, Evers JL. The expression of apoptosis-
related proteins Bcl-2 and Ki67 in endometrium of ovulatory
menstrual cycles. Gynecol Obstet Invest 2002; 53: 224-230
30 Mehta U, Kang BP, Bansal G, Bansal MP. Studies of apoptosis
and bcl-2 in experimental atherosclerosis in rabbit and influence
of selenium supplementation. Gen Physiol Biophys 2002; 21:
15-29
31 Chang WK, Yang KD, Chuang H, Jan JT, Shaio MF. Glutamine
protects activated human T cells from apoptosis by up-regulat-
ing glutathione and bcl-2 levels. Clin Immunol 2002; 104:
151-160
32 Chen GG, Lai PB, Hu X, Lam IK, Chak EC, Chun YS, Lau WY.
Negative correlation between the ratio of Bax to Bcl-2 and the
size of tumor treated by culture supernatants from Kupffer cells.
Clin Exp Metastasis 2002; 19: 457-464
33 Usuda J, Chiu SM, Azizuddin K, Xue LY, Lam M, Nieminen AL,
Oleinick NL. Promotion of photodynamic therapy-induced
apoptosis by the mitochondrial protein Smac/ DIABLO: depen-
dence on Bax. Photochem Photobiol 2002; 76: 217-223
34 Sun F, Akazawa S, Sugahara K, Kanihira S, Kawasaki E, Eguchi
K, Koji T. Apoptosis in normal rat embryo tissues during early
organogenesis: the possible involvement of Bax and Bcl-2. Arch
Histol Cytol 2002; 65: 145-157
35 Jang MH, Shin MC, Shin HS, Kim KH, Park HJ, Kim EH, Kim CJ.
Alcohol induces apoptosis in TM3 mouse Leydig cells via bax-
dependent caspase-3 activation. Eur J Pharmacol 2002; 449:
39-45
36 Tilli CM, Stavast-Koey AJ, Ramaekers FC, Nuemann HA. Bax
expression and growth behavior of basal cell carcinomas. J Cutan
Pathol 2002; 29: 79-87
37 Pettersson F, Dalgleish AG, Bissonnette RP, Colston KW.
Retinoids cause apoptosis in pancreatic cancer cells via activa-
tion of RAR-gamma and altered expression of Bcl-2. Br J
Cancer 2002; 87: 555-561