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RAPID COMMUNICATION

Anticancer activity of genistein on implanted tumor of human SG7901 cells in nude mice

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Abstract

AIM: To investigate genistein-induced apoptosis of implanted tumors of SG7901 cells in nude mice, and the relationship between this apoptosis and expression of Bcl-2 and Bax.

METHODS: Establishing a transplanted tumor model by injecting human SG7901 cells into subcutaneous tissue of nude mice. Genistein (0.5, 1 and 1.5 mg/kg) was directly injected adjacent to the tumor, six times at 2-d intervals. Then, changes in tumor volume were measured continuously and tumor inhibition rate of each group was calculated. We observed the morphological alterations by transmission electron microscopy (TEM), measured the apoptotic rate by the TUNEL staining method, and detected the expression of apoptosis-regulated gene Bcl-2 and bax by immunohistochemical staining and RT-PCR.

RESULTS: Genistein 0.5, 1 and 1.5 mg/kg significantly inhibited carcinoma growth when it was injected near the tumor by 10.8%, 29.9% and 39.6%, respectively. Genistein induced implanted tumor cells to undergo apoptosis, with apoptotic characteristics seen by TEM. The apoptosis index was increased progressively with increasing genistein dose (28.9% ± 1.2%, 33.8% ± 1.6% and 37.7% ± 1.2%). The positive rate of Bcl-2 protein was decreased progressively (11.9% ± 0.9%, 5.9% ± 0.7% and 4.2% ± 0.6%), and the positive rate of bax protein was increased progressively (0.9% ± 1.7%, 24.9% ± 0.8% and 29.6% ± 1.7%) by immunohistochemical staining, with increasing dose of genistein. The density of Bcl-2 mRNA decreased progressively and the density of bax mRNA increased progressively with elongation of time by RT-PCR.

CONCLUSION: Genistein was able to induce apoptosis of transplanted tumor cells. This apoptosis may be mediated by down-regulation of the apoptosis-regulated gene Bcl-2 and up-regulation of apoptosis-regulated gene bax.

Key words: Genistein; Gastric carcinoma; Nude mice; Apoptosis

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INTRODUCTION

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 including Bax and Bad[1-4]. In *in vitro* experiments, overexpression of Bcl-2 has been shown to inhibit apoptosis[5-9], but overexpression of Bax has been shown to promote apoptosis[10-14].

Genistein has estrogenic properties in receptor binding assays[15,16], cell culture[17,18], and uterine weight assays[19-21]. Genistein inhibits microsomal lipid peroxidation[22] and angiogenesis[23]. Genistein exhibits antioxidant properties[24-26] and has been reported to induce differentiation of numerous cell types[27-29]. Moreover, a recent report has shown that genistein is a potent cancer chemopreventive agent[30-32]. The anti-tumor activity of genistein might be related to the induction of apoptosis of tumor cells but the precise mechanism of its antitumor activity is not well understood.

This study investigated genistein-induced apoptosis of implanted tumors of SG7901 cells in nude mice, the relationship between this apoptosis and expression of Bcl-2 and bax *in vivo*, and the theoretical and methodological basis of the clinical application of genistein.

MATERIALS AND METHODS

Materials

Genistein was obtained from Sigma and dissolved in dimethylsulfoxide (DMSO). In situ cell detection kit, anti-Bcl-2 monoclonal antibody and anti-Bax monoclonal antibody were purchased from Beijing Zhongshan Biotechnology. Female Balb/C nude mice (4 wk old, 16-18 g) were obtained from Chinese Academy of Medical Science.

Cell culture

Fresh samples from a patient with low-differentiation gastric cancer were obtained in the operating room. A single-cell suspension of tumor cells with a concentration of 5×10^5/mL was prepared for seeding. SG7901 cells were artificially purified after culture by pancreatic proteinase.

Tumor implantation into nude mice

A transplanted tumor model was established by injecting 1×10^9 human SG7901 cells/L into the subcutaneous tissue of nude mice. After 10 d, 25 nude mice were randomly divided into five groups, and 0.2 mL normal saline solution, 1.5 mg/kg DMSO, or 0.5, 1 or 1.5 mg/kg genistein was directly injected adjacent to the tumor, six times at 2-d intervals. Changes in tumor volume [$V = (\pi/6) \times abc$] were measured at 11 d after drug treatment and the tumor inhibition rate of each group was calculated.

$$\text{Inhibitory rate (IR) of tumor growth} = \frac{C(V_1 - V_0) - T(V_1 - V_0)}{C(V_1 - V_0)}$$

where C is the control group; T is the treated group; V_1 the volume before treatment (mm^3); and V_0 the volume after treatment (mm^3).

Transmission electron microscopy (TEM)

The tumor samples were cut into 1 mm^3 blocks and fixed in 4% glutaraldehyde and immersed in Epon 821, and embedded for 72 h at 60℃. The cells were cut into ultrathin section (60 nm) and stained with uranyl acetate and lead citrate. Cell morphology was observed by TEM. made in Japan.

TUNEL assay

The tumor samples were cryopreserved in liquid nitrogen and cut into 8-μm-thick slices. Slices were fixed in ice-cold 80% ethanol for up to 24 h, treated with proteinase K and 0.3% H_2O_2, and labeled with fluorescein dUTP in a humid box for 1 h at 37℃. Slices were then combined with POD-horseradish peroxidase, stained with DAB, and counterstained with methyl green. Controls were treated the same except for labeling with fluorescein dUTP. Cells were examined by light microscopy. The apoptotic index (AI) was calculated as follows: AI = (number of apoptotic cells/total number of cells) × 100%.

Immunohistochemical staining

The tumor samples were cryopreserved in liquid nitrogen and cut into 8-μm-thick slices and fixed in acetone. After washing in PBS, slices were incubated in 0.3% H_2O_2 solution at room temperature for 5 min. Slices were then incubated with anti-Bcl-2 or anti-Bax monoclonal antibody at a 1:300 dilution at 4℃ overnight. After washing in PBS, the second antibody, biotinylated anti-rat IgG, was added and the cells were incubated at room temperature for 1 h. After washing in PBS, ABC compound was added and then incubated at room temperature for 10 min. DAB was used as the chromogen. After 10 min, the brown color signifying the presence of antigen bound to antibodies was detected by light microscopy. Controls were prepared in the same manner as the experimental group, except for incubation with the primary antibody. The positive rate (PR) was calculated as follows: PR = (number of positive cells/total number of cells) × 100%.

Table 1 Inhibition effect of genistein on implanted tumors in nude mice (mean ± SD)

Groups dosage	Number of animals		Volume of tumors (mm^3)		Inhibition rate (%)
	Beginning	Ending	Beginning	Ending	
Control group					
0.2 mL saline	5	5	20.6 ± 1.1	499.8 ± 11.8	
DMSO					
1.5 mg/kg	5	5	20.6 ± 1.3	509.4 ± 8.6	
Genistein					
1.5 mg/kg	5	5	20.3 ± 1.6	458.2 ± 6.7	10.8[a]
1.0 mg/kg	5	5	21.6 ± 1.6	359.7 ± 6.4	29.9[a]
1.5 mg/kg	5	5	21.5 ± 1.6	319.5 ± 10.6	39.6[a]

[a]$P < 0.05$ *vs* the control group.

RT-PCR

The tumor samples were cryopreserved in liquid nitrogen 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'-GTGGGGCGCCCCAGGCACCA-3', 5'-CTCCTTAATGTCACGCACGATTTC-3'; Bcl-2 (716 bp) 5'-GGAAATATGGCGCACGCT-3', 5'-TCACTTGTGGCCCAGAT-3'; bax (508 bp) 5'-CCAGCTCTGAGCAGATCAT-3', 5'-TATCAGCCCATCTTCTTCC-3'. PCRs were performed in a 50 μL reaction volume. RT-PCR reaction was run as follows: 94℃ for 7 min, one cycle; 94℃ for 1 min, 72℃ for 1 min, 30 cycles; 72℃ for 7 min, one cycle. Ten microliters PCR product was placed on to 15 g/L agarose gel and observed by ethidium bromide staining using a Gel-Pro analyzer.

Statistical analysis

Datas were analyzed by analysis of variance, and statistical significance was considered at $P < 0.05$.

RESULTS

Inhibitory rate of tumor growth

An inhibitory effect was observed in all therapeutic groups, and the IR for 0.5, 1 and 1.5 mg/kg genistein was 10.8%, 29.9% and 39.6%, respectively (Table 1).

Morphological changes

Control cells had a normal structure, but some cells in the therapeutic groups had apoptotic characteristics, including chromatin condensation, appearance of chromatin

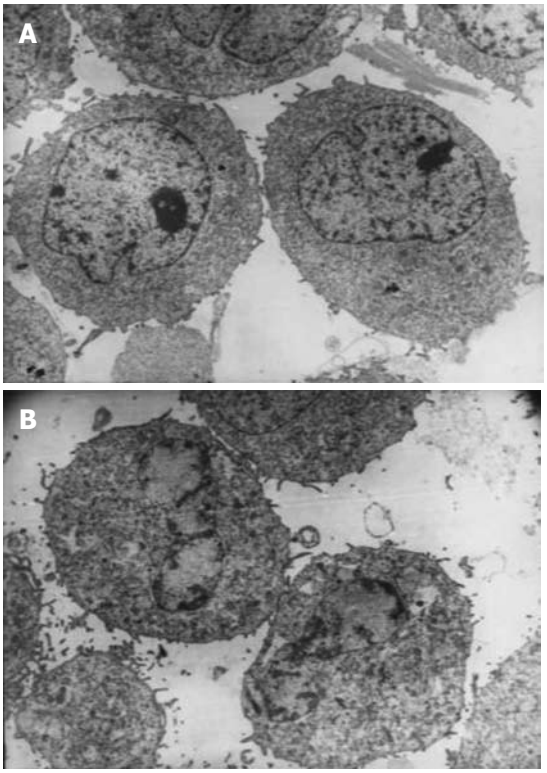

Figure 1 **A:** Ultrastructure of transplanted tumor cells (× 4800); **B:** Ultrastructure of apoptotic transplanted tumor cells induced by genistein (× 4800).

crescents, and nucleus fragmentation, which were seen by TEM (Figure 1A and B) .

TUNEL assay

Positive staining was located in the nucleus. The AI for 0.2 mL normal saline solution, 1.5 mg/kg DMSO, and 0.5, 1 and 1.5 mg/kg genistein was 12.6% ± 0.6%, 13.4% ± 0.7%, 28.9% ± 1.2%, 33.8% ± 1.6% and 37.7% ± 1.2%, respectively (Table 2) .

Expression of Bcl-2 proteins

Positive staining was located in the cytoplasm. The PR for Bcl-2 protein for 0.2 mL normal saline solution, 1.5 mg/kg DMSO, and 0.5, 1 and 1.5 mg/kg genistein was 18.4% ± 1.6%, 17.9% ± 0.7%, 11.9% ± 0.9%, 5.9% ± 0.7 and 4.2% ± 0.6%, respectively by immunohistochemical staining (Table 3) .

Expression of Bax proteins

Positive staining was located in the cytoplasm. PR for bax protein for 0.2 mL normal saline solution, 1.5 mg/kg DMSO, and 0.5, 1 and 1.5 mg/kg genistein was 11.2% ± 0.8%, 11.9% ± 0.5%, 20.9% ± 1.7%, 24.9% ± 0.8% and 29.6% ± 1.7%, respectively (Table 3).

RT-PCR

The density of Bcl-2 mRNA for 0.2 mL normal saline solution, 1.5 mg/kg DMSO, and 0.5, 1 and 1.5 mg/kg genistein decreased progressively, and the density of bax mRNA for 0.2 mL normal saline solution, 1.5 mg/kg DMSO, and 0.5, 1 and 1.5 mg/kg genistein increased progressively, with elongation of time by RT-PCR.

Table 2 Apoptotic index (AI) of implanted tumors in nude mice (%)

	AI
Control group	
0.2 mL saline	12.6 ± 0.6
DMSO	
1.5 mg/kg	13.4 ± 0.7
Genistein	
0.5 mg/kg	28.9 ± 1.2
1.0 mg/kg	33.8 ± 1.6
1.5 mg/kg	37.7 ± 1.2

Table 3 Positive rate of bcl-2 proteins and positive rate of bax proteins of implanted tumors in nude mice (%)

	Positive rate of bcl-2 proteins	Positive rate of bax proteins
Control group		
0.2 mL saline	18.4 ± 1.6	11.2 ± 0.8
DMSO		
1.5 mg/kg	17.9 ± 0.7	11.9 ± 0.5
Genistein		
0.5 mg/kg	11.9 ± 0.9	20.9 ± 1.7
1.0 mg/kg	5.9 ± 0.7	24.9 ± 0.8
1.5 mg/kg	4.2 ± 0.6	29.6 ± 1.7

DISCUSSION

Currently, only a few chemotherapeutic drugs are effective for the treatment of human primary gastric carcinoma, and there is a clear need to look for new anti-gastric carcinoma drugs. Genistein is a planar molecule with an aromatic A-ring, has a second oxygen atom from that in the A ring, and has a molecular mass similar to those of the steroidal estrogens. It has estrogenic properties in receptor binding assays[15,16], cell culture[17,18], and uterine weight assays[19-21]. Genistein inhibits topoisomerase Ⅱ[33], platelet-activating factor- and epidermal growth factor-induced expression of c-fos[34], diacylglycerol synthesis[35], and tyrosine kinases[36]. It also inhibits microsomal lipid peroxidation[22] and angiogenesis[23]. Genistein exhibits antioxidant properties[24-26] and has been reported to induce differentiation of numerous cell types[27-29]. Moreover, a recent report has shown that genistein is a potent cancer chemopreventive agent[30-32].

The Bcl-2 family plays a crucial role in the control of apoptosis. The family includes a number of proteins that have homologous amino acid sequences, including anti-apoptotic members such as Bcl-2 and Bcl-xL, as well as pro-apoptotic members including Bax and Bad[1-4]. Overexpression of Bax has the effect of promoting cell death[10-14]. Conversely, overexpression of anti-apoptotic proteins such as Bcl-2 represses the function of Bax[5-9]. Thus, the ratio of Bcl-2/Bax appears to be a critical determinant of a cell's threshold for undergoing apoptosis[37].

We found that genistein was able to induce apoptosis in SG7901 cells *in vitro*. This apoptosis might have been mediated by down-regulating the expression of the apoptosis-regulated gene Bcl-2 and up-regulating the

expression of apoptosis-regulated gene Bax. In this study, we evaluated the effectiveness of the gastric carcinoma apoptosis induced by genistein *in vivo*, and investigated the molecular mechanisms involved, and the theoretical and methodological basis for the clinical application of genistein, by using an animal model.

We demonstrated that inhibition was induced in all therapeutic groups. Control cells appeared normal in structure, but some cells in the therapeutic groups showed apoptotic characteristics. The AI of 0.5, 1 and 1.5 mg/kg genistein increased with the dose of genistein. Expression of Bcl-2 in the presence of genistein was decreased, but expression of bax was increased. The density of Bcl-2 mRNA decreased progressively with 0.5, 1 and 1.5 mg/kg genistein, and the density of bax mRNA increased progressively. The ratio of Bcl-2/Bax was decreased and triggered apoptosis of transplanted tumor cells. Our results demonstrated that genistein was able to induce apoptosis of transplanted tumor cells in nude mice. The apoptosis may have been mediated by down-regulating expression of apoptosis-regulated gene Bcl-2 and up-regulating expression of apoptosis-regulated gene Bax. Genistein may be potentially used as a chemotherapeutic drug in the anti-gastric carcinoma chemotherapy.

COMMENTS

Background

A recent study has shown that genistein is a potent cancer chemopreventive agent. The anti-tumor activity of genistein might be related to induction of apoptosis of tumor cells, but the precise mechanism of its anti-tumor activity is not well understood. This study investigated the genistein-induced apoptosis of implanted tumors of SG7901 cells in nude mice, the relationship between this apoptosis and expression of Bcl-2 and bax *in vivo*, and the theoretical and methodological basis for the clinical application of genistein.

Research frontiers

Our results demonstrated that genistein was able to induce apoptosis of transplanted tumor cells in nude mice. The apoptosis may have been mediated by down-regulating expression of apoptosis-regulated gene Bcl-2 and up-regulating expression of apoptosis-regulated gene Bax.

Related publications

For more information about each section, please refer to the studies cited in the reference list.

Innovations and breakthroughs

Genistein was able to induce the apoptosis of transplanted tumor cells in nude mice. The apoptosis may have been mediated by down-regulating expression of apoptosis-regulated gene Bcl-2 and up-regulating expression of apoptosis-regulated gene Bax. To the best of our knowledge, no similar studies are available at present.

Applications

According to the results of this study, genistein may potentially be used as a chemotherapeutic drug for gastric carcinoma.

Terminology

Bcl-2: This is a large family composed of various members that are key regulators of apoptosis. High levels and aberrant patterns of Bcl-2 expression have been reported in a wide variety of human caners. Bax: This is a pro-apoptotic member of the Bcl-2 family, which is thought to induce apoptosis. Loss of Bax in genetically engineered mice results in increased tumor incidence, which suggests that Bax may play a role in suppressing tumor growth *in vivo*.

Peer review

The study demonstrated that genistein was able to induce apoptosis of transplanted tumor cells in nude mice. The apoptosis may have been mediated by down-regulating expression of apoptosis -regulated gene Bcl-2 and up-regulating expression of apoptosis-regulated gene Bax. The authors conclude that genistein may potentially be used as a chemotherapeutic drug for gastric carcinoma.

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