

Anticancer activity of resveratrol on implanted human primary gastric carcinoma cells in nude mice

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Received: 2004-01-10 **Accepted:** 2004-03-12

Abstract

AIM: To investigate the apoptosis of implanted primary gastric cancer cells in nude mice induced by resveratrol and the relation between this apoptosis and expression of *bcl-2* and *bax*.

METHODS: A transplanted tumor model was established by injecting human primary gastric cancer cells into subcutaneous tissue of nude mice. Resveratrol (500 mg/kg, 1 000 mg/kg and 1 500 mg/kg) was directly injected beside tumor body 6 times at an interval of 2 d. Then changes of tumor volume were measured continuously and tumor inhibition rate of each group was calculated. We observed the morphologic alterations by electron microscope, measured the apoptotic rate by TUNEL staining method, detected the expression of apoptosis-regulated genes *bcl-2* and *bax* by immunohistochemical staining and PT-PCR.

RESULTS: Resveratrol could significantly inhibit carcinoma growth when it was injected near the carcinoma. An inhibitory effect was observed in all therapeutic groups and the inhibition rate of resveratrol at the dose of 500 mg/kg, 1 000 mg/kg and 1 500 mg/kg was 10.58%, 29.68% and 39.14%, respectively. Resveratrol induced implanted tumor cells to undergo apoptosis with apoptotic characteristics, including morphological changes of chromatin condensation, chromatin crescent formation, nucleus fragmentation. The inhibition rate of 0.2 mL of normal saline solution, 1 500 mg/kg DMSO, 500 mg/kg resveratrol, 1 000 mg/kg resveratrol, and 1 500 mg/kg resveratrol was 13.68±0.37%, 13.8±0.43%, 48.7±1.07%, 56.44±1.39% and 67±0.96%, respectively. The positive rate of *bcl-2* protein of each group was 29.48±0.51%, 27.56±1.40%, 11.86±0.97%, 5.7±0.84% and 3.92±0.85%, respectively by immunohistochemical staining. The positive rate of *bax* protein of each group was 19.34±0.35%, 20.88±0.91%, 40.02±1.20%, 45.72±0.88% and 52.3±1.54%, respectively by immunohistochemical staining. The density of *bcl-2* mRNA in 0.2 mL normal saline solution, 1 500 mg/kg DMSO, 500 mg/kg resveratrol, 1 000 mg/kg resveratrol, and 1 500 mg/kg resveratrol decreased progressively and the density of *bax* mRNA in 0.2 mL normal saline solution, 1 500 mg/kg DMSO, 500 mg/kg resveratrol, 1 000 mg/kg resveratrol, and 1 500 mg/kg increased progressively with elongation of time by RT-PCR.

CONCLUSION: Resveratrol is able to induce apoptosis of transplanted tumor cells. This apoptosis may be mediated

by down-regulating apoptosis-regulated gene *bcl-2* and up-regulating the expression of apoptosis-regulated gene *bax*.

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Key words: Gastric carcinoma; Resveratrol; Neoplasm Transplantation; Apoptosis

Zhou HB, Chen JJ, Wang WX, Cai JT, Du Q. Anticancer activity of resveratrol on implanted human primary gastric carcinoma cells in nude mice. *World J Gastroenterol* 2005; 11(2): 280-284 <http://www.wjgnet.com/1007-9327/11/280.asp>

INTRODUCTION

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*. In *in vitro* experiments, overexpression of *bcl-2* has been shown to inhibit apoptosis, but overexpression of *bax* has been shown to promote apoptosis.

Resveratrol, a phytoalexin found in grapes, fruits, and root extracts of the weed *Polygonum cuspidatum*, is an important constituent of Chinese folk medicine. Indirect evidence suggests that the presence of resveratrol in white and rose wine may be helpful to reduce risks of coronary heart disease which would be achieved by a moderate wine consumption. This effect has been attributed to the inhibition of platelet aggregation and coagulation, in addition to the anti-oxidant and anti-inflammatory activity of resveratrol. Moreover, a recent report showed that resveratrol was a potent cancer chemopreventive agent in three major stages of carcinogenesis. We found resveratrol was able to induce apoptosis in primary gastric cancer *in vitro*. This apoptosis may be mediated by down-regulating the expression of apoptosis-regulated gene *bcl-2* and up-regulating the expression of apoptosis-regulated gene *bax*.

This study was to investigate the apoptosis of implanted tumor of primary gastric cancer cells in nude mice induced by resveratrol and the relation between this apoptosis and expression of *bcl-2* and *bax* *in vivo* and to provide the theoretical and methodological basis for its clinical application.

MATERIALS AND METHODS

Materials

Resveratrol was obtained from Sigma Chemical Co. Ltd and dissolved in DMSO. *In situ* cell detection kit, anti-*bcl-2* and anti-*bax* monoclonal antibodies were purchased from Beijing Zhongshan Biotechnology Co. Ltd. Balb/C female nude mice (4 wk old, 16-18 g) were obtained from Chinese Academy of Medical Sciences.

Methods

Cell culture Fresh samples from a patient with low-differentiation gastric cancer were obtained at operating. A single-cell suspension of tumor cells with the concentration of 5×10^5 /mL

was prepared for seeding. Primary gastric cancer cells were artificially purified after cultured with pancreatic proteinase.

Tumor implanted into nude mice A transplanted tumor model was established by injecting 1×10^9 /L human primary gastric cancer cells into subcutaneous tissues of nude mice. After 10 d, 25 nude mice were divided into 5 groups at random and 0.2 mL normal saline solution, 1 500 mg/kg DMSO, 500 mg/kg resveratrol, 1 000 mg/kg resveratrol, and 1 500 mg/kg resveratrol were directly injected beside tumor body respectively 6 times at an interval of 2 d. Then changes of tumor volume ($V = (\pi/6) \times abc$) were measured 11 d after injecting drugs and tumor inhibition rate of each group was calculated according to the following formula.

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

Where C is control group, T is treated group, V_1 is the volume before treatment (mm^3), V_0 is the volume after treated (mm^3).

Transmission electron microscopy

Tumor samples were cut into $1 \text{ mm} \times 1 \text{ mm} \times 1 \text{ mm}$ sections and fixed in 4% glutaral and immersed with Epon 821, imbedded for 72 h at 60°C . 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

Tumor samples were cryopreserved in liquid nitrogen and cut into $8\text{-}\mu\text{m}$ thick slices. Slices were fixed in ice-cold 80% ethanol for 24 h, treated with proteinase K and 0.3% H_2O_2 , labeled with fluorescein dUTP in a humid box for 1 h at 37°C . Slices were then combined with POD-horseradish peroxidase, stained with DAB and counterstained with methyl green. Controls received the same management except the labeling of omission of fluorescein dUTP. Cells were visualized with light microscope. The apoptotic index (AI) was calculated as follows: $\text{AI} = (\text{number of apoptotic cells}/\text{total number}) \times 100\%$.

Immunohistochemical staining

Tumor samples were cryopreserved in liquid nitrogen, cut into $8\text{-}\mu\text{m}$ thick slices and fixed by acetone. After washed with 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°C overnight. After washed with PBS, the second antibody, biotinylated antirat IgG, was added and cells were incubated at room temperature for 1 h. After washed with PBS, ABC compound was added and incubated at room temperature for 10 min. DAB was used as the chromagen. After 10 min, the brown color signifying the presence of antigens bound to antibodies was detected by light microscopy. Controls were managed as the experimental group except the incubation of primary antibody. The positive rate (PR) was calculated as follows: $\text{PR} = (\text{number of positive cells}/\text{total number}) \times 100\%$.

RT-PCR

Tumor samples were cryopreserved in liquid nitrogen and total RNA was extracted. Concentration of RNA was determined by the absorption at 260 nm. The primers for bcl-2, bax and β -actin were as follows: β -actin (500 bp) 5' GTGGGGCGCCCCAGGCA CCA 3' (sense); 5' CTCCTTAATGTCACGCACGATTTTC 3' (anti-sense); bcl-2 (716 bp) 5' GGAAATATGGCGCACGCT 3' (sense); 5' TCACTTGTGGCCAGAT 3' (anti-sense); bax (508 bp) 5' CCAGCTCTGAGCAGATCAT 3' (sense), 5' TATCAGCCC ATCTTCTTCC 3' (anti-sense). Polymerase chain reactions were performed in a 50 μL reaction volume. RT-PCR reaction was run in the following conditions: at 94°C for 7 min, 1 circle; at 94°C

for 1 min, at 72°C for 1min, 30 circle; at 72°C for 7 min, 1 circle. Ten μL PCR products was placed onto 15 g/L agarose gel and observed by EB staining using the Gel-Pro analyzer.

Statistical analysis

Data were analyzed by analysis of variance, and $P < 0.05$ was considered statistically significant.

RESULTS

Inhibitory rate of tumorgrowth

An inhibitory effect was observed in all therapeutic groups and the inhibition rate of resveratrol at the dose of 500 mg/kg, 1 000 mg/kg and 1 500 mg/kg was 10.58%, 29.68% and 39.14% respectively ($P < 0.05$ vs the control group, Table 1).

Table 1 Inhibitory effect of resveratrol on implanted tumors in nude mice (mean \pm SD)

| Group | Number of animals | | Volume of tumors (mm^3) | | Inhibition rate % |
|---------------|-------------------|--------|------------------------------------|--------------------------------|-------------------|
| | Beginning | Ending | Beginning | Ending | |
| Control group | | | | | |
| 0.2 mL saline | 5 | 5 | 20.49 \pm 0.99 | 498.73 \pm 10.74 | |
| DMSO | | | | | |
| 1 500 mg/kg | 5 | 5 | 20.07 \pm 1.24 | 506.17 \pm 8.70 | |
| Resveratrol | | | | | |
| 500 mg/kg | 5 | 5 | 20.44 \pm 1.76 | 448.04 \pm 6.32 ^a | 10.58 |
| 1 000 mg/kg | 5 | 5 | 21.27 \pm 1.73 | 357.55 \pm 6.34 ^a | 29.68 |
| 1 500 mg/kg | 5 | 5 | 21.32 \pm 1.72 | 312.39 \pm 9.93 ^a | 39.14 |

^a $P < 0.05$ vs control group.

Morphological changes

The cells in control groups had normal structures, but some cells in therapeutic groups had apoptotic characteristics including chromatin condensation, chromatin crescent, nucleus fragmentation (Figure 1A, B).

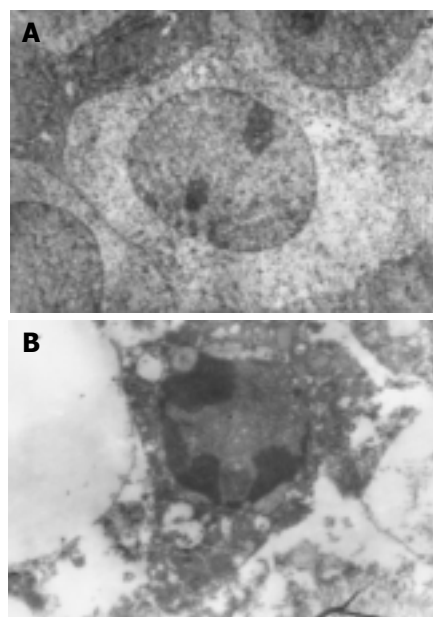


Figure 1 Ultra-microscopic structures of transplanted tumor cells and apoptotic transplanted tumor cells induced by resveratrol. A: Ultra-microscopic structure of transplanted tumor cells (Original magnification: $\times 4\ 800$); B: Ultra-microscopic structure of apoptotic transplanted tumor cells induced by resveratrol (Original magnification: $\times 4\ 800$).

TUNEL assay

Positive staining was located in nuclei (Figure 2). The apoptosis index of 0.2 mL normal saline solution, 1 500 mg/kg DMSO, 500 mg/kg resveratrol, 1 000 mg/kg resveratrol, and 1 500 mg/kg resveratrol was $13.68\pm 0.37\%$, $13.8\pm 0.43\%$, $48.7\pm 1.07\%$, $56.44\pm 1.39\%$ and $67\pm 0.96\%$, respectively ($P<0.001$ vs the control group Table 2).

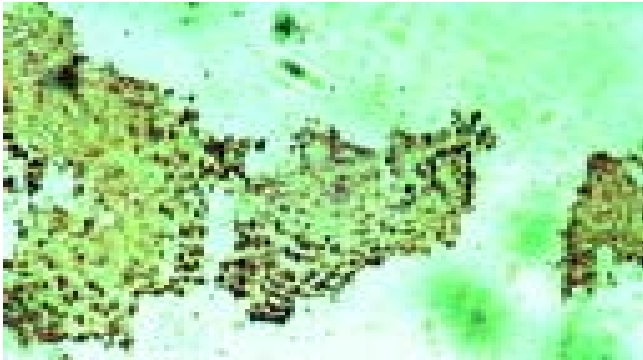


Figure 2 TUNEL assay of apoptotic transplanted tumor cells induced by resveratrol (Original magnification: $\times 200$).

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

| | Control | DMSO | 500 mg/kg | 1 000 mg/kg | 1 500 mg/kg |
|--------|-----------------|-----------------|----------------------|----------------------|----------------------|
| AI (%) | 13.68 ± 0.37 | 13.80 ± 0.43 | 48.70 ± 1.07 | 56.44 ± 1.39 | 67 ± 0.96 |
| F | | 0.13 | 1344.25 ^b | 2651.16 ^b | 7984.02 ^b |
| P | | >0.05 | <0.001 | <0.001 | <0.001 |

^b $P<0.001$ vs control group.

Expression of bcl-2 proteins

Positive staining was located in cytoplasm. The positive rate of bcl-2 protein of 0.2 mL normal saline solution, 1 500 mg/kg DMSO, 500 mg/kg resveratrol, 1 000 mg/kg resveratrol, and 1 500 mg/kg resveratrol was $29.48\pm 0.51\%$, $27.56\pm 1.40\%$, $11.86\pm 0.97\%$, $5.7\pm 0.84\%$ and $3.92\pm 0.85\%$ respectively by immunohistochemical staining ($P<0.001$ vs the control group Table 3).

Table 3 Positive rate of bcl-2 proteins of implanted tumors in nude mice

| | Control | DMSO | 500 mg/kg | 1 000 mg/kg | 1 500 mg/kg |
|--------|-----------------|-----------------|---------------------|----------------------|----------------------|
| PT (%) | 29.48 ± 0.51 | 27.56 ± 1.40 | 11.86 ± 0.93 | 5.70 ± 0.84 | 3.92 ± 0.85 |
| F | | 4.98 | 775.51 ^b | 1879.11 ^b | 1994.65 ^b |
| P | | >0.05 | <0.001 | <0.001 | <0.001 |

^b $P<0.001$ vs control group.

Expression of bax proteins

Positive staining was located in cytoplasm. The positive rate of bax protein of 0.2 mL normal saline solution, 1 500 mg/kg DMSO, 500 mg/kg resveratrol, 1 000 mg/kg resveratrol, and 1 500 mg/kg resveratrol was $19.34\pm 0.35\%$, $20.88\pm 0.91\%$, $40.02\pm 1.20\%$, $45.72\pm 0.88\%$ and $52.3\pm 1.54\%$ respectively ($P<0.001$ vs the control group Table 4).

RT-PCR

The density of bcl-2 mRNA in 0.2 mL normal saline solution,

1 500 mg/kg DMSO, 500 mg/kg resveratrol, 1 000 mg/kg resveratrol, and 1 500 mg/kg resveratrol decreased progressively and the density of bax mRNA in 0.2 mL normal saline solution, 1 500 mg/kg DMSO, 500 mg/kg resveratrol, 1 000 mg/kg resveratrol, and 1 500 mg/kg increased progressively with elongation of time by RT-PCR (Figure 3A, B).

Table 4 Positive rate of bax proteins of implanted tumors in nude mice

| | Control | DMSO | 500 mg/kg | 1 000 mg/kg | 1 500 mg/kg |
|--------|-----------------|-----------------|---------------------|----------------------|----------------------|
| PT (%) | 19.34 ± 0.35 | 20.88 ± 0.91 | 40.02 ± 1.20 | 45.72 ± 0.88 | 52.3 ± 1.54 |
| F | | 7.48 | 821.11 ^b | 2327.70 ^b | 1298.41 ^b |
| P | | >0.05 | <0.001 | <0.001 | <0.001 |

^b $P<0.001$ vs control group.

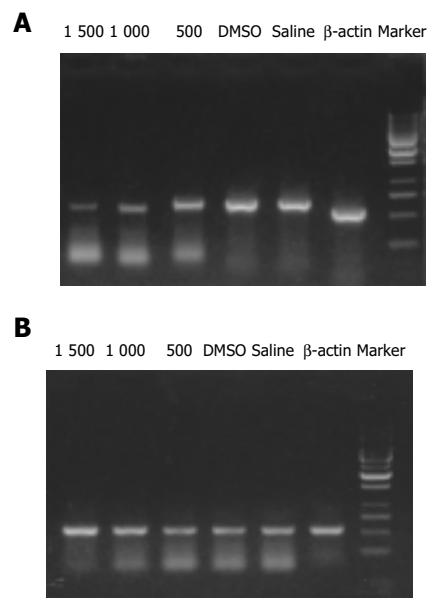


Figure 3 Expression of bcl-2 mRNA and bax mRNA in apoptotic transplanted tumor cells induced by resveratrol. A: Expression of bcl-2 mRNA in apoptotic transplanted tumor cells induced by resveratrol; B: Expression of bax mRNA in apoptotic transplanted tumor cells induced by resveratrol.

DISCUSSION

Currently, only few chemotherapeutic drugs are effective in the treatment of human primary gastric carcinoma and it is necessary to look for new anti-gastric carcinoma drugs. Resveratrol, a polyphenol has been found in various fruits and vegetables and grapes. The root extract from the weed *Polygonum cuspidatum*, an important constituent of Chinese folk medicine, is also an ample source of resveratrol^[1,2]. Several studies in the past several years have shown that resveratrol has cardioprotective and chemopreventive effects^[3-5]. This constituent might account for the reduced risk of coronary heart disease in humans which could be achieved by a moderate wine consumption^[6]. Resveratrol was able to inhibit the growth of a wide variety of tumor cells, including leukemic, prostate, breast and hepatic cells^[7-11]. The anti-tumor activity of resveratrol might be related to the induction of tumor apoptosis of tumor cells^[12-22].

Bcl-2 family plays a crucial role in the control of apoptosis. It has been found that the family includes a number of proteins

which have homologous amino acid sequences, including anti-apoptotic members such as bcl-2 and bcl-x_L, as well as pro-apoptotic members including bax and bad^[23-26]. Overexpression of bax could promote the cell death^[27-31]. Conversely, overexpression of antiapoptotic proteins such as Bcl-2 could repress the function of bax^[32-36]. Thus, the ratio of bcl-2/bax was a critical determinant of a cell's threshold for undergoing apoptosis^[37].

We found that resveratrol was able to induce apoptosis in primary gastric cancer in *in vitro* experiments. This apoptosis might be mediated by down-regulating the expression of apoptosis-regulated gene *bcl-2* and up-regulating the expression of apoptosis-regulated gene *bax*. In this study, we evaluated the effectiveness of apoptosis of gastric carcinoma induced by resveratrol *in vivo*, investigate the molecular mechanisms further and provide the theoretical and methodological basis for the clinical application of resveratrol.

We observed the inhibitory effect of resveratrol in all therapeutic groups. Cells in control groups had normal structures, but some cells in therapeutic groups had apoptotic characteristics. The apoptosis index of resveratrol at the dose of 500, 1 000, and 1 500 mg/kg was increased. Expression of bcl-2 of resveratrol at the dose of 500, 1 000, and 1 500 mg/kg was decreased, but expression of bax was increased. The density of bcl-2 mRNA induced by resveratrol at the dose of 500, 1 000, and 1 500 mg/kg decreased progressively and the density of bax mRNA increased progressively. The ratio of bcl-2/bax was decreased and triggered the apoptosis of transplanted tumor cells.

Our results demonstrated resveratrol was able to induce the apoptosis of transplanted tumor cells in nude mice. The apoptosis may be mediated by down-regulating the expression of apoptosis-regulated gene *bcl-2* and up-regulating the expression of apoptosis-regulated gene *bax*. Resveratrol may be potentially used as a chemotherapeutic drug in anti-gastric carcinoma chemotherapy.

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Edited by Wang XL Proofread by Chen WW