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

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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. We found resveratrol was able to induce apoptosis in primary gastric cancer in nude mice. World J Gastroenterol 2005; 11(2): 280-284

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 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 5×10⁶/mL

by down-regulating apoptosis-regulated gene bcl-2 and up-regulating the expression of apoptosis-regulated gene bax.
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 1×10⁶/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 = (π/6) × abc) were measured 11 d after injecting drugs and tumor inhibition rate of each group was calculated according to the following formula.

\[
\text{Inhibitory rate (IR) of tumor growth} = \frac{C (V₁-V₀) - T (V₁-V₀)}{C (V₁-V₀)}
\]

Where C is control group, T is treated group, V₁ is the volume before treatment (mm³), V₀ is the volume after treated (mm³).

**Transmission electron microscopy**

Tumor samples were cut into 1 mm×1 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-μm thick slices. Slices were fixed in ice-cold 80% ethanol for 24 h, treated with proteinase K and 0.3% H₂O₂, labeled with fluorescein dUTP, stained with DAB and counterstained with methyl green. Controls received the same management except the labeling of omission of fluorescein dUTP. Cells were observed by light microscope. The apoptotic index (AI) was calculated as follows: AI = (number of apoptotic cells/total number) ×100%.

**Immunohistochemical staining**

Tumor samples were cryopreserved in liquid nitrogen, cut into 8-μm thick slices and fixed by acetone. After washed with PBS, slices were incubated in 0.3% H₂O₂ solution at room temperature for 5 min. 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: AI = (number of apoptotic cells/total number) ×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' GTGGGCGGCCCAAGGCA CCA3' (sense); 5' TCTCTTAATGTACACCACTTTC3' (anti-sense); bcl-2 (716 bp) 5' GGAAA TA TGGCGCACGCT 3' (anti-sense); bax (508 bp) 5' CCAGCTCTGAGCAGA TCA 3' (sense); 5' TATCACGCCA ATCTTCTGCT 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 1 min, 30 circle; at 72 °C for 1 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 tumor growth

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±SD)

| Group          | Number of animals | Volume of tumors (mm³) | Inhibition rate |
|---------------|-------------------|------------------------|-----------------|
|               | Beginning | Ending | Beginning | Ending |                   |
| Control group | 5        | 5      | 20.49±0.99 | 498.73±10.74 |                   |
| 0.2 mL saline | 5        | 5      | 20.07±1.24 | 506.17±8.70 |                   |
| DMSO          | 5        | 5      | 21.32±1.72 | 312.39±9.93 |                   |
| 1 500 mg/kg   | 5        | 5      | 23.28±1.72 | 357.55±6.34 | 29.68              |
| Resveratrol   | 5        | 5      | 31.22±1.72 | 312.39±9.93 | 39.14              |
| 500 mg/kg     | 5        | 5      | 24.4±1.76 | 448.04±6.32 | 10.58              |
| 1 000 mg/kg   | 5        | 5      | 21.27±1.73 | 357.55±6.34 | 29.68              |
| 1 500 mg/kg   | 5        | 5      | 21.32±1.72 | 312.39±9.93 | 39.14              |

*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: ×4 800); B: Ultra-microscopic structure of apoptotic transplanted tumor cells induced by resveratrol (Original magnification: ×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±0.37%, 13.8±0.43%, 48.7±1.07%, 56.44±1.39% and 67±0.96%, respectively (P<0.001 vs the control group Table 2).

![Figure 2](image1.png) TUNEL assay of apoptotic transplanted tumor cells induced by resveratrol (Original magnification: ×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      | 13.8±0.43      | 48.7±1.07   | 56.44±1.39  |
| F        | 0.13            | 134.25b        | 2651.16b    | 7984.02b    |
| P        | >0.05           | <0.001         | <0.001      | <0.001      |

bP<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±0.51%, 27.56±1.40%, 11.86±0.93%, 5.70±0.84% and 3.92±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   |
| F        | 4.98       | 775.51b        | 1879.11b    | 1994.65b    |
| P        | >0.05      | <0.001         | <0.001      | <0.001      |

bP<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±0.35%, 20.88±0.91%, 40.02±1.20%, 45.72±0.88% and 52.3±1.54% respectively (P<0.001 vs the control group Table 4).

**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  |
| F        | 7.48       | 821.11b        | 2327.70b    | 1298.41b    |
| P        | >0.05      | <0.001         | <0.001      | <0.001      |

bP<0.001 vs control group.

**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, as well as pro-apoptotic members including bax and bad. Overexpression of bax could promote the cell death. Conversely, overexpression of antiapoptotic proteins such as Bcl-2 could repress the function of bax. Thus, the ratio of bcl-2/bax was a critical determinant of a cell’s threshold for undergoing apoptosis.

We found that resveratrol was able to induce apoptosis in primary gastric cancer 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, 1000, and 1500 mg/kg was increased. Expression of bcl-2 of resveratrol at the dose of 500, 1000, and 1500 mg/kg was decreased, but some cells in therapeutic groups had apoptotic characteristics.

The apoptosis index of resveratrol at the dose of 500, 1000, and 1500 mg/kg was increased. Expression of bcl-2 of resveratrol at the dose of 500, 1000, and 1500 mg/kg was decreased, but some cells in therapeutic groups had apoptotic characteristics.

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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