Acceleration of Apoptosis by Transfection of Bak Gene in Multi-drug Resistant Bladder Cancer Cells

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Abstract  Objective: To study the killing effects of bak gene on multi-drug resistant (MDR) bladder cancer cells and the mechanisms.  Methods: Bak gene was transfected into MDR bladder cancer cells by liposome. The expression of bak and Bcl-2 mRNA was detected by in situ hybridization. The expression of bak and Bcl-2 proteins was detected by SABC immunohistochemistry. The growth rate of human bladder cancer cells was studied by constructing the growth curve, cell apoptosis was measured by flow cytometry, and the morphology of cells was observed by fluorescence stain.  Results: The expression of bak mRNA was positive in EJ/bak cells (P<0.05). Bak protein expression of EJ/bak cells was positive and Bcl-2 protein expression was decreased (P<0.05). The growth of MDR bladder cancer cells was significantly inhibited after bak gene was transfected (P<0.05). Apoptosis cells were increased significantly. The apoptosis rate was 35%. Apoptotic bodies can be found in these cells by fluorescence stain.  Conclusion: Bak gene could inhibit the growth of MDR bladder cancer cells effectively. Inducing cell apoptosis by down-regulating the expression of Bcl-2 gene might be one of its mechanisms.

Key words: bak; bladder neoplasms; multi-drug resistance; apoptosis

Bak is one of Bcl-2 family members, which promotes cell apoptosis and counteracts the protection from apoptosis provided by Bcl-2. Bcl-2 functions as a potent factor resulting in multi-drug resistance (MDR) [1]. The purpose of this study was to observe how gene bak inhibited the growth of human MDR cells and discuss the possible mechanism.

Materials and methods

Cell line

The human MDR cell line of bladder cancer, EJ/MDR, was established in our laboratory. Cell line EJ/MDR was maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum and cultured in an incubator at 37 °C and with 5% CO₂.

Transfection

Cells were transfected by lipofectamine method. Briefly, EJ/MDR cells were seeded in 6-well culture plates at a density of 3×10⁶ cells/well in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum and transfected with 1.5 µg of pcDNA3 by lipofectamine. Six h later, culture medium was replaced by fresh medium and incubated for 24 h. EJ/MDR cells in the course of logarithmic growth phase were divided into two groups: trial group (EJ/MDR cells were transfected with pcDNA3-bak, named EJ/bak) and control group (EJ/MDR cells were transfected only with pcDNA3, named EJ/pcDNA3).

In situ hybridization

The two groups were culture for 48 h after the cells had attached to the slides. Then the slides were fixed with 0.1% formaldehyde for 30 min and washed with 0.01 M PBS. The probe labeled by digoxigenin was added to the slides for hybridization. The digoxigenin-labeled probe was detected using the DIG nucleic acid detection kit. Positive signals were dyed brown.

Immunohistochemical staining

The expression of bak protein was detected by streptavidin-biotin complex (SABC) method. Briefly, adhesive cells were fixed with freshly prepared 4% paraformaldehyde for 5–8 min. Before staining, the slides were treated with 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature and then washed with PBS. The slides were incubated with the primary antibodies, biotinylated rabbit anti-goat IgG and peroxidase-conjugated streptavidin one by one. The slides were then reacted with 0.02% diaminobenzidine tetrahydrochloride containing 0.005% hydrogen peroxide for 4 min, and counterstained with hematoxylin. Positive signals were dyed brown.
Cell counting
The cells were resuspended in serum-free RPMI1640 at a concentration of $1 \times 10^7$/L. Cell suspensions were seeded in 96-well plates (1 mL/well). The cells of 4 wells each 24 h were counted. Cell growth curve was drawn.

Inhibition rate = (1–Number of EJ/bak group/Number of blank control group)×100%

Flow cytometric analysis
The cells were incubated with PBS containing 5 µg/mL propidium iodide for 30 min at room temperature in the dark and analyzed on a flow cytometer as soon as possible.

Cell morphologic observation
The cells were cultured on the slides for 48 h. Slides were fixed by 0.1% formaldehyde and washed with PBS for 5 min. PI and RNA kinase were added to the slides in the dark. The cells were observed under fluorescence microscopy.

Statistical analysis
Statistical analysis was performed by using $t$ test.

Results

Expression of gene bak
The positive signals of gene bak expression were dyed brown and mainly located in the cell line of EJ/bak. The highest positive rate, which was observed on the third day, was 64% in EJ/bak cell line. Weak signals could be seen in EJ/pcDNA3 group. The positive rate in EJ/bak group was significantly different from EJ/pcDNA3 group ($P<0.05$).

Expression of bak protein
Bak protein was mainly located in EJ/bak cell cytoplasm. The highest positive rate, which was observed on the third day, was 60%. The positive rate in EJ/bak group was significantly different from EJ/pcDNA3 group ($P<0.05$). It was shown that bak protein was transfected successfully (Fig. 1, 2).

The expression of Bcl-2 was significantly inhibited in EJ/bak group. Bcl-2 protein was mainly located in EJ/pcDNA3 cell cytoplasm. There was significantly difference between two groups ($P<0.05$) (Fig. 5, 6).

Cell counting
The growth of EJ/bak cells was significantly inhibited and significantly different from EJ/pcDNA3 group ($P<0.05$) (Table 1). The highest growth inhibition rate, which was observed on the fourth day, was 32%. Cell growth curve was seen in Fig. 7.

Flow cytometric analysis
Peak sub-G of EJ/bak group was tall and sharp. The apoptosis rate of EJ/bak group was 35% and significantly different from EJ/pcDNA3 group (Fig. 8, 9).

Cell morphology
The cells of EJ/bak group showed jacinth and pyknosis. We could found apoptotic body in the cells. Only few apoptotic cells could be seen in EJ/pcDNA3 group (Fig. 10, 11).