THE APOPTOSIS OF EXPERIMENTAL COLORECTAL CARCINOMA CELLS INDUCED BY PEPTIDOGLYCAN OF BIFIDOBACTERIUM AND THE EXPRESSION OF APOPTOTIC REGULATING GENES

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ABSTRACT

Objective: To explore the antitumor mechanisms of whole peptidoglycan of bifidobacterium. Methods: The apoptotic cells and the positive expression of bcl-2 and bax oncoprotein were studied nude mice transplantation tumors of colorectal carcinoma by employing *in situ* end labeling technique and immunohistochemical staining. Results: The apoptotic cell density, the positive rate and the staining intensity of bax oncoprotein of the transplantation tumor of colorectal carcinoma in the whole peptidoglycan injection group were significantly higher when compared with the tumor control group. The positive rate of bcl-2 oncoprotein in the whole peptidoglycan injection group was obviously lower than that in the tumor control group ($P<0.01$). Conclusion: Whole peptidoglycan of Bifidobacterium bifidum could induce cell apoptosis of nude mice transplantation tumors of colorectal carcinoma by down-regulating the expression of the bcl-2 gene and up-regulating the expression of the bax gene.

Key words: Bifidobacterium, Whole peptidoglycan, Apoptosis, Colorectal carcinoma.

Bifidobacteria are predominant normal bacteria in the intestines of the human body. They play an important role on maintenance of the microbial balance in the intestine and the state of health of the body. Whole peptidoglycan (WPG) is a predominant proportion of the cell wall of bifidobacteria. It can inhibit the occurrence and development of many kinds of tumors *in vivo*, such as Meth A fibrosarcoma, mammary carcinoma, liver carcinoma and etc.[1] Our data also suggest that WPG of Bifidobacteria bifidum can markedly inhibit the growth of colorectal carcinoma transplantation tumors in nude mice. At present, the influence of WPG on the apoptosis of tumor cells has not been reported. In order to explore the antitumor mechanisms of WPG, the apoptosis of experimental colorectal carcinoma cells induced by WPG of Bifidobacteria bifidum and expression of bcl-2, bax oncoproteins of the tumors were studied by employing *in situ* end labeling (ISEL) and immunohistochemical staining.

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MATERIALS AND METHODS

Experimental Animals

BALB/c male nude mice were purchased from the experimental animal center of the First Military Medical University, at the age of 6–8 weeks, weighted in the range of 18–22 grams, and were housed in the SPF animal room.

WPG

WPG was extracted from the cell wall of Bifidobacteria bifidum, and evaluated depending on the method described by Sekine.[2] It was donated by Professor HU Hong, who worked at Department of Examination of Chongqing Medical University.

Colorectal Carcinoma Cell Line

The Lovo cell line was a human undifferentiated colorectal carcinoma cell line and was obtained from the Department of Pathology of the First Military Medical University. It was maintained, suspended in RPMI medium 1640 supplemented with 10% fetal calf serum routinely. When Lovo cells grew to form a monolayer, a single cell suspension of these cells was prepared by the trypsin digestion method. The cells were adjusted to a concentration of 1×10^7 ml.

Building of the Animal Model of Colorectal

Carcinoma Transplantation Tumors of Nude Mice and Antitumor Experiment of WPG

The animals were divided into two groups. (1) Tumor control group: 20 nude mice were inoculated with 2×10^6 (0.2 ml) Lovo cells in the left flank on day 1 subcutaneously. From day 2 after tumor inoculation, 0.2 ml isotonic phosphate buffered saline (PBS) was injected into each of these animals intraperitoneally 5 times continuously, every other day; (2) WPG injection group: the number, site and schedule of the inoculated Lovo cells was similar to tumor control group on day 1. From day 2 after tumor cell inoculation, 0.25 mg WPG equivalent to 1×10^9 Bifidobacteria for a single injection dose was injected into 20 nude mice intraperitoneally 5 times continuously, every other day. All animals were killed on day 21 after the tumor inoculations. The fresh tumor tissues extracted from the nude mice were fixed in 10% neutral formalin, and embedded in paraffin. Then paraffin sections were prepared, and dyed with haematin and eosin. At last histologically, the tumor tissues were demonstrated to be undifferentiated colorectal carcinoma.

ISEL Staining

The protocol described by TAN Xiao-hua was as follows:[3] (1) The tissue sections were deparaffinized, followed dipping in a series of different concentrations of ethanol, and hydrated; (2) The tissue sections were immersed in 2×SSC solution, incubated for 20 minutes at 80°C, rinsed with double distilled water; (3) The tissue sections were digested by 0.5% gastric protease (pH 2.0) for 10 min at 37°C, rinsed with double distilled water; (4) The sections were covered with Buffer A (50 mM/L Tris-HCl, 5 mM/L$MgCl_2$, 10 mM/L β-mercaptoethanol, 0.005% BSA, pH 7.5), incubated then for 5 min at room temperature; (5) Buffer A was discarded. 50 μl labeling fluid containing 0.005 mM/L dNTP (dATP, dCTP, dGTP), 0.005 mM/L Biotin-16-dUTP and 25 U/ml Klenow large segments were added to cover the sections; (6) The sections were rinsed with PBS for 3 min 2 times; (7) Endogenous peroxidase was inactivated by covering the sections with 1% H_2O_2-methanol solution for 15 min at room temperature; (8) The sections were rinsed with PBS for 3 min 2 times; (9) Horseradi shperoxidase labeled avidin (HRP-Avidin) was added to cover the sections and they were incubated for 30 min at room temperature; (10) The sections were rinsed with PBS for 3 min 2 times; (11) The sections were stained with DAB-H_2O_2 washed with water, stained with haematin repeatedly, dehydrated, cleared and mounted routinely. The staining procedures of the negative control sections were similar except that the labeling fluid, which didn't contain Klenow large segment was not added to cover the sections in the fifth step. The above main reagents, dNTP and biotin-16-dUTP were purchased from Boringer Manher Corporation. Klenow large segment and HRP-Avidin were produced by American Promega and ZYMED corporations respectively.

Immunohistochemical Staining

Immunohistochemical SP method was employed. Monoclonal mouse anti-human antibody bcl-2, monoclonal mouse anti-human antibody bax, biotinated horse anti-mouse antibody IgG and SP reagent box used were produced by Santa Cruz corporation. The protocol was made according to immunohistochemical SP method routinely. The staining procedures of negative control sections were to utilize PBS instead of the first antibody.

The Parameters and Statistic Analysis

In view of positive tissue sections of ISEL, bcl-2 and bax oncoproteins, the number of positive cells in the net squares under 400 fold magnification using vision class checking micronet was taken into account. The positive cells in 10 net squares were counted for each case. The average value was regarded as the positive cell density. Student's test was used to evaluate the results statistically. The expression intensity of bcl-2 and bax oncoproteins was divided into four grades depending on their staining intensity: (-) was known as negative. (+) was known as positive expression weakly. (++) was known as positive expression intermediately. (+++) was known as positive expression intensively. The difference of oncoprotein staining intensities between the control group and the WPG injection group was analyzed by means of rank sum test.

RESULTS

The Results of ISEL Staining of Colorectal Carcinoma Transplantation Tumors

The nuclei of ISEL stained positive cells were yellow. The staining background was clear. The ISEL positive cells of colorectal carcinoma transplantation tumors presented themselves in a scattered distribution in the tumor control group, which were rarely seen. The ISEL positive cells of the transplantation tumors were patchy or diffusely distributed in the WPG injection group. The number of ISEL positive cells of the tumors in the WPG injection group was significantly more when compared with the tumor control group ($P<0.01$). It is shown in Table 1 and Figure 1.

The Expression of bcl-2 and bax Oncoproteins of Colorectal Carcinoma Transplantation Tumors

The positive expression product of bcl-2 and bax oncoproteins was located in the cytoplasm of the neoplastic cells. A diffuse distribution of positive cells throughout the tumors was observed. The positive

expression rate of bcl-2 gene of the transplantation tumors in WPG injection group was 65%. It generally has a positive weakly expression; The positive expression rate of the transplantation tumors in the tumor control group was 90%. It mainly presented a positive expression intermediately and intensively. The positive cell density of bcl-2 oncoprotein in the tumor control group was markedly higher than that in the WPG injection group ($P<0.01$). The expresion of the bax gene was contrary to that of the bcl-2 gene. The positive cell density, expression rate and intensing of the bax oncoprotein were significantly lower in the tumor control group when compared with the WPG injection group. This is shown in Table 2 and Figure 2, 3.

Table 1. The number of apoptotic cells and bcl-2, bax positive cells of colorectal carcinoma transplantation tumors in nude mice($\bar{x}\pm s$)

Groups	n	ISEL positive cells	bcl-2 positive cells	bax positive cells
Tumor control group	20	20.62±8.80	162.34±20.31**	32.34±7.80
WPG injection group	20	248.30±15.36*	91.64±11.50	250.70±32.50***

Compared with tumor control group, *$P<0.01$, ***$P<0.01$; Compared with WPG injection group, **$P<0.01$

Table 2. The expression density and rate of bcl-2 and bax oncoprotein of colorectal carcinoma transplanationt tumors of nude mice

Groups	n	bcl-2 oncoprotein expression density				Percentage	bax oncoprotein expression density				Percentage
		−	+	++	+++		−	+	++	+++	
Tumor control group	20	2	2	6	10*	90%**	12	7	1	0	45%
WPG injection group	20	7	9	4	0	65%	1	1	9	9$^{\Delta}$	95%$^{\Delta\Delta}$

Compared with WPG injection group, *$P<0.01$, **$P<0.01$; Compared with tumor control group, $^{\Delta}P<0.01$, $^{\Delta\Delta}P<0.01$

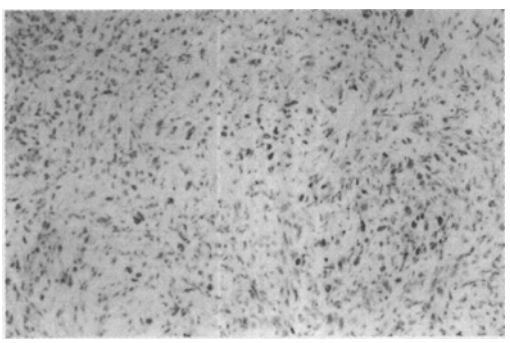

Fig. 1. The number of ISEL positive cells was more; their nuclei were yellow in WPG injection group, × 100.

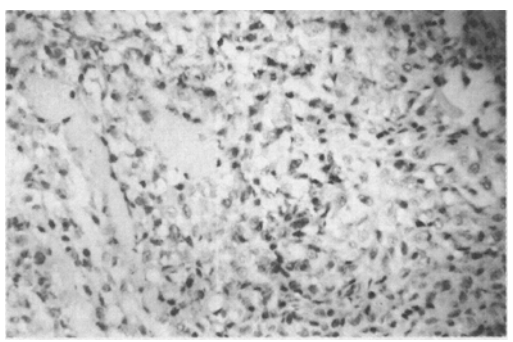

Fig. 2. bcl-2 oncoprotein shows a positive intensively expression in the tumor control group. SP immunohistochemical staining, × 200.

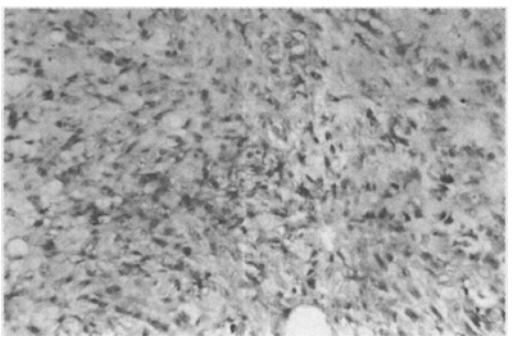

Fig. 3. bax oncoprotein shows a positive intensively expression. Their number of possitive cells was more. SP immunohistochemical staining, × 200.

DISCUSSION

Apoptosis is a specific mode of cell death triggered by intrinsic adjusting mechanisms under some conditions. It is well known that many antitumor factors are such as cytokines, chemotherapy drugs, irradiating ray, high heat as well as BCG able to induce apoptosis of neoplastic cells. Among the methods detecting apoptosis, the ISEL method had the advantages of intensive magnitude and sensitivity. Small DNA broken strands will give positive reactions with the ISEL method. The method could not only detect the existence of apoptotic cells *in situ*

specifically, but also arrest the early stage of apoptotic cells.[4] WPG of Bifidobacteria comprises polysaccharides and peptidoglycans. It contained some important biological functions of the whole bacteria. It has some important physiological functions of immunopotentiation, postponing senescence and antitumor. We established an animal model of colorectal carcinoma transplantation tumors of nude mice. The influence of WPG of Bifidobacteria bifidum on apoptosis of experimental colorectal carcinoma was studied. The results showed that ISEL positive cell density of colorectal carcinoma transplantation tumors in the WPG injection group was obviously higher than that in tumor control group. It was suggested that WPG of Bifidobacteria bifidum could induce apoptosis of colorectal carcinoma transplantation tumor cells *in vivo*.

Apoptosis occurres under the control of gene regulation. It is well known that the bcl-2 gene family are important apoptotic regulating genes. A bcl-2 gene could block apoptosis following a variety of stimuli. It extends cell survival rather than increasing proliferation.[5] The bax gene is a new member of bcl-2 gene family. It has 40% extensive amino acid homology with bcl-2, but its physiological functions are contrary. At present, the study has demonstrated that the expression degree of bcl-2 and bax genes and the ratio of bcl-2 to bax determine survival or death of cells following an apoptotic stimuli. When bax gene expresses predominantly, it could homodimerize to form a lot of bax-bax dimers, and heterodimerize with bcl-2 to form bax-bcl-2 dimers. At last, apoptosis triggered by bax gene occures. On the other hand, when bcl-2 gene expresses itself predominantly, cells survive continuously.[6] In our results, colorectal carcinoma transplantation tumors expressed bcl-2 gene mainly in the tumor control group, but colorectal carcinoma transplantation tumors expressed bax genes predominantly after tumor-bearing nude mice were treated with WPG of Bifidobacteria bifidum. By analysing apoptotic cell density, we found that ISEL positive cell density was proportional to the expression degree of bax gene, and contrary to the expression degree of bcl-2 gene. It was suggested that WPG of Bifidobacteria bifidum could augment the expression of bax gene of colorectal carcinoma transplantation tumors, and down-regulate the expression of bcl-2 gene.

It is widely acknowledged that WPG has the antitumor functions *in vivo*. Sekine found that WPG of Bifidobacteria infantis could significantly inhibit the growth of subcutaneously transplanted Meth A fibrosarcoma of mice. We also demonstrated that WPG of Bifidobacteria bifidum was able to counter the development of colorectal carcinoma transplantation tumors of nude mice. Many investigators reported that the antitumor mechanisms of WPG were the activativation of macrophages, natural killer cells and B lymphocytes of immune system to secrete large amount of IL-1, IL-6, TNF-α, IFN-γ and many kinds of factors which had antitumor activity.[7,8] According to the experimental results, we thought that another antitumor pathway of WPG was to induce apoptosis of tumors through regulating the expression of bcl-2 and bax genes.

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