BRM-SJS INDUCES PROGRAMMED CELL DEATH IN BcaP-37 HUMAN BREAST CANCER CELLS

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ABSTRACT

Objective: To investigate the antitumor activity and the mechanism of BRM-SJS on breast cancer cells. Methods: Flow cytometry, DNA agarose gel electrophoresis and other techniques were used to study the *in vitro* and *in vivo* inhibitory effect on BcaP-37 cells by BRM-SJS. Results: BRM-SJS showed an inhibitory rate of 33.8% on in vivo transplanted tumor (P<0.05, compared with control). The flow cytometry analysis of BRM-SJS treated BcaP-37 (2.5 ìmol/L, 5 ìmol/L, 10 ìmol/L for 48 h and 72 h) revealed typical sub-G_1 peak. The specific DNA Ladders were exhibited with BRM-SJS BcaP-37 cells treated. Conclusion: BRM-SJS has marked antitumor activity on BcaP-37 and its inhibitory effects on tumor were realized by both induction of apoptosis and necrosis of the tumor cells.

Key words: BRM-SJS; Apoptosis; Breast cancer

Today breast cancer displays a significant health problem in the world because the breast cancer morbidity is gradually increasing[1-7]. BRM-SJS has been shown to be capable of inhibiting the growth of several kinds of animal tumor cells in vitro and in vivo[8], the mechanism of which remains unknown. Cell death may occur by either of two mechanisms: apoptosis or necrosis. Necrosis, the first type of cell death is recognized by people, apoptosis, on the other hand, is a death process involving a series of well-organized events which require active cell participation and is primarily caused by physiological or chemical stimuli. It is well known that the anti-cancer effect of many chemotherapeutic agents is achieved via the induction of apoptosis in tumor cells[9-12].

Our previous study demonstrated that BRM-SJS could induce apoptosis in human breast cancer (MCF-7) and Souzhou human glioma (SHG-44) cell lines. In this experiment, we tried to explore the influence of BRM-SJS on cell cycle of tumor cells.

The present study was under taken by using flow cytometry, DNA agarose gel electrophoresis and mice tumor transplant techniques in vivo. We demonstrate that the growth inhibition in BcaP-37 human breast cancer cell strain is mainly accomplished via the induction of apoptosis and partially due to the occurrence of necrosis in these cells.

MATERIALS AND METHODS

Drugs and Tumor Strain

BRM-SJS and human breast carcinoma cell strain were provided by Beijing Clinical Tumor Gene Research Center, RPMI1640 medium was purchased from Sigma Co.

Cell Culture and Subculture

The monolayer BcaP-37 cells were cultured at 37°C in a humidified atmosphere containing 50 ml/L CO_2 in RPMI1640 supplemented with 100 ml/L heat inactivated fetal bovine serum, 100 U/ml of penicillin plus 100 ìg/ml streptomycin, 20 ml/L $NaHCO_3$, and 20 ml/L glutamine.

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In Vivo Inhibition Experiment of Tumor

BcaP-37 cells in mid-log phase were collected, washed with PBS and RPMI1640, and centrifuged at 1000 r/min for 10 min. The supernatant was discarded. After repeated washing, cells were suspended in sterile PBS, at a final concentration of 2.5×10^7 cells/ml. 18 inbred Balb/c female nude mice (nu/nu, BW 17-18 g) were divided into three groups. Each mouse received 0.2 ml (contain approximate 5×10^6 cells) of tumor cells suspension subcutaneously on right lateral of front leg armpit. One day later, BRM-SJS were administrated at concentrations of 800 mg/kg or 300 mg/kg, respectively, once daily for 14 days. After another 14 days the mice were killed and their subcutaneous tumor were collected to quantitate the inhibitory rate and fixed in 100 ml/L formalin. Paraffin sections were made and stained with HE. The morphology of cells were examined under a light microscope.

Flow Cytometric Analysis of Cell Cycle Status and Apoptosis[13-15]

The flow cytometric evaluation of the cell cycle status and apoptosis was performed. BcaP-37 cells (1×10^6) were incubated with BRM-SJS at concentrations of 2.5 μmol/L, 5 μmol/L and 10 μmol/L) for 24 h, 48 h, and 72 h respectively. Then the cells were harvested and washed twice with PBS and centrifuged at 1000 r/min for 5 min. The pelleted cells were stored in 700 ml/L cold ethanol at 4°C for 24 h, and centrifuged at 1000 rpm for 5 min, and the supernatant was removed completely. The pellets were resuspended in 0.5 ml PBS, then 50 μg Rnase was added and incubated at 37°C for 30 min, 50 μg propidium iodide (PI) was added and kept at the dark for 30 min, and 1 ml PBS was added, and filtered through 30 μmol/L mesh. The fluorescence of 10^4 cells was analyzed for each sample. Cells with DNA content below G_1 phase were regarded as apoptotic cells. The percentages of cells in the apoptotic sub- G_1 and G_2 and S phases were analyzed by flow cytometry.

DNA Fragmentation Assay in Agarose Gel[16, 17]

The cultured BcaP-37 cells (1×10^7) were treated with BRM-SJS at different concentrations (2.5 μmol/L, 5 μmol/L, 10 μmol/L) for 24 h, 48 h, or 72 h respectively. Then the cells were harvested, washed with PBS and centrifuged at 2500 r/min for 5 min. The sediments were added with 200 μl of lysis buffer (20 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 7.7, 10 ml/L NP-40) for 30 s, and recentrifuged. The supernatant was harvested and 10 ml/L SDS and Rnase (terminal concentration 50 μg/L) were added and incubated at 56°C for 2 h, and then treated with proteinase K (terminal concentration 25 μg/L) at 37°C for 2.5 h. 0.5 fold volume of 7 mol/L CH_3COONH_4 and 2.5 fold volume of ethonal were added and mixed, and the cells were stored at -20°C overnight. Next day, the DNA was extracted and electrophoresis was performed on 8 g/L agarose gel. The results were visualized by violet light.

RESULTS

Antitumor Activity Assay

The effect of BRM-SJS on transplanted BcaP-37 tumor cells in female nude mice was determined by weighing the tumor. The inhibitory rate in high dose group (800 mg/kg, PO, 14 d) was 33.8% (compared with control $P<0.05$), and in Low dose group was 20.9% (Table 1).

The pathological examination of High dose group showed cell nuclei condensation, heavy staining, and partly formed crescentic nucleuses, which demonstrated typical morphological changes of apoptosis, while the negative group displayed degeneration and necrosis (Figure 1, 2).

Cell Cycle Distribution Assay

When BcaP-37 tumor cells were exposed at different concentrations of BRM-SJS (2.5 μmol/L, 5 μmol/L, 10 μmol/L) for 24 h, 48 h or 72 h the apoptosis rates were correlated with exposure time and concentration of BRM-SJS. At the concentration of BRM-SJS of 10 μmol/L for 72 h, the apoptosis rate reached 61.4% (Table 2, Figure 3, Figure 4).

Table 1. Effect of BRM-SJS on transplanted BcaP-37 tumor in nude female mice

Groups	Dose (mg/kg)	Number of mice (n)	Mode of administration	Tumor weight ($\bar{x}\pm s$, g)	Inhibition of rate (%)	*P* value
Control	Distilled water	6	PO×14	1.48 ± 0.67		
BRM-SJS	800	6	PO×14	0.98 ± 0.37	33.8	<0.05*
BRM-SJS	600	6	PO×14	1.17 ± 0.57	20.9	>0.05*

*Compared with control

Table 2. Analysis of cell-cycle of BcaP-37 cells treated with BRM-SJS

Groups	Time (h)	Concentration of BRM-SJS (ì m)	Cell percentage (%)			
			G_1	G_2	S	Apoptosis
Blank Control			73.5	7.7	19.0	0
BRM-SJS	24	2.5	80.8	7.0	12.2	0
BRM-SJS	24	5.0	68.8	8.7	22.7	0
BRM-SJS	24	10.0	63.2	7.7	29.1	14.0
BRM-SJS	48	2.5	78.2	10.9	20.9	10.6
BRM-SJS	48	5.0	68.7	4.7	6.6	23.8
BRM-SJS	48	10.0	64.6	0.9	34.5	50.2
BRM-SJS	72	2.5	68.5	8.5	22.9	9.1
BRM-SJS	72	5.0	61.1	6.3	32.5	29.9
BRM-SJS	72	10.0	69.4	12.1	18.5	61.4

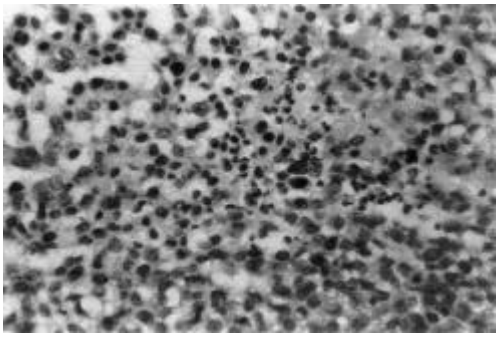

Fig. 1. Morphological changes of apoptosis in BcaP-37 solid tumor paraffin section, treated with BRM-SJS (800 mg/kg, PO×14). The apoptotic cell nuclei condensation and crescentic nucleuses could be seen. ×200

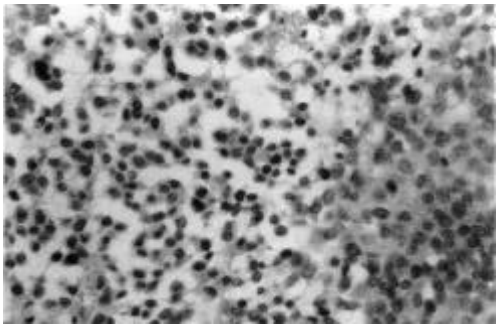

Fig. 2. The BcaP-37 tumor paraffin section of negative control: degeneration and necrosis. ×200

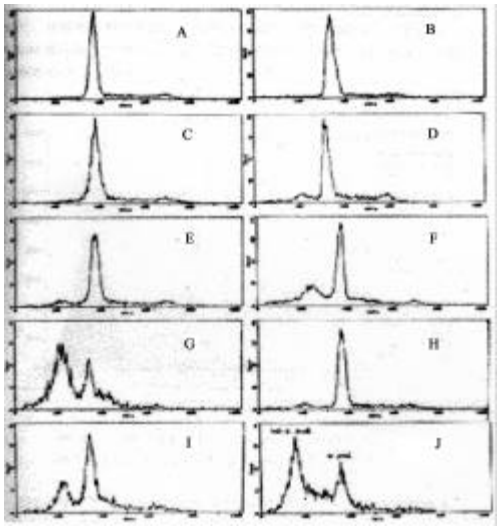

Fig. 3. Apoptotic cell cycle analysis induced in BcaP-37 cells by BRM-SJS (2.5-10 μmol/L) for 24 h to 48 h, respectively.

A: Blank control
B: BRM-SJS 2.5 μmol/L for 24 h
C: BRM-SJS 5 μmol/L for 24 h
D: BRM-SJS 10 μmol/L for 24 h
E: BRM-SJS 2.5 μmol/L for 48 h
F: BRM-SJS 5 μmol/L for 48 h
G: BRM-SJS 10 μmol/L for 48 h
H: BRM-SJS 2.5 μmol/L for 72 h
I: BRM-SJS 5 μmol/L for 72 h
J: BRM-SJS 10 μmol/L for 72 h

The DNA Ladder Examination

After treatment with different concentrations of BRM-SJS (2.5 ì mol/L, 5 ì mol/L, 10 ì mol/L) for 24 h to 72 h, all the DNA extracted from BcaP-37 cells presented characteristic apoptosis Ladders (Figure 5).

DISCUSSION

In this study, we investigated the *in vivo* inhibitory effect of BRM-SJS on BcaP-37 using transplanted tumor model of nude mice, and performed cell cycle analysis

and DNA Ladder assay on BcaP-37 cells using flow cytometry and DNA agarose gel electrophoresis. We found that in the *in vivo* experiment, the inhibitory rate of tumor weight was dose-dependent. Pathological microscopic examination revealed apoptosis in the paraffin sections of tumor cells. These results suggested that BRM-SJS may induce programmed cell death in BcaP-37.

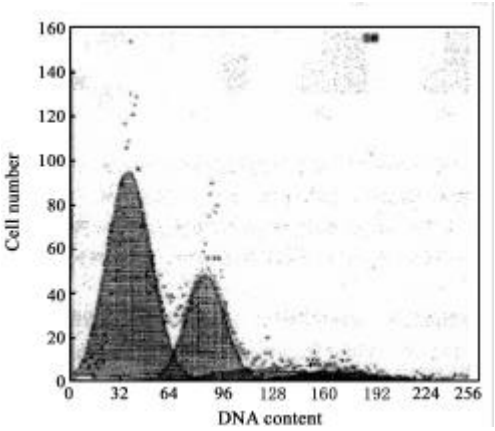

Fig. 4. The flow cytometric analysis of the BcaP-37 cells treated with BRM-SJS at 10 μmol/L for 72 h. The treated groups showed obvious apoptotic Sub-G1 peak, apoptosis rate reached 61.4%..

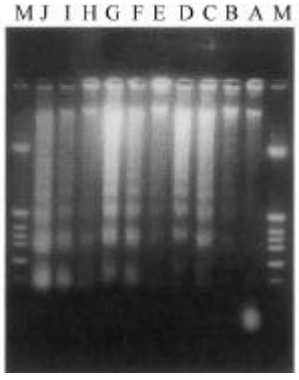

Fig. 5. Apoptosis DNA ladder induced in BcaP-37 cells by BRM-SJS (2.5-10 μmol/L for 24 h to 72 h).

A: Blank control
B: BRM-SJS 2.5 μmol/L for 24 h
C: BRM-SJS 5 μmol/L for 24 h
D: BRM-SJS 10 μmol/L for 24 h
E: BRM-SJS 2.5 μmol/L for 48 h
F: BRM-SJS 5 μmol/L for 48 h
G: BRM-SJS 10 μmol/L for 48 h
H: BRM-SJS 2.5 μmol/L for 72 h
I: BRM-SJS 5 μmol/L for 72 h
J: BRM-SJS 10 μmol/L for 72 h

Meanwhile cell cycle analysis and DNA electrophoresis of BRM-SJS treated BcaP-37 cells showed typical subG_1 peak and apoptosis Ladder in a time-dependent and dose- dependent manner. In addition BRM-SJS could also promote necrosis of BcaP-37 cells.

Previous studies exhibited that BRM-SJS had no toxicity in acute toxicity experiment and maximum tolerance dose assay in mice. Some studies also showed that BRM-SJS was effective in treating patients suffering from cancer and improving their quality of life. The underlying mechanism of the antitumor activity of BRM-SJS is largely unknown and needs further investigation.

REFERENCES

[1] Kothari AS, Beechey-Newman N, D' Arrigo C, et al. Breast carcinoma in women age 25 years or less [J]. Cancer 2002; 94:606.

[2] Bernstein JL, Lapinski R, Lynch C, et al. Factors influencing mortality among young women with second primary breast carcinoma [J]. Cancer 2002; 95:2051.

[3] Swanson GM, Lin CS. Survival patterns among younger women with breast cancer: the effects of age, race, stage, and treatment [J]. J Natl Cancer Inst Monogr 1994; 16:69.

[4] Nakachi K, Suemasu K, Suga K, et al. Influence of drinking green tea on breast cancer malignancy among Japanese patients [J]. Jpn J Cancer Res 1998; 89:254.

[5] Millikan R, Devoto E, Newman B, et al. Studying environmental influences and breast cancer risk: suggestions for an integrated population-based approach [J]. Breast Cancer Res Treat 1995; 35:79.

[6] Wolff MS, Collman GW, Barrett JC, et al. Breast cancer and environmental risk factors: epidemiological and experimental findings [J]. Annu Rev Pharmacol Toxicol 1996; 36:573.

[7] Hansen J. Breast cancer risk among relatively young women employed in solvent-using industries [J]. Am J Ind Med 1999; 36:43.

[8] Liu ZY, Wang ZG, Zhang W, et al. Antitumor effect of BRM [J]. Chin J Cancer Res 2002; 14 :195.

[9] Provinciali M, Stefano G, Ciavattini A. Baseline apoptosis of tumor cells as a response predictor to chemotherapy [J]. J Natl Cancer Inst 1997; 89:889.

[10] Kim R, Tanabe K, Uchida Y, et al. Current status of the molecular mechanisms of anticancer drug-induced apoptosis. The contribution of molecular-level analysis to cancer chemotherapy [J]. Cancer Chemother Pharmacol 2002; 5:343.

[11] Liang Y, Yan C, Schor NF. Apoptosis in the absence of caspase 3 [J]. Oncogene 2001; 20:6570.

[12] Toyoshima H, Hunter T. p27, a novel inhibitor of G_1 cyclin-cdk protein kinase activity, is related to p21 [J].

Cell 1994; 78:67.

[13] Rosfjord EC, Dickson RB. Growth factors, apoptosis, and survival of mammary epithelial cells [J]. J Mammary Gland Biol Neoplasia 1999; 4:229.

[14] Shao ZM, Nguyen M, Alpaugh ML. The human myoepithelial cell exerts antiproliferative effects on breast carcinoma cells characterized by p21-WAF1/CIP1 induction, G2/M arrest, and apoptosis [J]. Exp Cell Res 1998; 241:394.

[15] Yang C, Chang J, Gorospe M. Protein tyrosine phosphatase regulation of endothelial cell apoptosis and differentiation [J]. Cell Growth Differ 1996; 7:161.

[16] Wei YQ, Zhao X, Kariya Y, et al. Induction of apoptosis by quercetin: Involvement of heat shock protein [J]. Cancer Res 1994; 54:4952.

[17] Herrmann M, Lorenz HM, Voll R, et al. A rapid and simple method for the isolation of apoptotic DNA fragments [J]. Nucleic Acids Res 1994; 22:5506.