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Apoptosis-inducing effect of Jinke on Molt-4 cells and its mechanism*

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Abstracts ***Objective:*** To investigate the apoptosis-inducing effect of Jinke on Molt-4 cells and its possible mechanism. ***Methods:*** The Molt-4 cells were treated with different concentrations of Jinke and then cultured for necessary time. The Annexin-V / PI method was used to detect the apoptosis rate. The cell cycle was analyzed by DNA content with flow cytometry. Double parameters analysis of cyclins / DNA was performed to detect the expression of cyclin E. API method was used to confirm the cell cycle-specific apoptosis. The expressions of Bcl-2 and Bax were detected by western blot. ***Results:*** 24 h after the treatment of 0.5, 1.0, 1.5, 2.0 and 3.0 mg/mL Jinke, the apoptosis rate of Molt-4 cells was evaluated in a concentration-dependent manner, from 5.2% of the control group to 41.0% of the 3.0 mg/mL Jinke group. When the Molt-4 cells were cultured with 1.5 mg/mL Jinke, the apoptosis rate was evaluated in a time-dependent manner. DNA content analysis showed that G0/G1 phase of Molt-4 cells increased in a time-dependent manner. The expression of cyclin E increased gradually. API assay showed the apoptosis cells were almost in G0/G1 phase. Western blot showed the Bcl-2 was down-regulated and the Bax was up-regulated. ***Conclusion:*** Jinke could induce G1 phase-specific apoptosis in Molt-4 cells in time- and concentration-dependent manners involving G1 phase arrest. The mechanism of apoptosis inducing effect may be related to the up-regulation of Bax and the down-regulation of Bcl-2.

Key words Jinke; Molt-4 cells; apoptosis; cell cycle; flow cytometry; Bcl-2 family

Malignant tumor is a great threat to human health. Many ways have been used to treat tumor nowadays, surgical resection is one of the effective treatment measures, but the long-term effect should be improved. The main problems of drug treatment are that it often have serious adverse reactions, and drug resistance often happened easily. So, it is very urgent to develop new anticancer drugs. Basic research showed [1–3] that Jinke could induce apoptosis in human hepatocellular carcinoma cells, lung cancer cells, rectal adenocarcinoma cell line and so on.

Clinical application showed [4–6] that Jinke has unique effects on hepatoma, lung cancer, esophageal carcinoma and other neoplasms diseases and it also could enhance the effects of chemotherapy drugs and reduce drug resistance. Though it have been made some progression in basic research and clinical application, the mechanism of its anticancer effects was hardly reported. Our present research aimed at identifying the apoptosis-inducing effects on tumor cells and its mechanism with the Molt-4 cells as a model.

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Materials and methods

Materials

Propidium iodide, RNaseA, Bovine serum albumin (BSA), Triton X-100, FITC-conjugated goat anti-mouse, -rabbit IgG and formaldehyde were purchased from Sigma Chemica1 Co. (St Louis, USA). Annexin-V-FITC was provided by Kaiji Biological Development Co. Ltd. (China). Marker (SM0671) was purchased from Fermentas Company (USA). Rabbit-anti-human Bcl-2 and Bax antibody were provided by Cell Signaling Company (USA). Mouse anti-human cyclin E antibody was purchased from BD Pharmingen Company (USA). Jinke (PS-T) was provided by Gaitian Li Pharmaceutical Co. Ltd. (Jiangsu, China). Jinke was dissolved in RPMI-1640 to maintain the concentration was 0.1 mg/mL. The FACSort flow cytometry was provided by Bection Dickinson Company (USA).

Cell culture and treatment

Human acute lymphocyte leukemia (Molt-4) cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 1% (w/v) glutamine, 100 units/L benzylpenicillin and 100 mg/L streptomycin. For Annexin-V, the exponentia1 cells were treated with 0.5, 1.0, 1.5, 2.0, and 3.0 mg/mL Jinke for 24 h in RPMI-1640. Besides, the exponentia1 Molt-4 cells was treated with 1.5 mg/mL Jinke for different time.

Annexin-V / PI assay

The cells were collected and washed with PBS, followed by being resuspended in binding buffer (HEPES-NaOH 10 mmol/L, pH 7.4, NaCl 140 mmol/L, CaCl, 2.5 mmol/L). Cell concentration was adjusted to 1×10^6/mL, Annexin V-FITC 5 μL and PI 5 μL were added in 100 μL cells in dark for 15 min, then measured on FACSort f1ow cytometry.

DNA content and cell cycle analysis

Untreated and treated cells were collected after cultured in the presence or absence of Jinke for the indicated time, rinsed with PBS and suspended in 75% ethano1 at –20 ℃ overnight. Fixed cells were centrifuged at 1200 r/min and washed with PBS twice. For detecting DNA content, cells were contained in the dark with PI 50 mg/L and 0.1% RNAseA in 400 μL PBS at room temperature for 30 min. Stained cells were analyzed on FACSort (Becton Dickinson, USA).

Flow cytometry for cyclin E

Cells were washed with PBS and suspended in 75% ethanol at –20 ℃ over night. Fixed cells were centrifuged at 1200 xg, washed with PBS and treated with 0.25% Triton X-100 in PBS for 5 min. After the addition of PBS and centrifugation, the samples were incubated with mouse mAb to human cyclin E at 4 ℃ overnight. The antibody was diluted in PBS containing 1% BSA and applied at a ratio of 0.25 μg mAb / 5×10^5 cells. After washed with 1% BSA, the cells were incubated in the dark with FITC-conjugated goat anti-mouse IgG for 30 min. After being washed with 1% BSA again, the cells were stained with PI 50 mg/L and 0.1% RNaseA in 400 μL PBS for 30 min. Cyclin detection was performed with a FACSort f1ow cytometry.

API assay for cell cycle-specific apoptosis

The cells were collected and washed with PBS, followed by being resuspended in binding buffer (HEPES-NaOH 10 mmol/L, pH 7.4, NaCl 140 mmol/L, CaC1 2.5 mmol/L). The samples were incubated with 5 μL Annexin V in dark for 15 min, washed with binding buffer and re-suspended in l% formaldehyde in the binding buffer at 4 ℃ for 30 min. After washed with binding buffer again, the cells were stained with 500 μL PI in PIPES containing digitonin for 15 min then measured on FACSort f1ow cytometry.

Western-blot for Bax and Bcl-2

The cells were washed twice with PBS. The washed cells were lysed in 2 × SDS sample buffer and boiled for 5 min, then the lysates were centrifuged at 1800 r/min at 4 ℃ for 15 min. The protein concentration was determined by Bradford's method using bovine serum albumin as a standard. The supernatants were used for a western blot analysis. Equivalent protein extract (50 μL from each sample was subjected to electrophoresis using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were transferred to PVDF membranes at a constant current of 350 mA for 90 min. The membranes were blocked with 5% non-fat milk in TBST (Tris-HCl 10 mmo1/L. pH 8.0, NaCl 150 mmol/L, Tween-20 0.15%) at 25 ℃ for 1 h. They were subsequently incubated at 4 ℃ overnight with the primary monoclonal antibodies anti-Bax, anti-Bcl-2. All primary antibodies (in TBST plus 5% non-fat milk) were used at 1 mg/L. After the membranes were washed three times with TBST, they were incubated at 25 ℃ for 1 h with anti-mouse HRP polymer secondary antibody at a l:2000 dilution in TBST (plus 5% non-fat-milk). After being washed the membrane with TBST, the protein bands on the membranes were detected with the ECL system.

Results

Concentration-dependent apoptosis in Molt-4 cells induced by Jinke

We examined the apoptosis rate in Molt-4 cells treated with different concentrations of Jinke for 24 h. Annexin-V / PI assay showed that Jinke induced apoptosis in Molt-4 cells in a concentration-dependent manner (Fig. 1).

Time-dependent apoptosis in Molt-4 cells induced by Jinke

The apoptosis rate increased in a time-dependent manner when the Molt-4 cells were treated with 1.5 mg/mL Jinke for different times, from 4.8% in 0 h to 26.2% in 36 h (Fig. 2).

DNA content and cell cycle analysis

Fig. 3 illustrated changes in DNA content distribution in Molt-4 cells treated with 1.5 mg/mL Jinke for 12, 24 and 36 h. As the treatment time increased, the percentage of cells in G0/G1 phase increased accordingly, from 51.8% in 0 h to 58.6% in 36 h. And the percentage of cells in G2/M phase decreased gradually, from 7.8% in 0 h to 0.6% in 36 h.

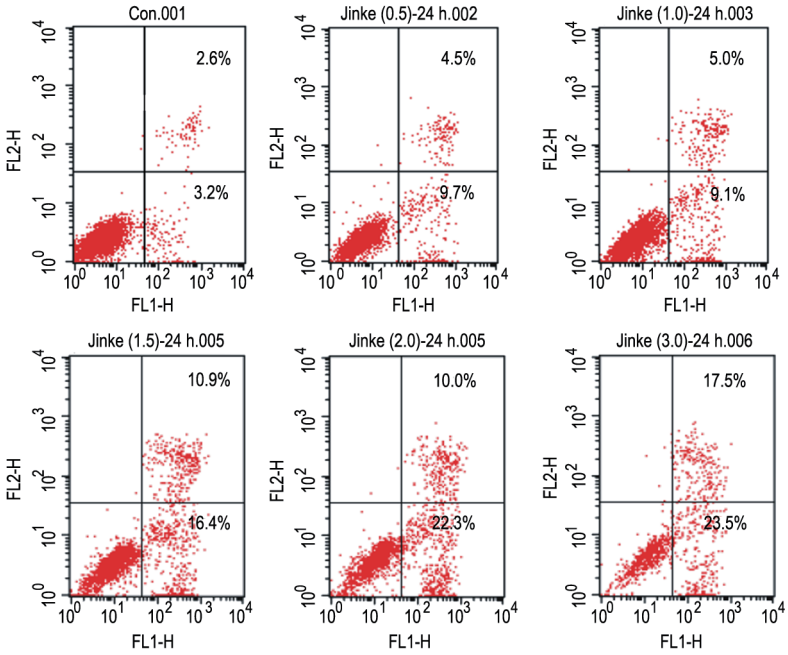

Fig. 1 Concentration-dependent apoptosis of Molt-4 cells 24 h after the treatment of Jinke

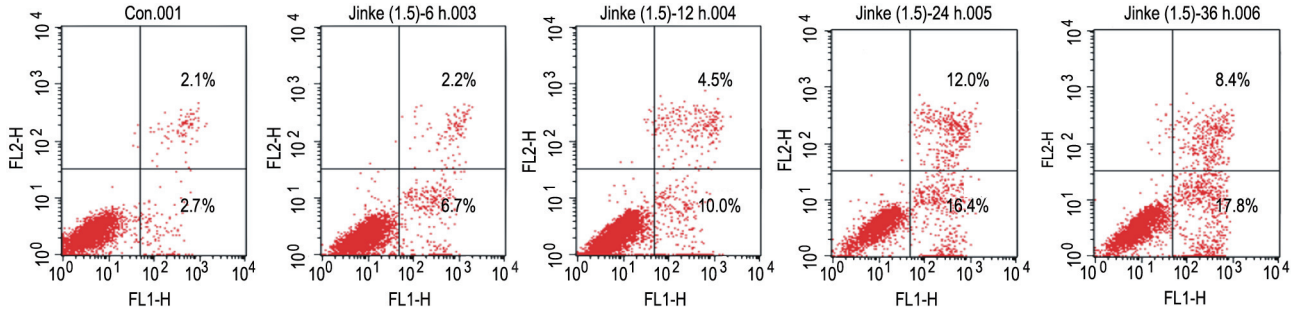

Fig. 2 Time-dependent apoptosis of Molt-4 cells after the treatment of 1.5 mg/mL Jinke

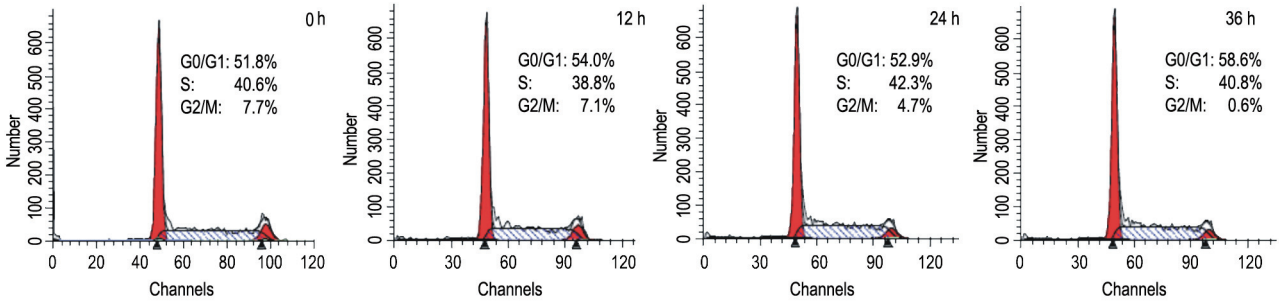

Fig. 3 Cell cycle was arrested in G0/G1 phase after the treatment of 1.5 mg/mL Jinke

Cyclin E analysis

The expression of cyclin E increased accordingly in Molt-4 cells treated with 1.5 mg/mL Jinke for 12, 24 and 36 h (Fig. 4).

Cell cycle-specific apoptosis in Molt-4 cells

Annexin-V positive cells could be seen in Molt-4 cells 12 h after treatment with 1.5 mg/mL Jinke. And almost all the apoptosis occurred in G0/G1 phase (Fig. 5). It meant that Jinke could induce G0/G1 phase-specific apoptosis.

Western blot for the expression of Bax and Bcl-2

Fig. 6 illustrated that the expression of Bcl-2 was down-regulation and the Bax was up-regulation in Molt-4 cells treated with 1.5 mg/mL Jinke 12, 24 and 36 h.

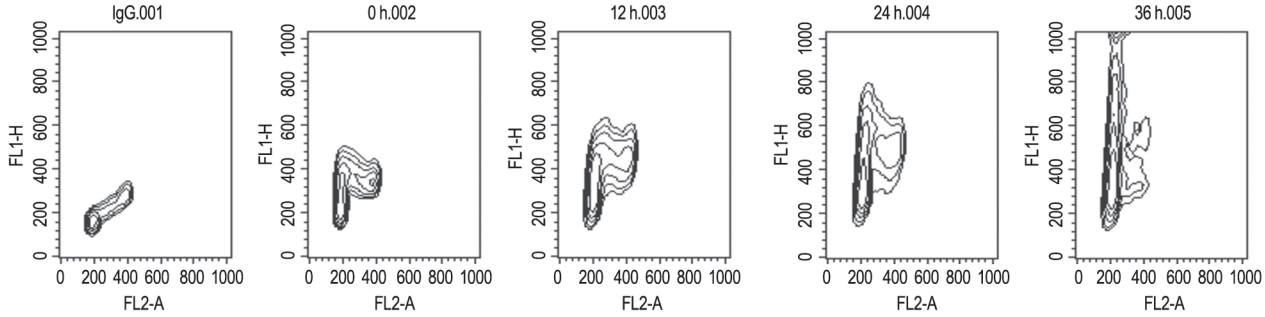

Fig. 4 Expression of cyclin E in Molt-4 cells after the treatment of 1.5 mg/mL Jinke

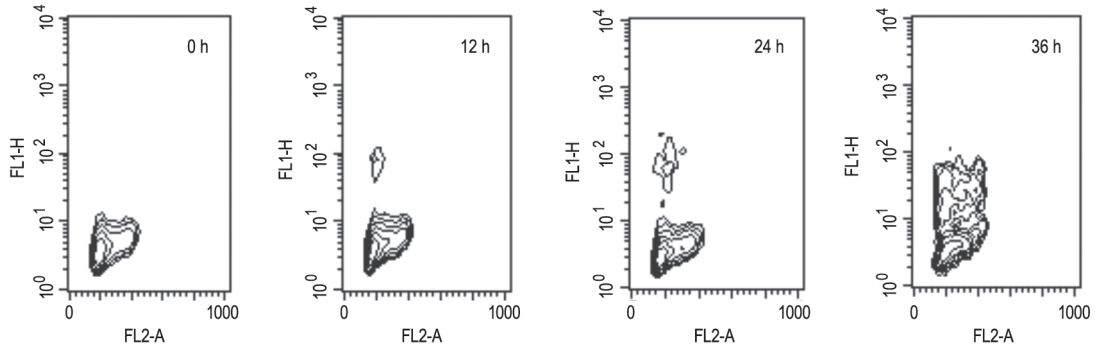

Fig. 5 Cell cycle-specific apoptosis of Molt-4 cells after the treatment of 1.5 mg/mL Jinke

Discussion

Jinke (polysaccharide of trametes robiniophila murr), which contains variety of organic ingredients and more than 10 kinds of mineral elements, was extracted from huaier with hot water. The main ingredient of Jinke is polysaccharide protein (PS-T), consists of 6 kinds of monosaccharides and 18 kinds of amino acids [7]. Jinke, the finished product of polysaccharide of trametes robiniophila murr, has been used in clinical as a new drug now.

Apoptosis was closely related to tumor disease and its therapy. So it's important to study the apoptosis-inducing effects of anti-tumor drugs on tumor cells. In this research, Annexin-V / PI showed that apoptosis rate increased with the increased concentration of Jinke in Molt-4 cells treated with different concentrations of Jinke for 24 h. And when the Molt-4 cells were treated with 1.5 mg/mL Jinke for different times, the apoptosis rate increased with time increased, too. So, Jinke could induce time- and concentration-dependent apoptosis in Molt-4 cells, which provided theoretical basis for clinical application.

The cell cycle checkpoint maintains the order and fidelity of cell cycle events in eukaryotes [8, 9]. DNA damage and mutations could be detected by it, and the damaged cells were arrested in specific checkpoint to repair. If the damaged DNA was repaired, the cells could enter into the next phase of cell cycle, or apoptosis would happen [10, 11]. In this research, DNA content analysis showed that the percentage of cells in G0/G1 phase increased gradually and the percentage of cells in G2/M phase decreased accordingly in Molt-4 cells treated with 1.5 mg/mL Jinke for different times. So, the cells were arrested in G0/G1 phase. With double parameters analysis of cyclins / DNA by flow cytometry, Gong *et al* [12, 13] probed the theory of scheduled expression of cyclins in cell cycle. Cyclin E begins to express in middle G1 phase, and it's negative in G0 phase, so the G0 and G1 phase can be distinguished by the expression of cyclin E. In this study, the expression

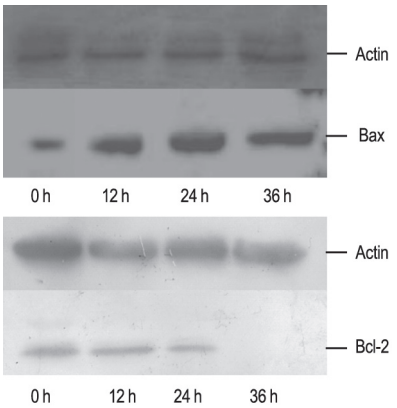

Fig. 6 Expressions of Bax and Bcl-2 in Molt-4 cells after the treatment of 1.5 mg/mL Jinke

of cyclin E increased with time increased in Molt-4 cells treated with 1.5 mg/mL Jinke. So it can be inferred the cell cycle was arrested in G1 phase, and the checkpoint in G1 phase was activated. API assay [14] showed that Jinke could induce G1 phase-specific apoptosis in Molt-4 cells.

The mechanism of checkpoint activation after DNA damage are p53-dependent or p53-independent. The mechanism of checkpoint activation in G1 phase is mainly p53-dependent [15]. ATM was activated after DNA was damaged, followed by p53 activated, which initiate the transcription of p21. The cyclin E couldn't be degraded as the CDK2-cyclin E complex was inhibited by p21. So the G1 phase block happened [16]. Besides [17], p53 could up-regulate the transcription of Bax, Cytc was released then, and the caspase-9 and caspase-3 were activated, which leaded to apoptosis happened. According to our research dates, after p53-normal Molt-4 cells were treated with Jinke, DNA damage happened (data were to be released), cell cycle was arrested in G1 phase, and the expression of Bax was up-regulated. It can be inferred that p53 was activated by ATM after DNA was damaged, and then the checkpoint in G1 phase was activated. The up-regulation of Bax may be because the p53 promoted the transcription of Bax.

Apoptosis was controlled by many genes, such as Bcl-2, Bax, c-myc and so on. The Bcl-2 family are the most important genes to regulate apoptosis. Bcl-2 family was mainly divided into two groups according to its function [18], the pro-apoptosis genes and the apoptosis-inhibiting genes. Bax and Bcl-2 could form heterodimers or homodimers. The regulation of Bax/Bcl-2 complex formation was a key regulator in apoptosis [19]. In this research, the Bcl-2 was down-regulated and Bax was up-regulated in Molt-4 cells treated with Jinke. The ratio of anti-apoptosis and pro-apoptosis declined lead to apoptosis happened.

The research of apoptosis-inducing effect of Jinke on tumor cells and its mechanism could provide theoretical basis for clinical application and the development for new kinds of Jinke. But the specific molecular mechanism should be further studied.

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