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Experimental Study on Apoptosis in Leukemia Cells Induced by Econazole*

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Abstract ***Objective:*** To investigate apoptosis in mouse leukemia cell (WEHI-3) induced by Econazole and its mechanism. ***Methods:*** Apoptosis induced by Econazole was examined by flow cytometry. Free calcium ($[Ca^{2+}]i$) was determined by Fura-2 fluorescein load technique. The protein was isolated from endoplasmic reticulum of WEHI-3 cells, and then the expression of caspase-12 and caspase-7 was detected by Western blot. ***Results:*** WEHI-3 cells exhibited typical change of apoptosis when they were treated by Econazole. $[Ca^{2+}]i$ was significantly higher in Econazole-treated group than in control group. The expression of caspase-12 and caspase-7 was increased with the increase of Econazole concentration. ***Conclusion:*** Caspase-12 may play a key role in WEHI-3 apoptosis induced by Econazole.

Key words: econazole; apoptosis; $[Ca^{2+}]i$; caspase-12

Econazole, antieumycetes drug, can cause endoplasmic reticulum (ER) stress by blockade of Ca^{2+} inflow, resulting in apoptosis[1]. But till now, whether it plays a role in apoptosis of leukemia cells has been not understood yet. Caspase-12 is a novel signal for apoptosis and is specifically activated in cells subjected to ER stress[2]. In this study, mouse leukemia cells (WEHI-3) apoptosis, caspase-12 protein and cytoplasm $[Ca^{2+}]i$ in WEHI-3 cells treated with Econazole were detected in order to investigate the mechanism of the WEHI-3 cells apoptosis induced by Econazole.

Materials and methods

Reagents

Econazole (Sigma, USA) was dissolve with methanol.

Cell culture

WEHI-3 cells were cultured in DMEM-HG (Gibco, USA) supplemented with 15% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2.

Apoptosis assay

Apoptosis was examined using Annexin-V/PI binding assay kit according to the instruction of the manufacturer.

Cytoplasm $[Ca^{2+}]i$ measurement

For measurement of cytoplasm $[Ca^{2+}]$ concentration ($[Ca^{2+}]I$), cells (3×10^6/mL) were loaded with the fluorescent indicator fura-2/AM (5 μM) taking precautions to avoid dye sequestration, then Hanks was added to stop the reaction. Fluorescent intensity (F) was measured at wavelength of 340 nm and 380 nm. The cell was added with 0.2% TritonX-100 to obtain maximum F (Fax) and added with 8 mmol/L (pH>8.5) EGTA to obtain minimum F (Fmin). Cytoplasmic Ca^{2+} concentration $[Ca^{2+}]$ was calculated according to the formula: $[Ca^{2+}]$=Kd (R-Rmin)/(Rmax-R)Ff2/Fb2. Correct parameter (Ff2/Fb2) was the ratio between the maximum (Ff2) and the minimum (Fb2), which was detected at 380 nm. Ff2 and Fb2 were free and saturated $[Ca^{2+}]$ respectively.

Western blot

Cells were resuspended in ice-cold fractionation buffer pH 7.4 (20 mM HEPES, 10 mM KCL, 250 mM sucrose, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitd, and protease inhibitor mixture). After cell lysis, nuclei were pelleted at 750 g for 10 min. The supernatant was centrifuged at 10 000 g for 30 min. The mitochondrial pellet was resuspended in the above buffer, and the supernatant contained the soluble cytoplasmic fraction. At the end of the reaction, samples were detected by Western blotting by loading equal amounts of total microsomal protein. An aliquot of 20 μg of protein was resolved

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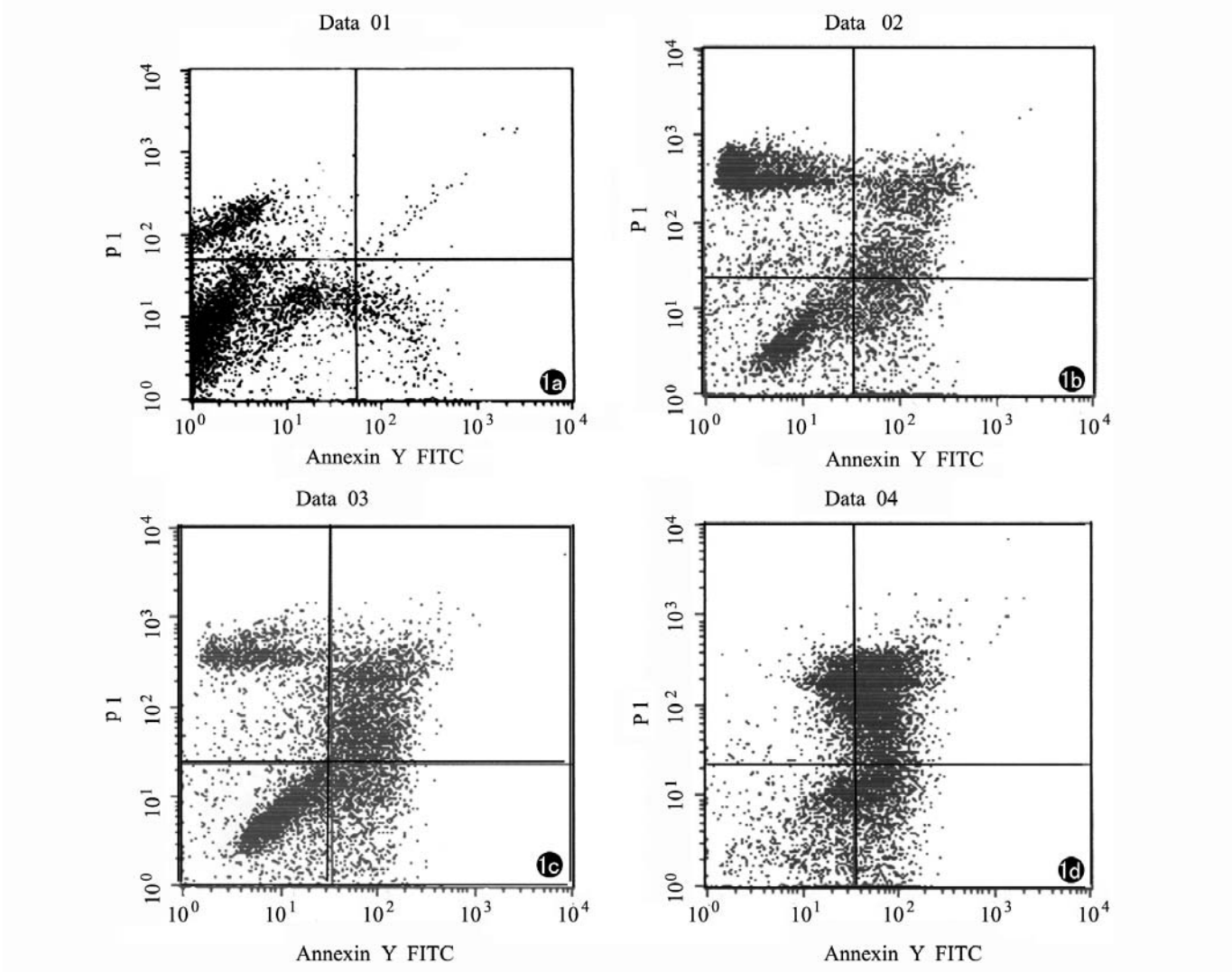

Fig. 1a–d The results of apoptosis by flow cytometry. (a) Control group; (b) Treatment with 5 μmmol/L Econazole for 24 h; (c) Treatment with 10 μmmol/L Econazole for 24 h; (d) Treatment with 15 μmmol/L Econazole for 24 h

on 12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto nitrocellulose membranes at 300 mA for 1 h at 4 ℃ with Towbin buffer. Membranes were blocked by incubation in TBS containing 5% dry low fat milk for 1 h at room temperature. Blots were probed with anti-caspase-12 rabbit polyclonal antibody (1:500, BioVision, France) and anti-capase-7 mouse polyclonal antibody (1:500, Neo Markers, USA). Immune complexes were detected using secondary antibody (1:10 000) and were visualized using ECL reagents (KPL, USA).

Statistical analysis

Data were analyzed by using analysis of variance.

Results

Econazole could induce apoptosis of WEHI-3 cells. Annexin-V/PI assay revealed that after treatment with 5, 10, 15 μmol/L for 24 h, the early apoptostic rate of WEHI-3 was 13.48%, 20.46%, 32.47% respectively in a dose-dependent manner (Fig. 1). The results indicated that different concentrations of Econazole could induce WEHI-3 cell apoptosis with the total apoptostic rate of WEHI-3 being (28.3±3.5)%, (42.6±2.9)%, (69.5±3.2)% respectively, significantly higher than in the control group [(7.3±0.5)%, F=28.34, P<0.01].

After the WEHI-3 cells were treated for 24 h with 5, 10, 15 μmmol/L Econazole respectively, the cytoplasm [Ca^{2+}]i was (131.2±11.2), (156.5±20.1), (182.1±13.6) nmol/L respectively, significantly higher than in the control group (80.6±15.3, F=21.26, P<0.01).

Western-blot revealed that after the WEHI-3 cells were treated with Econazole for 24 h, the expression of caspase-12 and caspase-7 was significantly increased as compared with that in control group (Fig. 2).

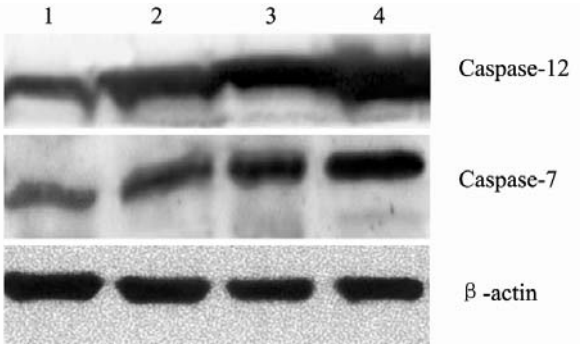

Fig. 2 The result of Western-blot. (1) Control group; (2) Treatment with 5 μmmol/L Econazole for 24 h; (3) Treatment with 10 μmmol/L Econazole for 24 h; (4) Treatment with 15 μmmol/L Econazole for 24 h

Discussion

Annexin-V/PI indicated that treatment with 5 μmol/L econazole for 24 h could induce the apoptosis of the WEHI-3 cells, which was consistent with those reported by Zhang *et al*[1]. Caspase-12 may play a key role in the apoptosis of WEHI-3 cells induced by Econazole.

Caspases are critical mediators of programmed cell death[3]. Caspase-12 is localized at the ER and activated by ER stress, including disruption of ER calcium homeostasis and accumulation of excess proteins in ER, but not by membrane-targeted or mitochondrial-targeted apoptotic signals. The endoplasmic reticulum is responsible for the synthesis, initial post-translational modification, and proper folding of proteins, as well as their sorting and export for delivery to appropriate cellular destinations. A variety of conditions, such as loss of the ER intraluminal oxidative environment or calcium content or the mutation or overexpression of relatively insoluble proteins, cause accumulation of misfolded proteins within the ER. Misfolded proteins are expelled from the ER and targeted for degradation by cytoplasmic proteasomes. Although these three protective responses control transiently the accumulation of misfolded proteins within the ER, they can be overcome by sustained ER stress, which leads to apoptosis[4]. Our findings suggest that ER stress plays a pivotal role in the activation of subset of caspase during ER stress-mediated cell death. A variety of toxic insults can result in ER stress that ultimately leads to apoptosis. Apoptosis is initiated by the activation of members of the caspase family and serves as a central mechanism in the cell death-process.

The Ca^{2+} is one of the most important and versatile intracellular messengers in eukaryotic cells. $[Ca^{2+}]$i elevations may not only activate a variety of cellular functions, but may also induce apoptosis[5]. In this study, after the WEHI-3 cells were treated with Econazole, the free Ca^{2+} concentration and the apoptotic rate of WEHI-3 cells were increased. These results suggested that Econazole could increase the $[Ca^{2+}]$i with the ER membrane activity was decreased. By increasing the Ca^{2+} permeability of the ER membrane, Econazole could increase the $[Ca^{2+}]$i within the lumen of the ER, and decrease the extracellular Ca^{2+} concentration. The imbalance of the intracellular Ca^{2+} homeostasis activated the Ca^{2+}-ATPase inhibitor, which would induce the apoptosis.

Caspase-12 is specifically involved in apoptosis that results from stress in the endoplasmic reticulum[6]. Treatment of cells with ER stress inducer such as Econazole induces the expression of caspase-12 protein and also leads to translocation of cytosolic caspase-7 to the ER surface. Caspase-7 is associated with caspase-12 and cleaves the prodomain to generate active caspase-12, resulting in increased cell death[7]. We demonstrate that stress not only induces the expression of caspase-12, but also causes translocation of cytosolic caspase-7 to the ER surface.

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