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Resistance to apoptosis of harringtonine-resistant HL60 cells induced by tetrandrine[1]

HE Qi-Yang, ZHANG Hong-Qing, PANG Da-Ben[2], CHI Xu-Sheng[2], XUE Shao-Bai[3]
(*Department of Biology, Beijing Normal University, Beijing 100875*; [2]*Institute of Basic Theory of TCM, China Academy of Traditional Chinese Medicine, Beijing 100700, China*)

KEY WORDS apoptosis; tetrandrine; harringtonines; leukemia HL60; c-*myc* genes; drug resistance; phosphorylation.

AIM: To study the mechanism of resistance to apoptosis in the harringtonine (Har)-resistant HL60 cells with tetrandrine (Tet). **METHODS**: Growth inhibition, flow cytometry, DNA agarose gel electrophoresis, protein phosphorylation, and RNA dot hybridization. **RESULTS**: The resistant cells had no cross resistance to Tet. Tet induced the sensitive but not the Har-resistant HL60 cells to apoptosis. The high phosphorylation of protein <30 kDa occurred when the resistant cells were treated with Tet. Tet and Har increased the expression of c-*myc* mRNA in the sensitive HL60 cells. The expression of c-*myc* mRNA in the resistant cells was obviously decreased and almost not changed in treatment with Tet and Har. **CONCLUSION**: Tet induced the sensitive but not the Har-resistant HL60 cells to apoptosis, and the resistance to apoptosis induced by Tet was associated with the high protein phosphorylation and reduction of the expression of c-*myc* mRNA.

Apoptosis differs from necrosis by chromatin condensation and blebbed into "Apo body." Many antitumor drugs cause tumor cells to apoptosis[1]. Harringtonine (Har), an antitumor drug, induced human leukemia HL60 cells to apoptosis[2], but the Har-resistant HL60 cells treated with Har 80 mg $\cdot L^{-1}$ for 48 h were not induced to apoptosis[3]. The non-growth-inhibitory concentrations of tetrandrine (Tet) abolished the drug resistance to doxorubicin in Chinese hamster ovary cells[4] and the Har-resistant HL60 cells[5]. The Har-resistant HL60 cells had no cross resistance to Tet. So we wanted to study the mechanism of the resistance to apoptosis with Tet in the Har-resistant HL60 cells.

MATERIALS AND METHODS

Drugs and cell culture Tet produced by Zhejiang Jinhua Pharmaceutical Factory, was dissolved in HCl 1 mol $\cdot L^{-1}$, adjusted pH to 6.7 with NaOH and added PBS buffer to a final concentration of 1 g $\cdot L^{-1}$. Har was purchased from Beijing Union Pharmaceutical Factory and made up into a solution of 1 g $\cdot L^{-1}$ with PBS buffer. Both drugs were stored at −20 ℃. Human leukemia HL60 cells and HT12 cells (Har-resistant HL60 cells established by us) were cultured in RPMI 1640 medium containing 10 % bovine serum with 95 % air + 5 % CO_2 at 37 ℃. The [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from Yahui Biomedical Engineering Company (Beijing).

Inhibition of cell growth The $4 \cdot 10^4$ cells/well were seeded on a 24-well plate. The cells were stained with trypan-blue after adding the drugs for 12, 24, 48 h, and non-stained cells were counted with a hemocytometer. The results were indicated with average cell numbers measured in 3 experiments.

Observation of chromatin condensation During the apoptosis, cellular chromatin was condensed and divided into several parts, but the cell membrane permeability remained unchanged. The condensed chromatin parts containing some cytoplasm which appeared as "dots" under a fluorescence microscope, were called "Apo bodies." The cells which kept the intact permeability of cell membrane were stained by Hoechst 33342 and excluded to be stained by propidium iodide (PI). The dead cells were stained by PI because they lost the cell membrane permeability. Using both Hoechst 33342 and PI dyes, the apoptosis was detected under a fluorescence microscope. The cells not stained by PI were indicative for apoptosis, because the necrotic cells lost the cell membrane permeability.

After the cells were stained with Hoechst 33342 (Sigma) 10 μmol $\cdot L^{-1}$ and PI (Sigma) 60 mg $\cdot L^{-1}$ for 0.5 h, they were washed twice with PBS buffer. The states of condensed chromatin were observed under a fluorescence microscope and taken microphotograph, and the cells not stained by PI were

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[3] Correspondence to Prof XUE Shao-Bai.
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counted at the same time.

Detection of apoptosis by flow cytometry Having been treated with the drugs for 6 h, the cells were processed and detected[2]. The DNA contents of "Apo bodies" were lower than those of the whole cells, and the peak lower than the G_1 peak, was called "Apo peak."

Extraction of cellular DNA Cellular DNA was isolated[6]. The DNA concentrations were determined by UV spectrophotometer Beckman DU50. DNA 5 μg per sample was loaded into 1.2 % agarose gel and electrophoresis was carried out in TAE buffer (Tris-acetate 0.04 mol·L^{-1}, edetic acid 0.001 mol·L^{-1} at 35 V for 2 – 3 h. DNA ladders were taken photographs under an UV light.

Protein phosphorylation *in vitro* The cells treated with the drugs for 4.5 h were washed with PBS buffer and suspended in M buffer containing Tris·HCl 20 mmol·L^{-1} pH 7.5, egtazic acid 5 mmol·L^{-1}, 1 % Triton X-100, phenylmethylsulfonyl fluoride 1 mmol·L^{-1}, and dithiothreitol 1 mmol·L^{-1}. The cells were broken down by supersonication and the lysates were centrifuged at 12 000 × g for 20 min. The supernatants were used for phosphorylated reaction. The protein concentrations were determined by Coomassie brillant blue method[7] with bovine serum albumin as a standard. Aliquots of 20 μL reaction system containing Tris·HCl 20 mmol·L^{-1} pH 7.5, $MgCl_2$ 20 mmol·L^{-1}, $MnCl_2$ 0.2 mmol·L^{-1}, [γ-^{32}P]ATP 185 kBq and protein 100 μg were incubated at room temperature for 30 min. The reaction was stopped by adding two-fold loading buffer. After incubation at 100 ℃ for 3 min, the samples were loaded into a SDS-polyacrylamide gel composed of 10 % separated gel. The electrophoresis was performed at 80 V for 6 h and the gel was autoradiographed with χ-film at −70 ℃ for 24 – 48 h.

Extraction of total cellular RNA and Dot blot hybridization The total RNA was isolated[8]. The nitrocellulose membrane was dotted with RNA 1, 5, 20 μg and hybridized with c-*myc* gene probe (2.2 kb) labelled by random primer method (Promega) with [α-^{32}P]dCTP 1.85 MBq, and autoradiographed[9].

RESULTS

Growth inhibition The sensitive HL60 cells and HT12 cells were all dead after they were treated with Tet 20 mg·L^{-1} for 24 h. The Tet inhibitory action was more apparent in HT12 cells than in HL60 cells. All the HT12 cells were stained with trypan blue after the cells were treated with Tet 5 mg·L^{-1} for 24 h, but HL60 cells remained 23 % not stained in treatment with Tet 5 mg·L^{-1} (Fig 1).

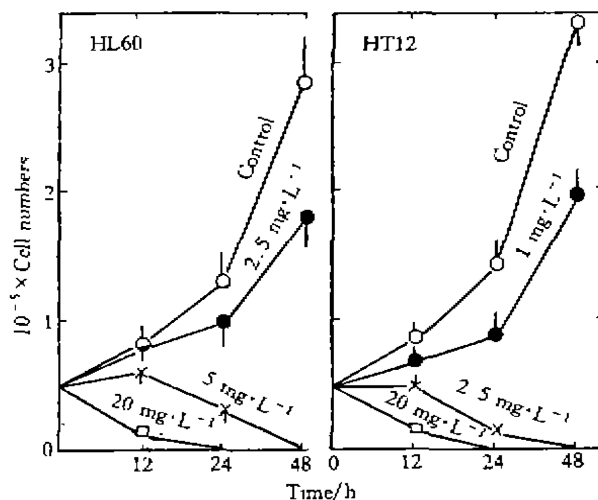

Fig 1. Growth-inhibitory actions of tetrandrine on HL60 and HT12 cells. $n = 9$, $\bar{x} \pm s$.

Detection of apoptosis by flow cytometry A typical "Apo peak" was detected after HL60 cells were treated with Tet 20, 15, 10 mg·L^{-1} for 6 h, respectively. The similar histograms were observed after HL60 cells were treated with Tet 20 mg·L^{-1} or Har 0.1 mg·L^{-1}. "Apo peak" was not detected when HT12 cells were treated with Tet 20, 10, 5 mg·L^{-1} and Har 40 mg·L^{-1}. But, the slight increment of S phase cells was observed in HT12 cells treated with Tet 20 mg·L^{-1} (Fig 2).

Chromatin condensation When Tet induced HL60 cells to apoptosis, condensed chromatin states were similar with those by Har, forming "dotted" chromatin, and then dividing into "Apo bodies." However, the time of forming the "Apo bodies" in HL60 cells induced by Tet was longer than that by Har for the condensed chromatin cells were not observed after HL60 cells were incubated with Tet 20 mg·L^{-1} for 4 h. HT12 cells did not occur chromatin condensation after the treatment with Tet or Har for 6 h (Fig 3). The cells not stained by PI were >95 % in the control groups and >90 % in the drug groups.

DNA agarose gel electrophoresis After HL60 cells were treated with Tet 20, 15, and 10 mg·L^{-1} for 6 h, typical DNA ladder bands were seen the same as those by Har 0.1 mg·L^{-1}. DNA ladder bands did not appear whenever HT12 cells were

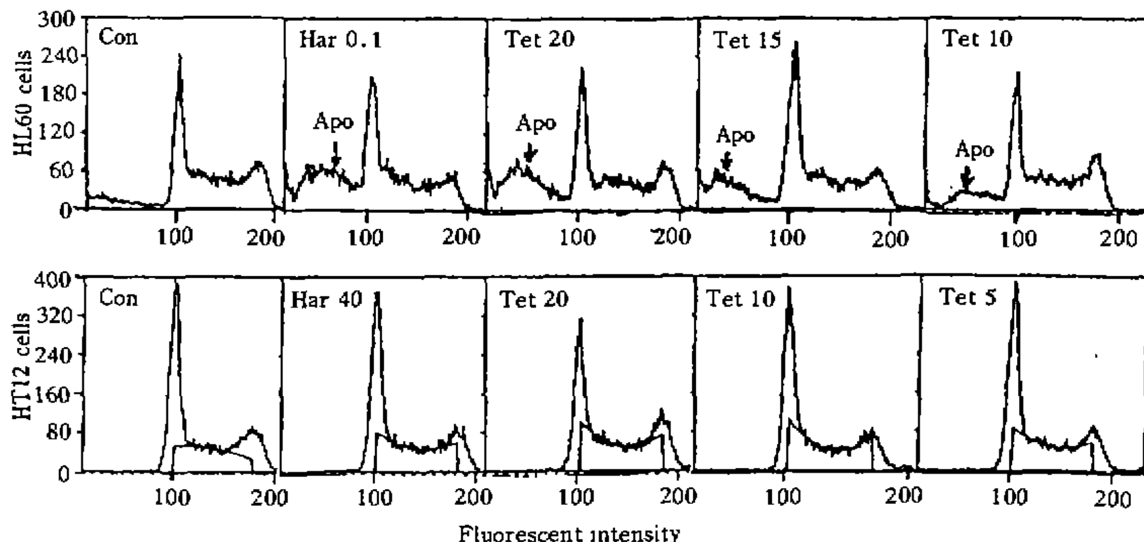

Fig 2. Detection of apoptotic cells by flow cytometry. Drugs/mg·L^{-1}.

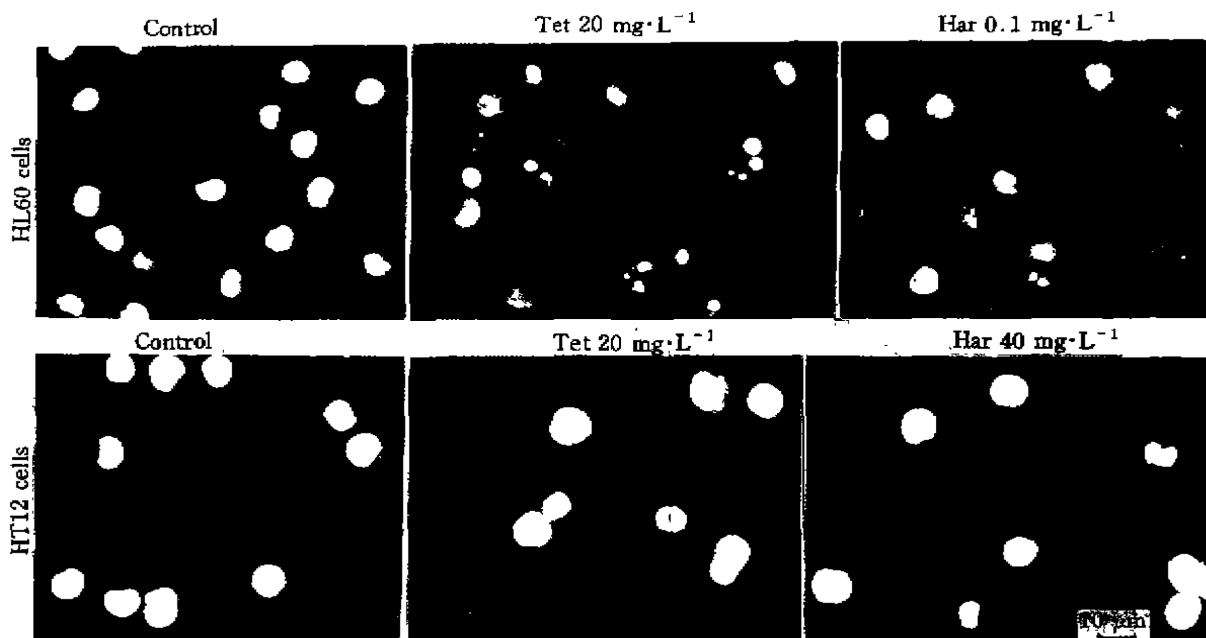

Fig 3. Nuclear morphological changes of HL60 and HT12 cells treated by tetrandrine and harringtonine. Cells were stained with Hoechst 33342 and propidium iodide. ×600.

treated with Tet and Har (Fig 4).

Protein phosphorylation The patterns of protein phosphorylation showed no changes after HL60 cells were treated with Tet 20 mg·L^{-1} or Har 0.1 mg·L^{-1} for 4.5 h. Protein 110 – 130 kDa was highly phosphorylated in HT12 cells, and another highly phosphorylated protein <30 kDa was observed, which was more apparent in treatment with Tet 20 mg·L^{-1}(Fig 5).

Expression of c-*myc* mRNA The expression of c-*myc* mRNA was increased after HL60 cells were incubated with Tet 20 mg·L^{-1} and Har 0.1 mg·L^{-1} for 3.5 h in contrast with control. The expression of c-*myc* mRNA was reduced in HT12

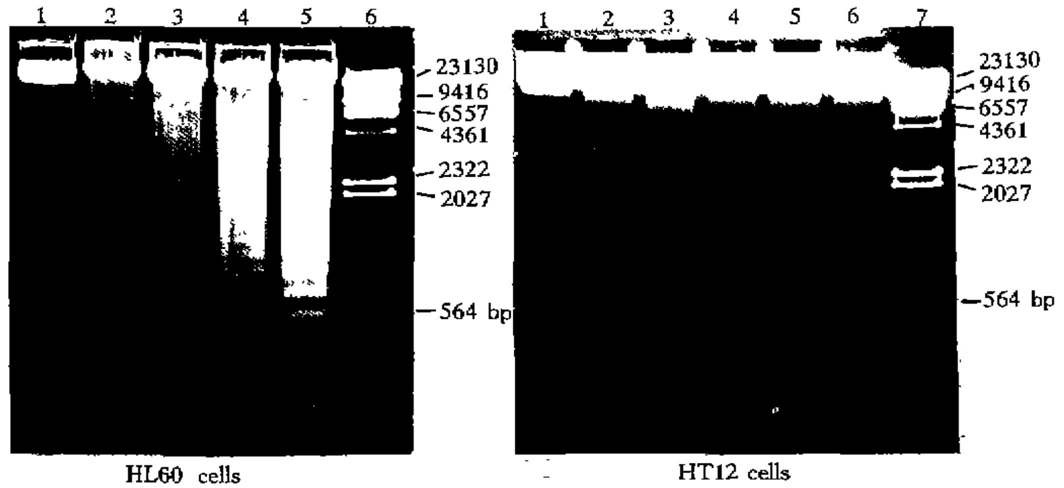

Fig 4. Agarose electrophoresis of cellular DNA. HL60 cells 1) control; 2) Tet 10 mg·L^{-1}; 3) Tet 15 mg·L^{-1}; 4) Tet 20 mg·L^{-1}; 5) Har 0.1 mg·L^{-1}; 6) DNA marker, λ DNA phage/Hind Ⅲ. HT12 cells 1) control; 2) Tet 2.5 mg·L^{-1}; 3) Tet 5 mg·L^{-1}; 4) Tet 10 mg·L^{-1}; 5) Tet 20 mg·L^{-1}; 6) Har 40 mg·L^{-1}; 7) DNA marker, λ phage DNA/Hind Ⅲ.

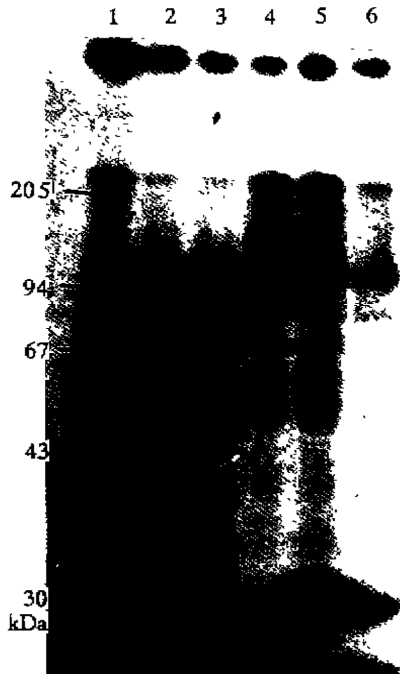

Fig 5. Protein phosphorylation of HL60 and HT12 cells treated with tetrandrine and harringtonine. Autoradiographs of the proteins separated by SDS-PAGE. 1) HL60 cells, control; 2) HL60 cells + Tet 20 mg·L^{-1}; 3) HL60 cells + Har 0.1 mg·L^{-1}; 4) HT12 cells, control; 5) HT12 cells + Tet 20 mg·L^{-1}; 6) HT12 cells + Har 40 mg·L^{-1}.

cells, had no change after HT12 cells were treated with Tet 20 mg·L^{-1} for 3.5 h, and was slightly increased by Har 40 mg·L^{-1} (Fig 6).

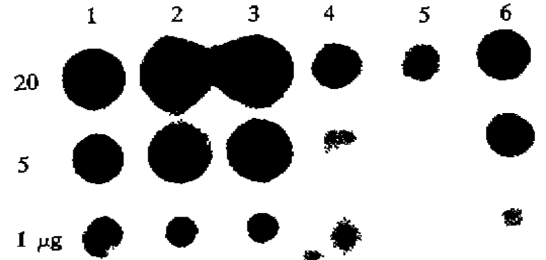

Fig 6. Expression of cellular c-*myc* mRNA from HL60 and HT12 cells treated by tetrandrine and harringtonine. Total RNA was dotted on the nitrocellulose filter and hybridized with c-*myc* probe for 24 h and autoradiographed. 1) HL60 cells, control; 2) HL60 cells + Tet 20 mg·L^{-1}; 3) HL60 cells + Har 0.1 mg·L^{-1}; 4) HT12 cells, control; 5) HT12 cells + Tet 20 mg·L^{-1}; 6) HT12 cells + Har 40 mg·L^{-1}.

DISCUSSION

Tet induced apoptosis in the sensitive HL60 cells, but not in the Har-resistant HL60 cells. When HL60 cells were induced to apoptosis by Tet and Har, expression of c-*myc* mRNA increased, which was in accordance with the results[10]. Reduction of the expression of c-*myc* mRNA in the Har-resistant HL60 cells may be one of the mechanisms of the resistance to apoptosis.

Protein phosphorylation is associated with apoptosis. The phosphorylation of tyrosine protein kinase increased in the course of human B-

lymphocyte precursors, FL-12 cells, and Nalm-6 cells to apoptosis, triggered by γ-ray[11]. However, when γ-ray and heat shock aroused B-cell lymphoma line, BM 13674 cells and T-cell leukemia line, as well as CEM-C7 cells to apoptosis, it was necessary to dephosphorylate some proteins, and prevention of apoptosis was through inhibition of dephosphorylation by okadaic acid, an inhibitor of phosphatase[12]. In our experiment, the highly phosphorylated proteins 110 – 130 kDa and about 30 kDa were observed in the Har-resistant HL60 cells, which is likely to be related to the resistance to apoptosis. In this paper we demonstrated that Tet aroused a typical apoptosis in the sensitive HL60 cells, but not in the Har-resistant HL60 cells. The mechanisms of the resistance to apoptosis may be related to protein phosphorylation and reduction of the expression of *c-myc* mRNA in the Har-resistant HL60 cells.

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抗三尖杉酯碱的 HL60 细胞抗粉防己碱诱导的细胞凋亡[1]

R977.1 R966

何琪杨，张鸿卿，庞大本[2]，池旭生[2]，薛绍白[3]

（北京师范大学生物系，北京 100875；

[2]中国中医研究院基础理论研究所，北京 100700，中国）

关键词 细胞凋亡；粉防己碱；三尖杉酯碱类；白血病 HL60；*c-myc* 基因；抗药性；磷酸化

A **目的**：用粉防己碱(Tet)研究抗三尖杉酯碱(Har)的 HL60 细胞抗细胞凋亡的机制 **方法**：生长抑制，流式细胞术、DNA 凝胶电泳、蛋白质磷酸化和 RNA 点杂交 **结果**：抗性细胞对 Tet 没有交叉抗性，Tet 诱导 HL60 细胞发生凋亡，但不能诱导抗性细胞发生凋亡. Tet 引起抗性细胞低于 30 kDa 的蛋白质高度磷酸化. Tet 和 Har 诱导 HL60 细胞 *c-myc* mRNA 表达增高，而抗性细胞 *c-myc* mRNA 表达明显降低，药物处理后变化也不明显. **结论**：Tet 诱导敏感的而不是抗 Har 的 HL60 细胞发生凋亡，抗凋亡的分子机制与蛋白质高度磷酸化和 *c-myc* mRNA 表达降低有关.