关键词 环磷酰胺；胚泡；抗体依赖性细胞毒性；细胞遗传毒性；微核；药源性异常

目的：以免疫外科法评价大鼠孕早期环磷酰胺染毒，对胚泡两群不同细胞的选择性影响. 方法：大鼠孕 d 3 ip 环磷酰胺(10, 20, 40 mg·kg^{-1})，孕 d 4 取胚泡行免疫外科术，分离胚泡内细胞团并检测其与胚泡对环磷酰胺损伤敏感性的差异. 结果：环磷酰胺组胚泡及内细胞团平均细胞数减少(35±3, 32±1, 30±1 及 14±2, 11±1, 9±2)，微核率增高(1.81 %, 2.27 %, 3.14 % 及 2.53 %, 2.98 %, 4.75 %)两者改变均呈剂量依赖性. 但胚泡与内细胞团受损随剂量增加不呈平行关系，后者改变更明显. 结论：环磷酰胺在大鼠胚泡着床前给药，对胚泡两群细胞呈剂量依赖性细胞毒性与遗传毒性，其中内细胞团受损尤甚.

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Cyclosporine inhibited calcium-mediated apoptosis of HL-60 cells[1]

HUANG Qi-Qing, FANG Min, ZHANG Hong-Qing, XUE Shao-Bai
(*Department of Biology, Beijing Normal University, Beijing 100875, China*)

KEY WORDS cyclosporine; calcimycin; harringtonines; camptothecin; thapsigargin; calcium; apoptosis; HL-60 cells; DNA; flow cytometry

AIM: To study the effects of cyclosporine (Cyc) on apoptosis of HL-60 cells. **METHODS**: Apoptotic cells induced by harringtonine (Har), camptothecin (Cam), or calcimycin (Cal), thapsigargin (Tha) were identified with DNA electrophoresis, morphology, and flow cytometry. Relative $[Ca^{2+}]_i$ alteration of apoptotic HL-60 cells were determined with flow cytometry. **RESULTS**: Cal 1 mg·L^{-1} or Tha 0.5 mg·L^{-1} induced apoptosis of HL-60 cells. This effect was inhibited by nontoxic concentration of Cyc 1 mg·L^{-1}. Cyc did not inhibit Har- or Cam-induced apoptosis of HL-60 cells. Both Cal and Tha increased intracellular calcium, whereas Har or Cam did not. **CONCLUSION**: Cyc inhibited apoptosis only induced by calcium increasement in HL-60 cells. The mechanism of apoptosis induced by Cal or Tha was different from that by Har or Cam.

Cyclosporine (Cyc), an immunosuppressive agent, inhibited T lymphocyte activation and activation-driven apoptosis of T-cell lines or T-cell hybridomas[1,2]. Cyc is a good drug of attenuating mutidrug-resistance of cancer cells[3]. Cyc blocks T cell activation pathways associated with an increase of intracellular calcium. Since an early increase of intracellular calcium was found in many types of apoptosis[4], Cyc may block apoptosis in other cell lines. In this study, we investigated whether Cyc prevented apoptosis triggered by drugs that increased intracellular calcium, and compared with apoptosis induced by anticancer drugs.

MATERIALS AND METHODS

Reagents Cyc was purchased from Sino-American Pharmaceutical Factory of East China. Propidium iodide (PI), calcimycin (Cal), thapsigargin (Tha), camptothecin (Cam), and Fluo 3-AM were from Sigma. Hoechst 33342 was from Molecular Probes Inc. Har was from Beijing Union Pharmaceutical Factory.

Cell culture and cell viability assessment HL-60 cells were grown at 37 ℃ in RPMI 1640 medium (Gibco) containing 10 % heat-inactivated fetal bovine serum in an atmosphere containing 5 % CO_2. Exponentially growing HL-60 cells were exposed to drugs. Cells after drug treatment were stained with Hoechst 33342 10 μmol·L^{-1} and PI 50 mg·L^{-1} for 20 min, then washed with PBS and resuspended in PBS. Morphological and quantitative analysis of apoptosis was performed with fluorescence microscopy (Olympus)[5].

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DNA extraction and electrophoresis The pattern of DNA cleavage was analyzed by agrose gel electrophoresis[6]. Briefly, cells (1×10^6) were lysed with 200 μL lysis buffer [edetic acid 10 mmol·L^{-1}; tris-HCl 50 mmol·L^{-1}; *N*-lauroyl sorcosine 0.5 % (wt/vol); proteinase K 0.5 g·L^{-1}] and incubated at 50 ℃ for 1 h. RNase A was added to a final concentration of 0.5 g·L^{-1}, and incubated at 50 ℃ for 1 h. After phenol extraction and ethanol precipitation, samples of 1.5 μg in each lane were subjected to electrophoresis on a 1.2 % agrose gel.

Flow cytometry Flow cytometry was made to identify apoptotic cells[5]. Briefly, cells were fixed in 70 % ethanol at 4 ℃ overnight. Cells after fixation were incubated in PBS containing RNase A 50 mg·L^{-1} at 37 ℃ for 1 h, and stained with PI 65 g·L^{-1} at 4 ℃ for 1 h, and then analyzed by a FACS 420 flow cytometer.

Determination of intracellular Ca^{2+} concentration Cells were loaded with Fluo 3-AM 10 μmol·L^{-1} at 37 ℃ for at least 30 min, then were washed by centrifugation at 400 × *g* for 3 min and resuspended in RPMI 1640 medium (37 ℃)[7]. After exposure to various drugs, Intracellular Ca^{2+} concentration alteration was determined by flow cytometry.

Experimental design Before apoptotic analysis, exponentially growing HL-60 cells on log phase were exposed to Cyc 1 mg·L^{-1} for 4 h, then were treated with Har 0.05 mg·L^{-1}, Cam 0.05 mg·L^{-1} or Cal 1 mg·L^{-1}, Tha 0.5 mg·L^{-1}. For determination of suitable concentration of Cyc, dosaged-reaction curve was made. Briefly, cells were planted to 24 well plate at the density of $20 \cdot 10^7$ cells·L^{-1}, various concentration of Cyc were added. Cell numbers were counted at 12, 24, and 48 h. Cell viability were assessed. The data from 6 experiments were expressed as $\bar{x} \pm s$ and compared with *t*-test.

RESULTS

HL-60 cell proliferation and apoptosis The maximal concentration of Cyc that did not obviously affect the growth of HL-60 cells was 3 mg·L^{-1}. Combined staining with PI and Hoechst 33342 revealed that within 24 h, cells treated with Cyc (< 3 mg·L^{-1}) showed no apoptosis or necrosis (Fig 1).

Exposure of HL-60 cells to Cal 1 mg·L^{-1} for 4 h or Tha 0.5 mg·L^{-1} for 2 h led to apoptosis. Combined staining with Hoechst 33342 and PI showed condensed nuclei in a large cell subpopulation (Fig 2), and the necrotic cells (PI positive) was <5 %. Agarose gel electrophoresis of DNA revealed a "ladder" pattern (Fig 3).

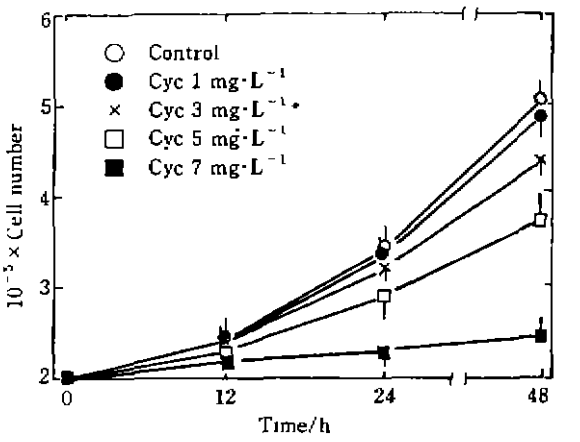

Fig 1. Growth of HL-60 cells treated with Cyc. $n = 6$, $\bar{x} \pm s$.

Apoptotic DNA peak was seen in DNA histogram after flow cytometric analysis (Fig 4).

Pretreatment of HL-60 cells with nontoxic Cyc 1 mg·L^{-1} for 4 h, then added Cal to a final concentration of 1 mg·L^{-1} or Tha to 0.5 mg·L^{-1}. Cells did not undergo apoptosis (Fig 2-4).

Pretreatment of HL-60 cells with CsA did not inhibit apoptosis induced by even very low concentration of Har or Cam (0.05 mg·L^{-1}) (Tab 1, Fig 5).

Tab 1. Effect of Cyc on apoptosis of HL-60 cells induced by Har or Cam. HL-60 cells were pretreated by Cyc for 4 h, then Har or Cam for 2 or 4 h. $n = 6$, $^aP > 0.05$ *vs* Har; $^dP > 0.05$ *vs* Cam.

Group	Cell number ($\bar{x} \pm s$) at 2 h	at 4 h
Control	5.1±1.2	5.8±1.7
Har	51.3±6.1	61.2±7.3
Cyc + Har	50.2±6.8^a	70.3±8.2^a
Cam	62.3±8.1	79.6±8.4
Cyc + Cam	60.6±8.6^d	81.0±9.5^d

Intracellular Ca^{2+} level Cal or Tha increased the intracellular Ca^{2+}, but Cyc did not prevent the rise of intracellular Ca^{2+}. However, cells treated with Har or Cam did not show increase of intracellular Ca^{2+} (Fig 6).

DISCUSSION

Our results showed that both calcium ionophore

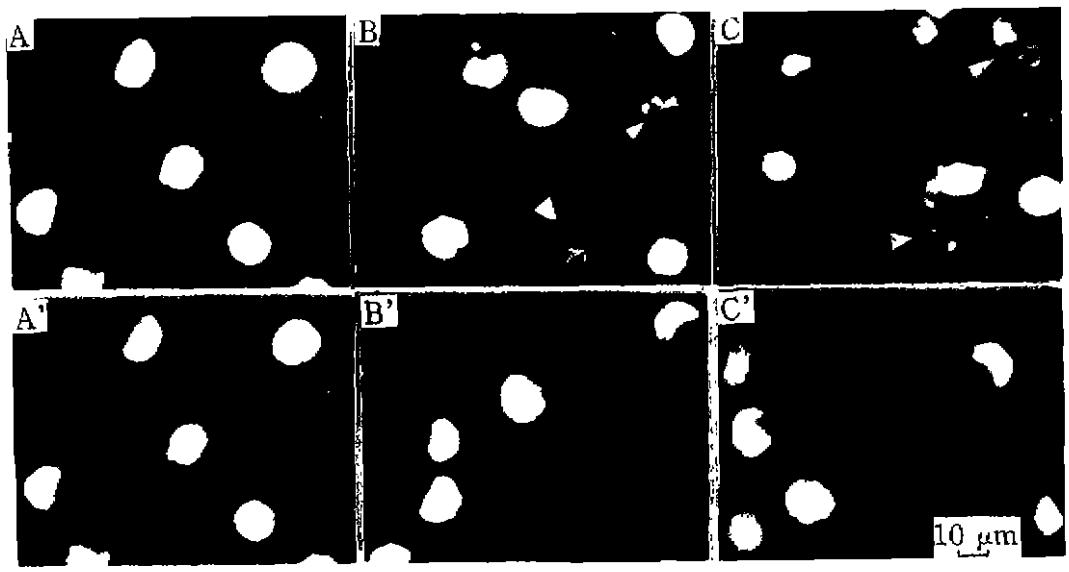

Fig 2. Fluorescence of HL-60 cells. (A) & (A′) control; (B) Cal 1 mg·L^{-1} for 4 h; (B′) Cyc 1 mg·L^{-1} for 4 h, then Cal 1 mg·L^{-1} for 4 h; (C) Tha 0.5 mg·L^{-1} for 2 h; (C′) Cyc 1 mg·L^{-1} for 4 h, then Tha 0.5 mg·L^{-1} for 2 h. Hoechst 33342 stain. ×400.

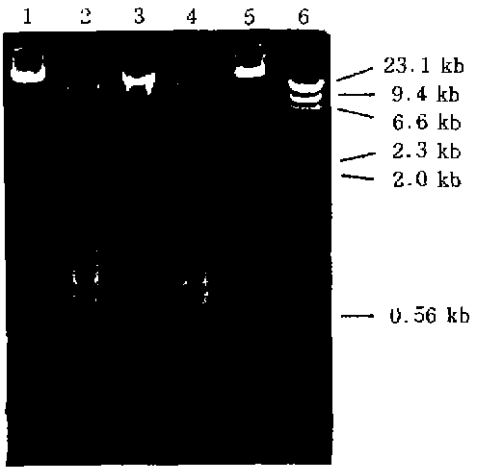

Fig 3. Agarose gel electrophoresis of DNA extracted from HL-60 cells. ① Control; ② Cal 1 mg·L^{-1} for 4 h; ③ Cyc 1 mg·L^{-1} for 4 h, then Cal 1 mg·L^{-1} for 4 h; ④ Tha 0.5 mg·L^{-1} for 2 h; ⑤ Cyc 1 mg·L^{-1} for 4 h, then Tha 0.5 mg·L^{-1} for 2 h; ⑥ λ DNA/Hind Ⅲ marker.

Cal and endoplasmic reticulum Ca^{2+}-ATPase inhibitor Tha could increase intracellular calcium, and induce apoptosis in HL-60 cells. Low dosage of Har or Cam could also induce apoptosis of HL-60 cells, but they did not increase intracellular calcium. Pretreatment with nontoxic concentration of Cyc inhibited apoptosis induced by Cal or Tha, but failed to inhibit apoptosis induced by Har or Cam. These evidence suggest that there may be different pathways led to apoptosis in HL-60 cells: the pathway mediated by Cal or Tha is calcium-dependent, and is sensitive to Cyc; the pathway mediated by Har or Cam is calcium independent, and is insensitive to Cyc.

It is not clear how Cyc blocks apoptosis. Our results showed that Cyc did not prevent the increase of intracellular calcium. So it may function on the downstream of calcium. Since Cyc is a specific inhibitor of protein phosphatase 2B (calcineurin)[8], it is possible that Cyc prevent apoptosis by inhibiting the activity of calcineurin[9]. However, Cyc was also found to affect other factors involved in apoptosis such as "tissue" transglutaminase (tTG), transcription factor Nur77[10,11].

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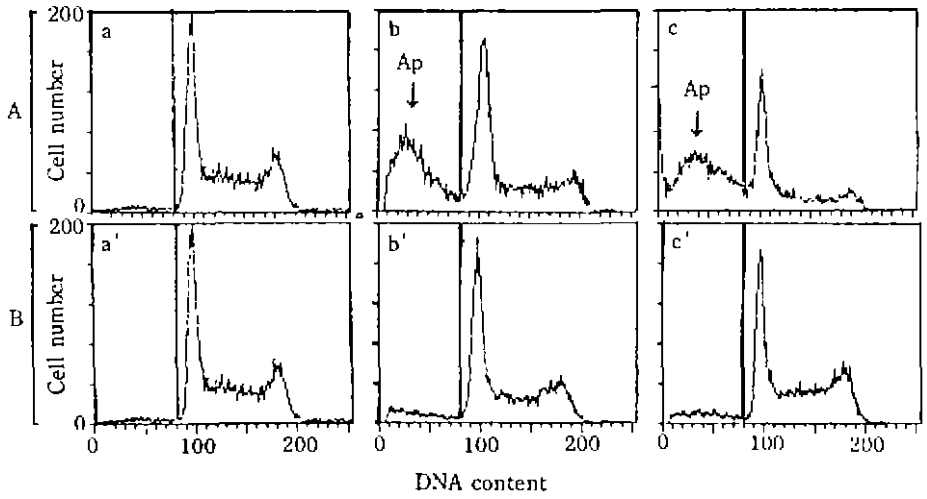

Fig 4. DNA contents of HL-60 cells (PI stain). The peaks in the frequency distribution of control cultures (channel 84 - 110) represent G1 cells, while those at twice the fluorescence intensity represent cells in G2 and motosis; S phase cells lie between the two populations. In b and c, a population with lower DNA stainability represents apoptotic cells (Ap). Cells were pretreated with (B) or without (A) Cyc 1 mg·L^{-1} for 4 h, then treated with Cal 1 mg·L^{-1} for 4 h (b, b') or Tha 0.5 mg·L^{-1} for 2 h (c, c'). Control (a and a').

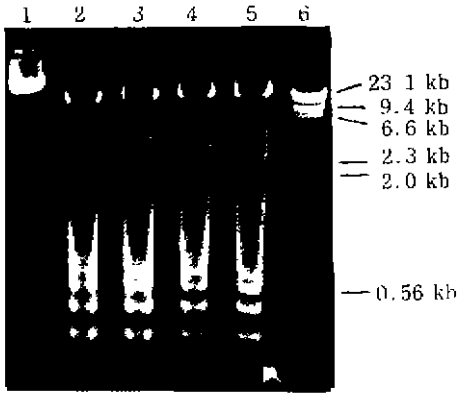

Fig 5. Agarose gel electrophoresis of DNA extracted from HL-60 cells pretreated with or without Cyc 1 mg·L^{-1} for 4 h, then treated with Har 0.05 mg·L^{-1} or Cam 0.05 mg·L^{-1} for 2 h. (1) Control; (2) Har; (3) Cyc + Har; (4) Cam; (5) Cyc + Cam; (6) λ DNA/Hind Ⅲ marker.

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环孢菌素抑制钙离子介导的 HL-60 细胞凋亡[1]

黄其擎, 方 敏, 张鸿卿, 薛绍白
(北京师范大学生物系, 北京 100875, 中国)

关键词 环孢菌素; 卡西霉素; 三尖杉酯碱类; 喜树碱; thapsigargin; 钙; 细胞凋亡; HL-60 细胞; DNA; 流动血细胞计数

目的: 研究环孢菌素(Cyc)在 HL-60 细胞凋亡中的作用. 方法: 通过 DNA 凝胶电泳、细胞形态观察及流式细胞术等方法确定药物三尖杉酯碱(Har),