BIBLID: ISSN 0253-9756 · Acta Pharmacologica Sinica 中国药理学报 1996 Sep; 17 (5): 463-467

Characteristics of harringtonine-resistant human leukemia HL60 cell[1]

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KEY WORDS harringtonines; leukemia HL60; multiple drug resistance; *mdr*-1 genes; glycoproteins; flow cytometry; polymerase chain reaction

AIM: To study the mechanisms of the resistance to harringtonine (Har) in the HL60 cells. **METHODS**: Growth inhibition, karyotype analysis, flow cytometry, Western blotting and polymerase chain reaction. **RESULTS**: The Har-resistant HL60 cell line, named HR20, showed cross resistance to homoharringtonine, doxorubicin, daunorubicin, vincristine, and colchicine. The growth doubling time and the cell numbers in G_1 phase were increased. The accumulation of cellular daunorubicin in the resistant cells was obviously reduced, but distinctly increased by tetrandrine and verapamil. The numbers of telocentromeric chromosome increased and the chromosomal aberration more occured in the resistant cells. The resistant cells overexpressed multidrug resistant *mdr*-1 gene and P-glycoprotein 150 kDa. **CONCLUSION**: The Har-resistant HL60 cell strain belonged to a multidrug resistance strain, overexpressing *mdr*-1 gene and P-glycoprotein.

Tumor cell multidrug resistance is a severe obstacle in clinical chemotherapy, especially occurring in the relapse of tumor patients. The overexpression of P-glycoprotein (Pgp) in the drug-resistant cell membrane is a main cause for drug resistance, and Pgp pumped the antitumor drugs out of the cells[1]. Harringtonine (Har), a domestic antitumor drug, has a sound effect for leukemia[2]. However, whether Har could arouse the drug resistance or not remains a suspensive problem in clinic. So we took human leukemia HL60 cell as a model and obtained a cell strain which was resistant to Har, using a method of stepwise drug induction. In this paper we studied the mechanisms of the resistance to Har in the HL60 cells.

MATERIALS AND METHODS

Drugs Har and homoharringtonine (Hom) were purchased from Beijing Union Pharmaceutical Factory and made up into a solution of 1 $g \cdot L^{-1}$ with PBS buffer. Doxorubicin(Dox) and daunorubicin (Dau) from Farmitalia Carlo Erba Ltd (Italy) were made up into the solutions of 2 $g \cdot L^{-1}$ with saline water. Vincristine (Vin), colchicine (Col), and verapamil (Ver) from Sigma were made up into the solutions of 1 $g \cdot L^{-1}$ with PBS buffer. Bleomycin (Ble) A5 from Huabei Pharmaceutical Factory was made up into a solution of 1 $g \cdot L^{-1}$ with non-serum 1640 medium. Tetrandrine (Tet) from Zhejiang Jinhua Pharmaceutical Factory was dissolved in HCl 1 $mol \cdot L^{-1}$, adjusted to pH 6.7 with NaOH, and added PBS buffer to a final concentration of 1 $g \cdot L^{-1}$. All the drugs were stored at -20 ℃.

Cell culture Human breast cancer cell line, MCF-7 cell and Dox-resistant MCF-7 cell were established by Dr Kenneth H COWAN in National Cancer Institute, USA. Human leukemia HL60 cell and Har-resistant HL60 cell (HR20) were cultured in RPMI 1640 medium containing 10 % bovine serum in 95 % air + 5 % CO_2 at 37 ℃.

Screening of Har-resistant cell line At the beginning the sensitive HL60 cells were treated with Har. After the cells acquired some degree of resistance, the cells were seeded into a 96-well plate for colonal purification. The cells from one of the clones was incubated in the fixed concentration of Har 10 $mg \cdot L^{-1}$ and tolerant only for 24 h at the first. After washing away the drug, the cells were restored to grow normally and then treated with Har. After about 4-month screening, the cells grew slowly in the medium containing Har 10 $mg \cdot L^{-1}$ and were seeded into a 96-well plate for second colonal selection with Har 10, 15, 20, 30, 40 $mg \cdot L^{-1}$. One clone obtained from the cells treated with Har 20 $mg \cdot L^{-1}$ was called HR20, which was continually cultured after incubation in the medium containing Har 10 $mg \cdot L^{-1}$ for 5 d per month.

Determination of drug resistance The logarithmic growth cells 4×10^4/well were seeded into a 24-well plate and added the drugs in triplicate per concentration. After 72 h, cell viability was detected by trypan blue exclusion and cell

[1] Project supported by the National Natural Science Foundation of China, No 39270785 and The Doctoral Programme Foundation for Institution of Higher Education, No9202702.
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Received 1995-08-29 Accepted 1996-05-22

numbers were counted by Coulter Counter Three separate experiments were taken for each drug and the IC_{50} value of each drug was calculated with weighted probit analysis.

Chromosome karyotype analysis[3] *HL60* cell and HR20 cell were treated with Col 0.05 mg·L^{-1} and 5 mg·L^{-1} for 6 h, respectively. The distribution of chromosome numbers was calculated by counting at random from 600 mitosis metaphases.

Flow cytometry The logarithmic growth cells were collected and the percent of cell cycle phases was assayed by flow cytometry[4].

Cellular Dau accumulation was detected by flow cytometry. The logarithmic growth cells were washed by centrifugation with PBS buffer and resuspended in RPMI 1640 medium. Adding Dau 10 mg·L^{-1} to all the samples and other drugs, the cells were incubated at 37 ℃ for 1 h. The viable cell numbers were more than 97 % by trypan blue exclusion. The samples were through filtration and measured. The results were treated by a computer and different diagrams were piled up into one for comparision.

Expression of *mdr*-1 gene detected by polymerase chain reaction (PCR) The primers of PCR were designed by computer assistance. The upstream primer of *mdr*-1 gene was 5′-GTA CCC ATC ATT GCA ATA GC-3′ and its downstream primer was 5′-CAA ACT TCT GCT CCT GAG TC-3′. The upstream primer of β_2-microglobulin (β_2m) as an internal control was 5′-ATG GCT CGC TCG GTG ACC CTAG-3′ and its downstream primer was 5′-TCA TGA TGC TTG ATC ACA TGT CTCG-3′. The primers were synthesized by an Applied Biosystems DNA synthesizer (model 220A, USA). Total cellular RNA was prepared[5]. cDNA synthesis was incubated at 37 ℃ for 30 min in the reation volume of 15 μL containing RNA 1 μg, random primers(Promega) 100 ng, Moloney murine leukemia virus reverse transcriptase 200 U, dNTP 0.2 mmol·L^{-1}, Tris·HCl 50 mmol·L^{-1} pH 8.3, KCl 50 mmol·L^{-1}, $MgCl_2$ 10 mmol·L^{-1}, dithiothreitol 10 mmol·L^{-1}. PCR was carried out in the reaction volume of 25 μL containing primers 0.2 μmol·L^{-1}, cDNA samples 5 μL, dNTP 0.2 mmol·L^{-1}, Taq polymerase (Promega) 1.5 U, KCl 50 mmol·L^{-1}, Tris·HCl 10 mmol·L^{-1} pH 8.3, and $MgCl_2$ 2.5 mmol·L^{-1}. PCR products were amplified by a DNA thermal cycler (Perkin-Elmer/Cetus) for 35 cycles as following parameters: 45 s at 95 ℃, 45 s at 55 ℃, 45 s at 72 ℃ and separated by 2 % agarose gel electrophoresis and taken photoes under uv light.

Western blotting Having been washed with PBS buffer twice, the cells were suspended in buffer A, containing Tris·HCl 10 mmol·L^{-1} pH 7.5, NaCl 10 mmol·L^{-1}, $MgCl_2$ 1.5 mmol·L^{-1}, phenylmethylsulfonyl fluoride 1 mmol·L^{-1}, leupeptin 10 mg·L^{-1}, and 1 % 3-[(3-cholamidopropyl) dimethyl-ammonio]-propansulfonate and broken down by ultrasound. The lysates were centrifuged at 12 000 × *g* for 10 min and taken the supernatant for the experiment. The protein concentration was determined[6]. Protein 100 μg/sample was for Western blotting[7]. The proteins were subjected to SDS-PAGE and transferred to the nitrocellulose membrane for 3 h at room temperature. The antibody to Pgp was purchased from Oncogene Science, Inc and the result was visualised with goat antibody conjugating horseradish peroxidase against rabbit IgG (Zhongshan Biotechnology Company).

RESULTS

Morphological feature and growth character In contrast with the sensitive HL60 cells, morphological feature of HR20 cells did not change. The extension of cell volume and slight adhesion were observed in HR20 cells. A few of HR20 cells extruded into spindles in treatment with Har and restored to sphere after washing away the drug. HR20 cells grew slowly in contrast with HL60 cells and the growth doubling time was 25.2 ± 2.4 h for HR20 cells and 20.4 ± 1.8 h for HL60 cells.

Change of drug resistance In comparison with the sensitive HL60 cells, the resistance to Har in HR20 cells was > 300 fold and > 400 fold to Hom. HR20 cells had cross resistance to Dox, Col, Dau, and Vin, but remained sensitive to Tet and sligbly to Ble A5 (Tab 1).

Tab 1. Cross resistance of HR20 cells. IC_{50} was calculated and compared by weighted probit analysis. The numbers in parentheses were 95 % confidence limits.

Drug	IC_{50}/μg·L^{-1} HR20	HL60	HR20:HL60
Har	3 128 (2792, 3505)	10.22 (9.87, 10.57)	306
Hom	1 275 (1058, 1537)	2.96 (2.12, 4.12)	432
Dox	976 (924, 1032)	8.43 (8.04, 8.83)	116
Dau	629 (390, 1014)	4.74 (4.39, 5.11)	133
Col	1 281 (1153, 1422)	3.02 (2.31, 3.95)	424
Vin	1 354 (1021, 1797)	3.06 (2.67, 3.50)	441
Ble A5	995 (580, 1705)	1 048 (976, 1125)	0.9
Tet	1 983 (1771, 2220)	2 629 (1635, 4226)	0.8

Chromosome analyses HL60 cells had 84 % chromosome numbers between 33 and 48, but the chromosomes in HR20 cells were distributed from 23 to 72, 45 % of which were between 53 and 68. In HR20 cells, aberrant chromosomes such as chromosomal chiasma and breakage increased, and

more telocentric chromosomes were observed. However, double minute chromosomes were not observed.

Change of cell cycle In contrast with HL60 cells, the obvious change of cell cycle in HR20 cells showed increment of about 20 % cells at G_1 phase and reduction of the cells at S and G_2M phases (Fig 1). When detecting PI fluorescence in the same parameters, the diagram of HR20 cells distinctly shifted right, indicating the DNA content increased.

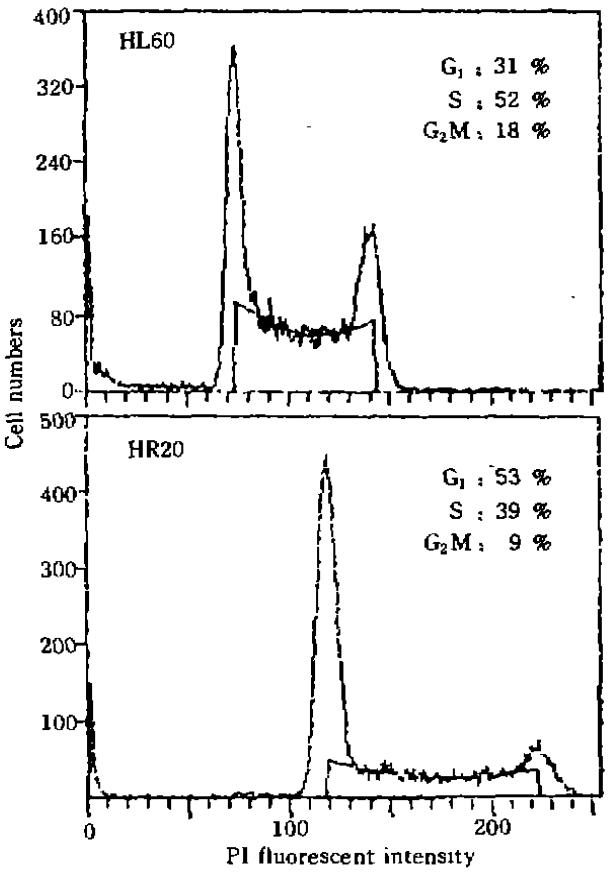

Fig 1. Cell cycle changes of HR20 and HL60 cells. The cells were fixed in 70 % ethanol for 24 h, stained by PI and analysed by flow cytometry.

Dau accumulation In viable cells, the fluorescent intensity of Dau accumulation in HR20 cells was lower than that in HL60 cells (Fig 2A) and obviously increased after HR20 cells were incubated with Ver 5 mg · L^{-1} and Tet 0.5 mg · L^{-1}. The action of Tet was more effective than that of Ver. HR20 cell population was unequal by judging from Dau fluorescent intensity, showing that the amount of Dau accumulation in some cells was similar to that in HL60 cells. The HR20 cells with high Dau accumulation not stained by PI were not dead, but the cell volume was expanded and the nuclei sphered. A few of the cells had 2 nuclei (Fig 2B). According to morphology of dead cells stained by PI, the cells might include the dying cells and a few of dead cells.

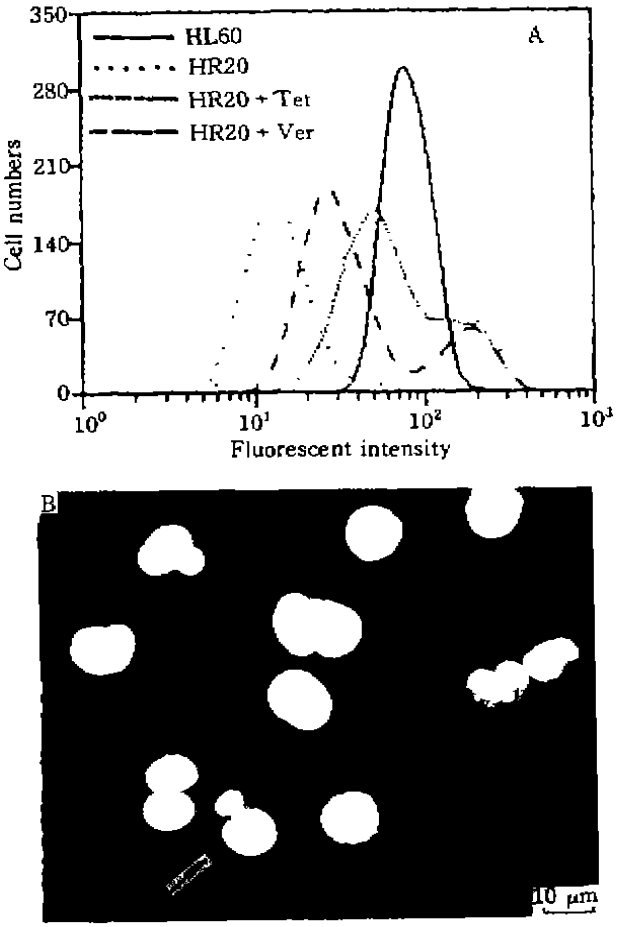

Fig 2. A) Cellular daunorubicin accumulations in HR20 and HL60 living cells analysed by flow cytometry. B) HR20 cells were stained with Hoechst 33342, PI and daunorubicin at the same time. (↑) Cells containing more daunorubicin. × 1000.

Expression of *mdr*-1 gene The overexpression of *mdr*-1 gene in HR20 cells and in Dox-resistant MCF-7 cells were detected by PCR, but not in the sensitive cells (Fig 3).

Expression of Pgp HR20 cells overexpressed Pgp 150 kDa and Dox-resistant MCF-7 cells did Pgp

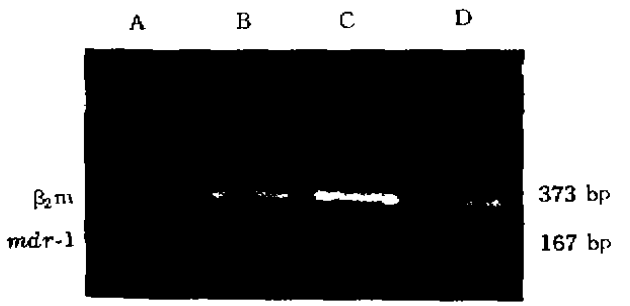

Fig 3. Expressions of *mdr* mRNA analysed by polymerase chain reaction from HL60 cells (A), HR20 cells (B), MCF-7 cells (C), and doxorubicin-resistant MCF-7 cells (D).

170 kDa. However, expression of Pgp was not observed in both sensitive cells.

DISCUSSION

The Har-resistant HL60 cells had cross resistance to Hom, a similar chemical structure with Har, and to Dox, Dau, and Vin, the normal used antitumor drugs in clinic. The results indicated that the Har-resistant HL60 cells was a multidrug resistant strain. Significant reduction of Dau accumulation in the resistant cells meant that it was difficult for Dau to kill the cells. The Har-resistant cells overexpressed Pgp 150 kDa and *mdr*-1 gene, which was in accordance with the results[8]. The results indicate that Har is a substrate excluded by Pgp.

In comparison with the established drug resistant HL60 cell strains, the overexpressed proteins showed great difference. The Vin-resistant HL60 cell overexpressed the proteins with 210, 180, 150 kDa[9], and the Dox-resistant HL60 cell did the proteins with 160, 110 kDa[10]. Another Dox-resistant HL60 cell overexpressed the proteins with 150, 50 kDa, and a 190 kDa protein in the endoplasmic reticulum played main role in the resistance to Dox[11]. The resistance to Har in HL60 cells may be related to other proteins besides Pgp. In this paper we demonstrated that Har aroused multidrug resistance of HL60 cells and the Har-resisant cell overexpressed Pgp and *mdr*-1 gene.

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抗三尖杉酯碱的人白血病 HL60 细胞的特性[1]

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关键词 三尖杉酯碱类；白血病 HL60；多种抗药性；*mdr*-1 基因；糖蛋白类；流式细胞术；聚合酶链反应

目的：研究人 HL60 细胞抗三尖杉酯碱(Har)的机制。方法：生长抑制，核型分析，流式细胞术，蛋白免疫印迹和聚合酶链反应。结果：抗性株(HR20)对高三尖杉酯碱、阿霉素、柔红霉素

R 979.1 R 915-

(Dau)、长春新碱和秋水仙碱均有交叉抗性. 抗性株倍增时间延长，G_1 期细胞增加，细胞内积聚的 Dau 明显减少，粉防己碱和维拉帕米增加 Dau 在抗性细胞内的积聚. 抗性细胞端着丝粒染色体增多，染色体畸变增加，过度表达多药抗性的 *mdr*-1 基因和 P-糖蛋白 150 kDa. 结论：抗 Har 的 HL60 细胞为多药抗药性株，过度表达 *mdr*-1 基因和 P-糖蛋白.

BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1996 Sep; 17 (5): 467 - 470

Oxidized low-density lipoproteins induce apoptosis in macrophages[1]

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KEY WORDS DNA; apoptosis; LDL lipoproteins; peritoneal macrophages

AIM: To examine whether oxidized low density lipoproteins (ox-LDL) might induce apoptosis in mouse peritoneal macrophages (MPM). **METHODS**: Low density lipoproteins (LDL) were isolated from healthy human plasma by ultracentrifugation and oxidized by $CuSO_4$ 10 μmol·L^{-1}. MPM were incubated in a medium containing ox-LDL, LDL, or phosphate-buffer solution (PBS) as control. DNA fragmentation was visualized by agarose gel electrophoresis and determined quantitatively using Hoechst 33258 fluorochrome. **RESULTS**: Ox-LDL, not LDL, elicited typical apoptotic morphological changes (shrinkage of cytoplasm and condensation of chromatin) and DNA fragmentation in a time- and dose-dependent manner. Incubation for 24 h was necessary for ox-LDL 200 mg protein ·L^{-1} to induce DNA fragmentation, and the maximal effect reached at 72 h. The DNA fragmentation after 24-h incubation with ox-LDL at concentrations of 100, 200, and 400 mg protein·L^{-1} amounted to 6.0 % ($P > 0.05$), 9.3 % ($P < 0.05$), and 30.9 % ($P < 0.01$), respectively *vs* PBS control. Dextran sulfate, a scavenger receptor blocker and cycloheximide, a protein synthesis inhibitor, exhibited no effect on DNA fragmentation. However, antioxidant butylated hydroxytoluene (BHT) 20 μmol·L^{-1} completely inhibited Cu^{2+}-mediated oxidation of LDL as well as the apoptosis-inducing effect of Cu^{2+}-exposed LDL. Lysophosphatidylcholine (LPC), an active component in ox-LDL, at concentration up to 60 μmol·L^{-1}, did not elicit DNA fragmentation in MPM. **CONCLUSION**: Ox-LDL induces apoptosis in MPM without involving LPC.

Macrophages can take up modified low density lipoproteins (LDL) to form foam cells[1]. As the atherosclerotic plaque develops, increasing number of macrophage derived foam cells appear in the artery wall. Some of these macrophages die and form the bulk of necrotic gruel at its base[2].

Apart from necrosis, there is an another type of cell death, *ie*, programmed cell death (PCD), and many pathological conditions and chemical agents, such as anticancer drugs, hyperthermia and viral infection can induce PCD[3,4].

Oxidized low density lipoproteins (ox-LDL), potent atherogenic lipoproteins, exist in atherosclerotic lesions, and can be regarded as endogenously biosynthesized cytotoxins. Its potential toxicity to endothelial cells and smooth muscle cells was demonstrated[5]. Ox-LDL triggered PCD in lymphoblastoid cells[6]. This study was aimed to investigate whether ox-LDL might induce apoptosis in macrophages.

[1] Project supported by the National Natural Science Foundation of China, № 39500058 & № 39200047.
[2] Correspondence to Prof GUO Zhao-Gui.
Received 1995-09-25 Accepted 1996-03-13