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Multidrug resistance in leukemic cell line K562/A02 induced by doxorubicin[1]

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AIM: To study the mechanism of the development of multidrug resistance in leukemic cells. **METHODS**: A human leukemic cell line K562/A02 was established by stepwise increase of concentrations of doxorubicin (Dox) in medium. P-glycoprotein was detected by immunohistochemistry assay. The mdr1 gene expression was measured by RT-PCR. The amplification of mdr1 gene in its genome, and DNA topisomerase I (Top I) gene expression were determined by dot-blot hybridization. **RESULTS**: K562/A02 was highly cross-resistant to vincristine (VCR), homoharringtonin (HHT), amsacrine (m-AMSA), daunorubicin (Dau) and etoposide (VP-16), slightly to cytosine arabinoside (Ara-C), but not cisplatin (Cis), methotrexate (MTX) and fluorouracil (5-FU), showing a typical phenotype of MDR. Intracellular accumulation of Dau in K562/A02 was 33 % as high as that in K562. P-glycoprotein P-170 was positive. In K562/A02, the mdr1 gene did not amplify, the mdr1 mRNA level was markedly higher, the Top I mRNA level was lower, and glutathione-*S*-transferase (GST) activity was higher than in K562. **CONCLUSION**: mdr1 mRNA was overexpression and thus the encoded P-170 was responsible for MDR in K562/A02 while Top II or GST may play a role in MDR.

KEY WORDS drug resistance; leukemia K562; glycoproteins; DNA topoisomerase; glutathione transferases; doxorubicin; antineoplastic agents

The anticancer drug resistance is often associated with cross-resistance to multiple, structurally diverse agents. This phenotype of drug resistance is known as multidrug resistance (MDR) and is the main cause of the chemotherapy failure. MDR may be related to mdr 1 gene expression, DNA topoisomerase I (Top I), glutathione-*S*-transferase (GST), DNA repair, *etc*[1-3]. In an attempt to understand the development of MDR, we established a MDR leukemic cell line K562/A02 induced by step-wise increase of concentrations of doxorubicin (Dox) in cell culture medium. The profile of cross-resistance for K562/A02 to other cytotoxic agents, intracellular concentrations of the agents, mdr1 gene expression, Top I mRNA level, the activities of GST in K562/A02 as well as in K562 were studied.

MATERIALS AND METHODS

Materials RPMI-1640 was from Gibco; newborn calf serum was from Tianjin Biochemistry Factory; Dox and daunorubicin (Dau) from Farmitalia Carloerbaltd, Italy; homoharringtonin (HHT) from Institute of Materia Medica, Chinese Academy of Medical Sciences; amsacrine (*m*-AMSA) from Hospital 307, Beijing; etoposide (VP-16) from Beijing Pharmaceutical Factory; vincristine (VCR) from Mingshen Pharmaceutical Factory; Fluorouracil (5-FU) from Shanxi Pharmaceutical Factory; cisplatin (Cis) from Shan-

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dong Pharmaceutical Factory; methotrexate (MTX) from Shanghai 12th Pharmaceutical Factory; cytosine arabinoside (Ara-C) from Experimental Pharmaceutical Factory, Beijing Medical University; Monoclonal antibody JSB-1 from Sanbio Co, Holland; [^{3}H]TdR from Beijing Institute of Atom Energy; verapamil (Ver) from Shanxi Fenghe Pharmaceutical Factory; M-MLV reverse transcriptase from Gibco; Taq Polymerase from Institute of Basic Medicine, CAMS; mdr 2 from American Type Culture Collection; pHaMDR1/A and PC15 were the gifts from Dr Michael M GOTTESMAN (NIH) and Dr Leroy F LIU (Johns Hopkins University), respectively.

Resistant leukemia cell line K562/A02 Cell line K562 was incubated in RPMI-1640 medium containing 20 % new-born calf serum and a sublethal concentration of Dox. By stepwise increasing the concentrations of Dox in the medium the cells became resistant to the inducer and then were selected with limited dilution. The selected cell line K562/A02 was incubated in the medium containing Dox 200 $\mu g\ L^{-1}$ for more than 60 passages and maintained the resistant characteristics in the medium without Dox for two months.

Inhibition of cell growth Using the [^{3}H]TdR incorporation method the IC_{50} concentrations required for 50 % inhibition of cell growth for 72 h were measured for the following drugs: Dox, Dau, HHT, VCR, *m*-AMSA, VP-16, Ara-C, 5-FU, MTX, and Cis. The ratio of IC_{50} between the resistant cell line K562/A02 and the cell line K562 is designated as "resistance fold."

Detection of P-glycoprotein P-170 Using indirect immunofluorescence assay and immunohistochemistry method with monoclonal antibody JSB-1 directed against P-glycoprotein P-170 as our previous report[4].

Determination of intracellular concentrations of drugs Using HPLC[5]. A) Accumulation. K562 and K562/A02 grew in medium containing Dau 2 $\mu mol\ L^{-1}$ and to the medium Cy-A or Ver was added at 90 min. B) Retention. After K562 and K562/A02 grew in the above condition for 90 min, the cells were washed with cold PBS three times at 4 C and then maintained in medium without drug. The intracellular concentrations of Dau were measured at different intervals.

Amplification and transcription of mdr1 and mdr2 genes Fragments 3.0 kilobases (kb) and 4.0 kb from mdr1 and mdr2 cDNA, respectively, digested by EcoRI were used as probes for dot blot hybridization[6]. Genomic DNA was loaded on NC membrane, hybrided with [α ^{32}P]-labelled probe and then radiophotographed. The ratio of areas of K562/A02 to K562 was determined with CS-9000 dual-wavelength flying-spot scanning densitometer. Total RNA was extracted by AGPC method[7] and was reversely transcripted into cDNA. The product was amplified by RT-PCR.

Expression of Top I gene Fragment 1.8 kb from pC15 digested by EcoRI was used as probe for dot blot hybridization[8]. Total RNA was loaded on NC membrane, hybrided with [α-^{32}p]-labelled probe and then radiophotographed. The ratio of areas of K562/A02 to K562 was determined with CS-9000 dual-wavelength flying-spot scanning densitometer.

Activity of GST The GST activities were determined[9].

RESULTS

Multidrug resistance for K562/A02

K562/A02 was highly cross-resistant to the MDR drugs such as VCR, HHT, VP-16, *m*-AMSA, and Dau; slightly to Ara-C besides Dox; but not resistant to non-MDR drugs such as Cis, 5-FU, and MTX, showing a classical phenotype of MDR (Tab 1).

Tab 1. Responses of K562 and K562/A02 to anticancer drugs. $\bar{x}\pm s$. $^*P>0.05$, $^{**}P<0.01$.

Drugs	$IC_{50}/\mu g\ L^{-1}$ K562	K562/A02	Resistance fold
VCR	15±0.7	7 500±820	500**
Dox	5.1±0.9	800±50	160**
HHT	3.3±0.4	390±40	130**
m-AMSA	13±0.2	92±10	71**
Dau	15±0.5	710±30	47**
VP-16	92±1.1	670±80	7**
Ara-C	2.3±0.5	10±3	5**
CPDD	1 160±89	1 300±70	1.12*
MTX	5.2±0.6	3±1	0.6*
5-FU	2 090±140	1 160±60	0.55*

Intracellular drug concentration in K562/

A02 with or without Ver or Cy-A Intracellular concentration of Dau in K562/A02 was about 33 % as high as that in K562 in accumulation and about 50 % in retention. Both Ver and Cy-A enhanced the intracellular accumulation of drug in K562/A02 by 1.38- and 2-fold, respectively, but did not work on K562. Cy-A also increased the intracellular concentration of drug retention by 1-fold (Fig 1).

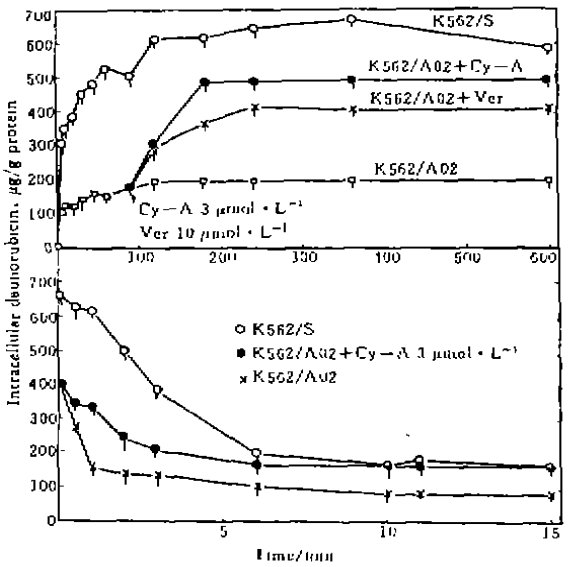

Fig 1. Intracellular concentration of daunorubicin (Dau) in K562 and K562/A02 growing in medium containing Dau 2 μmol L^{-1}, addition of Cy-A or Ver at 90 min.

Amplification of mdr1 gene By the dot blot hybridization the number of copy for mdr1 gene in K562/A02 was almost the same as that in K562, showing little amplification (Fig 2).

Transcription of mdr1 gene By RT-PCR the mdr1 mRNA was detected in K562/A02 but not in K562, indicating overexpression of mdr1 gene transcription in development of MDR (Fig 3).

Expression of P-glycoprotein P-170 K562/A02 showed a strong positive staining,

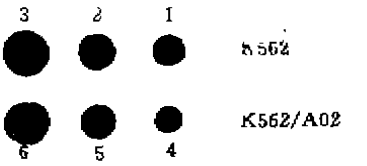

Fig 2. Dot hybridization analysis for mdr1 genomic DNA. 1) K562 DNA 1 μg. 2) K562 DNA 2 μg. 3) K562 DNA 4 μg. 4) K562/A02 DNA 1 μg. 5) K562/A02 DNA 2 μg. 6) K562/A02 DNA 4 μg.

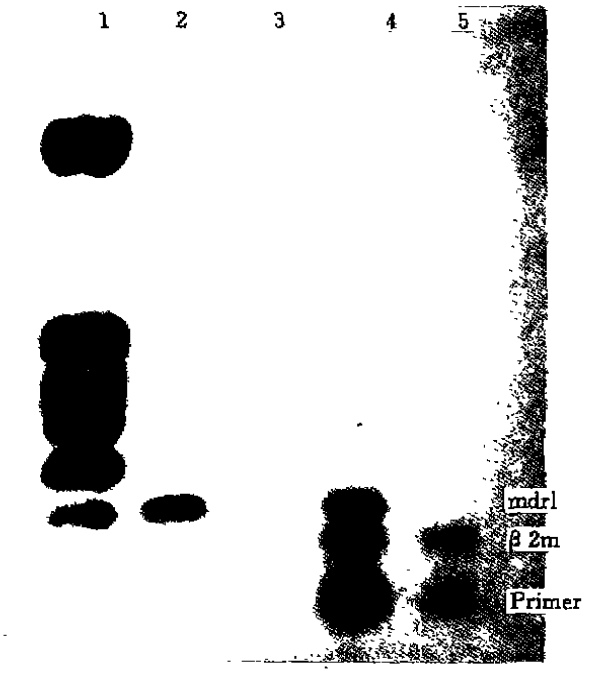

Fig 3. Assay of mdr1 gene expression in K562/A02 and K562 cell lines by RT-PCR. 1) marker. 2) mdr1 cDNA. 3) mdr2 cDNA. 4) K562/A02. 5) K562.

but K562 showed a negative staining. This is consistent with the result of RT-PCR.

Top Ⅱ gene expression The level of Top Ⅱ in K562/A02 was only 37.7 % as high as that in K562 by dot blot analysis (Fig 4).

GST activity The activities of GST were 372 ± 23 pmol L^{-1} $min^{-1}/10^7$ K562/A02 cells and 175 ± 9 pmol L^{-1} $min^{-1}/10^7$ K562 cells, respectively ($P<0.05$).

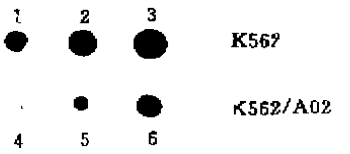

Fig 4. Dot hybridization analysis for Top I gene expression. 1) K562 RNA 1 μg. 2) K562 RNA 2 μg. 3) K562 RNA 4 μg. 4) K562/A02 RNA 1 μg. 5) K562/A02 RNA 2 μg. 6) K562/A02 RNA 4 μg.

DISCUSSION

The leukemic cell line K562/A02 induced by Dox showed cross-resistant to multiple, structurally diverse agents, such as Dau, HHT, VCR, VP-16 and *m*-AMSA, but still sensitive to Cis, 5-FU and MTX, being a typical phenotype of MDR. Our studies showed that P-glycoprotein P-170, an integral membrane protein, was accepted as an energy-dependent efflux pump and its level correlated with the decrease in accumulation of drug in the cells[1].

The overexpression of mdr1 gene showed high transcription instead of amplification and the encoded P-glycoprotein was detected in K562/A02, but not in wild cell line K562. The resistant characteristics of K562/A02 were consistent with those of human resistant tumor cells in patients. The reversal of MDR was performed by MDR-modulators, Ver and Cy-A, but the levels of the highest tolerated doses of Ver were below the optimal drug concentration used *in vitro* for MDR reversal. Therefore we used Cy-A as P-glycoprotein modulator in MDR reversal in a patient with refractory leukemia resulting in complete remission[3].

A second form of MDR, a typical MDR, is associated with drugs that interfere with Top I expression/activity. In this study, Dox, Dau, *m*-AMSA and VP-16 exert their cytotoxic effects by formation of a stable ternary complex. This interaction is believed to stabilize the DNA-Top I-cleavable complex, potentiating DNA damage — a process that interferes with replication and transcription, and leads ultimately to cell death. The fact that the Top I mRNA in K562/A02 was lower than in K562 suggested that Top I might play a role in MDR. GST functions as a detoxicant in cells. The increased activity in K562/A02 compared with that in K562 also suggested that the increase in GST activity might make some contributions to MDR mechanism.

The above results suggested that mechanisms of MDR might involve (1) mdr1 gene overexpression in transcription and the encoded P-glycoprotein responsible for the decrease in intracellular concentration of drug; (2) the lower level of the target enzyme Top I; (3) the increase in GST activity and (4) other unexplored factors.

The MDR mechanisms in K562/A02 showed an SOS for the cells against cytotoxic agents.

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阿霉素诱导的人白血病细胞系 K562/A02多药抗药性

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目的：研究白血病细胞多药耐药发生的机制. **方法**：用逐步增加培养基中阿霉素(Dox)浓度的方法，诱导出一株耐 Dox 的人白血病细胞系 K562/A02. 用[^{3}H]TdR 参入法测定 IC_{50}，HPLC 法测定细胞内药物浓度，免疫组织化学法检测 P-糖蛋白的表达，RT-PCR 法测定 mdr1基因表达，点杂交测定基因组中 mdr1 DNA 和拓扑异构酶 I （Top Ⅱ）基因表达，CDNB 法测定谷胱甘肽-*S*-转移酶(GST)活性. **结果**：K562/A02对 Dox、HHT、Dau、VCR、*m*-AMSA、VP-16具有较强的交叉耐药性，而对 Ara-C 耐药较弱，对5-FU、Cis 和 MTX 不交叉耐药，表现出典型的多药耐药表型. K562/A02细胞内药物浓度明显低于 K562细胞，P-170 阳性表达. K562/A02 细胞中 MDR1基因拷贝数与 K562的无差异，但 mdr1 mRNA 高表达. Top I 的 mRNA 水平低于 K562，GST 的活性增高. **结论**：白血病细胞多药耐药的机制与 mdr1基因表达密切相关，也与 Top Ⅱ和 GST 有关.

关键词 抗药性；白血病 K562；糖蛋白类；DNA 拓扑异构酶；谷胱甘肽转移酶类；阿霉素；抗肿瘤药

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Effects of *Rehmannia glutinosa* polysaccharide b on T-lymphocytes in mice bearing sarcoma 180

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AIM: To study immuno-antitumor action mechanism of RGP-b. RGP-b (*Rehmannia glutinosa* polysaccaride b) is a new component isolated from the herb, had an everage molecular mass of 160 kDa and 5 kinds of monosaccharides as acid-splitting products. Its HPLC showed a main sharp peak at 162 kDa. **METHODS**: The kinetic effects of RGP-b on IL-2 secretion, cytotoxic T-lymphocyte (CTL) activities and L3T4+, lyt-2+ T-lymphocyte subset in mice bearing S180 were observed. **RESULTS**: RGP-b 10 or 20 mg kg^{-1} ip obviously attenuated the decrease of CTL cytotoxity caused by excessive tumor growth on d 9 after the administration, but only partly ameliorated the descent of IL-2. Its effect on lyt-2+ subset was quite parallel with that

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