CHEMOSENSITIZING EFFECT OF VERAPAMIL ON SWISS-3T3 CELLS TRANSFECTED WITH HUMAN MDR1 GENE

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ABSTRACT To further understand the characteristics of drug resistance reversal of verapamil, the relationship between the level of doxorubicin resistance and the magnitude of modulation by verapamil was examined in multidrug resistant Swiss-3T3 cells transfected with human MDR1 gene. In independently isolated transfectants doxorubicin cytotoxicity decreased markedly compared with parent cells. Potentiation of doxorubicin toxicity by a noncytotoxic concentration of 3 $\mu mol \cdot L^{-1}$ of verapamil was much greater in transfectants than in parent cells, while the magnitude of reversal was inversely dependent on the level of resistance. Southern blot hybridization indicated the MDR1 cDNA integration in genomics of each transfectant. Defect in cellular accumulation of doxorubicin was restored by verapamil in transfected cells. The saturation of active drug transport that may involve the magnitude changes of potentiation by verapamil, and the mode of interaction between P-glycoprotein and drugs, were discussed.

Key words Drug resistance; Doxorubicin; Verapamil; MDR1 gene; Transfection; Chemosensitizing effect

Multidrug resistance (MDR) is usually associated with the expression of MDR genes encoding P-glycoproteins(Pgp) in cell plasma membrane[1]. Some agents called resistance modifiers or chemosensitizers, such as verapamil (Ver), completely or partially reverse MDR by competing for Pgp transport and affecting the drug accumulation defect in MDR cells, while often cause little potentiation of drug cytotoxicity in sensitive cells[2]. It is of interest to note the relationship between the magnitude of chemosensitizer effects and drug resistance level expressed, about which little is known. In this work the chemosensitizing effects of Ver on doxorubicin (Dox) cytotoxicity were investigated in human MDR1 cDNA transfected Swiss-3T3 cells.

MATERIALS AND METHODS

Materials The expression plasmid pHaMDR1/A containing human MDR1 cDNA[3] was a gift of

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Dr. M. M. Gottesman (NCI, NIH, USA). Doxorubicin (Dox), colchicine (Col), verapamil (Ver), polybrene and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA), Dimethyl sulfoxide (DMSO) was from E. Merck (Germany), Dulbecco's modified Eagle's medium (DMEM) was from GIBCO/BRL (UK), and calf serum was from the Institute of Hematology CAMS(Tianjin).

Cells and cell culture Mouse Swiss-3T3 cells (gift from Dr. HQ Zhang, Beijing Institute of Basic Medical Sciences, AMMS) were grown in DMEM supplemented with 10% calf serum in humidified air containing 5% CO_2 at 37℃.

Transfection of Swiss-3T3 cells Swiss-3T3 cells were transfected with pHaMDR1/A by polybrene mediated method[4]. Briefly, 4 ml DMEM containing polybrene 40 μg and plasmid DNA 40 μg was added to subconfluently cultured cells in 90 mm dish, and were incubated for further 6 h. The medium was drawn out and 4 ml DMEM containing DMSO 30% was added and cells were incubated for 5 min at 37℃. Then, the cells were washed and incubated in normal medium for 2 d. A plate of cells was transfected without plasmid DNA as control. The transfected and untransfected cells were grown in growth medium containing Col 0.3 μmol·L^{-1}. Two weeks later, the resistant clones were isolated in transfected cells while no resistant clones appeared in untransfected cells. The resistant clones were expanded and routinely cultured in the medium plus Col 0.3 μmol·L^{-1}.

Cytotoxicity assay The transfected clones of Swiss-3T3 cells were grown in a medium containing Col 0.3 μmol·L^{-1} for 2 months after being picked out and cytotoxicity determinations were performed using MTT assay[5]. Cells were plated into 96-well tissue culture plates and exposed to drugs continuously. After incubation for 4 d at 37℃, 50 μl of MTT dye (2 mg·ml^{-1} in Hanks solution) was added to each well and incubated for 4 h. The medium was then aspirated and 100 μl of DMSO was added. The plates were agitated for 5 min and optical density(OD) at 540 nm was read on a Titertek Multiscan MCC/340 plate reader (Flow Laboratories, UK). In all experiments 4 replicate wells were used for each drug concentration. IC_{50} values were obtained from dose-response curves fitted by logistic model[6]. The clones were tested simultaneously when their IC_{50}s were compared directly in order to avoid variations between batches of tests. The degree of drug resistance was expressed as relative resistance(RR) to this drug:

$$RR=\frac{IC_{50}\text{ of tested cells}}{IC_{50}\text{ of parent cells}}$$

The chemosensitizing or reversal effect of Ver on Dox resistance was expressed as reversal factor (RF):

$$RF=\frac{IC_{50}\text{ of Dox without Ver}}{IC_{50}\text{ of Dox with Ver}}$$

Cellular accumulation of Dox One of the transfectants (T9-3T3) was expanded for 10 d in a drug-free medium after 2 month culture in Col. Parent Swiss-3T3 line was used as control. Cells were treated with IC_{50} concentration of Dox in the absence or presence of Ver 3 μmol·L^{-1} (highest non

toxic concentration). Cellular content of Dox was measured as follows[7]. Drug treated cells cultured in 100 or 90 mm dishes were washed 4 times rapidly with 5 ml ice cold phosphate buffered saline and scraped into Eppendorf tubes. After centrifugation at 4℃, the cell pellet was resuspended in distilled water and left overnight in the dark to lyse the cells. Aliquots of 0.1 ml were suspended in 0.9 ml methanol and centrifuged at 4℃, the supernatant was read on a MPF-4 fluorescence spectrophotometer (Hitachi, Japan) (excitation wavelength 468 nm, emission wavelength 585 nm, slit 10 nm, and sensitivity 3.5). The amount of Dox was determined in comparison with a standard Dox curve. Cell lysate 5～10 μl was used for protein assay according to dye-binding method[8], modified to 96-well microplate scale. The Dox concentration was expressed as pmol of Dox/μg of protein. The final concentration of Dox in each sample was corrected by extracellular Dox concentration (IC_{50} value).

Southern blot hybridization Twenty μg of EcoRI-digested genomic DNA was electrophorised on 0.8% agarose gels, transferred to nylon membrane (Boehringer Mannheim, Germany), and hybridized to the full length MDR1 cDNA probe which was labeled with [α-^{32}P]dATP. High SDS concentration condition[9] was used in hybridization.

Statistics Linear correlation between IC_{50}s of Dox in Swiss-3T3 cells and the transfectants were analyzed. The difference of cellular content of Dox between absence and presence of Ver was determined by Student t test.

RESULTS

Effect of Ver on Dox cytotoxicity

The transfectants were grown in a medium containing Col 0.3 μmol · L^{-1} for two months, and MTT assay was performed 20 d after the withdrawal of Col. The results showed that doxorubicin cytotoxicity decreased markedly compared with parent cells(Fig 1).

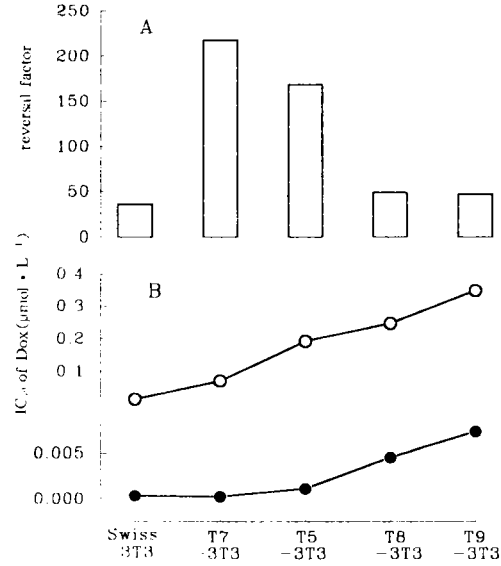

Fig 1 A: Reversal factor of verapamil on Swiss-3T3 cell and each transfectant. B: IC_{50} value of doxorubicin of Swiss-3T3 cell and each transfectant in the absence (○) and presence (●) of verapamil.

The reversal effect of Ver on Dox cytotoxicity was observed both in transfectants and in parent Swiss-3T3 cells. Potentiation of Dox toxicity by a noncytotoxic concentration (3 μmol • L^{-1}) of Ver was much greater in transfectants than in parent cells. A significant correlation between IC_{50} of Dox in Ver sensitization and IC_{50} of Dox in the absence of Ver was observed($P<0.05$). While the magnitude of reversal was inversely dependent on the level of resistance(Fig 1).

Cellular levels of Dox

Cellular accumulation of Dox in the transfectant T9/3T3 cell line decreased compared with its parent cell line. When incubated with equi-effective (IC_{50}) concentration of Dox in the presence of Ver (3 μmol • L^{-1}), intracellular accumulation of Dox in Swiss-3T3 cells did not change significantly at 1 h and 3 h, until at 6 h($P<0.01$), compared with in absence of Ver. Dox accumulation in T9/3T3 cell line was markedly enhanced by Ver at all the time points ($P<0.01$)(Fig 2).

Southern blot analysis

Southern hybridization showed low abundance of different integration pattern of MDR1 cDNA in each transfectant of different degrees of drug resistance(Fig 3).

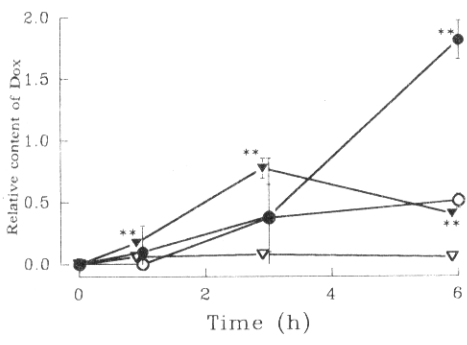

Fig 2 Swiss-3T3 cell (○) and T9-3T3 cell (△) were incubated in IC_{50} concentration of doxorubicin in the absence (open symbles) and presence (filled symbles) of verapamil. Cellular content of doxorubicin was measured and expressed as the ratio of doxorubicin accumulation (pmol/μg protein) to extracellular doxorubicin concentration (μmol • L^{-1}). ** $P<0.01$ absence vs presence of verapamil.

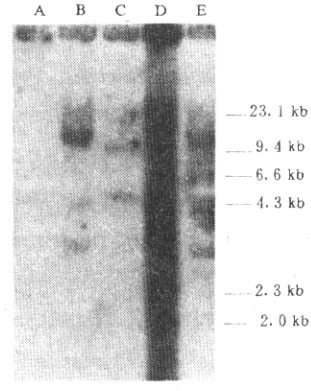

Fig 3 Southern blot hybridization detection of MDR1 gene. Twenty μg DNA from Swiss-3T3 (A), T5-3T3(B), T7-3T3(C), T8-3T3(D) and T9-3T3 (E) were digested by restriction endonuclease EcoRI. DNA was separated by agarose gel electrophoreses and blot-hybridized to the full length MDR1 cDNA.

DISCUSSION

It is generally accepted that chemosensitizers exert their effect by inhibiting Pgp binding with cytotoxic drugs, thus affecting the defect of drug accumulation in MDR cells[10]. Our results conformed to this assumption. We found that the magnitude of Ver reversal decreased on increasing

Dox resistance in these transfectants. This may be explained by a saturation of active drug transport, as it has been reported that a saturation level was easier to reach in less highly resistant cells[11].

The interaction between Pgp and reversing agents has not been fully understood. It has been shown that MDR modulators are divided into two types: one type is transported by Pgp and the other is not[12]. In a series of MDR mouse leukemia cells, it has been observed that the magnitude of modulation in Dox cytotoxicity by trifluoperazine, is dependent on the level of drug resistance, but that in vincristine cytotoxicity is not[13]. These results and the results shown here implied that different modes of interaction existed between Pgp and different drugs. The chemosensitizing effect of Ver may be a multifactorial process besides its interaction with Pgp[14]. We have observed a sensitizing effect of Ver on Dox toxicity in parent Swiss-3T3 cells (Acta Pharmacol Sin, in press). We postulate that an intracellular Ver dependent chemosensitizing effect presented in the parent cells, which may also involve in the modulation in Dox cytotoxicity of MDR1 gene transfected Swiss-3T3 cells.

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维拉帕米对 MDR1 基因转染的 Swiss-3T3 细胞的化疗增敏作用

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摘要 为进一步了解维拉帕米对抗药性逆转作用的特征，在人 MDR1 基因转染的 Swiss-3T3 多药抗药性细胞，观察了维拉帕米逆转幅度与阿霉素抗性水平的关系。各个转染细胞与母细胞相比，阿霉素毒性明显降低。非毒性浓度(3 μmol·L^{-1})的维拉帕米对阿霉素毒性的增强作用，在转染细胞均高于母细胞，但逆转幅度与抗性水平成反比。Southern 杂交显示，转染细胞基因组中有 MDR1 cDNA 整合。转染细胞的阿霉素蓄积障碍可被维拉帕米纠正。讨论了药物主动转运的饱和现象在维拉帕米增强效应中的作用，以及 P-糖蛋白与药物相互作用的方式。

关键词 抗药性；阿霉素；维拉帕米；多药抗药基因；转染；化疗增敏作用