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SHORT COMMUNICATION

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The structure of P-glycoprotein and the secretion of lysosomal enzymes in multidrug-resistant cells

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Abstract We have previously demonstrated that multidrug-resistant cells have a lower content of lysosomal enzymes, a consequence of an increased rate of secretion. The question was therefore to know whether an intact functional P-glycoprotein was necessary for expression of this property. Control NIH3T3 and *mdr1*-gene-transfected cells (pHaMDR1) were used together with 2 variants either lacking 23 amino acids at the carboxyl terminal (pHaMDRC 23) or in which 4 extra amino acids are inserted (pHaMDRBL2). Transfected and variant cells exhibited reduced uptake of [^{3}H]-vinblastine and [^{3}H]-daunomycin, a finding consistent with their drug resistance. By contrast, only pHaMDR1 cells had a reduced level of *N*-acetyl glucosaminidase that paralleled an increased rate of secretion of the same enzyme. The mutant cells secreted lysosomal enzyme at the same rate and had the same intracellular lysosomal enzyme content as NIH3T3 cells. Abnormal behavior of lysosomal enzymes in multidrug-resistant cells therefore seemed to require an intact P-glycoprotein molecule. Although sequestration in lysosomes and then secretion of drugs may possibly contribute to protection, it would not be an essential component of multidrug resistance.

Key words Multidrug resistance · Lysosomal enzymes Mutant P-glycoprotein

Introduction

Typical multidrug resistance (MDR) is associated with the overexpression of transmembrane glycoprotein P170 [2, 5, 15, 16]. It is widely believed that P170 acts mainly as a nonspecific efflux pump [6] through an adenosine triphosphate (ATP)-dependent process [1]. Nevertheless, recent works have focused on a decreased influx [13] that could be related to the presence of P170 [4]. Another basis for resistance is that the drugs that enter the cell by a diffusion process are compartmentalized and sequestered in intracellular vesicles [9, 12]. In support of this notion is our finding that MDR cells accumulate drugs in lysosomes. The level of their lysosomal enzymes is lower than that of their drug-sensitive counterparts, a consequence of an increased rate of secretion of their lysosomal content [8]. P170 is apparently capable of inducing the MDR cell to hypersecrete both drug and enzyme, lowering their intracellular concentrations. Furthermore, it has been found that verapamil, which is supposed to act mainly as a competitive inhibitor of the P170-mediated efflux, can interfere as well with the membrane traffic of resistant cells, thus disrupting the process of drug trapping and vesicular extrusion [10]. Some, but not all, lysosomotropic agents are capable of enhancing the cytotoxicity of chemotherapeutic drugs [8]. Monensin, a drug that is known to disrupt intracellular vesicular function, can overcome MDR [11].

In this paper we provide evidence that hypersecretion and lowered levels of lysosomal enzymes, linked to intact P170, are not essential components of drug resistance, although the putative protective effect of sequestration of drugs could nonetheless be operative.

Materials and methods

Cell lines

We used Wild-type NIH3T3 pHaMDR1 3T3 cells transfected with full-length cDNA encoding P170 [7, 14], pHaMDRC 23 cells bearing P170

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lacking 23 amino acids at the carboxyl terminal, and pHaMDRBL2 bearing P170 with the tetrapeptide histidine-proline-glycine-glycine inserted at amino acid 1179, at the second ATP-binding domain [3]. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Gibco, Grand Island, N.Y.) in Falcon 6- or 24-well plates.

Lysosomal enzymes

The secretion of lysosomal enzymes was studied by incubating the cells in 10 m*M* TRIS-HCl (pH 7.0), 1 m*M* $MgCl_2$, 250 m*M* sucrose (TMS), and 150 m*M* NaCl. After centrifugation, the cell pellet was homogenized in cold TMS and aliquots of supernatant and homogenate were assayed for NAGA at pH 4.7 using *p*-nitrophenyl-*N*-acetylglucosamine as the substrate [17].

Cytotoxicity studies

Cells (5×10^4) were grown in 24-well plates containing 1.5 ml medium/well in the presence of different concentrations of vinblastine or daunomycin (in triplicate). After 48 h, medium was replaced with 0.1 ml trypsin (0.025%) in phosphate-buffered saline containing Versene (0.15%). After incubation for 5 min at 37 °C, 0.9 ml DMEM containing 10% fetal calf serum was added and cells were counted in a Coulter counter. The IC_{50} is defined as the concentration of drug that inhibits growth by 50% as compared with the growth of cells in the absence of drugs.

Drug uptake

Uptake of [^{3}H]-vinblastine (Moravek Biochemicals, Brea, Calif.) and [^{3}H]-daunomycin (Dupont, NEN, Boston, Mass.) into cells was measured in 24-well plates. The medium was removed by suction and the cells in each well were washed once with 2.5 ml cold TMS and then incubated at 37 °C in 150 µl TMS containing 0.1 µg [^{3}H]-vinblastine or [^{3}H]-daunomycin (0.05 µCi). After incubation, the medium was removed by suction and the cells were washed with 2.5 ml ice-cold TMS, which was removed within 7 s. Cellular radioactivity was transferred from each well to vials by two 250-µl washes to 0.1 *N* NaOH and then measured. Each incubation was done in triplicate, and the results are expressed as the amount of drug taken up per milligram of cell protein. The bicinchoninic acid assay for proteins was used (Sigma Chemical Company, St. Louis, Mo.).

Results and discussion

The rate and extent of accumulation of [^{3}H]-vinblastine and [^{3}H]-daunomycin into drug-sensitive cells (NIH3T3) was considerably greater than that into MDR cells (pHaMDR1, pHaMDRC 23, and pHaMDRBL2). Accumulation by NIH3T3 was 5–8 times greater than that by pHaMDR1 cells containing wild-type P170, whereas cells bearing mutant P170 appeared to take up slightly more drugs, especially pHaMDRC 23 cells (Fig. 1).

From Table 1 it can be seen that the rate of secretion of NAGA was significantly enhanced in pHaMDR1 cells (wild-type) and the cellular content of enzyme was only 25% of that of the drug-sensitive parental line, NIH3T3. On the other hand, cells transfected with DNA coding for mutated human P170, although resistant to vinblastine and daunomycin, had levels of NAGA and secretion rates that were essentially the same as those of the drug-sensitive NIH3T3 cells.

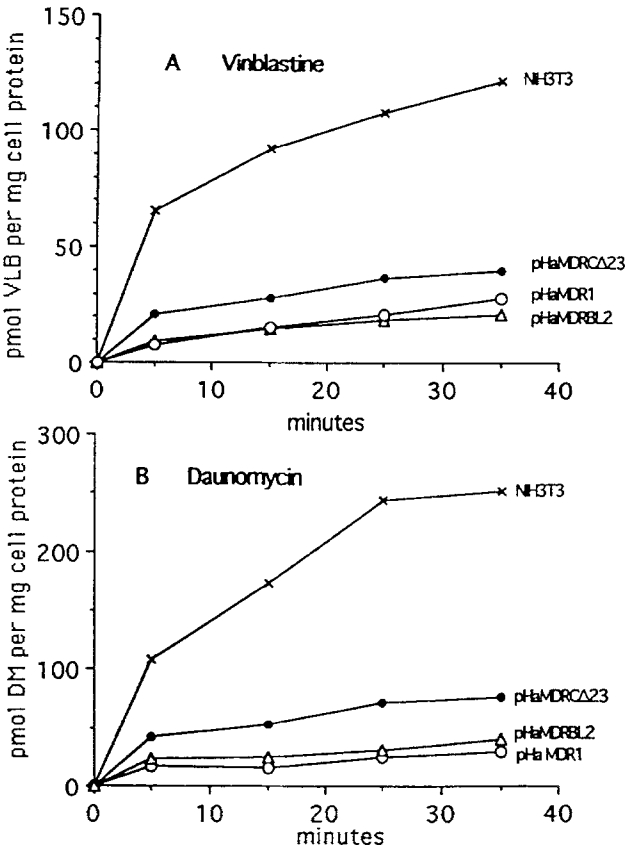

Fig. 1 A, B Accumulation of drugs by drug-sensitive and MDR cells. Uptake of **A** [^{3}H]-vinblastine and **B** [^{3}H]-daunomycin was measured as described in Materials and methods. Three experiments were carried out, each time point in triplicate (SD < 10%). ×, NIH3T3; ○, pHaMDR1; ●, pHaMDRC 23; △, pHaMDRBL2

Transfection of cells with DNA encoding the human *mdr1* gene endows them with a "pure" MDR phenotype. Experimentally, these cells are superior to MDR cells generated by selection of survivors repeatedly challenged by toxic levels of drugs, because survivors may carry along other unknown and undesirable characteristics, making their MDR phenotype much more complex to study. We had previously studied such cell lines and found MDR to be associated with increased secretion and lowered levels of lysosomal enzymes [18]. The same characteristics are exhibited by transfected pHaMDR1 cells. P170 could therefore be involved in this way. We have found additional evidence for that by electron microscope study revealing the presence of P170 in exocytosis vesicles of human leukemic MDR CEM cells (manuscript in preparation).

Other results from our laboratory (not shown) have demonstrated that MDR CEM cells have a reduced uptake of drugs that could be the consequence of a decreased influx and an increased efflux, a phenomenon that in both cases could be dependent on P170, according to Gottesman [4]. The same MDR cells exhibited an increased secretion of lysosomal enzymes [18], a mechanism that therefore remains efficient, even if drug influx is altered. Hence, whatever the mechanism of reduced drug uptake in

Table 1 Characteristics of the cell lines studied. Results are expressed as mean values ± SEM

Total enzyme activity and rate of secretion of cells sensitive and resistant to drugs:

Cell	Total NAGA[a]	Secretion of NAGA (%)[b]	Number of experiments
NIH3T3	300.0±26.4	10.8±1.5	6
pHaMDR1	71.0±11.2	22.7±2.1	6
pHaMDRC 23	259.5±18.1	11.9±1.9	3
pHaMDRBL2	271.6±44.2	14.5±1.8	3

[a] Substrate hydrolyzed (nmol/mg protein per 30 min)
[b] Percentage of total enzyme secreted in 30 min when cells are incubated in TMSucrose containing 150 m*M* NaCl

Cytotoxicity of drugs in drug-sensitive and -resistant cells, expressed as IC_{50} values:

Cell	Vinblastine (n*M*)	Daunomycin (n*M*)
NIH3T3	30.7±5.2	22.7± 6.3
pHaMDR1	>440	378.3±56.2
pHaMDRC 23	>440	330.0±61.0
pHaMDRBL2	>440	140.0±17.1

pHaMDR1 3T3 cells and mutants, it could not interfere with the exocytosis observed.

Transfection of cells with DNA encoding certain insertion and deletion mutations of *mdr1* has generated colchicine-resistant cells [3]. Currier et al. [3] have found that cells transfected with the *mdr1* gene lacking 23 amino acids at its carboxyl terminal nonetheless conferred a resistance to colchicine that was only slightly lower than that of cells transfected with unaltered *mdr1* itself. This may be reflected in the relatively higher rates of accumulation of vinblastine and daunomycin by pHaMDRCA 23 cells (Fig. 1) as compared with the pHaMDR1 and pHaMDRBL2 lines. The latter cell line was found to be fully resistant to colchicine [3], whereas our data suggest that it is not fully resistant to daunomycin (Table 1). These mutated P170-bearing cell lines do not demonstrate any alteration in lysosome activity. The evidence presented herein therefore suggests that this activity is dependent on an intact carboxyl-terminal segment of P170, for both cell lines bearing different mutations in this region of P170, one in an ATP-binding region, remain drug-resistant, but secretion and enzyme levels are the same as in the drug-sensitive parent, devoid of significant amounts of P170. Thus, the drug-resistance function of P170 is dissociated from its effect on lysosomes.

When NIH3T3 cells are cotransfected with a cloned gene for a major excreted protein (a precursor of lysosomal proteases) and the human *mdr1* gene, which serves as a selection marker, there is increased production and secretion of major excretory protein (MEP), equivalent to that of transformed NIH3T3 cells [7]. Increased secretion of MEP has been ascribed to its overproduction but, in fact, could be due to the mere presence of P170 in the membrane. It would be of real interest to cotransfect the MEP gene with DNA encoding mutant P170. This dominant selectable marker would continue to function, but perhaps secretion would be the same as in the parental NIH3T3 line.

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References

1. Ambukar SV, Lelong IH, Zhang J, Cardarelli CO, Gottesman MM, Pastan I (1992) Partial purification and reconstitution of the human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis. Proc Natl Acad Sci USA 89: 8472
2. Bradley G, Juranka PE, Ling V (1988) Mechanism of multidrug resistance. Biochim Biophys Acta 948: 87
3. Currier SJ, Ueda K, Willingham MC, Pastan I, Gottesman MM (1989) Deletion and insertion mutants of the multidrug transporter. J Biol Chem 264: 376
4. Gottesman MM (1993) How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal Foundation award lecture. Cancer Res 53: 74
5. Gottesman MM, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62: 385
6. Hammond JR, Johnstone RM, Gros P (1989) Enhanced efflux of [^{3}H]vinblastine from chinese hamster ovary cells transfected with a full-length complementary DNA clone for the *mdr1* gene. Cancer Res 49: 3867
7. Kane SE, Troen BR, Gal S, Ueda K, Pastan I, Gottesman MM (1988) Use of a cloned multidrug resistance gene for coamplification and overproduction of major excreted protein, a transformation-regulated secreted acid protease. Mol Cell Biol 8: 3316
8. Klohs WD, Steinkampf RW (1988) The effect of lysomotropic agents and secretory inhibitors on anthracycline retention and activity in multiple drug-resistant cells. Mol Pharmacol 34: 180
9. Marquardt D, Center MS (1991) Involvement of vacuolar H^+-adenosine triphosphatase activity in multidrug resistance in HL60 cells. J Natl Cancer Inst 83: 1098
10. Sehested M, Skovsgaard T, Van Deurs B, Winther-Nielsen H (1987) Increased plasma membrane traffic in daunorubicin resistant P388 leukaemic cells. Effect of daunorubicin and verapamil. Br J Cancer 56: 747
11. Sehested M, Skovsgaard T, Roed H (1988) The carboxylic ionophore monensin inhibits active drug efflux and modulates in vitro resistance in daunorubicin resistant Ehrlich ascites tumor cells. Biochem Pharmacol 37: 3305
12. Slapak CA, Lecerf JM, Daniel JC, Levy SB (1992) Energy-dependent accumulation of daunorubicin into subcellular compartments of human leukemia cells and cytoplasts. J Biol Chem 267: 10638
13. Stow MW, Warr JR (1993) Reduced influx is a factor in accounting for reduced vincristine accumulation in certain verapamil-hypersensitive multidrug-resistant CHO cell lines. FEBS Lett 320: 87
14. Ueda K, Cardarelli C, Gottesman MM, Pastan I (1987) Expression of a full-length cDNA for the human *mdr1* gene confers resistance to colchicine, doxorubicin, and vinblastine. Proc Natl Acad Sci USA 84: 3004
15. Van der Blick AM, Borst P (1989) Multidrug resistance. Adv Cancer Res 52: 165
16. Vendrik CPJ, Bergers JJ, Dejong WH, Steerenberg PA (1992) Resistance to cytostatic drugs at the cellular level. Cancer Chemother Pharmacol 29: 413
17. Warren L (1989) Stimulated secretion of lysosomal enzymes by cells in culture. J Biol Chem 264: 8835
18. Warren L, Jardillier J-C, Ordentlich P (1991) Secretion of lysosomal enzymes by drug-sensitive and multiple drug-resistant cells. Cancer Res 51: 1996