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Drug-transporter proteins in clinical multidrug resistance

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Summary

Upon exposure to chemotherapeutic drugs, mammalian cells can acquire resistance to structurally and functionally unrelated compounds, a property known as multidrug resistance (MDR). One MDR mechanism, i.e. by the overexpression of a plasma membrane protein, P-glycoprotein (P-gp), has been identified at the molecular level. The *mdr1* gene-encoded P-gp acts as a drug efflux pump, lowering intracellular drug concentration by active extrusion of drugs from the cell. The role of P-gp in determining clinical resistance to multiple anticancer drugs is likely to be largely different for various tumor types. Recently we selected a monoclonal antibody (mAb LRP56) for strong, granular cytoplasmic reactivity with MDR tumor cell lines without P-gp (over)expression. None or weak reactivity was observed with parental and P-gp positive cell lines. We hypothesize that as yet-undefined drug transport-mediating proteins are inserted in intracellular membranes lining the exocytotic compartment and thus may contribute to clinical multidrug resistance.

Multidrug Resistance

Drug resistance is a common problem in cancer chemotherapy. Some tumors

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never respond to cytostatic drug treatment; others initially respond well but eventually regrow and become resistant, with the possible emergence of resistant tumor cell subclones. That phenomenon may result from epigenetic or genetic mutations induced by the administered antitumor agents, or it may represent the selection of preexisting resistant cell populations in a heterogeneous tumor. The mechanisms which enable tumor cells to survive and proliferate in the presence of relatively high concentrations of toxic substances are not fully understood. A great number of *in vitro* biochemical studies have described differences between parent ('sensitive') and resistant cells selected by exposure to cytotoxic drugs of different chemical classes, i.e. altered enzyme activity (e.g. 7-dihydrofolate reductase, glutathione-*S*-transferase, topoisomerase II and stimulated DNA repair; for review see Ref. 1). These differences are thought to account for resistance to distinct, or closely related, cytostatic drugs. Frequently, however, upon exposure to chemotherapeutic drugs, mammalian cells can acquire resistance to many structurally and functionally unrelated compounds i.e. they display the phenomenon of multidrug resistance (MDR). The drugs included in the MDR spectrum have different targets and do not share a common metabolic activation or inactivation pathway. Major active anticancer agents such as anthracyclines, vinca alkaloids, podophyllotoxins and actinomycin D belong to this category of drugs. Therefore, MDR must be due to a decrease of drug concentration at the site of action.

P-glycoprotein

Thus far, one mechanism by which mammalian cells can acquire MDR, the overexpression of a plasma membrane protein, P-glycoprotein (P-gp), has been

TABLE I

P-gp expression in human tumors classified according to treatment status or putative resistance to cytostatic drugs: non-treated, primary tumors (or retrospectively, favorable outcome) or recurrent tumors (or retrospectively, unfavorable outcome).

Tumor type	P-gp positive fraction		Reference
	'Favorable'	'Unfavorable'	
Bladder cancer	3/12 (prim) ^a	4/5 (recurr)	Hikage [21]
Soft tissue sarcoma	1/21 (surv) ^b	8/9 (died)	Chan [18]
Renal cell cancer	0/4 (sens) ^c	10/17 (resist)	Mickisch [22]
Neuroblastoma	3/31 (untr) ^d	5/18 (tr)	Goldstein [20]
Ovarian cancer	0/40 (untr)	3/10 (tr)	Bourhis [19]
Breast cancer	2/7 (sens)	3/3 (resist)	Ro [23]
Esophageal cancer	0/6 (sens)	6/12 (resist)	Robey [24]
Myeloma/lymphoma	0/5 (sens)	7/7 (resist)	Salmon [25]

^aprim/recurr, in primary tumors/at recurrence.

^bsurv/died, patients who survived/died after subsequent chemotherapy.

^csens/resist, sensitive/resistant to subsequent chemotherapy.

^d(un)tr, (un)treated: patients had (not) previously received chemotherapy with relevant cytostatic drugs.

defined in considerable detail (for review see Ref. 2). P-gp acts as a drug efflux pump, lowering the intracellular drug concentration by active extrusion of drugs from the cell. P-glycoprotein is encoded by *mdr1*, a member of the *mdr*-gene family, in humans. High levels of P-gp expression are found in many noncancerous tissues (adrenal, colon epithelium, bile canaliculi, etc.) preventing too high intracellular concentrations of potentially toxic (mainly lipophilic) molecules. Cloning of *mdr1* cDNA and subsequent transfection experiments have confirmed that P-gp can confer MDR in vitro [5]. In addition to mRNA probes for Northern blotting, P-gp reactive mAbs for immunohisto-/ cytochemical staining, have recently greatly improved our knowledge about the clinical relevance of P-gp-mediated MDR. Table I summarises results from retrospective studies indicating that increased P-gp expression in these tumors, or positivity at recurrence, predicts poor responsiveness to treatment with MDR-related cytostatic drugs. Strikingly, however, the reverse is not the case, since resistance to multiple drugs in the clinic often does not relate to P-gp overexpression. No clear overexpression of P-gp is found in most patients with frequently occurring solid tumors, such as lung cancer and ovarian cancer, in contrast to tumors derived from normal tissues expressing P-gp, where P-gp expression is generally high (see also Ref. 26). In those tumors P-gp expression relates to differentiation and its measurement may have prognostic utility. Clearly other molecular mechanisms exist that can also mediate MDR. Intriguingly, such a role for the product of the other most closely related member of the *mdr*-gene family (*mdr3*) seems unlikely. Although the *mdr3* product has been suggested to contribute to MDR in B-cell lymphocytic leukemias [6], transfection of the *mdr3* gene did not confer drug resistance. Moreover, *mdr3* expression would have been noted in those clinical studies using the anti-P-gp mAb C219, since this mAb cross-reacts with the *mdr3* gene product [7].

Non-P-glycoprotein Mediated MDR

In the last few years several groups have independently generated MDR tumor cell lines which do not contain detectable levels of *mdr1* mRNA and stain negatively with all available anti-P-gp mAbs [4,8,9,10,11]. Importantly, these so-called 'non-Pgp' MDR tumor cell lines show resistance to the same panel of cytostatic drugs as their P-gp positive counterparts, although minor, but distinct, differences can be observed such as relatively strong etoposide resistance in non-Pgp positive tumor cell lines (Table II). Comparative mechanistic studies on these cell lines have revealed several interesting points. First, when generating SW1573 lung cell carcinoma-derived MDR cell lines we observed that non-Pgp MDR preceded the development of P-gp overexpression [9, 10]. The early MDR variants, selected at relatively low cytostatic drug (doxorubicin) concentrations, actually did not contain detectable *mdr1* mRNA in contrast to low level expression in the parent cell line. Only at relatively high drug concentrations *mdr1* message became upregulated. Secondly, several non-Pgp MDR cell lines show a reduced intracellular accumulation of cytostatic drugs which are energy dependent. This finding suggests that, like in P-gp positive MDR cells, the drugs are actively prevented from entering the cytoplasm, or constantly removed by a putative drug transporter. Third, in studying the non-Pgp mitoxantrone-resistant

TABLE II

Drug resistance patterns in various P-gp positive and negative *mdr* tumor cell lines

P-gp positive	Doxorubicin	Vincristine	VP-16	Gramicidin D
SW1573/2R16O (lung) ^b	35 ^a	480	120	146
MCF7/D40 (breast) ^c	75	190	11	8
H69/LX4 (lung) ^d	85	17–1060	88	—
LOVO/DR4 (Colon) ^e	56–129	200	63	>30
P-gp negative				
SW1573/2R120 (lung) ^b	4	17	45	2
MCF7/MITOX (breast) ^c	8	22	26	6
H69AR (lung) ^e	41–100	>55	16–59	>3
HT1080/DR4 (fibrosarcoma) ^f	222	25	837	1

^aResistance factors, defined as the concentration of drug inhibiting growth of resistant cells to 50% (IC50) divided by IC50 of the parent cells.

^bKuiper et al. [9].

^cTaylor et al. [27].

^dTwentyman et al. [28].

^eMirski et al. [29].

^fSlovak et al. [30].

gastric carcinoma cell line EPG85-257, Dietel et al., [4] observed a characteristic pattern of membrane vesicle formation. Vesicle formation and release was observed from the plasmalemma of the resistant cells, indicating an outward transport mechanism. This supports the view that vesicular drug binding, processing and extrusion may be involved in chemoresistance. Interestingly, such an exocytotic mechanism has been demonstrated to represent a key mechanism in intracellular routing for a great variety of molecules (see Ref. 3). Central in this mechanism are transporter molecules with structural resemblance to P-glycoprotein.

The Transmembrane Transporter Superfamily

It has now become clear that the *mdr1*-gene belongs to a much larger group of families; a superfamily of genes found both in prokaryotes and eukaryotes [3,12,13,14]. Its members are implicated in the ATP-dependent transport of various substrates across cell membranes. The most highly conserved portions of these molecules reside in cytoplasmic domains, around two short sequence elements believed to be involved in ATP binding. Most eukaryotic members of this superfamily probably function as units consisting of two transmembrane domains (each containing 6 segments that span the lipid bilayer) and two cytoplasmic domains containing the ATP binding sites. These include P-glycoprotein and the human cystic fibrosis transmembrane regulator (CFTR; possibly mediating chloride ion transport). Many of the prokaryotic members are produced as unit halves, with only one transmembrane and one ATP binding site; for example the white gene in *Drosophila melanogaster*, transporting eye pigment, and *oppD* and *oppF* in *S.*

typhimurium, transporting oligopeptides. Very recently genetic evidence was obtained that the transport of antigens, or peptide fragments thereof, into a membrane-bounded compartment in which insertion into the MHC-Class I grooves should take place, is also mediated by a related transport ATPase. The so-called HAM1 gene (coding for the putative Histocompatibility Antigen Modifier) spans approximately 10 kb of DNA and shows striking sequence homology (around 50%) to the *mdr*-gene products. The molecular mass of the HAM1 protein is predicted (assuming no glycosylation) to be around 65 kDa [3]. Apparently, the transmembrane transporter family provides more members than previously appreciated. Considering the similar patterns of drug resistance and transport in non-P-gp MDR as compared to P-gp-mediated MDR, we postulate the overexpression of another member of the superfamily of these transporters.

Fig. 1. Hypothetical model of Resistance Protein (RP) and P-glycoprotein (P-gp) mediated multidrug resistance. RP and P-gp do not belong to the same gene amplicon, since they can be independently upregulated under cytostatic pressure and may even be coded for by genes located on different chromosomes. Both RP and P-gp, however, would belong to the same superfamily of genes coding for ATP-dependent transporter proteins specialized in transport across membrane barriers. P-gp is primarily located in the outer membrane and removes toxic molecules directly into the extracellular space. In contrast, the putative RP transporter proteins are inserted in intracellular membranes lining the exocytotic compartment, thereby primarily removing toxic materials, such as cytostatic drugs and preventing these from harming cell function. However, P-gp may well be functioning in part intracellularly and RP may be present at the plasmamembrane as well.

Development of Reagents Reactive with Putative non-Pgp MDR Pumps

As yet no mAbs have been described that could identify such putative pumps in non-P-gp MDR cell lines. Clearly, the P-gp-specific mAbs available so far (MRK16, C219, JSB1, etc.) recognize epitopes not shared with the more remote members of the family [2,15,16,17]. A comparable situation exists for the immunoglobulin superfamily which includes not only all immunoglobulins, but also T cell-receptor molecules and various cellular adhesion molecules, all being recognised by different sets of monoclonal antibodies [32]. Nevertheless, recently a polyclonal (rabbit) antibody has been raised against a synthetic peptide corresponding to a P-glycoprotein sequence domain, which is highly reactive with 50-kDa and 190-kDa proteins contained in non-Pgp HL60 MDR tumor cells [8]. These proteins were not detected in drug-sensitive cells. Importantly, analysis of membrane subfractions showed that p190 is located primarily in the endoplasmic reticulum with only low levels contained in the plasma membranes. Very recently, using a non-P-gp MDR cell line (2R120, derived from the SW1573 non-small cell lung carcinoma cell line), we have been able to generate a monoclonal antibody (LRP-56) showing characteristic granular, cytoplasmic staining in all non-Pgp tumor cell lines tested so far (Scheper, Broxterman, Scheffer et al., unpublished data). These tumor cell lines have been derived from different histogenetic origins, including lung and breast cancer. The preliminary data favour the view that LRP-56 recognizes (a) putative pump(s) mediating non-Pgp MDR (see Fig. 1). Further studies are presently carried out to identify the antigen(s) recognized by this new mAb. These studies should define the contribution of a new drug transporter protein to clinical MDR and its relation to physiological processes such as cellular metabolite and/or peptide transport.

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