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Review

Cell biological mechanisms of multidrug resistance in tumors

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ABSTRACT **Multidrug resistance (MDR) is a generic term for the variety of strategies tumor cells use to evade the cytotoxic effects of anticancer drugs. MDR is characterized by a decreased sensitivity of tumor cells not only to the drug employed for chemotherapy but also to a broad spectrum of drugs with neither obvious structural homology nor common targets. This pleotropic resistance is one of the major obstacles to the successful treatment of tumors. MDR may result from structural or functional changes at the plasma membrane or within the cytoplasm, cellular compartments, or nucleus. Molecular mechanisms of MDR are discussed in terms of modifications in detoxification and DNA repair pathways, changes in cellular sites of drug sequestration, decreases in drug-target affinity, synthesis of specific drug inhibitors within cells, altered or inappropriate targeting of proteins, and accelerated removal or secretion of drugs.**

A central goal in the study of chemotherapy is to understand how tumor cells can become drug resistant by lowering the intracellular concentration of antitumor agents and/or altering the ability of these drugs to affect their targets. During the past 10 years, scores of different tumor and transformed cell lines have been studied, using equally as many different types of antitumor drugs. A general theme that has emerged from these investigations is that the mechanisms of multidrug resistance (MDR) are opportunistic in their manipulation and modification of normal pathways of cellular homeostasis. This article reviews both what we know—that a variety of changes in cellular mechanisms can produce MDR—and what we do not—that is, which, if any, cellular changes actually lead to MDR. In this article emphasis is given to experimental evidence that supports a variety of mechanisms for MDR, specifically: (*i*) altered composition and physical interactions of plasma membrane phospholipids; (*ii*) enhanced drug binding and cellular accumulation; (*iii*) changed levels of expression and activity of plasma membrane or endomembrane channels, transporters, and translocators; (*iv*) altered rates of endocytosis and subsequent subcellular targeting of endosomes; (*v*) altered rates and extent of exocytosis; (*vi*) modified ionic environments, such as pH, Ca^{2+} concentration of the extra-, intra-, or subcellular compartments; and (*vii*) alterations in the activity and expression of proteins necessary for drug detoxification and DNA replication and repair systems.

Our understanding of MDR in tumor cells has been limited by our lack of knowledge of the particular properties that make these cells more sensitive than surrounding normal tissue to cytotoxic drugs. More fundamentally, it is also unclear whether MDR is the consequence of tumors' losing a hypersensitivity to drugs or of alternative mechanisms that make the cells more resistant to the drugs. It has been suggested that tumors are extra sensitive to drugs because their replication rates are higher than normal cells. This may be the basis of their increased sensitivity to drugs that affect DNA replication and the cytoskeleton of the cell, which are the primary targets of chemotherapy. An alternative but not mutually exclusive explanation is based on the observation that chemotherapeutic drugs accumulate in tumor cells to higher concentrations than in normal cells. The higher sensitivity may reflect solely the higher intracellular drug concentration.

Drug resistance may subvert the same mechanisms that make tumor cells hypersensitive. If tumor cells are more sensitive because of changes in their cell cycle program, then MDR may result from cells remaining longer in a particular stage of the cell cycle. If tumor cells are hypersensitive because of their higher cytoplasmic/nucleoplasmic drug concentrations, then MDR may result from lowering intracellular drug levels. This could be accomplished by a number of strategies: preventing drug influx, limiting cytoplasmic accumulation, increasing efflux, or shifting the subcellular distribution of drugs away from their targets. Any or all cellular responses may separately or synergistically result in the MDR phenotype. Alternatively, MDR cells may use molecular modifications unrelated to the mechanisms that lead to hypersensitivity in tumor cells.

This article reviews the MDR literature to explore the changes in the cell biology of tumors that could result in MDR to chemotherapy. However, in any evaluation of this field, there are two issues that, although beyond the scope of this review, should be kept in mind. One concerns the potential differences between the MDR described for cells in tissue culture and the clinical MDR observed in tumors. A second concerns the functional interrelationships between effective drug structure or presentation (monomer, dimer, protein-associated, metal-associated), drug accumulation (plasma membrane, cytoplasm, Golgi, endoplasmic reticulum, lysosome, endosomes, secretory vesicles, nucleoplasm, nuclear envelope), and cytotoxicity (loss of membrane integrity, DNA damage, autophagy, initiation of apoptosis).

What Happens to Cells That Develop MDR?

There are three major changes in cells that develop MDR: (*i*) a decreased accumulation of cytotoxic drugs; (*ii*) changes in activity or expression of certain cellular proteins, including the P-glycoprotein (Pgp), MDR-associated protein (MRP), glutathione *S*-transferase π, protein kinase C, and DNA topoisomerase II; and (*iii*) changes in cellular physiology affecting the structure of the plasma membrane, the cytosolic pH, and the rates and extent of intracellular transport of membranes, as well as lysosomal structure and function. A dominant feature of MDR cell lines is a decreased accumulation of cytotoxic drugs (reviewed in refs. 1 and 2). This is in distinction to the considerable variability in the level of expression of proteins related to MDR and the diversity of physiological changes associated with the MDR phenotype. The observation that a particular protein or physiological change is not observed in a particular MDR cell line should be taken as evidence for the flexibility of adaptive responses available to the cell rather than proof that a particular protein or physiological change is not involved in some MDR mechanisms.

Abbreviations: MDR, multidrug resistance; Pgp, P-glycoprotein; MRP, MDR-associated protein; ABC, ATP-binding cassette.

A number of proteins have been observed to be overexpressed in MDR cell lines. The first protein to be associated with MDR was a 170-kDa transmembrane glycoprotein called the Pgp, the product of the *MDR1* gene (2, 3). This protein is strongly homologous (4, 5) to a family of ATP-binding cassette (ABC) protein membrane transporters which translocate proteins such as STE6 (which in yeast transports a small peptide **a** factor) (6, 7) and HlyB (which in *Escherichia coli* transports a large protein, hemolysin) (8). Although Pgp-mediated drug resistance is often used synonymously with MDR, there are non-*MDR1*-expressing drug-resistant cell lines. Two other proteins overexpressed in MDR cell lines are also members of the ABC family. MRP (9, 10) is 180 kDa and found predominately on intracellular organelles. A 110-kDa ABC protein is found primarily in lysosomes (11). Other proteins overexpressed in MDR include glutathione *S*-transferase π (12–16), catalase (16, 17), and thymidylate synthase and metallothionein (16), as well as a subunit of the vacuolar H^+-ATPase (18). In contrast, topoisomerase II expression appears to be down-regulated (19–21).

When examining studies of MDR that describe changes in protein expression, there are a number of issues that should be considered. Typically, the relationship between modifications in the expression of most of these proteins and the occurrence of MDR has been demonstrated indirectly through correlation. Only the Pgp has been transfected into cells and demonstrated to affect drug sensitivity. Second, there may be many more proteins, as yet to be identified, that play an active role in MDR. For many years, the Pgp (MDR1) was the only protein recognized to be overexpressed in tumor cells. Although non-MDR1 cell lines had been examined, it is only recently that a number of additional proteins have been found to be overexpressed in MDR cells. Underexpression of proteins may also lead to an MDR phenotype. This has been suggested for the "alternate" form of MDR that has been ascribed to a decreased expression of topoisomerase II (13, 16, 19–21). There may also be proteins whose level of expression is unchanged but whose activity is modified by co- or posttranslational modifications. Finally, cell lines often contain both over- and underexpressed proteins related to drug resistance. MDR may be a functional state which is the consequence of an interplay between the altered activities of these proteins.

Since enhanced nucleocytoplasmic drug accumulation appears to be a diagnostic parameter for sensitive cells, specific changes in transport and intracellular drug compartmentalization may have considerable influence on the development of MDR. Such observed changes include an alkaline shift of cytoplasmic pH, an increased transport of membrane through the endocytic system, and an increased vacuolization, including changes in lysosomal structure and physiology.

The Models

The most pronounced and consistent observation in MDR cell lines is a decreased accumulation of cytotoxic agents. Several investigations have indicated a decreased influx of drugs (22–29), an increased efflux of drugs (27, 28, 30–33), and a decreased trapping of drugs either in the cytosol (34–36) or in subcellular compartments (36, 37). Evidence has been published consistent with the plasma membrane, cytoplasm, cytoplasmic organelles, and the nucleoplasm all containing modified or under/overexpressed cellular proteins that may result in MDR. What follows is a description of some of these potential mechanisms. The relative contributions of each MDR-related change have not been determined and in any particular cell line any or all may be utilized.

Several models have been proposed to account for multidrug resistance: (*i*) an ATP-driven transporter that pumps drugs out of the cell; (*ii*) an increased trapping of drugs in intracellular organelles away from the cytosol and nucleoplasm; (*iii*) an increased rate of exocytosis that results in drug efflux from the intracellular organelles; (*iv*) an alkaline shift of cellular pH that reduces the accumulation of the drugs (most of which are weak bases) inside of cells; (*v*) nuclear mechanisms, including modifications of DNA binding, DNA repair, and permeability of the nuclear envelope; (*vi*) alternative cytoplasmic mechanisms, including detoxification pathways; and (*vii*) changes in plasma membrane structure which affect drug permeability.

(i) ATP-Driven Drug Efflux Model. The most generally accepted hypothesis for MDR suggests that Pgp uses ATP to power a molecular pump that then extrudes chemotherapeutic molecules from the cell (ref. 32 and reviewed in ref. 2). This model proposes that chemotherapeutic agents diffuse down a concentration gradient into the cell (or its membrane) and that the pump either transports the drugs out of the cytosol or serves as a "flippase" to expel them from the bilayer (38).

(ii) Compartmentalization. The target for most antitumor cells is the DNA within the nucleus. According to this model, changes in the ability of internalized drugs to reach critical concentrations at intranuclear sites can seriously limit the effectiveness of drug therapy and lead to MDR. Chemotherapeutic drugs primarily accumulate in the acidic compartments of the cell, the trans Golgi and lysosomal compartments (36, 37, 39, 40). Any effects on drug sequestration will affect the cyto- and nucleoplasmic concentration.

(iii) Exocytosis. Since anticancer drugs accumulate in the endocytic/exocytotic pathways, an increased rate of exocytosis (37) can result in greater trapping of drugs into sequestered transport compartments followed by expulsion of the drugs from the cell. This would decrease the drug concentration in the cytoplasm.

(iv) pH Hypothesis. Most cytotoxic drugs are weak bases with pK values between 7.4 and 8.2 (41–43). In their neutral form, they are hydrophobic and easily traverse membranes. In their protonated form, they are membrane impermeant. The relative distribution between the neutral and charged forms is determined by the concentration of protons. The pH within tumor cells is considerably more acidic than that of normal cells (44). This acidity results in the protonation of these molecules in the cytosol, thereby trapping them in their membrane-impermeant but biologically active form (45). Further, it is the protonated form of these drugs that can bind to their targets such as DNA (45–50), RNA (49, 51), and tubulin (52, 53). Thus, the acid pH of tumor cells could result in drug accumulation by ionic ligation (intracellular binding) and/or ionic trapping (more acidic cytoplasm) (36). This accumulation would favor the partitioning of drug into the nucleoplasm, leading to the cytotoxic effect. The pH of MDR cells is usually more alkaline (54, 55) than that of drug-sensitive cells, a condition that would act to reverse cellular accumulation of the drugs.

(v) Nuclear Mechanisms. Since the principal target for most presently employed chemotherapeutic agents is the DNA, it is reasonable to assume that MDR is achieved in cells by preventing drug accumulation in the nucleus and the resultant drug proximity or binding to DNA and associated nuclear proteins. Studies examining the interaction of acridines, actinomycins, and anthracyclines with DNA demonstrate that such drugs can inactivate the DNA template in transcription and replication by inducing topoisomerase-II-mediated single-strand breaks in the DNA (13, 21, 56). Potential nuclear targets of opportunity for the induction of MDR are through modifications in (*i*) mechanisms of drug translocation across the nuclear envelope/pore complex; (*ii*) binding sites in the DNA, RNA, nucleoli, and nuclear matrix; (*iii*) DNA repair mechanisms; and (*iv*) efficiency of mRNA export and protein import into the nucleus.

(vi) Cytoplasmic Mechanisms. There are a number of cytosolic mechanisms for eliminating toxic reagents, including the enhanced activity of the pentose phosphate shunt (57) and changes in glutathione metabolism (58–60). Enzymes involved in both of these pathways are modified in some MDR cell lines. A potential mechanism for drug cytotoxicity is related to the free-radical-forming ability of these drugs. Superoxide, hydrogen peroxide, and hydroxyl radicals are reactive oxygen species which can form in the reduction of oxygen by the anthracycline semiquinones. The Fe(III)–doxorubicin complex is able to catalyze the oxidation of thiol by O_2, promoting the oxidative destruction of macromolecular targets (13, 14, 16, 17, 58–60).

(vii) Plasma Membrane Mechanisms. The plasma membrane in MDR cells appears different from that in drug-sensitive parental cells (61). The alterations may reflect changes in specific phospholipid interactions with proteins associated with MDR. Alternatively, these differences in membrane composition and organization may be due to ionic interactions of drugs with the head groups of phospholipids which are asymmetrically distributed in either leaflet of the bilayer (62–64). Such changes could have a significant influence on the transmembrane partitioning of the drug by a non-channel diffusion-mediated process.

Evaluation of the Models

ATP-Driven Drug Efflux Pump. Shortly after the identification of the Pgp, it was noticed that, in the presence of azide, anticancer drugs accumulate in "drug-resistant" cell lines (32), suggesting an ATP-driven process. This model was significantly strengthened when the Pgp was cloned and demonstrated to have two ATP-binding sites and a strong homology to a family of membrane transporters (4, 8). Transfection of cells with Pgp is sufficient to confer an MDR phenotype—a significant observation which firmly establishes a role for Pgp in MDR (65, 66). A number of experimental observations support a plasma-membrane drug-transport role for this protein. The Pgp is found in the plasma membrane (a requirement for a pump), although it is also observed in numerous internal organelles. Mutational analysis of this protein has demonstrated that site-specific amino acid replacements can modify the specificity of Pgp for drugs (67–69) and inhibit ATP hydrolysis, a requirement for efflux activity. Photoaffinity labeling of Pgp can be demonstrated with derivatized drugs and efflux inhibitors. Monoclonal antibodies to Pgp have been shown to inhibit drug efflux (70–72) while also interfering with drug and inhibitor binding (73). Plasma membrane vesicles from MDR cells overexpressing Pgp showed a higher rate of drug transport than those vesicles from drug-sensitive cells (74).

There is considerable evidence to support the hypothesis that the Pgp functions as an ATPase to modify the cellular concentration of drugs. It remains unresolved whether it is a transport channel for the drugs. There are at least four diagnostic features used to identify an ATPase pump that remain to be demonstrated for the Pgp: (*i*) specificity; (*ii*) transport by the isolated protein; (*iii*) binding of substrate to the transporter; and (*iv*) fixed stoichiometry of ATP hydrolysis to transport. *Specificity.* It is proposed that the Pgp transports a diverse assortment of unrelated molecules (2). In contrast, all previously characterized pumps demonstrate quite limited and defined specificity. *Reconstitution.* Until recently it has not been possible to demonstrate transport by the purified reconstituted protein [although it has been possible to demonstrate drug-stimulated ATP hydrolysis by the Pgp (75)]. It is often difficult to find the proper solubilization and reconstitution conditions (76). However, even with crude membranes the maximum rates of transport are 5 pmol/mg of total membrane protein per 10 min (74), which is 1 molecule transported per Pgp molecule per 1.9 hr. [The same authors estimated that Pgp is 1% of their membrane protein (75).] Reconstitution of Pgp and drug transport in proteoliposomes was recently reported (77). However, when the colchicine concentration was 0.6 μM (a little higher than chemotherapeutic levels) the rate of transport was 1 molecule per Pgp molecule per 10 hr. These rates of transport are not likely to compensate for the relatively rapid rates at which these drugs accumulate in cells and are many orders of magnitude slower than the rates of all other transporters. *Binding.* It has been difficult to demonstrate clear binding of the substrates to the Pgp. While some binding has been reported, it was necessary to use high concentrations (30 μM doxorubicin, 160 μM colchicine, 170 μM puromycin, and 10 μM vanadate) to compete for the binding of only 7.5 nM vincristine (74). *ATP.* It has not yet been possible to demonstrate a clear stoichiometry of ATP hydrolysis to transport.

There are a number of additional problems with a drug-efflux pump. Drug influx is reduced in MDR cells (based on measurements of initial rates) (22–29). It is difficult to explain this with a drug-efflux model. A key piece of evidence supporting the drug-efflux model is the observation that verapamil reverses some forms of MDR and binds to the Pgp. However, if verapamil blocks a transporter, it should work at concentrations equivalent to the concentration of transporter, not the substrate to be transported. However, verapamil must be used at concentrations ≥10 μM to block transport of chemotherapeutic agents at 100 nM to 1 μM. This suggests that verapamil may produce its effect by other means—that is, by directly complexing with the drugs rather than the transporter. This is consistent with the observation that many of these anticancer drugs complex with agents such as chloroquine that can reverse MDR (78). While verapamil has been reported to bind to the Pgp, it requires a 200-fold molar excess of antitumor agents to compete for binding. There is the additional problem that MDR cells are usually more resistant to the drug to which they are exposed during selection or treatment than to other drugs (79). Even with cells that are selected on the same drug there are often variations in the sensitivity to other drugs (14).

Finally, there is the issue of Pgp's homology to the ABC proteins (5). This family includes STE6 (which in yeast transports the **a** mating factor) and HlyB (which in *E. coli* transports hemolysin, or any protein with a hemolysin signal sequence) (80–82). Indeed, the STE6 function can be complemented in yeast by transfection with mdr3 (83). Whether STE6 can induce the MDR phenotype in sensitive cells has, as yet, not been shown. A similar role for Pgp as a peptide translocator is inferred from experiments demonstrating its expression in CHO cells selected for their resistance to a cytotoxic tripeptide (*N*-acetylleucylleucylnorleucinol) (84). There is a growing body of evidence supporting a model by which peptide or protein translocation across membranes occurs through transmembrane protein-conducting channels (85–87). Similarly, overexpression of Pgp yields a concomitant increased chloride channel activity (88). This raises a number of mechanistic questions, central of which is how this protein can be both an aqueous channel and a pump.

Compartmentalization/Exocytosis/pH. The compartmentalization/exocytosis/pH models are closely intertwined. Many of the anticancer drugs are fluorescent, which has facilitated microscopic studies of their subcellular distribution. Their fluorescence is primarily punctate, an observation that is indicative of their localization to intracellular organelles—primarily Golgi and lysosomes (36, 40, 89). Any mechanism that increases the trapping of drugs in these compartments facilitates cell survival by decreasing drug concentration in the cytosol and nucleoplasm. An increased transport of membrane vesicles from the endocytic-secretory pathway to the surface exocytoses the drugs from the cell (37). One key mechanism that affects both trapping of these drugs in these compartments and the transport out of

the cell is pH. Many of these drugs are weak bases that accumulate in acidic membrane compartments. This was first demonstrated by showing the accumulation of these drugs in either erythrocyte ghosts (90, 91) or liposomes (92) is determined by the lumenal pH. Shifts of cellular pH also affect the rates of vesicular transport and exocytosis (93, 94).

Compartmentalization. Four different kinds of experiments suggest a role for subcellular compartmentalization of antitumor drugs in MDR. First, during MDR there is a shift of antitumor drug fluorescence from a nuclear to a peripheral staining, a shift which is reversed by verapamil (89, 95–97). Second, a number of the proteins overexpressed in MDR are localized to intracellular organelles. Third, drugs that reverse MDR, such as verapamil and chloroquine, accumulate in the lysosomes and affect the lysosomal structure and function. Fourth, there is an increase in the number of intracellular vacuolar membranes in some MDR cells.

(*i*) Shifts of subcellular fluorescence. An adriamycin-resistant human large cell lung cancer cell line that overexpresses MRP (190-kDa membrane protein) shows an altered pattern of subcellular drug localization (98). Fluorescent daunorubicin partitions into the cytoplasm and nucleus of the sensitive parent line, but it is confined to cytoplasmic perinuclear vesicles in resistant cells (98). The addition of verapamil increases nuclear fluorescence. Another study attempted a direct comparison of the subcellular localization of adriamycin between cell lines overexpressing Pgp (EMT6/AR1.0 mouse mammary tumor cell line) and cell lines overexpressing MRP (COR-L23/R human large cell lung cancer line previously mentioned). The sensitive parent EMT6 cell line demonstrates predominantly nuclear fluorescence, with some particulate cytoplasmic fluorescence and very low levels of plasma membrane fluorescence. The MDR line shows much fainter fluorescence, with the loss of nuclear fluorescence comparatively greater than the loss of cytoplasmic fluorescence. The MRP cell line shows reduced nuclear fluorescence when compared with the parental cell line. In agreement with the previously described results, an intense area of perinuclear staining is observed in the MRP line that is localized to the Golgi. Adding verapamil increases the intensity of fluorescence in both MDR lines, particularly in the nucleus.

(*ii*) Subcellular localization of MDR proteins. MRP has been localized to the endoplasmic reticulum and, to a lesser extent, the plasma membrane. A 110-kDa protein with homology to Pgp and MRP is overexpressed in lysosomal and vesicular membranes (11). The vacuolar H^+-ATPase subunit C is overexpressed in MDR HL60 cells (18). Even the Pgp is observed on intracellular membranes (S.M.S., unpublished observations). The number of intracellular organelles increases substantially in some MDR cell lines. Certain drugs that reverse MDR, such as verapamil, chloroquine, and acridine orange, accumulate in these compartments (99, 100). Verapamil accumulates in the lysosome and disrupts the functioning of a number of lysosomal enzymes (37, 101), some of which may be important for accumulation of the antitumor agents. Chloroquine and acridine orange are often used as indicators for the lysosomes and can cause an alkaline shift of organelle pH. This would block drug trapping and expose the nucleoplasm and cytoplasm to higher concentrations of antitumor drugs.

(*iii*) Lysosomal contributions. There have been a number of reports demonstrating the existence of more vacuolar compartments in MDR cells. The chemosensitizer SR33557 (a potent inhibitor of acid lysosomal sphingomyelinase) has been shown to modulate the subcellular distribution of adriamycin in MDR mouse leukemia cells (P388/ADR) (102). Incubation of these cells with SR33557 demonstrates that the adriamycin and the SR33557 are colocalized within large intracellular vesicles. Analysis with probes specific for the lysosomal and mitochondrial compartments suggests that these vesicles are neither mitochondria nor functional lysosomes. Pretreatment of cells with SR33557 completely inhibits sphingomyelin metabolism, which presumably results in the formation of laminated inclusions. It was suggested that the redistribution of adriamycin to these myeloid bodies prevented its expulsion by Pgp and is the key mechanism behind the action of SR33557 (102). A somewhat similar phenomenon is observed in MDR cells treated with chloroquine. This lysosomotropic agent can enhance the cytotoxicity of vinblastine in the vinblastine-resistant CEM/VLB100 human T-cell lymphobast leukemic cell line. The cytotoxic activities of vincristine, daunorubicin, and adriamycin are also elevated. A histological analysis demonstrated that the vinblastine-resistant cells contained more cytoplasmic vacuoles than the drug-sensitive parental line. Treatment with chloroquine, vinblastine, vincristine, adriamycin, and daunorubicin resulted in the appearance of many more cytoplasmic vacuoles in treated cells than in controls (100). These cells also stained more intensely for the lysosomal enzyme acid phosphatase. In light of these studies, it is possible to suggest that both SR33557 and chloroquine reverse MDR by a direct or indirect influence on lysosomal integrity, structure, physiology, and/or maturation/aging. Like SR33557, chloroquine inhibits the activity of lipid-degrading lysosomal enzymes. Such inhibition could directly lead to the intracellular accumulation of the type of lipophilic structures observed in Niemann–Pick disease. It may also lead to alterations in lysosome function/morphology that can enhance the accumulation of drug to higher levels, overloading the enhanced efflux activity of resistant cells. In this manner, chemosensitizers induce the creation of more drug "sink compartments" within cells that are capable of concentrating drugs against a gradient, resulting in considerably enhanced cellular accumulation within the cytoplasm. An interesting connection has been proposed between the modulation of MDR and ligand-toxin conjugate cytotoxicity. Pharmacological agents that reverse MDR and enhance cytotoxicity can equally enhance ligand-toxin conjugate cytotoxicity. Since ligand-toxin conjugates are generally activated within lysosomal or prelysosomal compartments, it appears that modifications in lysosomal activity/structure may influence a variety of drug uptake pathways that contain a concentration-dependent component that is presumably pH sensitive. The enhanced secretion of lysosomal enzymes observed to correlate with Pgp expression may be significant in this context (103). It is also important to note that sphingomyelin has been implicated as a cofactor in the transport of cholesterol from the Golgi to the plasma membrane (104). Changes in the metabolism of sphingomyelin may also influence the composition of the Golgi (enhanced cholesterol concentration) and plasma membrane (cholesterol depletion).

Exocytosis. Membrane recycling is increased in MDR cells (105, 106). Experiments with pleiotropically resistant Ehrlich ascites tumor cells show a significantly increased plasma membrane traffic to the endosomal compartment in comparison with drug-sensitive cells (105). Investigations with P388 cells show an approximately 4-fold increase in the plasma membrane area participating in recycling together with an increased endosomal volume, number, and membrane area in resistant cells (106). This plasma membrane traffic is significantly inhibited by the calcium channel blocker and inhibitor of MDR, verapamil, but is not affected by the antitumor drugs. The MDR human lymphoblastic leukemic cell line overexpresses Pgp and demonstrates a significantly enhanced secretion of lysosomal enzymes (103). The ability of this cell line to accumulate the drug vinblastine is significantly reduced. Addition of verapamil to these cells results in a decrease in both drug efflux and lysosomal enzyme secretion. These results suggest that in this cell line the presence of Pgp may, in some indirect manner, lead to increased exocytosis of lysosomal

enzyme, ultimately resulting in cellular depletion. The cationic drug vinblastine, which accumulates in the lysosomes and acidic vesicles, is then also eliminated from the cell by secretion mediated by exocytosis. This may provide either the principle or a supplementary pathway for drug efflux.

A number of the proteins overexpressed in MDR are members of the ABC family. They bear structural homology to the cystic fibrosis transmembrane regulator (CFTR) and, as with CFTR, overexpression of these proteins is correlated with an increased number of ion channels (88, 107–110). In the intestine, a switch occurs from CFTR to Pgp expression as the cells migrate across the crypt–villus boundary (111). A switch from CFTR to Pgp expression was also observed in the uterine epithelium with the onset of pregnancy. Considering the role of CFTR as a regulator of secretory activity in epithelia (as a cAMP-controlled Cl^- transport channel), it is possible to speculate that Pgp and MRP either directly or indirectly also influence secretory mechanisms leading to alterations in drug accumulation and the MDR phenotype (93). These results lead to the prediction that pathways for the development of MDR in cells may depend on the overexpression and/or modification of a number of ATPases or ATP-binding proteins that are directly or indirectly involved in maintaining transmembrane ionic equilibria or controlling cellular mechanisms of uptake and secretion. Overexpression of membrane ATPases could lead to significant changes in both transmembrane ionic fluxes and gradients and their regulatory machinery, resulting in significant modifications in the functioning of integrated pathways of coordinated transport. Such changes would affect symport and possibly substrate specificity of membrane transport proteins.

Cellular pH. This model is based on the observation that antitumor drugs are sufficiently hydrophobic to cross membranes. The asymmetric distribution of the drugs is assumed to be the consequence of an asymmetry of chemical potential (such as ΔpH, voltage, and ionic concentrations). For example, the higher rate of aerobic glycolysis in tumors causes an acidic environment (44), which should have two effects on the passive distribution of drugs. First, most of the chemotherapeutic agents are weak bases. When these drugs are neutral they can freely diffuse across membranes; when protonated, they are charged and significantly less permeant. These drugs are, thus, trapped in the acidic cytosol of tumors. Second, the binding of each of these drugs to their cytosolic targets, such as tubulin (52, 53) or DNA (45–49), has an acidic pH optimum. For example the cross-linking of DNA by mitomycin C is increased at acidic pH (50). Thus, an acidic environment simultaneously increases the amount of drug that is trapped inside the cells and the binding of drugs to their targets. Conversely, an alkaline shift of the pH both decreases the amount of drug inside the cell and reduces its potential for binding.

A role for pH in trapping drugs is consistent with a number of observations. In erythrocyte ghosts (91) and phospholipid vesicles (112) the transmembrane distributions of these drugs are determined by the ΔpH. In MDR cells the cellular pH often increases with increased drug resistance (54). The cytosolic pH becomes alkaline in drug-sensitive cells that are transfected with the Pgp (55). Changing the cytosolic pH of drug-sensitive cells to the pH of drug-resistant cells (in the absence of any MDR proteins) is sufficient to quantitatively account for the decreased drug accumulation observed in MDR (36). The same result is observed independent of the means by which the pH is changed (ammonium chloride or CO_2). Verapamil, which reverses MDR, partially reverses this shift of cytosolic pH (54). Drugs which acidify the cytosol, such as amiloride, reverse MDR (113).

Not all observations can be reconciled with the pH hypothesis. An MDR cell line has been identified whose cellular pH does not differ from the parental drug-sensitive lines (114). It is possible that there are some subcellular changes of pH that are not detected by a total cellular measurement. Alternatively, there may be forms of MDR that do not utilize pH.

It is also necessary to explain how expression of Pgp could affect cellular pH. Cells use a variety of mechanisms to keep their pH close to neutral (115–117). The cellular membrane potential is usually 50–80 mV (with the inside negative). Thus, if protons are allowed to equilibrate across the plasma membrane, the cytosol is significantly more acidic. The steady-state pH is a balance between proton influx (due to the electrochemical gradient), proton generation (from metabolism), and proton efflux (due to proton pumps, Na^+/H^+ exchangers, Cl^-/HCO_3^- exchangers, just to name a few). Tumor cells may use any or all mechanisms at their disposal to neutralize their pH and evade the chemotherapeutic agents. Depolarization of cells has been demonstrated to lead to alkalization of a number of different cell types (118–120). Overexpression of the Pgp in NIH 3T3 fibroblasts reveals a chloride conductance (88). Activation of this channel would depolarize the cell if the Nernst potential for chloride is more positive than the membrane potential. An immediate effect would be to decrease the driving force for proton entry into cells. However, depolarization has also been shown to cause an alkaline shift by modulating Na^+-lactate/H^+-lactate exchange (119, 120) and Na^+/HCO_3^- cotransport (121). Consistent with this hypothesis, experimental results using voltage-sensitive dyes suggest that the membrane potentials in drug-resistant cells are different from those in drug-sensitive cells (122, 123).

Nuclear Mechanisms. Since the principal target for most presently employed chemotherapeutic agents is the DNA and/or associated proteins, it is reasonable to assume that MDR can be conferred on cells by the prevention of drug accumulation in the nucleus or the desensitization of nuclear components to the drugs. An example of such altered drug sensitivity may be represented by the altered expression and/or activity of topoisomerase II (13, 16, 19, 21). Cells expressing the topoisomerase II-related form of MDR do not, apparently, express Pgp, are unaltered in drug accumulation and retention, and are unaffected by the chemosensitizer verapamil. MDR Ehrlich ascites tumor cells that overexpress Pgp accumulate only 20–30% of the daunorubicin taken up by sensitive cells. These MDR cells also exhibit decreased topoisomerase II activity. In addition, the amount of immunoreactive topoisomerase II from these cells is approximately one-third that which is observed in the drug-sensitive cell line. Although addition of verapamil enhances drug accumulation, it does not increase the number of topoisomerase II–DNA complexes in the drug-resistant cells to levels observed in the sensitive cells. It is important, therefore, to note that a single cell line can express multiple MDR mechanisms (19). A direct correlation between inhibition of topoisomerase II and resistance to topoisomerase II poisons is also observed in experiments that isolate genetic suppresser elements that can induce resistance to topoisomerase II-interactive drugs (124). Adriamycin has been demonstrated to form complexes in aqueous solution at 37°C (pH 7.3) with nucleotides, amino acids, proteins, and a broad range of biologically active compounds such as NAD and caffeine (78). Modifications in the drug or substrate competition may have considerable influence on the ability of pharmacologically active drugs to partition into the nucleus and bind to the target DNA. It has been suggested that the drugs may act only at the plasma membrane surface, since DNA-binding drugs ligated to beads have been demonstrated to kill cells at the plasma membrane (125, 126). However, this form of drug presentation may not be physiologically relevant (127, 128).

Cytoplasmic Mechanisms. The results of several studies suggest that cell detoxification mechanisms mediated by the pentose phosphate shunt may contribute

to the expression of MDR in tumors. Accumulation of 2-deoxyglucose 6-phosphate has been found to be much lower in a drug-resistant human T-lymphoblastoid cell line than in sensitive cells, indicating pentose phosphate shunt activation in the MDR line (57). There was unaltered hexokinase activity, higher glucose-6-phosphate dehydrogenase activity, increased glutathione, and marked increase of glutathione peroxidase activity after cell exposure to an oxidizing agent (57). Other experiments demonstrate correlations between enhanced Pgp expression and that of glutathione *S*-transferase π, thymidylate synthase, and metallothionein, suggesting multiple resistance mechanisms (14). In related studies, an MCF-7 human breast carcinoma cell line was selected for resistance to vincristine (59). Addition of a nontoxic concentration of verapamil significantly enhances vincristine-induced cytotoxicity. This resistance is associated with the overexpression of Pgp but without a concomitant increase in Pgp mRNA or gene amplification. Increased activity of cellular protein kinase C has been implicated in this increased activity of Pgp (129). Activities of total glutathione *S*-transferases (GSTs) and glutathione peroxidase are also elevated with overexpression of the GST-π isozyme and its associated mRNA. The data suggest a role for modifications in glutathione metabolism in MDR. Other more recent studies suggest a limited correlation between Pgp-mediated MDR and GST expression or activity (14).

Plasma Membrane Mechanisms. A decrease of drug influx has been observed in many MDR cell lines (22–32). Since most antitumor drugs can freely cross pure lipid bilayers, it is difficult to explain the decreased influx without postulating a change of membrane structure. Freeze–fracture studies reveal increases in the densities of protoplasmic face intramembrane particles in MDR Chinese hamster ovary (CHO) and human leukemic cells (61). This is not observed in revertants, suggesting that MDR may be associated with changes in plasma membrane architecture. Examinations of the effect of lipids and detergents on the ATPase activity of Pgp indicate a well-defined lipid preference for saturated phosphatidylethanolamine and fluid lipid mixtures (130). Treatment of cells with different detergents has also modified the accumulation and cytotoxicity of antitumor drugs (29, 131, 132). In a direct investigation of the interaction of daunomycin with the plasma membranes from tumor cells, it was found that drug binding is directly related to the availability of anionic phospholipid head groups (133). Previous studies of cell cytotoxicity with immobilized adriamycin show that the interaction of the drug with the plasma membrane is sufficient to kill cells (125–128). Thus, it can be argued that changes in the phospholipid composition or organization of the plasma membrane occurring in MDR cells could minimize drug binding. This can either prevent the membrane lytic activity of the drug or, alternatively, result in the inhibition of transmembrane signals that would lead to cell death.

Problems for the Future

Many changes in the basic cell biology of tumor cells can affect drug accumulation. Any or all of the above-described mechanisms could lead to the alterations in drug accumulation and cell sensitivity associated with MDR. Further, many of these mechanisms may be interdependent. Cellular pH may affect a drug-efflux pump, subcellular compartmentalization, and/or exocytosis of drug. Rates of membrane transport are affected by both the organellar and cytosolic pH (93). Trapping of the drugs in both the cytosol and organelles will largely be determined by the pH in each of these compartments. A key challenge is to determine the relative quantitative contributions of each of these mechanisms to the MDR phenotype. Such a quantification may help target efforts to reverse MDR.

A second challenge is to identify the normal physiological ligand for each of the proteins whose activity affects MDR. The subunit of the lysosomal H^+-ATPase is likely to be involved in organelle acidification. However, the functions are not known for the three proteins (Pgp, MRP, and the 110-kDa protein) belonging to the family of ABC proteins. Other members of this family are involved in translocating ligands, often proteins, across membranes. The homology between members of the family is both structural and functional. The functional consequences of deleting STE6 in yeast are reversed when the cells are transformed with the MDR3 protein (83). This suggests that the normal physiological ligands for these MDR proteins may be proteins or at least peptides. While most proteins exit the cell through the secretory pathway (134), many molecules are believed to be transported directly across the plasma membrane. In some cases the evidence is indirect: the protein is secreted without cleavage of a signal sequence and without glycosylation. However, this evidence can be misleading, since numerous proteins use the SRP-dependent translocation machinery of the endoplasmic reticulum without a cleavable signal sequence (135). There is strong evidence that interleukin 1 (136) and thioredoxin (137) are transported directly across the plasma membrane. They do not have a signal sequence and are not glycosylated, and blocking the secretory pathway with brefeldin A does not affect their export. Identification of the normal physiological ligands may suggest new strategies for reversing MDR and facilitate mechanistic studies on the transport properties of these proteins. This would then help resolve such issues as whether the Pgp is a drug-efflux pump, a transmembrane aqueous pore through which drugs diffuse, or a modifier of the transmembrane environment which only indirectly affects drug transport.

A third challenge is to devise new strategies for analyzing MDR that can relate the phenomenology of *in vitro* studies with clinically observed drug resistance. There are a number of problems associated with our current approaches. First, much effort is expended on developing cell lines that show greater and greater degrees of resistance to chemotherapy. This is based on the belief that it will be easier to study the mechanisms by overexpressing the responsible agents. Cells grown *in vitro* are exposed to ever-higher concentrations of antitumor drugs. However, resistant cells *in situ* are usually not more than 5- to 10-fold resistant. Indeed, such cells would never be exposed to higher concentrations because of drug cytotoxicity. The results described in this review indicate that many cell biological mechanisms can be subverted to enable a cell to escape from chemotherapy. Rather than focus on the mechanisms that can make an *in vitro* MDR line 100- or 1000-fold more resistant than normal cells, it may be more informative to delineate the mechanisms that make tumor cells hypersensitive and determine how that enhanced sensitivity is lost as a result of MDR. The principal challenge is to identify those mechanisms that are used by tumors *in situ* to acquire the characteristics of clinical MDR.

It is also important to note that many of these tumors grow as masses—not as isolated cells in a tissue culture dish. These masses present a completely different series of problems for chemotherapy (138, 139) and are relatively drug resistant. This is, in part, due to poor vascularization in the tumor, which reduces the influx of antitumor drugs, and a hypoxic acidic environment, which lowers growth rates. O_2 concentrations in tissue culture can approach 190–220 μM (140). Most normal tissues demonstrate an *in vivo* range from 25 to 50 μM. In tumor masses, O_2 can decline to 5 μM throughout the tumor. The pH within tumor masses can range down to 5.8–6.4 (140). The acidic environment should protonate many antitumor drugs, thereby blocking their entry into cells. The lowered levels of oxygen and nutrients have numerous effects on cell metabolism, cell cycle, and ultimately, drug sensitivity. The situation is further complicated in that tumor cells grow in a living orga-

nism. Altered patterns of cell communication and interaction through endocrine, paracrine, and gap junction-mediated processes could lead to types of MDR in tumor masses that are distinct from that observed in tissue culture. For example, the responsiveness of MCF-7 breast cancer cells to tamoxifen is affected by whether the cells are grown in tissue culture or as xenographs in mice (141). The challenge is now to devise experimental systems that more accurately reflect the *in vivo* environment of clinical MDR.

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