Role of organelle pH in tumor cell biology and drug resistance

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Multidrug resistance is a generic term for the variety of strategies that tumor cells develop to evade the cytotoxic effects of anticancer drugs. It is characterized by decreased cellular sensitivity, not only to the drug(s) employed in chemotherapy but also to a broad spectrum of drugs with neither obvious common targets nor structural homology. It is one of the major obstacles to the successful treatment of tumors. This review concentrates on some of the physiological changes observed in drug-sensitive and drug-resistant tumor cell lines that could account for their relative sensitivities to chemotherapeutics. These changes suggest alternative strategies for combating tumor cells in general and multidrug-resistant cells in particular.

Several different mechanisms have been proposed to account for multidrug resistance (MDR)[1]. MDR might result from structural or functional changes at the plasma membrane or within the cytoplasm, cellular compartments or nucleus. Molecular mechanisms include modifications in detoxification and DNA repair pathways, changes in cellular sites of drug sequestration, decreases in susceptibility to apoptosis, 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. Any or all of these could function to various extents in different tumors.

Numerous proteins have been characterized to increase, or decrease, their expression or their activity in multidrug-resistant tumor cell lines. These include changes in the expression or activity of P-glycoprotein (Pgp)[2,3], MDR-associated protein (MRP)[4,5], glutathione *S*-transferase[6–10], protein kinase C (Refs 11–16), DNA topoisomerase II (Refs 17–19) and proton ATPase[20]. In addition, the type and amount of cellular lipids are often altered. Many studies have examined the changes in proteins and lipids that occur during MDR and the reader is referred to several recent reviews[21–23].

The relevance of many of these changes to the clinical setting has yet to be clearly established. It is often unclear which of the changes are correlated with drug resistance and which are causally related. Often even the presence of these proteins in a clinical setting is not correlated with the eventual success rate of chemotherapy. Thus the strongest evidence implicating these proteins comes from studies on *in vitro* tumor cell lines. Some of the proteins have been purified, cloned and transfected into drug-sensitive tumor cell lines. The strongest evidence supports a role for Pgp in some forms of MDR. Cells transfected with Pgp are less sensitive to chemotherapeutic drugs[24–26]. Transfected cells are usually selected by growing cells in the presence of chemotherapeutics. However, even when selection for Pgp transfection is done in the absence of chemotherapeutics, the cells show a 2–4-fold decreased sensitivity to chemotherapeutics[27]. This value is very low compared with the 100–1000-fold levels of resistance observed in some tumor cell lines. However, this does not diminish the significance of the observation. Indeed, it might more closely resemble the situation in the clinical setting where the therapeutic index separating tumor and normal cells may be as low as 3–5-fold. Tumor cells may never experience levels of chemotherapeutics more than 2–4-fold higher; thus, higher levels of resistance may not be reflecting the mechanisms of resistance that develop *in situ*.

Our understanding of MDR is limited, in part, by our limited knowledge of what makes tumor cells more sensitive to chemotherapeutics. The rule of thumb that rapidly dividing cells are more drug sensitive does not always hold, as some slow-growing tumors are very sensitive (e.g. low-grade

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Hodgkin's disease) and other rapidly growing ones are resistant. Drug resistance might 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 might result from cells that remain longer in the G0 stage of the cell cycle or that arrest in response to damage. If tumor cells are hypersensitive because of their higher cytoplasmic/nucleoplasmic drug concentrations, then MDR might result from lower intracellular drug levels. This could be accomplished by several strategies: preventing drug influx, limiting cytoplasmic accumulation, increasing efflux, or shifting the subcellular distribution of drugs away from their targets. Any or all of these might separately or synergistically result in the MDR phenotype. Alternatively, MDR cells might use molecular modifications unrelated to the mechanisms that lead to hypersensitivity in tumor cells. Indeed, MDR is likely to be the consequence of a multitude of mechanisms. This review focuses on recent work examining one of these mechanisms: modulation of the cellular distribution of chemotherapeutics and thus their effective concentration at their targets within the cell.

Where are the chemotherapeutics in drug-sensitive and drug-resistant cells?

Many chemotherapeutics, such as Adriamycin (doxorubicin), daunomycin, mitoxantrone, vincristine and vinblastine, are naturally occurring weak bases with pK_as between pH 7.4 and 8.4. Some have heterocyclic groups, which make them fluorescent and allow their distribution to be quantified in living cells. The earliest observations using fluorescence microscopy on tissue culture cell lines indicated that chemotherapeutic drugs were spread diffusely throughout the cytoplasm and nucleus of drug-sensitive tumor cells. In sharp contrast, they were excluded from the nucleus of drug-resistant cells. Drug-sensitive cells were described as having a 'nuclear' distribution of chemotherapeutics (drugs in both the nucleus and the cytoplasm) compared with a 'cytoplasmic' distribution in drug-resistant cells (where the drugs were excluded from the nucleus)[28–31]. This distribution was diagnostic of the relative sensitivity of a cell to chemotherapeutics (see Fig. 1).

The distribution of Adriamycin has recently been further characterized in the drug-sensitive and drug-resistant MCF-7 human breast tumor cell line[32]. In short, the 'cytoplasmic' distribution in the drug-resistant cells is the result of localization in discrete punctate cytoplasmic organelles. Specifically, the chemotherapeutics co-localize with the recycling endosomes, the *trans*-Golgi network and the lysosomes. All of these compartments share one major

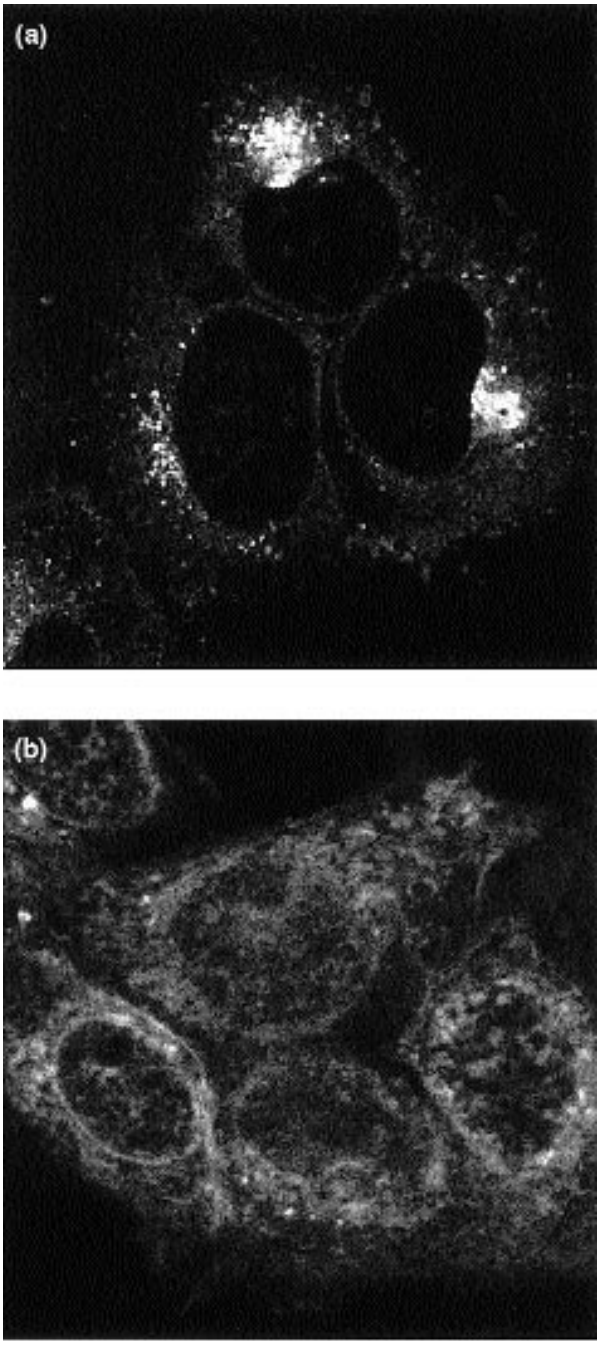

Figure 1. *The distribution of the weak base chemotherapeutic Adriamycin (doxorubicin) in the MCF-7 human breast tumor line. In the drug-sensitive cell line (b), Adriamycin is observed throughout the cytoplasm and nucleus. In contrast, in the drug-resistant MCF-7/ADR cell line (a), the chemotherapeutic is absent from the nucleus and is accumulated in punctate cytoplasmic organelles. Figure reproduced, with permission, from Ref. 32.*

feature: they are all acidified relative to the cytosol. The cytosol of most cells has a pH of 7.2–7.3. By contrast, the lumen of recycling endosomes and *trans*-Golgi network has a pH of 6.0–6.3 and that of the lysosomes is as low as 4.9. The distribution of chemotherapeutic drugs such as Adriamycin, daunomycin, vincristine and vinblastine in these compartments should have been expected from their weak base characteristics. In their neutral form they are relatively membrane permeable and in their protonated form relatively membrane impermeable. Thus, upon entering any acidified compartment the weak base chemotherapeutics will become protonated and thus sequestered in that compartment.

pH and distribution of chemotherapeutic drugs

The effect of pH on the distribution of chemotherapeutic drugs was first demonstrated by Dalmark, who showed that Adriamycin could accumulate in acidified red blood ghosts[33,34]. Subsequently it was shown that the concentration of Adriamycin in liposomes can be quantitatively predicted solely by the pH gradients across the liposome membrane[35–37]. These observations confirm that weak base chemotherapeutics will tend to accumulate in any membrane-bound compartment – whether the cell itself or cellular organelles – that has a lower internal pH than external pH.

There have been many observations and speculations implicating pH in tumor cells and MDR. Warburg had proposed that tumor cells became more acidified than normal cells as part of a key step in the shift from predominantly aerobic to anaerobic metabolism[38,39]. With the introduction of pH-sensitive fluorophores to cellular studies came the observation that tumor cells tend to be more acidic and multidrug-resistant cells less so[40–43]. Thus, consistent with the observations of Dalmark and Mayer, it was proposed that the increased accumulation of chemotherapeutics in tumor cells was the consequence of a more acidified cytosol[42,43]. However, there were two problems with this hypothesis. First, an acidic total cellular pH did not always correlate with drug sensitivity[44]. Second, a pH gradient across the plasma membrane could not account for the differences in subcellular distribution of chemotherapeutic drugs between drug-sensitive and drug-resistant cells.

The surprising observation was that the chemotherapeutic drugs did not accumulate in the lysosomes, *trans*-Golgi network and endosomes of the drug-sensitive tumor cell lines. One potential explanation would posit that the tumor cells have reduced acidification of these organelles. This was tested with acridine orange, itself a fluorescent weak base, which accumulates in acidified organelles resulting in

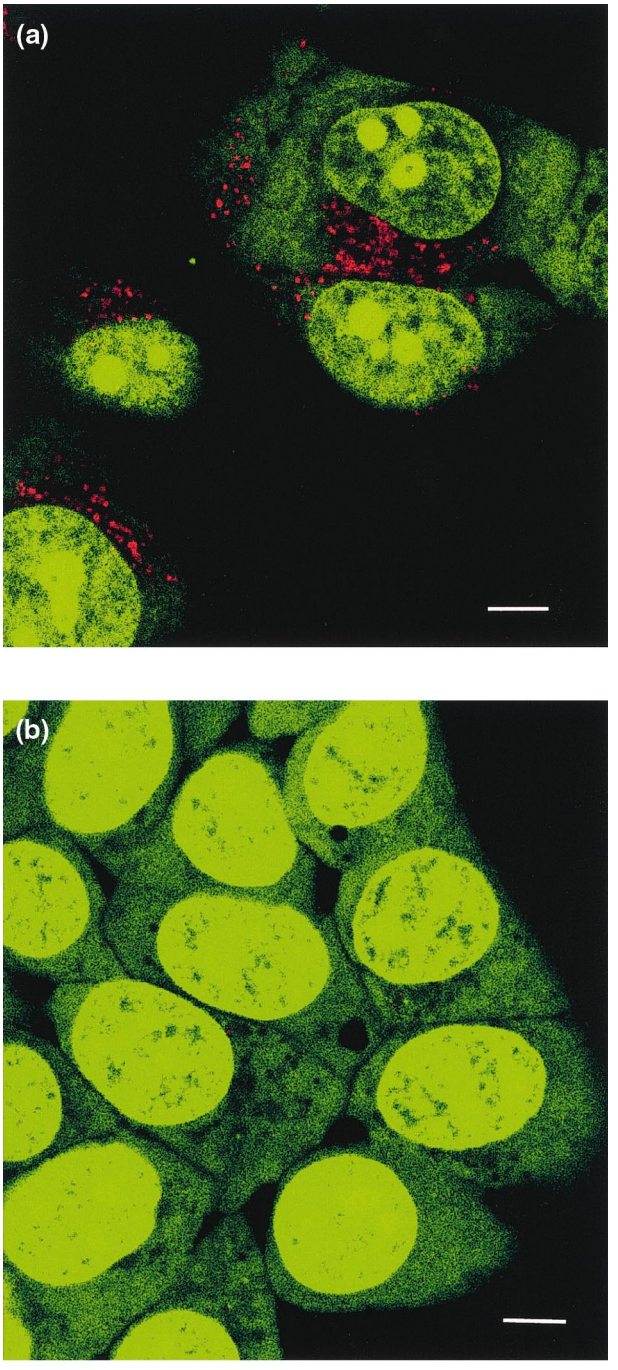

Figure 2. *Acridine orange is a vital stain for the presence of acidified organelles in living cells. Acridine orange in the MCF-7 cell line (b) shows a diffuse green fluorescence. In the MCF-7/ADR drug-resistant cell line (a) there is punctate red–orange fluorescence, which is diagnostic of acidified cytoplasmic organelles. Scale bar: 10 μm. Figure reproduced, with permission, from Ref. 32.*

a bright red–orange fluorescence. It is frequently used as a qualitative probe for acidified organelles[45–48]. In nontransformed cells or drug-resistant cells, acridine orange revealed the presence of many acidified cytoplasmic organelles[32,49,50]. The number of acidified cytoplasmic organelles was substantially reduced in drug-sensitive MCF-7 cells (a human breast tumor line) and K-562 cells (a human erythroleukemic cell line)[32,49,50] (see Fig. 2). This suggests that, while the drug-resistant cells resemble 'normal' cells of the body in the number of acidified organelles, there is a significant lack of acidification in the organelles of the drug-sensitive tumor cells.

The reduced acidification of cytoplasmic organelles in drug-sensitive cells was further characterized by generating a quantitative pH map of the intracellular compartments in drug-resistant and drug-sensitive cells. Quantifiable pH probes were selectively loaded into different intracellular compartments (Fig. 3). The pH profile of drug-resistant cells strongly resembled that of nontransformed cells: the cytosolic pH was neutral (7.2); the endosomes and Golgi were acidified (6.0) and the lysosomes were very acidic (4.9). These pH gradients were missing from the MCF-7 drug-sensitive human breast tumor line: both the cytosol and the organelles were at pH 6.6. This would explain the lack of fluorescent red acridine orange compartments in the cytoplasm and the failure to accumulate the chemotherapeutics in the cytoplasmic organelles.

The PSS hypothesis

These observations became the basis for the 'protonation, sequestration and secretion' hypothesis that was proposed to explain the sensitivity/resistance of cells to weak base chemotherapeutic drugs[32,49]. The neutral form of chemotherapeutics can freely enter cells and diffuse across membranes and through the cytosol and organelles. However, if they diffuse into a compartment that is acidified, the chemotherapeutics will become protonated – a form that cannot cross membranes so easily – and they will be sequestered in the organelle. If this organelle is part of the secretory pathway (Golgi, endosomes or secretory vesicles) then the chemotherapeutics will be secreted from the cell by exocytosis. If acidification is reduced in the organelles of the drug-sensitive cells, then sequestration of chemotherapeutics into these organelles will also be reduced. Thus, the sensitivity of tumor cells to chemotherapeutics would be the result of a reduced ability to sequester drugs away from the nucleus and cytoplasm. Acidification of the cytoplasmic organelles is normal in multidrug-resistant cells and thus they should sequester chemotherapeutics away from the cytosol, resulting in decreased sensitivity to chemotherapeutics.

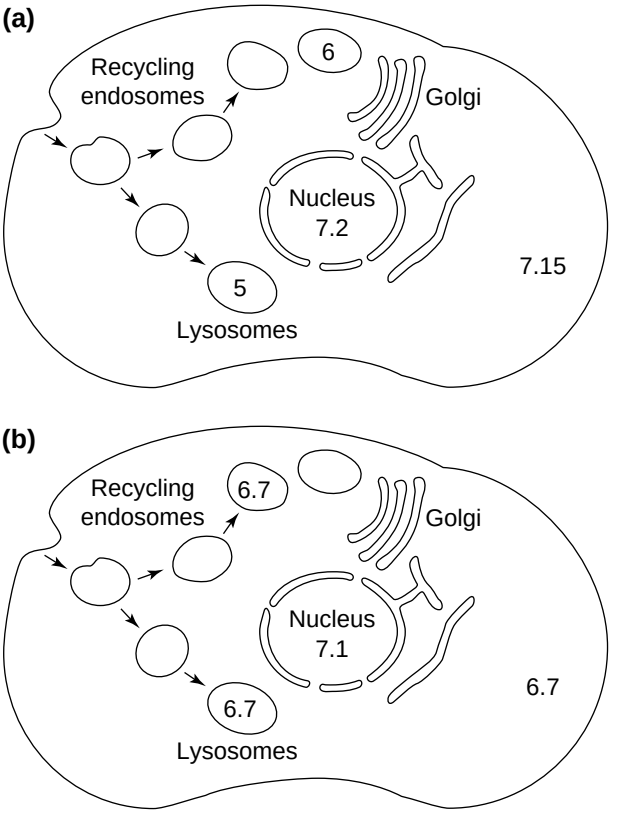

Figure 3. *Schematic of (a) a drug-resistant normal or MDR cell and (b) a drug-sensitive tumor cell showing the pH values measured in the various compartments. Figure adapted from the results of Altan* et al.*[32].*

This PSS hypothesis makes several experimental predictions. First, blocking acidification of the organelles in drug-resistant cells should reverse the accumulation of chemotherapeutics in the organelles and reverse the resistance to chemotherapeutic drugs. Second, agents that reverse drug resistance should affect either acidification of organelles or transport of organelles to the surface of the cell. The first of these predictions has recently been tested[32,49,51].

Acidification of organelles can be blocked either with a protonophore (such as the Na^+–H^+ exchanger monensin, or the K^+–H^+ exchanger nigericin), the weak base chloroquine or an inhibitor of the vacuolar H^+-ATPase (such as bafilomycin or concanomycin). Addition of any of these agents causes release of chemotherapeutic drugs from the cytoplasmic organelles and leads to substantially enhanced accumulation of these drugs in the nucleus[32,49]. Similarly,

the resistance of multidrug-resistant cells is substantially reduced when organelle acidification is blocked during treatment with chemotherapeutics[49,51].

These experimental results demonstrate that acidification of secretory organelles is sufficient to account for drug resistance in these cells (the MCF-7/ADR human drug-resistant breast tumor line). If there are other mechanism(s) of resistance in these cells (drug efflux pumps, permeability barriers) then they are not sufficient on their own to maintain drug resistance.

The PSS hypothesis suggests an alternative strategy for reversing drug resistance: blocking acidification of cytoplasmic organelles. However, such a strategy for reversing drug resistance would have to be carefully applied in a clinical setting: blocking acidification of cytoplasmic organelles will affect all cells of the body and thus the sensitivity of all cells to chemotherapeutics (both multidrug-resistant tumor cells and normal nontransformed cells) will be enhanced. A potential key to selectively sensitizing tumor cells is understanding the biochemical mechanism(s) that affect acidification in both drug-sensitive and drug-resistant tumor cells.

Mechanisms of organelle acidification

As protons are pumped into the lumen of the organelle, there is acidification of the lumen. However, there is also a build-up of positive charge which quickly generates a large membrane potential. For example, in a 200 nm microsome, transport of enough protons to raise the concentration 1 mM (total protons) will cause a membrane potential of over 100 mV. Given that the buffering capacity is likely to be many millimolar, there is only a minimal increase of free protons (or acidification of pH) even with the generation of a large membrane potential. It is necessary to have another ion freely permeating the membrane to dissipate the membrane potential to allow acidification. In most cells this is provided by a chloride conductance. In the absence of this conductance, the organelles cannot acidify. Thus, acidification of organelles requires a membrane that is relatively impermeable to protons, a transmembrane H^+-ATPase (to pump protons across) and a transporter for another ion such as chloride or potassium (to dissipate the voltage gradient).

It is not yet known what is responsible for the loss of acidification in drug-sensitive tumor cell lines. It may be a defect in the vacuolar H^+-ATPase, a defect in a counter-ion transport, or increased proton leakage across the membrane. Any or all of these might contribute to loss of acidification. Proper acidification in a particular multidrug-resistant drug cell line will require rectification, or subversion, of the defect in organelle acidification. For example, the gene encoding the vacuolar H^+-ATPase subunit C is overexpressed in multidrug-resistant HL60 cells[20]. It is not unreasonable to expect that there is a mutation affecting activity or expression of the native vacuolar H^+-ATPase in the parental tumor line, and that this mutation is compensated by overexpression of the H^+-ATPase in the drug-resistant offspring. There is some indirect evidence to suggest that loss of a counter-ion transport might, in some cases, be responsible for loss of acidification in drug-sensitive tumor cell lines. This is based on the observation that several drug-resistant cell lines express either the product of the *mdr1* gene, Pgp, or MRP. Both Pgp and MRP might affect the permeability of membranes to ions. MRP has been implicated in several studies as a potassium channel[52–54]. Pgp has been proposed to be a chloride channel[55,56], although questions have been raised about whether it forms a channel[57–60], or whether its activity regulates an anion channel[61].

pH and tumor cells in the clinic

The reduced acidification in drug-sensitive tumor cells is a surprising observation. All eukaryotic cells acidify their organelles. This observation raises some important questions. First, do these observations hold for tumors observed in a clinical setting (in contrast to these tumor cell lines)? If there are changes of acidification, are they found in all stages, or only in select stages of tumor cell growth? What are the implications for the reduced acidification for the physiology of tumor cells and the cell biology of cancer? Finally, can this reduced acidification be used as a weapon to attack tumor cells?

So far there have been no direct observations of the pH of secretory organelles in tumor cells *in situ*. However, as elaborated below, two independent, albeit indirect, lines of evidence suggest that there may be a defect in acidification of the secretory pathway of tumors *in situ*. First, several oncogenes found in human cancers have been cloned and shown to affect organelle acidification. Second, some of the physiological changes observed in tumor cells, such as secretion of lysosomal enzymes and increased response to cytokines, are diagnostic for cells that have defective organelle acidification.

Over the past ten years there has been a convergence in our studies on the cloning of genes found in human tumors, in our ability to characterize signal transduction pathways in living cells and in our cloning and characterization of the yeast genome. This convergence has allowed us to characterize rapidly the relative contributions of some of the genes found to be defective in human tumors. Some

of these genes affect DNA checkpoint repair. Other genes affect the ability of a cell to enter apoptosis. Still others, such as *ras* and *src*, overlap in a network of signal transduction pathways. When immortalized cells are transfected with *ras*, they lose acidification of the secretory pathway and lysosomes[62]. Likewise, the E5 protein of the papilloma virus, which is required for transformation, selectively binds the pore-forming subunit of the vacuolar H^+-ATPase and blocks acidification of organelles. Transformation of cells with different oncogenes has been shown to affect cytosolic pH (Refs 63–66).

Blocking acidification of organelles, either pharmacologically or with mutations, results in several distinct changes in cell physiology[67–74]. Reducing acidification affects glycosylation of membrane proteins and lipids. For example, the optimum pH for the α(2→6) sialyltransferase is 5.5 (Ref. 75). Raising the pH reduces the formation of α(2→6) sialic acid linkages on proteins. This, in turn, results in numerous compensatory changes in glycosylation and sulphonation of proteins. Reducing the acidification of secretory organelles also results in the secretion of lysosomal enzymes[76]. The mannose 6-phosphate receptor cycles between the *trans*-Golgi network and the endosomes and lysosomes. In the *trans*-Golgi network it binds to nascent lysosomal proteins, thereby blocking their egress through the secretory pathway. Upon shuttling proteins to the relatively acidified lysosomes/endosomes, the mannose 6-phosphate receptor releases the lysosomal enzymes. Reducing the acidification reduces the release of these proteins. Thus, the mannose 6-phosphate receptor becomes saturated with nascent lysosomal proteins, and newly synthesized lysosomal enzymes are no longer trapped in the *trans*-Golgi network but are secreted from the cell. Many of these alternations of cell physiology, which are diagnostic of defective acidification, have been observed in tumors. Tumors frequently secrete lysosomal enzymes, such as cathepsin D.

So far the evidence is only indirect for a relationship between organelle pH and tumors observed in the clinic. If such a relationship exists, it will remain to be determined whether the changes are found in all stages of tumor growth, or only in select stages of tumor cell growth. These studies will require assays to study selectively the steps of tumor angiogenesis[77], outgrowth and intravasation across blood vessels[78], and lodging into the blood vessels, extravasation back across and growth at a secondary location.

It is not yet clear what is responsible for the sensitivity of some tumor cells to chemotherapy. Acidification of cytoplasmic secretory organelles quite possibly contributes to the ability of all cells to cleanse themselves of alkaloids (environmental toxins that are weak bases). Many chemotherapeutic drugs are themselves alkaloids. The inability of drug-sensitive tumor cell lines to acidify secretory organelles may reduce their ability to cleanse themselves of all environmental toxins and thus enhance their sensitivity to chemotherapeutics. If the observed lack of acidification should generalize to tumors observed in the clinic, this might allow new strategies to combat tumors that take advantage of this aspect of tumor cell pathology.

Similarly, an understanding of the mechanism(s) responsible for the restoration of acidification in drug-resistant cell lines could help in design rationale strategies for enhancing the sensitivity of these cells to chemotherapeutics. Clearly, it would not be reasonable to use protonophores, weak bases, or blockers of the H^+-ATPase to sensitize drug-resistant tumors in a patient to chemotherapeutics, as these agents will work on all cells of the body and enhance the sensitivity of all cells to chemotherapeutics. However, an understanding of what has compromised the pH regulatory systems of these cells and what mechanism(s) restore pH regulation could allow rational design and screening of agents that could enhance the therapeutic index of chemotherapeutics.

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