



# Changes in lipid and protein constituents of rafts and caveolae in multidrug resistant cancer cells and their functional consequences

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The carcinogenic process involves a complex series of genetic and biochemical changes that enables transformed cells to proliferate, migrate to secondary sites and, in some cases, acquire mechanisms that make cancer cells resistant to chemotherapy. This phenomenon in its most common form is known as multidrug resistance (MDR). It is usually mediated by overexpression of P-glycoprotein (P-gp) or other plasma membrane ATPases that export cytotoxic drugs used in chemotherapy, thereby reducing their efficacy. However, additional adaptive changes are likely to be required in order to confer a full MDR phenotype. Recent studies have shown that acquisition of MDR is accompanied by up-regulation of lipids and proteins that constitute lipid rafts and caveolar membranes, notably glucosylceramide and caveolin. These changes may be related to the fact that in MDR cells a significant fraction of cellular P-gp is associated with caveolin-rich membrane domains, they may be involved in drug transport and they could have an impact on drug-induced apoptosis and on the phenotypic transformation of MDR cancer cells.

**Keywords:** Cancer, caveolae, caveolin, cholesterol, glucosylceramide, multidrug resistance, rafts, transformation

**Abbreviations:** ABC, ATP-binding cassette; GlcCer, glucosylceramide; MDR, multidrug resistance; P-gp, P-glycoprotein; SREBP, sterol regulatory element-binding protein

## Mechanisms of multidrug resistance in cancer cells

Anticancer drug resistance is the primary cause for the failure of chemotherapy in cancer patients [1,2]. One form of drug resistance, termed multidrug resistance (MDR), has been most studied, and was extensively characterized utilizing *ex vivo* models of multidrug resistant cancer cells in culture [3]. It was repeatedly observed that selection for resistance to any one particular drug is accompanied by resistance to a variety of other drugs. This general characteristic of cancer cells defines the MDR phenomenon.

Many cellular changes have been described that accompany the multidrug resistant phenotype. However, most studies have focused on the so-called 'classical' multidrug resistance protein termed P-glycoprotein (P-gp), which is the product of the MDR1 gene [4,5]. P-gp is a membrane-bound glycoprotein that belongs to the protein family of ATP-binding cassette (ABC) transporters. P-gp and other ABC drug

transporters act as ATP-dependent drug efflux pumps and are believed to mediate drug resistance by actively extruding drugs from the cells [4,6]. In support of a role for P-gp in mediating the MDR phenotype it has been demonstrated that P-gp can effect unidirectional drug transport and that it displays a drug-dependent ATPase activity and ATP-dependent drug binding. In addition, P-gp expression decreases cell sensitivity to drugs [1,7,8]. However, MDR1 transfection studies have demonstrated that drug resistance conferred by transfected P-gp is not as profound as that of drug-selected MDR cell lines (Ref. [9] and citations therein). It thus seems that while P-gp and other drug transporters do mediate drug efflux across the plasma membrane, they may not be sufficient for conferring a complete MDR phenotype in cancer cells. Acquisition of MDR probably involves additional mechanisms that often act concurrently in order to prevent or circumvent the lethal effects of chemotherapeutic drugs. In addition to expression of P-gp and related ATP-dependent drug efflux pumps, other mechanisms of MDR include: altered cellular pharmacokinetics of drug uptake; increased detoxification of drugs; increased DNA damage repair; and suppression of drug-induced programmed cell death [2,10].

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Recent studies have shown that acquisition of the MDR phenotype is accompanied by up-regulation of lipids and proteins that constitute lipid rafts and caveolar membranes, notably glucosylceramide and caveolin. These results are reviewed below.

### Membrane lipid changes associated with MDR

Lipids may play multiple and complex functions in MDR, e.g. by influencing drug transport across the plasma membrane and by modulating the activity of drug transporters. Lipids determine cell membrane fluidity (lipid bilayer structural order or membrane microviscosity) which, in turn, may affect the ability of MDR cells to accumulate drugs [11]. P-gp substrates initially interact with the lipid bilayer, by partitioning into the lipid phase, before gaining access to P-gp [12]. Indeed, recent 3-D structural data show that drugs may enter the hydrophilic core of the protein through a gap within the transmembrane domain of P-gp [7]. In addition, similar to other membrane-bound ATPases, P-gp must be reconstituted with bilayer phospholipids for full activity. Unsaturated phosphatidylcholine and phosphatidylserine are crucial for restoring P-gp ATPase activity, while saturated phosphatidylethanolamine is most effective in activating the ATPase [13]. Furthermore, lipids were suggested to play a role in the coupling between drug binding sites and catalytic sites of P-gp [14].

Studies on membrane lipid changes that accompany anticancer drug resistance date back to the early 1980s and have provided evidence for changes in membrane fluidity as well as membrane lipid composition. Whereas most studies reported that drug-resistant cells exhibit an increase in membrane fluidity, other studies have suggested the opposite (see Ref. [15] and citations therein). In parallel, chemical analyses of cell lipids, and quantitative comparison of various lipid types in drug-sensitive and drug-resistant cells, have indicated that the acquisition of MDR is associated with significant changes in cholesterol, sphingolipids and other lipids (see below). These alterations in lipid content might account for the changes in MDR cell-membrane properties and may affect drug uptake, transport, and extrusion.

*Changes in cholesterol.* Comparison of several drug-sensitive and drug-resistant cell lines with respect to their plasma membrane lipid composition revealed a significant elevation in free cholesterol level in vinblastine-resistant T lymphoblasts as compared with the vinblastine-sensitive parental cells [16]. Higher membrane cholesterol levels were also observed in MDR human ovarian cancer cells, when cultured under conditions of cholesterol deprivation [17]. Similarly, comparison of murine leukemic lymphoblasts cell lines revealed that cholesterol content was directly proportional to the relative resistance to vincristine [18]. These data indicate that cholesterol metabolism is modified in the MDR cells and suggest that MDR cells are better able to maintain high cholesterol levels. On the other hand, several other

studies reported no change in total cholesterol or cholesterol/phospholipid ratio in MDR cells [19–22].

*Changes in glycosphingolipids.* Patterns of glycosphingolipid expression are known to change profoundly upon oncogenic transformation of cells [23]. Intriguingly, more recent work has indicated that such changes occur also in association with the MDR phenomenon. Lavie and Cabot have shown that the precursor of all glycosphingolipids, glucosylceramide (GlcCer), is markedly elevated in various MDR cell lines, including MCF-7-AdrR, KB-V, and NIH:OVCA-3, as compared with the parental lines [24]. Additional studies demonstrated that higher activity of GlcCer synthase is the principal cause of this alteration in MDR GlcCer level [24] and that MDR cells efficiently convert exogenously added C<sub>6</sub>-ceramide into C<sub>6</sub>-GlcCer [25]. Analyses of tumor tissue samples delineated an inverse relationship between GlcCer levels and the patients' response to chemotherapy, complementing the *in vitro* data from cell lines and raising the possibility that GlcCer may serve as a marker molecule for multidrug resistant cancer [26]. MDR is also associated with changes in more complex glycosphingolipids such as gangliosides. In both actinomycin D- and daunorubicin-resistant DC-3F Chinese hamster lung cells there was a dramatic decrease in the complexity of the ganglioside labeling pattern, consistent with an apparent block in the synthesis of gangliosides at the level of G<sub>M3</sub> [27]. Vincristine-resistant DC-3F cells did not exhibit this general block but nevertheless displayed significantly more G<sub>M3</sub> than the parental cells [27]. Similarly, cisplatin-resistant small-cell lung cancer cell lines had a simplified ganglioside pattern (G<sub>M2</sub> and G<sub>M3</sub> only) and expressed higher G<sub>M3</sub> levels than the parental line [28]. Adriamycin-resistant small-cell lung cancer cell lines and MCF-7 AdrR breast adenocarcinoma cells were found to express higher G<sub>M2</sub> levels, while MCF-7 AdrR cells also exhibited higher G<sub>D2</sub> levels compared with drug-sensitive MCF-7 cells [29].

*Changes in other lipids.* Additional lipid compositional alterations that seem to accompany the MDR phenotype include elevated levels of ether-linked phospholipids, mainly in 1-O-alkyl phospholipids [16], as well as changes in fatty acyl group content, mainly elevations of arachidonic acid (C<sub>20:4</sub>) and docosahexaenoic acid (C<sub>22:6</sub>) [19]. Finally, sphingomyelin levels may also be elevated in some MDR cells [24,30].

In conclusion, reports from numerous studies in different MDR cells demonstrate significant changes in membrane lipid composition. Of particular interest are the elevated cellular levels reported for cholesterol (in some but not all MDR cell lines) and for GlcCer. Both cholesterol and GlcCer are enriched in the membrane microdomains termed lipid rafts and in plasma membrane invaginations known as caveolae [31,32]. The elevated levels of GlcCer and cholesterol, independently reported in different types of MDR cells, suggest that MDR might be associated with increased formation of these microdomains in the plasma membrane. Indeed, recent work

has provided direct evidence showing that caveolae and the caveolar coat protein caveolin are up-regulated in MDR cells.

### **Caveolae, caveolin and multidrug resistance**

Caveolae are defined as 50–100 nm nonclathrin-coated invaginations of the plasma membrane [33,34]. Caveolae have a characteristic striated coat structure, an essential component of which is a 21-kDa integral membrane protein called caveolin-1 [34–36]. Caveolin-1 forms high molecular weight homo- and hetero-oligomers (with caveolin-2), and acts as a 'scaffolding' protein for various other proteins [37]. Caveolae are commonly found in endothelial cells, adipocytes, pneumocytes, as well as differentiated epithelial cells and fibroblasts [37,38]. Caveolae have been implicated in transport processes such as nonclathrin-mediated endocytosis [39] and in cholesterol efflux [40]. In addition, caveolae play an important role in cellular signal transduction, probably as membranal platforms for receptor and signaling proteins complexes [37].

Recent studies have shown that multidrug resistant cancer cells express very high caveolin levels and exhibit a high surface density of caveolae [41,42]. Expression of caveolin-1 is dramatically up-regulated in numerous MDR human cancer cell lines, including: adriamycin-resistant MCF-7 AdrR breast adenocarcinoma cells and colchicine-resistant HT-29-MDR colon carcinoma cells [41]; vinblastine-resistant SKVLB1 ovarian carcinoma cells and taxol-resistant A549-T24 lung carcinoma cells [42]; and colchicine-resistant B16-MDR mouse melanoma cells (J. Troost and G. Faiman, unpublished results). Caveolin-2 expression was also up-regulated in HT-29-MDR, MCF-7 AdrR, and B16-MDR cells (Ref. [41]; J. Troost and G. Faiman, unpublished results). Thus, various cell types, while developing resistance to different drugs, acquire high caveolin expression. In HT-29-MDR cells the up-regulation of caveolin-1 expression is accompanied by a 5-fold increase in the number of juxtamembrane, 50 to 100-nm non-coated invaginations, with the characteristic flask shape morphology of caveolae. A comparable surface density of caveolae was also observed in MCF-7 AdrR cells, whereas the parental MCF-7 were virtually devoid of caveolae [41]. Phospholipase D2 which is another caveolae-resident protein is also up-regulated in MDR cells [43].

### **Possible consequences of high GlcCer and caveolin expression in MDR cells**

Do changes in lipid and protein constituents of caveolae confer any selective advantage upon cells exposed to cytotoxic agents? The answer seems to be yes. The elevated levels of GlcCer in MDR cells appear to be due to higher activity of GlcCer synthase, the enzyme which glucosylate ceramide to GlcCer [24,25]. Recent follow-up work has demonstrated that overexpression of recombinant GlcCer synthase confers resistance to adriamycin and to ceramide on human breast

cancer cells [44]. Drug-induced apoptosis involves an increase in cellular ceramide levels [45]. It was therefore suggested that drug resistance in these GlcCer synthase-transfected cells might be related to hyper-glycosylation of ceramide and the consequent attenuation of the apoptotic signal promoted by the chemotherapeutic drug [44]. In addition, accumulation of GlcCer may enhance cell survival upon exposure to chemotherapy. The contribution of GlcCer synthase to drug resistance was demonstrated directly by antisense inhibition of GlcCer synthase expression, which manipulation resulted in a 30% decrease in GlcCer synthase activity and a 28-fold increase in adriamycin sensitivity [46]. These results strongly support the hypothesis that GlcCer synthase contributes to drug resistance in MDR cells by clearing ceramide that carries a drug-induced apoptotic message.

Do caveolae play any role in drug resistance? Of the various functions assigned to caveolae, their proposed role in mediating cholesterol efflux seems most relevant in the context of MDR. The evidence in favor of a role for caveolin-1 in the transport of cholesterol to the plasma membrane may be briefly summarized as follows. First, transfection of caveolin-1 into cells results in an increase in the rate of transport of newly synthesized cholesterol to caveolar membranes, and a significant increase in cholesterol mass in these membranes [47,48]. Consistently, a dominant negative caveolin mutant appears to disrupt caveolar membranes by interfering with the transport of cholesterol [49]. Second, caveolin-1 expression and free cholesterol efflux rate are directly correlated under certain physiological conditions [48,50] and also following certain pharmacological manipulations [47,51,52]. Caveolin-1 expression is transcriptionally regulated by the sterol regulatory element-binding protein (SREBP). SREBP down-regulates caveolin-1 expression [53], an effect that is opposite in direction to the well known stimulatory effect of SREBP on sterol biosynthetic enzymes and low-density lipoprotein receptor expression. Caveolin-1 is a cholesterol-binding protein [54–56]. A soluble caveolin-1-chaperone complex that contains cholesterol, heat shock protein 56, cyclophilin 40 and cyclophilin A was recently demonstrated [57]. Pulse-chase and subcellular fractionation experiments indicate that caveolae act as plasma membrane terminals from where free cholesterol flows to non-caveolar regions of the membrane or is unloaded onto high-density lipoprotein molecules that interact with the scavenger receptor class B, type I [47,50,51,58,59]. In summary, these data suggest that a cholesterol efflux pathway exists that is up-regulated in caveolin-1-expressing cells. In this pathway caveolin-1 appears to act both as a component of plasma membrane-targeted cholesterol transport intermediates and as a structural coat protein of plasma membrane caveolae.

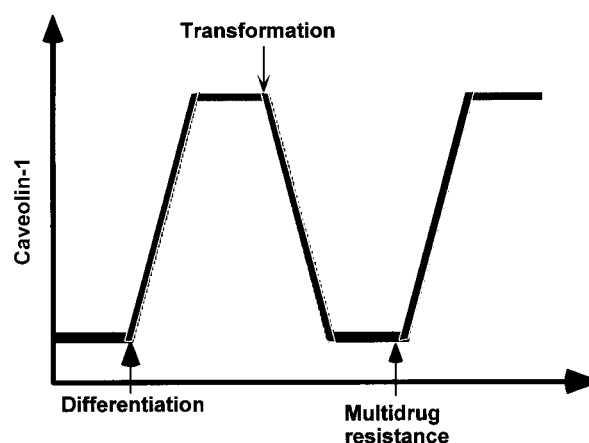
We have proposed recently that caveolin-dependent cholesterol efflux pathway(s) potentially may play a role in delivering drugs, from the various intracellular membranes into which they partition, to plasma membrane drug efflux pumps [60]. It was postulated that an intracellular cholesterol efflux pathway

could carry cytotoxic drugs (most of which are lipophilic compounds) upon the same platforms that deliver cholesterol and sphingolipids [60]. A highly active cholesterol efflux pathway would facilitate drug transport by accelerating the transport of drugs from intracellular membranes to the plasma membrane against a steep concentration gradient and by delivering drug-bearing rafts and possibly caveolin-chaperone complexes directly to plasma membrane cholesterol-rich domains. The latter is important because P-gp is localized in cholesterol- and caveolin-rich domains (Refs. [41,61]; G. Fiucci et al., unpublished results). The hypothesis that efficient drug transport is a two step process, requiring both an active intracellular cholesterol efflux pathway *and* a plasma membrane drug efflux pump, provides a rational explanation for the up-regulation of caveolin in MDR cancer cells.

### Reciprocal regulation of caveolin expression during cell differentiation and oncogenic transformation

Regardless of the reason for the up-regulation of caveolin in MDR cells, the fact of its presence at high levels in these cancer cells is likely to have a profound effect on their phenotypic transformation. Caveolae and caveolins are most abundantly expressed in terminally differentiated cells (e.g., adipocytes, endothelial cells and muscle cells). Adipose tissue was identified as the single most abundant source of caveolae [62]. Caveolin mRNA and protein are strongly induced during differentiation of 3T3-L1 fibroblasts to adipocytes [62]. Although it has previously been reported that little or no caveolin is detectable in neuronal cell lines, recent work has shown an up-regulation of caveolin-1 and -2 expression in differentiating PC-12 cells and dorsal root ganglion neurons [63]. Similarly, caveolin-1 expression is up-regulated during *in vitro* acquisition of an alveolar epithelial type I phenotype by progenitor lung cells [64]. Differentiated Schwann cells likewise express caveolin-1, the level of which is decreased when the cells revert to a premyelinating phenotype [65]. In summary, differentiation of various cells appears to be frequently associated with elevation in the expression of caveolin proteins and caveolae organelles. Conversely, caveolin-1 expression is down-regulated in most cancer cells (Figure 1).

Carcinogenesis may be considered as a multistep process of genetic alterations causing changes in cell proliferation, mobility, and invasiveness, leading to metastatic cancer. Of major importance in this process are expression of oncogenes and inhibition of tumor-suppressor genes. Comparisons between normal and transformed cells at the molecular level have provided a powerful analyzing tool in research of this phenomenon. Using molecular differential display techniques, Sager and co-workers have identified various putative tumor-suppressor genes [66]. In this study, caveolin-1 was identified as one of 26 gene products that are strongly down-regulated in human mammary carcinomas. In accordance with these findings, caveolin-1 gene is localized to a suspected tumor



**Figure 1.** Changes in caveolin-1 expression during differentiation, oncogenic transformation and acquisition of multidrug resistance. See details in text.

suppressor locus in humans (7q31.1/D7S522) that is deleted in many forms of cancer [67]. In addition, caveolin-1 mRNA and protein expression are reduced or absent in several oncogenically transformed cell lines and tumor cells. A direct correlation was found between caveolin reduction and a decline in caveolae organelle number in cells transformed with oncogenes [68]. Analysis of several human breast and human lung tumor cell lines also revealed the loss of caveolin-1 mRNA and protein expression [69,70]. Further analysis confirmed that most breast cancer cell lines, as well as most pancreatic tumor and all ovarian carcinoma cell lines expressed little or no caveolin-1; however, bladder carcinoma cells exhibited high caveolin-1 expression [71]. In contrast to the results obtained in breast cancer cell lines, 78–93% of malignant human breast tissues, including intraductal carcinoma, infiltrating ductal carcinoma and lymph node metastasis, were positively immunostained with a caveolin-1 antibody [72]. In prostate cancer tissue, however, the percentage of caveolin-1 positive samples was only 29% in T<sub>3</sub>N<sub>1</sub>-stage primary cancer and 56% in lymph node metastasis [72]. Collectively, these data suggest that down-regulation of caveolin-1 is not an essential requirement for cellular transformation. Yet, at least in certain cell types, antisense inhibition of caveolin-1 expression is sufficient for induction of phenotypic transformation, as shown in NIH 3T3 fibroblasts [73].

The suggested oncosuppressive activity of caveolin-1 is supported by the fact that expression of recombinant caveolin-1 has a growth-inhibitory effect on transformed cells [69,74]. Engelman et al. have shown that recombinant expression of caveolin-1 in oncogenically transformed NIH-3T3 cells abrogates anchorage-independent growth, thus depriving the transformed cells from one of their crucial tumorigenic abilities [74]. Accordingly, transfection of human mammary cancer cells with caveolin-1 cDNA results in substantial growth inhibition as demonstrated by a 50% decrease in

growth rate and a ~15-fold reduction in colony formation in soft agar [69]. Additional information from this study indicated that caveolin-1 expression is regulated during the cell cycle [69]. Altogether, a picture emerges whereby caveolin-1, within the context of its organelles—the caveolae, has important growth regulating function. This function of caveolin-1 is often repressed in oncogenic transformation and tumorigenesis but, as noted above, such repression is not obligatory.

At the molecular level, caveolin-1 may inhibit growth via its interaction with growth-promoting signaling molecules. Historically, caveolin-1 was first identified as a major tyrosine-phosphorylated, v-Src substrate in Rous sarcoma virus-transformed cells [35,75]. Various proteins that modulate cell proliferation were found to be localized within caveolae or to physically interact with caveolin-1 [38]. It has been suggested that caveolin forms a scaffold onto which the signaling molecules can assemble and thus co-localize within a distinct domain of the plasma membrane (i.e. caveolae), from which signaling cascades might be launched [37]. Caveolin-1 binding to these molecules may functionally regulate these molecules. Intriguingly, caveolin-1 exerts an inhibitory effect on the activity of H-Ras, c-Src, Raf-1, Mek, Erk2, EGF-R (ErbB1) and ErbB2 [37,38]. It was concluded that caveolin-1 exhibits a general pattern of negative modulation on pathways leading to cell proliferation and is consequently down-regulated in transformed cancer cells and up-regulated in slow-proliferating and terminally differentiated cells.

In view of the above discussion it is clear that, by expressing caveolin-1, multidrug resistant cancer cells reacquire a prominent marker of non-transformed/differentiated cells that has been lost upon oncogenic transformation (Figure 1). Given the dramatic effects that expression of caveolin-1 has on the phenotype of transformed cells [69,73,74], this fact is likely to have important consequences. Indeed, we have recently found that MCF-7 AdrR cells have lost the capacity for anchorage-independent growth, indicating that they have regained an essential requirement for an extracellular matrix-derived survival and/or growth signal (G. Fiucci and M. Liscovitch, submitted for publication). Previously it has been shown that certain MDR cell lines exhibit reduced tumorigenicity in athymic mice [76,77]. It thus seems that the acquisition of multidrug resistance, while allowing cells to survive in the presence of high concentrations of cytotoxic agents, is often accompanied by loss of at least some features of the transformed phenotype. This change may prove to be a previously unsuspected Achilles' Heel of multidrug resistant cells that will enable future development of much needed novel drugs for treatment of multidrug resistant cancer.

### Concluding remarks

Acquisition of multidrug resistance is clearly a multifactorial process. Whereas expression of drug transporters is a major factor, additional biochemical changes that are associated with

MDR may significantly contribute to the drug resistant phenotype. The results summarized above imply that there is a coordinated program for up-regulation of constituents of rafts and caveolae in MDR cells. The main lipids that seem to be involved in this phenomenon are GlcCer and cholesterol. A structural protein of caveolae, caveolin, is also dramatically elevated. Accordingly, the number of plasma membrane caveolae is greatly increased in MDR cells. The relationship between caveolin and MDR may be related to the function of caveolin in mediating cholesterol efflux, a pathway that is postulated to facilitate the delivery of drugs from intracellular compartments to the plasma membrane. An additional link seems to exist between the up-regulation of GlcCer synthase and attenuation of ceramide-mediated apoptotic signaling. These adaptations may promote cell survival under chemotherapy and, hence, be positively selected during cell exposure to cytotoxic drugs. However, the overexpression of caveolin-1, an oncosuppressive protein, may reverse important aspects of the phenotypic transformation of MDR cells.

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