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Topical Review

Rafts as Missing Link between Multidrug Resistance and Sphingolipid Metabolism

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Introduction

Since their discovery, detergent-insoluble glycosphingolipid-enriched membrane domains have accounted for several cellular functions. Besides their role in protein and lipid transport in polarized cells, most of the attention focuses on their organizing role in signal transduction. Given that virtually all multidrug-resistant cells exhibit a deviating sphingolipid composition, most typically increased levels of glucosylceramide, a possible role of sphingolipids in multidrug-resistance has been investigated. An increased conversion of cytotoxic drug-induced ceramide into glucosylceramide, thereby escaping ceramide-induced apoptosis, appeared as a novel and independent multidrug resistance mechanism. In addition, multidrug-resistant cells were found to have abundant caveolae, which harbored a large fraction of the cellular drug-efflux pump, P-glycoprotein. Soon thereafter, other drug-efflux pumps were shown to be located in membrane domains. Interestingly, alterations in cellular sphingolipid composition associated with multidrug resistance cells could largely be accounted for by these membrane domains. In this review, we present an overview of the current understanding of the relation between multidrug resistance and sphingolipid metabolism and the important role membrane domains appear to play in this respect.

Key words: Detergent-insoluble glycosphingolipid-enriched membrane domain — Raft — Sphingolipids — Glucosylceramide synthase — Multidrug resistance — ATP-binding cassette-transporters
Abbreviations: DIG, detergent-insoluble glycosphingolipid-enriched membrane domain; DRM, detergent resistant membrane domain; T_m, melting temperature

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Multidrug Resistance, Sphingolipids and ATP-binding Cassette Transporters

Chemotherapy is the primary approach towards the treatment of metastatic cancers. While initially tumor cells can respond well to treatment with chemotherapeutic agents, repeated drug administration often results in the selection of drug-resistant cells, and hence in incurable relapses. Very often these cells do not only gain resistance to the initially applied drugs, but also to a variety of (structurally unrelated) chemotherapeutic agents, a feature called multidrug-resistance [37]. Regarding the lipid composition of multidrug-resistant cell lines compared to drug-sensitive cell lines, changes have been reported for etherlipids, phospholipids, gangliosides, cholesterol and fatty acids [48, 49, 57]. Recent studies have indicated that virtually all multidrug-resistant cells exhibit a deviating sphingolipid composition, most typically, increased levels of glucosylceramide [30, 33, 34, 76]. At present it is not established with certainty in which way altered sphingolipid metabolism influences multidrug resistance characteristics. There are indications for an independent action of such sphingolipid alterations on the one hand, but on the other hand, sphingolipid changes may occur in cooperation with other multidrug resistance mechanisms, such as ATP-binding cassette protein overexpression [66].

Sphingolipids as Structural and Functional Membrane Components

Sphingolipids are primarily present in the external leaflet of the plasma membrane and in the equivalent luminal leaflet of functionally related organelles such as the Golgi apparatus, endosomes and lysosomes [50]. Whereas glycerol serves as the backbone for phosphoglycerolipids, sphingolipids have a long-chain sphingoid base as the central moiety, which

typically consists of a D-*erythro* C_{18} amine, with a trans double bond at the C_{4-5} position. The amino-group is generally acylated with a C_{16-24} fatty acid, yielding ceramide [51, 72]. Ceramide is synthesized at the endoplasmic reticulum and reaches the Golgi apparatus where sphingomyelin and glucosylceramide are synthesized [46]. Further glycosylation of glucosylceramide results successively in the formation of lactosylceramide and more complex glycosphingolipids, including gangliosides [51, 72]. The trans Golgi network is instrumental in the initial segregation and vesicular-mediated transport to apical and basolateral membranes of sphingolipid species [70, 75].

With respect to signaling, ceramide is the best studied sphingolipid. It plays a role in the regulation of key cellular processes such as growth inhibition, differentiation and apoptosis. Sphingomyelin breakdown by the activation of sphingomyelinases is generally considered as the main source of signaling-involved ceramide [20–22, 53], but de novo synthesis of ceramide has also been described in this respect [4].

Together with cholesterol, sphingolipids are the main contributors of so-called rafts, which can be isolated from cells as detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs) [56]. Being part of these rafts, sphingolipids play an additional role in signaling pathways and also in protein sorting [5, 58, 66, 70].

Glucosylceramide Synthase-mediated Multidrug Resistance?

It has been postulated that the increase of glucosylceramide observed in multidrug-resistant cells is due to an increased activity of glucosylceramide synthase, which converts excess ceramide into glucosylceramide, thereby circumventing the onset of ceramide-induced apoptosis. In this way, the metabolism of ceramide could function as an independent multidrug-resistance mechanism, acting separately from ATP-binding cassette transporter-mediated drug efflux [35, 43]. Overexpression of glucosylceramide synthase, indeed, conferred adriamycin resistance to human breast cancer cells, however, without apparent changes in cellular ceramide or glucosylceramide levels [39]. In accordance, expression of glucosylceramide synthase anti-sense resulted in reversal of adriamycin resistance, but cellular ceramide levels were only elevated after exposure to the drug [40, 41]. Recently, direct evidence was provided for upregulation of glucosylceramide synthase mRNA as well as enzyme activity in MCF7/ADR cells [17]. Furthermore, in HL-60/ADR cells evidence has been obtained for adriamycin-induced activation of Sp1 as the mechanism underlying adriamycin-induced upregulation of glucosylceramide synthase [74].

On the contrary, the multidrug resistant cancer cell lines $HT29^{col}$ and 2780AD, which over express multidrug resistance-associated protein1 and P-glycoprotein, respectively, displayed enhanced glucosylceramide levels without any change in glucosylceramide synthase expression or activity [29, 76]. Moreover, in $HT29^{col}$ cells the ceramide level was enhanced due to an increased rate of ceramide biosynthesis. The increased glucosylceramide levels in the 2780AD cell line appeared to be related to a decreased glucosylceramide availability for turnover to lactosylceramide in the Golgi apparatus. Furthermore, GM95 mouse melanoma cells deficient in glucosylceramide synthase expression, and therefore unable to metabolize excessive amounts of ceramide, did not show enhanced sensitivity towards chemotherapeutic drugs, compared to GM95 cells corrected for the deficiency by glucosylceramide synthase transfection [77]. This corresponds to the finding that glucosylceramide synthase introduced in jurkat cells did not attenuate the ceramide pool accumulating during apoptosis. While glucosylceramide synthase was able to convert de novo synthesized ceramide, it was unable to convert sphingomyelin-derived ceramide [73]. Moreover, the iminosugars C_4DNJ, C_4DGJ and C_9DGJ, which are potent and selective glucosylceramide synthase inhibitors, failed to cause any reversal of drug resistance in $HT29^{col}$, NCI/ADR^{res} (= MCF7/ADR) and MES-SA/DX-5 cells [29, 52].

Interestingly, GM95 cells expressed very low levels of the ATP-binding cassette transporter proteins P-glycoprotein and multidrug resistance-associated protein 1 [77]. Moreover, in $HT29^{col}$ cells, multidrug resistance-associated protein 1 expression and glucosylceramide levels developed simultaneously, suggesting that neither process is independent [29]. This corresponds to the finding that sphingolipid biosynthesis and drug efflux pump expression are coordinated at the DNA level in *Saccharomyces cerevisiae*. It was found that the transcription of drug efflux genes and that of the IPT1 gene, the product of which catalyzes the final step in the formation of the major yeast sphingolipid mannosyldiinositol phosphorylceramide, are regulated by the same transcription factors. Furthermore, loss of the IPT1 gene affected drug resistance of the resulting strain [19].

Sphingolipids as Constituents of Rafts

Rafts are subdomains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids. Rafts appear to be small in size, but together may constitute a relatively large fraction of the plasma membrane [5, 23]. Different proteins have been shown to be associated with rafts,

especially those involved in cell signaling. Many receptor tyrosine kinases, like the PDGF receptor and G protein-coupled receptors, such as β-adrenergic receptors, have been localized to rafts. Therefore, rafts are believed to play an important role in cell signaling [58]. In polarized cells, rafts are also believed to play an important role in the sorting of apical resident proteins. Glycosylphosphatidyl inositol-anchored proteins, like placental alkaline phosphatase (PLAP), were shown to be sorted to rafts during transport to the apical surface [5, 67]. A specific subclass of rafts is caveolae, which are defined by the presence of the cholesterol-binding protein caveolin. In contrast to other rafts, caveolae are the only ones that can be identified morphologically. Caveolae were known initially for their ability to transport molecules across endothelial cells, but many more functions have been and are still being discovered [1, 71].

The high content of glycosphingolipids and sphingomyelin gave rise to two different models for raft formation. The first model points out the importance of the relative long length and high saturation of the acyl chains of glycosphingolipids and sphingomyelin for raft formation. This allows close packing of the lipids, resulting in a high melting temperature (T_m). Self-aggregates of sphingolipids form a separate phase that is less fluid (liquid-ordered) than the bulk liquid-disordered phospholipids. Cholesterol is recruited to these aggregates, due to its ability to pack tightly with lipids of high T_m [6, 7]. According to the second model, rafts are primarily clusters of glycosphingolipids and sphingomyelin held together through hydrogen-bonding between glycosphingolipid head groups and close packing of the sphingolipids. Cholesterol fills up the gaps between the bulky heads of the glycosphingolipids [67].

Although glycolipids are highly enriched in DIGs they do not appear to be essential for the formation of rafts. It was shown that glycolipid-deficient GM95 melanoma cells had similar amounts of detergent-resistant membrane domains (DRMs) compared to control cells. While the fluidity of the DRMs isolated from both cell lines was similar, glycosphingolipids in DRMs of GM95 cells were substituted by sphingomyelin [54]. However, glycolipids were essential for Src kinase association to DIGs and hence appear to be essential for functional properties of rafts [26]. Furthermore, lactosylceramide was shown to promote detergent insolubility of porcine kidney dipeptidase reconstituted in liposomes. This effect was lactosylceramide-specific and could not be explained solely by the (length of the) acyl chain component of the lipid [55]. A similar effect was observed with ceramide in artificial vesicles [78], while ceramide was also found to compete with cholesterol for DIG association [42]. Furthermore, gangliosides, more complex glycosphingolipids, were able to displace glycosyl phosphatidylinositol-anchored proteins from lipid microdomains in live cells. The exogenous administration of these lipids probably enlarged the surface of the existing rafts and thus interfered with cross-linking of glycosyl phosphatidylinositol-linked proteins or attractive forces between glycosyl phosphatidylinositol anchors and surrounding lipids [68]. In mouse melanoma cells a distinct GM_3-rich glycosphingolipid signaling domain, with specific protein and lipid content, was isolated from a caveolin-containing membrane fraction [27, 28].

The tight packing of sphingolipids in rafts is probably the reason for their insolubility in nonionic detergents at low temperatures. Historically, lipid rafts have been defined operationally by their low density and insolubility in cold 1% Triton X-100 [7, 58]. Recently, a wide variety of detergents other than Triton X-100 have been used to isolate low-density detergent-insoluble membrane fractions [24, 32, 45, 62]. Several detergent-free preparations of membrane domains have also been reported [58]. In spite of the similarities in protein and lipid content between membrane domains isolated with different detergents, the significant differences between them suggest that different membrane domains are being isolated. Indeed, several studies indicate that different liquid-ordered domains co-exist in the plasma membrane [15, 27, 28].

Although sphingolipids mainly reside in the outer leaflet of the plasma membrane, the association of distinct inner-leaflet-associated lipids and signaling proteins with sphingolipid/cholesterol domains implies that membrane domains are probably bilayer structures [58, 75]. Accordingly, domains in the outer and inner leaflet perfectly matched in pure lipid membranes [11].

Rafts, Sphingolipids and ATP-binding Cassette Transporters

One of the best characterized multidrug resistance mechanisms is the overexpression of energy-dependent drug efflux proteins, which prevent intracellular drug accumulation. Of these proteins, all members of the ATP-binding cassette transporter protein superfamily, P-glycoprotein (or ABC B1) and multidrug resistance-associated protein 1 (or ABC C1) are the most widely studied [18, 25, 31]. ATP-binding cassette transporters are primary active transporters, which bind their substrate and move it through the membrane, using ATP hydrolysis to pump against a substrate gradient. Both P-glycoprotein and multidrug resistance-associated protein 1 mediate resistance to a broad range of structurally and functionally unrelated cytotoxic agents. The only common feature of the different substrates is their amphipatic nature. By this nature, once taken up by

the cell, most cytotoxic drugs preferably insert into the inner leaflet of the plasma membrane. This makes it most likely that the transporters function by either 'vacuuming' or by 'flipping' the drugs from the inner to the outer leaflet of the plasma membrane [2, 3, 8, 16].

P-glycoprotein and multidrug resistance-associated protein 1 are known to depend on their direct lipid environment for optimal functioning [14, 69]. Upon reconstitution in model membranes, the ATPase activity of both proteins is dependent on the close proximity of specific phospholipids, especially phosphatidylethanolamine and phosphatidylserine [9, 12, 38, 47, 60, 65]. Furthermore, P-glycoprotein was found to have a higher affinity for its substrates when the surrounding lipids are in gel phase rather than in liquid-crystalline phase [61]. This gel phase occurs when lipids have a high degree of saturation, like sphingolipids, which enables them to pack tightly. This is also an important characteristic of membrane microdomains or rafts, including caveolae [6, 64].

Lavie et al. [35] have shown for the first time the association of an ATP-binding cassette transporter protein with a detergent-resistant membrane domain. They found that a substantial fraction of P-glycoprotein was located in caveolin–1-containing Triton X–100-insoluble membrane domains in cells overexpressing P-glycoprotein. Furthermore, caveolae and the caveolin-1 protein were found upregulated [35]. More evidence for membrane-domain association of ATP-binding cassette transporters and its functional implication came from cholesterol depletion experiments. Cholesterol depletion not only resulted in a shift of P-glycoprotein out of DIG fractions, but P-glycoprotein-mediated drug transport was also affected [44]. In Caco-2 cell monolayers, cholesterol depletion significantly impaired the efflux activity of both P-glycoprotein and multidrug resistance-associated protein 2 [80]. P-glycoprotein association to caveolae and P-glycoprotein substrate levels were also found to be correlated [10].

In contrast, it was recently shown that P-glycoprotein and multidrug resistance-associated protein 1 were not associated with caveolae [24]. In 2780AD cells, which do not express caveolin-1 and hence lack caveolae, P-glycoprotein was still located in DIGs. $HT29^{col}$ cells do express caveolin-1, but multidrug resistance-associated protein 1 was only partly localized in caveolin–1-containing Triton X–100-based DIGs. Moreover, multidrug resistance-associated protein 1 and caveolin-1 were found to be dissociated, based on the absence of microscopical colocalization and absence of coimmunoprecipitation. While P-glycoprotein and multidrug resistance-associated protein 1 expression had increased dramatically during multidrug resistance acquisition, caveolin-1 expression remained unaltered relative to drug-sensitive cells. Both multidrug resistance-associated protein 1 and P-glycoprotein were found to be highly enriched in membrane domains defined by their insolubility in the non-ionic detergent Lubrol WX [24]. The different insolubility of caveolin-1 and ATP-binding cassette transporters in different detergents indicates an association with different membrane domains. Hence, it appears unlikely that caveolin-1 or caveolae play a significant role in the accommodation or function of ATP-binding cassette transporters.

Most multidrug-resistant cancer cells show elevated levels of glucosylceramide, an important constituent of rafts. In multidrug-resistant $HT29^{col}$ cells it was shown that the simultaneous development of multidrug resistance-associated protein 1 and glucosylceramide expression to a large extent occurred in DIGs [29]. Also in multidrug-resistant 2780AD cells, the ATP-binding cassette transporter (i.e., P-glycoprotein) and glucosylceramide are overexpressed and highly enriched in DIGs (Hinrichs, J.W.J., Klappe, K., van Riezen, M., and Kok, J.W., *unpublished observations*). This strongly suggests that sphingolipids are coordinately upregulated with ATP-binding cassette transporters in rafts.

Our studies revealed that several ATP-binding cassette transporters were predominantly located in Lubrol WX-based DIGs in both drug-selected and non-selected human tumor cell lines (Hinrichs, J.W.J., Klappe, K., and Kok, J.W., *unpublished observations*). These Lubrol WX-based DIGs were carefully analyzed and shown to be enriched in cholesterol and sphingolipids, the latter, however, to a lower extent than the sphingolipid enrichment in Triton X–100-based DIGs. In addition, Lubrol WX-based DIGs contained twice the amount of protein and phospholipid compared to Triton X–100-based DIGs. Moreover, concerning the phospholipid composition, Lubrol WX-based DIGs were enriched in phosphatidylethanolamine and phosphatidylserine, which is quite compatible with the well-known dependence of ATP-binding cassette transporters on these aminophospholipids for their ATPase activity [*see above*; 9, 12, 38, 47, 60, 65]. It is reasonable to assume that there is considerable overlap between Lubrol WX- and Triton X–100-based DIGs, based on calculations indicating that both types of DIGs contain a considerable ($> 50\%$) fraction of the total cellular pool of sphingolipids ([29] and Hinrichs, J.W.J., Klappe, K., van Riezen, M., and Kok, J.W., *unpublished observations*). In case of lactosylceramide, even nearly 100% of the cellular pool is found in DIGs. This is incompatible with the existence of Lubrol WX- and Triton X-100-based DIGs as completely separate membrane domains. Consistent with this notion, Drobnik et al. have shown that Lubrol WX-based DIGs contained at least 75% of Triton X-100-based DIGs [13].

We propose a hypothetical model, in which ATP-binding cassette transporters and sphingolipids

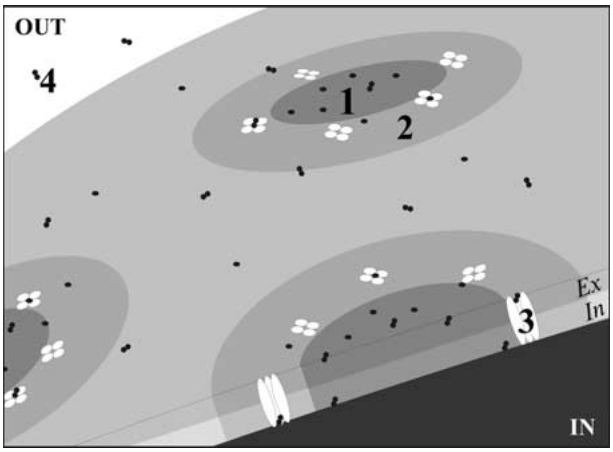

Fig. 1. Schematic representation of the plasma membrane domain organization of a multidrug-resistant cancer cell, according to the layered raft model. The Lubrol WX-based rafts consist of a sphingolipid-enriched Triton X–100-insoluble core (*1*), surrounded by a Triton X–100-soluble/Lubrol WX insoluble region (*2*), which is enriched in specific aminophospholipids and harbors most of the ATP-binding cassette transporter molecules (*3*). ATP-binding cassette transporter substrates (cytostatics) (*4*) concentrate, due to their amphipatic nature, in the hydrophobic sphingolipid-enriched Triton X–100-insoluble core of the raft. The ATP-binding cassette transporters, which optimally benefit from an ATPase activity-stimulating lipid environment in the core-surrounding raft layer, form a 'fence' and efficiently move cytostatic drugs, once they laterally diffuse out of the raft core, from the intracellular leaflet (*In*) to the extracellular leaflet (*Ex*) or out of the cell. *See* text for details.

co-exist in layered rafts (Fig. 1). The layered raft model has already been proposed in an elegant review by Pike [59] as one of three alternative models. All three models, including the homogeneous and heterogeneous raft models, could explain the variation in lipid and protein composition observed in rafts isolated by different protocols, using different detergents or detergent-free methods. These three models are not necessarily mutually exclusive and thus 'traditional' Triton X–100-based rafts may co-exist in cells with 'variant' rafts (e.g., Lubrol WX-based) as well as layered rafts. The layered rafts are composed of concentric layers of lipids ranging from a well ordered cholesterol- and glycosphingolipid-enriched core to less ordered regions and ultimately to the disordered structure of the bulk plasma membrane [59]. Consistent with this model are the observations that ganglioside-rich microdomains can exist within larger ordered domains in both natural and model membranes [63, 79]. In the multidrug resistance raft model, the Lubrol WX-based DIGs consist of a highly sphingolipid-enriched Triton X–100-insoluble core, surrounded by a Triton X–100-soluble region, which contains relatively high levels of specific aminophospholipids and harbors most of the ATP-binding cassette transporter molecules, which can optimally function in this lipid environment (Fig. 1). From the point of view of multidrug resistance, this model may also have interesting implications, which, however, remain to be proven. For example, ATP-binding cassette transporter substrates (cytostatics) may well, due to their amphipatic nature, concentrate in the hydrophobic sphingolipid-enriched Triton X–100-insoluble core. In this context, sphingomyelin has been shown to directly facilitate daunorubicin insertion within phosphatidylcholine-containing monolayers through hydrophobic interactions [36]. The ATP-binding cassette transporters, localized in the raft layer surrounding the sphingolipid-enriched Triton X–100-insoluble core could then act as a 'fence' and expel substrate molecules from the cell as soon as they diffuse laterally out of the raft core.

Conclusions

In the last 10 years it has become clear that glycosphingolipids play an important role in multidrug resistance. The discovery that sphingolipid metabolism could directly facilitate multidrug resistance, by escaping cytotoxic drug-induced apoptosis, was an important impulse for further research. Sphingolipids are also known to be important contributors to rafts and are enriched in detergent-insoluble glycolipid-enriched membrane domains (DIGs) isolated from cells. While rafts were already found to play diverse roles in cellular function, a role in multidrug resistance was postulated. DIGs were found to accommodate several multidrug resistance-associated drug efflux pumps. Importantly, drug efflux pumps and multidrug resistance-associated sphingolipids were found to be coordinately overexpressed during multidrug resistance acquisition and largely co-localized in DIGs. With the discovery of different types of rafts, drug efflux pumps appeared to localize in rafts with a specific lipid composition. One of the challenges lying ahead is to obtain more insight in the (co-)distribution of drug efflux pumps and their substrates in rafts and hence in the functional importance of raft localization of ATP-binding cassette transporters for multidrug resistance.

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