Chapter 6

Detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs) as missing link between multidrug resistance and sphingolipid metabolism

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ABSTRACT

Since their discovery, detergent-insoluble glycosphingolipid-enriched membrane domains have been 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, with 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 increased caveolar membrane domains, which harboured a large fraction of the cellular of the drug efflux pump, P-glycoprotein. Soon, 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 relations between multidrug resistance and sphingolipid metabolism and the important role membrane domains appear to play in this.
MDR, sphingolipids and ABC 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, called multidrug resistance (MDR) (Ling et al., 1983). Regarding the lipid composition of MDR cell lines compared to drug sensitive cell lines, changes have been reported for etherlipids, phospholipids, gangliosides, cholesterol and fatty acids (May et al., 1988, Mazzoni et al., 1993, Peterson et al., 1983). Recent studies have indicated that virtually all MDR cells exhibit a deviating sphingolipid composition, most typically, increased levels of GlcCer (Kok et al., 2000, Lavie et al., 1997, Veldman et al., 2002). At present it is not established with certainty in which way altered sphingolipid metabolism influences MDR 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 MDR mechanisms, such as ATP-binding cassette (ABC) protein overexpression (Sietsma et al., 2001).

Sphingolipids as structural and functional membrane components

Sphingolipids are primarily present in the external leaflet of the plasma membrane and in the equivalent lumenal leaflet of functionally related organelles such as the Golgi apparatus, endosomes and lysosomes (Merrill, 1991). 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 C18 amine, with a trans double bond at the C4-5 position. The aminogroup is generally acylated with a C16-24 fatty acid, yielding ceramide (Cer) (Merrill, 2002, Smith et al., 2002). Cer is synthesized at the endoplasmic reticulum and
reaches the Golgi apparatus were sphingomyelin (SM) and glucosylceramide (GlcCer) are synthesized (Mandon et al., 1992). Further glycosylation of GlcCer results successively in the formation of lactosylceramide (LacCer) and more complex glycosphingolipids, including gangliosides (Merril, 2002, Smith et al., 2002). The trans Golgi network is instrumental in the initial segregation and vesicular-mediated transport to apical and basolateral membranes of sphingolipid species (Slimane et al., 2002, van Meer et al., 2002).

With respect to signaling, Cer is the best studied sphingolipid. It plays a role in the regulation of key cellular processes such as growth inhibition, differentiation and apoptosis. SM breakdown by the activation of sphingomyelinases (SMases) is generally considered as the main source of signaling-involved Cer (Hannun et al., 1993, Hannun et al., 2000, Hannun et al., 2002, Obeid et al., 1993), but de novo synthesis of Cer has also been described in this respect (Bose et al., 1995).

Together with cholesterol, sphingolipids are the main contributors of so-called detergent-insoluble glycosphingolipid-enriched domains (DIGs) (Parton et al., 1995) or rafts. Being part of these rafts, sphingolipids play an additional role in signaling pathways and also in protein sorting (Brown et al., 1992, Pike, 2003).

**GCS-mediated MDR?**

It has been postulated that the increase of GlcCer observed in MDR cells is due to an increased activity of glucosylceramide synthase (GCS), which converts excess Cer into GlcCer, thereby circumventing the onset of Cer-induced apoptosis. In this way the metabolism of Cer could function as an independent MDR mechanism, acting separate from ABC transporter-mediated drug efflux (Lavie et al., 1998, Lucci et al., 1999). Overexpression of GCS indeed conferred adriamycin resistance to human breast cancer cells, however without apparent changes in cellular Cer or GlcCer levels (Liu et al., 1999). In accordance, expression
of GCS anti-sense resulted in reversal of adriamycin resistance, but cellular Cer levels were only elevated after exposure to the drug (Liu et al., 2000). Recently, direct evidence was provided for up-regulation of GCS mRNA as well as enzyme activity in MCF7/ADR cells (Gouaze et al., 2004).

On the contrary, the MDR cancer cell lines HT29col and 2780AD, which overexpress multidrug resistance associated protein1 (MRP1) and P-glycoprotein (Pgp), respectively, displayed enhanced GlcCer levels without any change in GCS expression or activity (Klappe et al., 2004, Veldman et al., 2002). Moreover, in HT29col cells the Cer level was enhanced due to an increased rate of Cer biosynthesis. The increased GlcCer levels in the 2780AD cell line appeared to be related to a decreased GlcCer availability for turnover to LacCer in the Golgi apparatus. Furthermore, GM95 mouse melanoma cells deficient in GCS expression, and therefore unable to metabolize excessive amounts of Cer, did not show enhanced sensitivity towards chemotherapeutic drugs, compared to GM95 cells corrected for the deficiency by GCS transfection (Veldman et al., 2003). This is corresponding with the finding that GCS introduced in jurkat cells did not attenuate the Cer pool accumulating during apoptosis. While GCS was able to convert novo synthesized Cer, it was unable to convert SM-derived Cer (Tepper et al., 2000).

Interestingly, GM95 cells expressed very low levels of the ABC transporter proteins Pgp and MRP1 (Veldman et al., 2003). Moreover, in HT29col cells MRP1 expression and GlcCer levels developed simultaneously, suggesting that both processes are not independent (Klappe et al., 2004). This is corresponding with 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 (Hallstrom et al., 2001).

**Sphingolipids as constituents of DIGs**

DIGs are subdomains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids. DIGs appear to be small in size, but together may constitute a relatively large fraction of the plasma membrane (Brown et al., 1992, Harder et al., 1997). Different proteins have been shown to be associated with DIGs, especially those involved in cell signalling. Many receptor tyrosine kinases, like the PDGF receptor and G protein coupled receptors, like β-adrenergic receptors have been localized to DIGs. Therefore DIGs are believed to play an important role in cell signalling (Pike, 2003). In polarized cells DIGs are also believed to play an important role in the sorting of apical resident proteins. Glycosylphosphatidylinositol (GPI) anchored proteins, like placental alkaline phosphatase (PLAP), were shown to be sorted to DIGs during transport to the apical surface (Brown et al., 1992, Simons et al., 1997). Caveolae belong to a specific subclass of DIGs, which have mainly the same lipid composition, but can be distinguished by the presence of the cholesterol binding protein caveolin. In contrast to other DIGs, caveolae are the only ones that can be identified morphologically. Caveolae were known initially for their ability to transport molecules across endothelial cell, but many more functions have been and are still discovered (Anderson, 1998, Smart et al., 1999).

The high content of glycosphingolipids gave rise to two different models for DIG formation. The first model points out the importance of the relative long length and high saturation of the acyl chains of glycosphingolipids for DIG 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 $T_m$ (Brown et al., 2000, Brown, 2002). According to the second model, DIGs are primarily clusters of glycosphingolipids held together through hydrogen-bonding between glycosphingolipid head groups. Cholesterol fills up the gaps between the bulky-heads of the glycosphingolipids (Simons et al., 1997).

Although glycolipids are highly enriched in DIGs they do not appear to be essential for their formation. It was shown that glycolipid-deficient GM95 melanoma cells had similar amounts of detergent-resistant membrane domains (DRM's) compared to control cells. While the fluidity of the DRM's isolated from both cell lines were similar, glycosphingolipids in DRM's of GM95 cells had been substituted by SM (Ostermeyer et al., 1999). However, glycolipids were essential for Src kinases association to DIGs and hence for functional properties of DIGs (Inokuchi et al., 2000). Furthermore, LacCer was shown to promote detergent insolubility of porcine kidney dipeptidase reconstituted in liposomes. This effect was LacCer specific and could not been explained solely by the (length of the) acyl chain component of the lipid (Parkin et al., 2001). A similar effect was observed with Cer in artificial vesicles (Xu et al., 2001), while Cer was also found to compete with cholesterol for DIG association (London et al., 2004). Furthermore, gangliosides, more complex glycosphingolipids, were able to displace glycosyl phosphatidylinositol (GPI) anchored proteins from lipid microdomains in live cells. The exogenous administration of these lipids probably enlarged the surface of the the existing DIGs and thus interfered with cross-linking of GPI-linked proteins or attractive forces between GPI anchors and surrounding lipids (Simons et al., 1999). 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 (Iwabuchi et al., 1998, Iwabuchi et al., 2000).
The tight packing of sphingolipids in DIGs 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 (Brown, 2002, Pike, 2003). Recently, a wide variety of detergents other than Triton X-100 have been used to isolate low-density detergent insoluble membrane fractions (Madore et al., 1999, Llangumaran et al., 1999, Roper et al., 2000, Hinrichs et al., 2004). Several detergent-free preparations of membrane domains have also been reported (Pike, 2003). 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 isolated. Indeed, several studies indicate that different liquid-ordered domains co-exist in the plasma membrane (Gomez-Muton et al., 2001, Iwabuchi et al., 1998, Iwabuchi et al., 2000).

Although sphingolipids mainly reside in the outer leaflet of the plasma membrane, the association of distinct inner-leaflet associated lipids and signalling proteins with sphingolipid/cholesterol domains implies that membrane domains are probably bilayer structures (Pike, 2003, van Meer et al., 2002). Accordingly, domains in the outer and inner leaflet perfectly matched in pure lipid membranes (Dietrich et al., 2001).

**DIGs, sphingolipids and ABC transporters**

One of the best characterized MDR mechanism is the overexpression of energy-dependent drug efflux proteins, which prevent intracellular drug accumulation. Of these proteins, all members of the ABC transporter protein superfamily, Pgp (or ABC B1) and MRP1 (or ABC C1) are the most widely studied (Gros et al., 1986, Hipfner et al., 1999, Konig et al., 1999). ABC 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 Pgp
and MRP1 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 (Bolhuis et al., 1996, Borst et al., 2000, Chang et al., 2001, Gottesman et al., 1993).

Pgp and MRP1 are known to depend on their direct lipid environment for optimal functioning (Dudeja et al., 1995, Sinicrope et al., 1992). The ATPase activity of both proteins is dependent on the close proximity of specific phospholipids, especially phophatidylethanolamine (PE) and phophatidylserine (PS) (Chang et al., 1997, Doige et al., 1993, Liu et al., 1998, Mao et al., 2000, Romsicki et al., 1998, Sharom et al., 1995). Furthermore, Pgp was found to have a higher affinity for its substrates when the surrounding lipids are in gel phase rather than in liquid-crystalline phase (Romsicki et al., 1999). 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 such as caveolae and DIGs (Brown et al., 2000, Schroeder et al., 1994).

Lavie et al. have shown for the first time the association of an ABC transporter protein with a detergent resistant membrane domain. They found that a substantial fraction of Pgp was located in caveolin-1 containing Triton X-100 insoluble membrane domains in Pgp over expressing cells. Furthermore, caveolin-1 expression, as well as caveolae themselves were found to be up-regulated (Lavie et al., 1997). More evidence for membrane domain association of ABC transporters, and its functional implication came from cholesterol depletion experiments. Cholesterol depletion not only resulted in a shift of Pgp out of DIG fractions, but Pgp-mediated drug transport was also affected (Luker et al., 2000). In Caco-2
cell monolayers, cholesterol depletion significantly impaired the efflux activity of both Pgp and MRP2 (Yunomae et al., 2003). Pgp association to caveolar membrane domains and Pgp-substrate levels were also found to be correlated (Demeule et al., 2000).

In contrast, it was recently shown that Pgp and MRP1 were not associated with caveolae (Hinrichs et al., 2004). In 2780AD cells, which do not express caveolin-1 and hence lack caveolae, Pgp was still located in DIGs. HT29<sup>col</sup> cells do express caveolin-1, but MRP1 was only partly localized in caveolin-1 containing Triton X-100-based DIGs. Moreover, MRP1 and caveolin-1 were dissociated on the basis of absence of microscopical colocalization and absence of coimmunoprecipitation. While Pgp and MRP1 expression had increased dramatically during MDR acquisition, caveolin-1 expression remained unaltered relative to drug-sensitive cells. Both MRP1 and Pgp were found to be highly enriched in membrane domains defined by their insolubility in the non-ionic detergent Lubrol (Hinrichs et al., 2004). The different insolubility of caveolin-1 and ABC 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 ABC transporters.

Most MDR cancer cells show elevated levels of GlcCer, an important constituent of DIGs. In MDR HT29<sup>col</sup> cells it was shown that the simultaneous development of MRP1 and GlcCer expression to a large extent occurred in DIGs (Klappe et al., 2004). Also in MDR 2780AD cells the ABC transporter (i.e. Pgp) and GlcCer are overexpressed and highly enriched in DIGs (Hinrichs, J.W.J., Klappe, K., van Riezen, M., Merrill, A.H., and Kok, J.W., unpublished observations). This strongly suggests that sphingolipids are coordinately up-regulated with ABC transporters in DIGs.

Our studies revealed that several ABC transporters were predominantly located in LU-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-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-based DIGs contained twice the amount of protein and phospholipid compared to Triton X-100-based DIGs. Moreover, concerning the phospholipid composition, Lubrol-based DIGs were enriched in PE and PS, which is quite compatible with the well-known dependence of ABC transporters on PE and PS for their ATPase activity (see above; Chang et al., 1997, Doige et al., 1993, Liu et al., 1998, Mao et al., 2000, Romsicki et al., 1998, Sharom et al., 1995). It is reasonable to assume that there is considerable overlap between Lubrol- 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 (Klappe et al., 2004 and Hinrichs, J.W.J., Klappe, K., van Riezen, M., Merrill, A.H., and Kok, J.W., unpublished observations). In case of LacCer, even nearly 100% of the cellular pool is found in DIGs. This is incompatible with the existence of Lubrol- and Triton X-100-based DIGs as completely separate membrane domains. Consistent with this notion, Drobnik et. al. have shown that Lubrol-based DIGs contained at least 75% of Triton X-100-based DIGs (Drobnik et al., 2002).

We propose a hypothetical model in which ABC transporters and sphingolipids co-exist in layered rafts (Fig. 1). The layered raft model has already been proposed in an elegant review by Pike (Pike, 2004) 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-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 through less ordered regions that ultimately grade into the disordered structure of the bulk plasma membrane (Pike, 2004). Consistent with this model are the observations that ganglioside-rich microdomains can exist within larger ordered domains in both natural and model membranes (Schnitzer et al., 1995, Yuan et al., 2002). In the MDR raft model, the Lubrol-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 ABC transporter molecules, which can optimally function in this lipid environment (Fig. 1). From the point of view of MDR, this model may also have interesting implications, which however remain to be proven. For example, ABC transporter substrates (cytostatics) may well, due to their amphipatic nature, concentrate in the hydrophobic sphingolipid-enriched Triton X-100 insoluble core. In this context, SM has been shown to directly facilitate daunorubicin insertion within phosphatidylcholine (PC) containing monolayers through hydrophobic interactions (Lecompte et al., 2002). The ABC 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.
Fig. 1. Schematic representation of the plasma membrane domain organization of a MDR cancer cell, according to the layered raft model. The Lubrol-based rafts consist of a sphingolipid-enriched Triton X-100 insoluble core (1), surrounded by a Triton X-100 soluble region (2), which is enriched in specific aminophospholipids and harbors most of the ABC transporter molecules (3). ABC transporter substrates (cytostatics) (4) concentrate, due to their amphipatic nature, in the hydrophobic sphingolipid-enriched Triton X-100 insoluble core of the raft. The ABC transporters, which optimally benefit from a ATPase activity stimulating lipid environment in the core-surrounding raft layer, form a ‘fence’ that efficiently move cytostatic drugs, once they laterally diffuse out of the raft core, from the intracellular leaflet to the extracellular leaflet or out of the cell. See text for details.
CONCLUSIONS

The last 10 years it has become clear that glycosphingolipids play an important role in MDR. The discovery that sphingolipid metabolism could directly facilitate MDR, by escaping cytotoxic drugs induced apoptosis, was an important impulse for further research. Sphingolipids are also known to be important contributors to detergent-insoluble glycolipid-enriched membrane domains (DIGs) or rafts. While rafts were already found to play diverse roles in cellular function, a role in MDR was postulated. Rafts were found to accommodate several MDR associated drug efflux pumps. Importantly, the expression of drug efflux pumps and MDR-associated sphingolipid alterations were found to be largely co-localized in DIGs and coordinately regulated during MDR acquisition. 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 DIGs and hence in the functional importance of DIG localization of ABC transporters for MDR.