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Topical Review

The Involvement of Sphingolipids in Multidrug Resistance

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Abstract. Administration of most chemotherapeutic agents eventually results in the onset of apoptosis, despite the agents' variety in structure and molecular targets. Ceramide, the central molecule in cellular glycosphingolipid metabolism, has recently been identified as an important mediator of this process. Indeed, one of the events elicited by application of many cytotoxic drugs is an accumulation of this lipid. Treatment failure in cancer chemotherapy is largely attributable to multidrug resistance, in which tumor cells are typically cross-resistant to multiple chemotherapeutic agents. Different cellular mechanisms underlying this phenomenon have been described. Of these the drug efflux pump activity of P-glycoprotein and the multidrug resistance-associated proteins are the most extensively studied examples. Recently, an increased cellular capacity for ceramide glycosylation has been recognized as a novel multidrug resistance mechanism. Indeed, virtually all multidrug-resistant cells exhibit a deviating sphingolipid composition, most typically, increased levels of glucosylceramide. On the other hand, several direct molecular interactions between sphingolipids and drug efflux proteins have been described. Therefore, in addition to a role in the multidrug resistance phenotype by which ceramide accumulation and, thus, the onset of apoptosis are prevented, an indirect role for sphingolipids might be envisaged, by which the activity of these efflux proteins is modulated. In this review, we present an overview of the current understanding of the interesting relations that exist between sphingolipid metabolism and multidrug resistance.

Key words: Sphingolipids — Ceramide — Sphingomyelin — Glucosylceramide Synthase — Multidrug resistance — P-glycoprotein

Chemotherapy Induces Apoptosis

Chemotherapy is the primary approach towards the treatment of metastatic cancer disease. For this, several different classes of cytostatic compounds are available to the treating clinician. Clinically, a vast amount of knowledge is available regarding the administration of (combinations of) these compounds to the patient [3]. From a molecular point of view, however, the exact mechanisms of action are poorly understood. Most chemotherapeutic agents are presumed to produce their cytotoxic effects by interfering at some stage with the synthesis or function of DNA or RNA. The anthracyclin doxorubicin for example, binds to and intercalates with DNA which, after local uncoiling of the double helix, results in an inhibition of DNA- and RNA synthesis [13]. Another cellular target of doxorubicin is the 170 kDa nuclear enzyme topoisomerase II. Being involved in

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Abbreviations: ABC, ATP-binding cassette; GlcCer, glucosylceramide; GCS, ceramide:UDP-glucose transferase (GlcCer synthase); GSL, glycosphingolipids; LacCer, lactosylceramide; MDR, multidrug resistance/resistant; MRP, multidrug resistance-associated protein; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; Pgp, P-glycoprotein; SM, sphingomyelin; SMase, sphingomyelinase; SPT, serine:palmitoyl-CoA transferase

DNA processing such as replication, transcription and recombination, inhibition of this enzyme results in DNA strand breaks [84]. Furthermore, several cytostatics give rise to the formation of free radicals, which in turn cause DNA damage, lipid peroxidation and alkylation of proteins. Compounds such as taxol and vincristine exhibit a different mechanism of action. By specifically interfering with tubulin dynamics, these agents effectively block mitosis and thus tumor growth.

The observation that etoposide administration to cultured leukemia cells induced a characteristic internucleosomal DNA fragmentation, suggested that apoptosis plays a vital role in chemotherapy-induced cell death [34]. Since then the onset of the apoptotic program has been implied in the action of numerous other chemotherapeutic agents [4, 14, 77]. Soon these initial *in vitro* studies were confirmed by *in vivo* observations [26]. In conclusion, the eventual onset of apoptosis appears to be a common theme in chemotherapy-induced cell death, independent of the agent, its direct molecular target and the mechanism of action.

Multidrug Resistance Occurs by a Variety of Mechanisms

Clinical resistance to anticancer drugs is the major reason for treatment failure. Generally, tumor cells initially respond well to chemotherapeutic agents. Unfortunately however, repeated drug administration provides a selective pressure which often results in the selection of drug-resistant cells, and hence in incurable relapses. Surprisingly, cells very often become resistant to a variety of structurally unrelated drugs after exposure to only one single drug. This phenomenon was first described by Ling and colleagues and is generally referred to as multidrug resistance (MDR; [6, 48]). Subsequent research has revealed a variety of mechanisms that are presumed to be responsible for the MDR phenotype of a cell. These mechanisms can be divided into two groups: those that reduce intracellular drug accumulation and those that reduce the consequences of intracellular drug accumulation.

Examples of the first group include a reduction in membrane permeability, which leads to a decreased entrance of xenotoxic compounds [9]. By far the best characterized mechanism leading to decreased intracellular drug levels, is the overexpression of energy-dependent drug efflux pump proteins such as P-glycoprotein (Pgp), the product of the MDR1-gene [7, 21, 24]. This 170 kDa integral membrane protein is a member of the ATP-binding cassette (ABC) transporter protein superfamily. Pgp consists of 1280 amino acids and its 12 transmembrane domains are present in two homologous halves, each containing six transmembrane regions and a large cytoplasmic loop with an ATP-binding cassette. Pgp shows very low substrate specificity since little structure-homology is observed within the broad range of different compounds subjected to the pump activity of Pgp. In fact, the only common feature of the different substrates is their amphipathic nature that elucidates the fact that many drugs, once taken up by the cell, preferably insert into the inner leaflet of the plasma membrane. Little is known with respect to the mechanism of Pgp action. The oldest and simplest model presents Pgp as a membrane pore, which selectively effluxes cytosolic compounds into the aqueous environment of the cell, a process accompanied by ATP hydrolysis. The 'hydrophobic vacuum cleaner' model provides an alternative mechanism [73]. According to this model, Pgp 'floats' in the lipid bilayer where it continuously removes drugs from the inner leaflet of the plasma membrane by expelling them into the extracellular space. Alternatively, Pgp acts as a flippase by translocating lipophilic compounds to the external leaflet of the membrane, from which they might eventually diffuse into the extracellular fluid [29]. In addition to Pgp, other members of the ABC transporter protein superfamily have also been identified as broad-range drug efflux pumps. The multidrug resistance-associated protein (MRP), for example, mediates cellular resistance to many structurally and functionally unrelated cytotoxic agents [30, 35, 38]. This 190 kDa protein shares only 15% amino acid homology with Pgp. Nevertheless, the two proteins confer resistance to a similar, though not identical, range of cytostatics [23].

The second group of MDR mechanisms does not lead to reduction of intracellular drug accumulation, but instead reduces the consequences of this intracellular action. An example is the conversion of a toxic drug into less harmful metabolites. In this respect, an enhanced cellular glutathione metabolism might be of great importance [85]. Other examples are the decreased expression of topoisomerase II, an important target for several cytostatic drugs [19], and the altered expression of β-tubulin isotypes, which mediates resistance to taxol [35]. Alternatively, target amplification processes decrease the drug-to-target ratio, which in turn improves the chance of survival [6]. Yet another option for MDR cells is provided by increased target repair mechanisms. With DNA as the primary target of many cytostatic drugs, increased DNA-repair activity helps to limit damage brought about by cytotoxic drugs [66]. Finally, since many cytostatics, independent of their exact mechanism of action, eventually induce apoptosis, inhibition of the apoptotic signalling pathways increases cell survival [26]. If a cell manages to avoid undergoing apoptosis, it might be defined as having acquired an MDR phenotype. In this context, regulatory proteins such as members of the Bcl2-family and p53 play an important role [42, 53, 86]. Adaptive changes in the regulation of $[Ca^{2+}]_i$ homeostasis also occur in MDR cells [18], in line with the notion that this

messenger molecule plays a role in the apoptotic process. In addition, the regulation of the pro-apoptotic sphingolipid ceramide might be critical in this respect. This, and other, possible relations between sphingolipid metabolism and the MDR phenotype of a cell will be extensively discussed in the following sections.

Sphingolipids are Involved in Apoptotic Signaling

Sphingolipids are a large family of lipids that reside primarily in the external leaflet of the plasma membrane. A long-chain sphingoid base, typically consisting of a D-*erythro* C_{18} amine with a *trans* double bond at the C_{4-5} position, serves as the central moiety of all sphingolipids. Although several forms of sphingoid bases exist as such in the cell, the amine group of the molecule is generally acylated with a C_{16} to C_{24} fatty acid, yielding a ceramide. This reaction takes place in the endoplasmic reticulum [55]. Further metabolism of ceramide into the phospholipid sphingomyelin (SM), by the addition of a phosphocholine headgroup, primarily occurs at the lumenal side of the Golgi membranes. In contrast, the formation of the cerebroside glucosylceramide (GlcCer), catalyzed by ceramide:UDP-glucose transferase (GlcCer synthase; GCS), occurs at the cytoplasmic side of the Golgi apparatus [20, 33]. GlcCer serves as the metabolic precursor for the synthesis of lactosylceramide (LacCer), and hence for all other neutral glycosphingolipids (GSL) and gangliosides. The externally orientated localization of GCS is exceptional since all other glycosyltransferases, and the sialyltransferases that are involved in ganglioside biosynthesis, are orientated towards the Golgi lumen [87]. This observation implies the presence of a GlcCer translocation mechanism in the Golgi membranes [41]. After their synthesis, sphingolipids are transported to their destination, *i.e.,* the plasma membrane, mainly by vesicular bulk flow [95]. However, also in this respect GlcCer is an exception since a substantial part of this particular lipid is transported by nonvesicular mechanisms [93, 96].

During the past decade an enormous increase in research effort on sphingolipids was triggered by discoveries that implicated several sphingolipid metabolites in signal transduction processes (for recent reviews *see* [47, 56, 68]). With respect to signaling, ceramide is the best-studied sphingolipid. Bioactivity of ceramide plays a role in the regulation of key processes such as growth inhibition, differentiation and apoptosis [27, 28, 63]. SM is generally considered as the primary metabolic source of this signaling-involved ceramide, although an increased *de novo* synthesis of ceramide has also been described in this respect [8]. Therefore, sphingomyelinases (SMases) are important in regulation of the cellular levels of this lipid. Lysosomal 'acidic' SMase is responsible for housekeeping SM catabolism during membrane turnover processes. Although acidic SMase has been implicated in the generation of signaling-involved ceramide, it is generally believed that specific neutral, membrane-bound SMases are responsible for this [47]. Depending on the cell type, activation of these enzymes might occur upon stimulation with ligands such as TNFα, IL-1β, Fas or neurotrophins. However, SMase activation is not necessarily a receptor-mediated process, since the administration of nonphysiological compounds such as chemotherapeutic drugs in some cases also results in the formation of ceramide (*see below*). Finally, several nonspecific stress factors such as UV-radiation, serum deprivation and ischemia also result in ceramide generation [25]. In many cases, the effects of these stimuli, including the onset of apoptosis, can be mimicked by the addition of cell permeable analogues of ceramide [63].

After generation, ceramide presumably exerts its effects by direct molecular interactions with specific target molecules, which in turn then activate further signaling cascades. The details of these downstream signaling events are not yet fully understood. Nevertheless, several molecules that might serve as direct targets for ceramide have thus far been identified. A 97 kDa plasma membrane-bound proline-directed protein kinase is activated by both ceramide and TNFα and has been designated as ceramide-activated protein kinase [49]. In addition, ceramide directly and specifically activates a serine/threonine protein phosphatase 2A of the heterotrimeric subfamily that is named ceramide-activated protein phosphatase [15]. Finally, it has been demonstrated that ceramide activates protein kinase ζ [61]. Further downstream, the (indirect) activation of a variety of signaling cascades, including MAPK, SAPK, NF-κB and/or the retinoblastoma gene product, has been attributed to ceramide [56]. With respect to apoptosis, the activation of caspases is interesting since these proteases are actively involved in the execution phase of the apoptotic process. However, it is not clear whether activation of this protease family is dependent on ceramide generation [81, 83]. Taken together, ceramide activates various signaling cascades that, in general, lead to growth inhibition and/or apoptosis.

In addition to ceramide, biological activity has been attributed to other sphingolipid metabolites such as sphingosine 1-phosphate and GlcCer, the latter being mainly implicated in the regulation of cell growth and differentiation [2, 76]. However, the underlying molecular mechanisms are even less understood than are those for ceramide action.

MDR Cells Display an Altered Sphingolipid Composition

It has been known for a long time that the etherlipid-, phospholipid- [58], triglyceride- [72] and cholesterol

composition [59, 60] of drug-resistant cells can differ from that of drug-sensitive cells. In addition, observations in the early 1980s [69] indicated differences in ganglioside composition of drug-resistant cells, when compared to sensitive cells. However, it was not until the end of the 1990s that MDR-related differences in simple sphingolipid composition were further investigated. Cabot et al. showed that in particular the levels of GlcCer, a precursor of all higher glycosphingolipids and a direct metabolic product of ceramide, was consistently elevated in several Pgp overexpressing cell lines [43]. It has therefore been suggested to consider increased GlcCer levels as a diagnostic marker for MDR tumors [54]. Recent work from our laboratory on human ovarian carcinoma cells demonstrated that, in addition to GlcCer, also SM and GalCer levels are significantly enhanced in Pgp overexpressing cells, when compared to its drug-sensitive counterpart (R.J. Veldman, H. Sietsma, K. Klappe and J.W. Kok; *unpublished observations*). Interestingly, LacCer and all higher GSL were substantially decreased in these cells. On the other hand, the levels of the bioactive sphingolipid metabolites ceramide and sphingosine were quite comparable when sensitive and resistant cell lines were compared. Furthermore, we showed that deviations in GlcCer levels are not restricted to Pgp overexpressing cells since also MRP1-overexpressing cells, such as the human colon carcinoma cell line HT29^{col}, show a 2- to 3-fold increase in this lipid [36].

Several metabolic mechanisms can be envisaged to underlie the observed differences in GlcCer levels. Although direct data on enzymatic activities are poorly available, an increased GCS activity is probably an important factor. In addition to an increased GCS activity, enhanced GlcCer levels might be explained by a decreased GlcCer translocation in the Golgi apparatus (R.J. Veldman, H. Sietsma, K. Klappe and J.W. Kok; *see below*). This latter finding might explain the decreased levels of LacCer and all higher GSL.

Chemotherapeutic Drugs Induce Ceramide Generation

In addition to receptor-mediated activation of the SM-cycle by physiological ligands, it has been shown that administration of a variety of chemotherapeutic agents leads to the production of apoptosis-involved ceramide [57, 82, 83]. However, the involved mechanism of ceramide production appears to differ between agents and possibly between cell types. For example, daunorubicin induces ceramide accumulation and subsequent cell death by either activation of a neutral SMase or, alternatively, by an increased *de novo* synthesis through ceramide synthase [8, 32]. This has also been described for camptothecin [92]. Yet another mechanism was recently provided by the observation of an increased activity of serine palmitoyltransferase (SPT), the first and rate-limiting step in the synthesis of all sphingolipids, upon administration of the topo-isomerase inhibitor etoposide [67]. In addition to chemotherapeutic agents which are known to be substrates for ABC transporters, the drug resistance modifier PSC833, a well-known Pgp inhibitor, has been shown to activate cellular ceramide formation [11].

Studies in yeast included unicellular organisms in our understanding of sphingolipid-mediated chemoresponsiveness. In a recent study, we compared the *Saccharomyces cerevisiae* 7R4 strain, which is mutated in the SPT-encoding LCB1-gene, with the 7R4/LCB1 strain, in which sphingolipid metabolism was restored by a transformation with an intact LCB1-gene. Whereas the transformed 7R4/LCB1 strain was sensitive to daunorubicin-induced growth suppression, the 7R4 strain, completely devoid of sphingolipids, appeared to be fully resistant. Interestingly, daunorubicin sensitivity could be restored in the 7R4 strain by co-administration of phytosphingosine. Clearly, these data indicate that phytosphingosine, and/or metabolites of this sphingolipid, mediate daunorubicin-induced cell death in yeast (H. Sietsma, G.M. Jenkins, W.A. Kamps, J.W. Kok, Y.A. Hannun, *unpublished observations*).

Ceramide Metabolism as a Novel Target for Chemosensitization

As outlined above, the mechanism of action of several chemotherapeutic drugs appears to implicate the generation of pro-apoptotic ceramide. Previous work demonstrated that the cellular ceramide levels are subjected to careful regulation. For example, when the human colon carcinoma cell line HT29 was treated with a bacterial SMase, rapid ceramide generation was observed. However, this excessive ceramide was efficiently metabolized into cerebrosides and higher GSL, and these cells survived the otherwise lethal treatment [90]. The importance of GCS in this respect was emphasized by experiments in which overexpression of this enzyme conferred drug resistance to previously sensitive cells [50]. Conversely, inhibition of GCS expression by antisense technology resulted in a clear increase in sensitivity for doxorubicin [52]. Furthermore, it was recently demonstrated that this mechanism might not only protect a cell from the damaging effects of xenotoxic compounds but also from physiological ligands such as TNFα [51].

In line with the described genetic approach are data from several pharmacological studies. For example, well-known drug resistance modulators such as tamoxifen, verapamil and cyclosporine A have been shown to exert (part of) their effect by inhibition of GCS [10, 44]. In addition, the effect of specific GCS inhibitors such as

1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP; [1, 70]) was tested. At high concentrations these compounds have been shown to preferentially kill multidrug resistant cells compared to drug-sensitive cells [62], whereas sublethal concentrations increased the sensitivity for the actions of vincristine and taxol [79]. Both vincristine and taxol are known to interfere with microtubule polymerization. Interestingly, the chemosensitizing effect of GCS inhibition could not be observed in combination with etoposide, a compound that has no known direct effects on cytoskeleton integrity. At present, the possible role of microtubuli in GSL metabolism in relation to drug sensitivity is not understood and awaits further investigation.

In conclusion, intracellular levels of the apoptosis mediator ceramide appear to be carefully regulated by an active metabolic mechanism. In particular, the activity of GCS seems to be critical in determining the drug sensitivity of a cell. This metabolic mechanism, which helps to avoid the onset of apoptosis, might thus represent a novel MDR mechanism. These findings are of potential clinical interest, since GCS might prove to be an interesting target for the development of novel resistance modifiers.

Is GCS-mediated Drug Resistance Dependent on ABC Transporter Proteins?

An important question arises when considering MDR-related GSL metabolism, i.e., whether increased GCS activity represents a novel MDR mechanism in its own right, as has been suggested [52], or whether changes in GSL metabolism facilitate the functioning of other MDR-involved mechanisms. In this context, especially the overexpression of ABC transporter proteins might be relevant since several interesting molecular interactions between GSL and this class of proteins have been described (*see below*). In fact, virtually all data obtained so far on GCS-related MDR have been obtained from cells that overexpress drug efflux proteins.

Therefore, we recently performed experiments on GM95 mouse melanoma cells. These cells lack functional GCS and hence are completely devoid of GlcCer and all higher GSL [31]. Consequently, these cells are seriously hampered in their capacity to metabolize excessive amounts of ceramide, as for example generated by bacterial SMase [37]. In the light of the putative MDR mechanism described above, it was to be expected that these cells exhibit an increased sensitivity towards chemotherapeutic drugs. Surprisingly however, when we compared mutated cells with GM95 cells that were corrected for the deficiency by a stable transfection with a functional GCS cDNA, no difference in viability was observed upon exposure to various drugs (R.J. Veldman, A. Mita, K. Klappe, J.W. Kok and T. Levade, *unpublished observations*). There was no difference in sensitivity despite the fact that all tested agents induced ceramide generation. In addition, no difference in activation of caspases or other apoptosis markers was detected between the two cell types. Interestingly, GM95 cells do not express detectable amounts of the ABC transporter proteins Pgp or MRP1. Therefore, these data support the hypothesis that GSL-mediated drug resistance is somehow dependent on the presence of drug transporter proteins. It should be noted that additional studies are required to test this hypothesis, since another study does show increased drug resistance upon GCS cDNA transfection in MCF-7 human breast cancer cells that do not abundantly express Pgp or MRP1 [50].

Sphingolipids Interact with ABC Transporter Proteins

A general property of drug efflux protein substrates is their amphipathic nature [21]. Therefore, the possibility existed that naturally occurring molecules could also be subjected to ABC transporter translocation and/or efflux activity. Indeed, it was discovered that several phospholipids are translocated by Pgp [17, 74, 80]. In addition, fluorescent short-chain analogues of both SM and GlcCer turned out to be translocated across the plasma membrane as well, in particular by MRP1 [71, 88, 89]. Conclusive evidence is still lacking for the hypothesis that endogenous long-chain sphingolipids are substrates for these proteins, but several studies have provided indirect evidence. (i) Although the majority of the cellular SM is located in the outer leaflet of the plasma membrane, the signaling-involved pool of SM is generally presumed to reside in the cytoplasmic leaflet [47]. (Over) expression of lipid translocators such as Pgp, might deplete this particular pool and therefore interfere with the SM-ceramide signaling pathway. In accordance with this is the finding that the Pgp blocker and chemosensitizer PSC833 restores the normal SM distribution over the membrane of TNFα-resistant KG1a cells. A pretreatment with PSC833 resulted in a restored ceramide production and subsequent cell death, upon TNFα administration [5]. (ii) In addition to its presence in the plasma membrane, a fraction of Pgp is present in the Golgi complex. It has been proposed that this Pgp mediates GlcCer translocation across the bilayer, from the cytosolic face of the Golgi to the lumen, to provide substrate for the lumenal synthesis of LacCer and higher GSL. In Pgp-transfected MDCK cells an increased biosynthesis of sphingolipids derived from GlcCer was observed, while Pgp was localized to the Golgi apparatus [40]. (iii) In accordance with this, we have obtained data that indicate a sphingolipid translocation function for Pgp present in other cellular membranes (R.J. Veldman, H. Sietsma, K. Klappe and J.W. Kok, *unpublished observations*). Intact

2780AD human ovarian carcinoma cells overexpress Pgp, which is localized primarily in the plasma membrane and intracellular, non-Golgi related, vesicular compartments. According to our model, this Pgp removes GlcCer from the cytosolic leaflet of the plasma/vesicular membrane and thereby creates a sink function for GlcCer synthesized on the cytosolic face of the Golgi apparatus. GlcCer is then transferred from the cytosolic face of the Golgi to the cytosolic leaflet of other cellular membranes in a nonvesicular fashion, resulting in decreased substrate availability for translocation to the Golgi luminal leaflet and hence LacCer biosynthesis. This model explains the reduced biosynthesis of LacCer and more complex sphingolipids we observed in these cells, and is further supported by experiments employing the Pgp inhibitor PSC833. This inhibitor blocked LacCer formation from exogenously added radiolabeled GlcCer in intact cells, but not in a cell-free assay or in isolated Golgi membranes, indicating that PSC833 does not affect the activity of the LacCer synthase enzyme directly. These observations are in line with the notion that LacCer biosynthesis in intact cells depends on Pgp-mediated GlcCer translocation, while in a cell-free system the substrate is not limited due to loss of membrane integrity. The latter study shows an indirect effect of PSC833 on sphingolipid metabolism, via modulation of Pgp function. It should be noted that not all effects of PSC833 on sphingolipid metabolism are related to Pgp function. PSC833-induced accumulation of ceramide in cells appears to occur through increased *de novo* biosynthesis [11] and independent of Pgp [22].

Taken together, it can be assumed that these ABC transporter proteins, among others, play an important role in the active maintenance of lipid asymmetry across the plasma membrane, and of other cellular membranes as well. It is to be expected that competition occurs between apparent natural substrates such as GlcCer and xenotoxic substances such as chemotherapeutic drugs. However, when cellular GlcCer levels were decreased by incubating Pgp overexpressing ovarium carcinoma cells with the GCS inhibitor PDMP, no difference in Pgp efflux activity was observed, as measured with a rhodamine 123 efflux assay [91]. The apparent absence of competition between the natural GlcCer substrate and rhodamine 123 may be related to the presence of different binding sites within the ABC transporter molecule. Noncompetitive interactions of two different compounds, including rhodamine 123, with Pgp have been reported [78].

ABC transporter proteins are likely to be involved in particular membrane regulation processes. For optimal functioning on the other hand, these proteins will, like most other membrane proteins, be dependent on their immediate lipid environment [12, 16]. To investigate the latter issue, we functionally assayed Pgp activity in the presence of various GSL [91]. Employing rhodamine 123 as a fluorescent substrate, we determined that its Pgp-mediated efflux could be inhibited by short-chain analogues of GlcCer, SM and GalCer, whereas more complex GSL, such as GM_2 and GM_3, were not effective. Ceramide was also ineffective, which is in accordance with previous observations that this lipid is not a substrate for Pgp-mediated efflux (R.J. Veldman and J.W. Kok, *unpublished observations*). Interestingly, sphingosine enhanced Pgp activity. Sphingosine is a known inhibitor of protein kinase C activity. Therefore, the observed Pgp activity modulation by sphingosine might be indirect since PKC-mediated phosphorylation of Pgp affects its activity [75]. The inhibitory effect of the GlcCer analogue on Pgp was functionally translated to an increased cellular toxicity of doxorubicin, which indicates potential physiological relevance. During the described experiments, loading of the cells with the lipid analogues occured at 4°C and the rhodamine 123 efflux itself took place within several minutes. Therefore, it is reasonable to assume that the lipid analogues did not gain access to the inner leaflet of the plasma membrane and could thus not be flipped by Pgp. Thus, competition between rhodamine 123 and the lipid analogues can be excluded as an explanation for the observed effects. Rather, we propose that specific lipid-protein interactions in the outer leaflet of the membrane underlie the observed effects.

Membrane Domains May Be Involved in MDR-related Alterations in Sphingolipid Composition

The observation that MDR cells consistently overexpress GlcCer raises questions concerning the cellular localization of this increased lipid pool. Detergent-insoluble glycosphingolipid-enriched complexes (DIG), which are present in virtually all cell types, provide an interesting possibility. Among other lipids such as SM and cholesterol, these membrane domains are highly enriched in GlcCer [39]. Caveolae are a specialized form of DIG. These flasked-shaped invaginations of the plasma membrane contain high quantities of caveolin, a family of proteins which, in addition to a structural role, are involved in a variety of signal transduction routes [64]. Interestingly, caveolin expression, and thus the presence of caveolae, is substantially upregulated in Pgp-overexpressing cells [45, 46, 94]. Of this Pgp, a substantial fraction was present in these GlcCer-enriched membrane domains. This relationship between Pgp and caveolae was further established by the recent observation that the absence of biologically active Pgp results in defects in the processing of caveolae and budding from the Golgi complex, suggesting a functional association

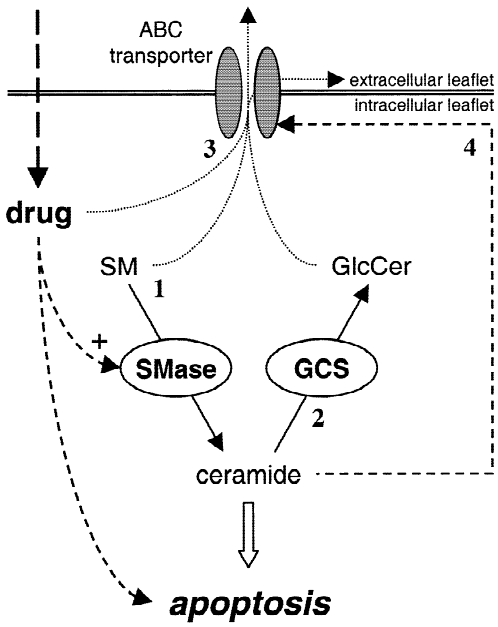

Fig. 1. Schematic representation of four possible mechanisms by which sphingolipids might be involved in the regulation of drug resistance. (1) Drug transporter-mediated translocation of SM over the plasma membrane, rendering it inaccessible to SMases and thus interrupting a ceramide-mediated apoptotic program. (2) Increased GCS expression or activity controls drug-induced ceramide accumulation. (3) Competitive inhibition between xenotoxic substances and membrane lipids results in increased sensitivity. (4) Sphingolipid-mediated signal transduction processes might regulate the activity of ABC-transporter proteins and/or other MDR mechanisms. *See* text for details.

between the transport of caveolae to the plasma membrane and Pgp [65]. Taken together, several interesting relationships appear to exist between GlcCer, Pgp and caveolae. (i) GlcCer is a substrate for Pgp. (ii) GlcCer is highly enriched in caveolae and (iii), Pgp is present in caveolae and is involved in caveolae transport from the Golgi apparatus to the plasma membrane. Further research is required to understand the functional relevance of these correlations.

Conclusions

Taken together, we envisage four different mechanisms by which sphingolipids might determine the drug-resistant phenotype of a tumor cell. These mechanisms, which might act independently, synergistically or even opposing each other, are summarized in Fig. 1. Upon entering a cell, most cytotoxic drugs elicit an apoptotic program, either or not via ceramide generation. In the case of ceramide-mediated apoptosis, an important role is played by SMases that release ceramide from its metabolic precursor SM. A specific pool of signaling-involved SM is presumed to reside in the inner leaflet of the plasma membrane. The first mechanism is based on the finding that SM is probably a substrate for ABC transporters (Fig. 1, item 1). Therefore, the presence of these proteins might cause a depletion of SMase substrate, and hence may prevent the initiation of ceramide-mediated apoptosis. The second mechanism is not dependent on ABC transporter protein expression, since it is purely metabolic by nature. Namely, once generated, ceramide might be efficiently converted into GlcCer and higher GSL (Fig. 1, item 2). Therefore, despite drug-induced activation of SMases or other ceramide generating mechanisms, apoptosis-inducing threshold levels of ceramide will not be exceeded. A third mechanism, which in contrast to the others is chemosensitizing by nature, again relies on the presence of drug transporters. Obviously, these proteins will expel most drugs directly. However, with natural lipids such as SM and GlcCer as a substrate, competitive inhibition of the pump might be envisioned when a cell expresses high amounts of these lipids (Fig. 1, item 3). This in turn will lead to an accumulation of the drug and thus to increased cell death. The least understood possibility, which nevertheless cannot be excluded, is that sphingolipid metabolites such as ceramide and sphingosine 1-phosphate, are involved in the regulation of membrane pumps, or other MDR mechanisms, by means of signal transduction, for example, by interacting with the regulatory domains of MDR proteins (Fig. 1, item 4). If applicable, these interactions might result in either an increased drug resistance or sensitivity. Apart from these mechanisms, it should be noted that MDR-related alterations in sphingolipid metabolism and composition may be secondary to the expression of ABC transporter proteins or other MDR mechanisms. ABC transporters functioning as translocators of sphingolipids can thereby (drastically) influence the subcellular distribution of sphingolipids and hence their metabolic conversions. Alternatively, upregulation of specific membrane domains, such as DIG and caveolae, may be an autonomous MDR mechanism (or needed to accommodate ABC transporter proteins), which concomitantly leads to increased sphingolipid expression.

Although the described mechanisms are intriguing, very little is known regarding their relative contribution to the actual MDR phenotype. In addition, it is not known to what extent these different mechanisms act independently, synergistically or even opposing each other. Nevertheless, we consider the current developments as very promising, since manipulation of the GSL metabolism of MDR tumor cells, by pharmacologic or genetic means, might prove to be a novel target for cancer therapy and the circumvention of MDR.

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