Bioactivity of sphingolipids

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8.1 - GENERAL SUMMARY

The main objective of the research described in the present thesis was to increase our understanding concerning the functional relationship between cellular sphingolipids and the regulation of processes such as cell differentiation, apoptosis and multidrug resistance (MDR). The general approach we employed was to modulate the sphingolipid metabolism of cultured cells by pharmacological- and biochemical means, and to study the subsequent effects of these treatments on various cell physiological phenomena.

In chapter 2 studies are described which were performed with the glucosylceramide (GlcCer)-synthase inhibitor D,L-*threo* 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP). Generally, the metabolic effect of enzyme inhibitors such as PDMP is not limited to one particular lipid. In a series of control experiments however, we were able to pinpoint the decreasing levels of GlcCer as the primary cause for the observed onset of cell differentiation, whereas bioactive effects of lipids such as ceramide (Cer) could be excluded in this respect. Therefore, this observation suggests that GlcCer is actively involved in the regulation of cellular differentiation processes. On the other hand, an increase in the levels of Cer induced apoptosis (chapter 3). However, this only occurred on the condition that Cer reached intracellular sites, since Cer which was generated in the outer leaflet of the plasma membrane, and topologically corresponding cellular sites, failed to induce apoptosis. Chapter 4 describes an attempt to modulate (Glc)Cer levels by γ-interferon (IFNγ), a differentiation-, and apoptosis-inducing ligand. However, in the presently employed HT29 model system, no such changes were observed (see discussion below). In chapter 5 data are presented which suggest that, in addition to Cer and GlcCer, free sphingoid bases might be involved in cellular growth regulation, and that the metabolism of these lipids is susceptible to pharmacological intervention.

Chapter 6 describes experiments which demonstrate that sphingolipids might be involved in the regulation of P-glycoprotein (Pgp)-mediated MDR, in addition to their above-described role in cellular growth regulation. In line with these studies, we established a novel MDR cell line, *i.e.,* HT29 cells which specifically overexpress MRP1 (chapter 7). Interestingly, also these cells showed alterations in their sphingolipid composition, which indicates that a sphingolipid-MDR link is not restricted to Pgp-overexpressing cells.

8.2 - CONCLUDING DISCUSSION

The ceramide:glucosylceramide ratio; a model - Data from the literature show that high cellular levels of GlcCer are generally associated with a high proliferation activity. In addition to this, the results described in chapter 2 indicate that a decrease in the levels of this particular lipid leads to differentiation. On the other hand, an

increase in Cer results in the onset of the apoptotic program. In terms of cell physiology, proliferation, differentiation and apoptosis can be considered as increasing states of cellular maturation, occurring on a continuous scale. *E.g.*, concomitant with the onset of differentiation, the proliferation activity of a cell population decreases. In addition, apoptosis can be considered as the terminal stage of differentiation. The present data suggest that, in the employed HT29 model system, the state of maturation is determined by the levels of Cer and GlcCer. In other words, sphingolipid levels do not change as a consequence of a changed cell physiological state, but *vice versa*; there appears to exist a causal relation between the levels of particular sphingolipids and cellular growth. Based upon the present observations we therefore propose a model in which the balance between Cer and GlcCer is a determining factor for the state of cellular maturation. A low Cer:GlcCer ratio will result in a high proliferation activity of the cells. An upward shift in this ratio will induce differentiation of cells, which thereby gradually lose their proliferation activity. Finally, at high Cer:GlcCer ratios, cells will irreversibly go into apoptosis. Future research will prove the general validity of this model.

Physiological modulation of sphingolipids and signaling - The ratio between Cer and GlcCer is determined by the activity of GlcCer-synthase and GlcCer-glucosidase. In addition, the levels of Cer are regulated by sphingomyelinases (SMases), ceramidases and Cer synthase. The model proposed above suggests the involvement of signal transduction events both upstream- and downstream of these enzymes.

In chapter 4 radiolabeling studies are described in which we tested the sphingolipid-modulating ability of IFNγ, a known enterocyte differentiation-, and apoptosis-inducing factor. However, neither short- nor long term exposure of HT29 cells to this cytokine had any affect on the cellular pools of SM, Cer or GlcCer. Similar results were obtained in experiments in which we employed other ligands (TNFα, IL1, NGF), other cell types (HL60, U937, oligodendrocytes, neurons) and/or alternative methods of sphingolipid analysis (NBD-prelabeling, measurement of endogenous lipid pools; unpublished observations). Taken together, it is our experience that reported data which suggest the presence of ligand-responsive sphingolipid-metabolizing enzymes (*i.e.* SMases), are particularly difficult to reproduce, which as such raises questions concerning the validity of these claims. Due to this discrepancy, no experimental data are presently available concerning signaling mechanisms upstream to Cer and GlcCer in HT29 cells.

With respect to sphingolipid-initiated downstream signaling events, abundant literature is available on Cer (see Introduction). However, the presently suggested role for GlcCer in growth-related signaling is a novelty and at this moment nothing is known concerning direct molecular targets for GlcCer and/or further downstream signaling events. In future studies on GlcCer-mediated signal transduction, the involved molecular mechanisms will therefore be a central theme.

Subcellular localization of bioactive sphingolipids and ABC transporters - In addition to the metabolic aspects described above, also the subcellular localization is a determining factor for bioactivity of sphingolipids. In this respect, an important observation is described in chapter 3 in which it is reported that Cer only induces apoptosis on the account that it reaches the appropriate intracellular target site (presumably the inner leaflet of the plasma membrane). In analogy to this, and also to other lipid-mediated signaling systems (e.g. the activation of protein kinase C by diacylglycerol), it is tempting to speculate that bioactive GlcCer should also reside in the cytoplasmic leaflet of the plasma membrane to ensure interaction with its (hypothetical) signaling-targets.

Like all other sphingolipids however, GlcCer is preferentially present in the outer leaflet of the plasma membrane. Biosynthesis of sphingolipids mainly occurs at the lumenal site of the Golgi apparatus, which, given the vesicular transport mechanism, topologically corresponds with the outer leaflet of the plasma membrane. With its site of synthesis at the cytoplasmic site of the Golgi apparatus, which is a topological equivalent of the inner leaflet of the plasma membrane, GlcCer biosynthesis forms a major exception to this (see also the Introduction chapter). This suggests that, after transport, at least some GlcCer might end up at the inner leaflet. Translocation over the membrane is conceived to be mediated by ABC-transporter proteins (see also chapter 6), which thus suggests a role for these proteins in the regulation of the size of the inner leaflet GlcCer pool. In addition to metabolic regulation of cellular GlcCer levels, it can thus be envisaged that GlcCer-translocation activity plays a role in determining the state of cell differentiation. On the other hand, data from chapter 6 strongly suggest that sphingolipids might be involved in the regulation of ABC-transporter protein activity.

Multidrug resistance and apoptosis - It is now shown for the first time that a functional relationship exists between cellular sphingolipid expression patterns and the activity of the MDR protein P-glycoprotein (Pgp). In addition to Pgp overexpression, other MDR mechanisms exist, one of which is the ability of some cells to escape from apoptosis. From the present-, and many other studies, it is clear that Cer is actively involved in the onset of this apoptotic process. Interestingly, we made several observations which indicate that (HT29) cells actively avoid cellular accumulation of Cer. For example, when cells were treated with the GlcCer-synthase inhibitor PDMP (chapter 2) or with a bacterial SMase (chapter 3), Cer did not reach the expected values. In contrast, the initial excess in Cer was actively metabolized and the cells survived both treatments. These observations made us speculate whether this Cer-metabolizing capacity might represent a novel apoptosis-escape mechanism. The present data therefore suggest the existence of two functional links between sphingolipid metabolism and the MDR phenotype.

Taken together, sphingolipid metabolism might provide a suitable point of application for the development of a new type of anti-cancer drugs. A two-cutting aspect can be envisioned since prudential manipulation of tumor cell sphingolipid

metabolism should not only provide the possibility of growth inhibition and the onset of cell death, but also evade the often encountered obstacle of drug resistance. Obviously, many questions will have to be elucidated, both *in vitro* and *in vivo*, before applications as outlined above can become reality.

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