Spingolipids in childhood malignancies.

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Introductory remarks

During the last decades a noteworthy progress has been made in the treatment of childhood cancer, mainly through the development and clinical use of various classes of chemotherapy in combination with surgery and radiation therapy. However these multi modality treatment regimens did not lead to a 100% cure rate of this disease. Two common malignancies of childhood are leukemia and neuroblastoma. Although they are very different with regard to epidemiology, biology, pathogenesis and treatment they share important properties. Bone marrow suppression is a common presenting characteristic of both leukemia and neuroblastoma patients. In addition, relapses are characterized by a poor prognosis due to the occurrence of multidrug resistance, which renders tumor cells less prone to apoptotic induction. Cancer cells of both tumors produce and shed specific sphingolipids, which can interfere with the development of other cells. In addition, sphingolipids play an important role in proliferation, differentiation, cell cycle process, cellular senescence and apoptosis, processes that are usually deregulated in tumor cells. The experiments described in this thesis were performed in order to increase our understanding of the inter- and intracellular functions of tumor cell sphingolipids.

Intercellulair effects of sphingolipids; inhibition of normal hemopoiesis

Tumor cells have been shown to display an altered sphingolipid metabolism. Moreover, many tumor cells, including leukemia and neuroblastoma cells, produce specific complex glycosphingolipids (gangliosides), which they shed into their microenvironment. This results in inhibition of several immune responses. Leukemia and neuroblastoma patients with bone marrow infiltration by malignant cells usually present with pancytopenia, indicative for impaired hemopoiesis. Normal hemopoiesis is marked by a tightly regulated process of proliferation, differentiation and maturation. Because of the known involvement of gangliosides in these biological processes, their possible influence on normal hemopoiesis was studied. First we studied the effects of gangliosides on erythropoiesis since anemia is the most pronounced manifestation of tumor associated bone marrow suppression. In ***chapter 3*** we show a dose-dependent inhibition of exogenously added gangliosides on *in vitro* murine erythroid colony formation. Moreover, similar results were obtained with plasma derived gangliosides from anemic leukemia patients which were much more inhibitory than gangliosides from healthy individuals. Mechanistically, gangliosides appear to interfere with ongoing maturation, resulting in the accumulation of less mature cells. Since erythropoietin is very important in the production of mature red blood cells, dose response curves with this growth factor were performed. The observed inhibitory effects of gangliosides, however, could not be abolished by high doses of erythropoietin. Therefore, mechanisms other than direct interactions with growth factors, such as modulation of downstream signalling routes may be involved. The data presented in this chapter strongly suggest that gangliosides shed by malignant cells are responsible for the observed pancytopenia *in vivo*. Additional evidence is described in

chapter 4. PDMP, a potent inhibitor of glucosylceramide synthase and therefore an inhibitor of the synthesis and shedding of gangliosides, abolished the inhibitory effects of shed gangliosides of murine neuroblastoma cells (Neuro-2a) on hemopoiesis *in vitro*. Furthermore, the observations on inhibitory effects by plasma samples of leukemia patients (***chapter 3***) could be extended to neuroblastoma patients. Neuroblastoma-derived plasma gangliosides also inhibited hemopoietic colony formation to a higher extent than control plasma gangliosides. Thus, inhibitory actions of tumor-derived gangliosides on hemopoiesis seem to be a general phenomenon. In order to translate the *in vitro* results into an *in vivo* tumor model, we evaluated the effects of neuroblastoma cells (Neuro-2a) on murine hemopoiesis as described in ***chapter 5***. Intravenous injection of Neuro-2a cells resulted in bone marrow and spleen metastasis. Tumor bearing mice showed reduced numbers of bone marrow and spleen progenitor cells. Both erythropoiesis and myelopoiesis were affected. However, no decreases were seen at the more mature precursor level, resulting in a normal pheripheral output. Thus the pancytopenia seen in neuroblastoma patients was not observed in neuroblastoma bearing mice. Studies concerning the mechanisms underlying this discrepancy may provide additional information about the pathogenesis of neuroblastoma in patients.

Intracellulair effects of sphingolipids; involvement in chemoresponsiveness

Sphingolipids play a prominent role in the induction of apoptosis. Since most of the anti-cancer agents induce cell death via the induction of apoptosis, we studied the role of ceramide in chemosensitivity of neuroblastoma cells, in which high amounts of sphingolipids are produced. In ***chapter 6*** we demonstrate that the capacity of tumor cells to metabolize ceramide is determinative for their chemoresponsiveness. By blocking the synthesis of glucosylceramide and therefore increasing the ceramide levels with the use of PDMP, neuroblastoma cells became highly sensitive to taxol and vincristine. Interestingly, this was not observed in the case of etoposide. The treatment with a combination of PDMP and a chemotherapeutic agent that affects microtubules, such as taxol and vincristine, appears to be more effective, suggesting that impaired transport of ceramide is essential in order to achieve a synergistic cytotoxic effect. Indeed, upon treatment with PDMP and taxol/vincristine a sustained elevation of ceramide was observed. In addition, PDMP by itself also influenced drug efflux activity by Pgp and MRP as revealed by mimicking the effects of specific inhibitors of Pgp and MRP on the efflux of taxol and vincristine. These results indicate that the ability of cells to remove excess ceramide may constitute a MDR mechanism. To investigate whether sphingolipids are essential in chemoresponsiveness we used *Saccharomyces cerevisiae* as a model. This model has proven its usefullness in studying the involvement of sphingolipids in other stress responses such as heat and osmotic pressure. In ***chapter 7*** we demonstrate that sphingolipids are necessary for daunorubicin induced growth inhibition. A mutated strain without sphingolipids due to the lack of serine palmitoyl transferase activity, the first enzyme of sphingolipids synthesis, was not sensitive to daunorubicin in contrast to the

wild type cells. In the mutated cells, sensitivity was restored by the addition of the sphingolipid precursor phytosphingosine. Interestingly, an opposite effect was observed with actinomycin D. The mutated strain was sensitive in contrast to the wild type cells being resistant to actinomycin D. In ***chapter 8***, we studied the mechanisms of etoposide-induced cell death regarding the involvement of *de novo* generated ceramide. Fumonisin B1, an inhibitor of ceramide synthase, inhibited the etoposide-induced *de novo* generation of ceramide. However, the appearance of apoptotic features, such as phosphatidylserine exposure, release of membrane lipids, loss of mitochondrial transmembrane potential, and PARP-cleavage could not be inhibited. In contrast, Zvad, a pancaspase inhibitor, inhibited these apoptotic features. Interestingly, cell death determined with trypan blue exclusion was inhibited by fumonisin B1. When added together with Zvad the percentage of trypan blue positive cells was further reduced, indicating that *de novo* generated ceramide results in a caspase independent signalling route leading to disruption of the plasma membrane integrity. Similar results as compared to fumonisin were obtained with the use of clastolactocystin, a specific proteasome inhibitor. Clastolactocystin by itself did not lead to generation of ceramide. These data suggest a common effector pathway involving *de novo* ceramide and the proteasome distinct from a caspase activated pathway.
In conclusion, the experiments described in this thesis demonstrate the importance of tumor cell sphingolipids in inter- and intracellular communication. Complex sphingolipids, gangliosides, shed by tumor cells inhibit normal hemopoiesis and contribute to tumor associated bone marrow suppression. Altered sphingolipid metabolism contributes to drug resistance, which is likely due to a change in susceptibility to apoptotic induction.

Future perspectives

The data summarized in the previous section demonstrate a close relationship between sphingolipids and cancer biology. In addition, normal cell physiology appears to be regulated by sphingolipids as well.
Important issues for future study include the screening of all sphingolipid molecules, in addition to ceramide, for excerting specific cell-biological effects. This should be integrated with studies on the subcellular and intramembrane topology and transportmechanism of sphingolipids to obtain information as to how and where these molecules excert their specific activities in relation to their site of production. In order to study these processes at the molecular level, it will be essential to further dissect complex phenomena, such as apoptosis, into individual biochemical events with their own regulatory features. Again, subcellular localisation of these processes is of great importance. Another major challenge is to elucidate the downstream events that couple the generation of bioactive sphingolipid species to the cell-biological effect. More attention needs to be given to the interaction of specific sphingolipids with (protein) targets or of which only a few have been proposed, which are not very well characterized yet. Novel targets will without doubt be described in the future. In addition, sphingolipid