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Biochemical, pathological and oncological relevance of Gb3Cer receptor

D. Đevenica · V. Čikeš Čulić · A. Vuica · A. Markotić

Abstract Glycosphingolipids are amphipathic molecules composed of hydrophilic oligosaccharide chain and a hydrophobic ceramide part, located primarily in the membrane microdomains of animal cells. Their oligosaccharide chains make them excellent candidates for the cell surface recognition molecules. Natural glycosphingolipid, globotriaosylceramide (Gal α1-4, Gal β1-4, Glc β1-1, ceramide), is also called CD77 and its expression was previously associated with proliferating centroblasts undergoing somatic hypermutation, but it has been demonstrate that globotriaosylceramide is not a reliable marker to discriminate human centroblasts from centrocytes. Globotriaosylceramide constitutes rare P k blood group antigen on erythrocytes, and it is also known as Burkitt's lymphoma antigen. On endothelial cells, globotriaosylceramide plays as the receptor for bacterial toxins of the Shiga family, also called verotoxins. Precise biological function and significance of globotriaosylceramide expression on endothelial cells remains to be the subject of many studies and it is believed globotriaosylceramide represents an example of a glycolipid antigen able to transduce a signal leading to apoptosis. In past decade, cancer researches put a great afford in determining new therapeutic agents such as bacterial toxins against tumor malignancies. Reports have demonstrated that verotoxin-1 induces apoptosis in solid tumor cell lines expressing globotriaosylceramide such as astrocytoma, renal cell carcinoma, colon cancer and breast cancer due to verotoxin-1 high specificity and apoptosis-inducing properties, and therefore, it is suggested to be an anticancer agent. Verotoxins have been investigated weather they could reduce treatment side-effects and toxicity to normal tissues and become a new oncological tool in cancer labeling.

Keywords Glycosphingolipids · Verotoxins · Lipid rafts · Endothelial apoptosis · Antibody-based cancer immunotherapy

Introduction

In mammalian cells, the ceramide moiety of glycosphingolipids is typically generated from the long-chain aminoalcohol sphingosine (d18:1), which is linked with a fatty acid varying in chain length from C16 to C24. Within the cell membrane, glycosphingolipids are clustered as membrane microdomains, also called lipid rafts [1, 2], and they function as attachment platforms for host pathogens and their toxins [3].

Natural glycosphingolipid, globotriaosylceramide (Gb3Cer), is implicated in the pathogenesis of *Escherichia coli* 0157:H7 and *Shigella dysenteriae* type 1 that cause gastrointestinal diseases, including hemorrhagic colitis and more serious hemolytic uraemic syndrome (HUS) as the cell surface receptor for the Shiga-like toxin (Stx) [4, 5].

Gb3Cer as Shiga-like toxin receptor

Stx is a member of the AB5 family of protein holotoxins that consist of an enzymatic A-subunit non-covalently

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attached to B-subunit that binds to its receptor globotria-osylceramide (Gb3Cer) on the surface of susceptible endothelial cells [6]. Shiga toxins, also known as vero-toxins (Vt-s), named for their select cytotoxic activity on vero cells, an African green monkey renal tubule epithelial cell line [7], have been divided into two families: Vt-1 and Vt-2, each of which consists of the major Vt type and several variants [8]. Vt-2 binds to Gb3Cer more slowly than Vt-1 but when bound, Vt-2 is difficult to dissociate. Differential binding properties may affect toxicities of Vt-1 and Vt-2 in vivo. It is believed that both the oligosaccha-ride part and the ceramide lipid anchor of the receptor are important for toxin binding [9]. The degree of unsaturation and hydroxylation of the sphingosine base can vary, but the primary source of heterogeneity of the ceramide lies in the fatty acid composition. It has been shown that the length of the fatty acyl chain of Gb3Cer influences receptor function [10], intracellular sorting and retro-translocation of Vt to the cytosol [11] and nuclear targeting [12]. Those data provide a molecular basis for the different pathology in vivo [8].

The B-subunit of Vt (Vt B-subunit) is pentamer responsible for cellular targeting and intracellular transport of the holotoxin and can bind to up to 10–15 Gb3Cer molecules [13]. The toxin–receptor complex undergoes retrograde transport through the Golgi network to the endoplasmic reticulum. Proteolytic cleavage of the A-sub-unit in a loop formed by the internal disulfide bond and reduction of this disulfide bond facilitate rapid intoxication [14]. In most cells, the cleavage into an enzymatically active A1 fragment (27.5 kDa) and a small A2 fragment (4.5 kDa) is performed by the enzyme furin [15] located in the Golgi apparatus and in endosomes. The A1 fragment possesses the rRNA N-glycosidase activity [16] and depu-rinates adenosine at position 4324 of the 28S ribosomal RNA causing apoptosis of the target cell through inhibition of protein synthesis [17].

Gb3Cer in lipid rafts

Lipid rafts are heterogeneous, dynamic domains enriched in glycosphingolipids, cholesterol, various cellular proteins (e.g., doubly acylated Src-type kinases) and transmem-brane proteins. The biophysical mechanisms by which cells regulate the size, lifetime, and spatial localization of these domains are rather poorly understood at the moment [18]. Recent experiments indicate that non-equilibrium cellular processes regulate formation of lipid rafts. Cells maintain a non-equilibrium lipid raft composition via lipid recycling to and from the membrane either by coupling to a lipid reservoir or by vesicular and non-vesicular lipid transport. Formation of rafts can be induced by passive or active

processes, whereas stabilization of raft properties occurs upon CD28 T-cell costimulation [19]. This stabilization requires the actin-cytoskeleton regulating protein filamin-A [20]. Cholera toxin binding to its receptor located in lipid raft, acidic glycosphingolipid GM1, causes pentameric clustering of GM1 and further enhancement of the parti-tioning of GM1 (now a toxin—GM1 complex) into lipid raft microdomains [21].

Vt B-subunit mediates attachment and internalization of the Vt-Gb3Cer complex by receptor-mediated endocytosis via clathrin-coated vesicles [22] or by endocytic routes that do not involve clathrin-coated pits [23–25]. As with cyto-toxicity, Vt-1-induced transmembrane signaling also requires a Gb3Cer detergent-resistant microdomains format [26]. It has been demonstrated with detergent-resistant microdomains on the cell surface of human renal tubular epithelium-derived cells that Vt-1 ligation of cell mem-brane Gb3Cer within detergent-resistant microdomains results in transmembrane signaling and activation of Src family kinases [27]. The retrograde transport of the Vt-Gb3Cer complex from the cell surface to endosomes, Golgi and endoplasmic reticulum is dependent on the ini-tial Gb3Cer being present within detergent-resistant microdomains [28]. Studies that investigated the role of lipid rafts in Vt-1-interaction with various cell types demonstrated the clustering of Gb3Cer in lipid rafts [29] and also binding of Vt B-subunit with raft-localized receptors as a requirement for the retrograde transport. That suggests that only glycosphingolipids that associate strongly with lipid rafts carry AB5 toxins retrogradely from the plasma membrane and sort the toxins to the endoplas-mic reticulum [30]. This retrograde pathway is required for A-subunit cytosolic transit and cytotoxicity [31].

Gb3Cer binding of Vt B-subunit induces narrow tubular membrane invaginations in human and mouse cells. Vt B-subunit could induce membrane invaginations even when the cellular membrane deformation machinery is inhibited. To study whether Vt B-subunit induces invag-inations in the total absence of cellular proteins and to analyse the physical and biochemical conditions of tubule formation in a controlled manner, the process was recon-stituted in a minimal system. Cytosol-free giant unilamellar vesicles contained defined molecular Gb3Cer species by chemical synthesis. Tubule formation could readily be observed on vesicles containing Gb3Cer with a C22:1 (Δ^{13}) single unsaturated acyl chain in cis-geometry. In contrast, no tubules were formed when Gb3Cer was made with a saturated C22:0 acyl chain or lyso Gb3Cer, lacking acyl chain. Inhibition of membrane scission by dynasore (dyn-amin inhibitor) partially protected cells from Vt-induced protein biosynthesis inhibition indicating that a sizable fraction of toxin traffics through dynamin-dependent pathways [25]. Using Gb3Cer from erythrocytes or HeLa

cells, rich in hydroxylated Gb3Cer, Romer et al. found that cortical actin shell leads to the cholesterol-dependent scission of Vt B-subunit-induced invaginations, even in the absence of dynamin [32].

Binnington et al. showed increased binding of both Vt-1 and Vt-2 for the receptor glycolipid, Gb3Cer containing an α-hydroxy C22 fatty acid. Hydroxylation of C18Gb3Cer had minimal stimulatory effect on receptor activity. Vt-binding affinity for Gb3Cer in general increases at lower receptor concentrations showing dose-dependent apparent cooperative-binding kinetics at select Gb3Cer concentrations. These results indicate that Gb3Cer is not behaving as a single species but that some parameter of organization of the receptor, important for binding, is changing with concentration [33]. Hydroxylated Gb3Cer is obviously important for Vt cell entry, because children with HUS showed lower non-hydroxylated fatty acyl-Gb3Cer to non-hydroxylated fatty acyl-lactosyl-ceramide ratio in erythrocytes than control children and Vt-infected diarrheal children without subsequent HUS [34]. Thus, erythrocyte glycolipid levels could be use in Vt-infected diarrheal individuals to predict those who are at high risk of developing HUS.

Mass spectrometric analysis showed expression of Gb3Cer with long (C24)- and short-chain fatty acids (C16) in malignant tissues and pointed out the presence of hydroxylated fatty acid lipoforms. Therefore, the lipoform-dependent traffic of Gb3Cer from the cell surface to the endoplasmic reticulum has been proposed as a signal transduction pathway [35]. Interestingly, increased number of Gb3Cer carrying hydroxylated fatty acids has been evidenced in ovarian carcinoma-derived cells. Those cells had selectively survival in the presence of taxol and cis-platin, and therefore, the mentioned modification of the GSL structure is put in association with anticancer-drug resistance [36].

Studies showed that the targeting of the toxin to a specific intracellular transport pathway and the affinity of Vt-1 toxin toward Gb3Cer can be determined not only by the presence or absence of Gb3Cer in the lipid raft microdomains of the membrane but also by the Gb3Cer isoforms expressed on the cell surface. Gb3Cer species with long-chain fatty acids have been associated with greater toxicity because they possibly better mediate the localization of internalized toxin to the endoplasmic reticulum [37].

Intracellular and cell surface–bound Vt-1 is more resistant to detergent extraction than Vt-2, suggesting that a greater proportion of Vt-1-bound Gb3Cer is within lipid rafts when compared to Vt-2 [38]. Cholesterol contributes to fluidity and bilayer thickness of the membrane. It has been showed on Burkitt's lymphoma cells that constitutive localization of Gb3Cer to lipid rafts was unperturbed by either Vt B-subunit binding or cholesterol depletion, and

also how the presence of cholesterol has been shown to differentiate between the binding of two ligands (anti-Gb3Cer and Vt B-subunit) to a Gb3Cer in Burkitt's lymphoma cells [39, 40]. Studies showing that the depletion of glucosyl ceramide resulted in the loss of Gb3Cer from the detergent resistance membrane fraction imply that glycolipid interactions may be necessary to resist detergent extraction. While most glycosphingolipids are enriched in detergent-resistant microdomains, it is clear that Gb3Cer can be found in both the detergent-resistant microdomains and non-detergent-resistant microdomains fraction [41]. For cells in which Gb3Cer is present in the non-detergent-resistant microdomains plasma membrane fraction, the toxin–receptor complex is internalized and trafficked to lysosomes where the toxin is degraded and cells therefore survive without cytotoxic effect. Thus, not all Gb3Cer-containing cells are sensitive to Vt cytotoxicity. This provides a possible explanation as to why bovine animal reservoir gastrointestinal mucosal epithelial cells that express Gb3Cer are nevertheless resistant to cytotoxicity [42].

Lingwood's group [43] reported the role of Gb3Cer as a signal transducer in CD19 and interferon-α2 and that the extracellular domain of CD19 has a potential Gb3Cer-binding site with extensive sequence similarity to the Vt B-subunits. These observations provide evidence that CD19/Gb3Cer interactions function in adhesion and signal transduction at a specific stage in B-cell development. By targeting Gb3Cer+ B cells, Vt-s may suppress the humoral arm of the immune response during infection [44].

Vt bindings to Gb3Cer on different cells

Glycosphingolipid composition of endothelial cells has generally received low attention. On the basis of histo-pathologic findings and in vitro experiments, it has been generally believed for some time that vascular endothelial cells are a prime target for Vt-mediated cytotoxicity [45]. However, reports have shown that Vt-s induce cell death in various other cells, such as intestinal epithelium, Burkitt's lymphoma cells, Vero- and MDCKrenal-derived cells, ACHN renal adenocarcinoma cells, renal epithelial cells and human umbilical vein endothelial cells (HUVECs). It has been shown that Vt-mediated killing of endothelial cells depends on the level of Gb3Cer and is up-regulated by tumor necrosis factor-alpha, interferon-gamma and lipo-polysaccharide. Studies concluded that the susceptibility of human vascular endothelial cells to Vt differs remarkably depending on their cellular origins. The same group showed later that cutaneous microvascular endothelial cells prepared from both adult and neonate are high sensitive on Vt-s [46].

Globotriaosylceramide synthase, the enzyme required for synthesis of the Gb3Cer receptor, was not expressed by normal or inflamed human colon epithelium in vivo. Therefore, Vt did not bind to the epithelium in normal or inflamed human colon in vivo. These results are supported by biochemical and immunochemical studies that have failed to detect Gb3Cer in human intestinal epithelium [47, 48].

Upon stimulation with inflammatory mediators, an enhanced expression of Gb3Cer has been reported for HUVECs and primary human brain endothelial cells from various sources. It is been reported that microvascular endothelial cells from the human brain (HBMECs) are insensitive to Vt-1 and to require incubation with proinflammatory cytokines to overcome this resistance [49–52]. Human glomerular epithelia and renal tubular epithelia appear to constitutively express higher levels of Gb3Cer and might be considerably more responsive to Vt-s than HUVEC. Pulmonary epithelium and human lung carcinoma cell lines contain Gb3Cer-expressing cells also. These cells are highly sensitive to Vt-mediated cytotoxicity and can be directly damaged during *Escherichia coli* infection. The authors conclude that in lung, both vascular endothelium and lung epithelium are the major targets for Vt-s [53].

The expression of Gb3Cer in the human central nervous system still remains unclear but researches on rabbits proved that the Gb3Cer was expressed on endothelial cells in the brain parenchyma, but not on neurons or glial cells. It has been found that vascular damages and induction of endothelial apoptosis can be caused by direct cytotoxic action of Vt-2 via up-regulation of tumor necrosis factor-alpha and interleukin-1 beta transcripts that occurs in the brain parenchyma of rabbits treated with Vt-2 [54].

Studies showed that Gb3Cer co-localize with the multidrug resistance protein P-glycoprotein and have thus been suggested as a possible target for multidrug-resistant cells [55].

Apoptosis induction via Gb3Cer

The mechanisms of apoptosis induction suggest that Vt-induced apoptosis may contribute to the pathogenesis of HUS caused by Vt-s [56–58]. Vt-s causes sublethal cell injury by altering cell adhesive properties and by increasing endothelial susceptibility to leukocyte-mediated injury [59]. Injured endothelium changes its normal thromboresistant phenotype and becomes thrombogenic, initiating microvascular thrombus formation [60]. Many studies have showed that Gb3Cer is able to transduce apoptotic signals via binding of various ligands: Vt-s, the recombinant binding B-subunit of Vt and anti-Gb3Cer mAb. It has been

demonstrated on Burkitt's lymphoma cells that the Vt-1 and anti-Gb3Cer-induced pathways are completely independent: Vt-1-induced apoptosis involves caspase activation and mitochondrial depolarization, whereas oxidative stress seems to mediate anti-Gb3Cer mAb-induced cell death [61–63]. The anti-Gb3Cer antibody has some similarities with Vt B-subunit, but has been shown to trigger different apoptotic pathways in cell lines that were originally established from endemic or sporadic cases of Burkitt's lymphoma. While Vt-1 triggers a caspase-dependent pathway, the anti-Gb3Cer antibody induces ROS-dependent pathway [63].

Moreover, it has been considered that Vt-1-induced apoptosis in Burkitt's lymphoma cells goes trough degradation of the caspase-8 inhibitory molecule c-FLIPL and this degradation occurs through the ubiquitin–proteasome pathway. The same group showed that mitochondrial activation is mainly due to i) cleavage and activation of the pro-apoptotic Bcl-2 family member Bid by caspase-8 and ii) Bax relocalization to mitochondrial membranes which lead to cytochrome c release [64].

Studies done on the THP1 myelogenous leukemia cell line and epithelial cell lines showed how Vt induces an apoptotic pathway dependent on caspases and mitochondria [65]. Researches found out that apoptosis in endothelial cells occurs because the toxin specifically inhibits the expression of Mcl-1, an antiapoptotic member of the Bcl-2 family [66]. Interestingly, in HeLa cells, Vt induces apoptosis via activation of caspase-8, 6 and 3 but apoptosis seems to be independent of the mitochondrial pathway [67].

Oncology interests and therapeutic possibilities of Gb3Cer ligands

Vt receptor Gb3Cer has been reported to be increased on the surface of several tumor cells lines originating from hematological malignancies, astrocytoma tumors [68, 69] malignant meningioma [70] neuroblastomas, centrofollicular and Burkitt's lymphomas [22, 71], and testicular seminomas [72]. The expression of Gb3Cer was also increased in liver metastases, in small number of rare human small intestinal tumors of different origin, lymphomas, epidermoid carcinomas, neuroendocrine tumors, leiomyosarcomas and tumor cells in glioma cryostat sections [73]. Primary ovarian tumors, metastases and drug-resistant ovarian cancers also contain elevated Gb3Cer levels [74].

Vt and its B-subunit or B-subunit derivatives could be new therapeutic strategies for the treatment or oncological applications of malignancies which express Gb3Cer [75]. The Vt receptor is currently under investigation as a

potential target candidate for toxin-based therapeutics [70, 76–78].

The potential use of Vt-1 in the past is been considered to be limited to an ex vivo application, because its toxicity includes endothelial cell damage and HUS observed in patients after gastrointestinal infections with Vt-1-producing *Escherichia coli* or *Shigella dysenteriae* but studies reported that only 5% of patients with HUS had antibodies to Vt-1 [79]. In another study, six out of 21 patients with diarrhea induced by *Shigella dysenteriae* had antibodies to Shiga holotoxin, but none to Vt B-subunit-derived peptides [80]. Preclinical data on mouse models indicate that repeated uptake of Vt B-subunit by the oral or intravenous route is well tolerated and does not cause adverse side-effects [78]. It is been considered that *Escherichia coli* or *Shigella dysenteriae* enteropathogenic toxins have naturally evolved to resist digestive enzymes and pH changes in the intestinal lumen and are able to cross the intestinal barrier and spread in the organism [81].

Vt B-subunit has been used as an antigen delivery tool to dendritic cells for the development of immunotherapy protocols [82]. Gb3Cer is almost exclusively expressed on antigen-presenting cells such as dendritic cells and B cells among hematopoietic cells. Dendritic cells are necessary for efficient priming of cytotoxic T lymphocytes. Immunization with peptide or recombinant proteins generally fail to elicit cytotoxic T lymphocytes, which are thought to play a key role in the control of virus-infected cells and tumor growth. Vt B-subunit fused to a tumor peptide derived from the mouse mastocytoma P815 can induce specific cytotoxic T lymphocytes in mice without the use of adjuvant [83]. Successful targeting of antigen to dendritic cells has also been associated with antibody induction [84], which may explain results regarding the ability of Vt B-subunit-ovalbumin complex to increase the amount of ovalbumin-specific IgG2a antibodies in mice [85].

LaCasse et al. demonstrate the expression of Vt-1 receptors on three of the human cancers commonly treated by autologous stem cell transplant (breast cancer, lymphoma and multiple myeloma) and proves the absence of Vt-1 receptor on human CD34+ stem cells. That evidence supports the clinical use of Vt-1 as an ex vivo purging agent to deplete malignant cells expressing receptors for Vt-1 from autologous stem cell grafts. The expression of Gb3Cer was assessed on human breast cancer cell lines and clinical biopsies of primary human breast cancer tissues in light of the growing number of breast cancer patients treated each year with stem cell transplants. Eighty percent of primary breast cancer biopsies were Gb3Cer+, indicating the potentially broad expression of Gb3Cer on tumor cells at both primary and metastatic sites. These findings identify Gb3Cer as a marker on clinical specimens of breast cancer and indicate that Vt-1 could be used to purge these cells [69].

Using primary short-term cultures of human tumors, researches found that Vt B-subunit accumulates in the tumors cells of epithelial origin. Vt B-subunit remained associated with tumor cells of epithelial origin for several days. The investigators conclude that Gb3Cer expression by human digestive cancers may be used for noninvasive tumor labeling, imaging or drug delivery using natural Gb3Cer ligands, such as verotoxin from *Shigella dysenteriae* and *Escherichia coli*. The fact of Vt B-subunit presence in tumor cells after several days may be of great value for the use of Vt B-subunit for noninvasive tumor imaging because the contrast agents may be cleared from non-tumor tissues before image acquisition is started. A possible imaging application of the Vt B-subunit delivery technology concerns small intestine tumors that cannot be reached by fibroscopy from the stomach or by colonoscopy from the rectum. An important finding of Falguères's study is the robust up-regulation of Gb3cer expression by liver metastases and the uptake of Vt B-subunit by primary cultures from liver metastasis. In colorectal cancer, liver metastases are present in 25% of cases at the time of initial diagnosis and they caused the death of a majority of all patients. Therefore, early and reliable detection of hepatic colorectal metastasis is of great clinical importance. Gb3Cer in metastasis may allow their detection through noninvasive imaging approaches, such as Vt B-subunit-vectorized positron emission tomography imaging. Authors speculate that the naturally evolved targeting properties of Vt B-subunit could be diverted for the in vivo delivery of contrast agents or therapeutic compounds to colorectal primary carcinomas and metastases [86]. These important results obtained in vitro are strongly supported by results of in vivo study. Vt B-subunit accumulates in the tumor area of human colorectal carcinoma xenografted to nude mice as well as in the epithelial cells of the neovascularization and the monocytes and macrophages surrounding the xenografts [87]. Iodine (125)-Vt-1 autoradiography identified the lungs and nasal turbinates as major targets for Vt-1, while kidney cortex and the bone marrow of the spine, long bones and ribs are also significant targets [88]. Vt-2 does not target the lung, but accumulates in the kidney to a greater extent than Vt-1.

Besides Vt, in past decade, lectins of *Pseudomonas aeruginosa* have been investigated by many researches in association with Gb3Cer. This opportunistic bacterium is responsible for numerous infections in immunocompromised patients. It produces a wide variety of carbohydrate-binding proteins, including the soluble lectins I (PA-IL; gene lecA) and II (PA-IIL; gene lecB), which are specific for galactose and fucose, respectively [89]. PA-IL is the first *Pseudomonas aeruginosa* lectin to be isolated by

affinity chromatography. It consists of 121 amino acids (12.75 kDa) associated in homotetramers. It is been showed that PA-IL recognizes Galα1-4Gal and Gal1α-3Gal epitopes, and it is been considered that lectin can be used for differentiation between P-positive and P-negative red blood cells and has potential applications for cell typing and/or tumor targeting [90].

The potential of Gb3Cer ligands for treatment of non-hematological malignancies

Recently, reports showed that 62% of pancreatic and 81% of colon adenocarcinomas have increased Gb3Cer expression [91] and that Vt B-subunit is able to induct an apoptosis in colorectal cancer cell lines. Study on T84 colon cancer cells found out that Vt-2 was harmful to intestinal epithelial cells in an organ culture system, even though these cells are Gb3Cer negative [48]. It was shown that Gb3Cer expression in human colorectal cancer correlates with ability to form metastasis [76]. Interestingly, normal human intestinal epithelia do not express Gb3Cer nor do they bind Vt [92].

Vt-1 has been shown to induce apoptosis and rapid elimination of mice tumor xenografts in Gb3Cer-expressing human renal cell carcinoma, colon carcinoma, glioblastoma and malignant meningiomas [93–95].

Researchers report a highly significant up-regulation of the glycosphingolipid Gb3Cer in human colorectal cancer and liver metastases, when compared with normal colon tissue and benign lesions. In fact, on average, human colon carcinomas expressed 3-fold more Gb3Cer when compared with normal tissue. Of note, the authors observed a correlation between tumor progression along the adenoma/carcinoma sequence and Gb3Cer expression: only malignant lesions expressed high levels of Gb3Cer, whereas adenomas showed Gb3Cer levels that were comparable to normal colon. However, there was no significant difference of Gb3Cer expression between metastasized and non-metastasized tumors. It is concluded that Vt B-subunit does not induce apoptosis in primary colorectal cancer cells. Therefore, it has been stated that cell type–specific differences may be the most likely explanation for the apparent differences in apoptosis induction [86].

The demonstration of Gb3Cer expression in breast cancer tissue by Johansson et al. indicates that it is possible to use Vt-1 in the treatment of breast cancer. The authors demonstrated that Vt-1 induced a dose-dependent decrease of cell viability in Gb3Cer-expressing T47D cells, but not in MCF-7 cells that lacked Gb3Cer expression. Caspases-3 and 9, but not caspase-8 was found to be activated in both cell lines indicating that the intrinsic pathway to apoptosis was activated by Vt-1. There was no Vt-1-induced cellular

activation of caspase-3, 8, or 9 after preincubation with a chemical inhibitor of glucosylceramide synthesis; PPMP. The mode of Vt-1 cytotoxicity at low concentrations is still unclear. The high specificity and the ability of Vt-1 to selectively induce breast cancer cell death indicate that Vt-1 may be used as a potential antineoplastic agent for treatment of Gb3Cer-positive breast cancers [96].

The potential of Gb3Cer ligands for treatment of hematological malignancies

Burkitt's lymphoma is a human B-cell malignancy with characteristic epidemiologic features. The tumor is frequent in children in equatorial Africa and New Guinea and occurs with a lesser incidence all over the world. Almost all tumors from high incidence areas are Epstein-Barr virus (EBV)-positive, whereas only about 15% of tumors from low-incidence areas are associated with EBV [97]. It has been stated by the authors of the WHO classification that BL is a single disease with multiple variants, including endemic BL, sporadic BL and 'atypical' BL, having in common an exceedingly high Ki-67 index and a translocation t [8, 14] involving the MYC locus [98]. In contrast to eBL, the immuno-profiles of the European lymphomas were less homogeneous and inconsistent for CD10, CD38, Gb3Cer, IgM and bcl-2 expression, while eBL was positive for the same markers.

Authors showed that Vt induces cell death in Gb3Cer+ Burkitt's lymphoma cells, not only by inhibiting protein synthesis but also through an additional mechanism, DNA fragmentation characteristic of apoptosis. It has been shown by several studies that recombinant B-subunit of Vt is capable of apoptosis induction in Burkitt's lymphoma cells, although it is not able to inhibit protein synthesis [99]. In fact, Vt-induced apoptosis was shown to be accompanied by increased expression of the proapoptotic protein Bax and inhibited by over expression of the anti-apoptotic protein Bcl-2 [17]. Interestingly, the incubation of cell lines that were originally established from endemic or sporadic cases of Burkitt's lymphoma cells with anti-oxidant compounds strongly protected against anti-Gb3Cer-induced cell death [63]. Mutants of the Burkitt's lymphoma-derived Daudi cell line that is deficient in Gb3Cer showed to be resistant to the cytotoxic effects of Vt [100].

The prototype Gb3Cer mAb, 38.13, was initially designated as defining a moiety termed 'BLA', or Burkitt's Lymphoma Antigen [101]. A large number of follicular lymphoma appears to remain faithful to their normal counterparts by displaying Gb3Cer on the tumor cell surface [102]. It was reported that on crosslinking 38.13 antibody bound to Gbr3Cer, an intracellular signaling

cascade is initiated involving a rise in cytosolic Ca^{2+} and the generation of ceramide [103]. Those components have been implicated in apoptotic cell death in B lymphoma cells [104, 105]. Studies found that megakaryoblastic leukemia cells also express high levels of Gb3Cer based on the up-regulation of the $\alpha1,4$Gal-T gene, and that MEG-01, one of megakaryoblastic leukemia lines, underwent apoptosis with Vt-s via the caspase pathway [106].

Cell lines established from multiple myeloma patients express Gb3Cer [107]. Circulating clonotypic B cells previously shown to be part of the myeloma clone [108] also bind Vt-1.

Two malignancies frequently associated with Epstein-Barr virus (EBV) are classic Hodgkin lymphoma (HL) and post-transplantation lymphoproliferative disease (PTLD), in which the tumor cells often appear to derive from B-cell receptor-deficient and therefore preapoptotic germinal center (GC) B cells. Human tonsillar Gb3Cer+ GC B cells were infected in vitro with EBV and findings support the idea that EBV plays a central role in the pathogenesis of classic HL and PTLD by rescuing BCR-deficient, preapoptotic germinal center B cells from apoptosis [109]. Previous work showed the basis of a possible novel approach to PTLD therapy utilizing the specific targeting of Vt to the infiltrating lymphoma cells. Biopsies of adenoid, kidney or liver tissue of four PTLD patients were stained with Vt to determine expression of Gb3Cer. In each PTLD case, the infiltrating EBV-positive B lymphoma cells were strongly and selectively stained with Vt, identifying Gb3Cer as a new marker for these cells. For such individuals, Vt might provide the basis of an approach to control their malignancy [110].

CD40 activation is necessary for rescuing phenotypically immature lymphoma cells from B-cell antigen receptor-induced cell death [111]. Gb3Cer expressing group I Burkitt's lymphoma cells that are normally susceptible to activation-induced death on binding Vt-1B chain are protected in the presence of CD40 ligand. Group I Burkitt's lymphoma cells that ectopically express Bcl-2 are protected from Vt B-subunit-induced apoptotic cell death [99]. These cells were chosen because overexpression of bcl-2 decreases the proportion of Fas+ B-lymphocyte lineage cells [112]. Fas is a cell membrane protein involved in programmed cell death. A dissociation exists between the rescue from apoptosis achieved by CD40 stimulation and induction of bcl-2 [113]. Bcl-2 is not induced in Burkitt's lymphoma cell lines rescued from apoptosis by CD40 activation [114]. Therefore, experiment on group I Burkitt's lymphoma cells that ectopically express Bcl-2 enables clear distinguishing between endogenous survival pathways from therapeutic modalities based on the use of Verotoxin-1 B chain and exogenous survival pathways supported by CD40 signaling.

Recently, authors searched dynamic interplay between the Gb3Cer and the therapeutic antibody target CD20 within the lipid bilayer of model B lymphoma cells. Ligating Gb3Cer with the bacterial toxin Vt B-subunit promotes a rapid and marked reduction in the availability of important B-cell surface proteins CD20 and CXCR4. CD20 availability may play an important role in the use of anti-CD20 antibody (Rituximab) as a treatment of B-cell malignancy [115].

Gb3Cer has also been implicated in the survival of a reservoir of infectious human immunodeficiency virus (HIV) in B cells. It is been found significantly more CD20/CD77 B cells in HIV Africans compared to HIV-uninfected individuals or European patients [116]. Variable Gb3Cer expression may provide a natural HIV resistance factor in the general population, and pharmacological manipulation of Gb3Cer levels may provide an approach to induction of HIV resistance [117].

Investigations of cancer therapies should ensure that the cytotoxic effect of Vt-1 does not lead to severe side-effects due to targeting of normal Gb3Cer-expressing cells outside of the tumor especially microvasculature of the kidney and tonsil germinal center B lymphocytes which regularly express Gb3Cer [71]. Targeting neovascular cells is another approach to inhibit tumor growth that might ''starve'' tumors as cancer cells are deprived of blood flow. Lingwood et al. [35] showed the complete long-term elimination of human astrocytoma xenografts in nude mice after Vt-1 administration. Massive apoptosis was observed in both tumor and vascular cells within the treated xenograft, suggesting tumor elimination not only by antineoplastic but also by antiangiogenic activity.

In conclusion, combined approaches for therapeutic use of Gb3Cer ligands, that involve interplay of cell endogenous and exogenous pathways, deserve attention in future experiments. The potential of Gb3Cer ligands for treatment of malignancies has to be estimated in relationship to expression of variety of molecules, like proapoptotic and antiapoptotic proteins, surface markers etc., in individual patient.

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