Minireview

Shiga toxin and its use in targeted cancer therapy and imaging

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Summary

Shiga and the Shiga-like toxins are related protein toxins produced by *Shigella dysenteriae* and certain strains of *Escherichia coli*. These toxins are composed of two non-covalently attached, modular parts: the A moiety (StxA) containing the enzymatically active A$_1$ fragment, and the non-toxic, pentameric binding moiety (StxB). Stx binds specifically to the glycosphingolipid globotriaosylceramide (Gb3) at the surface of target cells and is then internalized by endocytosis. Subsequently, in toxin-sensitive cells, the Stx/Gb3 complex is transported in a retrograde manner via the Golgi apparatus to the endoplasmic reticulum, where the enzymatically active part of Stx is translocated to the cytosol, enabling it to irreversibly inhibit protein synthesis via modification of ribosomal 28S RNA. Whereas Gb3 shows a relatively restricted expression in normal human tissues, it has been reported to be highly expressed in many types of cancers. This review gives a brief introduction to Stx and its intracellular transport. Furthermore, after a description of Gb3 and the methods that are currently used to detect its cellular expression, we provide an updated overview of the published reports on Gb3 overexpression in human cancers. Finally, we discuss the possibility of utilizing Stx or StxB coupled to therapeutic compounds or contrast agents in targeted cancer therapy and imaging.

Introduction

Shiga toxins, their intracellular transport and involvement in human disease

The Shiga toxins comprise a family of related protein toxins secreted by certain types of bacteria. Shiga toxin (Stx) is produced by *Shigella dysenteriae*, whereas the Shiga-like toxins, Stx1 and Stx2, with a few known isoforms, are secreted by specific strains of *Escherichia coli* named Shiga-toxin-producing *E. coli* (STEC). Stx1 is virtually identical to Stx, differing in only one amino acid residue, whereas the Stx2 isoforms share less sequence similarity with Stx (~60%) and are immunologically distinct. In spite of the differences in their amino acid sequence, all the Stx isoforms share the same overall toxin structure and mechanism of action, and unless otherwise specified, for the remainder of this review the singular term 'Stx' will refer to the family of Shiga toxins in general (for recent reviews on Stx see Sandvig *et al.*, 2009; Johannes and Römer, 2010).

Gastrointestinal infection with STEC serotypes might be followed by the life-threatening complication haemolytic uremic syndrome (HUS), which is defined by haemolytic anaemia, thrombocytopenia and acute renal failure (Palermo *et al.*, 2009). HUS predominantly affects young children, and although the mechanism is not entirely resolved, the disease involves Stx-induced damage to kidney cells. The STEC serotypes associated with HUS produce isoforms of Stx1 or Stx2, or a combination of these, and the most severe disease outcome is associated with Stx2 (Boerlin *et al.*, 1999). It should be noted that, unlike STEC, *S. dysenteriae* bacteria invade host cells, and only *S. dysenteriae* serotype 1 expresses Stx (for a recent review about diseases caused by *S. dysenteriae* see Schroeder and Hilbi, 2008).

The Shiga toxins are composed of an enzymatically active A moiety that is non-covalently attached to a pentameric binding moiety (Fig. 1A–C). The whole natural toxin is commonly referred to as the 'holotoxin'. The B moiety binds specifically to the sugar domain of the glycosphingolipid globotriaosylceramide (Gb3) (Fig. 1D) in the plasma membrane of target cells and mediates uptake and intracellular transport of the toxin (Sandvig *et al.*,
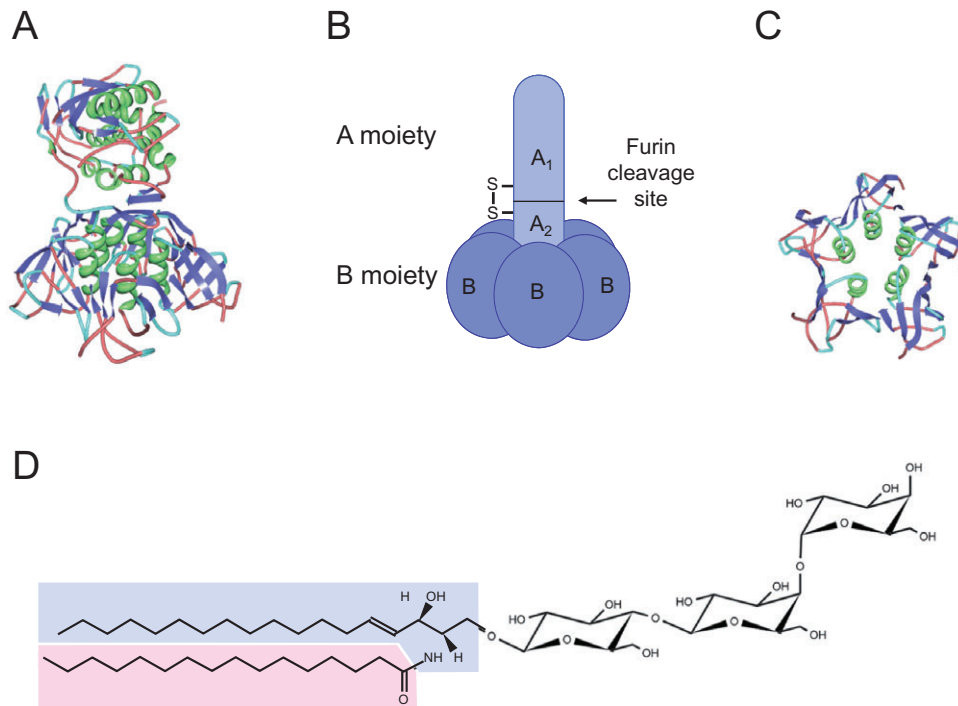
A

B

C

D

Fig. 1. The structure of Shiga toxin and its receptor globotriaosylceramide (Gb3).
A. Side view of the 70 kDa holotoxin (PDB protein data bank 1DM0).
B. Schematic presentation of Shiga toxin.
C. Top view of the doughnut-shaped B moiety composed of five identical 7.7 kDa subunits.
D. Chemical structure of Gb3. The sphingosine backbone (indicated in blue) has a relatively invariable chain length of 18 C-atoms, whereas the fatty acyl chain (indicated in pink) occurs with both variable lengths and degrees of saturation, here shown as C16:0.
The structures in (A) and (C) were prepared by PDB ProteinWorkshop 3.6 (Moreland *et al.*, 2005), whereas the structure in (D) was prepared at LIPID MAPS, the Nature Lipidomics Gateway.

2009; Johannes and Römer, 2010). More than one endocytic pathway seems to be involved in Stx entry, as both clathrin-dependent and clathrin-independent toxin uptake has been identified in different cell types (Sandvig *et al.*, 1989; Khine and Lingwood, 1994; Schapiro *et al.*, 1998; Nichols *et al.*, 2001; Lauvrak *et al.*, 2004; Saint-Pol *et al.*, 2004; Torgersen *et al.*, 2005; Römer *et al.*, 2007). The exact contribution of each pathway to toxin uptake is difficult to determine, as inhibition of one pathway might lead to upregulation of another (Damke *et al.*, 1995). Notably, Stx seems to have the ability to stimulate its own uptake (Torgersen *et al.*, 2005; Lauvrak *et al.*, 2006), and recently, the toxin was reported to induce tubule formation (Römer *et al.*, 2007). To which extent tubule-induction contributes to toxin uptake is unresolved and has been suggested to be minor (Hansen *et al.*, 2009).

The sorting of Stx into the retrograde transport pathway from early endosomes to the Golgi apparatus is a highly regulated process (for recent reviews, see Johannes and Popoff, 2008; Sandvig *et al.*, 2009; Torgersen *et al.*, 2010). An overview of components reported to be involved in this process is shown in Fig. 2. These include, for instance, SNARE proteins, Rabs, kinases, sorting nexins, scaffold proteins and cytoskeletal elements. During intracellular transport, primarily in the endosomes, the Stx A moiety is cleaved by furin into the A_1 and A_2 fragments (Fig. 1B) (Garred *et al.*, 1995). The enzymatically active A_1 fragment remains bound to the A_2 fragment via a disulfide bond during the transport from the Golgi apparatus to the endoplasmic reticulum (ER), and is released upon exposure to the reducing conditions in the ER. The A_1 fragment is then translocated into the cytosol, possibly via the Sec61 complex involved in ER-associated degradation of misfolded proteins (Yu and Haslam, 2005). The A_1 fragment is able to escape ubiquitination and cytosolic degradation, due to the virtual absence of lysine residues, and its N-glycosidase activity irreversibly modifies the ribosomal 28S RNA, leading to inhibition of protein synthesis. Although this may by itself lead to cell death, Stx has also been shown to induce apoptosis via induction of ribotoxic- and ER stress signals (Smith *et al.*, 2003; Lee *et al.*, 2008), or even via signal transduction induced by Gb3-ligation (Mangeney *et al.*, 1993; Taga *et al.*, 1997; Tetaud *et al.*, 2003; Kovbasnjuk *et al.*, 2005). The mechanism(s) whereby Stx kills cells still needs further clarification and seems at least in some
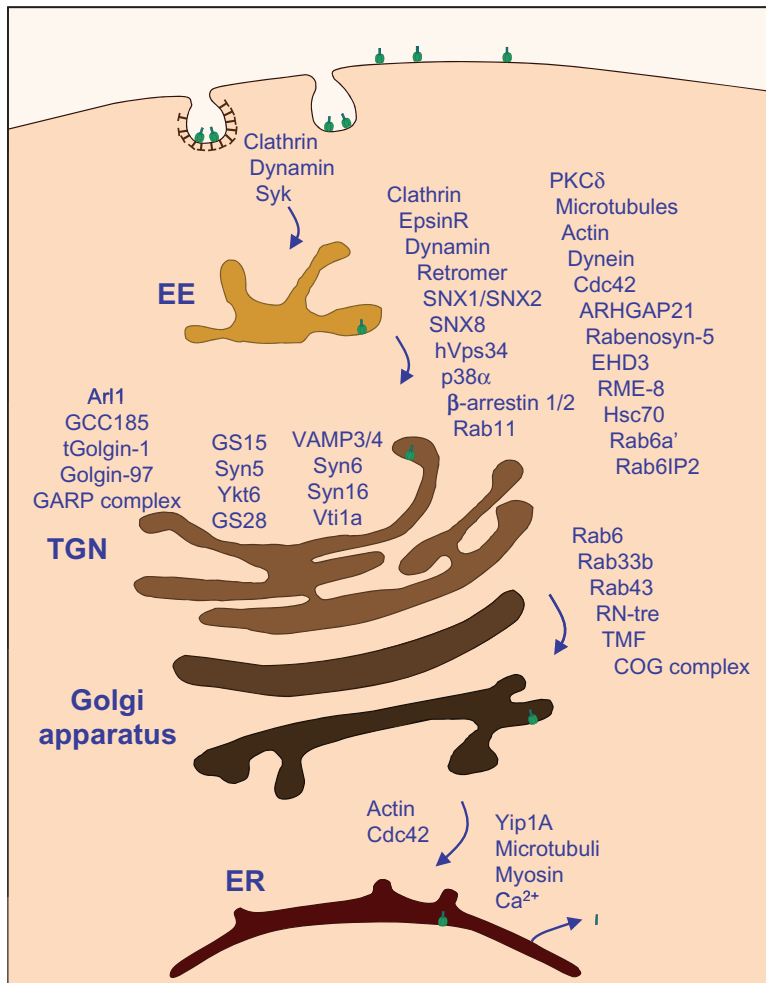
Fig. 2. The intracellular transport pathway of Shiga toxin. Upon binding to the receptor Gb3, Shiga toxin is taken in by both clathrin-dependent and clathrin-independent pathways, and the toxin–receptor complex is transported in a retrograde manner from early endosomes (EE) to the *trans*-Golgi network (TGN), the Golgi apparatus and further to the endoplasmic reticulum (ER). The enzymatically active A_1 fragment is then translocated into the cytosol where its N-glycosidase activity irreversibly modifies the ribosomal 28S RNA, which leads to inhibition of protein synthesis. Components reported to be involved in Stx transport are indicated at each step along the pathway (for references see Johannes and Popoff, 2008; Sandvig *et al.*, 2009; Johannes and Römer, 2010; Starr *et al.*, 2010; Torgersen *et al.*, 2010).

cases to depend on the cell type (Tesh, 2010). However, in most cell types, retrograde transport of the toxin to the ER is a prerequisite for Stx toxicity.

The Shiga toxin receptor, Gb3

The receptor for Shiga toxin in human cells is Gb3. The glycosphingolipids (GSLs) are a subtype of glycolipids that are synthesized by the addition of sugar molecules to a ceramide backbone. The metabolic pathways of GSLs branch at the point of lactosylceramide (Gal-β1→4Glc-β1→Cer) into the lacto-, ganglio-, and globo-series (Hakomori, 2008). The globoseries of GSLs are unique in having an α1→4Gal structure at the internal core, resulting in an unusual conformational structure distinct from that of the other series. Gb3 is the first product in the globoseries of GSLs, and is synthesized by the addition of galactose to lactosylceramide in a reaction catalysed by Gb3 synthase (α-1,4-galactosyltransferase). For the molecular structure of Gb3 see Fig. 1D.

Binding of Shiga toxin to Gb3

Binding of Shiga toxin to Gb3 is complex (Peter and Lingwood, 2000; Pina *et al.*, 2007; Lingwood *et al.*, 2010b), and although much has been learned, the Stx–Gb3 interaction is far from being completely understood. Crystallographic studies have indicated that in the context of the StxB pentamer, each of the five B-chains has three potential Gb3 binding sites (Ling *et al.*, 1998), so that at least in theory, one Stx molecule can simultaneously bind up to 15 Gb3s. Mutational analysis of the Gb3 binding sites in StxB indicates that at least two of the sites are required for StxB to be able to bind to Gb3, whereas optimal binding involves all three binding sites (Soltyk *et al.*, 2002). Furthermore, other studies have indicated that optimal interaction between Stx and Gb3 requires a mixture of Gb3 species with different fatty acid chain lengths in their ceramide backbone moieties (Pellizzari *et al.*, 1992) combined with an optimal organization of Gb3 species (Nyholm *et al.*, 1996), as well as a favourable surrounding lipid environment in the plasma membrane itself (Arab and

Table 1. Advantages and disadvantages of analysing Gb3 by different methods. Examples of published articles where the given technique has been applied are indicated for each method.

Method	Advantages	Disadvantages
Mass spectrometry (Müthing and Distler, 2010; Raa *et al.*, 2009)	Identification and quantification of all Gb3 species. Very sensitive.	Expensive equipment. Special knowledge needed. Sample must be homogenized and extracted before analysis.
TLC with orcinol or overlay assays (Lingwood *et al.*, 2010a; Müthing and Distler, 2010)	Rapid visualization of several samples. Do not need expensive equipment.	Relationship between signal intensity and amount of Gb3 present may be complicated to interpret when using overlay assays. Precise species composition not obtained. Sample must be homogenized and extracted before analysis.
Fluorescence microscopy/ Immunohistochemistry (Salhia *et al.*, 2002; Falguières *et al.*, 2008)	Direct visualization of cells. Discriminate between tumour cells and surrounding tissue.	Not possible to obtain reliable quantitative data of total Gb3 content or the species composition.
Flow cytometry (LaCasse *et al.*, 1999; Tetaud *et al.*, 2003)	Measure distribution of cellular Gb3 expression in a sample. Possible to estimate the surface level of Gb3 of non-permeabilized cells.	Not possible to obtain reliable quantitative data of total Gb3 content or the species composition. Expensive equipment.

Lingwood, 1996). As one example of the possible implication of differences in such factors *in vivo*, a recent study reported that due to differential membrane Gb3 organization in paediatric versus adult renal glomeruli, Stx binds stronger to the former (Khan *et al.*, 2009). These findings may at least in part explain why STEC-induced HUS is mainly a paediatric disease.

Differences in the fatty acid chain lengths in the ceramide backbone of Gb3 may alter not only the binding characteristics of Gb3 to Stx, but also the intracellular routing of the Gb3/Stx complex. Whereas the sphingosine part of the ceramide backbone in general appears with a constant number of 18 carbon atoms, the number of carbon atoms in the fatty acid part varies, normally appearing within a range of 16–24 carbon atoms (C16-C24). There are large cell type-dependent differences in the species composition of Gb3 (Raa *et al.*, 2009). In general, however, the most abundant Gb3 species contains C24, whereas the second most abundant species contains C22, C18 or C16. Gb3 species with short fatty acid chain lengths (C16 or C18) have been associated with enhanced retrograde transport of Stx (Arab and Lingwood, 1998; Raa *et al.*, 2009). So far there is little information available regarding the species composition of Gb3 in different tissues.

Methods to detect the Stx receptor Gb3 in cells and tissues

To evaluate the possibilities of using Stx for imaging or therapy, one needs to investigate the distribution of Gb3, and the ability of Gb3 to bind Stx in human cells and tissues. Different methods have been used for this purpose, and we therefore provide a brief overview of those that are most commonly used. Advantages and disadvantages of these methods are summarized in Table 1.

Mass spectrometry. Due to recent development within the field of mass spectrometry (MS), the total amount of Gb3 and the relative content of different Gb3 species in tissue or cell extracts can now be routinely analysed by MS. Using high-resolution MS, the analysis may be performed with direct injection of the extracts into the MS (so-called 'shot-gun analysis'), i.e. without any chromatographic separation of the samples. The MS analysis also offers the possibility to obtain information about the total cellular/tissue lipidome, including the content of other GSLs, which may give additional important information about the samples (Raa *et al.*, 2009). We anticipate that direct MS will play an increasing role in analysis of Gb3 in the future, but so far most studies have used the more traditional methods described below.

TLC with orcinol staining or overlay assays. Analysis of lipid extracts from cells or tissues may be performed by various chromatographic methods combined with different types of detection. In practice, chromatographic analysis of Gb3 is today mainly performed by thin layer chromatography (TLC) (for reviews see Lingwood *et al.*, 2010a; Müthing and Distler, 2010). Usually high-performance TLC (HPTLC) plates are used. Glycolipids can be visualized by staining carbohydrates with a mixture of orcinol and sulfuric acid. Information about the identity and approximate quantity of the bands can be obtained by comparing the mobility of the band of interest with a reference, or a set of known glycolipid standards.

Alternatively, to obtain information about the amount of binding of Stx or anti-Gb3 antibodies to the Gb3 species on the TLC plate, the plate can be overlaid with Stx, a Stx derivative or an anti-Gb3 antibody, followed by overlay with a secondary antibody conjugated to a detection moiety for visualization of the bound molecules. Thus, the TLC overlay methods are used to indicate the total cellular levels of Gb3 in the cells/tissue sample. Based on the differential motility of Gb3 species with, e.g. long or short fatty acid chain lengths in their ceramide backbone, one can also obtain indications on the relative expression of different Gb3 species, which can be further identified by MS. The MS identification is important, since a single band on a HPTLC plate is likely to contain different species of Gb3 (Arab and Lingwood, 1998; Lingwood *et al.*, 2010b).

Fluorescence microscopy/immunohistochemistry of cryosections. These methods are based on visualization of cellular Gb3 by use of Stx/StxB or anti-Gb3 antibodies followed by secondary antibodies conjugated to fluorescent molecules or enzymes, to allow for detection. Alternatively, Stx/StxB or the anti-Gb3 antibodies may be directly coupled to fluorescent/enzymatic moieties. These methods give an indication of the *in situ* total cellular level of Gb3 if the cells are permeabilized before staining (which is normally the case), or *in situ* surface levels of Gb3 if staining is performed at 4°C on non-permeabilized preparations. Importantly, in the case of a tumour sample, one can obtain information on the origin of the Gb3-expressing cells, i.e. if they are the cancer cells and/or cells from the surrounding tissue. This is in contrast to MS and chromatographic methods, which only aim at measuring the total levels of Gb3 in the sample.

Flow cytometry. By use of flow cytometry one can obtain information on single-cell expression levels of Gb3 in a large number of cells (normally ≥ 10 000 cells are analysed), and thereby obtain information on the distribution of cellular Gb3 expression levels in the sample. Similarly to the method mentioned above (fluorescence microscopy/immunohistochemistry of cryosections), this method is based on staining of cellular Gb3 by use of Stx/StxB or anti-Gb3 antibodies followed by fluorescently labelled secondary antibodies, or alternatively the use of directly coupled Stx/StxB or anti-Gb3 antibodies. Surface levels of Gb3 can be determined if staining is performed at 4°C on non-permeabilized cell preparations. The origin of the Gb3-expressing cells can be assessed by immunofluorescence-based counterstaining with antibodies towards known cell markers.

Function and expression of Gb3 in humans

Apart from its suggested involvement in negative selection of tonsillar B cells during affinity maturation (Taga *et al.*, 1997), the normal function of Gb3 in humans is unknown. Although not fully characterized, Gb3 appears to show a relatively restricted expression in normal human tissues, being mostly found in kidney epithelium and endothelium (Lingwood, 1994; Khan *et al.*, 2009), in microvascular endothelial cells in intestinal lamina propria (Miyamoto *et al.*, 2006; Schuller *et al.*, 2007), in platelets (Cooling *et al.*, 1998), and in subsets of germinal centre B lymphocytes (Murray *et al.*, 1985; Gregory *et al.*, 1988; Mangeney *et al.*, 1991). A low level of Gb3 expression has been reported in monocytes (van Setten *et al.*, 1996), and in monocyte-derived macrophages and dendritic cells (Falguières *et al.*, 2001). Recent reports indicate that Gb3 is also expressed by intestinal pericryptal myofibroblasts (Schuller *et al.*, 2007), neurons (Obata *et al.*, 2008) and endothelial cells in the central nervous system (Johansson *et al.*, 2006; Obata *et al.*, 2008), and the possible implications of this needs to be further clarified. For example, since primary cultures of human cerebral capillary and microvascular endothelial cells are found to be largely resistant to Stx (Arab *et al.*, 1998; Ramegowda *et al.*, 1999; Hughes *et al.*, 2002), it remains to be determined to what extent Gb3 is expressed at the cell surface of human brain endothelial cells, and whether Stx is toxic to these cells *in vivo*. It should be kept in mind that with regard to the sensitivity of cells to the toxic action of Stx, cell surface expression of Gb3 is not always sufficient (Sandvig *et al.*, 1992; Jacewicz *et al.*, 1994), since in general the retrograde transport step to the Golgi and ER is required for Stx toxicity. Moreover, as pointed out above, the expression of different species of Gb3 may alter the binding and intracellular routing of Stx.

Expression of Gb3 in human cancers

Aberrant glycosylation appears to be a universal feature in carcinogenesis, and may alter cell signalling, growth, adherence and motility (Hakomori, 1989; 1996; Hakomori and Zhang, 1997). Remarkably, essentially all experimental and human cancers are found to exhibit alterations in GSL composition and metabolism (Hakomori and Zhang, 1997), and several tumour-associated antigens have been found to be GSLs. These include GSLs of the lacto-, ganglio- and globo-series (Hakomori, 1989; Hakomori and Zhang, 1997). Interestingly, Gb3 was identified as a fibrosarcoma-associated antigen in rats (Ito *et al.*, 1984). Moreover, the antigen defined by a rat monoclonal antibody (referred to as 38.13) directed to a Burkitt's lymphoma cell line was identified as Gb3 (Nudelman *et al.*, 1983), and was found to be highly accumulated in a number of Burkitt's lymphoma cell lines, as well as in 4 out of 8 primary Burkitt-like B cell lymphomas (Wiels *et al.*, 1981) (and see Table 2). Later, Gb3 was found to be expressed in a large proportion of several other types of B

cell lymphomas (Murray *et al.*, 1985; LaCasse *et al.*, 1996; 1999). Using another approach, analysing the overall GSL expression pattern in lipid extracts from primary human testicular cancer tissues compared with normal testicular tissue, a marked accumulation of Gb3 was found to be the most conspicuous and consistent change in testicular tumour extracts (Ohyama *et al.*, 1990; 1992). It should be noted that the patient sample size was relatively low in these studies, especially for the normal samples (only four normal tissue samples in each study; see Table 2). The cancer type for which the highest number of patients has been analysed for Gb3 expression is colorectal carcinoma. Here, three separate studies, which include 141 patients in total, all show that Gb3 expression is significantly elevated in the tumours relative to normal or benign colonic tissue (Kovbasnjuk *et al.*, 2005; Falguières *et al.*, 2008; Distler *et al.*, 2009). For other cancer types, the patient sample sizes have in general been too small to test for statistical significance. However, as shown in Table 2, a clear tendency towards enhanced Gb3 expression in tumour tissues compared with the corresponding normal tissue has been observed in cancers of the ovary (Farkas-Himsley *et al.*, 1995; Arab *et al.*, 1997), breast (LaCasse *et al.*, 1999; Johansson *et al.*, 2009) and pancreas (Distler *et al.*, 2009), as well as in gliomas (Arab *et al.*, 1999; Johansson *et al.*, 2006), malignant meningiomas (Salhia *et al.*, 2002) and acute non-lymphocytic leukaemia (Cooling *et al.*, 2003).

For several cancer types, the overexpression of Gb3 reported in patient tumour samples is reflected in cultured cell lines from the same cancer type. For example, in one study, 13 out of 18 breast cancer cell lines examined (72%) were found to express Gb3 at their cell surface (LaCasse *et al.*, 1999), fitting with the findings by the same authors that 8 out of 10 primary breast cancer tumours (80%) expressed Gb3 (LaCasse *et al.*, 1999). Moreover, a recent study reported Gb3 expression in 17 out of 25 (68%) breast cancers (Johansson *et al.*, 2009). In another study, 23 out of 23 testicular embryonal carcinoma cell lines tested showed high expression of Gb3 (Wenk *et al.*, 1994), in agreement with findings by others that testicular cancers in general overexpress Gb3 (Ohyama *et al.*, 1990; 1992), and in particular, that the Gb3 level in 3 out of 3 testicular embryonal carcinomas and 4 out of 4 testicular embryonal carcinomas plus teratomas was found to be strongly elevated compared with that in normal testicular tissue (Ohyama *et al.*, 1990). Interestingly, in several cancer types, not only the cancer cells themselves are found to overexpress Gb3, but also the tumour vasculature (Arab *et al.*, 1997; 1999; Salhia *et al.*, 2002; Johansson *et al.*, 2006; 2009). Moreover, in some ovarian and breast carcinoma patient samples only the tumour vasculature, but not the cancer cells, was found to express Gb3 (Arab *et al.*, 1999; Johansson *et al.*,

2009), and in studies of gliomas and malignant meningiomas, Gb3 was predominantly found in the tumour vasculature (Salhia *et al.*, 2002; Johansson *et al.*, 2006). From these results, it is tempting to speculate that the cancer cells secrete factors that induce Gb3 expression in the tumour vasculature, or alternatively that a host response towards the tumours may result in such factors being released to the tumour vasculature. Notably, several cytokines have been shown to induce Gb3 expression in endothelial cells (van de Kar *et al.*, 1992; van Setten *et al.*, 1997; Lingwood, 1999).

Given the relative restricted expression of Gb3 in human tissues, the above-mentioned reports on Gb3 expression in human cancers raise the possibility that Stx or Stx derivatives, which specifically bind to Gb3, may be used for targeted therapy and imaging of tumours and tumour vasculature. The feasibility of such a medical application depends on several factors, among others to what degree Gb3 is expressed at the cell surface of various types of normal cells, and to what degree and how Stx and Stx derivatives are bound to, taken up and transported inside normal and malignant cells. These and other aspects related to imaging and targeted cancer therapy of Gb3-expressing cancers are discussed below.

Use of Stx for targeted imaging and therapy of cancer

Imaging using labelled StxB

Cancers with overexpression of Gb3 are possible to image by injecting non-toxic StxB labelled with some type of imaging agent (Janssen *et al.*, 2006; Viel *et al.*, 2008); it is not necessary to use the intact toxin for this purpose. The imaging modalities most useful for targeted imaging are PET (positron emission tomography), SPECT (single photon emission-computed tomography) and optical/fluorescence based techniques due to their high sensitivity (Weissleder and Pittet, 2008). The positron emitters most commonly used for PET are ^{18}F and ^{11}C (half-lives of 110 and 20 min respectively), whereas the gamma emitter ^{99m}Tc (half-life of 6 h) is by far the most used isotope for SPECT. These three imaging techniques all have some advantages and disadvantages. Using the combination of PET with the X-ray based modality CT (Computed Tomography), PET contributes with an extreme sensitivity of the imaged lesion (10^{-11}–10^{-12} M of the probe (Phelps, 2000) in a tissue volume of approximately 1 cm^3), whereas CT gives a nice anatomical picture clearly visualizing where the lesion is located. A disadvantage with PET is the very short half-lives of the positron emitters, which therefore need to be produced by a cyclotron nearby the imaging centre. Although SPECT in general is 10–100 times less sensitive than PET, it has the advantage that the gamma emitter ^{99m}Tc is readily available at most hospitals.

Table 2. Overview of published reports on Gb3 expression levels in primary human cancers.

	Total number of patients	Number of healthy/benign patient tissue samples	Number of malignant patient tissue samples	Proportion of malignant samples exhibiting enhanced expression of Gb3	Fold average increase in Gb3 expression	P-value	Method used to determine Gb3 expression level	Reference
Colon carcinoma	97	31[1]	66[2]	ND	3[3]	0.002[4]	StxB TLC overlay	Falguières *et al.* (2008)
	28	18[5]	10[6]	7/10 = 70%[7]	3[8]	< 0.05	Immunofluorescence[8]	Kovbasnjuk *et al.* (2005)
	16	16[9]	16[9]	13/16 = 81%	1.54	0.021	Stx1 TLC overlay[10]	Distler *et al.* (2009)
B cell lymphomas								
Malignant lymphoma[11]	63	11	52	23/52 = 44%	ND	ND	Flow cytometry using StxB–FITC[12]	LaCasse *et al.* (1996)
Follicular lymphoma	≥ 27	11[13]	16	11/16 = 69%[13]	ND	ND	Flow cytometry using StxB–FITC[12]	LaCasse *et al.* (1996)
Follicular lymphomas, grade I-III	≥ 43	NS	43	31/43 = 72%[13]	ND	ND	Flow cytometry using StxB–FITC[12]	LaCasse *et al.* (1999)
Small lymphocytic lymphoma[14]	≥ 46	NS	46	15/46 = 33%	ND	ND	Flow cytometry using StxB–FITC[12]	LaCasse *et al.* (1999)
Diffuse large B cell lymphoma	≥ 12	NS	12	5/12 = 42%	ND	ND	Flow cytometry using StxB–FITC[12]	LaCasse *et al.* (1999)
Non-Burkitt-like lymphoma[15]	≥ 12	NS	12	8/12 = 67%[16]	ND	ND	Immunohistochemistry[17]	Murray *et al.* (1985)
B cell Burkitt-like lymphoma	≥ 8	NS	8	4/8 = 50%	ND	ND	Immunofluorescence[18]	Wiels *et al.* (1981)
Ovarian carcinoma	15	5	10[19]	5/10 = 50%[20]	ND	ND	Stx1 TLC overlay	Farkas-Himsley *et al.* (1995)
	26	10	16	12/16 = 75%[21]	23[22]	ND	Stx1 TLC overlay[23]	Arab *et al.* (1997)
Breast cancer	≥ 10	NS	10	8/10 = 80%[24]	ND	ND	Flow cytometry using StxB–FITC[12]	LaCasse *et al.* (1999)
	25	0	25	17/25 = 68%[25]	ND	ND	Immunohistochemistry[26]	Johansson *et al.* (2009)
Testicular cancer	17	4	13	12/13 = 92%[27]	ND	ND	TLC orcinol staining[28]	Ohyama *et al.* (1990)
	18	4	14	12/14 = 86%[29]	ND	ND	TLC orcinol staining	Ohyama *et al.* (1992)
Gliomas	14	7	7	2/7 = 29%[30]	ND	ND	Immunohistochemistry[26]	Johansson *et al.* (2006)
	4	0	4	3/4 = 75%[31]	ND	ND	Immunofluorescence[32]	Arab *et al.* (1999)
Malignant meningioma	16	5[33]	11	9/11 = 82%[34]	ND	ND	Immunohistochemistry[35]	Salhia *et al.* (2002)
Pancreatic Cancer	21	21[9]	21[9]	13/21 = 62%	1.42	0.189[36]	Stx1 TLC overlay[10]	Distler *et al.* (2009)
Acute non-lymphocytic leukaemia	≥ 11	NS	11	9/11 = 82%[37]	ND	ND	Stx and anti-Gb3 Ab TLC overlay[38]	Cooling *et al.* (2003)

[1]19 normal and 12 benign colonic adenomas.
[2]31 with metastasis and 35 without metastasis.
[3]Range: 0.4–14 fold. Authors state that this may be an underestimation, since normal colonic epithelial tissue was found to be negative for Gb3 in immunoflourescence studies of cryosections using StxB–Cy3. Authors speculate that contaminating myofibroblasts, endothelial and/or immune cells in the samples may be the source of the Gb3 detected in normal and benign adenoma tissues.
[4]Comparing Gb3 levels in tissues from cancer patients with that from patients with normal/benign adenomic colons. There was no significant difference between Gb3 levels in non-metastatic and metastatic cancers.
[5]15 normal and 3 benign colonic adenomas.
[6]3 non-metastatic cancers, 5 primary lesions of metastatic colon cancer and 2 liver metastases.
[7]Gb3 was not enhanced in the 3 non-metastatic tumour samples.
[8]Based on StxB–Alexa 488 fluorescent stain of pancreatin-positive cells in 8 μm cryosections. Tissues were fixed and permeabilized with 4% PFA and 0.1% saponin, i.e. total *in situ* levels of Gb3 were assessed. Fluorescence was enhanced threefold compared with background autofluorescence in normal cells, which were reported to be negative for Gb3.
[9]Malignant and adjacent healthy tissues were obtained from the same patient for each of the patients.

[10]Similar results were obtained with Stx1 and polyclonal anti-Gb3 antibody TLC overlays, the latter showing a little higher higher sensitivity in tracing Gb3 species.

[11]Includes non-Hodgkin's lymphoma, acute lymphocytic leukaemia and B cell chronic lymphocytic leukaemia.

[12]Cell surface Gb3 expression was measured, since the cells were not permeabilized and simultaneously stained for cell surface markers, e.g. CD19 to gate for B cell lymphoma cells. Patient samples in which > 15% of the cells stained positively with StxB–FITC were defined as positive for the Stx receptor Gb3. This percentage value (15%) was calculated from the average percentage of StxB–FITC-positive cells (3% ± 4%) observed in samples of non-cancerous patients plus 3 SDs. It is not stated exactly how (from where) the cells from the 11 non-cancerous control patients were collected. The samples may originate from peripheral blood, fine-needle aspirates, and lymph node- and bone marrow biopsies (see fig. 4 in LaCasse *et al.*, 1996).

[13]Normal follicle centre cells reportedly express Gb3. It is not stated exactly how (from where) the cells from the 11 non-cancerous control patients were collected, i.e. it is unclear whether the level of Gb3 in normal follicle centre cells was tested, and if so, from how many individuals. These studies have therefore not evaluated whether follicle centre lymphoma cells express enhanced levels of Gb3 compared with normal follicle centre cells.

[14]With or without chronic lymphocytic leukaemia.

[15]Malignant lymphomas classified as centroblastic/centrocytic.

[16]Authors state and show some examples that lymphoma centrocytes and centroblasts generally reacted more strongly with the anti-Gb3 antibody than cells in normal germinal centres.

[17]Staining of 4–5 μm cryosections with anti-Gb3 antibody (clone 38.13) and detection using an avidin-biotin peroxidase complex immunoperoxidase technique. Cryosections were fixed in acetone, which also permeabilizes cells, i.e. total *in situ* Gb3 levels were assessed.

[18]Staining with monoclonal rat IgM anti-Gb3 antibody (clone 38.13) and fluorescein-conjugated anti-rat IgG on living lymphocytes at 4°C, i.e. cell surface levels of Gb3 were analysed.

[19]Samples from cysts/benign tumours were omitted from the table, since the cysts showed increased levels of Gb3 expression compared with normal ovaries. Samples from patients with cysts/benign cancers are only included in the table if the cysts/benign tumours do not show elevated levels of Gb3 compared with the corresponding normal tissue. The reason is to make the table shorter and easier to follow.

[20]Authors state that in 8 out of 10 ovarian cancer cases, a significant increase in Gb3 expression was observed. However, after a closer inspection of the data, and after dividing the bands into not detectable (no band visible on the Stx1 TLC overlay), weak (much weaker than 0.5 nmol of the standard), intermediate (comparable with 0.5 nmol of the standard), and strong (much stronger than 0.5 nmol of the standard) Gb3 expression, our estimates are as follows for normal ovaries: not detectable: 2/5, weak: 2/5, intermediate: 1/5; for ovarian cancers: not detectable: 2/10, weak: 3/10, intermediate: 4/5, strong: 1/10. Thus, 5 out of 10 ovarian cancers express intermediate-to-high levels of Gb3, whereas 1/5 normal ovaries expresses intermediate levels of Gb3. Thus, we propose that a reasonable estimate from these data is that 5 out of the 10 ovarian cancers expressed more Gb3 than the average from the normal ovaries. Alternatively, another way to interpret the data: 8/10 (80%) ovarian cancers expressed Gb3, whereas 3/5 (60%) normal ovaries expressed Gb3.

[21]Our own calculation based on numbers in Table 1 in the publication. Samples showing ≥ sixfold higher Gb3 levels than the average Gb3 level in normal ovaries are regarded as samples with enhanced Gb3 expression.

[22]Our own calculation based on numbers in Table 1 in the publication.

[23]Binding of StxB–FITC to frozen tumour or normal tissue from selected patients generally correlated with the levels of Gb3 extracted from the tissue (as measured by Stx1 TLC overlay). Little or no staining with StxB–FITC was seen in sections of normal ovary. Extensive binding of StxB–FITC to the lumen of blood vessels that vascularize the tumour was also observed, even in Gb3-negative tumours.

[24]No Gb3' (0–15% StxB–FITC-positive cells): 2/10. 'Weak Gb3' (15–40% StxB–FITC-positive cells): 3/10. 'Intermediate Gb3' (41–70% StxB–FITC-positive cells): 4/10. 'Strong Gb3' (71–100% StxB–FITC-positive cells): 1/10. That is, 5/10 breast cancers were intermediate to strongly positive for cell surface Gb3.

[25]Gb3 expression was found in one tumour cells in 17 of the 25 breast cancer specimens. It was not further stated how extensive the Gb3 expression was in these 17 specimens, and also not stated if there were differences within these 17 specimens, e.g. if some showed higher expression than others. Gb3 expression was detected in vascular endothelial cells in all 25 tumour specimens.

[26]Using anti-Gb3 antibodies on cryosections. A rat monoclonal IgM antibody from Immunotech, Marseille, was used (this is most likely the 38.13 clone). Cryostat specimens were fixed and permeabilized with acetone, i.e. total *in situ* levels of Gb3 were analysed.

[27]5 out of 6 seminomas, 3 out of 3 embryonal carcinomas, and 4 out of 4 embryonal carcinomas + teratoma, showed strongly elevated Gb3 levels.

[28]For selected samples, elevation of Gb3 expression was confirmed with anti-Gb3 antibody (1A4 = clone 38.13) TLC overlay. The identity of Gb3 was also confirmed by purification and subsequent degradation with α-galactosidase.

[29]12 out of 12 seminomas, and 0 out of 2 testicular malignant lymphomas showed strongly elevated Gb3 levels.

[30]Gb3 was detected only in a fraction of tumour cells in any one section. In normal brains, Gb3 was expressed only in endothelial cells. In 4/7 tumour sections, the endothelium stained positive for Gb3.

[31]Extensive binding of StxB–FITC to tumour cells was observed in 3/4 tumours. Also the endothelial cells in the tumour sections stained positive for Gb3.

[32]StxB–FITC on 5 μm cryosections. Thawed cryosections were stained for 1 h at room temperature in a humidified chamber, i.e. total *in situ* levels of Gb3 were analysed.

[33]From benign meningiomas.

[34]Malignant meningioma: very strong staining, 3; strong staining, 2; intermediate, 2; weak, 3; no staining, 2. Benign tissue: weak staining, 1 (was extremely faint); no staining, 4. Gb3 was detected in the tumour vasculature, and this staining was included in the scoring just mentioned. In all positive samples, Gb3 was primarily localized to the microvasculature. In two tumours with strong staining, Gb3 was also found within cancer cells.

[35]Stx overlay followed by overlay with mouse anti-Stx antibody and biotinylated anti-mouse antibody on 5 μm cryosections.

[36]Gb3 expression was significantly higher ($P = 0.039$) in less differentiated tumours (histopathologic grade, g > 2) ($n = 6$) compared with more differentiated tumours (g ≤ 2) ($n = 14$). Gb3 species most likely corresponding to hydroxylated Gb3 (d18:0, h16:0 and d18:1, h16:0) were expressed in as many as 18/21 (86%) of the tumours, whereas it was not expressed at all in the healthy tissue. It seems that the slower migrating band (mostly C16 Gb3) was much more increased than the faster migrating band (mostly C22-24 Gb3), but the statistical significance of this was not addressed.

[37]Staining was faint in two of the positive samples. Samples contained minimal amounts of contaminating Gb3-expressing cells. Gb3 was not detected in normal mature neutrophils.

[38]Essentially the same results were obtained using either Stx or anti-Gb3 antibodies in the TLC overlay assay. The identity of the Gb3 bands was confirmed by mass spectrometry.

ND, not determined; NS, not stated; TLC, thin layer chromatography; Ab, antibody.

Moreover, the longer half-life of ^{99m}Tc means that it is possible to extend the time between injection and imaging to allow more of the free, unbound probe to be excreted before imaging. This is very important as a main challenge of targeted imaging is to reduce the signal from tissue nearby the lesion such that a good signal-to-noise ratio is obtained. A rapid excretion of the targeting compound not bound to the lesion (normally less than 2–3% of the injected dose is retained at the molecular target) is therefore important.

It has been shown that it is possible to detect tumours in the digestive tract of mice (spontaneous tumorigenesis models were used) after force-feeding the animals with a single dose of 300–500 μg fluorescence-labelled StxB using confocal laser endoscopy, or after retro-orbital injection of ^{18}F-labelled StxB followed by PET imaging 1–2 h after injection (Janssen *et al.*, 2006) (the precise dose of ^{18}F-labelled StxB was not stated, but was referred to as a 'tracer' dose). Later the same group reported imaging of tumours subcutaneously implanted in mice (specimen from human colon cancer) from 5 h to 9 days after giving 50 μg of fluorescence-labelled StxB by oral, intraperitoneal or intravenous administration (Viel *et al.*, 2008). The best signal-to-noise ratio was obtained 2–4 days after intravenous administration. The images presented in these two studies showed considerable background signals from various tissues, such that it could have been a challenge to detect the tumours if not knowing where to look for them. It should be noted that a biodistribution study performed in mice using autoradiography (cryosections of 40 μm) following intravenous injection of ^{125}I-labelled Stx1 or Stx2 showed considerable amounts of radioactivity in several tissues. Thus, Stx1 was found, e.g. in lungs, kidney cortex and bone marrow; whereas Stx2 did not target the lung, but accumulated in kidneys to a greater extent than Stx1 (Rutjes *et al.*, 2002).

Ideally targeted imaging in the clinic should be performed using intravenous injection (simple administration, low dose and a homogenous delivery), and the targeting substance should be as small as possible to allow for rapid renal excretion of free, unbound substance. The StxB pentamer has a 'doughnut' shaped structure with a central pore (Fig. 1C), and thus its hydrodynamic size is larger than expected for a globular protein with a mass of 38.5 kDa. StxB may therefore be too large to obtain a rapid renal excretion of the free, unbound substance, and future studies are needed to learn if StxB clearance is fast enough to be useful for PET or SPECT imaging or if smaller Gb3-binding substances have to be investigated. The longer half-life for ^{99m}Tc than the isotopes used for PET imaging makes it more likely that StxB can be successfully used with SPECT than with PET. Both these imaging techniques have the advantage that deep-seated lesions (the whole body) can be imaged.

One advantage with optical and fluorescent probes is that it is possible to extend the time between injection of the substance and imaging, thus allowing more time for clearance of the free, unbound probe. The time needed for clearance/excretion of a substance normally increases with the size of the animal. Although it is difficult to predict elimination half-lives across species, an indication of this species difference is that a substance cleared by glomerular filtration is excreted approximately 5 times faster in rats than in humans (Lin, 1998). Optical/fluorescence-based imaging techniques have the disadvantage that it is not possible to image deep into tissues; most of these techniques allow imaging of only 1–2 cm into the tissue. These techniques are therefore most useful for imaging of surface areas, including those that can be reached by detecting probes in body cavities or during surgery. Thus, intravenous injection of StxB labelled with a fluorescent probe seems to be an attractive opportunity for targeted imaging of cancers with overexpression of Gb3, which are close to a surface that can be reached with the detecting probe. It should be noted that all three imaging techniques described above should be expected to give similar images whether the labelled substance is bound to the cell surface or taken up into the cell; it is the concentration within a given tissue volume that matters.

Targeted therapy using Stx or StxB

The reported overexpression of Gb3 in many cancers opens for the possibility of using either the holotoxin (Stx) or StxB coupled to therapeutic drugs in targeted cancer therapy. Whereas the first option in general requires that the cancer cells are able to transport the toxin to the ER and translocate the toxic A_1 fragment into the cytosol, for the second option, the ability of the cancer cells to bind or take up StxB may be sufficient for a therapeutic effect. The two options are expected to give different therapeutic effects as well as different side effects, and this is discussed below.

The simplest choice for targeted therapy of Gb3 overexpressing cancers would be to utilize the natural holotoxin, the crucial point being whether it can be tolerated by humans (see discussion below). Stx harbours several advantageous properties compared with conventional chemotherapeutic drugs. It kills cells in an extremely effective manner (theoretically, one toxin molecule is enough to kill a cell), and thus, Stx could possibly have therapeutic effects at low doses and with only one or a few rounds of treatment. The latter has indeed been shown to be the case in several murine cancer models. Thus, intratumoral or intraperitoneal injection of Stx1 inhibited tumour growth in a murine metastatic fibrosarcoma model (Farkas-Himsley *et al.*, 1995), as well as in mouse xenograft models of human malignant meningiomas

(Salhia *et al.*, 2002), atypical human bladder carcinoma with endothelial characteristics (Heath-Engel and Lingwood, 2003), human renal carcinoma (Ishitoya *et al.*, 2004) and human astrocytoma (Arab *et al.*, 1999). Remarkably, in the two latter cases, complete regression of the tumours was reported 7–10 days after a single intratumoral injection of Stx1, and apoptosis was shown to occur in both tumour cells (Arab *et al.*, 1999; Ishitoya *et al.*, 2004) and vascular cells (Arab *et al.*, 1999) within the treated grafts. Moreover, no side effects were reported in these murine models. In the latter mentioned study, the mice were followed up for more than 50 days post treatment, during which the animals remained tumour free and were without apparent side effects (Arab *et al.*, 1999). Another advantage with Stx compared with conventional chemotherapeutic drugs is the fact that Stx kills cells by a distinct mechanism that is independent of most drug-resistant cancer cell phenotypes. Thus, Stx might be able to kill cancer cells that would otherwise escape chemotherapeutic treatment and lead to a relapse. Intriguingly, some reports indicate that drug-resistant cancer cells are in fact hypersensitive to the toxic effect of Stx (Farkas-Himsley *et al.*, 1995; Arab *et al.*, 1997; 1998; 1999), implying that Stx may be extremely effective in the treatment of multidrug-resistant cancers. One could imagine a form of treatment that combines chemotherapeutic drugs with very low doses of Stx (the latter to kill drug-resistant cancer cells).

A major concern with the use of the active Stx in cancer therapy would be the putative side effects, given that upon infection with bacteria that produce Stx1/Stx2 (STECs), life-threatening complications arising from HUS can occur, in which Stx in most instances in believed to play a crucial etiological role. Of note, however, serious complications due to STEC infection are very rare, and occur primarily in a minor fraction of young children and the elderly. Moreover, purified Stx is less likely to induce HUS than an STEC infection, since other virulence factors than Stx such as adhesins, other toxins and proteases also contribute to STEC-induced disease (Palermo *et al.*, 2009). Furthermore, not only Stx, but also other bacterial factors such as lipopolysaccharide and flagellin are believed to contribute to enhance the damaging effect of Stx via induction of cytokines that upregulate Gb3 expression in endothelial cells (Proulx *et al.*, 2001; Tarr *et al.*, 2005; Palermo *et al.*, 2009). Thus, one would expect Stx-mediated damage to the endothelium to be more pronounced in the context of an STEC infection than when highly purified (lipopolysaccharide-free) Stx is administered alone. Moreover, administrating Stx alone allows for controlled toxin dosage, localization and choice of Stx isoform. Of note, Stx1 is generally found to be more toxic to cultured cancer cells than Stx2. Interestingly, however, STECs that produce Stx2 are much more frequently asso-

ciated with HUS than STECs producing only Stx1 (Boerlin *et al.*, 1999), indicating that Stx1 may be less toxic *in vivo* than Stx2. This was indeed shown to be the case for juvenile baboons [in which tissue localization and quantity of Gb3 is similar to humans (Tesh *et al.*, 1994)] developmentally comparable to 3- to 6-year-old humans, as these animals tolerated four consecutive intravenous injections with 25 ng kg^{-1} Stx1, but not Stx2 (Siegler *et al.*, 2003). Given the observation that Stx-hypersensitive cancer cells can be killed by as little as 1 pg ml^{-1} Stx1 *in vitro*, a dose of 25 ng kg^{-1} Stx1 could be clinically efficient. Moreover, adult baboons (and adult humans) are likely to tolerate higher doses of Stx1 than juvenile baboons, and higher doses of Stx can be expected to be tolerated upon e.g. intratumoral than upon intravenous injection of Stx. In conclusion, it is conceivable that e.g. Stx1 could be used in the treatment of non-elderly, adult cancer patients. However, more studies are required to assess the short- and long-term effects of Stx in animals and humans.

If adverse reactions should turn out to prohibit the direct use of active holotoxin in cancer therapy, an alternative for some cancer types could be to purge the cancer cells *ex vivo*. *Ex vivo* Stx1 treatment has indeed been shown to be highly efficient in eradicating malignant B cells from lymphoma and myeloma patient samples, whereas at the same time leaving haematopoietic progenitor cells unaffected (LaCasse *et al.*, 1999). This indicates that Stx1 may be used as an *ex vivo* purging agent to eliminate malignant cells from autologous stem cell grafts.

As an alternative to using the holotoxin for targeted cancer therapy, the non-toxic StxB could be used to bring drugs to the cancers the same way as described above for bringing imaging modules to cancers. If used therapeutically instead of for imaging, there is no need for rapid excretion of the targeting molecule, and it is probably an advantage if the substance is circulating for a longer time. StxB should in principle be useful to bring therapeutic isotopes, normally alfa or beta emitters (Dahle and Larsen, 2008; Dancey *et al.*, 2009), or other drugs to cancers with overexpression of Gb3. Using therapeutic isotopes it should be sufficient with surface-binding of isotopes to kill the cells overexpressing Gb3. This approach would be expected to give different and perhaps additional side effects than if using the holotoxin, since it will potentially be toxic to all normal cells that express Gb3 at the cell surface, whereas the holotoxin in general is toxic only to cells that in addition are able to transport the toxin in a retrograde manner to the ER and the cytosol. For example, isotope-coupled StxB, but not the holotoxin, might kill monocyte-derived macrophages and dendritic cells, as it has been reported that these cells take up StxB, but direct it to the degradative pathway instead of to the Golgi (Falguières *et al.*, 2001). A different approach would be to conjugate StxB to a drug that has a thera-

peutic effect only after endocytosis and release of the drug to the cytosol. Strategies to avoid the drugs from killing normal cells include the use of phototoxic compounds or the use of drugs that preferentially kill cancer cells. In the former case, StxB-mediated targeting of Gb3-expressing cancer cells is refined with a second level of specificity by local illumination of the tumour. The release of drugs to the cytosol is in general quite a challenge. Thus, the combined property of StxB both to target Gb3-expressing cancer cells and to transport drugs to the cytosol via retrograde transport to the ER is rather unique. However, in some Gb3-expressing cancer cells, the retrograde transport pathway for StxB is inefficient. Photochemical internalization includes use of substances (e.g. porphyrins) that are taken up into the endosomal membrane and after light treatment generate free radicals, which destroy the endosomes (Berg *et al.*, 2007). Thus, in addition to adding a second level of specificity in the targeting of cancers (as mentioned above), linking StxB to photochemical substances can improve the efficiency of drug delivery to the cytosol. StxB photosensitizer conjugates have indeed been shown to be highly effective in killing cancer cells *in vitro* (Tarrago-Trani *et al.*, 2006; Amessou *et al.*, 2008). StxB coupled to drugs that preferentially kill cancer cells have also been synthesized and tested on cancer cells *in vitro*. In one study, the active principle of the topoisomerase I inhibitor camptothecin 11, which is used in the treatment of colorectal cancers, was bound to StxB through a disulfide linkage, the idea being that the drug would be released after retrograde transport to the reducing environment in the ER (El Alaoui *et al.*, 2007). This conjugate was indeed shown to be highly cytotoxic to HT29 colorectal carcinoma cells *in vitro* (El Alaoui *et al.*, 2007). The selection of cancer cells would, in this case, not only be due to the fact that the topoisomerase inhibitor preferentially kills cancer cells, but also that a prodrug was used (due to conjugation to StxB), so that the active drug would only be released in cells that are able to transport StxB to the ER. As a general note, as is the case with the holotoxin, more studies are required to assess the short and long-term effects of StxB and the various StxB derivatives in animals and humans.

Concluding remarks and future perspectives

The numerous reports that indicate a selective overexpression of Gb3 in various types of human cancers warrant further research into the potential application of Stx or StxB derivatives in cancer medicine. Several issues need to be clarified. First, many of the studies on Gb3 expression in cancer have analysed too few patient samples to be able to test for statistical significance. Thus, there is a need for studies with larger patient sample sizes. There is also a general need for better characterization of

the Gb3 expression levels in non-malignant human tissues. Given the complexity of Stx binding to Gb3, a future challenge also lies in the accurate detection and discrimination between different Gb3 species and their plasma membrane environment in cancer cells versus non-malignant cells, as well as in the interpretation of such information. Thus, we also need to learn more about the implications of Stx binding to different kinds of Gb3s, both concerning Stx binding and endocytosis, but also concerning the effects on the intracellular trafficking of Stx.

Using StxB coupled to contrast agents, we expect imaging of Gb3-expressing cancers to be more feasible with SPECT than with PET, due to the likely relatively slow renal clearance of StxB. Even more feasible, in theory, is the use of StxB coupled to optical or fluorescent probes, although this limits the application to imaging of cancers that can be reached with the detecting probe, i.e. within body cavities or during operations. Although StxB is non-toxic *in vitro*, the short- and long-term effects of StxB and its derivatives need to be tested further in animal models. This is also a critical issue concerning the potential use of Stx, or StxB coupled to therapeutic agents, in the treatment of cancer patients. The exceptionally potent anticancer effect of Stx observed in murine cancer models justifies further research into clarifying whether Stx or Stx derivatives can be exploited in the cancer clinic.

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