Targeting glycosphingolipids for cancer immunotherapy

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(Received 2 July 2020, revised 20 August 2020, accepted 20 August 2020, available online 9 September 2020)

doi:10.1002/1873-3468.13917

Edited by Giovanni D’Angelo

Glycosphingolipids (GSLs) consist of glycans linked to C-1 hydroxyl group of ceramide in all eukaryotic plasma membranes [1]. Many reports suggest that GSLs display diverse functions in normal development and cancer growth [1–3]. However, the analysis of GSLs remains challenging due to their amphiphilic nature and inherent complexity. Antibodies against several GSLs, designated as stage-specific embryonic antigens (SSEAs), have been widely used to characterize the GSL profiles of embryonic stem cells (ESCs). Since these antibodies often display cross-reactivities [4], GSL profiles in human ESCs and differentiated derivatives are more accurately delineated by systematic survey using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) and tandem mass spectrometry analyses. We previously reported that during differentiation of ESC to embryoid body, the core structures of GSLs switched from globo- and lacto- to ganglioseries [5]. Lineage-specific differentiation was also marked by alterations of specific GSLs [6].

Aberrant expression of glycoconjugates is a common feature of cancer cells [7–11]. The unique sugar signatures in the embryonic stage during normal embryogenesis often resurge during the development of cancer cells and may be exploited as glycan targets for immunotherapeutics.
Expression of GSLs during ontogenic development

Globo H ceramide, which consists of hexasaccharide moiety \((\text{Fuc}_{\alpha1-2}\text{Gal}_{\beta1-3}\text{GalNAc}_{\beta1-3}\text{Gal}_{\alpha1-4}\text{Gal}_{\beta1-4}\text{Glc}_{\beta1}\text{Cer})\) attached to ceramide, was initially identified in human breast cancer cell line MCF-7 [19]. The biological roles of GHCer in normal cells are largely unknown in the literature. As to the mechanism underlying GSL profile changes, \(A4GALT\) and \(B3GNT5\) encode enzymes \(\text{Gb3Cer}\) synthase and \(\text{Lc3Cer}\) synthase to convert \(\text{LacCer}\) to \(\text{Gb3Cer}\) and \(\text{Lc3Cer}\), respectively. Then, \(B3GALT3\), which catalyzes \(\text{SSEA}-3\) (Gb5Cer) and \(\text{Lc4Cer}\) synthesis, led to the expression of \(\text{SSEA}-3\) and \(\text{Lc4Cer}\). Finally, \(FUT1\) and \(FUT2\) which encode two fucosyltransferases catalyze synthesis of Globo H and Fuc-Lc4Cer by adding a terminal fucose moiety via \(\alpha1-2\) linkage [8,11,15].

Disialoganglioside GD2 is a \(\beta\)-series ganglioside, which contains two \(\alpha\)-2, 8 sialic acids. It is an acidic GSL consisting of five carbohydrate moieties [\(\text{GalNAc}_{\beta1-4}\left(\text{NeuAc}_{\alpha2-8}\text{NeuAc}_{\alpha2-3}\right)\ \text{Gal}_{\beta1-4}\text{Glc}_{\beta1-1}\)] attached to a ceramide through a glycosidic linkage. A series of specific glycosyltransferases (GTs) are involved in the synthesis of GD2. First, a \(\beta\)-linked glucose ceramide (GlCer) in the early Golgi apparatus receives a galactose through galactosyltransferase I to form a lactosylceramide, followed by sequential addition of two sialic acids to lactosylceramide by ST3 \(\beta\)-galactoside \(\alpha\)-2,3-sialyltransferase 5 (ST3GAL5 or GM3 synthase) and ST8 \(\alpha\)-N-acetyleneuraminic \(\alpha\)-2,8-sialyltransferase 1 (ST8SIA1 or GD3 synthase). Finally, N-acetylgalactosamine (GalNAc) is added by \(\beta1, 4\)-N-acetylgalactosaminyltransferase (\(\beta4\text{GalNAcT1, GM2/GD2 synthase}\)) [20,21].

Although the biological roles of GHCer and GD2 in normal cells are largely unknown, many studies have addressed GSL profile changes in mouse ESCs [22–26]. For example, globo-series GSLs including Globo H are expressed at high levels at four cell stages and then decline during mouse embryogenesis [27]. On the other hand, ganglio-series GM3, GD3, GT3, GM2, and GD2 are expressed at later stages during neural crest formation in mice [28,29]. For studies of human ESCs, we used MALDI TOF MS and tandem MS/MS analyses, in combination with immunostaining/flow cytometry to systematically evaluate changes of GSL expression profiles in the human undifferentiated ESCs, differentiated embryoid body, neural progenitor cells, and definitive endoderm [5,6]. Consistent with the observed changes during mouse embryonic development [27], globo- and lacto-series GSLs were identified abundantly in the undifferentiated human ESCs. During differentiation to embryoid body, a ‘makeover’ change of GSL core structures from globo- to ganglio-series was observed [30]. Reflecting a unique stage-specific transition characterizing this ontogenic development, this switching of GSLs could be attributed to the changes of expression of key GTs involved in GSL biosynthetic pathways [30].

We analyzed expression of GTs involved in GSL biosynthesis to elucidate the mechanism underlying GSL changes during hESC differentiation. \(A4GALT\) and \(B3GNT5\), the genes that encode enzymes \(\text{Gb3Cer}\) synthase and \(\text{Lc3Cer}\) synthase, which convert \(\text{LacCer}\) to \(\text{Gb3Cer}\) and \(\text{Lc3Cer}\), were downregulated, respectively, to 30% and 50% of levels for differentiated embryoid body outgrowth cells. \(B3GALT3\), which catalyzes \(\text{SSEA}-3\) (Gb5Cer) and \(\text{Lc4Cer}\) synthesis, was reduced to 30%, presumably contributing to a decrease of expression of \(\text{SSEA}-3\) and \(\text{Lc4Cer}\) in the...
differentiated embryoid body outgrowth cells. FUT1 and FUT2, the genes encoding two fucosyltransferases that catalyze synthesis of Globo H and Fuc-Lc4Cer, were both downregulated to 20% during differentiation. These changes may account for inefficient conversion of LacCer to globo- and lacto-series GSLs during hESC differentiation. In contrast to the reduced expression of GTs involved in biosynthesis of globo- and lacto-series GSLs, two GTs involved in biosynthesis of ganglioside-series GSLs (gangliosides), GM2 synthase (B4GALNT1) and GM3 synthase (ST3GAL5), increased 8.4- and 14.2-fold, respectively, during hESC differentiation. These changes are consistent with the increased expression of gangliosides and reduced expression of globo- and lacto-series GSLs in differentiated embryoid body outgrowth cells. Increased expression was also observed for other GTs involved in ganglioside biosynthesis: GD3 synthase (8.9-fold), GT3 synthase (ST8SIA1; 8.9-fold), and sialyltransferase 4 (ST3GAL1; 18.3-fold). The altered expression of these key GTs may account for the switch in core structures of GSLs in favor of ganglioside-series GSL biosynthesis during hESC differentiation.

Furthermore, for differentiation of ESCs into neural progenitor cells, core structures continued to shift primarily to ganglioside-series dominated by GD3 [5,6]. In contrast to the reduction of GTs related to globo-/lacto-series GSLs, increases were observed for genes encoding GTs related to ganglioside-series GSLs: ST3GAL5 (2.7-fold), ST8SIA1 (10.1-fold), and ST3GAL1 (1.5-fold). These changes explain the increased expression of gangliosides in differentiated neural progenitor cells. On the other hand, as human ESCs differentiated into endodermal lineage, there was prominent expression of Gb4 ceramide with little expression of other globo-series in endodermal cells [5,6]. These ‘makeover’ changes in GSL profiles were again due to alterations of glycosyltransferase in GSL biosynthetic pathways [5,6]. Reduction in genes encoding GTs related to globo- and lacto-series GSLs included B3GALT5 (0.2-fold), FUT1 (0.6-fold), and FUT2 (0.9-fold). Genes encoding GTs related to ganglioside-series GSLs (ST3GAL5, ST8SIA1, ST3GAL1) were reduced to 0.9-, 0.7-, and 0.8-fold, respectively. These findings explain the decreases in all three types of GSLs in definitive endoderm cells.

Epithelial–mesenchymal transition (EMT) is another important phenomenon that occurs during embryonic development [31] and plays a role in metastatic progression of primary tumors [30–33]. GSLs are also involved in the EMT process [34–36]. Guan et al. [34,35] demonstrated that specific GSLs were downregulated during EMT and that the enhanced expression of these GSLs blocked the EMT process [34,35]. In addition, ganglioside GM3 was involved in regulation of TGF-β1-induced EMT in human lens epithelial cells [36]. These studies demonstrated that specific GSL patterns and clustered GSL-enriched microdomains at the cell surface serve as cell-specific markers and complexes that initiate intracellular signaling through interaction with other functional membrane components. A useful in vitro EMT model, the HMLE-Twist-ER cell, was developed by Weinberg et al. in 2008 [37,38] and adapted by others [38,39]. Upon induction of Twist expression, epithelial cells develop mesenchymal morphology and acquire cancer stem cell (CSC) properties. This model had been used for comparison of GSL expression profiles in breast CSCs vs. non CSCs [40]. These studies provided information on GSL profiles in human breast CSCs, a possible functional role of GD2/GD3 in CSCs, and a novel approach to preventing cancer recurrence by targeting CSCs.

In addition, we examined the expression of these unique surface GSLs during reprogramming of human fibroblasts into induced pluripotent stem cells (iPSCs) by transduction with the four reprogramming transcription factors Oct4, Sox2, Klf4, and c-Myc [41]. The starting population of fibroblasts expressed almost no Globo H, Lc4Cer, and H type 1 antigens, but fully reprogrammed iPSCs that had reactivated an endogenous Oct4 gene displayed levels of these GSL markers comparable to those in ESCs. In contrast, after differentiation of iPSCs, the expression levels of these markers declined significantly to levels typical of fibroblasts before reprogramming [8,11,15]. Collectively, these data point to a strong association of GSL surface markers with the pluripotent state in both ESCs and iPSCs and an association of GSLs with the undifferentiated state.

These GSL markers have long been hypothesized to play crucial roles in a variety of cellular functions, such as cell migration, adhesion, signal recognition, and differentiation. Now, based on findings of GSL markers in ontogenic development of ESCs from various research groups, we have keen interest in the relationship of the stage-specific glycans present in ESCs and the expression of onco-fetoproteins in cancers/CSCs [5]. It was suggested that cancer cells often possess traits reminiscent of those ascribed to normal early embryonic cells; indeed, cancer cells express many onco-fetoproteins that are found in ESCs. Thus, these stage-specific glycan entities in ESCs may serve as markers for cancer detection or as targets of cancer therapy.
Current cancer immunotherapeutics

Immunotherapy of cancer is a rapidly evolving field. There are several types of cancer immunotherapy, including monoclonal antibodies (mAbs), vaccines, immune system modulators, and immune effector-cell therapy. The most commonly available cancer immunotherapeutics are mAbs against tumor antigens or immune checkpoint molecules. In 1997, the United States Food and Drug Administration (US FDA) approved rituximab, a mAb against CD20, for treating patients with non-Hodgkin’s lymphoma. Since then, many therapeutic mAbs targeting tumor-associated antigens have been developed and approved for cancer treatment (Table 1). These mAbs can kill tumor cells by antibody-dependent cell cytotoxicity (ADCC), or in conjugation with radio-isotopes, chemotherapeutics, or toxins. The recent approval of T cell-engaging bispecific antibodies and chimeric antigen receptor (CAR) T cells for the treatment of acute lymphoblastic leukemia opens up opportunity to redirect immune effector cells with mAbs. The approval of ipilimumab for advanced melanoma in 2011 inaugurates a new era of cancer immunotherapeutics, which target immune checkpoints such as cytotoxic T lymphocyte-associated antigen 4, programmed cell death protein 1 (PD-1), programmed cell death-ligand 1 (PD-L1) that compromise the ability of the immune system to mount an effective antitumor response. These immune checkpoint blocking antibodies can reinvigorate dysfunctional/exhausted T cells to restore tumor immunity and represent paradigm-shifting therapeutic strategies for cancer treatment. In addition, dendritic cell-based vaccines have been developed for enhancing the priming of T cells toward unknown, patient- and tumor-specific antigens. Dendritic cells (DCs) are potent antigen-presenting cells, which present the processed epitopes to activate CD4 and CD8 T cells. So far, only one dendritic cell therapy, provenge (sipuleucel-T), has been approved by FDA for patients with metastatic castrate-resistant prostate cancer. Of note, a new strategy has been developed for enhancing the presentation of the tumor antigens in extracellular vesicles (EVs) released from tumors by DCs. Transduction of DCs with a lentivirus-coded chimeric receptor termed EV-internalizing receptor endows DCs with the capacity to specifically uptake cancer-derived EVs. This in turns enhances dendritic presentation of the EVs associated tumor antigens to CD8+ T cells, thereby enhancing the priming of CD8+ T cells to tumor-derived antigens in patients [42]. On the other hand, although the development of cancer immunotherapeutics targeting tumor-associated antigens and immune checkpoints is now flourishing, the list of targets of the commercially approved agents has been short (22 as of this writing) and all, but one, are protein molecules (Table 1). The approval of dinutuximab, a chimeric anti-GD2 antibody ch14.18, for the treatment of high-risk neuroblastoma in 2015 marks the first new agent targeting a glycolipid molecule, thereby widening the net of potential pharmaceutical targets.

Biological roles of GD2 in cancer

As discussed previously, GD2 is not expressed in normal tissues except weak expression in brain, peripheral sensory fibers, and skin melanocytes [43,44]. In contrast, various cancers of neuroectodermal origin, such as neuroblastoma (> 98%) [45], glioma, melanoma [46], and small cell lung cancer, highly express GD2, with as much as ~ 10^7 GD2 molecules on a primary neuroblastoma cell [47]. GD2 is also expressed in a variety of sarcomas [48]. Moreover, cancer cells expressing GD2 within adenocarcinoma and malignant phyllodes tumor of breast exhibited CSCs-like characteristics [40,49,50]. Hence, GD2 is an excellent target for cancer immunotherapy.

GD2 has also been considered as a marker for embryonic neural stem cells [51], mesenchymal stem cells (MSCs) [52,53], and neural progenitor cells [54]. For example, a subpopulation of GD2-expressing mouse bone marrow-derived mesenchymal stem cells (BM-MSCs) exhibited greater proliferative and clonogenic capabilities as well as better differentiation potential to adipocytes and osteoblasts, as compared to unsorted BM-MSCs [55]. We showed that GD2+ALDH cells isolated from malignant phyllodes tumor of the breast were able to not only differentiate into neural cells of various lineages but also display greater mammosphere forming ability than ALDH GD2− cells. Importantly, xenograft studies showed a higher tumor-initiating frequency of GD2+ALDH cells compared to ALDH GD2− cells [50]. Similarly, greater mammosphere forming capacity was observed in GD2-expressing H-Ras oncogene-transformed human mammary epithelial (HMLER) cells than GD2− HMLER cells. Furthermore, GD2+ HMLER cells were very similar in the gene signature to CD44+CD24− breast CSCs [49]. On the other hand, there was no significant difference in neurosphere formation capacity between GD2high and GD2low glioblastoma multiforme cancer cells [56]. In general, GD2 may serve as a stem cell maker for BM-MSCs and breast cancer, but not glioblastoma.

Overexpression of GD2 in tumors may occur as a consequence of aberrant expression of GD2.
Table 1. mAbs for cancer immunotherapy approved by FDA.

| Name                  | Malignancy                  | Antigen target(s) | Approval  |
|-----------------------|----------------------------|-------------------|-----------|
| Rituximab             | B-cell lymphoma/ B-CLL      | CD20              | 1997,     |
| Ofatumumab            |                            |                   | 2009,     |
| Obinutuzumab          |                            |                   | 2013,     |
| Ibritumomab          |                            |                   | 2002,     |
| Trastuzumab           | Breast, lymphoma           | Her-2/neu         | 1998,     |
| Pertuzumab           |                            |                   | 2012,     |
| Pertuzumab           |                            |                   | 2012,     |
| Brentuximab           | B-CLL                      | CD52              | 2001,     |
| Alemtuzumab          | B-CLL                      |                   | 2001,     |
| Tuxetan               |                            |                   | 2002,     |
| Sacituzumab          | Colorectal                 | VEGF              | 2004,     |
| Enzastuzumab         | Neutrophilic leukemia      |                  | 2004,     |
| Inotuzumab           | Hodgkin’s lymphoma, ALCL   | CD30              | 2011,     |
| Ipilimumab           | Melanoma                   | CTLA4             | 2011,     |
| Blinatumomab         | Acute lymphoblastic leukemia | CD19, CD3         | 2014,     |
| Ramucirumab          | Gastric cancer             | VEGF2             | 2014,     |
| Pembrolizumab        | Melanoma, squamous cell carcinoma | PD-1        | 2014, 2017, |
| Nivolumab            | PDGF Receptor              | PD-L1             | 2016, 2017, |
| Cemiplimab           | Soft tissue sarcoma       |                  | 2016, 2017, |
| Atezolizumab         | Bladder cancer             |                  | 2016, 2017, |
| Avelumab             | Meckel carcinoma           |                  | 2016, 2017, |
| Durvalumab           |                            |                  | 2017,     |
| Inotuzumab           | Acute lymphoblastic leukemia | CD22+ calicheamin | 2017, 2018 |
| Ozogamicin,          | Lymphoblastic leukemia, hairy cell leukemia | CD33+ calicheamin | 2017, 2018 |
| Moxetumomab         |                            |                  | 2017,     |
| Pasudotox            |                            |                  | 2017,     |
| Gemtuzumab           | Acute myeloid leukemia     |                  | 2017,     |
| Ozogamicin           |                            |                  | 2017,     |
| Mogamulizumab       | Mycosis fungoides, Sezary syndrome | CCR4        | 2018,     |
| Polatuzumab         | DLBCL                      | CD79b MAAE        | 2019,     |
| Enfortumab          | Urothelial cancer          | Nectin-4 MAAE     | 2019,     |
| Sactizumab          | Triple negative breast cancer | Trop2- SN38    | 2020,     |
| Govitecan-hzyi      | Multiple myeloma           |                  | 2020,     |
| Belantamab           |                            |                  | 2020,     |
| Mafodotin-blmf       |                            |                  | 2020,     |

*22 antigens are successfully targeted so far, all except GD2 are protein antigens.

GD2 can be shed from cancer cells to the circulation of patients or tumor microenvironment [62,63]. Insertion of the shed GD2 from renal cell carcinoma to T-cell surface-induced apoptosis of the T cells [64]. Only a few studies used purified GD2 ceramide to investigate the biological functions of GD2. Purified GD2 ceramide inhibited T-cell proliferation [13] and inhibited differentiation of human CD34+ cells into mature DCs [14]. On the other hand, GD2 promoted adhesion of platelet to collagen-coated wells via increasing phosphorylation of focal adhesion kinase [65], implying possible involvement of GD2 in tumor metastasis (Fig. 1A).

Many studies used mAbs against GD2 to examine the role of GD2 in cancer cells. GD2-specific mAb 14G2a induced apoptotic cell death of GD2-expressing mouse lymphoma EL4 cells [66] and human neuroblastoma IMR-32 cells [67]. The latter was associated with decreased levels of p-aurokinases and MYCN and increased level of P53 and PHLDA1 proteins [68]. Furthermore, proteome array analysis and immunoblotting showed that the mAb 14G2a decreased the levels of p-AKT (S473 and T308), p-mTOR (S2448), p-p70 S6 kinase (T389 and S371), p-4E-BP1 (T37 and T46), and p-AMPK (S108) in IMR-32 cells (Fig. 1B) [69]. Microarray study revealed increased expression of SVIL (supervillain), RASSF6 (Ras association RafGDS/AF-6 domain family member 6), and JUN, and decreased expression of ID1 (inhibitor of DNA-binding 1, dominant negative helix-loop-helix protein) and TLX2 (thyroid adenoma associated) in the mAb 14G2a-treated IMR-32 cells (Fig. 1B) [70]. mAb 14G2a also inhibited invasion of three human osteosarcoma cell lines, Saos-2, MG-63, and SJS-1, through reducing the levels of metalloproteinase-2 mRNA and protein, phosphatidylinositol 3 kinase, and p-Akt [71]. Another anti-GD2 mAb 3F8 biosynthetic enzymes. For example, ST8SIA1 regulated GD2 expression and breast CSC function [40,57]. Overexpression of ST8SIA1 in MDA-MB-231 breast cancer cells increased GD2 at the cell surface and enhanced proliferation of the cells. Upon ST8SIA1 overexpression, phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase pathways and c-Met signaling were activated [58]. As to the transcriptional regulation of ST8SIA1, promoter analysis revealed the presence of a NK-kB-binding site located at the 5'-flanking region of the ST8SIA1 gene [59], and tumor necrosis factor- could induce ST8SIA1 gene expression via upregulation of NF-kB transcription factor [60,61]. Thus, the ST8SIA1 can regulate GD2 expression and might serve as a drug target for cancer therapy.

**References**

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FEBS Letters 594 (2020) 3602–3618 © 2020 The Authors. FEBS Letters published by John Wiley & Sons Ltd on behalf of Federation of European Biochemical Societies
was shown to activate caspase pathways, inhibit cytochrome c, and increase caspase 9 independent apoptotic-inducing factor (AIF) from mitochondria to induce apoptosis of human melanoma HTB63 cells [72]. Moreover, mAb 3F8 increased Src-family kinase activity, NMDA receptor NR2B subunit phosphorylation, and Ca++ fluxes in human neuroblastoma SH-SY5Y-TrkB cells (Fig. 1B) [73]. These studies showed that binding of mAb to GD2 on the tumor cell surface could directly interfere with various signal pathways and reduce the cell viability. However, the molecular mechanisms underlying the anti-GD2 antibody-induced signaling pathways remain to be elucidated.

Upon stimulation with anti-CD3/anti-CD28 mAbs, human CD4 T cells upregulated ST8SIA1, with a consequent expression of GD2 on cell surface. Notably, confocal microscopy revealed colocalization of GD2 with T-cell receptors in the activated CD4 T cells [74], suggesting possible involvement of GD2 in TCR-mediated activation. Silencing of the GM2/GD2 synthase resulted in inhibition of anti-CD3/anti-CD28 mAbs induced proliferation of human CD4 T cells [74]. However, in GM2/GD2 synthase null mice which did not express GD2, the proliferation of mouse CD4 T cells in response to anti-CD3/anti-CD28 mAbs was normal [75]. This discrepancy may stem from differences in the human and mouse CD4 T cells.

Cell adhesion to extracellular matrix has critical effects on cell survival [76]. Immunofluorescence staining with an IgM anti-GD2 mAb 126 revealed the presence of GD2 in the focal adhesion plaques after detachment of Melur melanoma cells from glass coverslips by ethylenediaminetetraacetic acid [77]. Furthermore, binding of GD2 to extracellular matrices, including collagen type I and IV and fibronectin, enhanced the adhesion of SK-MEL-28 cells transfected
from apoptosis, making GD2 an excellent target for dermal origin. Thus, targeting GD2 may not only kills find overexpression of GD2 in tumors of neuroecto-
and differentiated neural cells, it is not a surprise to the known expression of GD2 in neuronal stem cells GD2 in promoting cancer cell survival, coupled with some cancers. In view of these desirable features of cells. In addition, it is a marker to enrich stem cells in When shed by tumor cells, it induces apoptosis of T signaling pathways upon anti-GD2 antibody binding. When shed by tumor cells, it induces apoptosis of T cells. In addition, it is a marker to enrich stem cells in some cancers. In view of these desirable features of GD2 in promoting cancer cell survival, coupled with the known expression of GD2 in neuronal stem cells and differentiated neural cells, it is not a surprise to find overexpression of GD2 in tumors of neuroectodermal origin. Thus, targeting GD2 may not only kills cancer cells, including CSCs, but also prevent T cells from apoptosis, making GD2 an excellent target for cancer immunotherapy.

**Biological roles of Globo H in cancer**

Globo H ceramide, which consists of Globo H (Fucz1-2Galβ1-3GalNAcβ1-3Galβ1-4Galβ1-4Glcβ1) linked to ceramide, was initially identified in human breast cancer cell line MCF-7 [19]. It was the most prevalent cancer-associated GSL [79,80] and over-expressed in many epithelial cancers such as breast, prostate, lung, colon, liver, ovary, and uterus cancers. Currently, it is being pursued as the next nonprotein target for anticancer therapy [81]. It was also shown to be expressed in breast CSCs and Globo H high cells are more tumorigenic than Globo Hlow cells [7,17]. However, the biological functions of GHCer remain under-explored.

Since CSCs have been considered to be a promising therapeutic target for cancer therapy, the identification of the CSC-specific surface biomarkers will be important for cancer therapy. We have shown the expression of Globo H and its precursor SSEA3, in 61% and 77%, respectively, of breast cancer tissues, and 20% and 62.5%, respectively, of breast CSCs [7]. In addition, both α1, 2-fucosyltransferase 1 (FUT1) and FUT2 are involved in the biosynthesis of Globo H in breast cancer cell lines. The gene expression of FUT1/ FUT2 correlated with the expression of Globo H in these CSCs. Furthermore, with the sorting of breast cancer cells using two cell lines and one patient-derived xenograft, the breast cancer cells expressing high levels of Globo H displayed more significant tumor growth with higher blood vessel densities when compared with cells expressing low Globo H [17]. Besides, the expression of Globo H in normal tissues is restricted to the luminal surface of glandular tissues; thus, it is usually not accessible to the immune system, which makes GHCer an ideal target for anticancer therapy. These findings provide the impetus for the development of Globo H-based immunotherapeutics and FUT1/FUT2-targeted therapy for breast cancer [7].

Expression of FUT1 and FUT2 is regulated by pro-inflammatory cytokines. IL-1β significantly increased the mRNA levels of FUT1 and FUT2, and IL-6 enhanced the level of FUT1 mRNA but not FUT2 mRNA [82]. These cytokines induced the NF-κB signaling pathway to modulate the mRNA expression levels of FUT1 and FUT2 [82]. In addition, ETS like-1 protein (Elk-1) has been shown to transcriptionally regulate the FUT1 gene expression [83]. B3GALT5 is essential for the synthesis of stages specific embryonic antigen-3 (SSEA3), the pentasaccharide precursor of Globo H. This gene has three native promoters and an integrated retroviral long terminal repeat (LTR) promoter. A recent study showed that B3GALT5-LTR is regulated by lamin A-NFYA and SIRT1-STAT3 signaling [84]. Further studies are needed to explore the involvement of transcription factors in the regulation of Globo H antigen expression during tumorigenesis.

Interestingly, it was observed that Globo H was detected not only on tumor cells of the clinical specimens but also on some of the tumor-infiltrating lymphocytes in the tumor microenvironments [8,11,17]. On the other hand, when peripheral blood mononuclear cells (PBMC) from healthy donor were co-incubated with Globo H breast cancer cells, GHCer shed from tumor cells were taken up by T and B lymphocytes [18], with subsequent inhibition of their proliferation and cytokine/immunoglobin production. These results suggest that Globo H could be transferred from cancer cells to be incorporated into nontumor cells in tumor microenvironments.

In addition, it was found that the addition of synthetic GHCer to PBMC suppressed their proliferative response toward anti-CD3/CD28 or LPS stimulation [18]. We also showed that incorporation of GHCer to T and B cells led to the inhibition of interleukin (IL)-2, interferon-γ, and IL-4 secretion by T cells and IgM/IgG production by plasma cells [18]. We also showed evidences that such GHCer-induced immunosuppression could be attributed to lymphocyte exhaustion and senescence via (a) the downregulated expression of Notch, which is crucial for T cell activation, and (b) the upregulated expression of its regulatory molecules, inhibitor of DNA-binding protein 3, early growth response 2/3, itchy E3 ubiquitin protein (itch) [18]. These data thus indicated for the first time that GHCer could act as an immune checkpoint molecule, facilitating the escape of cancer cells from immune surveillance [18].

with ST8SIA1 cDNA and β4GalNAcT1 cDNA [78]. Thus, GD2 might be important for cell survival by increasing cell adhesion to the extracellular matrix.

To sum up, GD2 is involved in cell adhesion to maintain cancer cell survival and can trigger cell signaling pathways upon anti-GD2 antibody binding. When shed by tumor cells, it induces apoptosis of T cells. In addition, it is a marker to enrich stem cells in some cancers. In view of these desirable features of GD2 in promoting cancer cell survival, coupled with the known expression of GD2 in neuronal stem cells and differentiated neural cells, it is not a surprise to find overexpression of GD2 in tumors of neuroectodermal origin. Thus, targeting GD2 may not only kills cancer cells, including CSCs, but also prevent T cells from apoptosis, making GD2 an excellent target for cancer immunotherapy.
In addition to immunosuppressive effects of GHcer, we also provided evidence for the proangiogenic activities of GHcer based on in vitro and in vivo experiments [8,11,17]. It was found that the addition of synthetic GHcer induced migration, tube formation, and intracellular Ca2+ mobilization [11,17]. The injection of GHcer-containing matrigel plug in vivo subcutaneously into mice promoted greater formation of new blood vessels than those with ceramide or PBS [17]. Furthermore, with the sorting of breast cancer cells using cell lines and patient-derived xenografts, the breast cancer cells expressing high levels of GHcer displayed greater tumor growth with higher blood vessel densities when compared with cells expressing low Globo H [17]. This observation was recapitulated by the finding that clinically, GHcer-expressing breast cancer specimens displayed higher vessel density than GHcer-negative breast cancer specimens.

Mechanistic investigations linked the angiogenic effects of Globo H-ceramide to its binding to translin-associated factor X (TRAX) in endothelial cells, which was identified as Globo H interactive protein [17]. In addition, the angiogenic effects of GHcer on human umbilical vein endothelial cells (HUVECs) were compared with Gb5 Ceramide (Gb5Cer), a precursor of GHcer which was also reported to be overexpressed in cancers, including breast CSCs [7,85,86]. Interestingly, only GHcer induced calcium influx, tube formation, and mRNA expression of vascular endothelial growth factor A, kinase insert domain receptor (also known as VEGFR2), fibroblast growth factor factor 2, and fibroblast growth factor factor 13; in contrast, Gb5Cer had no effects on HUVECs.

As illustrated in Fig. 2, GHcer was incorporated into endothelial cells (HUVEC) through the uptake of microvesicles shed from Globo H expressing tumor cells [11,17]. Previously, it was reported that TRAX could capture phospholipase Cβ1 (PLCβ1) in the cytosol of endothelial cells, preventing activation of this phospholipase by Gq protein alpha subunit (Gαq) protein [87]. GHcer showed strong binding affinity with TRAX by immunoprecipitation, immunofluorescence staining, and fluorescence resonance energy transfer in HUVECs, which interrupted the TRAX-PLCβ1 interaction with consequent release and activation of PLCβ1 [17]. Indeed, the level of PLCβ1 in the immunoprecipitate of TRAX was disrupted by GHcer with ensuing angiogenesis [17]. As shown in Fig. 2, the GHcer fitted into a binding site on TRAX, resulting in the release of PLCβ1 and its subsequent activation by Gαq protein. The activated PLCβ1 then promoted the hydrolysis of phosphatidylinositol 4,5-bisphosphate and formation of inositol 1,4,5-trisphosphate and diacylglycerol, thereby eventually inducing Ca2+ mobilization and angiogenesis in the tumor microenvironment [88]. In other words, the competition between PLCβ1 and GHcer for binding to TRAX forms the basis for a proposed link to the proangiogenic mechanism of GHcer. On the other hand, Gb5Cer did not show binding in TRAX by Biacore analysis and did not compete with PLCβ1 binding to cause Ca2+ mobilization and angiogenesis (Fig. 2).

In short, GHcer shed by cancer cells can modulate tumor microenvironment to the benefit of cancer cells by promoting angiogenesis and suppressing immune effector functions. Whether GHcer exerts any survival advantages to the tumor cells per se awaits further investigation. Since epithelial cancers derive mostly from endodermal lineage, and prominent expression of Gb4 ceramide is noted during differentiation of human ESCs into endodermal lineage, it seems rational for epithelial cancers to upregulate the expression of downstream globo-series GSLs so as to exploit the survival advantage of GHcer. Thus, it is imperative to develop Globo H-targeted therapeutics, which should have wide application for epithelial cancers, the most prevalent types of human cancers.

GD2-targeted immunotherapies

Both active and passive immunotherapeutic strategies have been pursued to target GD2 for cancer treatment. These include mAbs, bispecific antibodies, immunocytokine, and CAR T cell, as well as vaccines consisting of synthetic GD2, peptide mimotopes, and anti-idiotypic antibody. GD2 is a poor antigen and is hard to stimulate a GD2-specific immune response in vivo. To improve its immunogenicity, the keyhole limpet hemocyanin (KLH) and QS-21 as carrier protein and adjuvant, respectively, are used to generate a GD2-linked KLH+QS-21 vaccine. Currently, a bivalent vaccine, which contains GD2- and GD3-linked KLH and QS-21, has been evaluated in a phase II trial (NCT00911560). This review will focus on the most successfully implemented strategies, namely, anti-GD2 mAbs.

Potential clinical value of murine anti-GD2 monoclonal antibodies 14G2a and 3F8

Several anti-GD2 antibodies have been generated. Among these antibodies, two murine mAbs, 14G2a and 3F8, have been extensive examined in vitro and evaluated in clinical trials. mAb 14G2a was developed by isotype switching of an IgG3 mAb14.18, which was
generated by immunizing mice with human neuroblastoma cell LAN-1 [89,90]. Besides the aforementioned effects of 14G2a on cancer cells in vitro, mAb 14G2a was effective in suppression growth of human neuroblastoma tumor in athymic mice [90]. In phase I clinical trials of mAb 14G2a in neuroblastoma and osteosarcoma, dose- and infusion rate-dependent toxicities were observed [91–93]. In these early-phase trials, clinical benefits were observed in some patients despite their advanced disease status.

mAb 3F8 is a murine IgG3 mAb directed against GD2, which was generated by immunized mice with human neuroblastoma cells [94]. It has been assessed in sequential nonrandomized clinical trials, which demonstrated its antitumor effect in patients with relapsed neuroblastoma and melanoma, and showed similar side effects as the mAb 14G2a [95] for a few patients with stage IV neuroblastoma in phase II trials of mAb 3F8 was reported to have long-term survival [96,97]. In addition, tumor imaging and tumor shrinkage were observed in clinical trials of 131I-labeled mAb 3F8 [98–100].

**Chimeric and humanized anti-GD2 monoclonal antibodies**

Murine antibodies can be recognized by the human immune system and induce human anti-mouse antibodies (HAMA), which may compromise anticancer efficacy. To reduce HAMA responses, chimeric and humanized mAbs have been developed.

A chimeric anti-GD2 mAb ch14.18 was generated by combining the variable region of the heavy chain (VH) and light chain (VL) of murine mAb14.18 with the constant regions of human IgG1 and k light chain, using recombinant DNA technology to transfect the hybridoma Sp2/0 Ag14 [101]. mAb ch14.18 was not only active in complement-dependent cytotoxicity (CDC) against neuroblastoma cells [102] but also mediated ADCC through neutrophils, natural killer (NK), and lymphokine-activated killer cells [103]. Importantly, mAb ch14.18 displayed higher ADCC activities than mAb 14G2a [104]. Investigational new drug (IND) application for ch14.18 was filed in 1989, marking the first IND application for chimeric mAb generated by recombinant DNA technology. As expected, phase I trials showed that mAb ch14.18 had longer half-life (66.6 ± 27.4 h) than mAb 14G2a (18.3 ± 11.8 h) [105,106] but had a similar toxicity profile [105,107]. Of note, some patients with refractory/relapsed neuroblastoma achieved complete and partial response in the phase I trials [105,107].

Based on preclinical data showing improved antitumor activity of mAb ch14.18 when combined with granulocyte–macrophage colony-stimulating factor (GM-CSF) or IL-2, phase I clinical trials of combining ch14.18 with GM-CSF [108] and IL-2 [109] were conducted to determine the optimal dosage of ch14.18. These studies paved the way to a randomized phase III clinical trial to evaluate the anticancer efficacy of ch14.18 + GM-CSF + IL-2 in patients with high-risk neuroblastoma. Among 226 randomized patients, significantly improved EFS and OS were observed in patients treated with ch14.18 + GM-CSF + IL-2 when compared with those treated with standard therapy [12]. This encouraging result formed the basis for the
subsequent FDA approval of ch14.18 (dinutuximab) in 2015, after the successful completion of an FDA-required clinical trial (COG ANBL0931) of the safety and toxicity of the immunotherapy in patients with high-risk neuroblastoma in 2014 [110]. Dinutuximab became the first approved immunotherapeutics targeting a glycan antigen, and this immunotherapy has since become the new standard of care for high-risk neuroblastoma. Subsequently, dinutuximab beta, which is a clone of mAb ch14.18 generated in Chinese hamster ovary cells instead of murine hybridoma, was approved in Europe in 2017, based on similar survival outcome in patients with high-risk neuroblastoma. Interestingly, no survival benefit of adding IL2 to dinutuximab beta was observed, suggesting that the IL-2 might not be necessary for the anticancer effect of dinutuximab [111]. Furthermore, although ADCC is considered to be the primary mechanism of action in dinutuximab-mediated anticancer effects [104], new evidence suggested that NK/plasmacytoid dendritic cell axis [112] and CD105+ cells [113], such as mesenchymal stromal cells and monocytes, might also be involved.

Strategies to improve the efficacy of dinutuximab by combination with chemotherapy are ongoing. An open-label, randomized, phase 2 selection design trial tested the addition of temsirolimus or dinutuximab to irinotecan–temozolomide in patients with relapsed/refractory neuroblastoma and found that 9/17 patients in the irinotecan–temozolomide-dinutuximab treatment group had objective responses, as compared to 1/18 in the irinotecan–temozolomide–temozolomide group [114]. This finding paves the way to an ongoing pilot study of combining chemotherapy and immunotherapy in frontline induction therapy.

Humanized anti-GD2 mAb hu14.18 was generated by complementarity determining regions (CDR) grafting of 14.18 V regions into human frameworks. The engineered sequences were further modified with a K322A mutation of the hu14.18 Fc region to limit the ability of complement fixation [115] so as to reduce neuropathic pain associated with anti-GD2 due to complement fixation. Indeed, mAb hu14.18K322A induced less allodynia than ch14.18 in a rat model while its ADCC activity was maintained [115]. This allowed higher doses of Hu14.18 to be administered clinically [116]. In a phase II clinical trial, combination of hu14.18K322A with frontline induction chemotherapy led to tumor shrinkage and an encouraging 2-year event-free survival (85.7% with 95% CI: 70.9–93.3) [117].

To improve antitumor effects, hu14.18 was linked to IL-2. Clinical studies demonstrated that hu14.18-IL2 had some antitumor activities, but the maximal tolerable dosage was lower than ch14.18 due to IL2-associated toxicities [118].

Humanized 3F8 (hu3F8) generated by CDR grafting of 3F8 had better ADCC and worse CDC activity than mAb 3F8 [119]. A phase I study of hu3F8 + GM-CSF in patients with refractory or recurrent neuroblastoma showed durable clinical responses in some patients [120]. To further improve the binding affinity, random mutagenesis of the hu3F8-scFv was performed, and hu3F8 mutants with high GD2-binding affinity were selected from the yeast libraries. Several potential mutants were analyzed using molecular modeling and energy calculations. A D32H mutant was expected to strengthen its interaction with the sialic acid of GD2. Indeed, hu3F8.D32H showed greater binding, ADCC, and CDC activities, as well as antitumor efficacy in the IMR-32-bearing xenograft mouse model when compared with the mAb hu3F8 [121].

**Globo H-targeted immunotherapies**

GHCer was expressed abundantly in the undifferentiated human ESCs, which disappeared after differentiation to form various tissues at the stage of gastrulation. In the normal adult tissues, Globo H epitope was not detected except for a few secretory borders of epithelium, where access to the immune molecules is restricted [7]. Such unique GHCer expression is reminiscent of the expression pattern of oncofetal proteins of tumors, making GHCer a potential target for anticancer immunotherapy. It was reported that immunization of mice with Globo H conjugated to KLH mixed with α-GalCer induced production of antibodies reactive with Globo H and SSEA3, suggesting that a Globo H-based vaccine would target tumor cells expressing Globo H or SSEA3, including breast CSCs [7]. Subsequently, we demonstrated that GHCer played two important regulatory roles in tumor microenvironments: as an immune checkpoint molecule [18] and an proangiogenic factor [17]. These findings provide further impetus for clinical development of Globo H-targeted immunotherapy [122].

Two phase I clinical trials of Globo H-KLH/QS-21 vaccine had been carried out in patients with relapsed prostate cancer (n = 18) [123] and metastatic breast cancer (n = 27), respectively [124], which demonstrated the safety of the vaccine, along with induction of humoral antibody responses. Recently, a multinational randomized phase II clinical trial of Globo H-KLH vaccine was conducted in 348 patients with metastatic breast cancer [16]. Although there was no difference in PFS between patients treated with Globo H vaccine
and those treated with placebo, in the vaccinated group those patients who mounted anti-Globo H responses had significantly better PFS than the placebo group [16]. Based on these promising results, a global phase III trial in triple negative breast cancer is ongoing [125–127].

In addition, a new generation of Globo H vaccine was developed, in order to optimize the vaccine and obviate the need for extracting natural products to produce the carrier protein KLH and the adjuvant OPT-821, used in the current Globo H-KLH + QS-21 vaccine. In a preclinical study, three carrier proteins, including bovine serum albumin, tetanus toxoid, and genetically modified cross reacting material of diphtheria toxoid (CRM197 DT), together with various adjuvants (α-GalCer and its analogs 7DW8-5, C34 and C17, alum, and MF59), were evaluated. Immunization with the Globo H-CRM197 DT + C34 vaccine induced greatest level of anti-Globo H IgG and IgM, equivalent to Globo H-KLH + QS-21 vaccine [128]. Thus, Globo H-CRM197 DT+C34 vaccine is now under early-phase clinical development. Furthermore, a new anti-Globo H antibody is now undergoing phase II clinical trial (ClinicalTrials.gov Identifier: NCT03573544), and an antibody drug conjugate is in preclinical development.

Conclusions and perspectives

The anti-GD2 mAb dinutuximab is the first immunotherapeutic agent targeting a glycan antigen that has received approval by FDA for cancer immunotherapy. New strategies for improving the efficacy of anti-GD2 immunotherapy, such as combining anti-GD2 with chemotherapy or immune checkpoint blockade for patients with high-risk neuroblastoma, are under development. Globo H is another cancer-associated glycan which is being pursued as a promising target for immunotherapy. Studies from our group revealing the roles of GHCer as an immune checkpoint and angiogenic factor in the tumor microenvironment have provided sound scientific rationales for targeting Globo H. The demonstration of detailed molecular interactions between GHCer and the intracellular protein TRAX not only offers an insight into the molecular mechanisms underlying the functions of GHCer, but also facilitates rational design of new glycan-targeted anticancer therapeutics using small molecular compounds to disrupt glycan–protein interactions. Besides, development of therapies against GD2/Globo H might offer an opportunity to eradicate cancer stem cells, which are resistant to radiation and chemotherapy. Further investigation of the impacts of GD2 and Globo H on tumor cells per se and the molecular mechanisms involved may help to elucidate the role of these molecules during tumorigenesis and provide insights for developing new strategies for cancer treatment.

Acknowledgements

This work was supported by Ministry of Science and Technology of Taiwan (MOST 109-2321-B-182A-005 to JY), Ministry of Health and Welfare of Taiwan (MOHW109-TDU-B-121-134010 to ALY), and Chang Gung Memorial Hospital at Linkou of Taiwan (OMRPG3C0047 and CMRPG 3F0973 to JY, OMRPG3C0018 to ALY, and CMRPG3G1531-CMRPG3G1533 to JTH). We thank H.W. Wu for her assistance with graphic illustration.

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