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Lack of iGb3 and Isoglobo-Series Glycosphingolipids in Pig Organs Used for Xenotransplantation: Implications for Natural Killer T-Cell Biology

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Abstract

α-1,3-Terminated galactose residues on glycoproteins and glycosphingolipids are recognized by natural anti-α-1,3-galactose antibodies in human serum and cause hyperacute rejection in pig-to-human xenotransplantation. Genetic depletion of α-1,3-galactosyltransferase-1 in pigs abolishes the hyperacute rejection reaction. However, the isoglobotriosylceramide (iGb3) synthase in pigs may produce additional α-1,3-terminated galactose residues on glycosphingolipids. In both α-1,3-galactosyltranserase-1 knockout mice and pigs, cytotoxic anti-α-1,3-galactose antibodies could be induced; thus, a paradox exists that anti-α-1,3-galactose antibodies are present in animals with functional iGb3 synthases. Furthermore, iGb3 has been found to be an endogenous antigen for natural killer T (NKT) cells, an innate type of lymphocyte that may initiate the adaptive immune responses. It has been reasoned that iGb3 may trigger the activation of NKT cells and cause the rejection of α-1,3-galactosyltransferase-1-deficient organs through the potent stimulatory effects of NKT cells on adaptive immune cells (see ref.[20]). In this study, we examined the expression of iGb3 and the isoglobo-series glycosphingolipids in pig organs, including the heart, liver, pancreas, and kidney, by ion-trap mass spectrometry, which has a sensitivity of measuring 1% iGb3 among Gb3 isomers, when 5 μg/mL of the total iGb3/Gb3 mixture is present (see ref.[35]). We did not detect iGb3 or other isoglobo-series glycosphingolipids in any of these organs, although they were readily detected in mouse and human thymus and dendritic cells. The lack of iGb3 and isoglobo-

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[1]We previously developed a method to detect iGb3 among iGb3/Gb3 mixtures, which has a sensitivity of measuring 1% iGb3 among Gb3 isomers, when 5 μg/mL of total iGb3/Gb3 mixture is present.[35] We typically analyze the permethylated GSL samples at a concentration of at least 250 μg/mL; thus, the concentration of Hex-Hex-Hex trisaccharideceramide GSLs per analysis is above 5 μg/mL.

[2]By both MS and anti-Galα3Gal antibody staining, Diswall et al. found that lacto-series GSLs, but not isoglobo-series GSLs, carry the Gal α3Gal epitope in pig organs. Galα3Gal epitope-bearing GSLs such as Galα3nLc4 and Galα3nLc6 are present in wild-type pigs but completely absent in α3GalT1 knockout pigs.[36-39]

AUTHOR CONTRIBUTION

FT, YL, DH, ICA, SBL and DZ conceived and designed the experiments. FT, YL, LG, and DZ performed the experiments. FT, YL, DH, ICA, SBL, and DZ analyzed the data. DH, IAC, SBL and DZ contributed reagents/materials/analysis tools. FT, YL and DZ wrote the paper.

FINANCIAL DISCLOSURE

DZ is an inventor involved in patents related to technologies mentioned in this article, issued or in application.

series glycosphingolipids in pig organs indicates that iGb3 is unlikely to be a relevant immune epitope in xenotransplantation.

Keywords

Natural killer T cells; Isoglobotriaosylceramide; CD1d; α1,3-Galactosyltransferase-1; iGb3 synthase

INTRODUCTION

The major barriers to solid organ xenotransplantation occur at the interface between the human blood supply and the pig organ, where human blood flows through pig blood vessels. Galα3Gal epitopes are the major xenoantigens causing hyperacute rejection (HAR) in pig-to-human xenotransplantation;[1] these epitopes are also implicated in the acute vascular rejection of xenografts.[2] Anti-Galα3Gal antibodies play a central role in rejection by activating endothelial cells.[3,4] There is also evidence that anti-Galα3Gal immunoglobulin G (IgG) is required for human natural killer (NK) cell migration across pig aortic endothelial cells in vitro, a process that is independent of antibody-dependent cellular cytotoxicity,[5] and the role of Galα3Gal has been reported to be a ligand directly recognized by NK cells.[6]

The enzyme α1,3-galactosyltransferase enzyme 1 (α1,3GalT1, EC 2.4.1.51) synthesizes Galα3Gal epitopes (Galα3Galβ4GlcNAc-R) on the cell surface in almost all mammals with exceptions of humans, apes, and Old World monkeys.[7,8] α1,3GalT1 is not the sole enzyme able to synthesize Galα3Gal. Isoglobotriaosylceramide (iGb3) synthase (iGb3S, EC 2.4.1.87),[9-11] another member of the α1,3-glycosyltransferase family, initiates the synthesis of isoglobo-series glycosphingolipids (GSLs). Starting from iGb3, a series of α3Gal-terminated GSLs, B4, B5, and B6, can be synthesized.[12] This raises the possibility that iGb3S may synthesize the Galα3Gal structure in α3GalT1$^{-/-}$ animals.

Recently, several independent research groups reported that iGb3 is recognized by invariant natural killer T (NKT) cells in mice and humans.[13-16] Invariant NKT cells are innate immune cells functionally similar to NK cells. The semi-invariant NKT-cell receptor recognizes glycolipid antigens presented by the monomorphic CD1d molecule. iGb3 is a first-identified glycolipid antigen with characteristics of the natural iNKT cell-selecting ligand in the thymus.[17] These investigations laid groundwork for later studies of the trimeric crystal structures for T-cell receptors recognizing CD1d-presented iGb3 published by other independent groups,[18,19] indicating a surprising conformation that T-cell receptors of NKT cells “flatten” the sugar part of iGb3 and bind to antigen and CD1d in an “induced-fit” conformation. The terminal α3-Gal binds to a “pocket” area of the CD1d molecule and allows an optimal TCR recognition. There is also in vivo evidence that its increased expression in dendritic cells is associated with NKT activation in peripheral tissues.[15] Subsequently, iGb3 was proposed by Christiansen et al. to be a stimulatory ligand in pig organs that causes rejection of α1,3-galactosyltransferase-1-deficient, xenotransplanted organs by causing NKT cells to exert potent stimulatory effects on adaptive immune cells in hosts receiving xenotransplants.[20]

Anticarbohydrate antibodies are evolutionarily conserved in humans and nonhuman primates as a consequence of their function in innate immune responses.(21) Humans have natural antibodies to the carbohydrate Galα3Gal, mainly immunoglobulin M (IgM) and to a lesser extent IgG.[22,23] These antibodies initiate hyperacute rejection of vascularized pig organs that have been grafted to nonhuman primates and humans.[3,4] Investigators have

produced α3GalT1 knockout pigs to eliminate Galα3Gal antigen. However, α3GalT1 knockout pig kidneys and hearts have been found to undergo acute humoral xenograft rejection (AHXR) and thrombosis at 3 to 6 months after transplantation in primates despite extensive immunosuppression. Thus, it has been proposed that additional epitopes may be responsible for binding to recipient antibodies.[24-27]

The induction of anti-α-3Gal antibodies in both mouse and pig α3GalT1 knockout (KO) animals without causing autoimmune attacks indicates that the presence of anti-αGal antibodies and functional iGb3 synthase is not mutually exclusive.[28-31] Cytotoxic anti-α3Gal antibodies (both IgM and IgG) can be induced in α3GalT1 KO mice by oral inoculation of *Escherichia coli* O86:B7, consistent with the hypothesis that enteric exposure to α3Gal-like bacteria induces anti-α3Gal antibodies in humans.(29) Repeated immunization of α3GalT1 KO mice may induce 0.6% of total IgGs that are specific for α3Gal, with affinity/avidity even higher than those in human serum.[30] Similar findings were reported in α3GalT1 KO pigs, that the presence of anti-αGal antibodies in these animals is similar to that in humans.[31] However, both mouse and pig express functional iGb3 synthase,[21,27] producing α3Gal epitopes in cell transfection experiments in vitro, which may react with anti-αGal antibodies. There exist several possible mechanisms for this paradox: (1) the anti-αGal antibodies induced by αGal epitopes in α3GalT1 KO animals and human do not bind to iGb3; (2) the iGb3 epitope in α3GalT1 KO animals is converted to nonreactive epitopes such as iGb4; and (3) the iGb3 synthases in α3GalT1 KO animals are not synthesizing iGb3 in multiple organs.

Advancement of mass spectrometry technologies allows not only molecular profiling of glycosphingolipids but also structural information for sugar sequence, linkage, and branching.[32] To determine more precisely the potential mechanisms involved in acute rejection of xenotransplanted pig organs, in this study, we used a sensitive and specific ion-trap mass spectrometry (MS) technology[33-35] in combination with immuno-staining to study whether isoglobotriosylceramide and isoglobo-series glycosphingolipids exist in pig organs that are used for xenotransplantation.

METHODS AND MATERIALS

Extraction of Glycosphingolipids

Human monocyte-derived dendritic cells were prepared as described,[11] using peripheral blood mononuclear cells from healthy donors (Gulf Coast Regional Blood Center, Houston, TX). Briefly, monocytes were attached to 75-mm Falcon cell culture flasks (BD Biosciences, San Jose, CA) for 2 h and cultured in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA) with 2% human serum (Valley Biomedical Products & Services, Winchester, VA) with 800 U/mL granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN) and 1000 U/mL interleukin-4 (IL-4) (R&D Systems, Minneapolis, MN) for 5 d. At day 6 of culture, 10 ng/mL IL-1β, 10 ng/mL tumor necrosis factor-α (TNF-α), 15 ng/mL IL-6 (all from R&D Systems), and 1 μg/mL prostaglandin E2 (EMD Chemicals, Gibbstown, NJ) were added to trigger the maturation of DCs. The DCs were harvested on day 8 of culture to be used for glycosphingolipid analysis. Kidneys, pancreas, hearts, and livers from wildtype pigs were purchased from Pel-Freez Biologicals (Rogers, AR). GSLs were extracted as described.[33-35] Lipids were extracted by extensive sonication four times with solvents of mixed polarity. The solvent used in the first and second rounds of sonication was chloroform-methanol 1:1 (v/v). The solvent used in the third and fourth sonication was isopropanol-hexane-water 55:25:20 (v/v/v). Sonication was followed by centrifugation to pellet the insoluble material. The supernatants were pooled and dried by centrifugal evaporation at −58°C and subjected to preliminary analysis by high-performance thin-layer chromatography (HPTLC).

Separation of Neutral and Acidic Lipids

Neutral and acidic lipids were fractionated by anion-exchange chromatography on a small column of DEAE Sephadex A-25 (Sigma, St. Louis, MO). The acidic lipid fraction was eluted with 0.8 M sodium acetate in methanol. Both neutral and acidic fractions were dried, desalted by dialysis, dried, and analyzed by HPTLC.

Florisil Fractionation of Neutral GSLs

The DEAE Sephadex A-25 pass-through fraction was dried under vacuum in the presence of P_2O_5 for 3 h and peracetylated with 1 mL of pyridine and 0.5 mL of acetic anhydride in the dark at rt overnight. The peracetylated material was dried, with the addition of 2 mL toluene to ensure complete evaporation. A Florisil (Sigma-Aldrich, St. Louis, MO) column (30–60 mesh, 10 × 80 mm) was equilibrated in 1,2-dichloroethane-hexane 4:1 (v/v). The peracetylated sample was applied in this solvent, and the column was then washed with 10 mL of the same solvent, followed by 10 mL 1,2-dichloroethane. Neutral peracetylated GSLs were eluted with 10 mL of 1,2-dichloroethane-acetone 1:1 (v/v). The fractions were dried and deacetylated with 1 mL of 0.5 M sodium methoxide in 2 mL of methanol for 3 h at rt. The mixture was neutralized with methanolic acetic acid, dried, and then desalted by dialysis. The GSL contents of all three fractions were compared by HPTLC. GSLs were quantified by heating in 0.2% Orcinol in 50% sulfuric acid solution, as described.[33]

Purification of Human Anti-Galα3Gal Antibody

Anti-Galα3Gal was isolated by affinity chromatography from the plasma of healthy individuals with blood type AB. Batches of 200 mL of AB plasma (Valley Biomedical Products & Services, Winchester, VA) were loaded onto a protein G-Sepharose column equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column-bound IgGs were eluted with 100 mM glycine-HCL, pH 2.8, and each fraction was neutralized with 1 M Tris buffer. The anti-Galα3Gal IgG was purified from the pooled IgGs using a Galα3Galβ4GlcNAc-OGr-ss column (V-Labs, Covington, LA). The pooled IgGs were applied onto a Galα3Galβ4GlcNAc-OGr-ss-equilibrated column with 20 mM Tris-HCl and 150 mM NaCl, pH 7.4. After extensive washing with 20 mM Tris-HCl and 150 mM NaCl pH7.4 the first time, with 20 mM Tris-HCl pH7.4 containing 0.5 M NaCl the second time, and with 20 mM Tris-HCl and 150 mM NaCl, pH 7.4, for the last time, the column-bound IgG was eluted with 100 mM glycine-HCl, pH 2.8, and each fraction was neutralized with 1 M Tris buffer.

HPTLC and Immunostaining of Glycolipid Fractions

Neutral GSL fractions were separated on aluminum-backed silica gel 60 HPTLC plates (Macherey-Nagel, Bethlehem, PA) using chloroform-methanol-water, 65:25:4. After drying, the gel was blocked in 5% (v/v) fish gelatin in phosphate-buffered saline overnight at rt. Anti-Gb3 antibody (Seikagaku Biobusiness Corporation, Tokyo, Japan) was used at 0.2 μg/mL final concentration and incubated with HPLTC-separated GLSs for 2 h at rt. The human anti-Galα3Gal antibody was used at a final concentration of 0.5 μg/mL and incubated with HPTLC-separated GSLs for 4 h at rt. After washing with phosphate-buffered saline, the gel was incubated with goat antimouse IgG or goat anti-human IgG secondary antibodies conjugated with horseradish peroxidase (Jackson ImmunoResearch Inc., PA). After 2 hours at room temperature, the peroxidase was visualized with 0.06% (wt/vol) 4-chloro-1-naphthol (Bio-Rad, Hercules, CA) diluted in 200 mM methanol containing 0.033 mM H_2O_2.

Per-N,O-Methylation of GSLs

GSLs (20 μg) were introduced into a conical-glass vial, and dimethyl sulfoxide (150 μL) was added without using special drying conditions or an inert gas atmosphere. Powdered

sodium hydroxide (40 to 60 mg) was then added to the sample solution and was stirred at rt until completely dissolved. Iodomethane (80 μL) was added with a syringe, and the mixture was shaken at rt for 1 h. The methylation reaction was quenched with water (2 mL). The permethylated products were extracted three times by adding dichloromethane (2 mL each round). The combined dichloromethane extracts were then washed three times with water (2 mL each wash). Following the final wash, the samples were transferred to a new tube and dried under nitrogen stream at 35°C to 40°C.

Electrospray Ionization-ion-Trap-mass Spectrometry (ES-LIT-MS^n) of Permethylated Neutral GSLs

Mass spectrometry was carried out on a linear ion-trap mass spectrometer (LTQ, ThermoFinnigan, San Jose, CA) using a nanoelectrospray source, 0.30 μL/min flow rate at 230°C capillary temperature in positive ion mode with 30% to 50% collision energy. Collision energies were set to leave a minimal residual abundance of precursor ion. All ions were detected as sodium adducts.

The iGb_3 in isobaric mixture of Gb_3 and iGb_3 standards was identified by comparing the different patterns of MS^4 product ions from the sodiated molecular ion via the glycan fragment *m/z* 667 and the terminal disaccharide 1-ene ion *m/z* 445 (i.e., X→667→445→) of pure permethylated iGb_3 standards via ESI-LIT-MS with those of permethylated Gb_3.[33-35]

Molecular Cloning and Characterization of Human iGb3 Synthase

The cDNA of human iGb3 synthase (Genbank accession No. NM 0010 80438) was cloned from thymi of pediatric patients by reverse transcription PCR. cDNA was prepared from thymi of pediatric patients (aged 2 months to 2 years old) by a first-chain synthesis kit from Invitrogen (Carlsbad, CA). A cDNA fragment containing exon 2 to the beginning of exon 5 was amplified by PCR primers F84 (5′ CCCTAAATTCAGGCATCTGG 3′) and R335 (5′ GGAAGCGCTCCAGGTACTTCTCCAGGTATC 3′). A cDNA fragment containing exon 5 was amplified by PCR primers F335 (5′ GGAAGCGCTCCAGGTACTTCTC 3′) and R1001 (5′ CTAGTTCCGCAGCAGCCGGTACC 3′). The above two cDNA fragments were digested by restriction enzyme Eco47III (underlined in primers R335 and F335) and ligated to one cDNA fragment containing exon 2 to the end of the gene. The exon 1 and the beginning of exon 2 were directly added by PCR reaction using a long primer F1 (ATGGCTCTCAAGGAGGGACTCAGGGCCTGGAAGAGAATCTTCTGGCGGCAGAT CCTACTTACACTTGGCCTCTTAGGCCTGTTTCTGTATGGCCTCCCTAAATTCAGG CATCTGGAAGCC) and the primer R1001 using the cDNA (exon 2 to the end of the gene) as the template. The full-length human iGb3 synthase gene was transfected into Chinese hamster ovary (CHO) cells by a pIRES2-EGFP plasmid (Clontech, Mountain View, CA). GFP-expressing cells were sorted and analyzed for expression of iGb3 and iGb4 according to the published linear ion-trap mass spectrometry method.[34,35] GFP-expressing CHO cells transfected by mock pIRES2-EGFP plasmid were used as negative control.

RESULTS

Since iGb3 was proposed as a xenoantigen presented by human dendritic cells to trigger the activation of NKT cells, we measured the endogenous concentration of iGb3 in human monocyte-derived dendritic cells.[11] iGb3 signature ions were present in all of the five human dendritic cell samples generated from monocytes obtained from five different healthy donors (Fig. 1A), representing 1.2% to 10.8% of the iGb3/Gb3 isomer mixtures in some iGb3-containing MS1 trihexosylceramide ions. Not surprisingly, we only found iGb3 in a few of the MS1 trihexosylceramide ions, so the overall ratio of total iGb3 versus Gb3 is

much lower. iGb4 was also found in human monocyte-derived dendritic cells (Fig. 1B), while other isoglobo-series GSLs, such as B4 (Galα3Galα3Galβ4Glc-Cer), B5 (Galα3Galα3Galα3Galβ4Glc-Cer), and B6 (Galα3Galα3Galα3Galα3Galβ4Glc-Cer), were not found (Table 1).

Next, we examined iGb3 and related GSLs in pig organs. Table 2 shows the pig GSLs identified by mass spectrometry analysis, as well as the relative abundance of molecular ions. Figure 2 shows the MS^1 profile of total neutral GSLs from pig tissues. Glc-Cer (Glcβ1Cer), Lac-Cer (Galβ4Glc-Cer), Gb3 (Galα4Galβ4Glc-Cer), Gb4 (GalNAcβ3Galα4Galβ4Glc-Cer), and nLc4(Gal β4GlcNAcβ3Galβ4Glc-Cer) were found to be the major GSLs. Molecular-ions representing Hex-Hex-Hex trisaccharide-ceramide GSLs are 42%, 29%, 49%, and 9% in pig heart, kidney, liver, and pancreas, respectively (Table 2). We studied the presence of Hex-Hex-Hex trisaccharide-ceramide GSLs, including both Gb3 and iGb3, using an ion-trap MS method.[1][33-35] Different organs from six individual pigs were studied, both separately and as pooled tissues. Despite extensive searching, we could not find a signal for characteristic MS^4 fragment ions for iGb3 (211 and 371). Figure 3 shows the negative finding for iGb3 in pig heart. Six molecular ions, 1215, 1243, 1271, 1299, 1313, and 1327, representing Hex-Hex-Hex-Cer with different lipid portions of the ceramide part, were subjected to MS^4 analysis, and only characteristic ions for Gb3 were detected.

Since iGb3 can be subsequently converted to iGb4, we studied whether iGb4 exists in pig organs by published methods[33,34] using MS^5 analysis of HexNAc-Hex-Hex-Hex-Cer representing Gb4 or iGb4 (Fig. 4). We could not find a signal for characteristic MS^5 fragment ions for iGb4 in any examined pig organs.

We further studied whether Galα1,3Gal-terminated isoglobo-series GSLs that stem from iGb3 are present in pig organs. For this, we searched for B4 (Galα3Galα3Galβ4Glc-Cer), B5 (Galα3Galα3Galα3Galβ4Glc-Cer), and B6 (Galα3Galα3Galα3Galα3Galβ4Glc-Cer) GSLs by the precursor ion mapping method, according to respective neutral loss of each GSL's sugar part (Table 2). None of the B4, B5, or B6 GSL structures could be found. In contrast, αGal-nLc4 (GalαGalβ4GlcNAcβ3Galβ4Glc-Cer), which is an Galα3Gal structure GSL formed by α1,3GalT1, could be easily detected by both MS1 analysis (Fig. 2) and precursor ion mapping (precursor ions were found as 1664, 1692, 1720, 1748, 1776, and 1805, representing Hex-Hex-HexNAc-Hex-Hex-Cer, with different lipid portions of the ceramide part).

To determine whether iGb3 exists in pig organs and binds to human natural anti-Galα3Gal antibodies as suggested by Christiansen et al.,[20] we studied the binding of pig GSLs to anti-Galα3Gal immunoglobulin purified from pooled human serum. Since iGb3 synthase only initiates synthesis of αGal epitopes on isoglobo-series GSLs,[9,10] we focused our study on neutral GSLs. Immunostaining of separated neutral glycolipids with anti-Gb3 antibody revealed the abundant presence of Gb3 in the glycolipids extracted from wild-type pig kidney, pancreas, heart, and liver (Fig. 5). The immunostaining with anti-αGal antibodies purified from pooled human serum (Fig. 5) revealed that iGb3 did not bind to human natural anti-Galα3Gal antibodies. Galα3Gal epitope antigens were present in extracted neutral glycolipids from wild-type pigs. However, those bands represented Galα3Gal epitopes present on lacto-series (stemming from GlcNAcβ3Galb4GlcCer) glycolipids with more than three sugar units, a result that is consistent with our MS analysis and that supports previous studies by others.[2][36-39]

To explore the enzymatic mechanism of iGb3 expression in human dendritic cells, we have cloned the human iGb3 synthase. Previously, Christiansen et al. reported that they failed to

clone human iGb3 synthase.[20] We have overcome the technical barriers through cloning the cDNA fragments of exons 1 to 4 and exon 5 separately, and joining the two cDNA fragments through an internal restriction enzyme site (Eco47 III). We have transfected the CHO cells with mouse, rat, and human iGb3 synthase genes. To standardize the transfection efficiency, we have cloned each iGb3 synthase in a pIRES2-EGFP plasmid. The IRES-EGFP sequence encodes a reporter gene that allows us to determine the expression efficiency. We also sorted mock pIRES2-EGFP plasmid-transfected, EGFP-positive cells as negative control, to exclude the possibility that CHO cells mutate or that serum glycosphingolipids contaminate our mass spectrometry assays. Our results showed that human iGb3 synthase initiated the synthesis of both iGb3 (Fig. 6A) and iGb4 (Fig. 6B), as measured by LIT-MS^n technology. However, the human iGb3 synthase showed very weak activity even in the CHO cell transfection experiment and could not further elongate iGb3 to B4, B5, and B6 GSLs (Table 1).

DISCUSSION

Carbohydrate antigens with Galα3Gal epitopes are recognized by natural antibodies in humans and pose a major barrier for pig-to-human xenotransplantation. To avoid hyperacute rejection of xeno-organs, knockout pigs have been produced for α3GalT1, which transfer α3Gal to N-acetylactosamine-(LacNAc)-type oligosaccharides.[24-26] Yet Milland et al. reported that a low level of Galα3Gal is still expressed in α3GalT1 knockout animals in the form of a lipid, isoglobotriosylceramide (iGb3), and suggested that iGb3 synthase can synthesize such structures on isoglobo-series GSLs.[27] To examine this possibility, we used an ultra-sensitive MS method that can measure minute quantities of iGb3 in mouse and human dendritic cells[33-35] (Fig. 1). Using the same method in this study, we could not find any signal suggesting the presence of iGb3 in examined pig organs.

We further used human anti-Galα3Gal antibodies to search for Galα3 Galterminated neutral lipids in pig organs including the kidney, pancreas, heart, and liver. We found several Galα3Gal-terminated compounds, containing more than three sugar units. On the other hand, we did not find staining of iGb3, although Gb3 was detected. This result contradicts with the immunohistochemical staining results published by Milland et al., which showed binding of human anti-Galα3Gal antibodies to iGb3 lipid or to tissues in α3GalT1$^{-/-}$ pigs.[27] Our finding of a lack of iGb3 staining by anti-Galα3Gal antibodies purified from human serum along with data from other groups[36-39] suggests that it is not necessary to eliminate iGb3 synthase for xenotransplantation.

A series of papers by Sandrin's group have focused on the central hypothesis that pig organs and endothelial cells should express iGb3, which crossreacts with human xenoantibodies.[20,27,40-42] Thus, Sandrin et al. reasoned that in addition to the well-known α1,3-galactosyltransferase-1 (α3GalT1), iGb3 synthase must be depleted in pig organs before they can be transplanted to humans. In support of this hypothesis, Milland et al. reported that the Galα3Gal epitopes produced by iGb3 synthase could be detected by immunohistochemical staining by a 15.401 antibody in the pancreas and kidneys of α3GalT1 knockout mice.[27] Endothelial cells were reported as the major cell type that expresses Galα3Gal epitopes produced by iGb3 synthase.

However, both biochemical and immunological studies have seriously challenged the idea that Galα3Gal epitopes are present in α3GalT1 knockout tissues and cells. After extensive biochemical fractionation and characterization of GSLs extracted from the pancreas of α3GalT1 knockout pigs, Diswall et al. could not detect iGb3 or any isoglobo-series GSL terminated by Galα3Gal epitopes.[36-39] α3Gal-containing GSLs were found in neither neutral nor acidic GSL fractions. In a separate study,[43] Kiernan et al. elegantly showed that

immunization of monkeys by α3GalT1$^{-/-}$ pig endothelial cells elicited anti-non-Gal xenoreactive antibodies that are related to but distinct from those elicited by the Galα3Gal epitope. Furthermore, no expression of iGb3 synthase was found in pig epithelial cells (43).

iGb3 has been described as another source of foreign Galα1,3Gal xenoantigen with obvious significance in the field of xenotransplantation,[20] for which evidence in pig organs (kidneys, pancreas, heart, and intestine) is currently lacking. This is the most controversial idea in the field and had been contradicted by other groups. Evidence for iGb3 in pig endothelial cells is not merely lacking, but instead there is strong immunologic,[43] genetic, and biochemical counterevidence from several sources.[36-39]

It has also been asserted that the presence of iGb3 must be mutually exclusive from the presence of Galα3Gal-reactive antibodies,[20] suggesting that iGb3 must be absent from animals bearing Galα3Gal-reactive antibodies. However, Thall et al.,[28] Posekany et al.,[29] Chiang et al.,[30] and Dor et al.[31] in multiple studies found that cytotoxic anti-Galα3Gal antibodies may be induced in concentrations at levels as high as milligrams per milliliter in α3GalT1$^{-/-}$ mice and pigs without acute or chronic rejection of self-organs or self-blood vessels, opposite to the implication of this assertion. Paradoxically, Christiansen et al.[20] acknowledged that they had generated anti-iGb3 monoclonal antibodies in α3GalT$^{-/-}$ mice that express the functional iGb3 enzyme. There are still questions about the specificity of these antibodies and whether such antibodies with multiple cross-reactivities such as 15.101 is justifiable for use as standards for biochemical relevance.[12]

In conclusion, our data and studies by multiple other laboratories using a variety of sensitive MS technologies as well as antibody staining and nuclear magnetic resonance spectrometry analysis[36-39] do not support the presence of iGb3 and iGb3-derived GSLs in pig organs that are relevant to transplantation immunology. There are three possible mechanisms that may explain the paradox of coexisting functional iGb3 synthase and anti-α-Gal antibodies in animals: (1) the anti-αGal antibodies induced by αGal epitopes in α3GalT1 KO animals and humans do not bind to iGb3; (2) the iGb3 epitope in α3GalT1 KO animals is converted to nonreactive epitopes such as iGb4; and (3) the iGb3 synthases in α3GalT1 KO animals are not synthesizing iGb3 in multiple organs. Currently, the first mechanism is supported by data from multiple groups.[11,36-39] The iGb3 epitope in α3GalT1 KO animals is not converted to nonreactive epitopes[36-39] (Fig. 1), thus excluding the second mechanism. The absence of iGb3 in animal organs with functional iGb3 synthases is a puzzle that remains to be solved, and the presence of iGb3 is not correlated to the mRNA level of iGb3 synthase[11,27,43-45] (Table 3). Transfection of pig, rat, or mouse iGb3 synthase into CHO cells led to high expression of iGb3 and the B4, B5, and B6 series of iGb3-derived GSLs[12] (Table 1). Transfection of a human iGb3 synthase cDNA resulted in iGb3 production in CHO cells only measurable by mass spectrometry, and the human enzyme could not further elongate the synthesis of poly αGal-GSLs, indicating much weaker iGb3 synthase activity than was observed in the rat or mouse enzyme.[12,20] We reason that there may exist a critical chaperone protein necessary for the enzyme's activity, as exemplified by the α-lactalbumin, which is a component of lactose synthetase,[46] and the *cosmc* gene required for core-1 β3 galactosyltransferase.[47] On the other hand, there may exist suppressive chaperone proteins for iGb3 synthase. Alternatively, there may exist critical partners that are responsible for binding to iGb3 synthase and directing its trafficking to the correct cellular compartments, presumably in the *trans* region of the Golgi complex, where GSLs such as Gb3 are synthesized.[48,49] It may not be surprising that the expression of iGb3 is so restricted in the organs of mice, pigs, and humans. Since iGb3 is a stimulatory ligand for NKT cells, high-level expression of iGb3 in thymocytes may lead to the deletion of NKT cells during their development. High-level expression of iGb3 in peripheral immune organs may lead to hyperactivation of NKT cells, as reported by Darmoise et al. in a mouse model

of Fabry disease in which iGb3 accumulates because of a defect in α-galactosidase.[15] Glycosyltransferases involved in generating NKT cell ligands (e.g., iGb3 synthase) may be regulated in a highly restricted manner, in clear contrast to other glycosyltransferases, which are often housekeeping genes.[50]

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ABBREVIATIONS

αGalCer	α-galactosylceramide
α1,3GalT1	α1,3-galactosyltransferase enzyme 1
B4	Galα3Galα3Galβ4Glc-Cer
B5	Galα3Galα3Galα3Galβ4Glc-Cer
B6	Galα3Galα3Galα3Galα3Galβ4Glc-Cer
Gb3	globotriaosylceramide, Galα4Gal β4Glc-Cer
Gb4	GalNAcβ3Galα4Galβ4Glc-Cer
GSL	glycosphingolipid
HAR	hyperacute rejection
iGb3	isoglobotriaosylceramide, Galα3Galβ4Glc-Cer
iGb4	GalNAcβ3Galα3Galβ4Glc-Cer
IgG	immunoglobulin G
MS	mass spectrometry
NK	natural killer cells
NKT	natural killer T cells
nLc4	Gal β4GlcNAcβ3Galβ4Glc-Cer

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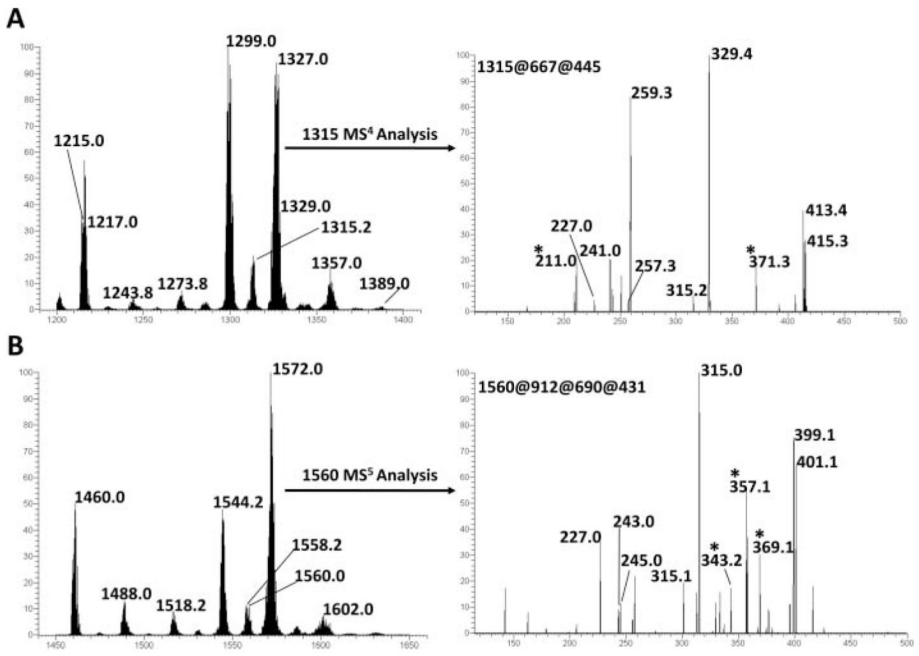

Figure 1.

Linear ion-trap mass spectrometry detection of iGb3 and iGb4 in human dendritic cells. GSLs purified from human dendritic cells[11] were subjected to linear ion-trap MS^n analysis as described. Left panels show MS^1 profile of iGb3 **(A)** and iGb4 **(B)** generated by a "precursor ion mapping method,"[34,35] containing multiple lipoforms due to the variation of length and unsaturation of fatty acyl chains. Right panel shows representative MS^4 profile of human dendritic cell iGb3 **(A)** and MS^5 profile of iGb4 **(B)**. Stars indicate signature ions generated from iGb3 **(A)** and iGb4 **(B)**.

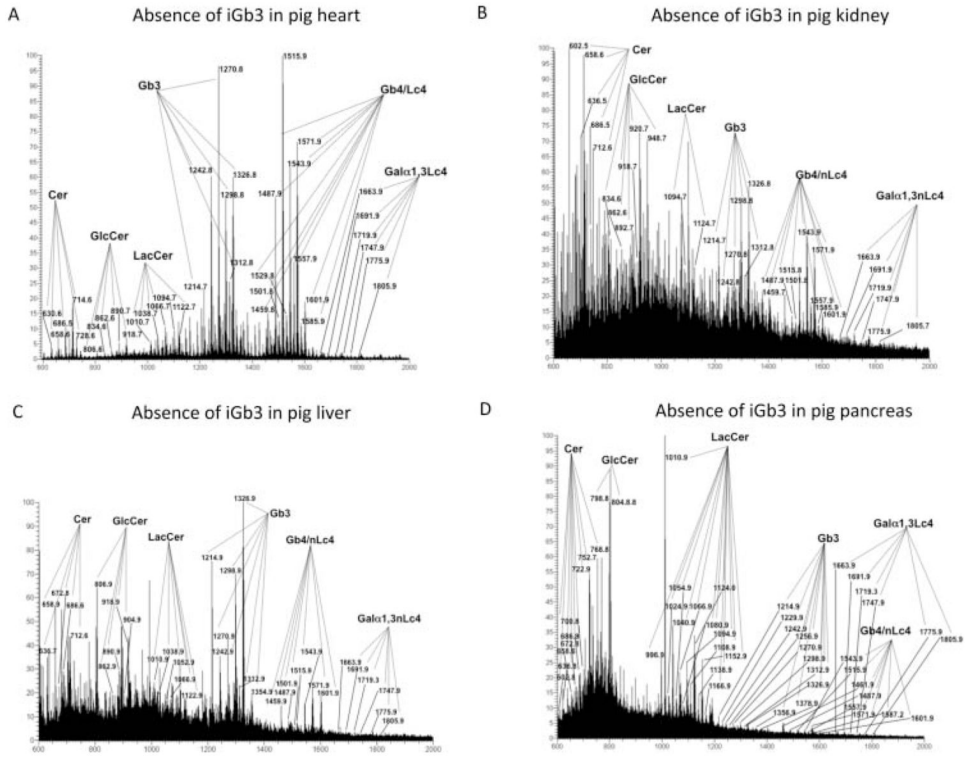

Figure 2.

Absence of iGb3 and isoglobo-series GSLs in pig heart, kidney, liver, and pancreas as measured by ion-trap mass spectrometry. GSLs were extracted as described in the text. Permethylation was performed on GSLs to enhance the sensitivity of detection and allow better fragmentation assays. Ceramide, glucosylceramide (GlcCer), lactosylceramide (LacCer), Gb3, Gb4 (and nLc4), and Galα1,3nLc4 were detected. To detect whether the Hex-Hex-Hex-Cer (possibly representing Gb3 or iGb3) contains iGb3, we subjected all MS^1 ions to MS^4 analysis (see below, Fig. 3). No characteristic fragment ions for iGb3 were found.

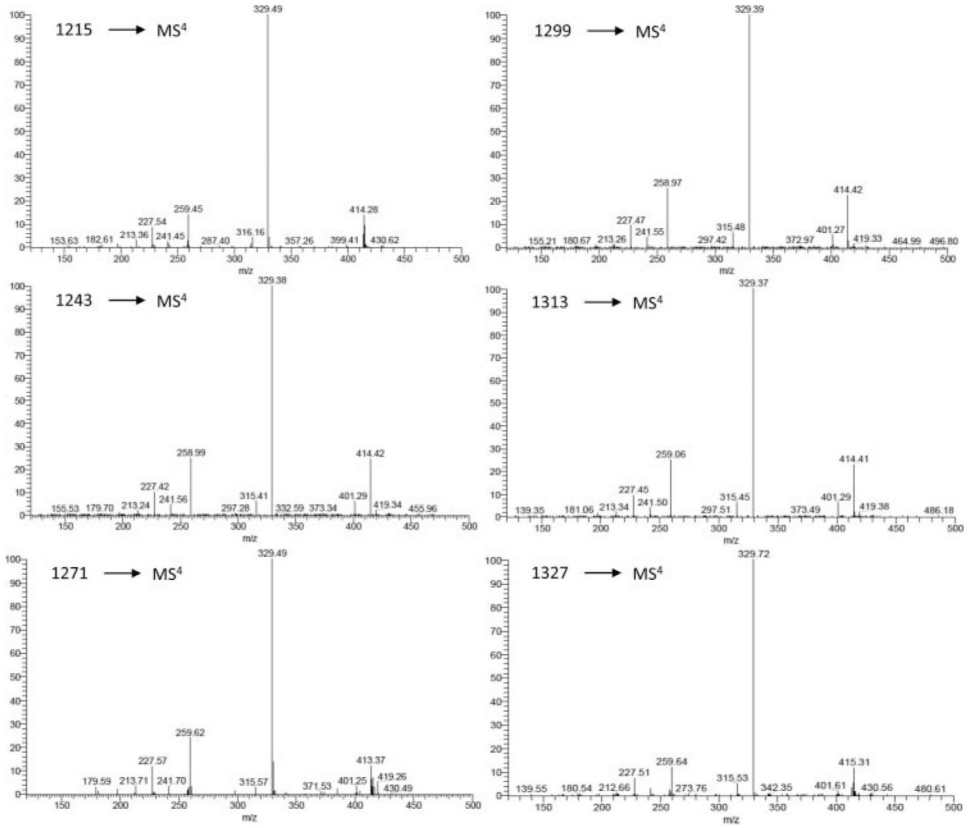

Figure 3.

Absence of characteristic ions for iGb3 in MS^4 analysis. Six molecular ions, 1215, 1243, 1271, 1299, 1313, and 1327, representing Hex-Hex-Hex-Cer with different lipid portions of the ceramide part, were subjected to MS^4 analysis, and only characteristic ions for Gb3 were detected. Characteristic ions for iGb3, 211 and 371, were found in human monocyte-derived dendritic cells (Fig. 1A).

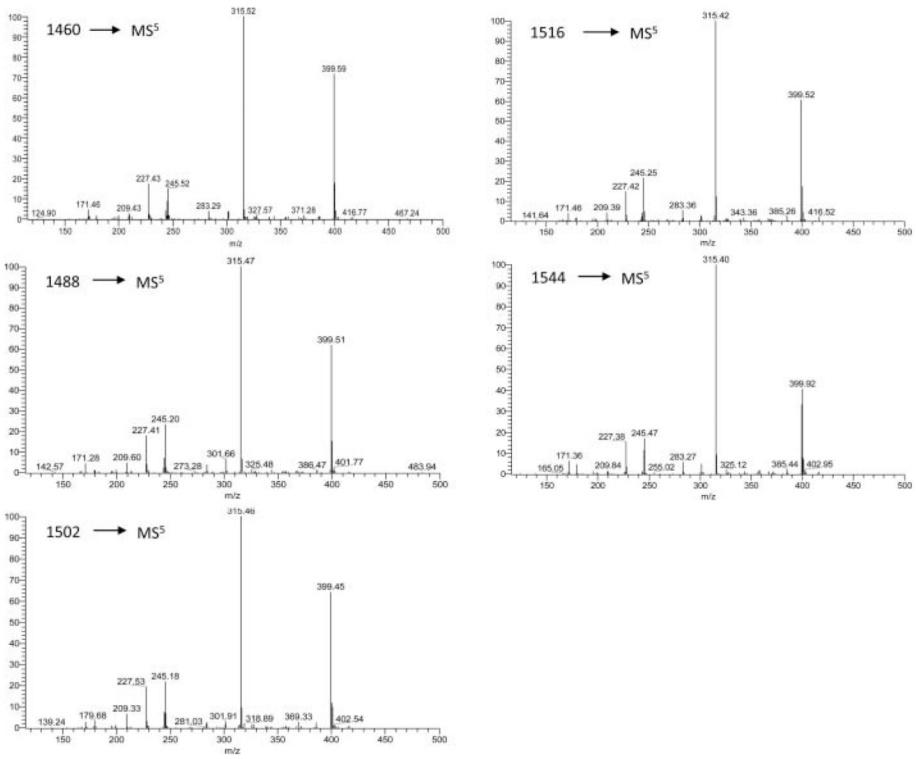

Figure 4.

Absence of characteristic ions for iGb4 in MS^5 analysis. Five molecular ions, 1460, 1488, 1502, 1516, and 1544, representing HexNAc-Hex-Hex-Hex-Cer with different lipid portions of the ceramide part, were subjected to MS^5 analysis, and only characteristic ions for Gb4 were detected. Characteristic ions for iGb4, 343, 357, and 369, were found in human monocyte-derived dendritic cells (Fig. 1B).

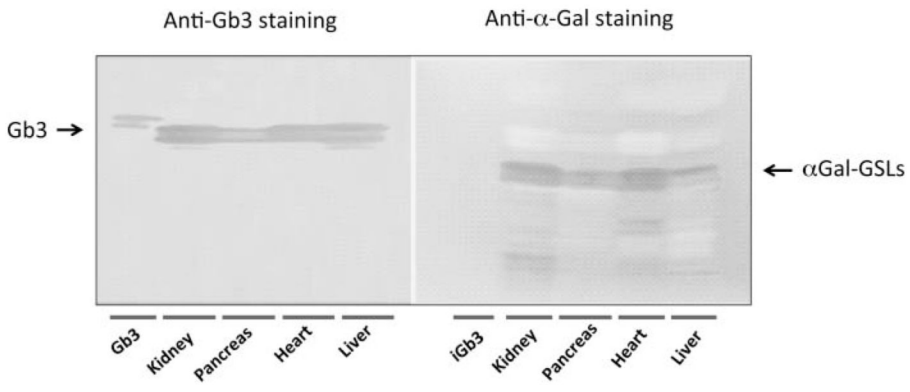

Figure 5.

Absence of iGb3 in pig organs as determined by anti-Galα3Gal antibody staining. Gb3 and iGb3 staining of neutral glycolipids isolated from pig kidney, pancreas, heart, and liver. **A:** The lipids were stained using anti-Gb3 (clone BGR-23, 51) as primary antibody and donkey antihuman IgG as second antibody, and 5 μg of GSLs were loaded with 5 μg of Gb3 as standard per lane of silica gel. **B:** The lipids were stained using AB human serum anti-Galα3Gal as primary antibody and donkey antihuman IgG as secondary antibody, and 5 μg of each lipid fraction was loaded per lane with 5 μg of iGb3 as standard. For both antibody stainings, GSLs were separated on HPTLC plates under the same conditions. The double-band appearance is due to structural heterogeneity in the ceramide part. The staining signal was specific when primary antibody was used at 0.5 μg/mL. At higher antibody concentrations, we observed a high background, consistent with studies published by other investigators,[39] who could not show the difference in staining between iGb3 and Gb3 (negative control).

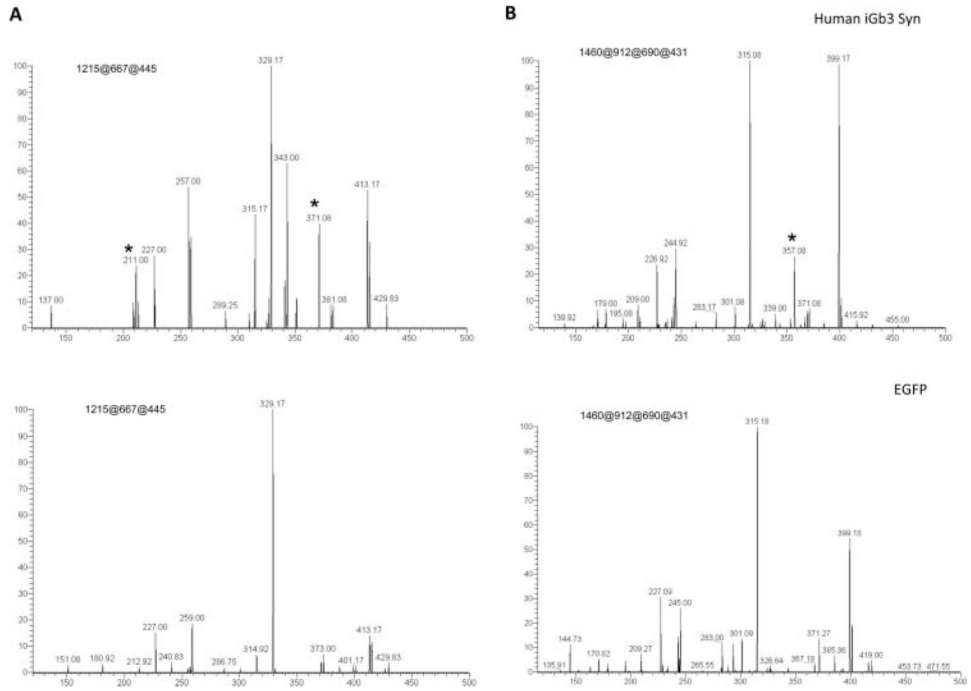

Figure 6.

Synthesis of iGb3 and iGb4 by human iGb3 synthase. **A:** Synthesis of iGb3 by human iGb3 synthase. iGb3 was measured by linear ion-trap MS^4 technology as described.[35] Stars indicate signature ions generated from iGb3. Human iGb3 Syn: CHO cells transfected by human iGb3 synthase cloned in pIRES2-EGFP plasmid and sorted by EGFP expression. EGFP: CHO cells transfected by mock pIRES2-EGFP plasmid and sorted by EGFP expression. **B:** Synthesis of iGb4 in human iGb3 synthase0transfected CHO cells. iGb4 was measured by linear ion-trap MS^5 technology as described.[34] Stars indicate signature ions generated from iGb4. Human iGb3 Syn: CHO cells transfected by human iGb3 synthase cloned in pIRES2-EGFP plasmid and sorted by EGFP expression. EGFP: CHO cells transfected by mock pIRES2-EGFP plasmid and sorted by EGFP expression.

Table 1

α-Gal glycosphingolipids studied by mass spectrometry

	MS^2 ion (Neutral loss of sugar part of GSL)
Isoglobo-series	
Galα3Galβ4Glc-Cer (iGb3)	667
Galα3Galα3Galβ4Glc-Cer (B4)	871
Galα3Galα3Galα3Galβ4Glc-Cer (B5)	1075
Galα3Galα3Galα3Galβ4Glc-Cer (B6)	1279
Lacto-series	
Galα3Galβ4GlcNAcβ3Galβ4Glc-Cer (α3Gal-nLc4)	1116

Table 2

Ion abundance of neutral GSLs ($[M+Na]^+$) in pig organs

GSL	m/z	Mass intensity	Structure[a,b]	Relative abundance (%)
		Pig heart		
GlcCer	806.68	69.1	(d18:1/16:0)	5.1
	834.64	59.7	(d18:1/18:0)	
	862.66	87.3	(d18:1/20:0)	
	890.71	157.3	(d18:1/22:0)	
	918.72	185.6	(d18:1/24:0)	
LacCer	1010.67	103.5	(d18:1/16:0)	5.5
	1038.69	126.5	(d18:1/18:0)	
	1066.69	182.5	(d18:1/20:0)	
	1094.7	124.9	(d18:1/22:0)	
	1122.75	147.5	(d18:1/24:0)	
Gb3	1214.72	377.6	(d18:1/16:0)	42.5
	1242.75	977.3	(d18:1/18:0)	
	1270.81	1581.1	(d18:1/20:0)	
	1298.84	880.9	(d18:1/22:0)	
	1312.81	449.5	(d18:1/23:0)	
	1326.84	984.7	(d18:1/24:0)	
Gb4/Lc4	1459.74	217.9	(d18:1/16:0)	46.0
	1487.84	770.9	(d18:1/18:0)	
	1501.88	165	(d18:1/19:0)	
	1515.87	1452.4	(d18:1/20:0)	
	1529.87	214.4	(d18:1/21:0)	
	1543.9	902.6	(d18:1/22:0)	
	1557.87	458.3	(d18:1/23:0)	
	1571.88	1055.8	(d18:1/24:0)	
	1585.85	159.5	(t18:1/23:1)	
	1601.88	285.7	(d18:0/26:0)	
Galα1,3Lc4	1663.72	16.5	(d18:1/16:0)	0.80
	1691.76	22.3	(d18:1/18:0)	
	1719.86	21.5	(d18:1/20:0)	
	1747.99	18.4	(d18:1/22:0)	
	1775.89	12.3	(d18:1/24:0)	
	1805.48	7.5	(d18:0/26:0)	
		Pig kidney		
GlcCer	834.55	779365.9	(d18:1/18:0)	35.0
	862.59	796032.7	(d18:1/20:0)	
	892.67	1022847.4	(d18:0/22:0)	
	918.67	890849.9	(d18:1/24:0)	
	920.71	1851002.5	(d18:0/24:0)	

GSL	m/z	Mass intensity	Structure[a,b]	Relative abundance (%)
	948.72	1740219.8	(d18:0/26:0)	
LacCer	1094.7	497521.2	(d18:1/22:0)	6.0
	1124.81	714168.7	(d18:0/24:0)	
Gb3	1214.72	931295.4	(d18:1/16:0)	29.1
	1242.49	504777.8	(d18:1/18:0)	
	1270.77	900490.7	(d18:1/20:0)	
	1298.78	1369690.9	(d18:1/22:0)	
	1312.8	629376	(d18:1/23:0)	
	1326.82	1552890.4	(d18:1/24:0)	
Gb4/nLc4	1459.7	575410.7	(d18:1/16:0)	25.2
	1487.78	346444.7	(d18:1/18:0)	
	1501.79	295976.7	(d18:1/19:0)	
	1515.77	762632.1	(d18:1/20:0)	
	1543.84	1103266	(d18:1/22:0)	
	1557.9	449664.5	(d18:1/23:0)	
	1571.87	954779	(d18:1/24:0)	
	1585.61	257169.3	(d18:1/h23:1)	
	1601.85	352508.2	(d18:1/26:0)	
Galα1,3nLc4	1663.72	145373.3	(d18:1/16:0)	4.8
	1691.84	208087.2	(d18:1/18:0)	
	1719.88	194130.2	(d18:1/20:0)	
	1747.56	151514.6	(d18:1/22:0)	
	1775.97	196452.8	(d18:1/24:0)	
	1805.51	82542.8	(d18:0/26:0)	
		Pig liver		
GlcCer	806.89	2172136.1	(d18:1/16:0)	23.3
	862.87	711836.2	(d18:1/20:0)	
	890.96	1003582.6	(d18:1/22:0)	
	904.92	667953.7	(d18:1/23:0)	
	919.01	1566675.7	(d18:1/24:0)	
LacCer	1010.96	878515.7	(d18:1/16:0)	11.6
	1039.04	491513.2	(d18:1/18:0)	
	1052.44	520762.1	(d18:1/19:0)	
	1067.08	495547.5	(d18:1/20:0)	
	1123.09	645843	(d18:1/24:0)	
Gb3	1215.05	2723757.7	(d18:1/16:0)	49.9
	1243.02	1348374	(d18:1/18:0)	
	1271.08	1552353.2	(d18:1/20:0)	
	1299.14	2505075.5	(d18:1/22:0)	
	1313.13	820384.9	(d18:1/23:0)	
	1327.14	3533102.8	(d18:1/24:0)	
	1355.08	611654.9	(d18:1/26:0)	

GSL	m/z	Mass intensity	Structure[a,b]	Relative abundance (%)
Gb4/nLc4	1460.11	535114.6	(d18:1/16:0)	12.8
	1488.1	305549.2	(d18:1/18:0)	
	1501.8	184057.5	(d18:1/19:0)	
	1516.19	333314.9	(d18:1/20:0)	
	1544.19	552035.9	(d18:1/22:0)	
	1572.25	780407.8	(d18:1/24:0)	
	1602.24	655170.4	(d18:0/26:0)	
Galα1,3nLc4	1664.17	116122.6	(d18:1/16:0)	2.4
	1691.29	161591.6	(d18:1/18:0)	
	1719.36	64106.7	(d18:1/20:0)	
	1747.36	120742.8	(d18:1/22:0)	
	1776.1	96489.7	(d18:1/24:0)	
	1805.83	81214.2	(d18:0/26:0)	
		Pig pancreas		
GlcCer	798.73	1128598.2	(d18:0/15:0)	22.9
	804.26	650301.3	(d18:1/16:1)	
LacCer	996.99	383669.6	(d18:1/15:0)	62.7
	1010.96	1445301.2	(d18:1/16:0)	
	1024.95	202225.4	(d18:1/17:0)	
	1040.97	537083.1	(d18:0/18:0)	
	1054.96	182535.9	(d18:0/19:0)	
	1066.98	315017	(d18:1/20:0)	
	1080.96	159241.4	(d18:1/21:0)	
	1095.02	364338.4	(d18:1/22:0)	
	1109.04	251678.3	(d18:1/23:0)	
	1124.06	342079.6	(d18:1/24:0)	
	1139.02	196906.4	(d18:1/25:0)	
	1153.06	377711.7	(d18:1/26:0)	
	1166.99	111445	(d18:1/h25:1)	
Gb3	1215.01	105470	(d18:1/16:0)	8.6
	1229.94	72702.1	(d18:1/17:0)	
	1242.99	74323.4	(d18:1/18:0)	
	1256.93	56919.1	(d18:1/19:0)	
	1271.01	71989.9	(d18:1/20:0)	
	1299.06	73940.3	(d18:1/22:0)	
	1313.01	51368.5	(d18:1/23:0)	
	1327.1	72030.2	(d18:1/24:0)	
	1357.06	48551.6	(d18:0/26:0)	
	1378.96	43777.4	(t18:1/26:2)	
Gb4/nLc4	1462.17	75440.9	(d18:0/16:0)	4.4
	1488.09	42258.7	(d18:1/18:0)	
	1516.1	39960.4	(d18:1/20:0)	

GSL	m/z	Mass intensity	Structure[a,b]	Relative abundance (%)
	1544.21	42546.7	(d18:1/22:0)	
	1558.18	32966.2	(d18:1/23:0)	
	1572.23	54989.9	(d18:1/24:0)	
	1587.16	27378.5	(d18:0/25:0)	
	1602.21	29640.4	(d18:0/26:0)	
Galα1,3Lc4	1664.05	21747.4	(d18:1/16:0)	1.3
	1692.15	21028.8	(d18:1/18:0)	
	1720.1	18850.9	(d18:1/20:0)	
	1748.07	14547.2	(d18:1/22:0)	
	1775.98	15612.6	(d18:1/24:0)	
	1806.06	11845	(d18:0/26:0)	

For some ceramide forms, alternate fatty-N-acyl/sphingosine compositions are possible.

[a]For fatty acids, the number before the colon refers to the carbon chain length. The number after the colon gives the total number of double bonds. Fatty acids with a 2-hydroxyl group are denoted by the prefix “h” before the abbreviation.

[b]For the sphingoid base, the number before the colon refers to the carbon chain length. The number after the colon gives the total number of double bonds. “d” denotes dihydroxyl and “t” denotes trihydroxyl.

Table 3

mRNA expression of iGb3 synthase gene in different species

	Pig[a]	Mouse[b]	Human[c]
Heart	+	+	−
Liver	+	+	−
Kidney	+	+	−
Thymus	+	+	+
Pancreas	+	+	−
Endothelial cell line	+	Unknown	Unknown
Monocyte derived dendritic cells	Unknown	Unknown	+

[a] Data from ref. [20,27] and Mauro Sandrin, patent application, DNA molecules encoding iGb3 synthase, and uses thereof for the disruption of glycosyltransferase genes in xenotransplantation tissues and organs (WO2002081688).

[b] Data from ref.[11,20,27,43,45]

[c] Data from ref. [20,27] and current study. cDNAs were prepared by a SuperScript III cDNA Synthesis Kit (Invitrogen), and two nested PCR reactions were used to amplify gene segments of iGb3 synthase. The first pair of primers consisted of 5′CCCTAAATTCAGGCATCTGG3′ and 5′CCACATCTGGGTCGAAAGAG3′. PCR products generated by first pair of primers were diluted 10-fold and used for second PCR amplification by primer pairs 5′TGTCCCAGCTGAGAGACAAC3′ and 5′AAGAGCCATCCCAAATAATG3′. The 116 bp product generated by second PCR was sequenced and found to match GenBank cDNA sequence NM 001080438.