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Characterization of porcine kidney neutral glycosphingolipids: identification of a carbohydrate antigen recognized by human natural antibodies

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We have investigated the glycosphingolipids of pig kidney with a special interest in identifying compounds which may be involved in the rejection of tissue in xenotransplantation. Nine neutral glycosphingolipids have been characterized in porcine kidney and structurally characterized by a combination of techniques including ^{1}H-NMR, permethylation analysis and thin-layer chromatography (TLC) immunostaining with carbohydrate sequence-specific monoclonal antibodies. The major components are members of the globo family and represent the human p^k and P antigens. Three other compounds were found to contain a neolacto core structure; the major neolacto compound carries a nonreducing terminal epitope (Galα1-3Galβ1-4GlcNAc) recognized by the naturally-occurring human antibody, anti-Gal, and a second neolacto compound carries the blood group A trisaccharide (GalNAcα1-3(Fucα1-2)Gal). These results are discussed with respect to tissue transplantation.
Keywords: kidney; natural antibody; transplantation

Introduction

The pig has been identified as the likely donor for xenotransplantation in humans. A recent study by Good *et al.* [1] has demonstrated that humans express natural antibodies that bind to carbohydrate structures present in pig tissue. The predominant carbohydrate structure identified was a linear B component, with a Galα1-3Gal nonreducing disaccharide. Previous studies from our laboratory have demonstrated a unique evolutionary distribution of Galα1-3Galβ1-4GlcNAc glycosphingolipids and a corresponding naturally occurring antibody among mammals [2, 3]. Recently, we [4] have shown that specific patterns of expression of Galα1-3Galβ1-4GlcNAc glycosphingolipids occur in kidney of different animal species, and obtained preliminary evidence for the occurrence of glycosphingolipids with a Galα1-3Galβ1-4GlcNAc epitope in several animals including the pig. However, in a recent report by Holgersson *et al.* [5] no evidence for such a structure was presented. In light of the importance of this structure for successful xenotransplantations of pig kidney into humans, we have done a complete evaluaton of the types of neutral glycosphingolipids expressed in pig kidney. The results of these analyses have demonstrated that porcine kidney contains members of several glycosphingolipid families including blood group P and A antigens. Most importantly, our work illustrates that pig kidney contains a Galα1-3Galβ1-4GlcNAc pentasaccharide glycosphingolipid.

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Materials and methods

Materials

Frozen porcine (*mixed breed*) kidney was obtained from Pel-Freeze (Rogers, AK). Glycosidases and DEAE-Sephadex were purchased from Sigma (St Louis, MO). Vector Labs (Burlingame, CA) was the source of the immunostaining reagents as previously described [6]. NMR reagents were purchased from Cambridge Isotope Labs (Woburn, MA). An anti-A antibody was purchased from Dako (Carpenteria, CA); others were prepared as previously described [2, 7–9]. An A-positive, neutral glycosphingolipid preparation from dog intestine [10] was kindly supplied by Dr Hakon Leffler, University of California, San Francisco. Florisil was purchased from Fisher Scientific Co. (Fair Lawn, NJ) and BioSil A was purchased from Bio-Rad Laboratories (Richmond, CA).

Purification of glycosphingolipids

Porcine kidney (approximately 300 g) was homogenized with water and lyophilized. Glycosphingolipids were extracted

and purified as previously described [11–15]. Glycosphingolipids were analysed by TLC on silica gel 60 HPTLC plates (Kieselgel, EM Reagents, Germany) using the solvent system chloroform:methanol:water 60:35:8. Orcinol reagent was used as a detection reagent. Densitometry was used to estimate the relative quantities of the major glycosphingolipid components. TLC plates were scanned with an HP ScanJet IIc Flatbed scanner and the results were analysed with the Image 1.41 program on a Macintosh LC II. A standard curve was generated by scanning a series of known quantities of standard glycosphingolipids and used to estimate the quantity of the purified glycosphingolipids [16]. Immunostaining of TLC plates (aluminium-backed silica gel 60) was done as previously described [6]. The conditions for glycosidase treatment of glycosphingolipids were as previously reported [15]. Permethylated glycosphingolipids and partially methylated alditol acetates were prepared and analysed as previously described [17]. The type of sugar residue and its glycosidic linkage position was determined by its column retention time and characteristic mass fragmentation pattern. PMAA were analysed on a 25 m capillary column (HP-1, cross-linked methyl silicone gum as the stationary phase) installed in a Hewlett-Packard GC-MS.

Nuclear magnetic resonance (NMR) of glycosphingolipids

Glycosphingolipid samples were lyophilized twice from D_2O overnight. DMSO-d_6 was added to the dried glycosphingolipid samples and then transferred to NMR tubes. Samples of <100 μg were diluted to 100 μl and analysed in microsample NMR tubes. Larger samples were diluted with 500 μl of DMSO-d6 and 1.5 μl of D_2O. ^{1}H-NMR spectra were recorded with either a 300 MHz General Electric QE or GN spectrometer at 50 °C.

Results

Nine neutral glycosphingolipids were characterized in porcine kidney. Five components were obtained as homogeneous components by repeated HPLC and structurally characterized. Four other compounds were detected, in fractions containing a mixture of two glycosphingolipid components, by immunostaining, or ^{1}H-NMR and methylation analysis. Repeated HPLC was used to obtain a homogeneous preparation of several glycosphingolipid components (Fig. 1). Figure 1 shows an initial HPLC separation of the total neutral glycosphingolipid material obtained from porcine kidney. The TLC analysis of this initial HPLC separation shows the presence of glycosphingolipids with one to approximately seven sugar residues. To obtain homogeneous components, fractions from the initial HPLC separation which contained glycosphingolipids with similar TLC mobility were pooled and rechromatographed using gradient conditions that were optimized for separating components with different carbohydrate chain lengths. The glycosphingolipids in each of the purified fractions were evaluated by TLC immunostaining for compounds with specific nonreducing terminal carbohydrate sequences. Fractions which contained compounds with the same chromatographic and immunostaining properties were combined. Figure 2 shows the thin layer chromatographic behaviour of each of the purified compounds obtained from the HPLC separations (the purified GalCer fraction is not shown).

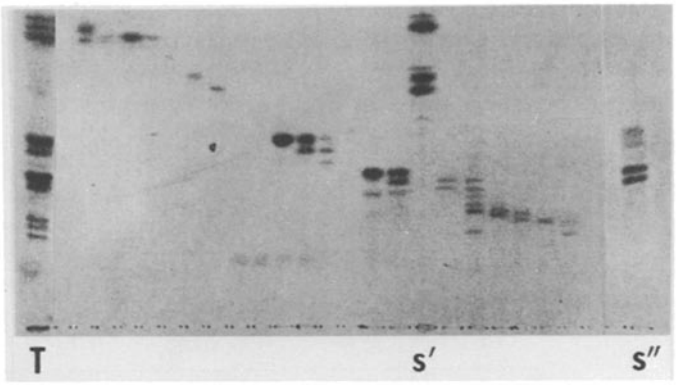

Figure 1. Thin layer chromatogram of porcine kidney neutral glycosphingolipids. The total neutral glycosphingolipid fraction (labelled T) from porcine kidney was separated by HPLC on an Iatrobead column (1 × 50 cm) using a gradient of isopropanol: hexane:H_2O (55:44:1 (solvent A) to 55:30:15 (solvent B)). The gradient was developed linearly (0%B to 100%B) over a period of 150 min. Every third fraction (starting with fraction 1) was spotted and the thin layer chromatogram was developed in $CHCl_3$:MeOH:H_2O (60:35:8, v/v/v). Glycosphingolipids were visualized with Orcinol reagent. The lanes labelled S′ (GlcCer, LacCer) and S″ (Gb_3Cer and Gb_4Cer) on the bottom of the chromatograms indicate the lanes in which glycosphingolipid standards were spotted.

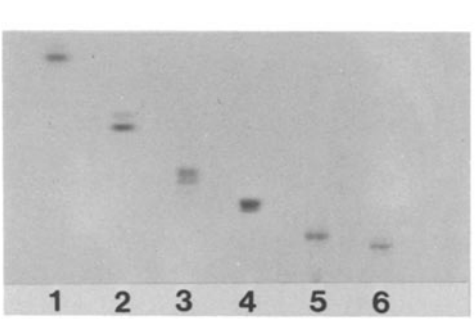

Figure 2. Thin layer chromatogram of purified porcine kidney neutral glycosphingolipids. Lane 1, GL_1 (GlcCer); lane 2, GL_2 (LacCer); lane 3, GL_3 (Gb_3Cer); lane 4, GL_4 (Gb_4Cer); lane 5, GL_5 ($IV_3\alpha$GalnLc$_4$Cer) and lane 6, GL_6 (blood group A positive glycolipid). In some cases (e.g. lane 3) more than one band is seen and is due to differences in ceramide composition.

Two GL_1 and two GL_2 compounds were identified in porcine kidney. These compounds were characterized by ^{1}H-NMR and methylation analyses (Tables 1 and 2) and shown to be GlcCer, GalCer, and LacCer (structures of all glycosphingolipids identified in this study are shown in Table 3). The ratio of the two GL_1 species was 8:2 for GlcCer:GalCer as estimated from the methylation analysis of a crude fraction representing the total GL_1 fraction. The total GL_1 fraction accounts for about 15% of the total neutral glycosphingolipids in porcine kidney. Ga_2Cer was

Table 1. Methylation analysis of porcine kidney neutral glycosphingolipids.

Sugar	*Substitution*	*Glycolipid*						
		[a]GL_{1a}	[a]GL_{1b}	[b]GL_2	GL_3	[c,d]GL_4	[d]GL_5	[d]GL_6
Glc	Terminal	1.0	0	0	0	0	0	0
Glc	4	0	0	1.0	1.0	1.0	1.0	1.0
Gal	Terminal	0	1.0	1.0	1.0	0	1.2	0
Gal	3	0	0	0	0	1.0	2.0	0.9
Gal	2, 3	0	0	0	0	0	0	0.6
Gal	4	0	0	0	1.0	0.8	0	0
Fuc	Terminal	0	0	0	0	0	0	0.8
GlcNAc	4	0	0	0	0	0	1.0	0.7
GalNAc	Terminal	0	0	0	0	0.9	0	0.7

[a] Two forms of GL1 were obtained. To distinguish them they were labelled as GL_{1a} and GL_{1b}.
[b] Some GL_2 fractions contained 4-linked Gal indicating the presence of Ga_2Cer which was confirmed by ^{1}NMR.
[c] Result obtained for the major GL_4 component.
[d] Values for amino sugars, trisubstituted Gal and terminal Fuc were corrected by comparisons with standard glycosphingolipids.

Table 2. Chemical shifts (δ) and coupling constants ($J_{1,2}$) of anomeric protons of neutral glycolipids from pig kidney. Chemical shifts were measured relative to the solvent peak (2.49 ppm).

Glycolipid	*Chemical shifts (ppm) and $J_{1,2}$ coupling constants (Hz)*				
	V	*IV*	*III*	*II*	*I*
GL_1 (GalCer)					4.12(7.3)
GL_1 (GlcCer)					4.09(7.8)
GL_2 (LacCer)				4.20(7.5)	4.15(7.9)
GL_3 (Gb_3Cer)			4.78(3.5)	4.26(7.1)	4.15(8.1)
GL_4 (Gb_4Cer)		4.53(8.1)	4.82(4.1)	4.27(7.7)	4.16(7.8)
GL_5 (IV^3αGal-nLc4Cer)	4.83(2.9)	4.32(7.8)	4.72(8.3)	4.29(7.8)	4.16(7.8)

The chemical shifts and coupling constants for each kidney glycosphingolipid agree with those previously reported for these compounds (Dabrowski *et al.* [21, 22] and refs. therein). For example, the anomeric protons of the α-linked galactose residues of GL_3, GL_4 and GL_5 gave chemical shifts of between 4.7 and 4.9 ppm, with coupling constants of 2.9 to 4.1 Hz due to their equatorial configurations. β-linked amino sugars showed chemical shifts in the range of 4.5–4.7 ppm, due to the deshielding effect of the amino group on the anomeric proton.

Table 3. Structural formulas of porcine kidney neutral glycosphingolipids.

Glycolipid	*Structure*
GL_1	Galβ1-1Cer (GalCer) and Glcβ1-1Cer (GlcCer)
GL_2	Galβ1-4Glcβ1-1Cer (LacCer) and Galα1-4Galβ1-1Cer (Ga_2Cer)
GL_3	Galα1-4Galβ1-4Glcβ1-1Cer (Gb_3Cer)
GL_4	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer (Gb_4Cer) and Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer (nLc_4Cer)
GL_5	Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer (IV^3αGal-nLc_4Cer)
GL_6	GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer (IV^3αGalNAc,IV^2αFuc-nLc_4Cer)

detected in some HPLC fractions of LacCer by NMR and methylation analysis. It was estimated to account for less than 10% of the total GL_2 material of porcine kidney. The GL_2 components were 2–3% of the total neutral glycosphingolipid fraction.

A single GL_3 component was detected in porcine kidney and was immunostained with the mouse monoclonal antibody 38.13, which has a specificity for Gb_3Cer [7]. ^{1}H-NMR (Table 2) and methylation analyses (Table 1) confirmed the identity of the GL_3 compound as Gb_3Cer or the human blood group p^k antigen. This glycosphingolipid was a major component of porcine kidney neutral glycosphingolipid, accounting for about 30% of the total material.

A major (>98%) and a minor GL_4 component were detected in porcine kidney. The major GL_4 was purified to homogeneity and characterized by ^{1}H-NMR, methylation analyses, and glycosidase treatment and shown to be Gb_4Cer or the human blood group P antigen. Gb_4Cer accounts for more than 40% of the total neutral glycosphingolipid or porcine kidney. The minor GL_4 component could not be separated from the large quantity of Gb_4Cer, but could be detected by TLC immunostaining with the mouse monoclonal antibody 1B2. This antibody binds to glycosphingolipids with a nonreducing terminal Galβ1-4GlcNAc epitope [8]. This result suggests that this compound is nLc_4Cer, the precursor for the biosynthesis of several antigens, including the ABO antigens.

A single GL_5 component, accounting for about 10% of the total neutral glycosphingolipids, was purified from porcine kidney and structurally characterized. This compound was positively immunostained by the mouse monoclonal antibody Gal-13, which binds to glycosphingolipids with a nonreducing terminal trisaccharide epitope of Galα1-3Galβ1-4GlcNAc [9]. The GL_5 compound was also immunostained (result not shown) by the naturally occurring antibody, anti-Gal [2]. ^{1}H-NMR (Fig. 3 and Table 2) and methylation analyses (Table 1) confirmed that the GL_5 compound in porcine kidney is $IV^3\alpha$Gal-nLc_4Cer or a compound referred to as a linear B structure. α-Galactosidase treatment of the GL_5 compound produced a GL_4 compound with properties identical to nLc_4Cer (results not shown).

Another glycosphingolipid (GL_6 in Table 3) was purified from porcine kidney and characterized by immunostaining and methylation analysis. Figure 4 shows the results of TLC immunostaining of this compound with an anti-A mouse monoclonal antibody. This compound (Fig. 4, Lane 2) was intensely stained with the anti-A antibody and had a TLC migration similar to an A-positive neolactohexosylceramide, $IV^3\alpha$GalNAc,$IV^2\alpha$Fuc-nLc_4Cer (Fig. 4, Lane 1), from dog intestine [10]. Results from permethylation analysis (Table 1) suggested that the porcine kidney, A-positive compound was $IV^3\alpha$GalNAc,$IV^2\alpha$Fuc-nLc_4Cer. Another more polar A-positive compound (not shown) was also

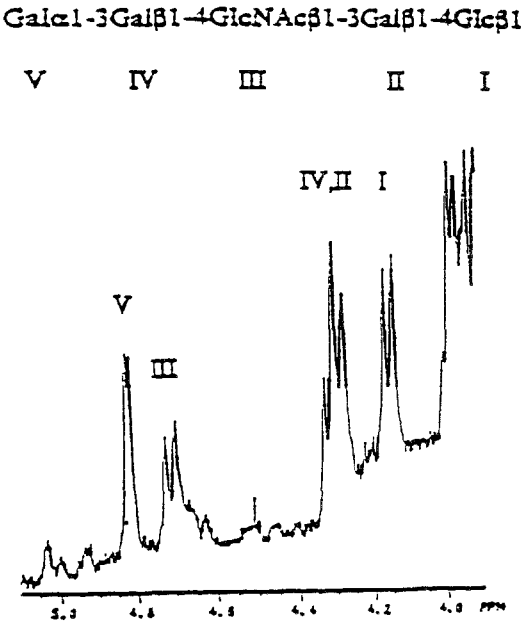

Figure 3. Proton NMR of porcine kidney GL_5. The assigned structure of each compound is shown and the position of the signals for the anomeric proton of each sugar residue are designated with Roman numerals.

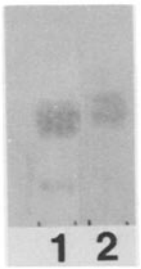

Figure 4. Anti-blood group A immunostained thin layer chromatogram of porcine neutral glycosphingolipid GL_6. Lane 1, blood group A glycosphingolipids from dog intestine. The upper component is $IV^3\alpha$GalNAc,$IV^2\alpha$Fuc-nLc_4Cer. Lane 2, porcine kidney blood group A positive glycosphingolipid (GL_6).

detected in porcine kidney, but was not obtained in sufficient quantities for structural characterization. GL_6 components accounted for about 1% of the total neutral glycosphingolipids of porcine kidney.

Discussion

The results of our study demonstrate that pig kidney contains several glycosphingolipid structures which are known to have antigenic properties in humans. Two members of the human P antigen family (Gb_3Cer and Gb_4Cer) are major neutral glycosphingolipids in pig kidney. Minor quantities of two blood group A glycosphingolipids were also detected in pig kidney. Finally, a glycosphingolipid

(linear B-like antigen) that is not found in humans, but is commonly found in non-primate mammals was found in substantial quantities in pig kidney. Interestingly, this was the only Galα1-3Gal neutral glycosphingolipid detected in porcine kidney. In a previous study, we [4] also have shown that the α1-3galactosyltransferase responsible for the final step in the biosynthesis of this compound is present in porcine kidney. Furthermore, we have demonstrated that all humans (regardless of blood type) produce a naturally occurring antibody to the epitope carried on this antigen throughout life in a concentration of >1 μg ml^{-1} serum. It is now clearly established that the synthesis of this antigen is prevented in humans by a mutation in the gene encoding the α1-3galactosyltransferase responsible for the last step in the biosynthesis of this antigen [18].

The recent work of Good *et al.* [1] has demonstrated that pig tissues, including kidney, absorb several types of anti-carbonate antibodies from human blood. Most prominent among these were antibodies against the linear B-like antigen. This was true regardless of the ABO blood type of the human donor. In addition, these workers detected human antibodies to A antigen, as well as antibodies to antigens of the P blood group family. All of these antigens have been detected in pig kidney in the current study and thus our results correlate well with those presented by Good *et al.* [1]. In contrast, Holgersson *et al.* [5] have previously reported the presence of A and P type glycosphingolipids, but not the linear type B-like antigen in porcine kidney.

Some preliminary studies with pig-to-monkey transplantation have shown that there is a high rate of tissue rejection, however the nature of the rapid rejection is unknown (see discussion in [5]). It is speculated that the presence of preformed antibodies against pig antigens is the primary reason for acute rejection. As we [3] have shown, Old World primates (as well as humans) produce high levels of a naturally occurring antibody which specifically recognizes the terminal epitope found on the linear B-like antigen. Therefore, it is possible that the presence of this compound in porcine kidney accounts for the acute rejection of this tissue when transplanted into monkeys. A recent study by Oriol *et al.* [19] reinforces this concept. They have screened various pig tissues with anti-Gal by immunofluorescence to detect the distribution of the correponding antigen. Intense staining was detected in several tissues including kidney. Vascular endothelium was extensively stained, and it was suggested that the presence of the Galα1-3Gal-R antigens in the cells of the vascular endothelium may play a major role in the hyperacute rejection of pig to man xenotransplantations.

Good *et al.* [1] also found that pig tissues absorb human antibodies against Forssman antigen from some O type human plasma. This antigen was found in the kidney of pigs analysed by Holgersson *et al.* [5], but this antigen was not present in the pig kidney extracted in our study. The reason(s) for the difference between our results and those of Holgersson *et al.* [5] is not clear, but it is possible that different strains of pig express different antigens.

Both our study and that of Holgersson *et al.* [5] demonstrate that porcine kidney contains blood group A active glycosphingolipids based on the type 2 core. Blood group A glycosphingolipids based on other core structures (type 1, 3 and 4) were also detected by Holgersson *et al.* [5] in porcine kidney. It is not clear whether these same components are present in the tissue we have analysed. However, two distinct A positive glycosphingolipids were detected by TLC immunostaining in the present study. Interestingly, analysis of porcine erythrocyte glycosphingolipids has recently been done by Sako *et al.* [20] and it was found that these cells only express type 1, blood group A glycosphingolipids. These workers proposed that the blood group active glycosphingolipids of porcine erythrocytes were acquired from serum and thus probably originated from an intestinal source. Therefore, it appears that the expression of blood group A glycosphingolipids (with different core structures) in pig occurs in a tissue specific manner.

Acknowledgements

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