DK3-1 Scriptaid (-)		DK3-1 Scriptaid (+)		DK3-2 Scriptaid (-)		DK3-9 Scriptaid (+)	
Weight (g)	Length (cm)	Weight (g)	Length (cm)	Weight (g)	Length (cm)	Weight (g)	Length (cm)
780	23	900	28	720*	23	1000	25
820	24	840*	25	480*	19	1000	26
820*	24	600*	23			1100	27
900	25	700*	25			900	25
1120	29	1500	27			700	23
580	23	1200	28			500	20
560	22	900	23			380	19
730	25	800	24				
1060*	27	900	25				
700	22	600	22.5				
700	23	600	24				
600	22	700*	26				
920	25	700	24				
1100	29						
900	25						
1120	29						
580	23						

*stillborn piglets

Groups	Average body weight (g) (mean± SEM)	Average body length (cm) (mean± SEM)
DK3-1 / Scriptaid (-)	822.9± 47.3	24.7± 0.6
DK3-1 / Scriptaid (+)	841.5± 72.0	25.0± 0.5
DK3-2 / Scriptaid (-)	600.0**	21**
DK3-9 / Scriptaid (+)	797.1± 104.2	23.6± 1.2

** SEM was not given due to limited sample numbers

Table 5. Body weight and length of the cloned piglets derived from the three types of the GalT-KO pigs

When 176 cloned embryos derived from DK3-2 were transferred into 2 recipients, both became pregnant, and 2 stillborn piglets were delivered (Table 4). The body weights and crown-rump lengths of these piglets were also much less than those of the normal piglets: 480 g compared with 720 g and 19 cm compared with 23 cm, respectively (Table 5). These stillborn piglets were delivered 2 days later than the expected date of farrowing. This delay may explain the weak DNA amplification signal obtained by PCR (Fig. 10).

Homozygous knock-out of the GalT gene was confirmed in all of the cloned pigs by PCR (Fujimura et al., 2008a; Fujimura et al., 2008b; Takahagi et al., 2005) (Fig. 10).

Transmission of the two transgenes, i.e., hDAF and GnT-III, was also confirmed in the cloned offspring. In the present study, we did not examine expression of these transgenes. However, faithful expression of transgenes in the cloned offspring produced by SCNT of a polytransgenic pig has been demonstrated in our previous studies (Fujimura et al., 2008a; Fujimura et al., 2008b; Takahagi et al., 2005). The influence of epigenetic modification on gene expression in cloned pigs needs to be investigated.

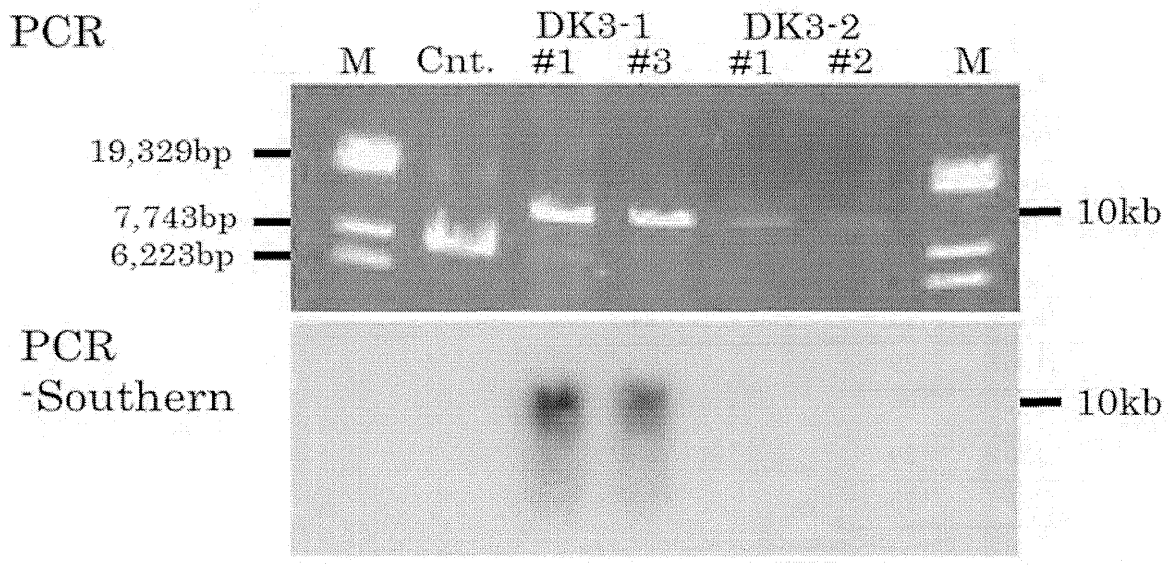

Fig. 10. PCR and PCR-Southern analysis of cloned pigs derived from homozygous GalT-KO pig somatic cells

Cloned pigs were produced from cells derived from DK3-9 (Table 4). DK3-9 is a GalT-KO homozygote and has no other genetic modification or transgene integration. The cloned embryos were treated with 500 nM Scriptaid for 15–20 hours. When 377 SCNT embryos were transplanted at the early cleavage stages (day 1 to 3), all 4 recipients became pregnant, and one sow farrowed seven live offspring. The average body weight and crown-rump length of the cloned piglets derived from the DK3-9 cells were 797.1+/- 104.2 g and 23.6 +/- 1.2 cm (Table 5). The other recipients had miscarriages. In the cloning experiments with DK3-9 cells, several miscarriages occurred, although the SCNT embryos had been treated with HDACi Scriptaid. This result is in contrast to the results of the cloning of DK3-1. It may be pertinent that the experiments with DK3-9 were performed during one of the hottest summers recorded in Japan, and this may have affected the recipients' pregnancies; further investigation will be necessary to identify the causes of the difference in experimental outcomes. Most of the cloned offspring with a normal range body weight were healthy and grew normally (Figs. 11, 12).

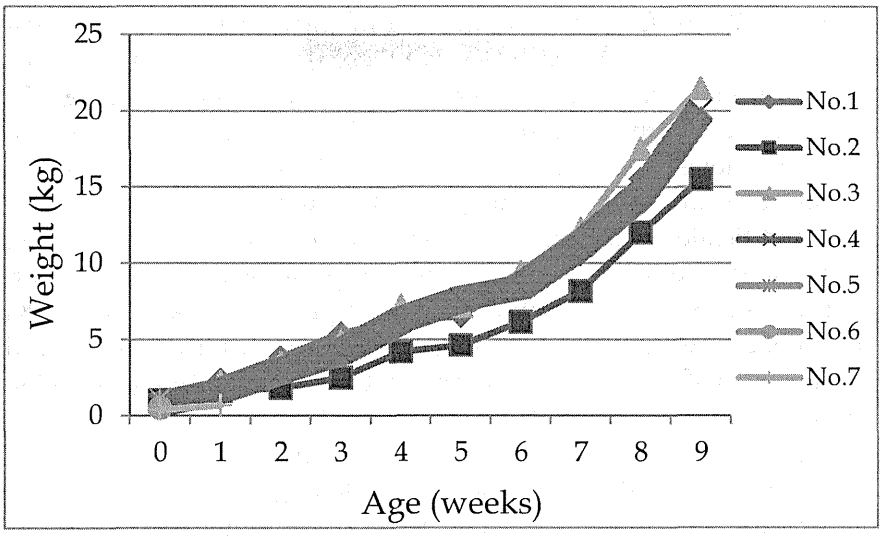

Fig. 11. Growth of the cloned pigs derived from the GalT-KO cells (DK3-9)

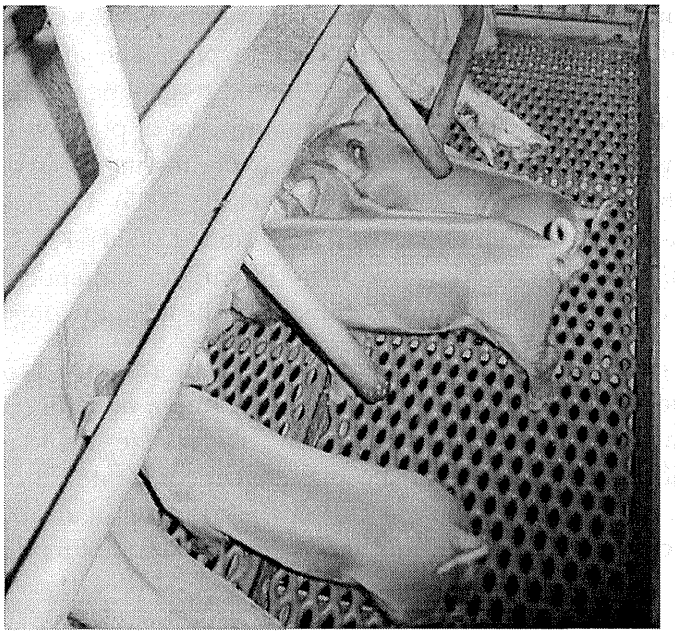

Fig. 12. Live offspring of a GalT-KO pig (DK3-9) produced by somatic cell cloning

The GalT-KO pig (DK3-9) cells that we created were transported by air from Japan to Germany and used to produce cloned pigs. As shown in Table 4, 214 cloned embryos were produced and transferred into 2 recipient sows. Both recipients became pregnant, and one cloned offspring was produced.

6. The prospect of the development of genetically modified pigs and the development of "rainbow pigs"

To bring xenotransplantation into clinical application, it is essential to overcome xenograft rejections, such as hyperacute rejection, delayed xenograft rejection, and cellular rejection

(Ekser and Cooper, 2010). Genetic modification of pigs by technologies based on SCNT would be the most promising approach to solve these issues. Furthermore, the advantage of SCNT is that additional genetic modification can be achieved based on the analysis of phenotypic expression of the resulting genetically modified pigs (Fig. 1). Genetically modified pigs so far developed as xenotransplantation donors including ours (Fujimura et al., 2008a; Fujimura et al., 2008b; Takahagi et al., 2005) will be further improved. With multiple gene modifications in pigs, the feasibility of serial somatic cell cloning (multi-generational cloning) would have important implications. In this regard, we have successfully produced the 4th generation of cloned pigs by serial cloning (Kurome et al., 2008a; Matsunari et al., 2008a). We have developed a process for multi-generational cloning of pigs, that is, a means of proceeding from the first clone generation (G1) to the second (G2) and then to the third generation (G3), and the efficiency of producing cloned pigs throughout this process was almost constant (Kurome et al., 2008a; Matsunari et al., 2008a). Notably, the cloned offspring did not show any shortening of their telomeres (Kurome et al., 2008a) and grew normally. Our results indicate that serial cloning is a feasible option for production of specifically designed pigs with multiple genetic modifications.

In this study, we investigated the feasibility of cloning the GalT-KO pigs by focusing on the introduction of multiple genetic modifications into pigs. The application of somatic cell cloning in xenotransplantation research is not limited to genetic modifications. Somatic cell cloning could also be used for reproducing large numbers of pigs with a preexisting genetic modification for use in organ transplantation experiments in primates and for other preclinical research (Kuwaki et al., 2005; Yamada et al., 2005). We have already started an organ transplantation experiment using the cloned GalT-KO pigs (unpublished). Additionally, as already discussed, while it is difficult to transport genetically modified pigs, it is a comparatively simple matter to transport cells from the pigs by air to overseas.

Development of genetically modified pigs has been attempted in several countries, including Australia, Britain, Germany, Japan, South Korea and the USA, with the aim of applying such animals in xenotransplantation. To realize their potential for clinical applications, it will be necessary to create pigs that incorporate all of the desired genetic modifications. We shall continue our pursuit of the ultimate goal of producing "rainbow" pigs—cloned pigs covering every possible genetic modification of medical interest.

7. Conclusion

In conclusion, the data presented in this chapter demonstrate that homozygous GalT-KO pigs can be efficiently reproduced by SCNT; hence, cloning technology is suggested to be a feasible option for proliferation of the genetically modified pigs. As part of the process of developing organ donors for xenotransplantation, somatic cell cloning provides an efficient and superior technology with high reliability and reproducibility for creating and reproducing pigs with multiple genetic modifications.

8. Acknowledgement

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ORIGINAL ARTICLE

Trial using pig cells with the H–D antigen knocked down

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Abstract
Purpose This report describes an attempt to reduce the expression level of Hanganutziu–Deicher (H–D) antigens by small interfering RNA (siRNA) for pig cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (pCMAH).
Methods A pig endothelial cell (PEC) line, and PEC and fibroblasts from an α1,3galactosyltransferase knockout (GalT-KO) piglet were used. Real-time PCR was used to evaluate the degradation of mRNA by siRNA. The H–D antigen was stained, and then the cells were incubated with human serum for the FACS analysis. The extent of lysis in human serum was next calculated using an LDH assay.
Results Suppression of the mRNA of pCMAH by each siRNA was first determined. The mixture of siRNAs for pCMAH reduced the expressions of the H–D antigen on the PEC and fibroblasts to a considerable extent. The further reduction in the xenoantigenicity for human serum of the GalT-KO cells was then confirmed. In addition, the PEC line showed a significant downregulation in complement-dependent cytotoxicity by the siRNAs, thus indicating that the anti-H–D antigen in human serum is capable of causing lysis of the pig cells.
Conclusion pCMAH silencing by siRNA reduced the expression of the H–D antigen and its antigenicity, thus confirming that the H–D antigen is one of the major non-Gal antigens in this situation.

Keywords Xenotransplantation · Hanganutziu–Deicher (H–D) antigen · CMAH gene · Knockdown · Non-Gal antigen

Abbreviations

H–D	Hanganutziu–Deicher
siRNA	Short interfering RNA
CMAH	Cytidine monophospho-*N*-acetylneuraminic acid hydroxylase
PEC	Pig endothelial cell
PCR	Polymerase chain reaction
FACS	Fluorescence-activated cell sorter

Introduction

The potential for clinical applications of xenotransplantation has generated considerable interest in recent years. However, hyperacute rejection (HR) and humoral rejection continue to represent formidable obstacles to the successful clinical use of this technique, especially in the case of organ transplantation [1, 2].

The major xenoantigen responsible for hyperacute rejection is the α-Gal epitope (Galα1–3Galβ1–4GlcNAc-R), which is expressed in most mammalian cells, including the pig [3]. Knocking out the α-Gal epitope prevents hyperacute rejection [4–6], and it appears that the Hanganutziu–Deicher (H–D) antigen could thus be the cause of

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the strong humoral response observed in pig-to-human transplantation. The H–D antigen, with a terminal *N*-glycolyl neuraminic acid (NeuGc), is widely distributed in the animal kingdom, with the exception of humans and chickens. The expression of NeuGc is controlled by the action of the enzyme, cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (CMAH) [7, 8].

On the other hand, a small interfering RNA (siRNA) represents a mechanism for posttranscriptional gene silencing that has been described in plants, invertebrates and in mammalian cells [9]. It has been applied to inhibit the integration of HIV-1, and also the porcine endogenous retrovirus (PERV) [10].

This study reports on an attempt to reduce the expression levels of H–D antigens by siRNA for pig CMAH and a study of the antigenicity of the H–D antigen in human serum, as the first step in producing H–D antigen knockdown or knockout pigs.

Materials and methods

Cell line

The heterozygous α1,3galactosyltransferase knockout (GalT-KO) pigs were successfully cloned by somatic cell nuclear transfer (SCNT). Natural breeding of the heterozygous GalT-KO pigs also gave rise to homozygous GalT-KO pigs [6].

A pig endothelial cell (PEC) line, MYP30 [11], and PEC and fibroblasts from GalT-KO piglets were used in the study. Culture media were supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and kanamycin/amphotericin B. The cultures were maintained in a 5 % CO_2/95 % air atmosphere at 37 °C.

Transfection of synthetic siRNA

The pig cells were seeded in a 10-cm dish with D-MEM containing 10 % FBS. The medium was replaced by FBS-free D-MEM the next day. Four siRNAs (21 mer) (Gene Design, Osaka, Japan) and three siRNAs [25 mer: Stealth (S)] (Life Technology, Tokyo, Japan) were designed, #1: GC CAAUCUCAAGGAAGGAAUC, #3: GCCUGAAGGCAU GUAAGAACA, #4: GCUGAGCCGCGCAGAUUUAAU, #5: GGAGCUUCAGGCUUUCCAAUG, #S2: CACAUGC ACUCAGACCACCUGAGUU, #S6: CCAAUGGAGGA AGGCUGCCUAUGAA, #S8: GAAUCCCACCCAGCAG ACAAGUAUA. The nominated siRNAs were diluted with Opti-MEM and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and incubated for 20 min at room temperature, and added to the cells, thus resulting in a final siRNA concentration of 20 nM. The medium was replaced with D-MEM containing 10 % FBS after 4 h [9].

Quantitative real-time RT-PCR

Total RNA was collected from the transfectants, using the TRIZOL LS Reagent (Invitrogen). The total RNA was used in reverse transcriptase (RT) reactions.

SYBR-Green real-time polymerase chain reaction (PCR) was performed using a Smart Cycler II System (Takara, Tokyo, Japan) and the SYBR premix Taq (Takara), to evaluate the degradation of the mRNA. The pig CAMH sequence was amplified using the two primer pairs below: 5′-AGACCAAGTCGGGAACATCCA-3′ (sense) and 5′-CCTATGTACAAGTCATCCCAGAGCA-3′ (antisense). RT was carried at 42 °C for 15 min, followed by 95 °C for 2 min, using random primers, followed by 45 cycles of PCR at 95 °C for 5 s and 60 °C for 20 s.

The amount of mRNA of the pig CAMH in the transfectants was normalized with the level of GAPDH RNA: 5′-GACAACTTCGGCATCGTGGA-3′(sense) and 5′-CTT GGCAGCACCAGTAGAAGCA-3′(antisense).

The extent of mRNA degradation in the transfectants with siRNA was then calculated [12].

Flow cytometry analysis

The cell-surface H–D antigens were stained with a chicken anti-H–D antigen polyclonal antibody (a gift from Prof. N. Wakamiya, Asahikawa Medical College, Hokkaido, Japan) for 1 h at 4 °C, and FITC-conjugated rabbit anti-chicken IgG (Cappel) as a second antibody for 1 h at 4 °C.

The pig cells were also incubated with a 10 % solution of normal human pooled serum (NHS) from type O blood for 1 h at 4 °C, washed and then incubated with 1.25 mg of FITC-conjugated anti-human IgG and IgM (Cappel, West Chester, PA, USA) as a second antibody for 1 h at 4 °C. The stained cells were analyzed with a fluorescence-activated cell sorter (FACS) Calibur flow cytometer (Nippon Becton Dickinson, Tokyo, Japan).

Lactate dehydrogenase (LDH) assay for the complement-dependent cytotoxicity

This assay was performed following a previously described method, using a MTX-LDH kit (Kyokuto, Tokyo, Japan). The cells were plated at a concentration of 2×10^4 per well in a 96-well tray, 1 day prior to the assay. After 15 h, the plates were then incubated with 20 or 40 % NHS for 2 h at 37 °C, and the released LDH was then determined. The spontaneous release of LDH activity from the target cells was <5 % of the maximal release of LDH activity, determined by the complete target cell lysate by treatment with 10 % TritonX [10].

Statistics

Data are presented as the mean ± SD. Student's t test was used to ascertain the significance of differences within groups. Differences were considered to be statistically significant when $p < 0.05$.

Results

Localization and efficacy of the siRNA targeting pig CMAH

Several synthetic siRNAs to target the gene were designed according to the published guidelines. A schematic presentation of the pig CMAH with localization of the siRNA is shown in Fig. 1. Two types of siRNA were used in this experiment. The expression of the mRNA of the pig CMAH was then measured by real-time RT-PCR. Each siRNA for the pig CAMH substantially reduced the expression of the mRNA in PEC, MYP-30.

In addition, the maximum reduction in the mRNA of pCMAH remained high from day 3 to day 5 after the transfection of each siRNA (data not shown).

The expression of the H–D antigen is inhibited by the siRNA for pig CMAH

PECs and fibroblasts from a GalT-KO pig were used to check the changes in the expression of the H–D antigen by knocking down the CMAH mRNA, using a mixture of the siRNAs (10 nM of #1, #5 and #S8 in Fig. 1). The percentage expression of the H–D antigen in the cells with the siRNAs is indicated in comparison to those in the parental cells (without siRNA), based on data from a FACS analysis. The siRNA for pig CMAH reduced the expression of the H–D antigen of PEC and fibroblasts to a considerable extent (Fig. 2).

The reduction in the antigenicity of the pig cells in human serum by the siRNA for pig CMAH

PECs and fibroblasts from a GalT-KO pig were next used to check the changes in total antigenicity for the human natural antibody by knocking down the CMAH gene, using the same mixture of the siRNAs as shown in Fig. 2. The percentage expression of the total antigenicity of the pig cells to human serum was compared with that for parental cells (without siRNA). The xenoantigenicity of the PECs from a GalT-KO pig was 18.5 ± 8.5 % ($n = 3$), in comparison to wild-type cells, MYP-30. FACS analyses clearly confirmed the further reduction in the xenoantigenicity of the GalT-KO cells by the siRNA for pig CMAH. These data indicated that pCMAH silencing by siRNA resulted in a reduction in not only the H–D antigen, but also in the total xenoantigenicity in human serum as well (Fig. 3).

LDH assay for the pig cells

Amelioration of the complement-dependent cytotoxicity of human serum to the PECs by downregulating the H–D antigen was studied next. MYP-30, a PEC line, was used in these experiments. The results showed that the siRNA mixture caused a significant downregulation in cytotoxicity for pig CMAH by human serum (Fig. 4).

The net values for the lysis of GalT-KO cells by NHS were so low that significant differences between the parental cells and those with siRNAs could not be determined (data not shown).

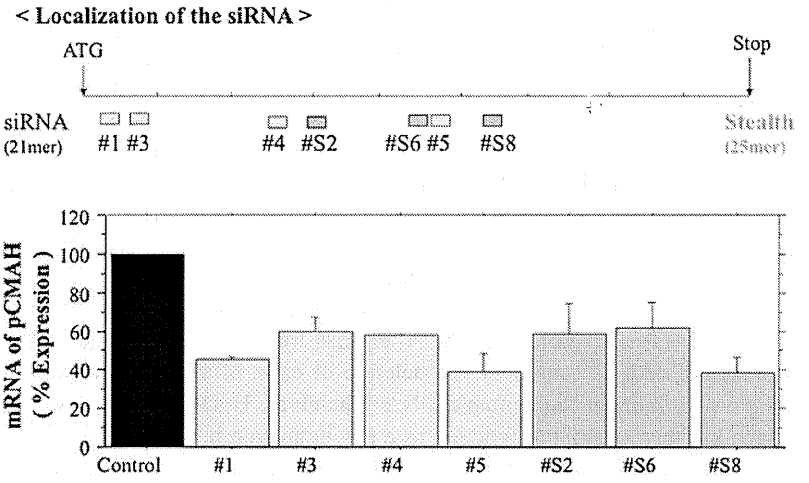

Fig. 1 Localization of siRNA and the percentage expression of the mRNA of CMAH by each siRNA. Schematic presentation of pig CMAH with the localization of the siRNA was indicated. The efficacy of each siRNA for the mRNA of the pig CMAH, in a PEC line, MYP-30, was measured by real-time RT-PCR ($n = 3$)

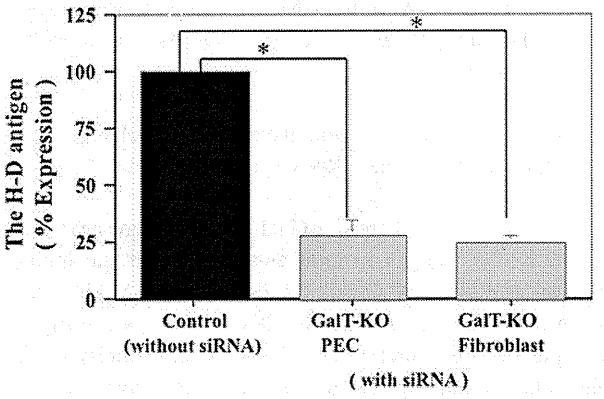

Fig. 2 The effect of siRNA on the expression of the H–D antigen by pig cells. PECs and fibroblasts from a GalT-KO pig were used to check the changes in the H–D antigen for the mixture of the siRNAs (#1, #5 and #S8 in Fig. 1). The percentage expression of the H–D antigen is indicated in comparison to the that for parental cells (without siRNA), using FACS analysis ($n = 5$). The *asterisks* indicate significant differences versus parental controls (without siRNA; $*p < 0.05$)

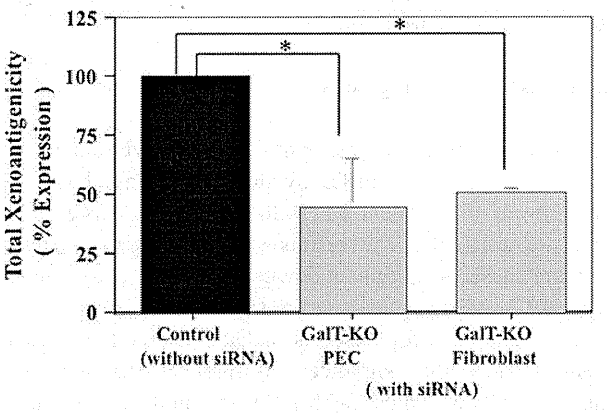

Fig. 3 Deducing the antigenicity on pig cells for human serum by the downregulation of the H–D antigen. PECs and fibroblasts from a GalT-KO pig were next used to check the changes in antigenicity for the human natural antibody by knocking down the CMAH gene using FACS analysis. The percentage expression of antigenicity is indicated in comparison with the parental cells (without siRNA; $n = 5$). The asterisks *indicate* significant differences versus controls ($*p < 0.05$)

Discussion

H–D antigen is widely distributed in mammalian species, including monkeys and apes, but it is not found in humans and birds. The expression of NeuGc is controlled by the enzymatic activity of CMAH. Two different groups, Irie et al. [13] and Chou et al. [14] have cloned the cDNA for human CMAH and reported that the N-terminal truncation of human CMAH is caused by the deletion of exon 6, a 92-base long pair, in the genomic DNA. Therefore, H–D antigen has the potential to function as a potent antigen in pigs to human xenotransplantation after α1,3GT is knocked

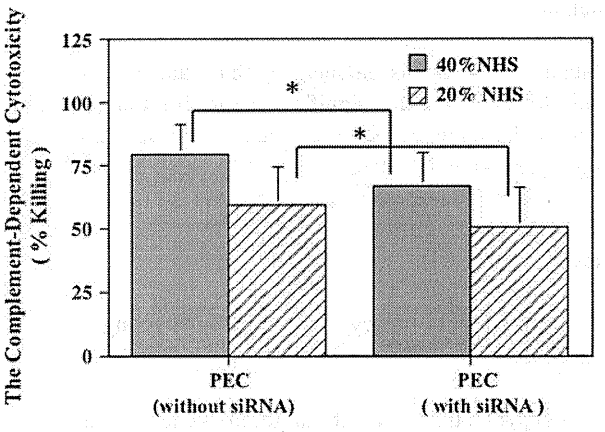

Fig. 4 Amelioration of the complement-mediated cytotoxicity of human serum to pig cells by the downregulation of the H–D antigen. Wild-type PECs, MYP-30, were incubated with 20 or 40 % NHS for 2 h at 37 °C, and the amount of LDH released was then determined ($n = 8$). The *asterisks* indicate significant differences versus controls (without siRNA; $*p < 0.05$)

out. On the other hand, Zhu et al. [15, 16] indicated that the majority of human anti-non-Gal antibodies are specific for the H–D antigen (NeuGc). These experiments involved the use of flow cytometry and pig red blood cells that had been pre-incubated with sialidase. Furthermore, this anti-NeuGc activity was detectable in 85 % of healthy humans. Magnusson et al. [17] reported that, the staining of both α-Gal terminal structures as well as the H–D reactive gangliosides, NeuGc-GM3 and NeuGc-GD3, increased in a patient who had been extracorporeally perfused with a pig kidney. Another study, using ELISA, also showed the existence of anti-H–D IgM and IgG in all naive human sera [18]. In addition, Basnet et al. [19] using a mouse strain lacking both the α-Gal and H–D antigen, reported that the antigenicity of the H–D antigen in mice cells for human serum was significant but weaker in comparison with α-Gal. They also suggested that it is unlikely that the anti-H–D antibody is involved in hyperacute rejection, but that the H–D antigen may elicit a significant humoral response and could thus play a role in a delayed form of rejection.

On the other hand, several strategies for reducing the H–D antigen levels in pig cells are now under consideration and include knocking down and knocking out the CMAH gene. Findings reported in a previous study indicated that the overexpression of *N*-acetylglucosaminyltransferase-III (GnT-III) could possibly reduce the levels of H–D antigen. However, GnT-III acts only on an N-linked sugar of a glycoprotein, even if it clearly has antigenic properties, including the H–D antigen [20]. Song et al. [21] previously reported on the knockdown of CMAH expression in pig kidney cell line, PK15. The siRNA for pig CMAH in PEC line also substantially reduced the expressions of the mRNA and the H–D antigen. The further reduction in the

xenoantigenicity of the GalT-KO PECs and fibroblasts from in vivo by the siRNA for pig CMAH was confirmed in this study. In spite of the fact that changes in the cytotoxicity of the GalT-KO cells, as a result of knocking down the H–D antigen is not clear, the silencing of pig CMAH by siRNA resulted in a reduction in the H–D antigen and the antigenicity to human serum, thus confirming that the H–D antigen produced by pig CMAH is one of the major non-Gal antigens.

In addition, the significance of the H–D antigen in the area of in vivo rejection remains a controversial topic. Studies using large animals, such as pig to monkey organ transplantation, are therefore required to assess the role of this antigen in xenograft rejection. However, the fact that many animals besides humans expresses this antigen indicates the difficulty in the design of such in vivo experiments.

The current data are consistent with the previous studies mentioned above [15–21], thereby suggesting the H–D antigen to be one of the major non-Gal antigens and the next knockout target. The results are thus considered to represent useful information for future clinical xenotransplantation studies.

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Conflict of interest None of the authors has any conflict of interest to declare.

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A comparison of the main structures of *N*-glycans of porcine islets with those from humans

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After producing α1-3-galactosyltransferase knockout (GKO) pigs, most of the organs of these pigs showed less antigenicity to the human body. However, wild-type adult pig islets (API) that originally contained negligible levels of α-galactosidase now showed a clear antigenicity to human serum. In this study, *N*-glycans were isolated from both APIs and human islets. Their structures were then analyzed by a mapping technique based on their high-performance liquid chromatography elution positions and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometric data. Both preparations contained substantial amounts of high-mannose structures. The *N*-glycans from human islets were separated into 17 neutral, 8 mono-sialyl and 4 di-sialyl glycans, and the API glycans were comprised of 11 neutral, 8 mono-sialyl, 3 di-sialyl, 2 mono-sulfated, 3 mono-sialyl-mono-sulfated and 1 di-sulfated glycans. Among them, the API preparation contained one neutral, five mono-sialyl glycans and six sulfated glycans that were not detected in human islets. The structures of 9 of these 12 could be clearly determined. In addition, a study of the sulfate-depleted API suggests that sulfate residues could be antigenic to humans. The data herein will be helpful for future studies of the antigenicity associated with API.

Keywords: *N*-glycan / pig islets / sulfated glycan / xenotransplantation

Introduction

The increasing challenges associated with the worldwide shortage of donor organs have led to a renewed interest in xenotransplantation. The pig pancreas is considered to be the most suitable source of islets for clinical xenotransplantation. Some clinical trials have resumed in New Zealand, Russia, etc., using islets from a wild-type pig via the use of an immuno-isolation technique (Elliott 2011). In addition, based on data collected from the "Inventory of human xenotransplantation practices" (http://www.humanxenotransplant.org/index.html), many clinical trials appear to be ongoing.

On the other hand, after producing α1-3-galactosyltransferase knockout (GKO) pigs (Dai et al. 2002; Takahagi et al. 2005), most of the organs from these pigs were found to show less antigenicity to the human body. However, wild-type adult pig islets (API) that originally contained negligible levels of α-galactosidase (α-Gal) (Galα1-3Gal) (Galili et al. 1987) showed a clear antigenicity to human serum (Komoda et al. 2004), and this fact represents a significant obstacle to successful xenotransplantation (Thompson et al. 2011).

Concerning the so-called non-Gal epitopes, many studies related to glycoproteins and glycolopids are on-going in attempts to identify them. However, our knowledge of non-Gal glycoantigens is still incomplete. That is, previous analyses of *N*-glycans from pigs included the use of additional tissues, in addition to islets. However, besides α-Gal and Hanganutziu-Deicher (Varki et al. 2009; Yamamoto et al. 2013) antigen expression, the glycosylation of API remains relatively unclear (Breimer 2011; Byrne et al. 2011; Miyagawa et al. 2012).

We wish to report herein on the analysis of the glycosylation of the *N*-linked sugars of API, compared with the corresponding values for human islets, using a high-performance liquid chromatography (HPLC) technique, which is capable of providing reliable data. The collected data will be of use in future research concerning non-Gal antigens and promises to provide us with clues for producing new types of immuno-modified pigs with less antigenicity than GKO pigs.

Results

Isolation of N*-glycans of the porcine and human islets*

N-glycans derived from porcine (11.9 mg of protein) and human islets (12.47 mg of protein) were separated into five peaks, based on increasing acidity using a diethylaminoethyl (DEAE) column. The following peaks were produced: Neutral

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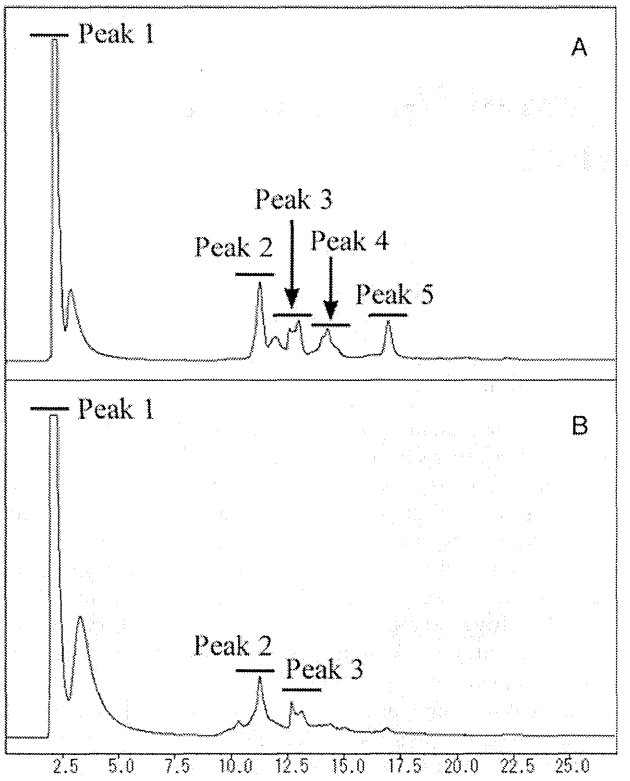


Fig. 1. Anion exchange DEAE elution profiles of PA-glycans derived from porcine islets (**A**) and the human islets (**B**). The PA-glycans were fractionated according to their sialic acid content and sulfate residues as neutral (Peak 1), mono-sialyl (Peak 2), di-sialyl or mono-sulfate (Peak 3), mono-sialyl-mono-sulfate (Peak 4) and di-sulfate (Peak 5) oligosaccharide fractions as indicated.

(N), Peak 1; mono-sialyl (M), Peak 2; di-sialyl (D) or mono-sulfate (S1), Peak 3; mono-sialyl-mono-sulfate (MS2), Peak 4 and di-sulfate (S2), Peak 5; glycan fractions with molar ratios (peak areas) of 84.4, 3.6, 2.6, 2.2 and 7.2% from APIs, and 94.0, 4.0, 2.0, 0 and 0% from the human islets, respectively (Figure 1).

Concerning API, when an octa decyl silyl (ODS) column was used, it was possible to separate the neutral fraction into fractions N1–N9, the mono-sialyl fraction into fractions M1–M6, the di-sialyl or mono-sulfate fraction into fractions D1–D3 and S1, the mono-sialyl-mono-sulfate fraction into fractions MS1–MS3 and the di-sulfate fraction into fraction S2. On the other hand, in the case of human islets, the neutral fraction was separated into N1–N13, the mono-sialyl into M1–M6 and the di-sialyl into D1–D4, as shown in Figure 2A–H.

Further analysis with GALAXY database

These ODS fractions were individually fractionated on an amide column and further subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF-MS) analysis. The porcine N2, N6, M2, M3 and S1 and the human N2, N5, N6, N12, M2 and M4 fractions were found to contain two kinds of *N*-glycans (Figures 3 and 4).

The coordinates of all of the *N*-glycans coincided with those for known references in the glycoanalysis by the three axes of MS and chromatography (GALAXY) database except for several *N*-glycan fractions including human fractions N5-1, N5-2, N6-1, N6-2, M1, M2-2 and porcine S1-1, MS1and MS3. Most of the *N*-glycan structures were then identified by the mapping technique on the basis of their HPLC elution positions and MALDI-TOF-MS data.

Structural analysis of each sample

Pyridylamino (PA)-glycans, which did not correspond to any of the *N*-glycans so far registered in GALAXY, were trimmed by treatment with an exoglycosidase, which produced known glycans (Yagi et al. 2005).

In the case of S1-2, no reactivity was detected by β-acethyl-hexiominidase. Next, a methanolysis treatment induced the conversion of S1-2 into S1-2a, but additional treatment with β-galactosidase resulted in no change to S1-2a. Moreover, the β-*N*-acethylhexosaminidase treatment converted S1-2a into S1-2b, and S1-2b was proved to be the same structure as M4.1 in GALAXY, as evidenced by the observation that samples of S1-2b and M4.1 co-chromatographed (Figure 5).

The MS2 sample was analyzed following a similar procedure. The sample did not serve as a substrate for β-galactosidase and α2,3-sialydase, but was converted into MS2a by treatment with α-sialydase. Further methanolysis and β-galactosidase converted MS2a into MS2b and MS2c, respectively. MS2b was next verified to be 210.4a in GALAXY by the co-chromatography of both samples. On the other hand, MS2c, when treated with β-*N*-acethylhexosaminidase and methanolysis, was converted into MS2d and MS2e, respectively. MS2e was also proved to be 110.4a in GALAXY by the co-chromatography of both samples (Figure 6).

Concerning S2, the sample was unchanged as a result of a β-*N*-acethylhexosaminidase treatment. On the other hand, a methanolysis treatment cleaved two sulfate residues from S2 and produced S2a, which was shown to be 210.4b in GALAXY by the co-chromatography of both samples (Figure 7).

In the analyses, a total of 28 and 29 *N*-glycan structures of API and human islets, respectively, were identified and the findings are summarized in Tables I–VI (Supplementary data, Figure S1).

Sodium chlorate treatment on pig islets

The effect of removal of the sulfate structures of pig islets on the antigenicity to human serum was investigated. The use of a sodium chlorate and a sulfate-free medium led to a significant reduction in antigenicity to human serum, suggesting that the sulfate structures in adult islets are targets for human natural antibodies (Figure 8).

Discussion

Twenty-eight kinds of *N*-linked glycans were identified in the case of the API glycans and 29 were identified from human islets, based on their HPLC elution peaks. While the human preparation contained neutral, mono-sialyl, di-sialyl *N*-linked glycans, the API sample contained not only these three types, but mono-sulfate, MS2 and di-sulfate types of *N*-linked glycans

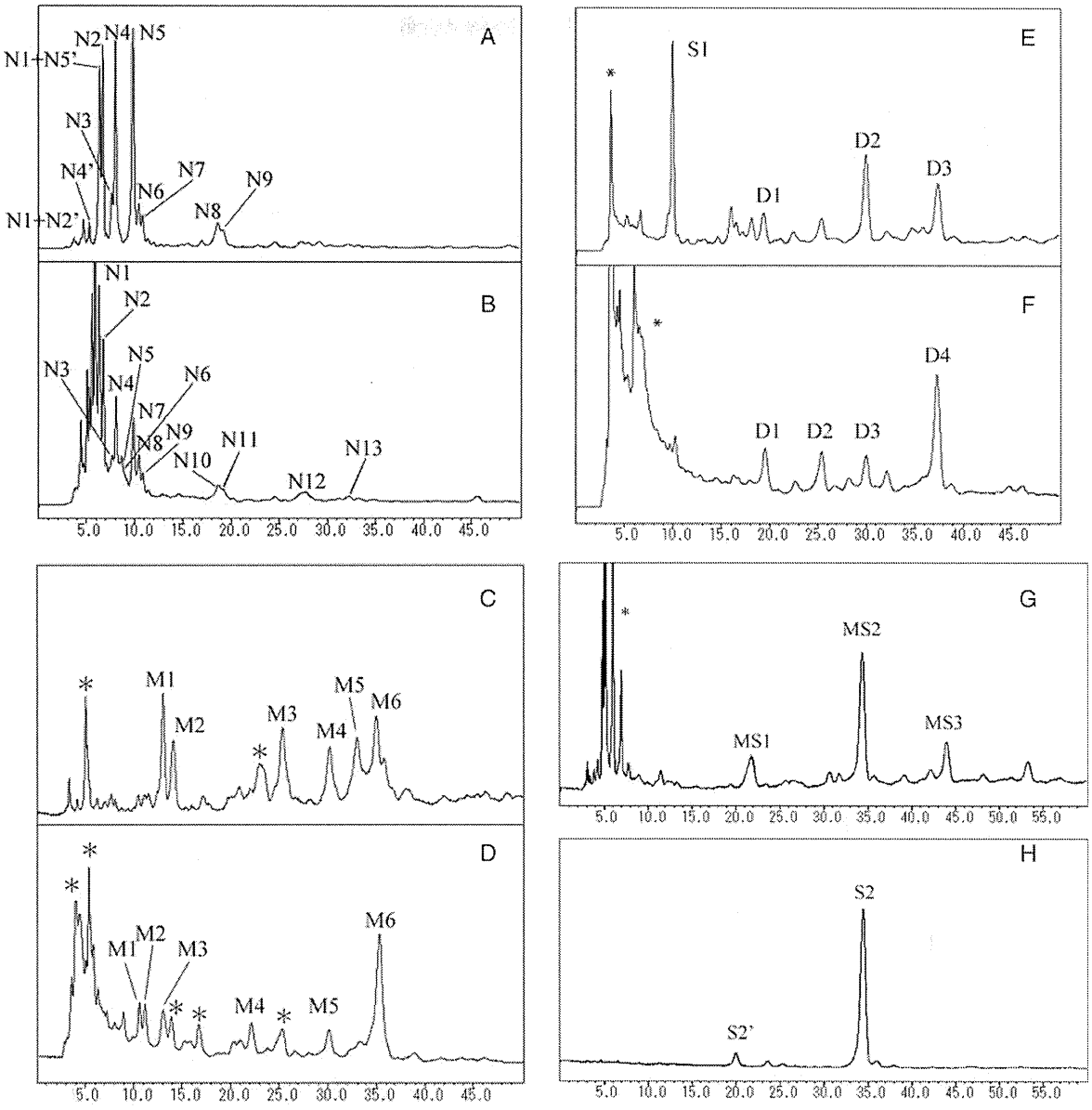


Fig. 2. Reverse-phase ODS elution profiles of PA-glycans obtained from each different fraction separated on the DEAE column. The neutral, mono-sialyl, di-sialyl or mono-sulfate, mono-sialyl-mono-sulfate and di-sulfate fractions were individually applied to the ODS column and gave elution profiles according to their hydrophobicity. (**A**) pig Peak 1, (**B**) human Peak 1, (**C**) pig Peak 2, (**D**) human Peak 2, (**E**) pig Peak 3, (**F**) human Peak 3, (**G**) pig Peak 4 and (**H**) pig Peak 5. N2′: Epimerization of N2; N4′: Epimerization of N4; N5′: Epimerization of N5; S2′: Epimerization of S2. Asterisks indicate the fractions containing no detectable PA-oligosaccharides.

as well. Among them, one neutral, five mono-sialyl and six sulfates of *N*-linked glycans in the API preparation were not detected in human islets. The structures of 9 of these 12 glycans were clearly identified in this study.

Concerning the characteristics of the *N*-glycans identified in the API preparation, the neutral glycans contained relatively high levels (%) of high-mannose type glycans. In comparison with the *N*-glycans from human islets, the high-mannose type of *N*-glycan found in API contains high levels (5 or 6) of mannoses. In addition, glycans with structures of fractions N6-2 were not detected in human islets. On the other hand, in the case of API, the relative content of sulfated *N*-glycans approached 10%. In addition, the di-sulfate type glycans represented 7% of the relative quantity, indicating that sulfated *N*-glycans are a common structure in *N*-glycans of API but do not appear to be produced by human islets. In addition, all the sulfates are attached to a β-linked *N*-acetylgalactosamine (GalNAc).

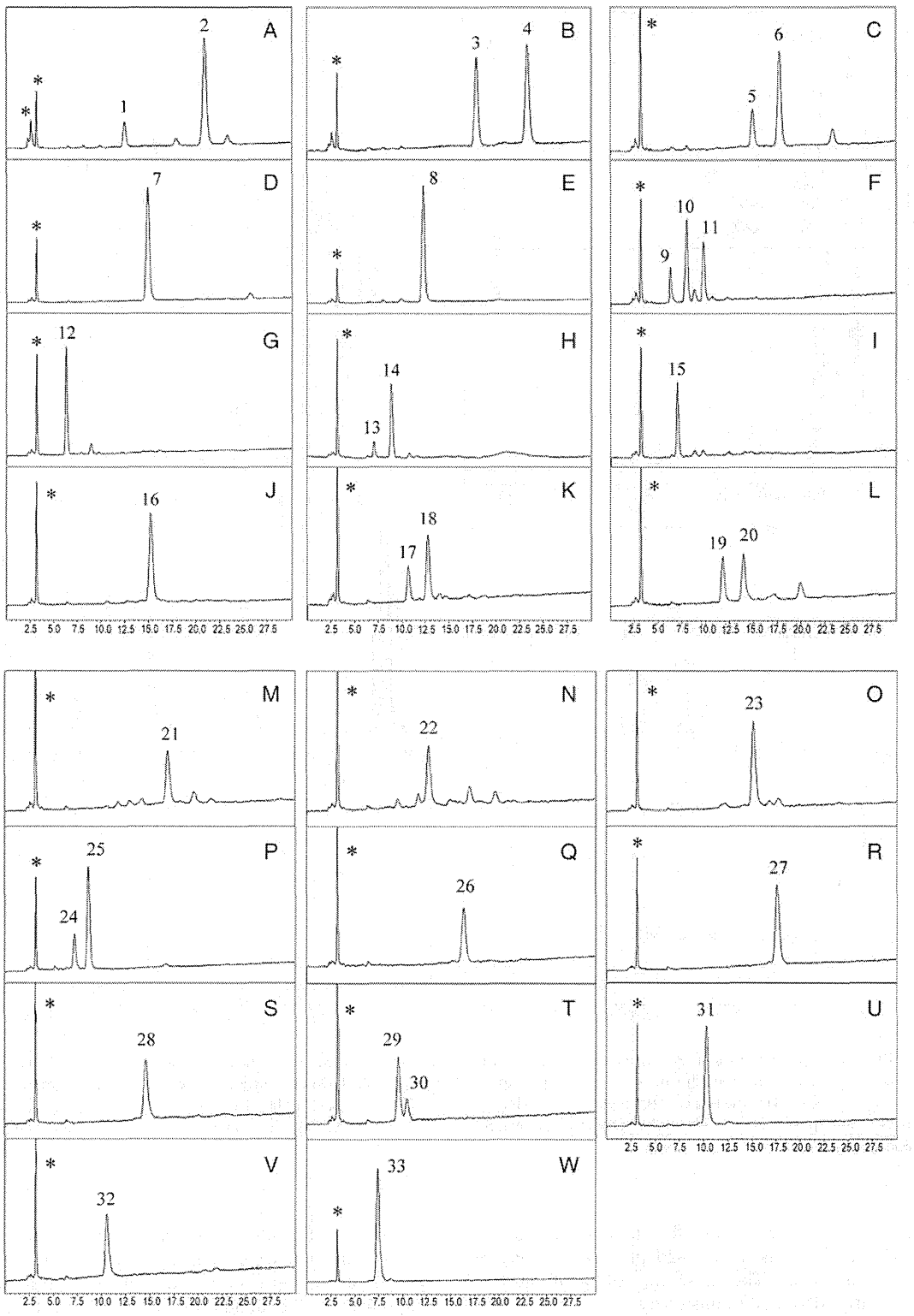


Fig. 3. Amide column elution profiles of PA-glycans of pig islets from each different fraction separated on the ODS column. (**A**) ODS peak-N1 + N5′. Peak 1 is the epimerization of the ODS peak-N5. Peak 2 was then settled as N1. (**B**) ODS peak-N2. Peaks 3 and 4 correspond to N2-1 and N2-2, respectively. (**C**) ODS peak-N3. Peak 5 was contamination of the ODS peak-N4. Peak 6 corresponds to N3. (**D**) ODS peak-N4. Peak 7 corresponds to N4. (**E**) ODS peak-N5. Peak 8 corresponds to N5. (**F**) ODS peak-N6. Peak 9 was contamination of ODS peak-N7. Peaks 10 and 11 correspond to N6-1 and N6-2, respectively. (**G**) ODS peak-N7. Peak 12 corresponds to N7. (**H**) ODS peak-N8. Peak 13 was contamination of ODS peak-N9. Peak 14 corresponds to N8. (**I**) ODS peak-N9. Peak 15 corresponds to N9. (**J**) ODS peak-M1.

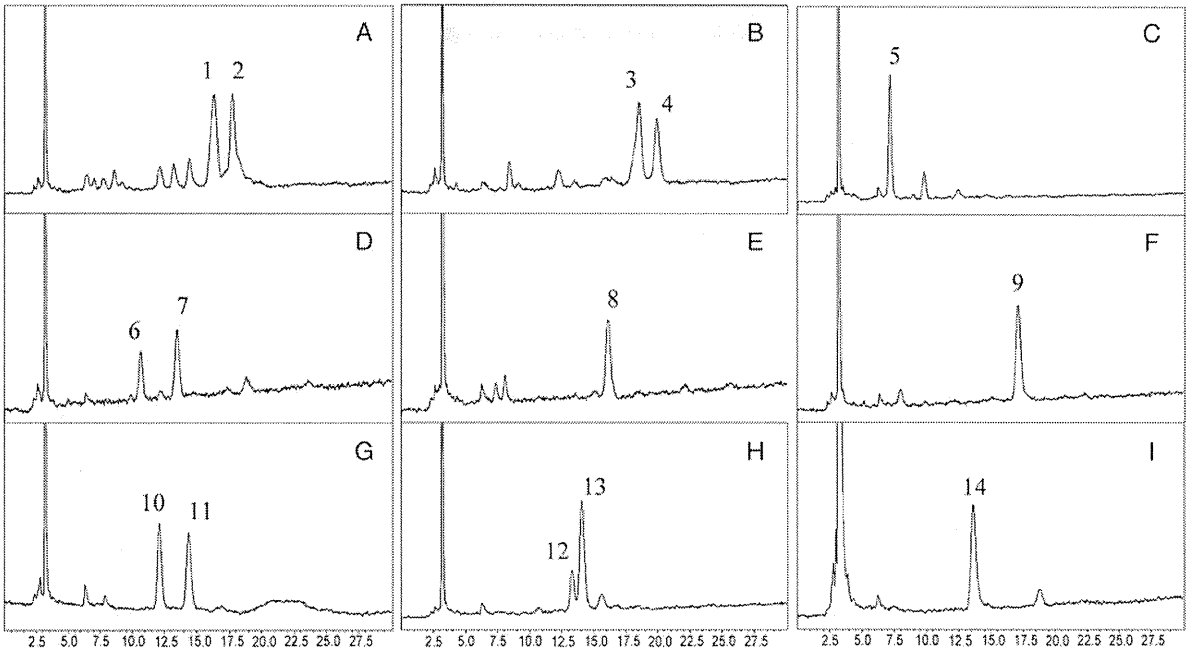


Fig. 4. Amide column elution profiles of PA-glycans from each fraction separated on the ODS column of human islets. (**A**) ODS peak-N5. Peaks 1 and 2 correspond to N5-1 and N5-2, respectively. (**B**) ODS peak-N6. Peaks 3 and 4 correspond to N6-1 and N6-2, respectively. (**C**) ODS peak-N11. Peak 5 corresponds to N11. (**D**) ODS peak-N12. Peaks 6 and 7 correspond to N12-1 and N12-2, respectively. (**E**) ODS peak-N13. Peak 8 corresponds to N13. (**F**) ODS peak-M1. Peak 9 corresponds to M1. (**G**) ODS peak-M2. Peaks 10 and 11 correspond to M2-1 and M2-2, respectively. (**H**) ODS peak-M4. Peaks 12 and 13 correspond to M4-1 and M4-2, respectively. (**I**) ODS peak-D2. Peak 14 corresponds to D2.

No terminal fucose was detected in the *N*-glycans from either type of islets in this study.

Previous studies reported by other groups concluded that many kinds of *N*-glycans are found in API, using MALDI-TOF/MS and MS/MS (Kim, Gil et al. 2008; Kim, Gil et al. 2009; Kim, Harvey et al. 2009). The difference in the number of detected *N*-glycans in this study can be attributed to the sensitivity of the MS method and HPLC. It, thus, appears that the accuracy of the data presented here using HPLC mapping in conjunction with a MALDI-TOF technique provided much more detailed information. That is, MS data are sensitive and can be rapidly obtained, but indicate only a glycan structure based on the calculated molecular weight. Therefore, discriminating between isomeric structures becomes difficult (Wheeler and Harvey 2001). In addition, except for N-glycolylneuraminic acid (NeuGc), it does not indicate the specific structure of sialyl acids present. On the other hand, the data reported herein can be used to identify the representative features of each *N*-glycan in the API preparation. However, the possibility that several glycans, such as pN6-2, pM2-1, pM2-2, pM3-1, pM3-2 and pM5, that were not detected in humans islets as major *N*-glycans are expressed in human islets at very low levels cannot be completely excluded. In addition, concerning the sulfated *N*-glycans such as S1-1, S1-2, S2, MS1 and MS3, the accuracy in identifying the position of the SOH3 attached to β1-4GalNAc was not clear in this study, and it is possible that these sulfated glycans also may be produced in human islets or other tissues, because humans produce several sulfotransferase enzymes that can catalyze the attachment of a sulfate to GalNAc (Boregowda et al. 2005).

Chlorate is a selective inhibitor of adenosine triphosphate sulfate adenylyltransferase, the first enzyme in the sulfate activation pathway (Girard et al. 1998). It inhibits all sulfotransferases. Therefore, although API had a diminished antigenicity to human serum, especially IgM, as a result of the presence of sodium chlorate treatment, a structural analysis of the changes on the sulfated *N*-glycans and other nonsulfated glycans of the API after the treatment might be needed to assess antigenicity issues. On the other hand, it was not possible to determine the binding site of the sulfate residue to GalNAc using this method. However, the possibility that the sulfate residue is one of the non-Gal antigens in pig islets cannot be excluded based on the data presented herein. Further study will be needed to analyze the non-Gal antigen in pig islets, especially to sulfotransferase enzymes.

In comparison with a report concerning the pig lung and trachea, using exactly the same HPLC mapping in conjunction with the MALDI-TOF technique, Sriwilaijaroen et al. (2011) reported a relatively small percent of high-mannose type

Peak 16 corresponds to M1. (**K**) ODS peak-M2. Peaks 17 and 18 correspond to M2-1 and M2-2, respectively. (**L**) ODS peak-M3. Peaks 19 and 20 correspond to M3-1 and M3-2, respectively. (**M**) ODS peak-M4. Peak 21 corresponds to M4. **N**: ODS peak-M5. Peak 22 corresponds to M5. (**O**) ODS peak-M6. Peak 23 corresponds to M6. (**P**) ODS peak-S1. Peaks 24 and 25 were identified as S1-1 and S1-2, respectively. (**Q**): ODS peak-D1. Peak 26 corresponds to D1. (**R**) ODS peak-D2. Peak 27 corresponds to D2. (**S**) ODS peak-D3. Peak 28 corresponds to D3. (**T**) ODS peak-MS1. Peak 29 corresponds to MS1. Peak 30 is the epimerization of ODS peak-MS2. (**U**) ODS peak-MS2. Peak 31 corresponds to MS2. (**V**) ODS peak-MS3. Peak 32 corresponds to MS3. (**W**) ODS peak-S2. Peak 33 corresponds to S2. *Not a sugar.

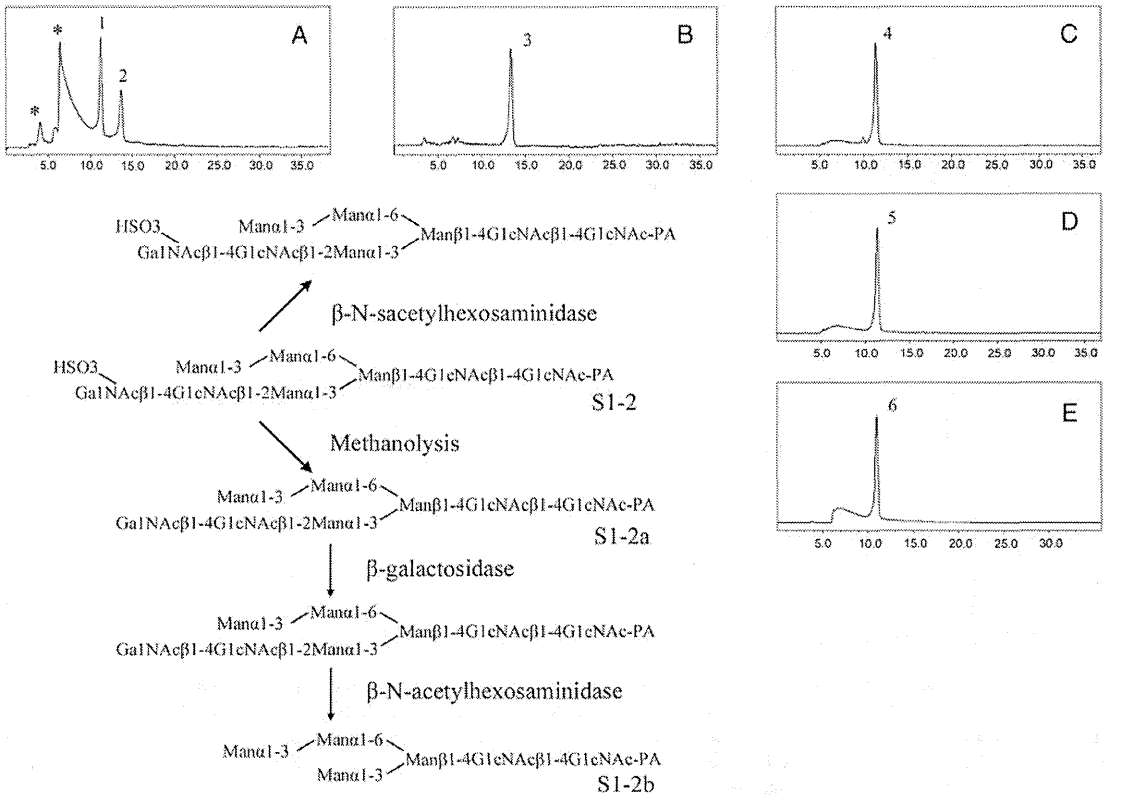


Fig. 5. Structural analysis of S1-2. (**A**) ODS peak after methanolysis treatment of S1-2. Peak 1 is the nonreacted sample, S1-2 (7.5 GU and 1641 Da). Peak 2 corresponds to S1-2a (8.3 GU and 1557 Da). (**B**) ODS peak after β-galactosidase treatment to S1-2a. Peak 3 is identical to S1-2a in ODS (GU) and molecular weight. (**C**) ODS peak after β-*N*-acetylhexosaminidase treatment of S1-2a. Peak 4 corresponds to S1-2b (7.5 GU and 1151 Da). (**D**) ODS peak after co-chromatography of S1-2b and M4.1. S1-2b was then proved to be the same structure as M4.1 in GALAXY. (**E**) ODS peak after β-galactosidase treatment to S1-2. Peak 6 is just the same as S1-2 in GU and molecular weight. * Not a sugar.

N-glycans. However, in this study, pig islets contain a relatively large percent of *N*-glycans, 81%, and human islets also contain 76.7%. Therefore, this evidence related to high-mannose types was assumed to be a typical feature of islets. It is noteworthy that in this pig islets study no evidence was found for the presence of α-Gal and NeuGc structures, while the pig lung and trachea clearly produce both antigens. Concerning α-Gal, as has been indicated in many reports, pig islets express very low levels of α-Gal. On the other hand, concerning NeuGc, our previous study reported that NeuGc is expressed on the *N*-glycans of API (Komoda et al. 2004). Therefore, pig islets must contain NeuGc in relatively minor amounts and, as a result, were not detected in this study, because pig lung and trachea contain relatively minor levels of NeuGc structures.

In addition, NeuGc-Gal-GlcNAc and Galα1-3 Lewis x (Lew^x) were recently reported as novel antigens, as evidenced by a structural analysis of *N*-glycans from the miniature pig kidney (Kim et al. 2006). However, neither of these antigens was detected in this study.

Blixt et al. (2009) reported on the carbohydrate specificities of sera obtained from clinical patients in whom neonatal bone pig islet-like cell clusters (NPCC) had been intraportally injected, using a printed covalent glycan array with 200 structurally defined glycans. Besides α-Gal and NeuGc, the patients had Abs against terminal α-linked GalNAc, β3-linked Gal especially Galβ1,3GlcNAc even if terminally sulfated or sialylated, β-GlcNAc except for β1,3-linked, oligomannosyl compounds, some neuranimic acid (NeuAc) and Galα1-3Lew^x. Compared with the data reported here, pM5 has β-GlcNAc, might be applicable for the target structure of the patients. In addition, N6-2, pM2-2 and pM3-2, which contain Manα1-3Manα1-6Man structures, are also potential target antigens. However, the antigenicity of NPCC may slightly be different from that for API.

As the other non-Gal antigens, the Forssman, the terminal GalNAc related to the Tn-antigen (GalNAcα-*O*-Ser/Thr), T-antigen (Thomsen-Friedenreich; Galβ1,3GalNAcα-*O*-Ser/Thr) and sialyl-Tn antigen (NeuAcα2,6GalNAcα-*O*-Ser/Thr) are also reported to be important (Ezzelarab et al. 2005). However, these glycans are related to *O*-glycans and glicolipids (Diswall et al. 2011).

In summary, as a feature, pig islets are rich in high-mannose type *N*-glycans, especially relatively low amounts of mannose. Several API structures, such as N6-2, pM2-1, 2-2, 3-1, 3-2, and pM5, and the sulfate structure, β-linked GalNAc-SOH3, were not detected in human islets. In addition, it is possible that the sulfated glycans of API are involved in the observed antigenicity to human serum. The data herein provide important information that can be useful to future clinical xenotransplantation studies.

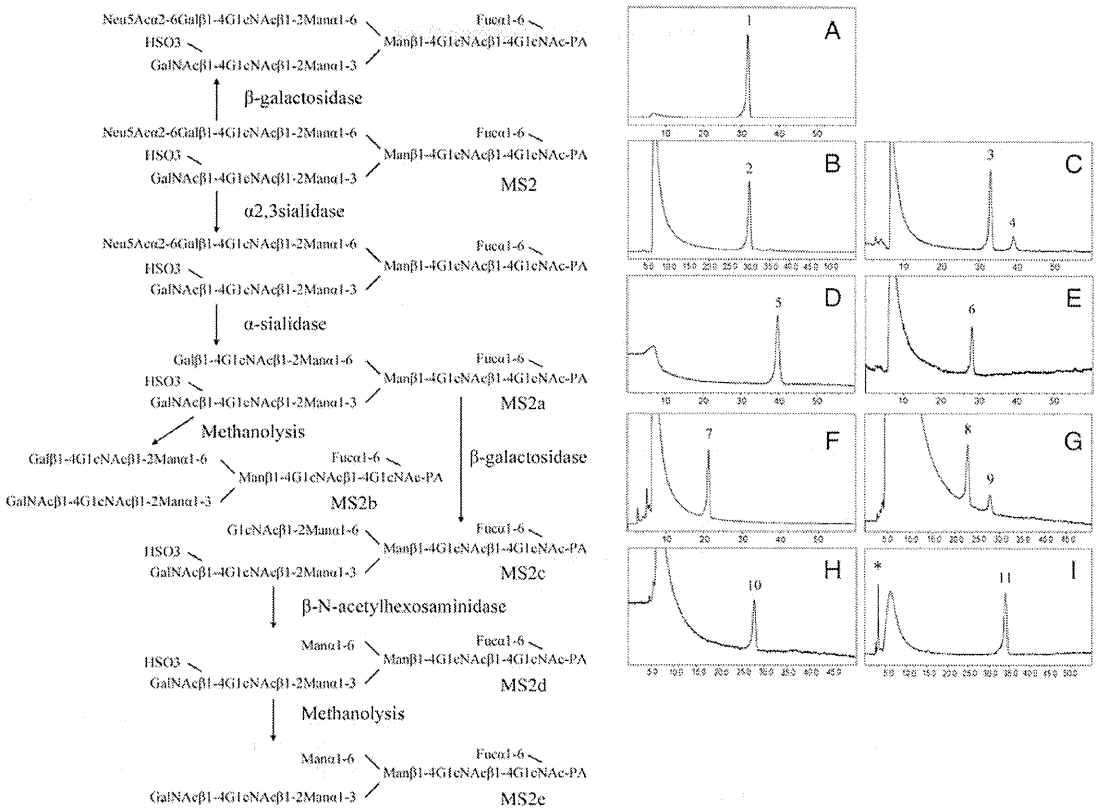


Fig. 6. Structural analysis of MS2. (**A**) ODS peak after α2,3-sialidase treatment to MS2. Peak 1 was just the same as MS2 in GU and molecular weight. (**B**) ODS peak after α-sialidase treatment to MS2. Peak 2 corresponds to MS2a (12.1 GU and 1988 Da). (**C**) ODS peak after methanolysis treatment to MS2a. Peak 3 is the nonreacted sample. Peak 4 lacked one sulfate residue from MS2a and corresponds to MS2b (13.9 GU and 1907 Da). (**D**) ODS peak after co-chromatography of MS2b and 210.4a in GALAXY. MS2b was proved to be the same structure as the 210.4a in GALAXY. (**E**) ODS peak after β-galactisidase treatment to MS2a. Peak 6 lacked one galactose from MS2a and corresponds to MS2c (11.4 GU and 1826 Da). (**F**) ODS peak after β-*N*-acetylhexosaminidase treatment to MS2c. Peak 7 corresponds to MS2d (9.7 GU and 1622 Da). (**G**) ODS peak after methanolysis treatment of MS2d. Peak 8 was the nonreacted sample. Peak 9 lacked one sulfate residue from MS2d and corresponds to MS2e (11.0 GU and 1541 Da). (**H**) ODS peak after co-chromatography of MS2e and 110.4a in GALAXY. MS2e was proved to be the same structure as the 110.4a in GALAXY. (**I**) ODS peak after β-galactisidase treatment to MS2. Peak 11 is identical to MS2 in GU and molecular weight.
* Not a sugar.

Materials and methods

Pig islet isolation

Pancreatic glands were removed from several pigs at a slaughterhouse that handles young market weight pigs (Large White/Landrace x Duroc, 6 months old, ~100 kg). Isolation of porcine islets was performed using the Islet Isolation Technique (Goto et al. 2004), with minor modifications. Purified islet fractions were pooled and cultured at 37°C in a humidified atmosphere with 5% CO_2 in CMRL1066 medium (Biochrom, Berlin, Germany) supplemented with 20% heat inactivated porcine serum, 2 mm *N*-acetyl-L-alanyl-L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*1-2-ethanesulfonic acid, 100 IU/mL penicillin, 100 μg/mL streptomycin (Biochrom) and 20 μg/mL ciprofloxacin (Bayer, Leverkusen, Germany).

Human islet isolation

The method used to isolate islets has been reported previously (Matsumoto et al. 2002). In brief, the pancreas was distended with a cold enzyme solution through the pancreatic duct using a pressure-controlled pump system. In all cases, the distended pancreata were digested using the semi-automated method (Matsumoto et al. 2006). All centrifuged pellets were collected in cold storage/purification stock solution (Mediatech, Inc., Manassas, VA).

Islet isolations were conducted based on the Edmonton protocol with our modifications. The results of the isolations were evaluated based on the Edmonton protocol. Islets were purified with a COBE 2991 cell processor (CaridianBCT, Inc., Lakewood, CO) using density-adjusted iodixanol-based continuous density gradient. The final preparation of islets was assessed using dithizone staining (Sigma Chemical Co., St. Louis, MO) for islet yield and purity. Islet yield was converted into a standard number of islet equivalents (diameter standardizing to 150 μm). Islet viability was evaluated with fluorescein diacetate (10 μmol/L) and propidium iodide (15 μmol/L) staining. All procedures were done at the Baylor Research Institute, TX.

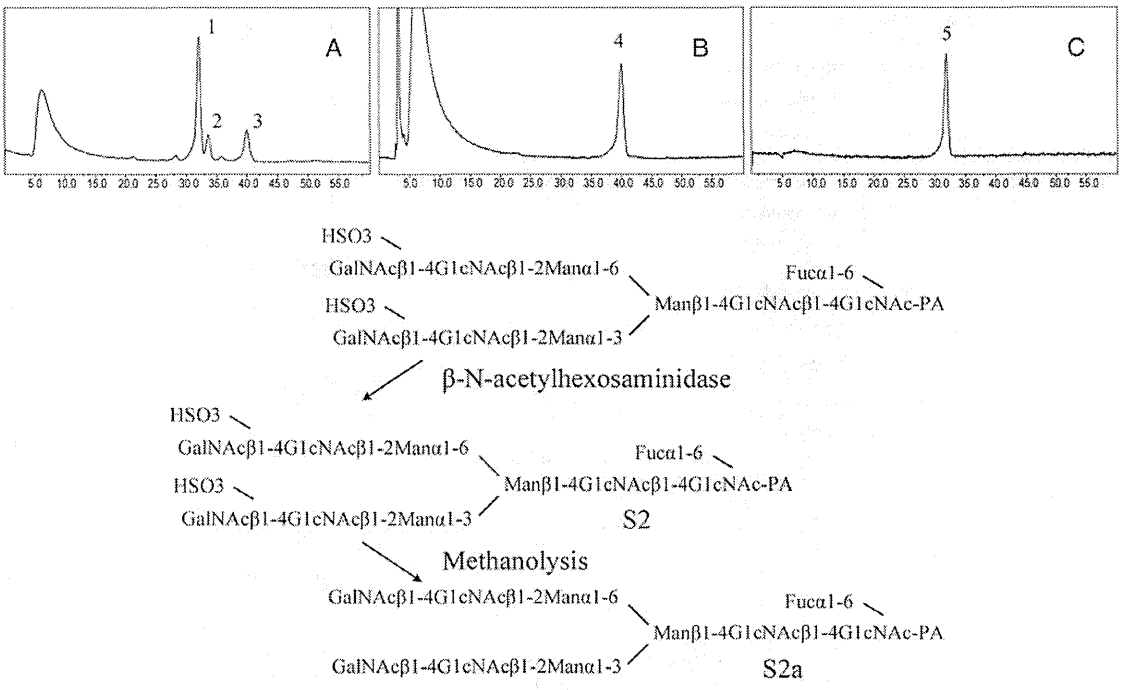


Fig. 7. Structural analysis of S2. (**A**) ODS peak after methanolysis treatment to S2. Peak 1 was the nonreacted sample, S2 (12.7 GU, 2110 Da). Peak 2 lacked one sulfate residue from S2, 13.2 GU and 2029 Da. Peak 2 lacked two sulfate residues from S2, corresponding to S2a (15.1 GU and 1948 Da). (**B**) ODS peak after co-chromatography of the samples of S2a and210.4b. S2a was the same structure as the 210.4b in GALAXY. (**C**) ODS peak after β-*N*-acetylhexosaminidase treatment of S2. Peak 5 was identical to S2 in GU and molecular weight.

Materials for analyses

Glycoamidase A from sweet almond, α-manosidase, β-galactosidase and β-*N*-acetylhexosaminidase from jack bean were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). α-Gal from coffee bean was purchased from Oxford GlycoSciences, Inc. (Oxford, UK). Trypsin and chymotrypsin were obtained from Sigma (St. Louis, MO). Pronase protease from *Streptomyces griseus* was from Calbiochem (San Diego, CA). The PA derivatives of isomalto-oligosaccharides 4–20 (indicating the degree of polymerization of glucose residues) and reference PA-oligosaccharides were purchased from Seikagaku Kogyo Co.

Characterization of N-glycan derived from islets

The residue after extracting each islet with a chloroform–methanol solution was used as the starting material. All experimental procedures used, including the chromatographic conditions and glycosidase treatments, have been described previously (Takahashi et al. 2001). The extract was proteolyzed with chymotrypsin and trypsin mixture and further digested with glycoamidase A to release *N*-glycans. After the removal of the peptide materials, the reducing ends of the *N*-glycans were derivatized with 2-aminopyridine (Wako, Osaka, Japan). This mixture was applied to a DEAE column (Tosoh, Tokyo, Japan) or a TSK-gel Amide-80 column (Tosoh), and each fraction that was separated on the amide column was applied to a Shim-pack HRC-ODS column (Shimadzu, Kyoto, Japan). The elution times of the individual peaks onto the amide-silica and ODS columns were normalized with respect to a PA-derivatized isomalto-oligosaccharide with a known degree of polymerization, and are represented in units of glucose unit (GU). Thus, a given compound from these two columns provided a unique set of GU values, which corresponded to the coordinates of the two dimension HPLC map. The PA-oligosaccharides were identified by comparison with the coordinates of <500 reference PA-oligosaccharides in a homemade web application, GALAXY (http://www.glycoanalysis.info/) (Takahashi and Kato 2003). The calculated HPLC map based on the unit contribution values was used to estimate some high-mannose type PA-oligosaccharides. The PA-oligosaccharides were co-chromatographed with the reference to PA-oligosaccharides on the columns to confirm their identities.

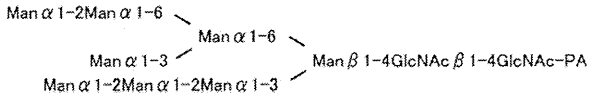
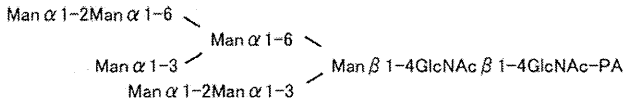
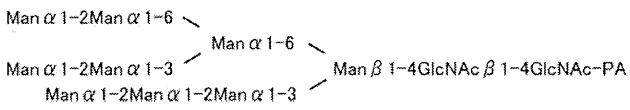
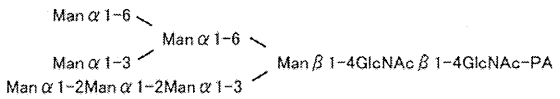
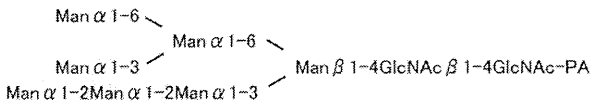
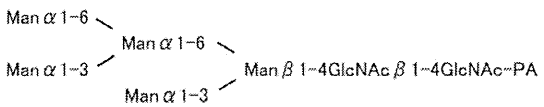
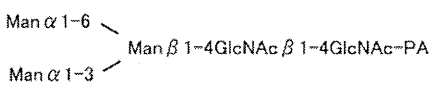
MS analyses of PA-glycans

PA-oligosaccharides were subjected to MALDI-TOF-MS analysis. The matrix solution was prepared as follows: 10 mg of 2,5-dihydroxybenzoic acid (Sigma) was dissolved in 1:1 (v/v) of acetonitrile/water (1 mL). Stock solutions of PA-glycans were prepared by dissolving them in pure water. One microliter of sample solution was mixed on the target spot of a plate with 1 μL of matrix solution and then allowed to air-dry. MALDI-TOF-MS data were acquired in the positive modes using AXIMA-CFR (Shimadzu) operated in the linear mode.

Single islet cell preparation

Single-cell suspensions were prepared by the method described by Ono et al. (1977). Isolated islets were exposed to 0.04% ethylenediaminetetraacetic acid for 5 min at room temperature

Table I. Structures and relative quantities of neutral, mono-sialyl, di-sialyl or mono-sulfate, mono-sialyl-mono-sulfate and di-sulfate PA-oligosaccharides derived from human and porcine islets

Peak code number	GU[a] ODS (Amid)	Molecular[b] mass (Da)	Structure[c]	Relative quantity (%)[d]	
					Pig
Neutral glycan					
N1	4.9 (8.8)	1800		11.6	24.9
N2-1	5.3 (7.9)	1638		5.8	3.5
N2-2	5.3 (9.5)	1962		7.4	9.5
N3	6.0 (7.9)	1638		3.0	1.9
N4	6.2 (7.0)	1475		16.7	10.1
pN5 = hN7	7.3 (6.1)	1313		24.0	11.3
pN6-1 = hN8	7.5 (4.2)	989		2.3	6.2

Continued