Remodeling of the Major Pig Xenoantigen by N-acetylglucosaminyltransferase III in Transgenic Pig

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RUNNING TITLE: Transgenic Pigs with human N-acetylglucosaminyltransferase III gene
SUMMARY

We have been successful in generating several lines of transgenic mice and pigs that contain the human \( \alpha \)YD-mannoside \( \alpha \)Y1,4-N-acetylglucosaminyltransferase III (GnT-III) gene. The overexpression of the GnT-III gene in mice and pigs reduced their antigenicity to human natural antibodies, especially the Gal \( 1-3 \) Gal \( 1-4 \) GlcNAc-R (Gal), as evidenced by immunohistochemical analysis. Endothelial cell studies from the GnT-III transgenic pigs also revealed a significant downregulation in antigenicity, including Hanganutziu-Deicher (H-D) antigen, and dramatic reductions in both the complement- and NK cell-mediated pig cell lyses.

Changes in the enzymatic activities of other glycosyltransferases, such as \( \alpha \)1,3 galactosyltransferase, GnT-IV and GnT-V, did not support cross talk between GnT-III and these enzymes in the transgenic animals.

In addition, we demonstrated the effect of GnT-III in down-regulating the xenoantigen of pig heart grafts, using a pig to cynomolgus monkey transplantation model, suggesting that this approach may be useful in clinical xenotransplantation in the future.
INTRODUCTION

The increasing problem of the worldwide shortage of donor organs has led to a revival of interest in xenotransplantation. The expression of complement regulatory proteins, such as membrane cofactor protein (MCP; CD46) (1), decay accelerating factor (DAF; CD55) (2) and CD59 (MACIF (3), HRF20 (4)) in transgenic pigs, has been shown to be very effective in protecting against hyperacute rejection in a xenograft (5-8).

However, since Galili et al reported that the Gal |1-3Gal|Y4GlcNAc-R (|-Gal) is the major antigen to human xenotransplantation in pig, genetic approaches to modify this glycoantigen have been the focus of xenotransplantation studies. This antigen was first described as a internal type 1 chain, but was later corrected as a linear type 2 oligosaccharide, the |-Gal which is synthesized by |1,3 galactosyltransferase (|1,3GT) (9-13). The human sequence, however, has suffered a deletion of a single nucleotide at two separate positions which disrupt the translational reading frame (14, 15). As a result, humans produce a natural antibody which comprises as much as 1% of the circulating IgG, and which is also found in significant amounts as an IgM antibody (16).

The most reliable approach for the elimination of |-Gal from pig tissue, is to disrupt the pig |1,3GT gene, via homologous recombination and/or gene transfer. However, gene targeting is not feasible at the present time. Another strategy for downregulating the |-Gal involves taking advantage of enzymatic competition involving terminal glycosylation, between |1,3GT and other glycosyltransferases for the common acceptor substrate in the trans Golgi stack and network. Several glycosyltransferases, such as |1,2 fucosyltransferase (|1,2FT) (17, 18), |1,3
fucosyltransferase (|1,3FT), |2,3 sialyltransferase (|2,3ST) (19), as well as |2,6 sialyltransferase (|2,6ST) represent possibilities (20).

The strategy, we present herein, involves controlling sugar chain biosynthesis using the |D-mannosyl|1,4-N-acetylglucosaminyltransferase III (GnT-III) (21, 22), which leads to a remodeling of the total antigenicity of the cell surface (23). The mechanism by which the introduction of the GnT-III gene significantly suppresses xenoantigens is not fully understood, but its suppression could, in part, be due to the inhibition of further branching of the carbohydrate moieties and/or a lack of maturation in processing. That is, once a bisecting GlcNAc residue is added to the core mannose by GnT-III, competitive enzymes, including |3-D-mannoside |-1,4-N-acetylglucosaminyltransferase IV (GnT-IV) and |6-D-mannoside |-1,6 N-acetylglucosaminyltransferase V (GnT-V) are prevented from introducing any further tri-structures in the Golgi stack (24). Our previous structural analysis of N-linked sugars of the pig endothelial cell (PEC) transfectant with GnT-III revealed that the complex-type oligosaccharides with bi-, tri-, and tetraantennary structures, which contained |Gal, decreased markedly with a parallel increase in bisected structures that contained no |galactosyl residues (24, 25).

In the present study, transgenic mouse and pig lines carrying GnT-III were produced, and the expression levels of GnT-III, as well as changes in antigenicity in the various tissues were analyzed.
EXPERIMENTAL PROCEDURES

Construction of plasmids. A cDNA of human GnT-III, Act-5, was subcloned into the pCX site; a β-actin promoter and a cytomegalovirus enhancer (26). The plasmid was separately transformed into Escherichia coli C600 and then amplified using standard techniques.

Transgenic mice and pigs. B6C3F1 female mice were induced to superovulate and then crossed with B6C3F1 males. Microinjection and embryo transfer were performed by standard methods to generate transgenic mice. The DNA fragments for microinjection were prepared by digesting the plasmids with SalI and HindIII to remove the vector sequences. DNA fragments were microinjected into mouse ova (C57BL/6 × C3H), resulting in transgenic mice. Genomic DNA from the tail tips of newborn mice was analyzed by PCR and Southern blots to identify the produced transgenic animals. Mice carrying these pCX-GnT-III plasmids were crossed with B6 to obtain offspring.

The pCX-GnT-III gene was also used to produce transgenic pigs. Prepubertal crossbred gilts (Landrace and Large White) were used as embryo donors and recipients. Methods used in the superovulation for gilts have been presented previously (27). Embryo donors were artificially inseminated, and embryos were collected 50 to 54 h after human chorionic gonadotropin (hCG) injection. Embryos were centrifuged at 12000 × g for 8 min to visualize the pronuclei and microinjected with approximately several thousand copies of each hybrid gene. Microinjected embryos were then transferred to unmated synchronized recipients or the embryo donors (donor-recipients). Transgenic pigs were identified by PCR and/or southern...
blot analysis with genomic DNA extracted from the tail tips of the newborn pigs. Founder transgenic pigs were bred with non-transgenic boars or gilts to obtain the second generation.

Glycosyltransferase assays. For the assay of enzyme activity, tissues were sonicated and lysated in PBS. The enzyme activities of GnT-III, GnT-IV(28), or GnT-V (29) were determined, using the pyridylaminated biantennary sugar chain (GlcNAc β 1-2Man α 1-6 (GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA) as a substrate (30).

The reaction buffer for the GnT-III assay consisted of 125 mM 2-[N-morhpolino] ethane sulfonate buffer (pH 6.25) containing 40 mM UDP-GlcNAc, 20 mM MnCl₂, 400 mM GlcNAc, and 1% TritonX-100. The reaction mixture for GnT-IV contained 250 mM Mops buffer (pH 7.3), 80 mM UDP-GlcNAc, 15 mM MnCl₂, 400 mM GlcNAc, and 1.0% (W/V) Triton X-100. Assayed GnT-V activity employed (pH 6.25), 250 mM 2-[N-morhpolino] ethane sulfonate buffer, containing 80 mM UDP-GlcNAc, 20 mM EDTA, 400 mM GlcNAc, and 1.0% (W/V) Triton X-100. It should be noted that Mn²⁺ is not essential for GnT-V activity. 20 mM EDTA, contained in the reaction mixture, completely inhibited GnT-III activity. To 25 µl of these solutions were added 10 µl of 100 µM substrate followed by 15 µl of cell lysate. The assay mixture was then incubated at 37°C for 3 h (31).

The acceptor substrate, pyridylaminated lacto-N-neotetraose (LNnT-PA) (Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc-PA) at a final concentration of 10 µM was employed in the α1,3GT activity assays. Lacto-N-neotetraose was purchased from Seikagaku Kogyo (Tokyo, Japan) and pyridylaminated according to the method of Kondo et al. (32). α
1,3GT activity was assayed in a reaction mixture containing 10 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic Acid (HEPES), pH 7.2, 20mM UDP-Galactose, 10mM MnCl₂, 33mM NaCl, 3mM KCl. 10 μl of 50 μM substrate and 15 μl of cell lysate were added to this mixture, which was then incubated at 37°C for 3h (19).

The enzyme reactions were quenched by boiling for 5 min. The samples were then centrifuged at 12000 x g for 5 min and an aliquot of each supernatant was subjected to HPLC analysis, using a TSK-gel ODS-80Tm column (4.6 x 250 mm). The reaction products were eluted with 20 mM acetate buffer, pH 4.0 containing 0.01 % n-butanol at a flow rate of 1.0 ml/min at 55°C and were monitored with a fluorescence spectrophotometer (Shimadzu model RF-10AXL, Tokyo, Japan) using excitation and emission wavelengths of 320 and 400 nm, respectively. The specific activity of the enzyme is expressed as moles of product produced per h of incubation per mg of protein. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA), using bovine serum albumin as a standard.

**Immunohistochemical detection of β-Gal.** Various organs were excised from transgenic mice and pigs. A portion of each organ was fixed with 4 % paraformaldehyde/ Dulbecco’s PBS (D-PBS) for 30 min. The fixed sections were incubated with blocking solution (2% BSA/D-PBS) for 1 h and then reacted with normal human pooled serum (NHS) of blood type O, or a mouse mAb anti-the β-Gal, M86 (a generous gift from Dr. U. Galili) (33). After removal of excess antibody, the sections were reacted with FITC-conjugated goat anti-human Ig (Cappel, West Chester, PA, USA), or FITC-conjugated rabbit anti-mouse IgM (Cappel ICN, Aurora, OH, USA), respectively. Each section was also reacted FITC-conjugated *Griffonia simplicifolia* I
(GS-IB4) lectin which binds the \(\alpha\)-Gal (Honen, Tokyo, Japan). Double staining of pancreas islets was also carried out using anti-GnT-III mAb (Fujirebio, Tokyo, Japan) and anti-pig insulin polyclonal Ab (DAKO Japan, Kyoto, Japan), and subsequently stained with FITC-conjugated anti-mouse Ig (Cappel ICN, Aurora, OH, USA) and Alexa Fluor 594 labeled Goat anti-Guinea Pig IgG secondary antibody (Molecular Probes Europe BV, Leiden, the Netherlands), respectively. For the detection of monkey C3 and C5b-9 deposition, mouse anti-human C3 mAb and mouse anti-human C5b-9 mAb (DAKO, Japan, Kyoto, Japan) were used as the first antibody, and subsequently stained with FITC-conjugated rabbit anti-mouse IgG Cappel ICN, Aurora, OH, USA). The slides were viewed by means of a Zeiss Axioplan 2 universal microscope (Jena, Germany).

**Flow cytometry.** The PEC from transgenic pigs with or without human GnT-III was removed from aorta and cultured in DMEM containing 10% FBS with L-glutamine (Gibco / BRL, Rockville, MD, USA) and penicillin/streptomycin (Meiji, Tokyo, Japan) (34). The PECs were incubated with various dilutions of NHS at 4°C for 1 h, washed and then incubated with 1.25 mg of FITC-conjugated anti-human Ig (Cappel, West Chester, PA, USA) as a second antibody for 1 h at 4°C. The cell-surface carbohydrate epitopes were also examined with an FITC-conjugated GS-IB4 lectin (Honen, Tokyo, Japan), and chicken anti-Hanganutziu-Deicher (H-D) antigen polyclonal Ab (a Gift from Dr. N. Wakamiya, Osaka University, Osaka, Japan), and FITC-conjugated rabbit anti-chicken IgG (Cappel, West Chester, PA, USA). The stained cells were analyzed with a FACS Calibure flow cytometer (Nippon Becton Dickinson, Tokyo, Japan).

**Lactate dehydrogenase (LDH) assay.** This assay was performed according to the manufacture's recommended protocol, using an MTX"LDH" kit (Kyokuto, Tokyo,
Japan). The PEC from transgenic pigs was plated at $2 \times 10^4$ cells per well in flat-bottomed gelatin-coated 96-well trays 1 day prior to assay. Fifteen hours after plating the cells, the wells were washed twice with serum-free D-MEM to remove the LDH, which is present in fetal calf serum, and incubated with several concentrations of NHS which had been diluted with D-MEM. The plates were incubated for 2 h at $37^\circ C$ and the released LDH was then measured. The percent cytotoxicity was calculated using the formula:

$$\text{Cytotoxicity} = \frac{E - N - S}{M - N - S} \times 100$$

where E is the experimentally observed release of LDH activity from the target PEC, N the LDH activity in each concentration of NHS, S the spontaneous release of LDH activity from target PEC incubated in the absence of NHS, and M the maximal release of LDH activity, as determined by sonication.

The spontaneous release of LDH activity from PEC was less than 5%, compared to the maximal release obtained by sonication (34).

**NK cell-mediated cytotoxicity assay.** The PEC from transgenic pigs were plated at $2 \times 10^4$ cells per well in a flat-bottomed gelatin-coated 96-well plate. Fifteen hours after plating the cells, the plates were incubated with effector cells, an NK-like cell line, YT cells, which were kindly provided by Drs. Junji Yodoi and Keisuke Teshigawara (University of Kyoto, Kyoto, Japan) (35), at various effector: target (E:T) ratios. Each assay was performed in triplicate. After a 4-h incubation at $37^\circ C$, the released LDH was measured using an MTX"LDH" kit (Kyokuto, Tokyo, Japan). The spontaneous release of LDH activity from effector cells and target cells were less than 10% and 5%, respectively. The results are expressed as the percent of specific lyses (36).

**Experimental Animals.** Examinations were carried out for according to the
guidelines for the handling of animals from the Research Institute of the HAMRI Co., LTD (Ibaraki, Japan). The heterozygous transgenic pigs with GnT-III and wild-type controls, either sex (age 18-22 days old, weight 2.5-6.0 kg), were used as donors for all experiments. Cynomolgus monkeys, *Macaca fascicularis*, obtained from HAMRI Co., LTD. (Ibaraki, Japan), weighing 2.5-7.0 kg were used as recipients. Preoperative serum from all monkeys was assayed for the anti-pig endothelial cell antibody titer and complement hemolytic activity (CH50 unit)(37).

*Anti-PEC natural antibodies and CH50 in cynomolgus monkey.* The anti-PEC antibodies, IgG and IgM, were checked, using PEC as a target. The PECs from control pigs were incubated with 10% serum from each recipient monkey at 4 °C for 1 h, washed and then incubated with 1.25 mg of FITC-conjugated anti-human IgG or IgM (Cappel, West Chester, PA, USA) as a second antibody for 1 h at 4 °C. Stained cells were analyzed with a FACS Calibur flow cytometer (Nippon Becton Dickinson, Tokyo, Japan).

CH50 was determined by a microtiter method, according to the methodology described by Mayer (37, 38). In this procedure, CH50 was assayed in gelatin veronal buffer (GVB) by using sensitized sheep erythrocytes (EA). After incubation at 37 °C for 60 min, 50 µl of the same buffer was added and the mixtures were centrifuged. The haemoglobin content of each supernant was estimated spectrophotometerically. The CH50 unit was defined as the serum volume sufficient to lyse 50% of the erythrocytes added to each well, and the complement activity in a test serum was then calculated as the number of CH50 units.

*Heterotopic Heart Transplantation.* After the donor pigs had been anesthetized
with thiopental sodium (20mg/kg) and stresnil (2mg/kg), a median sternotomy was performed. The inferior vena cava was divided above the diaphragm, and 200 ml of glucose-potassium cardioplegic solution with heparin (1000 IU) (39) was then infused from the ascending aorta. The heart was excised under topical cooling with PBS (4 °C) after division of the superior vena cava, the pulmonary aorta and veins, and the ascending aorta.

Recipient cynomolgus monkeys were anesthetized using ketamine hydrochloride (10 mg/kg) and xylazin (1 mg/kg). A heterotopic heart transplantation was performed in the abdomen, according to the Ono-Lindsey method (40). A midline abdominal incision was used to expose the aorta and inferior vena cava below the renal vessels. The donor heart was placed in the abdomen of the recipient monkey, the end-to-side anastomosis being donor aorta to recipient abdominal aorta and donor pulmonary aorta to recipient inferior vena cava. No immunosuppression and anti-coagulants were administered before or after the procedure.

The rejection of the cardiac transplants was defined based on the cessation of beating. The following time was 4 hours after the operation. Samples of graft tissue were removed at the time of rejection or 4 hours after transplantation for immunohistochemical analysis.

Statistics. Data are presented as the mean ± SEM. The Student $t$ test was used to ascertain the significance of differences within groups. Differences were considered statistically significant when $p < 0.05$. 
RESULTS

Establishment of transgenic mice and pigs. The pCX promoter was used for the ubiquitous expression of GnT-III in transgenic mouse. Eight founder pCX-GnT-III transgenic mice were obtained from 73 live pups from 576 microinjected oocytes. A founder which was determined to express high levels of human GnT-III was mated with B6 mice to propagate transgenic offsprings for the analysis of transgene expression in various tissues.

Transgenic pigs were also obtained by mean of the pCX-GnT-III construct. Five founders of pCX-GnT-III transgenic pigs were obtained from 59 live pups from 583 microinjected oocytes. Of the founder pigs, one was stillborn, and another died shortly after birth. Two founders, Gx-1 and Gx-2, showed GnT-III enzyme activity in their tails and the other, Gx-3, had only a very low level of GnT-III activity. The transgenic pig, Gx-1, was successfully bred, and three offsprings, 3wk (pig#1), 3mo (pig#2) and 6mo (pig#3), with the transgene were examined with two, 3wk, being used in the transplantation experiments.

The copy number of transgenes. The copy number of transgenes in hemizygous offsprings (F1) of transgenic mouse and pig lines was examined by Southern hybridization. The copy number of transgenic mouse, line#2 which was used in this study, has 7 copies of pCX-GnT-III constructs, and the pig lines Gx-1 and Gx-2 have 3 and 2 copies, respectively. The level of activity of GnT-III in each animal was not correlated with the copy numbers of the transgene.

Profiles of the GnT-III transgenic mice and pigs. The profiles of the GnT-III activities of each organ in wild-type and the GnT-III transgenic animals were
investigated. While the wild-type mouse showed GnT-III activity only in kidney
tissue, GnT-III was ubiquitously expressed in the GnT-III transgenic mice (Fig. 1 A).

As shown in Figure 1 B, pigs show very low levels of GnT-III activity in kidney, but
a slightly higher level than of expression in the brain. The CAG promoter leads to the
nearly ubiquitous expression of GnT-III in the organs of the transgenic pig.

**Immunohistochemical study.** To analyze the alteration of antigenicity in the
transgenic mouse, immunostaining of each organ was performed using NHS and GS-
IB4 lectin and M86 monoclonal antibody (Table I). Characteristic of the transgenic
mouse, changes of antigenicity were found in most organs except for the kidney
which has endogenous GnT-III activity (Fig. 2 c, d). In particular, the antigenicity of
the liver from the GnT-III transgenic mouse is clearly downregulated in spite of its
relatively lower expression of GnT-III activity (Fig. 2 e, f).

In the case of pigs, compared with wild-type pig, GnT-III transgenic pigs indicated
a lower susceptibility to NHS, GS-IB4 lectin and M86 mAb in many organs except brain
which also has GnT-III endogenous enzyme activity (Fig. 2 m, n).

**Double staining of pancreas tissue.** To determine whether the pancreatic islets
from the transgenic pig, have elevated GnT-III activity, double staining with anti-
GnT-III Ab and anti-insulin Ab was carried out. Double staining of the pancreas
revealed that the islets from the transgenic pig have a high level of expression of the
GnT-III enzyme (Fig. 3).

**Cross talk of the enzymes.** The 1,3 GT and GnT-IV, and GnT-V enzyme activity
in the control animals and the influences of an excess of GnT-III over the enzymes in
each organ were measured.

The average GnT-IV activities in many organs of the transgenic mouse and pig
were lower than those in wild, but the differences were not significant (Fig. 4 A, D). It
was not possible to predict changes in GnT-V and |1, 3 GT activities in both mice and pigs.

Profiles of PEC from the transgenic pigs. GnT-III and |1,3GT activities in the PEC from transgenic piglets were examined by HPLC, and the amelioration of antigenicity of the PEC were also analyzed by flow cytometry, using NHS, GS-IB4 lectin and M86 (Fig. 5).

While control parental PEC reacted strongly with human natural antibodies in NHS, the PEC from GnT-III transgenic pigs showed a diminished reactivity. Consistent with the previous in vitro study, the PEC from in vivo also ascertained the clear downregulation of antigenicity. The percent reduction in xenoantigenicity to human antibodies was approximately 50 %, as evidenced by mean fluorescence intensity. The |Gal was approximately 50% by GS-IB4 and 70% by M86 downregulated in PEC from the GnT-III transgenic pigs.

H-D antigen. The remodeling of pig antigenicity, by overexpression of GnT-III is directed, not only at the |Gal, but to other unknown epitopes, as well. The influence on the H-D antigen in the PEC isolated from GnT-III transgenic pig was then examined. As expected from our previous study, the H-D antigen on PEC was significantly downregulated (Fig. 5 f).

Complement-mediated and NK-mediated cytotoxicities of the PEC. The amelioration of complement-mediated lysis as a result of the overexpression of GnT-III was determined. In these experiments, NHS was used as a source of natural antibody and complement, as an in vitro model of hyperacute rejection. An approximate 40% inhibition of cytotoxicity was observed in the PEC derived from the GnT-III transgenic pig, GX-1 (Fig. 5 g). These results also suggest that GnT-III is quite effective in the remodeling of PEC.
In order to examine the involvement of the remodeling of oligosaccharides in cell-mediated cytotoxicity, assays for NK cell-mediated direct cytotoxicity were also carried out using the PEC from the GnT-III transgenic pigs as a target. As expected, cytotoxicity to the PEC isolated from GnT-III transgenic pig, was substantially decreased, compared to those from the wild-type pig.

Experimental xenotransplantation of pig to cynomolgus monkey. Experimental xenotransplantation, pig to cynomolgus monkey, was performed, using the heart from the GnT-III transgenic pigs. The natural antibody titer of each cynomolgus monkey was evaluated using the PEC, which is derived from the wild-type pig. The CH50 unit was also measured, and these data are summarized in Table II.

An abdominal heterotopic heart transplant was achieved. The ischemic time of the transplant organ varied from 35 to 60 minutes. Two hearts from wild-type pigs which were transplanted to cynomolgus monkey were hyperacutely rejected.

On the other hand, one heart graft carrying human GnT-III transplanted into the monkey with high natural Ab, especially IgM, was also rejected hyperacutely, whereas another heart graft from a transgenic pig continued to beat normally, when removed at 4 h after transplantation for purposes of histological analysis.

In addition, immunostaining of C3 and C5b-9 deposition on the graft after rejection or 4 h after transplantation, were performed. Compared with the rejected grafts, the specimen from the transgenic pig #2 organ showed a lower deposition of C3, as well as C5b-9 (Fig. 6).
DISCUSSION

The pCX promoter expressed human GnT-III ubiquitously, and intensely in the vascular endothelia of transgenic pigs. With this construct, the frequency of live births was slightly decreased, suggesting the possibility of a slight deleterious effect of human GnT-III expressed under the regulation of this promoter on the early development of pigs. We were able to produce transgenic mice carrying the same constructs used in the present study with nearly equal frequency.

Immunohistochemical profiles revealed that this strategy, i.e., remodeling by GnT-III, is feasible in transgenic animals. While each organ in the wild-type animals reacted strongly with human natural antibodies in NHS and GS-B4, most organs in the transgenic pig showed a diminished reactivity, because surface xenoantigens, especially |-Gal, were reduced. It is particularly noteworthy that the endogenous mouse GnT-III activity avoids remodeling the antigenicity in the transgenic mouse kidney, in spite of the high elevated level of expression of human GnT-III activity. On the contrary, the liver, with a relative low elevated human GnT-III activity in the transgenic mouse, clearly showed a downregulation in antigenicity.

On the other hand, the wild-type pigs, in which low endogenous GnT-III activity was observed in the kidney, led to an apparent downregulation of antigenicity in the kidney of transgenic pigs. In the case of pig brain, the level of GnT-III is not distinct, but is higher than that of other tissues. Therefore, similar to the mouse kidney, endogenous GnT-III activity might have hindered the remodeling of glycoantigen in this organ. Fortunately, the wild-type pig brain itself did not indicate a high level of antigenicity to human serum in the immunostaining procedure. These data suggest that transgenic pigs with GnT-III are suitable for kidney xenotransplantation and also have a possibility of therapeutic use for Parkinson’s disease using the brain.
To address the issue of whether pancreatic islets from the GnT-III transgenic pig are able to be used for pancreatic islet transplantation, double staining was carried using anti-pig insulin and anti-GnT-III Abs. Fortunately, in addition to the high level of GnT-III activity in entire pancreas tissue of the transgenic pig, the pancreatic islets clearly expressed GnT-III.

In our previous study, using the PEC transfectants with GnT-III, several clones with a high level expression of GnT-III indicated that the enzyme activity of 1,3GT, GnT-IV, and GnT-V is diminished(19). The possibility of enzymatic cross talk between GnT-III and these enzymes was considered. However, in vivo results of these enzymes between control and transgenic animals failed to support this in vitro hypothesis. Concerning GnT-IV and 1,3GT, the average enzyme activities of most tissues, including PEC from transgenic animals were lower than those from the wild-type. However, no significant differences were found in this study.

In terms of 1,3GT activity in organs, the mouse expresses a relatively lower 1,3GT activity than the pig in many organs, but the differences are in the 10-fold range, except for the lung. On the contrary, some mouse tissues, such as the pancreas, revealed a higher 1,3GT activity than those in pigs. The data may not support the reports by Galili, who concluded that |-Gal epitope expression in pig organs is up to 500-fold higher than in mouse organs (41). However, the amount of |-Gal epitopes in each tissue may not be directly related to 1,3GT enzyme activity.

We also demonstrated the remodeling of antigenicity to human serum in PEC derived from the transgenic pigs, which is in agreement with conclusions reached in our previous in vitro study. The PEC from transgenic pig showed an approximate 50% reduction in the antigenicity by GnT-III, not only |-Gal but also H-D antigen. The Hanganutziu-Deicher antigen, which contains N-glycolyl neuraminic acid (NeuGc)
is widely distributed in mammalian species, except for humans. The expression of NeuGc is controlled by cytidine monophospho-N-acetylmuramic acid (CMP-NeuAc) hydroxylase activity. The absence of NeuGc in human glycoconjugates is due to a partial deletion in the gene that encodes CMP-NeuAc hydroxylase (42-45). Therefore, this epitope has the potential to become one of the largest epitopes in the pig to human xenotransplantation after 1,3GT is knocked out. Fortunately, it is possible for GnT-III to reduce the levels of the H-D antigen, because GnT-III acts, not only an 1,-Gal but any other unknown epitopes as well.

Increasing evidence suggests that NK cells play a critical role in swine to human xenotransplantation (46, 47). Thus, an investigation of the effect of enzymatic remodeling of a glycoantigen, especially 1,-Gal, to NK-mediated direct cytotoxicity was carried, using the PEC from the transgenic pig. The downregulation of NK cell-mediated direct killing was also observed in the PEC, consistent with the in vitro data reported in our previous study (36).

Some reports revealed that the cynomolgus monkey may not be an ideal substitute for the human recipient, in terms of the hyperacute rejection of pig organs. The natural antibody titer to PEC and the classical pathway of the complement, CH50, were therefore assessed and compared with several human volunteers. However, these data, relative to the cynomolgus monkey that we used here is no lower than those of humans (data not shown). Other reports have indicated that transplanted pig hearts were rejected within 1 h, in the case of recipient cynomolgus monkeys. In this study, the control #1 donor heart was rejected at 25 min in the recipient cynomolgus monkey with an average CH50 unit and a slightly high IgM titer. Therefore, the case of control #2, with a relatively low level of IgM and IgG titer (48), that was rejected in 145 min after transplantation, might be a rare case for the pig to
cynomolgus monkey combination.

The transplantation experiments do not permit specific conclusions to be drawn, because of the number of experimental animals used. However, the graft survivals can be inferred from the effect of the downregulated antigenicity of GnT-III. Approximately half diminished antigenicity of GnT-III prolonged the heart graft survival of the pig in the cynomolgus monkey in the case of transgenic pigs. The first case, transgenic #1 graft, rejected at almost the same time as the control #2. However, the recipient monkey in which the transgenic #1 graft was transplanted showed a slightly lower CH50 unit, but had 4.6 times and 3.8 times higher IgM and IgG titers, respectively, than that of control #2. On the other hand, in the case of transgenic #2, in spite of the fact that the recipient monkey had a IgM titer nearly equal to control #2, the graft heart showed a good contraction during the follow-up time, 4h, without any sign of rejection. The outcome of pig xenografts in primate models has also been shown to correlate with in vitro human serum-mediated cytolysis and other assays performed using pig endothelial cells from the transgenic pig.

A variety of strategies have been pursued to eliminate |-Gal from swine tissue including the knock out of the |1,3GT gene. Among these, the gene transfection of |1,2FT has been reported to result in a drastic suppression of |-Gal. Sandrin, M.S. et al. reported an approximately 70% reduction in |-Gal expression in a pig kidney fibroblast cell. Moreover, Sharma, A. et al demonstrated that this approach is effective in transfected CHO cells with |1,3GT and |1,2FT. Both reports also indicated that this approach to reduce the |-Gal is quite effective in the transgenic mouse. In addition, transgenic pigs which express |1,2FT have already been
reported by several groups (49-52). However, as a disadvantage, Sepp, A. et al. demonstrated that Lewis\textsuperscript{X} expression was reduced to background levels, while the Lewis\textsuperscript{Y} neoepitope was induced in the case of 1,2FT expressing pig cells (53).

On the other hand, as we have previously reported, 2,3ST and 2,6ST dramatically suppresses the antigenicity of pig cells to human natural antibodies, better than the 1,2FT (19, 20). In addition, we have demonstrated that the combined transfer of GnT-III and 2,3ST appears to comprise approximately 80% of the downregulation in xenoantigenicity (54). However, the transgenic mouse and pig carrying a high level of 2,3ST activity or 2,6ST was difficult to produce, judging from our previous experience (data not shown).

Another study suggests that a combined transgenic approach using \{-galactosidase and 1,2FT results in the continuous suppression of the \(-Gal of donor tissue (55). However, in spite of the drastic elimination of the \(-Gal in the pig cells with the \(-galactosidase gene, the transgenic mouse carrying the \(-galactosidase alone showed only a 15-25% reduction in the \(-Gal (55). At the present time, none of these approaches except for the knockout completely eliminate the expression of the \(-Gal. Galili, U. suggests that even a decrease of 95% in the \(-Gal expression may not suffice for prevention the rejection of a xenograft (56). Further approaches might be required to effectively suppress the xenoantigenicity of pig cells and grafts.

Regarding transgenic pig with a glycosyltransferase, four groups have reported on a transgenic pig with 1,2FT. The first report was from Nagoya’s group. They actually produced transgenic pigs with 1,2FT, but could not maintain the transgenic line long enough for a clear analysis (49). The report from Nextran only analyzed a tail section of the transgenic pig (50). The next case was reported by Alexion Pharmaceuticals Inc. They used a H2K\textsuperscript{P} and a CMV promoter and obtained a good
expression of the 1,2FT gene in most of the transgenic pig tissues, and analyzed the modified cell surface carbohydrates, mainly fibroblasts. A 50% reduction of the |-Gal epitopes of the PEC from the 1,2FT transgenic pigs was also shown, but they did not perform the transplantational experiment (51). The other report by Cown, P.J. et al. described the combined transgenic pigs with CD55(DAF)/1,2FT and CD55/CD59/1,2FT. While the 1,2FT expressions were relatively weak and did not significantly reduce the |-Gal epitopes, the transgenic kidney expressing these genes, when transplanted into baboon, survived for as long as 5 days (52). This report suggests the importance of the combination of the complement regulatory proteins and the glycosyltransferases.

The combination approach within glycosyltransferases, such as 2,3ST and GnT-III, or Ygalactosidase and 1,2FT, have already been demonstrated to cause the effective suppression of YGal. Therefore, the triple combination of GnT-III, fucosyltransferase or sialyltransferase, and Ygalactosidase, might be the best approach to achieving the downregulation of xenoantigenicity at the present time. Needless to say, to achieve the best approach for overcoming hyperacute rejection, two or three complement regulatory proteins must be simultaneously added to the combined glycosyltransferases.

Collectively, the advantage of the present approach in reducing xenoantigens is that: 1) GnT-III is relatively easy to express in transgenic pig. 2) This approach leads to a remodeling of the total antigenicity, that is, not only the |-Gal but also H-D antigen and other unknown epitopes as well. 3) the combind transgenic approach of GnT-III with the other glycosyltransferase, such as 2,3ST or 1,2 FT, is very effective (54). 4) Therefore, after a 1,3GT knockout pig becomes feasible, it would be advisable to add this gene to the knockout pig to reduce xenoantigenicity, in addition
Finally, the expression of the GnT-III in transgenic pigs clearly modifies the surface glycoantigen in most tissues and cells, especially |-Gal, and confers resistance to human natural immunity.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Milton S. Feather for his editing this manuscript.
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FOOTNOTES

This work was supported, in part, Grants-in-Aids from the Ministry of Education, Science, and Culture, Japan, and The Program for the Promotion of Basic Research Activities for Innovative Biosciences.

The abbreviations used are:
MCP, membrane cofactor protein; DAF, decay accelerating factor; \(\beta\)-Gal, the Gal \(1\rightarrow3\) Gal \(\rightarrow\) 1-4 GlcNAc-R; \(\beta\)-1,3GT, \(\beta\)-1,3Gal transferase; \(\beta\)-1,2FT, \(\beta\)-1,2 fucosyltransferase; \(\beta\)-1,3FT, \(\beta\)-1,3 fucosyltransferase; \(\beta\)-2,3ST, \(\beta\)-2,3 sialyltransferase; \(\beta\)-2,6ST, \(\beta\)-2,6 sialyltransferase; GnT-III, \(\beta\)-D-mannoside \(\beta\)-1,4-N-acetylglicosaminyl transferase III; GnT-IV, \(\beta\)-3-D-mannoside \(\beta\)-1,4-N-acetylglicosaminyltransferase IV; GnT-V, \(\beta\)-6-D-mannoside \(\beta\)-1,6 N-acetylglicosaminyltransferase V; PEC, pig endothelial cell; NHS, normal human serum; GS-IB4, \textit{Griffonia simplicifolia} I; H-D, Hanganutziu-Deicher; LDH, lactate dehydrogenase; CH50, complement hemolytic activity.
FIGURE LEGENDS

FIG. 1. **Features of GnT-III enzyme activity in transgenic mice and pigs.** GnT-III activity in each organ of wild-type and transgenic animals, mice (A) and pigs (B). GnT-III activity (p mol/ h / mg of protein) was examined. Values are the mean ± SEM for three mice in each group.

FIG.2. **Immunostaining of tissue sections from control and transgenic animals, mice and pigs.** Staining with GS-IB4 lectin (a, b, e, f, m, n), M86(c, d, g, and h) and NHS (i, j, k, l) is shown on tissue sections, heart (a, b, g, h), kidney (c, d, l, j), liver (e, f, k, l), and brain (m, n), from the wild-type mouse (a, c, e), the GnT-III transgenic mouse (b, d, f), the wild-type pig (g, i, k, m), and the transgenic pig (h, j, l, n). Representative fields from each section were examined.

FIG.3. **Double staining with anti-pig insulin Ab and anti-GnT-III.** Staining with anti-pig insulin (a, c, d), and anti-GnT-III (b, c, d) are shown on pancreas tissue sections from control and transgenic pigs. Representative fields from each section were examined.

FIG.4. **Changes in several enzyme activities in GnT-III transgenic animal tissues.** To assess the influence of the GnT-III transgene on intrinsic GnT-IV, GnT-V, and 1,3GT activity, relevant enzyme activities were measured. Compared with the enzyme activity in wild-type organs, those in transgenic animals indicated small and variable changes.

FIG.5. **Features of the PEC from transgenic pigs.** Enzyme activities, GnT-III (A) and 1,3GT (B), of PECs from transgenic pigs were measured by HPLC. Each value is
expressed as the mean ± SEM of three to four independent experiments.

Xenoantigenicity of transfectants to NHS (C), GS-IB4 (D) and M86 (E), as investigated by flowcytometry. PECs from control and transgenic pigs were treated with 10 or 20% NHS as the first antibody and anti-human immunoglobulin second Abs. The reduction of βGal on the cell surface was also analyzed using GS-IB4 lectin and M86 mAb. PECs from control and transgenic pigs were treated with FITC-conjugated GS-B4 lectin, M86 mAb and FITC-conjugated anti-mouse IgM secondary antibody. The FACS mean shift value of the PEC treated with polyclonal chicken anti-H-D antigen antibody is indicated (F). The H-D antigen of the PEC from transgenic pigs were significantly downregulated. Each value is expressed as the mean ± SEM of six to eight independent experiments.

Amelioration of complement-mediated lysis by from wild-type and transgenic pigs were estimated by 20 or 40 % NHS, which served as a source of natural antibodies and complement (G). The percentage of inhibition of NK cell-mediated lysis are also presented. YT cells were incubated with PEC at E:T ratios of 10:1 or 5:1 (H). The resulting cytolysis is expressed as the mean percent of specific lysis ± SEM of six to eight independent experiments. *: significant (p < 0.05).

FIG.6 Deposition of activated complement components in pig heart xenograft. Staining with monkey C3 (a, b) and C5b-9 (c, d) is shown for the pig tissue section from the control #2 (a) and #1 (c), and Gnt-III transgenic #2 (b, d). Representative fields of coronary and ventricle from each section were examined. C3 and C5b-9 are both reduced on the specimen for transgenic pig #2, compared with wild-type controls.
TABLE I. Immunohistochemical analysis

A. Transgenic mice

<table>
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<th>NHS</th>
<th>GSIB4</th>
<th>M86</th>
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<tbody>
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<td></td>
<td>Wild-type</td>
<td>Transgenic</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Heart</td>
<td>#1</td>
<td>+++</td>
<td>+〜±</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>#1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>#1</td>
<td>+</td>
<td>±〜</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>Lung</td>
<td>#1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Pancreas</td>
<td>#1</td>
<td>±〜</td>
<td>-</td>
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<tr>
<td></td>
<td>#2</td>
<td>+〜±</td>
<td>-</td>
</tr>
<tr>
<td>Muscle</td>
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</tr>
<tr>
<td></td>
<td>#2</td>
<td>++</td>
<td>+〜±</td>
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</tbody>
</table>

B. Transgenic pigs

<table>
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<tbody>
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<td>Transgenic</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Heart</td>
<td>#1</td>
<td>+++</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>+</td>
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<td></td>
<td>#3</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>Kidney</td>
<td>#1</td>
<td>+〜〜</td>
<td>±</td>
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<td>+</td>
<td>±</td>
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<td></td>
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<td>±</td>
<td>+~±</td>
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<td>N.D.</td>
<td>N.D.</td>
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<td>±</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td></td>
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<td>+</td>
</tr>
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<tr>
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<td>+</td>
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<td>++~+</td>
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<td></td>
<td>+</td>
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<td>-</td>
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<tr>
<td></td>
<td>+</td>
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<td>±</td>
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<tr>
<td></td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aorta</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Grading scale:

(-)= not stained. (±)= stained equivocally or weakly.

(+) = stained moderately. (++)= stained intensely.
TABLE II. Heterotopic heart transplantation, from pig to cynomolgus monkey.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Anti-PEC titer (IgM)</th>
<th>Anti-PEC titer (IgG)</th>
<th>CH50 (unit)</th>
<th>Survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1.</td>
<td></td>
<td>83.1</td>
<td>27.8</td>
<td>149.6</td>
<td>25 min</td>
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<tr>
<td>#2.</td>
<td></td>
<td>38.1</td>
<td>18.8</td>
<td>200.3</td>
<td>2 h 45 min</td>
</tr>
<tr>
<td>Transgenic</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>#1.</td>
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<td>175.3</td>
<td>71.1</td>
<td>128.4</td>
<td>2 h 33 min</td>
</tr>
<tr>
<td>#2.</td>
<td></td>
<td>33.5</td>
<td>32.4</td>
<td>164.3</td>
<td>&gt; 4 h*</td>
</tr>
</tbody>
</table>

*: the graft was beating normally when removed.
Figure 1

A

![Bar graph showing GnT-III activity in different tissues for Wild-type and Transgenic mice.](image)

B

![Bar graph showing GnT-III activity in different tissues for Wild-type, Transgenic GX-1, and Transgenic GX-2 mice.](image)
Figure 2
Figure 3
Figure 4

A

B

C

GnT-I activity (pmol/h/mg-protein)

Heart Kidney Liver Lung Muscle Pancreas

Wild-type Transgenic

GnT-II activity (pmol/h/mg-protein)

Heart Kidney Liver Lung Muscle Pancreas

Wild-type Transgenic

GnT-I activity (pmol/h/mg-protein)

Heart Kidney Liver Lung Muscle Pancreas

Wild-type Transgenic

GnT-IV activity (pmol/h/mg-protein)

Heart Kidney Liver Lung Muscle Pancreas

Wild-type Transgenic

1,3GT activity (pmol/h/mg-protein)

Heart Kidney Liver Lung Pancreas Muscle

Wild-type Transgenic
Figure 4

GnT-IV activity (pmol/h/mg-protein)

Heart Kidney Liver Lung Spleen Pancreas Muscle Skin Brain Aorta

Transgenic Wild-type

0.05
0.1

F

1,3GT activity (pmol/h/mg-protein)

Heart Kidney Liver Lung Spleen Pancreas Muscle Skin Brain Aorta

Transgenic Wild-type

0.05
0.1

E

GnT-V activity (pmol/h/mg-protein)

Heart Kidney Liver Lung Spleen Pancreas Muscle Skin Brain Aorta

Transgenic Wild-type

0.05
0.1

D

GnT-IV activity (pmol/h/mg-protein)

Heart Kidney Liver Lung Spleen Pancreas Muscle Skin Brain Aorta

Transgenic Wild-type

0.05
0.1

C
Figure 5

A

![Graph A: GnT-III activity](image1)

B

![Graph B: 1,3GT activity](image2)

C

![Graph C: FACS mean shift](image3)

D

![Graph D: FACS mean shift](image4)
Figure 5

E

F

G

H

E:T=10:1

E:T=5:1

Wild-type 

Transgenic

Wild-type 

Transgenic

Wild-type 

Transgenic

Wild-type 

Transgenic

FACS mean shift

FACS mean shift

% Lysis

% Lysis
Remodeling of the major pig xenoantigen by N-acetylglucosaminyltransferase III in transgenic pig

Shuji Miyagawa, Hiroshi Murakami, Yoichi Takahagi, Rei Nakai, Mako Yamada, Ayako Murase, Souichirou Koyota, Masaru Koma, Katsuyoshi Matsunami, Daisuke Fukuta, Tatsuya Fujimura, Tamotsu Shigehisa, Masaru Okabe, Hiroshi Nagashima, Ryota Shirakura and Naoyuki Taniguchi

*J. Biol. Chem.* published online August 2, 2001

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