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H. 知的財産権の出願・登録状況

特になし。

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分担研究報告書

バイオ人工細胞・臓器の開発による糖尿病その他の疾患の治療に関する研究

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研究要旨
医療用バイオ人工細胞・臓器の開発である。主眼をバイオ人工膵島とし、その細胞供給用の遺伝子改変ブタの作出をめざす。CRISPR (Clustered Regularly Interspaced Short Plidromic Repeats) /Cas (CRISPR Associated) システムでのブタCMAH遺伝子のknockoutに一定の目途がたった。

研究協力者
蓮輪英毅（助教)、江崎陽子（研究員)

A. 研究目的

目的は、医療用バイオ人工細胞・臓器の開発である。主眼をバイオ人工膵島とし、その細胞供給用の遺伝子改変ブタの作出をめざす。

B. 研究方法

1). CMAH遺伝子の検索およびCRISPR法の適応。

CRISPR (Clustered Regularly Interspaced Short Plidromic Repeats) /Cas (CRISPR Associated) システムでのブタCMAH遺伝子のKO siteを検索した。

① CMAHのexon7部位に焦点をあて、そこにまず８カ所のsiteを設定し、さらに４カ所に絞り込んだ。

② この４カ所を含む遺伝子を約４９０bpをcloningし、pCAG-EGxxFP vectorに組み込んだ。(pCAG-EGxxFP-CMAH(Ex7)作成)

③ これをCHO細胞に遺伝子導入し、lineを確立した。

④ 設定したKO siteをpX330のBbsl siteに挿入し、この遺伝子を樹立したCHO細胞のlineに対して導入し、４８時間後のGFPの発現量を検定した。

C. 結果

1). off-target siteの検定。

設定したCMAH(ex7)のoff-target siteとしては、gS01<N12: 23><N13: 2><N14: 0>、

gS03<N12: 3><N13: 0><N14: 0>、gAS04<N12: 0><N13: 0><N14: 0> gSA08<N12: 20><N13: 6><N14: 0>であった。

2). Validation in vitro

GFP強度は、gS01がbestと考えられた。

D. 考察

作製したhybrid遺伝子構築に関しては，既にin vitroでの発現の確認を終え，動物個体（マウス）での発現を確かめている。また新しいKO方法である今回のCRISPH/CS法は、相同組み換え法に比べて、かなり容易にKOできると考えられた。 CRISPH/CS法はダブルKOクローン個体の作出に有効であると判断できた。

E. 結論

超急性拒絶反応克服においてαGalに次ぐ糖鎖抗原であるH-D抗原の除去に目途がたった。

CRISPR/CS法は、今後のブタでのKOに有効と考えられた。

G. 研究発表

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H.　知的所有権の出願・登録状況

特に無し。

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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).

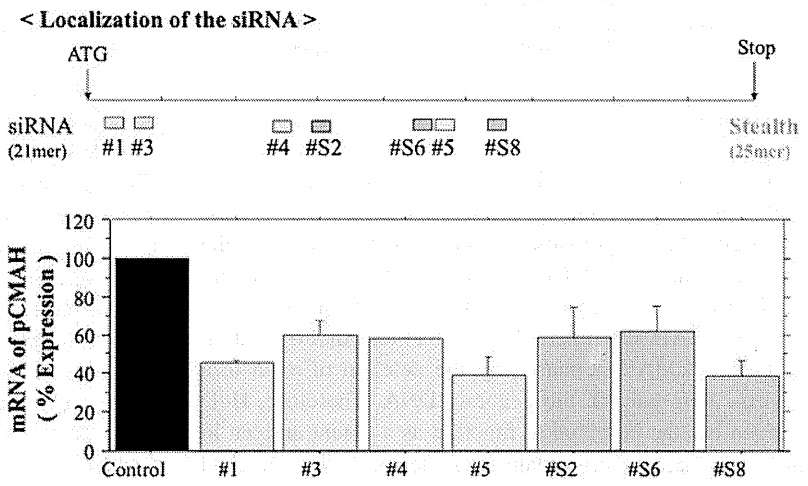

Fig. 1 Localization of siRNA and the percentage expression of the mRNA of CMAH by each siRNA. Schematic presentation of pig CMAH with the localization of the siRNA was indicated. The efficacy of each siRNA for the mRNA of the pig CMAH, in a PEC line, MYP-30, was measured by real-time RT-PCR ($n = 3$)

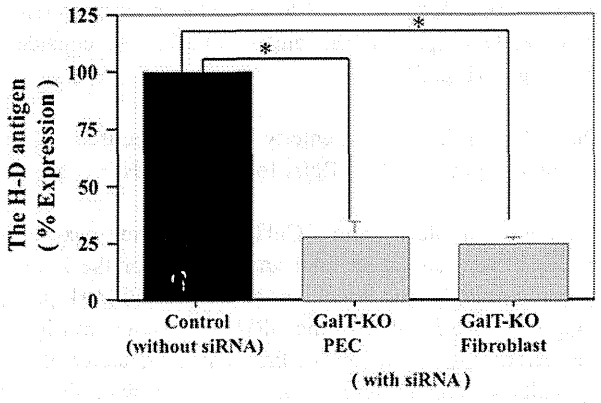

Fig. 2 The effect of siRNA on the expression of the H–D antigen by pig cells. PECs and fibroblasts from a GalT-KO pig were used to check the changes in the H–D antigen for the mixture of the siRNAs (#1, #5 and #S8 in Fig. 1). The percentage expression of the H–D antigen is indicated in comparison to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

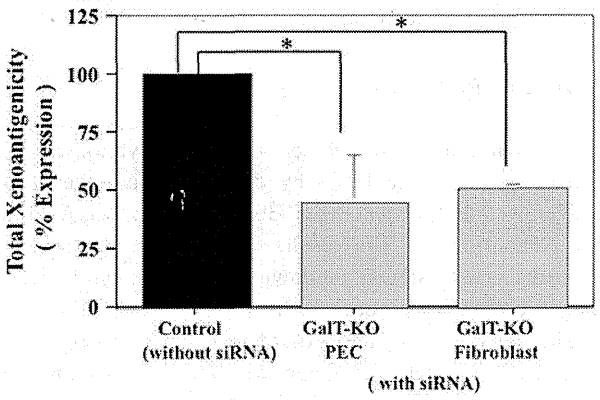

Fig. 3 Deducing the antigenicity on pig cells for human serum by the downregulation of the H–D antigen. PECs and fibroblasts from a GalT-KO pig were next used to check the changes in antigenicity for the human natural antibody by knocking down the CMAH gene using FACS analysis. The percentage expression 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

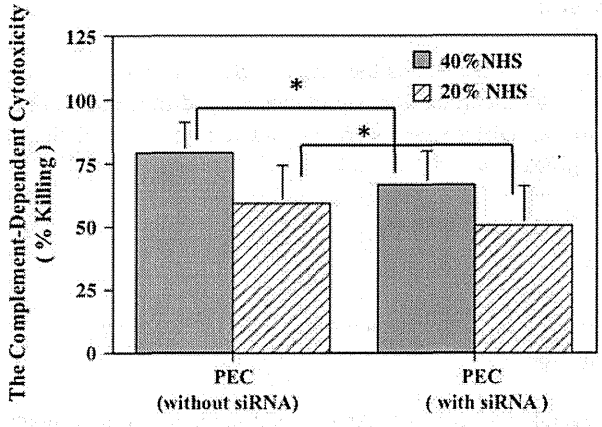

Fig. 4 Amelioration of the complement-mediated cytotoxicity of human serum to pig cells by the downregulation of the H–D antigen. Wild-type PECs, MYP-30, were incubated with 20 or 40 % NHS for 2 h at 37 °C, and the amount of LDH released was then determined ($n = 8$). The *asterisks* indicate significant 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 lectin microarray study of glycoantigens in neonatal porcine islet-like cell clusters

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ABSTRACT

Background: Besides α-Gal expression, the differences of glycosylation and antigenicity between adult pig islets (APIs) and neonatal porcine islet-like cell clusters (NPCCs) are altogether unclear. In this study, lectin microarray analyses of NPCCs were performed and the results compared with the corresponding values for wild-type APIs and NPCCs from α-Gal transferase knockout (GalT-KO) pig.

Methods: NPCCs were isolated from 1–3-d-old neonatal wild-type pigs and cultured for 1 d, 5 d, and 9 d, using a previously described technique. Alternatively, the isoration of APIs were isolated based on the method for human islets.

Results: In a comparison between NPCCs and APIs, all of the NPCCs showed higher signals for *Sambucus nigra*, *Sambucus sieboldiana*, and *Trichosanthes japonica I* and the binding of α2,6 sialc acid, whereas the APIs showed stronger signals for *Lotus tetragonolobus*, *Aleuria aurantia*, *Narcissus pseudonarcissus*, and *Galanthus nivalis*, suggesting that APIs contain high levels of high-mannose forms. Among the NPCCs, NPCC (day1) appeared to be richer than the others in *Lotus tetragonolobus*, *Narcissus pseudonarcissus*, *Galanthus nivalis*, and *Urtica dioica*, implying the presence of high-mannose forms. However, as a whole, the signals for many lectins for NPCCs were very similar. The NPCCs from a GalT-KO pig indicated not only the down-regulation of α-Gal expression but α-GalNAc as well, and α2-6 sialic acid was upregulated.

Conclusions: The results reported herein contain useful information for the future production of immunomodified pigs with less antigenicity than GalT-KO pigs toward clinical applications of NPCCs.

1. Introduction

The increasing problem of the worldwide shortage of donor organs has led to a revival of interest in xenotransplantation. Xenografting can be classified as either discordant or concordant, such as pig to human or monkey to human, respectively, based on the severity and pattern of the graft rejection. The pig represents an ideal animal for discordant

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xenografts for a variety of reasons, including anatomical, physiological, and ethical considerations. The exposure of pig cells, tissues, and organs to human blood, however, results in a hyperacute rejection, which is mediated by naturally occurring high-titer antibodies and complements that are produced by humans [1]. The major xenoantigen responsible for this type of rejection is a single carbohydrate structure, the α-Gal epitope (Galα1-3Galβ1-4GlcNAc-R) [2], which is expressed by most mammalian cells, including the pig. Old World monkeys, apes, and humans lack this enzyme activity because the α1,3-galactosyltransferase gene is inactivated, and, in contrast, produce large amounts of antibodies, designated as anti-Gal, against the α-Gal epitope. Human anti-pig antibodies are not only IgG, but also include IgM and IgA, and these bind most strongly to oligosaccharides that contain this alpha galactose terminal residue [3].

On the other hand, using somatic cell nuclear transfer technology [4,5], we successfully produced genetically engineered pigs by heterozygous knockout (KO) of the α1-3galactosyltransferase (GalT) gene (GalT-KO). However, even after GalT-KO pigs were produced, most antigens, including the so-called non-Gal antigen, were still present on the surface of the pig cells, and this fact represents a significant obstacle to successful xenotransplantation. That is, although the production of GalT-KO pigs appeared to eliminate the problem of hyperacute rejection, acute vascular rejection/acute humoral xenograft rejection, which involves the production of xenoreactive antibodies related to glycoantigens and subsequent activation of the graft endothelium, containing the system that controls activation of the complement and coagulation, was defined as a new obstacle for clinical xenotransplantation [6].

The pig pancreas is now considered to be the most suitable source of islets for clinical xenotransplantation, especially for patients with type I diabetes, which is a very common disease (approximately one million people have been diagnosed in the United States). Two types of islet transplantations are under consideration. One is the transplantation of adult pig islets (APIs) [7]. The other is the transplantation of neonatal porcine islet-like cell clusters (NPCCs) [8]. Several clinical trials [9] and preclinical experiments [10] using either type of islets are currently in progress. However, in addition to the α-Gal epitope, our knowledge of the extent of glycosylation and xenoantigenicity of pig islets is far from clear.

Regarding the extent of α-Gal expression on APIs, the results of a number of published studies indicate that the expression of α-Gal in adult pig islet cells is negligible. However, APIs have non-Gal antigenicity, including the Hanganutziu-Deicher (H-D) antigen, especially in terms of *N*-linked sugars. Evidence collected in our previous study showed that human natural antibodies IgG and IgM react with adult islets. Neuraminidase-sensitive sialic acid antigens other than H-D antigens are related to the binding of non-Gal antibodies. On the other hand, concerning the expression of α-Gal on NPCCs, NPCCs clearly express α-Gal, and the existence of the H-D antigen on NPCCs was also demonstrated. NPCCs have a strong antigenicity derived from *N*-linked sugars, but at the same time the cell surface glycolipids of these cells are also antigenic. Available data indicate that pig sialic acids, besides the H-D antigen, are clearly antigenic to human serum [7,8].

In this study, we took advantage of the fact that lectins are available that bind to unique glycostructures. Lectin microarray analyses [11] for NPCCs cultured for 1, 5, and 9 d were performed and the results compared with the corresponding values for islets from wild-type APIs. In addition, changes in the glycoantigens of the NPCCs from a GalT-KO pig that was produced as a pig line by our group were also analyzed.

2. Materials and methods

2.1. *Preparation of NPCC*

Pancreases were isolated using the technique described by Korbutt *et al.* [12]. Pancreases were obtained from 1–3-d-old neonatal pigs (Large White/Landrace × Duroc) of either sex. A pig cloned from a homozygous GalT-KO pig and wild-type pigs were used. The pancreas was dissected and stored in cold Hanks' balanced saline solution (HBSS; GIBCO Labs, Grand Island, NY) supplemented with 0.25% bovine serum albumin (Nakalai Tesque, Kyoto, Japan), 10 mm N-2-hydroxyethylpiperazine-N1-2-ethane-sulfonic acid (HEPES, Nakalai Tesque), and antibiotics until processed. In a typical preparation, a pancreas was minced into 1–2 mm^3 fragments and then digested with type V collagenase (2.5 mg/mL; Sigma, St Louis, MO) at 37°C with gentle shaking. After filtration through a nylon screen (500 μm), the tissue was washed in HBSS three times and cultured at 37°C in a humidified atmosphere with 5% CO_2 for 7–9 d in Ham's F10 Medium (GIBCO) containing 10 mmol/L glucose, 50 μmol/L isobutylmethylxanthine (Nakalai Tesque), 0.5% bovine serum albumin (Nakalai Tesque), 2 mmol/L l-glutamine (Nakalai Tesque), 10 mmol/L nicotinamide (Sigma), and antibiotics. The culture medium was changed on the first day after isolation and every other day thereafter [8].

2.2. *Adult pig islet isolation*

Porcine islet isolation and cultures were performed as previously described [13], with minor modifications. In brief, the duodenal-splenic lobe and connective lobe were distended intraductally by treatment with a cold enzyme solution of LiberaseHI (Roche Molecular Biochemicals, Indianapolis, IN) diluted with HBSS. The distended glands were digested by the semi-automated continuous-filtration method at 37°C.The cleaved islets were purified from non-islet tissue with a continuous density gradient in a COBE 2991 cell processor (COBE Blood Component Technology, Lakewood, CO). Isolated islets were cultured overnight at 37°C in a humidified atmosphere with 5% CO_2 in Tohoku culture bags (Nipro, Osaka, Japan) [14] with medium 199 (Gibco) supplemented with 10% heated inactivated porcine serum, 2 mM N-acetyl-l-alanyl-l-glutamine, 10 mM HPEPES (Nakarai Tesque), 100 IU/mL penicillin, 100 μg/mL streptomycin (Biochrom), and 20 μg/mL ciprofloxacin.

2.3. *Preparation of the samples*

Each sample was washed with phosphate-buffered saline and sonicated in PBST (phosphate-buffered saline containing 1.0%

Triton X-100). The whole-cell lysate was immediately frozen at −80°C. Each sample was thawed on ice, and after centrifugation at 14,000 *g* for 5 min, the supernatant was collected.

2.4. *Lectin microarray*

The lectin microarray was prepared as previously described, with minor modifications [11,15]. The glycoprotein from each cell was incubated at room temperature for 60 min in the dark with 10 μg Cy3-succimidyl ester (Amersham Biosciences, Tokyo, Japan). The resulting reaction product was loaded on a Sephadex-G25 spin column to remove the excess fluorescent reagent. After centrifugation, the volume of the collected solution was adjusted to 200 μL with probing buffer (Tris-buffered saline containing 1.0% Triton X-100 and 500 mM glycine) and then incubated for 2 h at room temperature, in order to completely inactivate residual fluorescent reagent. To each well on the glass slide, 60 μL of the Cy3-labeled glycoprotein solution was applied in triplicate and the solution was incubated overnight at 20°C in a humid chamber. After the binding reaction was complete, a fluorescence image of the array was acquired using an evanescent-field fluorescence scanner, Glycostation Reader (Moritex, Yokohama, Japan). All data were analyzed with Array Pro analyzer version 4.5 (Media Cybernetics, Bethesda, MD). The acquired data were differentially analyzed using normalized signals obtained for all lectins, with the lectin showing the strongest signal intensity (max intensity) being assigned a value of 1.0 (Table).

2.5. *Statistics*

Data are presented as the mean ± SD. The Student t-test was used to ascertain the significance of differences within groups. Differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. *Comparison between NPCCs and APIs*

All of the NPCCs (days 1–9) showed significantly higher signals for the binding of α2,6 sialic acid (Sia) with SNA ($P < 0.05$), SSA ($P < 0.05$), and TJA-I ($P < 0.05$), and slightly higher for the binding of α2,3Sia, whereas the APIs showed generally stronger signals for lectins that bind high-mannose forms, such as NPA, Con A, GNA, and Calsepa.

Concerning lectins related to α-GalNAc, VVA, DBA, and HPA were high in API, whereas SBA and GSI-A4 showed stronger signals in NPCCs (Fig. 1).

3.2. *Among the NPCCs*

NPCC (day 1) showed a tendency to be richer than NPCC (day 5 and day 9) in LTL, NPA, and GNA, implying the presence of high-mannose structures. However, as a whole, the signals corresponding to NPCCs were nearly the same for many lectins (Fig. 1).

3.3. *Difference between NPCCs from wild-type and GalT-KO pig*

In comparison with the wild-type NPCCs, the NPCCs (day 9) from the GalT-KO pig were diminished, not only for EEL and GSI-B4, which bind α-Gal, but SBA, PTL, and GSI-A4, which bind GalNAc. On the other hand, the GalT-KO NPCC showed stronger signals for SNA, SSA, and TJA-I, which bind α2-6Sia (Fig. 2).

4. Discussion

Glycoantigens are major obstacles to xenotransplantation. Even after producing the GalT-KO pigs, the so-called non-Gal antigens, especially glycoantigens, were still present on the surface of pig cells. These are also responsible for several other types of rejection, such as humoral rejection, based on an antibody reaction. Lectin microarray analyses to detect the non-Gal antigens of NPCCs from wild-type pigs were performed in this study and the results were compared with the corresponding values for wild-type APIs and an NPCC from a GalT-KO pig.

In general, including reports concerning sera obtained from clinical patients in whom NPCC had been intraportally injected [16] and a high-performance liquid chromatography (HPLC) analysis of pig cells [17], several glycans could function as a non-Gal glycoantigen that could be recognized by an antibody reaction of humans, including a terminal α-linked GalNAc, linear and branched oligomannose structures, Galα1-3Lew^x, NeuAcα2-3Galβ1-3GlcNAc, and a variety of structures that are terminated with α2-3- and α2-6-linked NeuGc (N-glycolylneuraminic acid: H-D antigen) units [18–21].

The H-D antigen, one of the best-known non-Gal antigens, is widely distributed in mammalian species including monkeys and apes, but is not found in humans and birds. The production of NeuGc from NeuAc (N-acetylneuraminic acid) is controlled by the enzymatic activity of cytidine monophospho-N-acetylneuraminic acid hydroxylase. Humans lack this enzyme activity because of the partial deletion of exon 6. As a result, humans can produce antibodies to this epitope. However, unfortunately, the lectin analysis failed to detect this glycoantigen because no lectin can specifically bind to NeuGc.

On the other hand, in our previous study concerning the antigenicity of NPCC, neuraminidase-sensitive sialic acid antigens, other than the H-D antigen, were related to the binding of non-α-Gal antibody, especially IgG [8]. Moreover, the results of the NPCC were essentially identical to those for API [7]. In this study, in comparison with API, NPCC was found to be very rich in α2-6Sia, including 2-6NeuAc and 2-6NeuGc, and the differences are quite evident. In addition, the expression of sialic acid antigens, such as 2-6Sia, was unfortunately upregulated on the NPCC from the GalT-KO pig. Some types of glycostructures containing 2-6NeuAc might be candidates for functioning as a non-Gal antigen.

Concerning α2-3NeuAc, such as NeuAcα2-3Galβ1-3GlcNAc, the signals of several lectins for α2-3Sia were also stronger in NPCC and the tendency appeared to be steady.

Regarding the other differences, high-mannose forms were upregulated in API. The difference is relatively clear. The high expression of the high-mannose form in API may have some relationship to the antigenicity to humans, because the

Table – List of lectins used and their reported specificities.

	Abbreviation	Lectin	Reported specificity
1	LTL	*Lotus tetragonolobus*	Fucα1-3(Galβ1-4)GlcNAc, Fucα1-2Galβ1-4GlcNAc
2	PSA	*Pisum sativum*	Fucα1-6GlcNAc, α-D-Glc, α-D-Man
3	LCA	*Lens culinaris*	Fucα1-6GlcNAc, α-D-Glc, α-D-Man
4	UEA-I	*Ulex europaeus* I	Fucα1-2Galβ1-4GlcNAc
5	AOL	*Aspergillus oryzae*	Fucα1-6GlcNAc (core fucose)
6	AAL	*Aleuria aurantia*	Fucα1-6GlcNAc, Fucα1-3(Galβ1-4)GlcNAc
7	MAL	*Maackia amurensis*	Siaα2-3Galβ1-4GlcNAc
8	SNA	*Sambucus nigra*	Siaα2-6Gal/GalNAc
9	SSA	*Sambucus sieboldiana*	Siaα2-6Gal/GalNAc
10	TJA-I	*Trichosanthes japonica* I	Siaα2-6Gal/GalNAc
11	PHA(L)	*Phaseolus vulgaris* (L)	Tri/tetra-antennary complex-type *N*-glycan
12	ECA	*Erythrina cristagalli*	Galβ1-4GlcNAc
13	RCA120	*Ricinus communis*	Galβ1-4GlcNAc
14	PHA(E)	*Phaseolus vulgaris* (E)	Bi-antennary complex-type *N*-glycan with outer Gal and bisecting GlcNAc
15	DSA	*Datura stramonium*	(GlcNAcβ1-4)n, Galβ1-4GlcNAc
16	GSL-II	*Griffonia simplicifolia* II	Agalactosylated tri/tetra antennary glycans, GlcNAc
17	NPA	*Narcissus pseudonarcissus*	High-mannose, Manα1-6Man
18	ConA	*Canavalia ensiformis*	High-mannose, Manα1-6(Manα1-3)Man
19	GNA	*Galanthus nivalis*	High-mannose, Manα1-3Man
20	HHL	*Hippeastrum hybrid*	High-mannose, Manα1-3Man, Manα1-6Man
21	ACG	*Agrocybe cylindracea*	Siaα2-3Galβ1-4GlcNAc
22	TxLC-I	*Tulipa gesneriana* I	Manα1-3(Manα1-6)Man, bi- and tri-antennary complex-type *N*-glycan, GalNAc
23	BPL	*Bauhinia purpurea alba*	Galβ1-3GalNAc, GalNAc
24	TJA- II	*Trichosanthes japonica* II	Fucα1-2Galβ1- > or GalNAcβ1- > groups at their nonreducing terminals
25	EEL	*Euonymus europaeus*	Galα1-3Gal, blood group B antigen
26	ABA	*Agaricus bisporus*	Galβ1-3GalNAc
27	LEL	*Lycopersicon esculentum*	GlcNAc trimers/tetramers
28	STL	*Solanum tuberosum*	GlcNAc oligomers, oligosaccharide containing GlcNAc and LacNAc
29	UDA	*Urtica dioica*	GlcNAcβ1-4GlcNAc, mixture of Man5 to Man9
30	PWM	*Phytolacca americana*	(GlcNAcβ1-4)n
31	Jacalin	*Artocarpus integrifolia*	Galβ1-3GalNAc, GalNAc
32	PNA	*Arachis hypogaea*	Galβ1-3GalNAc
33	WFA	*Wisteria floribunda*	GalNAcβ1-4GlcNAc, Galβ1-3(-6)GalNAc
34	ACA	*Amaranthus caudatus*	Galβ1-3GalNAc
35	MPA	*Maclura pomifera*	Galβ1-3GalNAc, GalNAc
36	HPA	*Helix pomatia*	α-linked terminal GalNAc
37	VVA	*Vicia villosa*	α-linked terminal GalNAc, GalNAcα1-3Gal
38	DBA	*Dolichos biflorus*	GalNAcα1-3GalNAc, blood group A antigen
39	SBA	*Glycine max*	α- or β-linked terminal GalNAc, GalNAcα1-3Gal
40	Calsepa	*Calystegia sepium*	Mannose, maltose
41	PTL-I	*Psophocarpus tetragonolobus* I	α-linked terminal GalNAc
42	MAH	*Maackia amurensis*	Siaα2-3Galβ1-3(Siaα2-6)GalNAc
43	WGA	*Triticum unlgaris*	chitin oligomers, Sia
44	GSI-A_4	*Griffonia simplicifolia* I-A_4	α-linked GalNAc
45	GSI-B_4	*Griffonia simplicifolia* I-B_4	α-linked Gal

These data were collected by Moritex Corporation.

high-mannose forms may include both linear and branched oligomannose structures. In general, N-linked sugars (Asn-linked) are classified as high-mannose forms, complex forms, and hybrid forms. The high-mannose form normally consists of five to nine mannose residues and two N-acetylglucosamine residues and is a biosynthetic precursor of hybrid-form and complex-form N-glycans.

In addition, GalNAc forms related to the Tn antigen were rich in API, but also not measurably different. The Tn antigen was named from the observation by Moreau *et al.* [22] based on the observation that it was similar yet distinct from the Thomsen-Friedenreich antigen (T(F)-antigen; Galβ1-3GalNAcα1-O-Ser/Thr) [23]. It has a simple structure composed of an N-acetyl-D-galactosamine unit that is linked *via* an α glycosidic linkage to serine/threonine residues in glycoproteins (GalNAcα1-O-Ser/Thr).

On the other hand, concerning the changes during the 9 d of incubation on the NPCC, the intensities of most of the signals were, unfortunately, not clear in this study, suggesting that NPCCs contain numerous immature islets and cells other than islets.

Finally, NPCC from a GalT-KO pig was analyzed and the results compared with those for wild-type pigs. Concerning the T antigen detected by Jacalin, PNA and MPA indicates that the T antigen is present at lower levels in the GalT-KO pig, but this may not be significant. On the other hand, clear differences between wild-type NPCC and GalT-KO were found among the samples for DBA, SBA, PTL-I, and GSI-A4, which bind GalNAc, including the Tn antigen. Regarding the Gal-T KO pig, only one NPCC could be analyzed in this study. Therefore, it would be speculative to draw firm conclusions regarding the differences between wild-type NPCC and Gal-T

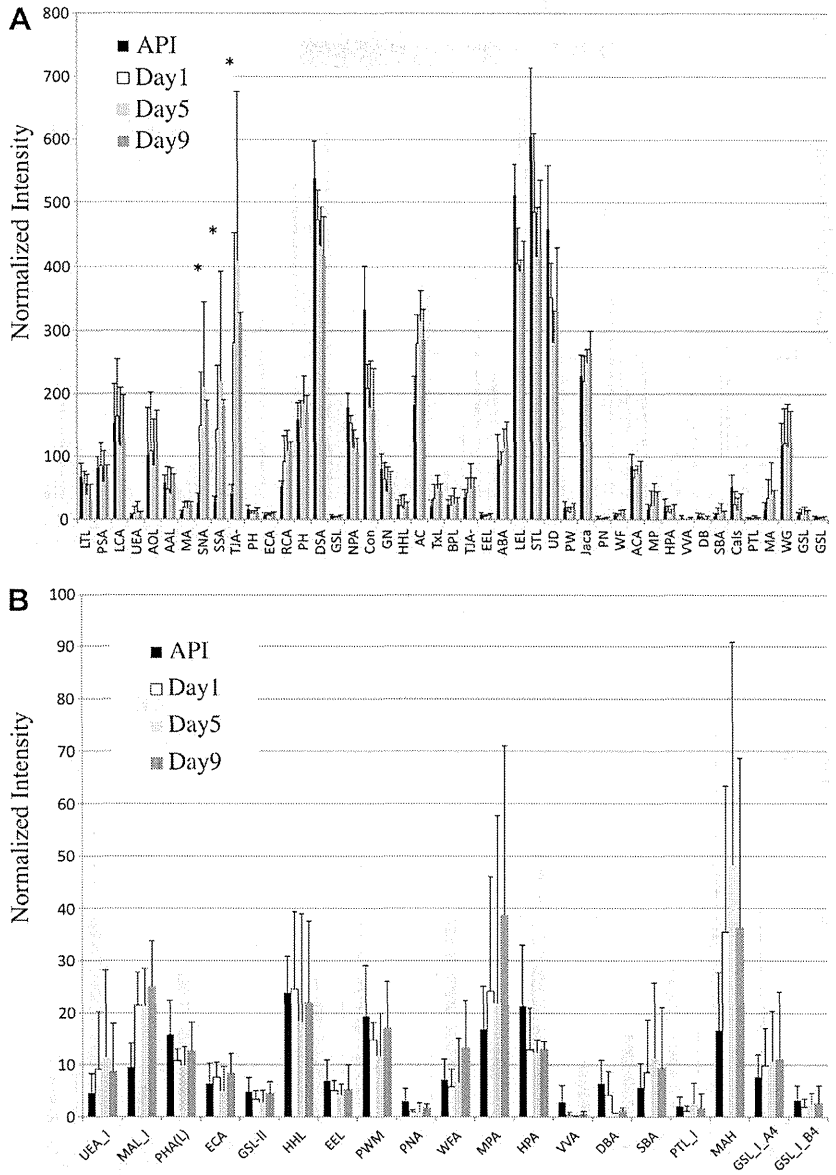

Fig. 1 – (A) Comparison of glycan profiles between API and NPCC. Summary of the normalized glycan intensity. The difference in islets between API and NPCC was subsequently studied. The net intensities of the obtained signals were normalized by the total intensity of each experiment, and the average value of three experiments was calculated. (B) Summary of the normalized glycan intensity for small peaks. Columns indicate the signal intensity and are the average ± standard deviation of six experiments for API and three experiments for NPCC. Asterisk indicates $P < 0.05$, compared with API.

KO. However, the differences in signal intensity between them were similar to the results reported for endothelial cells and fibroblasts in our previous study [11]. In addition to α-Gal or terminal GalNAc, not only H-D antigen but also some NeuAc structures represent possible targets for human natural antibodies. Therefore, even after α-GalT was knocked out, it remains possible that these epitopes could still remain in NPCC. The findings of a recent report from Edmonton indicate that NPCC from GalT-KO donors can improve the outcome of an NPCC transplantation to a nonhuman primate. However, large quantities of drugs, such as CTLA4Ig, anti-CD154, anti-LFA-1, and MMF, are needed for a favorable outcome. In addition, in spite of the heavy drug treatment, one of five recipients failed to engraft and another graft was rejected within 50 d after transplantation [24].

Future studies will include HPLC analyses in an attempt to identify the key glycan of NPCC that produces antigenicity in humans, comparing with those of APIs and human islets. Based on the data reported herein, and further HPLC analyses of NPCC, we plan to attempt to identify the pig genes that produce the non-Gal antigenicity, especially relating to Sia. As the next step, we plan to produce new KO pigs that lack one of the non-Gal antigens using somatic cell nuclear transfer technology.

In summary, although the levels of high-mannose forms are high in APIs, NPCCs are rich in α2-3 and α2-6Sia expression in comparison with API. The signals for lectins, which bind not

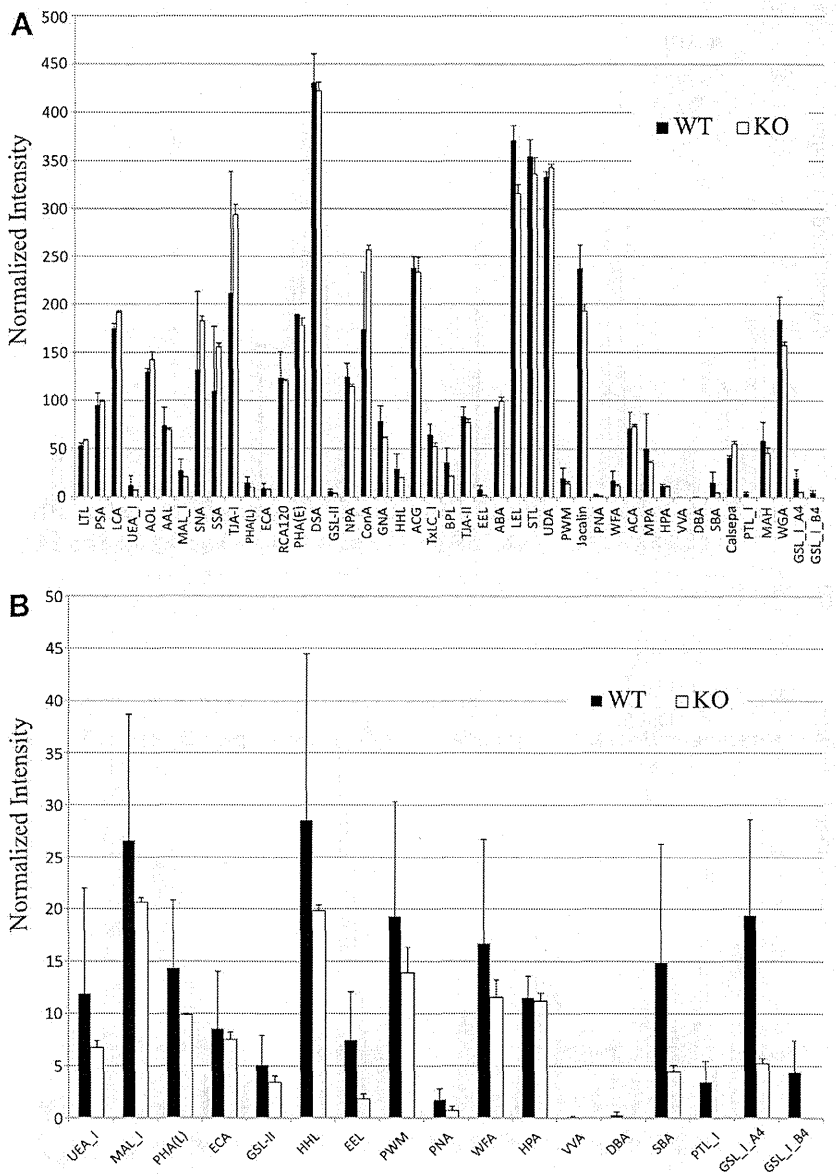

Fig. 2 – (A) Comparison of glycan profiles between NPCC (day 9) from wild-type and GalT-KO pigs. Summary of the normalized glycan intensity. The difference in islets between NPCC (day 9) from wild-type and GalT-KO pigs was subsequently studied. The net intensities of the obtained signals were normalized by the total intensity of each experiment. (B) Summary of the normalized glycan intensity for small peaks. Columns indicate the signal intensity and are the average ± standard deviation of two experiments for wild-type and the average ± standard deviation of triplicate determination of one experiment for a GalT-KO pig.

only to α-Gal but also to (terminal) GalNAc, were clearly diminished in the NPCC from the GalT-KO pig. However, the expression of α2-6Sia was upregulated.

These findings support a future plan that involves the production of immunomodified pigs with less antigenicity to humans than GalT-KO pigs for clinical applications of NPCCs.

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The suppression of inflammatory macrophage-mediated cytotoxicity and proinflammatory cytokine production by transgenic expression of HLA-E☆

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ABSTRACT

Background: Macrophages participate in xenogenic rejection and represent a major biological obstacle to successful xenotransplantation. The signal inhibitory regulatory protein α (SIRPα) receptor was reported to be a negative regulator of macrophage phagocytic activity via interaction with CD47, its ligand. Because a majority of human macrophages express the inhibitory receptor CD94/NKG2A, which binds specifically to the human leukocyte antigen (HLA)-E and contains immunoreceptor tyrosine-based inhibition motifs (ITIMs), the inhibitory function of HLA class I molecules, HLA-E, on macrophage-mediated cytolysis was examined. The suppressive effect against proinflammatory cytokine production by macrophages was also examined.
Methods: Complementary DNA (cDNA) of HLA-E, and CD47 were prepared and transfected into swine endothelial cells (SEC). The expression of the modified genes was evaluated by flow cytometry and macrophage-mediated cytolysis was assessed using in vitro generated macrophages.
Results: Transgenic expression of HLA-E significantly suppressed the macrophage-mediated cytotoxicity. HLA-E transgenic expression demonstrated a significant suppression equivalent to CD47 transgenic expression. Furthermore, transgenic HLA-E suppressed the production of pro-inflammatory cytokines by inflammatory macrophages.
Conclusions: These results indicate that generating transgenic HLA-E pigs might protect porcine grafts from, not only NK cytotoxicity, but also macrophage-mediated cytotoxicity.

1. Introduction

Xenotransplantation is one of the most attractive strategies for overcoming the worldwide shortage of organs. However, immune rejection due to species disparity is a major hurdle in successful xenograft transplantation. Hyperacute rejection (HAR), mediated by pre-existing natural antibodies against the galactose-α-1,3-galactose (α-Gal) antigen and the activation of a complement cascade, usually occurs immediately after xenotransplantation [1,2]. The successful production of α-1,3-galactosyltransferase knockout (GT-KO) cloned pigs by somatic cell nuclear transfer (SCNT) was recently reported [3–5]. However, Shimizu et al. demonstrated the acute humoral xenograft rejection (AHXR) of xenografts from GT-KO pigs in baboons [6]. After overcoming HAR and acute humoral xenograft rejection (AHXR), the xenograft might become susceptible to cellular rejection, which is mediated by monocytes, macrophages, and NK cells.

Recently reported findings confirm the role of innate cellular immunity in xenograft rejection, induced by a combination of xenoantigen recognition by activating receptors, and incompatibility in inhibitory receptor-ligand interactions. It should be noted in this respect that a considerable body of evidence exists regarding NK-mediated cytotoxicity against porcine cells [7–13]. Xenografts are believed to be more susceptible to NK-mediated cytotoxicity due to the inability of porcine MHC (SLA) class I molecules to deliver inhibitory signals to human NK cells. Actually, we (and others) have shown that the overexpression of human MHC I molecules on porcine cells resulted in a recuction in xenocytotoxicity mediated by NK cells (10–13). It is also well known that macrophages induce the rejection of donor cells in highly disparate xenogeneic settings [14,15] Ide et al. recently reported that the incompatibility of CD47 is responsible for the in vitro phagocytosis of xenogeneic porcine cells by human macrophages [16]. CD47 is a member of an immunoglobulin superfamily that serves as a ligand for an inhibitory receptor, the signal regulatory protein (SIRP)α. The binding of CD47 to SIRPα plays important roles in hematopoietic and immunological regulation [17–21]. Furthermore, CD47 inhibits both Fcγ and complement receptor-mediated phagocytosis via its ability to bind to SIRPα [22]. Furthermore, Vaithilingam et al. examined xenogenic immunoreactions against microencapsulated foetal pig islet-like cell clusters [23]. In this study, an enhanced intragraft mRNA expression of pro-inflammatory factors such as MIP-1α, which are associated with the recruitment of macrophages, was detected within the first two

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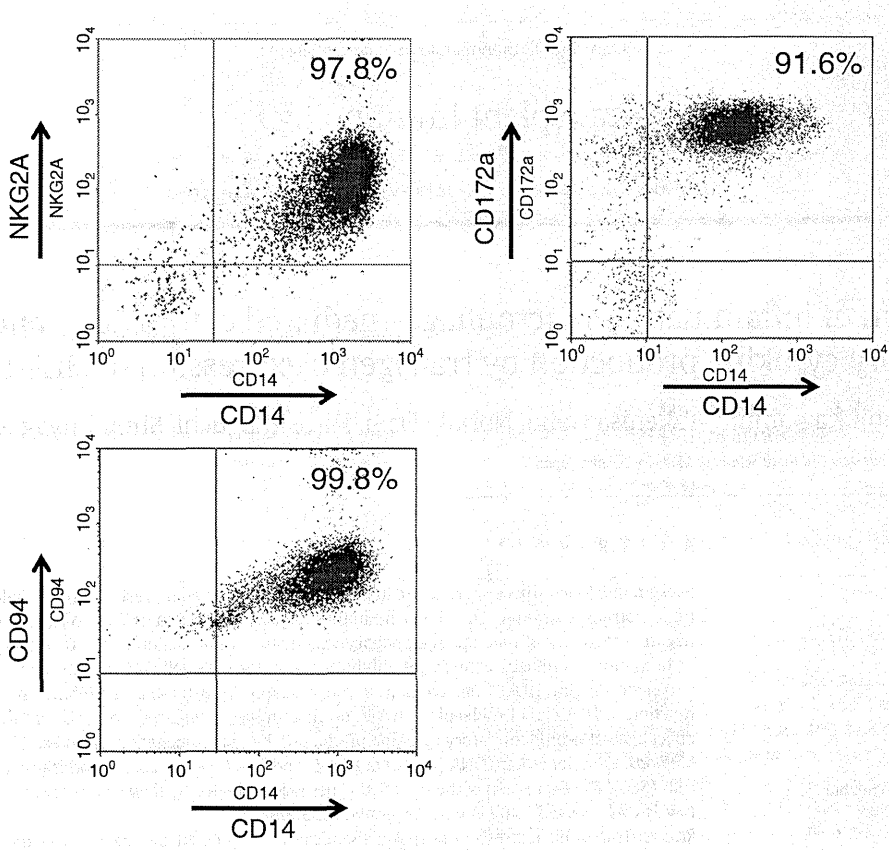

Fig. 1. Expression of inhibitory receptors on in vitro generated macrophages. Human monocytes were harvested from PBMC after adhering to the plastic vial. Cells were cultured in the presence of 100 ng/ml GM-CSF for 5 days. After LPS stimulation for 24 h, cells were harvested and the expressions of NKG2A/CD94 and SIRPα were analysed by flow cytometry. The vast majority of in vitro generated macrophages expressed NKG2A/CD94 and SIRPα.

weeks post transplantation. These results indicate that macrophages represent a potential obstacle in xenogenic conditions without HAR and AHXR. Therefore, it may be necessary to regulate macrophages in human recipients to achieve the successful engraftment of xenografts.

In previous reports, we (and others) demonstrated that the transgenic expression of HLA-E and G suppresses NK-mediated cytotoxicity (10–13). Therefore, the expression of inhibitory receptors in inflammatory macrophages was assessed. The vast majority of inflammatory macrophages express inhibitory receptors, such as the CD94/NKG2A heterodimer. We hypothesized that the transgenic expression of HLA-E might suppress, not only NK cell-mediated cytotoxicity, but also macrophage-mediated cytotoxicity. Due to the extensive molecular incompatibilities between donor and host, innate cellular immune responses are more serious aspects in the rejection of xenografts than in allograft rejection. Activated innate immune cells destroy xenografts, not only by direct cytotoxicity, but also by augmenting subsequent T cell xenoresponses.

In this study, the suppressive effect of the transgenic expression of HLA-E in pig cells against macrophage-mediated xenocytotoxicity and pro-inflammatory cytokine production by macrophages was evaluated and the results are reported herein.

2. Materials and methods

2.1. Cells and reagents

A SEC (swine endothelial cell) line, MYP30, was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO). A fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG, a monoclonal antibody to MHCI (clone: B9.12.1), a monoclonal antibody against human CD94 were purchased from Beckman Coulter (Marseille, France) and a fluorescein isothiocyanate (FITC)-labelled anti-human CD47, Phycoerythrin (PE)–anti-human CD172a (signal-regulatory proteinα, SIRPα) and Allophycocyanin (APC)-labelled anti-human CD14 were purchased from Bio Legend (San Diego, CA). PE-labelled anti-NKG2A was purchase from R&D (Minneapolis, MN).

2.2. Construction of hybrid genes

cDNAs for the HLA-E (HLA-Ev(147)) gene [21] and b2-microglobulin (hb2m) were prepared. The hybrid gene, HLA-Ev(147) + IRES + hb2m, was then constructed and cloned into the pCXN2L (β-actin promoter + neomycin resistance) expression vector.

Concerning the CD47 molecule, the cDNAs of PI-anchored CD47 with a FLAG-tag epitope, consisting of the eight-amino acid sequence, DYKDDDDK, was established. In brief, pCXN2L/CD47-PI was initially constructed, as follows. The cDNA for the extracellular domain of CD47 with a FLAG-tag epitope in N-terminus was synthesized (MBL, Nagoya, Japan), and then connected to the GPI-anchor region of CD55 with an Xho I site. The cDNA was also subcloned into the expression vector pCXN2L. All sequences were verified by means of an ABI 310 autosequencer (Perkin-Elmer Corporation, Norwalk, CT).

2.3. Transfection experiments

The purified plasmids (5 μg) were introduced into MYP-30 by pid-mediated DNA transfection using the Lipofectamine™ reagent (Invitrogen, Carlsbad, CA). The transfected MYP-30 cells were maintained in complete medium for 24 h in an atmosphere of humidified 5% CO^2 at