Reduction of the Major Swine Xenoantigen, the α-Galactosyl Epitope by Transfection of the α2,3-Sialyltransferase Gene*

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α2,3-Sialyltransferase represents a putative enzyme that reduces the GaⅢ1Gal β1-4GlcNAc-R (the α-galactosyl epitope) by intracellular competition with the α2,3-galactosyltransferase for a common acceptor substrate.

This study demonstrates that the overexpression of the α2,3-sialyltransferase gene suppresses the antigenicity of swine endothelial cells to human natural antibodies by 77% relative to control cells and by 30% relative to cells transfected with α2,1-fucosyltransferase, and in addition, it reduces the complement-mediated cell lysis by 75% compared with control cells and by 22% compared with cells transfected with α2,1-fucosyltransferase. The mechanism by which the α-galactosyl epitope was reduced was also studied. Suppression of the α2,3-galactosyltransferase activity by 30–63% was observed in the transfectants with α2,3-sialyltransferase, and mRNA expression of the α2,3-galactosyltransferase gene was reduced as well.

The data suggest that the α2,3-sialyltransferase effectively reduced the α-galactosyl epitope as well as or better than the α2,1-fucosyltransferase did and that the reduction of the α-galactosyl epitope is due not only to substrate competition but also to an overall reduction of endogenous α1,3-galactosyltransferase enzyme activity.

Xenotransplantation has been proposed as the most promising procedure to alleviate the world-wide shortage of organs for transplantation (1–3). However, in a vascularized organ, a xenograft between discordant species, such as swine to human (4), hyperacute rejection occurs within minutes due to the destruction of endothelial cells, a process that is mediated by fixation of natural antibodies and/or complement activation (5). Initial attempts to prevent hyperacute rejection have focused on modification of the donor xenograft via the expression of human complement regulatory proteins (6–10). This strategy has proven to be very useful in prolonging swine grafts survival (11–14).

Since Galili et al. (15–17) reported that Galα1-3Gal β1-4GlcNAc-R (the α-galactosyl epitope) represents the major antigen in swine to human xenotransplantation, genetic approaches to modify this glycoantigen have been the focus of numerous xenotransplantation studies. This antigen is a linear type 2 oligosaccharide, the α-galactosyl epitope of which is synthesized by UDP-Gal:β1-4GlcNAc α1-3-galactosyltransferase (α1,3GT). Two deletion mutations in the human α1,3GT DNA sequence cause the inactivation of α1,3GT in humans (18–20). Therefore, humans produce a natural antibody that makes up as much as 1% of the circulating IgG in humans and is also found in significant amounts as an IgM antibody (15–17).

One strategy to down-regulate the α-galactosyl epitope is to knock out the α1,3GT gene using embryonic stem cells (21, 22) or somatic cells and nuclear transplantation techniques (23). In the case of swine, however, these systems are not available. Another strategy for down-regulating the α-galactosyl epitope is to take advantage of enzymatic competition involving terminal glycosylation between the α1,3GT and other glycosyltransferases for the common acceptor substrate. Several glycosyltransferases, such as α1,2 fucosyltransferase (α1,2FT) (24), α1,3 fucosyltransferase (α1,3FT) (25), α2,3-sialyltransferase (α2,3ST) (26–29), and α2,6-sialyltransferase (α2,6ST) (30, 31) represent possibilities. Among these, the gene transfection of α1,2FT has been reported to result in a drastic suppression of the α-galactosyl epitope (32–34).

The purpose of this study is to investigate whether the antigenicity of swine endothelial cells is affected by α2,3ST, an enzyme that catalyzes the sialylation of N-acetyllactosamine (Gal β1-4GlcNAc β1-) to form α2,3-sialyl N-acetyllactosamine, the precursor of the sialyl Lewis X structure (35) and to assess the mechanisms by which the α-galactosyl epitope is reduced. In the case of the sialyltransferase family, three types of sialyltransferases (26–31, 36) are known to participate in the intracellular competition with α1,3GT for the common acceptor substrate. Our previous study, using β-1, 4-mannoside β-1, 4,N-acetylglucosaminyltransferase III, which catalyzes the β-1,4 addition of N-acetylglucosamine to the β-linked mannose of the trimannosyl core of N-linked sugar chains, demonstrated that N-linked sugars were the main source of xenotrigenicity (37–41). Therefore, in the present study, we employed ST3Gal III, which catalyzes the transfer of sialic acids to a lactosamine structure, mainly to N-linked terminal sugar residues.

**EXPERIMENTAL PROCEDURES**

Endothelial Cell Culture—A swine endothelial cell (SEC) line, MYP30, was cultured in Dulbecco's modified Eagle’s medium containing 10% FBS with l-glutamine and penicillin/streptomycin (Life

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1 The abbreviations used are: α1,3GT, UDP-Gal:β1-4GlcNAc α1,3Gal-transferase; α1,2FT, α1,2 fucosyltransferase; α1,3FT, α1,3 fucosyltransferase; α2,3ST, α2,3 sialyltransferase; SEC, swine endothelial cell; β1,4GT, β-1,4 N-galactosyltransferase; PBS, phosphate-buffered saline; NHS, normal human serum; LDH, lactate dehydrogenase; GlcNAc, N-acetylglucosamine; IB4, G. simplicifolia 1.
Swine Endothelial Cells Transfected with the α2,3ST Gene

Technologies, Inc.) (9).

Construction of Plasmids—A cDNA of mouse α2,3ST (ST3Gal III) was subcloned into the site of pCXN2 (42), which is a β-actin promoter and a cytomegalovirus enhancer with a neomycin-resistant gene. The plasmid was separately transformed into Esherichia coli C6000 and amplified using standard techniques (43) to obtain the cDNA of human α1,2FT, a gift from Dr. John B. Lowe (University of Michigan), was also subcloned into pCXN2.

Expression of cDNAs—The cDNAs (20 μg) were introduced into MYP-30 by lipid-mediated DNA transfection with lipofectamine (Life Technologies, Inc.) (53). Transfected MYP-30 were cultivated in complete medium containing 0.4 mg/ml G418 (Life Technologies, Inc.) for selection (9). Expression of plasmids was confirmed by high performance liquid chromatography as described below.

Enzyme Activity—The enzyme activities of α2,3ST, α1,2FT, β1,4-galactosyltransferase (β1,4GT), and α1,3GT in each cell line were assayed by high performance liquid chromatography as described (44, 45).

For the assay of enzyme activity, cells were washed twice with phosphate-buffered saline (PBS) and then centrifuged at 1500 × g for 10 min. The precipitated cells were resuspended in 100 μl of PBS and sonicated. The same acceptor substrate, pyridylaminated lacto-N-neotetraose (Galβ1-4Glcnacβ1-3Galβ1-4Glc-PA) at a final concentration of 10 μM was employed in α2,3ST, α1,2FT, and α1,3GalT activity assays. Lacto-N-neotetraose was purchased from Seikagaku Kogyo (Tokyo) and pyridylaminated according to the method of Kondo et al. (46).

α2,3ST activity was assayed in a reaction mixture that contained 0.1 mM cymodilactone buffer, pH 6.8, 0.01 mM MnCl₂, 0.45% Triton X-100, 10 mM CMP-sialic acid (Sigma). 10 μl of 50 μM substrate and 15 μl of cell lysate were added to 25 μl of this solution. The mixture was then incubated at 37 °C for 3 h.

The assay for 1,2FT activity employed 50 mM potassium phosphate buffer, pH 6.1, containing 0.2% Triton X-100, 2 mM GDP-fucose, 25 mM phenyl-galactoside, and 10 mM ATP. To 25 μl of this solution was added 10 μl of 50 μM substrate followed by 15 μl of cell lysate. The assay mixture was then incubated at 37 °C for 3 h.

α1,3GT activity was assayed in a reaction mixture containing 10 mM HEPES, pH 7.2, 20 mM UDP-galactose, 10 mM MnCl₂, 3 mM NaCl, 3 mM KCl. 10 μl of 50 μM substrate and 15 μl of cell lysate were added to this mixture, and the mixture was then incubated at 37 °C for 3 h.

The enzyme activity of β1,4GT was determined according to methodology described previously (47), using the pyridylaminated biantennary sugar chain (GlcNacβ1-2-Manα1-6 (GlcNacβ1-2-Manα1-3)-Manβ1-4GlcNAcβ1-4Glc-PA) as an acceptor substrate. The same reaction mixture as for the α1,3GT assay was used. 100 μl of 100 μM substrate and 15 μl of cell lysate were added to 25 μl of this solution, and the mixture was then incubated at 37 °C for 3 h.

The enzyme reactions were quenched by boiling for 5 min. The samples were then centrifuged at 12000 × g for 5 min, and an aliquot of each supernatant was subjected to high performance liquid chromatography analysis, using a TSK-gel ODS-80TM column (4.6 × 250 mm). The reaction products were eluted with 20 mM acetate buffer, pH 4.0, containing 0.01% 1-butanol at flow rate of 1.0 ml/min at 55 °C and were monitored with a fluorescence spectrophotometer (Shimadzu, model RF-10AXL) 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 hour of incubation per mg of protein.

Protein concentrations were determined with a BCA protein assay kit (Pierce) using bovine serum albumin as a standard.

α2,3ST and α1,2FT Activity of Parental SECs and Transfectants—Four positive stable clones of α2,3ST and several positive clones of α1,2FT were established. Of the α1,2FT transfectants one hybrid, moderate activity whereas the other had a higher activity. These were used as controls in comparison with the α2,3ST transfectants. The enzyme activities of these α2,3ST and α1,2FT transfectants are shown in Table I.

**RESULTS**

| Cells          | α2,3ST | α1,2FT |
|----------------|--------|--------|
| Parental SECs  | ND     | ND     |
| A-9 ST         | 30 ± 7.4| ND     |
| A-4 ST         | 150 ± 15| ND     |
| B-11 ST        | 167 ± 13| ND     |
| A-2 ST         | 200 ± 35| ND     |
| A-3 FT         | ND     | 220 ± 25|
| B-11 FT        | ND     | 1104 ± 25|

*ND, not detected.*

**Fluorescence Histograms of Parental SECs, α2,3ST-transfected SECs, and α1,2FT-transfected SECs Stained with NHS and IB4 Lecitin—** Whereas the parental SECs reacted strongly

Hydrate epiteps was also examined with an fluorescein isothiocyanate-conjugated IB4 lectin (Honen Co. Ltd., Tokyo, Japan) that binds the α-galactosyl epitope.

Lactate Dehydrogenase (LDH) Assay—The assay was performed according to a modified version of the method of Korzeniewski and Callewaert (48), using a Rugby LTX LDH kit. The transfected cells were plated at 2 × 10³ cells/well in 96-well trays 1 day prior to assay. The next morning, the wells were washed twice in serum-free Dulbecco’s modified Eagle’s medium to remove the LDH that is present in fetal calf serum and incubated in several concentrations of NHS diluted with Dulbecco’s modified Eagle’s medium. The plates were incubated for 2 h at 37 °C, and the released LDH was measured. The percentage cytotoxicity was calculated using the formula: cytotoxicity = |(E – N – S)/(M – N – S)| × 100, where E is the experimentally observed release of LDH activity from the target SECs, N is the LDH activity in each concentration of NHS, S is the spontaneous release of LDH activity from target SECs incubated in the absence of NHS, and M is the maximal release of LDH activity, as determined by the addition of 2% Nonident P-40 (49).

**Western Blotting—** Total cell lysates (3 μg) from parental or transfected SECs were subjected to 12% SDS-PAGE under reducing conditions using the methods of Laemmli (50) and then transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked in PBS containing 3% bovine serum albumin and incubated for 1 h with 0.2% NHS. After washing, the blots were incubated with horseradish peroxidase-avidin complex (Vector) and developed using an ECL detection system (Amersham Pharmacia Biotech).
with human natural antibodies in NHS, the α2,3ST- or α1,2FT-transfected SECs had a decreased reactivity. The percentage of reduction of xenotransgenic to human natural antibodies was 42–77% in the α2,3ST transfec-
tants and 32–66% in the α1,2FT transfec-
tants, as judged by mean fluorescence intensity (Fig. 1).

The α-galactosyl epitope, as judged by IB4 lectin binding was approximately 27–81% down-regulated in the α2,3ST transfec-
tants and 5–61% down-regulated in the α1,2FT transfec-
tants. α2,3ST appeared to be more effective in the down-regulation of xenotransgenic to human natural antibodies and to the α-galactosyl epitope than α1,2FT (Fig. 2).

**LDH Assay of α2,3ST- or α1,2FT-transfected SECs—Amelioration of complement-mediated lysis by the SEC transfec-
tants and parental SECs was determined. The control parental SEC lysis resulting from 20% and 40% NHS was found to be 19.1 ± 5.5% and 35.2 ± 9.2%, respectively (Fig. 3A).**

From the results of SEC transfec-
tants, B-11, α1,2FT was very effective in reducing the xenoepitope of SECs. However, α2,3ST was also quite effective, and it appeared to be more efficient in down-regulating the xenoepitope of SECs. An inhibition of over 70% of cytotoxicity was observed in the α2,3ST transfec-
tants B-4, B-11, and A-2 (Fig. 3B).

Western and Lectin Blotting—Western and lectin blotting were performed in order to analyze the alterations of reactivity to human natural antibodies and the IB4 in α2,3ST transfec-
tants. Evaluation of these blot profiles revealed that proteins with molecular masses under 66 kDa derived from the α2,3ST transfec-
tants had reduced reactivity to NHS, especially IgG, compared with the parental SECs. However, no detectable differences were observed between the α2,3ST and α1,2FT transfec-
tants.

Similar to Western blotting patterns, proteins with molecu-
lar masses under 66 kDa in both transfected cells reduced reactivity to IB4, as evidenced by lectin blotting (Fig. 4).

**α,1,3GT Activities of Parental SECs and Transfectants—To assess the influence of the transfected gene on intrinsic α,1,3GT and β,1,4GT activity, enzyme activities were measured. Parental SECs and the mock samples contained relatively high α,1,3GT and β,1,4GT activities. Whereas the α2,3ST and α1,2FT transfec-
tants had an elevated β,1,4GT activity, the intrinsic activity of the α,1,3GT was clearly present in both the α2,3ST and α1,2FT transfec-
tants (Table II).** The degree of down-regulation of α,1,3GT activity in each transfec-
tant was less pro-
nounced than that of antigenicity to NHS and to the comple-
ment mediated lysis.

The Influence of an Excess of Other Transferases on α,1,3GT Measurement in SECs—To ascertain whether or not overex-
pressed α2,3ST or α1,2FT down-regulates the α,1,3GT value of each transfected SECs, the lysates of parental SECs were mixed with several COS 7 cell lysates that contained excess α2,3ST or α1,2FT activities, and α,1,3GT enzymatic activities were then estimated and compared with those in parental SECs with PBS.

Initially, the enzymatic activities of α,1,3GT, α,2,3ST, and α,1,2FT in parental SECs, parental COS, and COS transfectants with α2,3ST or α1,2FT were determined, and the results are shown in Table III. The α,1,3GT enzymatic activities of
Northern blot analysis was performed in order to determine the alterations in mRNA production. The introduction of the glycosyltransferases, which are capable of competing with 1,3GT for the same substrate (Table III), decreased the 1,3GT enzyme activity. This is consistent with data reported by Galili et al. (15–17).

**TABLE II**

| Cells            | 1,3GT | 1,4GT |
|------------------|-------|-------|
|                  | pmol/h/mg of protein | pmol/h/mg of protein |
| Parental SECs    | 433 ± 86 | 1982 ± 415 |
| MOCK             | 453 ± 60 | 2100 ± 350 |
| B-9 ST           | 303 ± 75 | 1762 ± 941 |
| A-4 ST           | 202 ± 42 | 2134 ± 93  |
| B-11 ST          | 183 ± 46 | 2504 ± 409 |
| A-2 ST           | 160 ± 38 | 2909 ± 80  |
| A-3 FT           | 289 ± 40 | 3536 ± 220 |
| B-11 FT          | 178 ± 59 | 3167 ± 50  |

**DISCUSSION**

The amelioration of antigenicity of SECs by overexpression of α2,3ST was examined as a possible approach to provide a permanent solution for overcoming hyperacute rejection in clinical xenotransplantation.

As expected, the down-regulation of xenogeneic epitopes was quite obvious for the case of the α2,3ST transfectants as judged by flow cytometric analysis and a cytotoxicity assay. However, no measurable differences between α2,3ST and α1,2FT transfectants were observed. Therefore, Western and lectin blot analysis of the α2,3ST transfectants was carried out. However, both α2,3ST and α1,2FT also reduced the the extent of reactivity to human IgG and IgM, and they reduced the reactivity to α-galactosyl epitopes in nearly the same manner (Fig. 4). Interestingly, the changes in the bands for these transfectants were more evident for IgG and IB4 lectin than for IgM blotting. This is consistent with data reported by Galili et al. (15–17). Nearly the same results were obtained for the α1,2FT transfectants.

It might be generally thought that α1,2FT is effective in modulating N-linked sugars as well as O-linked sugars and glycolipids, whereas α2,3ST;ST3GalIIIm might have affected N-linked sugars (31). However, at the present time, there are no specific data to support this hypothesis. Therefore, it is possible that α2,3ST;ST3GalIIIm might have affected O-linked sugars and glycolipids, as well as N-linked sugars, on the SECs. On the contrary, the results in this study are consistent with findings in our previous study of SEC transfectants with β-1,4-mannoside β-1,4-N-acetylgalactosaminyltransferase III, which acts on an N-linked sugar (41). A possible explanation for this is that the α2,3ST;ST3GalIIIm and α1,2FT gene acts largely on the same xenoepitopes of N-linked sugars.

In addition, regarding α1,2FT-transfected SECs, Sepp et al.
which the production. The results relative to the mRNA levels of approximately 70% decrease in the value for enzymatic activity was confirmed by the fact that the overexpressed glycosyltransferases. The reliability of each enzymatic activity of 1,3GT in the transfectants were remarkably low as compared to those of parental SECs and mock

Regarding the β1,4GT activity, the up-regulation of the enzyme activities of these transfectants is shown in Table II. However, because the gene code of this molecule in swine has not yet been reported, it is impossible to verify the mRNA levels of this enzyme. To understand the mechanism will require more detailed investigations into the influence of, for example, 1,3GT and β1,4GT activities, message formation, gene transcription, and so forth, by the overexpression of other glycosyltransferase genes.

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