Expression of α-1,3-Galactose and Other Type 2 Oligosaccharide Structures in a Porcine Endothelial Cell Line Transfected with Human α-1,2-Fucosyltransferase cDNA*

Armin Sepp, Patricia Skacel‡, Ragnar Lindstedt, and Robert I. Lechler§

From the Departments of Immunology and Haematology, Royal Postgraduate Medical School, DuCane Road, London W12 0NN, United Kingdom

The binding of xenoreactive natural antibodies to the Galα1–3Galβ1–4GlcNAc (α-galactose) oligosaccharide epitope on pig cells activates the recipient’s complement system in pig to primate xenotransplantation. Expression of human α-1,2-fucosyltransferase in pigs has been proposed as a strategy for reducing the expression level of the α-galactose epitope, thereby rendering the pig organs more suitable for transplantation into humans. The aim of this study was to examine how the cell surface expression of α-galactose, H, and related fucosylated and sialylated structures on a pig liver endothelial cell line is affected by transfection of human α-1,2-fucosyltransferase cDNA. Nontransfected and mock-transfected cells expressed α-galactose, α-2,3-sialylated, and α-2,6-sialylated epitopes strongly, with low level expression of type 2 H and LewisX. By contrast, expression of the H epitope was increased 5–8-fold in transfected cells with a 40% reduction in the expression of α-galactose epitope and a 50% decrease in sialylation, as measured by binding of Maachaia amurenensis and Sambucus nigra agglutinins. LewisX expression was reduced to back-
fected with human
are absent from the parent cells.

Extensive down-regulation of α-Gal epitope expression in the pig kidney fibroblast cell line PLL-K1 was achieved by expression of human blood group H GDP-Fuc:Galβ-R α-1,2-fucosyltransferase (EC 2.4.1.69) by Sandrin et al. (33). This was the result of competition between the human α-1,2-fucosyltransferase (α-1,2FT) and endogenous pig α-1,3-galactosyltransferase (α-1,3GT) for the same type 2 lactosamine acceptor substrate (α-1,3GT cannot α-galactosylate type 2 H structures (34)). The net result of decreased expression of the α-Gal epitope was increased resistance to lysis by human complement. However, other glycosyltransferases can also utilize lactosamine as their acceptor substrate as shown in Fig. 1. Several α-1,3-fucosyltransferases (α-1,3FTs) can fucosylate the GlcNAc residue of terminal lactosamine sequences to synthesize the LewisX (35, 36) epitope. The same acceptor substrate can also be sialylated by α-2,3-sialyltransferases to produce α-2,3-sialyllectosamine, the precursor of sialyl LewisX (35, 36). Sialylation of lactosamine by α-2,6-sialyltransferase leads to CDw75, CD76, and HB-6 carbohydrate epitope expression (37).

Hyperacute rejection of an organ in pig to primate xenotransplantation is triggered by the binding of the recipient’s natural preformed antibodies to the antigens expressed on the endothelium of the graft (1). Within minutes, this results in the activation of endothelial cells, fixation of complement (2, 3), and blood coagulation (4). The main epitope for the human natural preformed IgM and IgG antibodies on the pig endothelium has been identified as a linear type 2 oligosaccharide Galα1–3Galβ1–4GlcNAc1-R (α-Gal)5–8. This α-Gal epitope is synthesized by UDP-Gal:Galβ1–4GlcNac-R (Gal to Gal) α-1,3-galactosyltransferase (EC 2.4.1.124/151), an enzyme that is expressed in all mammals except Old World primates (9–12). In pigs, the α-Gal epitope is expressed in a variety of tissues including endothelium (13), where it can be present on N-linked (14) and O-linked oligosaccharides (15) as well as on glycolipids (16).

Several human complement control proteins, such as CD46, CD54, and CD59, have been found to protect porcine cells against human complement attack in vitro (17–20) and in vivo (7, 21–23). Nonetheless, inhibition of the expression of the α-Gal epitope is a highly desirable goal in that it would greatly reduce preformed human natural antibody binding and consequently reduce the risk of endothelial cell activation (2, 24, 25) and antibody-dependent cell-mediated cytotoxicity (26, 27). Given that genetic knockout is not currently possible in pigs, due to the absence of suitable embryonic stem cells, several strategies have been considered for preventing the binding of human preformed IgM antibodies to the α-Gal epitopes expressed on pig endothelial cells. Besides IgM antibody depletion (28–30), oligosaccharides such as Galα1–3Galβ (31) and to delay hyperacute rejection in pig to baboon cardiac transplantation (32).

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Complex Effects of Human α-1,2FT Expression in Porcine Cells

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To investigate the consequences of human α-1,2FT expression on the overall glycosylation profile of porcine cells, we have transfected a pig liver endothelial cell line (PLECT) with a cDNA encoding human α-1,2FT. We studied cell surface expression of H, α-Gal, and several fucosylated and sialylated oligosaccharide epitopes.

EXPERIMENTAL PROCEDURES

DNA Subcloning—For α-1,2-fucosyltransferase, a 3.4-kilobase pair Xho1 fragment encoding α-1,2FT was excised from pCDM7-α1,2FT and subcloned into Xho1-digested, dephosphorylated pREP10 (Invitrogen) expression vector. Clones with a correctly oriented insert were identified as the ones where Xho1 digestion produced 2.1 kilobase pairs, 10.8 kilobase pairs, and several 200–500-base pair fragments.

Cell Lines, Transfection, and Selection—The pig liver cell line used in the expression studies was established from the liver of a male inbred blood group H SLA/b pig developed at the Babraham Institute (Cambridge, UK). Briefly, 2 g of freshly collected tissue were minced with a scalpel and then pushed gently through 250-μm stainless steel mesh. The disrupted tissue was collected and washed four times with PBS containing 10 mM glucose, 100 units/ml penicillin, and 100 units/ml streptomycin (Sigma). Washed cells were digested with 0.1% collagenase from Vector Laboratories. Reagent grade human IgM was from Sigma.

The disrupted tissue was collected and washed four times with PBS containing 10 mM glucose, 100 units/ml penicillin, and 100 units/ml streptomycin (Sigma). Washed cells were digested with 0.1% collagenase type V (Sigma) in PBS/glucose/penicillin/streptomycin for 1 h at 37 °C. Finally, the sieved and collagenase-digested cells were fractionated using Dolichos biflorus agglutinin-coated Microcelllars (Applied Immune Services, Inc.), since D. biflorus agglutinin is known to be an endothelial cell-specific marker in mice (38–41) and pigs (42, 43). Purified cells were maintained in EC-SPM (Life Technologies, Inc.) containing 5% heat-inactivated (30 min at 56 °C) FCS (GlobePharm, UK) and penicillin/streptomycin as above. Immortalized cell lines were established by transfection with psV3neo using Lipofectin (Life Technologies, Inc.). Selection for transfomants was performed by adding G418 (Boehringer Mannheim) at 200 μg/ml to the growth medium. Cells were plated into 96-well plates (Nunc, Denmark) at 200 cells/well and selected by brief treatment with 5 mM EDTA in PBS, centrifuged for 5 min at 500 g, and left to bind at room temperature for 1 h. After several washes, bound lectin was detected using Streptavidin–alkaline phosphatase conjugate (Sigma). Alkaline phosphatase activity was detected using nitro blue tetrazolium–4-bromochloro-3-indolyl phosphate substrate as recommended by the manufacturer (Promega).

Glycosylation Assays—Aliquots of cell suspensions (2 × 105 in 150 mM NaCl) were stored at −70 °C prior to assay. Cells were solubilized in 0.2% Triton X-100 for 1 h at 4 °C. α-1,3-Fucosylation was assayed with the following 8-methoxy carbonyloctyl (R) acceptors: Fuca1→2Galβ1→4GlcnAcβ1→R (type 2-R), Galβ1→4GlcnAcβ1→R (LacNAc-R), NeuAcα2→3Galβ1→4GlcnAcβ1→R (3′-sialyl LacNAc-R), and Galβ1→3Galβ1→4GlcnAcβ1→R (LNB1-R). Incubation mixtures contained [3H]GDP-fucose (50,000 dpm; NEN Life Science Products; specific activity, 6.95 Ci/mmol), 400 μM acceptor, sodium cacodylate buffer 0.1 M, pH 7.0, 0.2% bovine serum albumin, and 10 mM MnCl2. The reaction volume was 20 μl. Assays were incubated for 15 min or 3 h (to obtain adequate incorporation of radioactive sugar) at 37 °C and stopped by the addition of 0.5 ml of water. Radioactive product was separated from the nucleotide sugar donor using Whatman Sep-Pak C18 cartridges and eluted in 3.5 ml of methanol for scintillation counting (46).

α-1,2-Fucosylation assay was assayed with 5 mM phenyl-β-D-galactoside as the acceptor substrate. To compare the levels of enzyme activity in transfected cells, saturating concentrations (50 μM) of GDP-fucose were achieved by adding cold GDP-fucose (Sigma).

α-1,3-Galactosyltransferase was assayed with LacNAc-R as acceptor and [14C]-UDP-galactose (50,000 cpn) (Amersham Corp.; specific activity, 330 mCi/mmol) as radioactive substrate. Other reaction conditions were the same as for fucosylation assays.

α-2,3- and α-2,6-sialyltransferases were measured with LacNAc-R and LNB1-R as acceptor substrates on 4 × 106 cells. Incubation mixtures contained [3H]CMP-sialic acid (200,000 dpm) (NEN Life Science Products; specific activity, 20.6 Ci/mmol), 1 μM acceptor, 0.1 mM sodium cacodylate buffer, pH 6.5, 0.2% bovine serum albumin, and 5 mM MnCl2. The reaction volume was 20 μl. Assays were incubated for 2 h.

Flow Cytometry—Cells were detached from the tissue culture flasks by brief treatment with 5 mM EDTA in PBS, centrifuged for 5 min at 256 × g in a Heraeus 2.0R Megafuge in the presence of 10% FCS, and resuspended at 105 cells/ml in cold (4 °C) PBS, 0.1% bovine serum albumin, 0.1% NaN3 (Sigma, UK). 50 μl of cells in triplets or quadruplets were incubated in U-well Falcon microtiter plates with the primary antibody added at 10 μg/ml, or at the dilutions recommended by the manufacturer, for 1 h with shaking at 4 °C. Cells were washed three times with cold PBS/FCS/NaCl, before being resuspended in the same buffer containing second-stage reagents at the dilutions recommended by the manufacturer. Following a 1-h incubation with shaking at 4 °C in the dark, the samples were again washed three times with cold PBS/FCS/NaCl, and resuspended in 50 μl of ice-cold PBS, 1% formaldehyde. FITC-conjugated Ulex europaeus, D. biflorus agglutinin, and B. simplicifolia 1/B lectins were used at 5 μg/ml in PBS/FCS/NaCl. Cells were labeled for 1 h with shaking at 4 °C and then washed and mixed as above. Each individually labeled sample was analyzed separately by flow cytometry using a Coulter XL cytemeter. The errors reported are S.D. values.

Labeling of PLECT cells with 3,3′-diododecylindocarboxylic conjugated low density lipoprotein and flow cytometric analysis was performed as recommended by Biogenesis.

Cell membrane extracts were prepared using n-octylglucopyranoside (Sigma). 2 million cells were detached from the plastic by brief treatment with cell culture 5 mM EDTA in PBS and washed into 200 μl of 150 mM NaCl solution. Papain digestion was carried out at 37 °C for 2 h after addition of an equal volume of Papanic reagent to the cell suspension. Cells were resuspended from time to time and, at the end of the incubation, were washed several times with cold PBS before lectin binding experiments for flow cytometry or preparation of cell membrane extracts as described above.

Western Blots—The Mini-protein II Western blotting system was used to transfer 10 μl of 10% SDS-polyacrylamide gel electrophoresis-separated PLECT cell membrane-extracted membranes on to nitrocellulose membrane (Bio-Rad) at 100 V for 1 h or at 20 V overnight. Background binding was blocked using 1% bovine serum albumin in Tris-buffered saline for 2 h at room temperature. Biotinylated lectin was applied at 5 μg/ml in Tris-buffered saline, 9 mM CaCl2, 4 mM MgCl2, and left to bind at room temperature for 1 h. After several washes, bound lectin was detected using Streptavidin–alkaline phosphatase conjugate (Sigma). Alkaline phosphatase activity was detected using nitro blue tetrazolium–4-bromochloro-3-indolyl phosphate substrate as recommended by the manufacturer (Promega).

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RESULTS

Cell Surface Expression of H, α-Gal, and Related Structures—Untransfected PLECT cells and cells transfected with the pREP10 vector (mock-transfected) expressed low levels of H and Lewis^\text{X} epitopes. No blood group A, B, VIM-2, Lewis^\text{a}, sialyl Lewis^\text{X} or sialyl Lewis^\text{a} antigens were present on the parent cell line or on any of the human α-1,2FT-expressing clones. Transfection of PLECT cells with human α-1,2FT cDNA resulted in the isolation of several stable clones displaying increased levels of type 2 H epitope. Clones D and E, displaying the highest staining with anti-H mAb and \textit{U. europaeus} lectin using flow cytometry (Figs. 2, A and B), were chosen for further study. Increased staining with anti-H reagents correlated with increased α-1,2FT activity as measured in vitro using β-phenylgalactoside as acceptor substrate (Table I). However, there was no significant difference between the level of α-1,2FT activity in clones D and E despite measurable two-fold difference in \textit{U. europaeus} lectin or anti-H mAb binding (Fig. 3). Increased expression of H was accompanied by a decrease in the expression of the α-Gal epitope as detected by the binding of \textit{B. simplicifolia} 1B3 (Fig. 3). There was no change in the α-1,3GT activity (data not shown).

The low levels of cell surface expression of Lewis^\text{X} antigen detected on untransfected and mock-transfected PLECT cells were reduced to background on the cells transfected with human α-1,2FT cDNA. By contrast, the Lewis^\text{Y} antigen, which

| Cell line              | α-1,2FT activity\(^\text{a}\) |
|-----------------------|-----------------------------|
| PLECT (control)       | <2                          |
| Clone D               | 500                         |
| Clone E               | 580                         |

\(^{a}\) Activity measured with β-phenylgalactoside is expressed as pmol of fucose transferred/h/10^6 cells.
Complex Effects of Human α-1,2FT Expression in Porcine Cells

Endogenous α-1,3-fucosyltransferase activity in the PLECT cell line measured with a panel of low molecular weight acceptor substrates

| Substrate         | α-1,3FT activity<sup>a</sup> % |
|-------------------|---------------------------------|
| H type 2-R        | 189%                            |
| LacNAc-R          | 100                             |
| 3'-SialylLacNAc-R | 0                               |
| H type 1-R        | 0                               |
| LNB1-R            | 0                               |

<sup>a</sup> Activity expressed relative to that measured with LacNAc-R (100%).

was not previously detectable, appeared following transfection (Fig. 4A). α-1,3FT activity, which is necessary for the synthesis of Lewis<sup>a</sup> and Lewis<sup>a</sup> structures on PLECT cells, was present with a substrate specificity similar to that of human Fuc-TIV (Table II). There were no differences in activity measured with H type 2-R between the control and α-1,2FT-transfected cells (data not shown). These results suggest that down-regulation of Lewis<sup>a</sup> expression in transfected cells was a result of direct competition between human α-1,2- and pig α-1,3-fucosyltransferases occurring in the α-1,2FT-transfected cells.

Maackia amurensis agglutinin and Sambucus nigra agglutinin lectins were used to detect terminal α-2,3- and α-2,6-linked sialic acid on control transfected and α-1,2FT-expressing PLECT cells. Binding of α-2,3-linked sialic acid-specific <i>M. amurensis</i> agglutinin was reduced by approximately 50%, and binding of the α-2,6-linked sialic acid-specific <i>S. nigra</i> agglutinin was reduced by 20 and 60% in clones E and D, respectively (Fig. 5). Sialyltransferase activity was detectable with LacNAc-R and LNB1-R. The former is an acceptor for both α-1,3- and α-2,6-sialyltransferases, whereas activity measured with LNB1-R is indicative of α-1,3-sialyltransferase only. There was no difference in activity between control and transfected cells measured with either acceptor.

The Binding of Human IgM to Pig Endothelial Cell Transfectants—Human IgM binding was studied by flow cytometry using total purified human IgM. At concentrations up to 700 μg/ml the mean fluorescence intensity of IgM bound to the PLECT cells was proportional to the IgM concentration in the incubation buffer (data not shown). It was not possible to obtain saturating conditions with the IgM preparation used (supplied at 1 mg/ml). Therefore, at the concentrations used (100 and 200 μg/ml), the mean fluorescence intensity measures the level of xenoreactive natural antibody present in the IgM preparation rather than the level of xenoreactive epitope expression on the PLECT cells. Contrary to expectation, the human α-1,2FT-expressing clones D and E bound more human IgM than the mock-transfected PLECT cells (Fig. 6).

Oligosaccharide inhibition studies of human IgM binding to the PLECT cells by sucrose, type 2 H trisaccharide, Lewis<sup>a</sup> tetrasaccharide, and α-Gal trisaccharide were carried out to characterize the xenoreactive epitopes on mock-transfected and human α-1,2FT-expressing PLECT cells. Only α-Gal trisaccharide inhibited human IgM binding to PLECT cells, by 85% in mock transfected cells and by 80% in the α-1,2FT-expressing PLECT cells (Fig. 7). This suggests that the increased binding of human IgM to the α-1,2FT-expressing PLECT cells was still largely due to the α-Gal-related epitopes. This is further supported by a similar pattern of inhibition achieved by α-Gal trisaccharide for the mock-transfected and the α-1,2FT-expressing PLECT cells (Fig. 7).

Western Blot Analysis of Changes in the α-1,3-Galactosylation and α-1,2-Fucosylation of Cell Membrane Glycoproteins—Western blots of cell membrane extracts from human α-1,2FT-expressing and control cell lines were probed with biotinylated <i>U. europaeus</i> and <i>B. simplicifolia</i> IB<sub>1</sub> lectins to identify the glycoprotein substrates of α-1,3GT and α-1,2FT. Although lactosamine is a substrate for both α-1,3GT and α-1,2FT in vitro, it can be seen that the glycoprotein substrate repertoires of these two porcine enzymes in the PLECT cells are not identical (Fig. 7, lanes 1 and 3). It was also noted that expression of human α-1,2FT in PLECT cells did not affect either overall intensity or distribution of H or α-Gal epitopes on the cell surface proteins (Fig. 8, lanes 2 and 4). To further distinguish between H and α-Gal epitopes on glycoproteins and glycolipids synthesized by PLECT cells, the cells were subjected to papain digestion. Preincubation of mock-transfected and α-1,2FT-expressing PLECT cells with papain was found to decrease sig-
significantly the amount of *U. europaeus* lectin- and *B. simplicifolia* I/B4-reactive glycoproteins in cell membrane extracts (Fig. 8, lanes 5–8). Although there was a marked decrease in the lectin binding to blotted proteins from papain-treated cells, binding to the cell surface, as measured by flow cytometry, was virtually unchanged (Fig. 9). This difference may be explained by the fact that flow cytometric analysis measures antigen expression on both glycoprotein and glycolipid components of the cell surface, whereas Western blotting characterizes primarily glycoproteins. These data, together with the lack of increase in H staining and decrease in α-Gal staining on Western blots from transfected cells, suggests that a significant fraction of the increased expression of H may have been on glycolipids.

**DISCUSSION**

In the present study, we have established an immortalized pig liver endothelial cell line (PLECT) and stably transfected it with human α-1,2FT, an intracellular class II transmembrane Golgi-located glycosyltransferase that directs the expression of type 2 H epitopes (47). Increased expression levels of α-1,2FT activity in PLECT cells led to increased H epitope expression and a decrease in the expression of α-Gal, Lewis^X^, and α-2,3- and α-2,6-linked sialic acid. Our results suggest that new type 2 H epitopes may be associated predominantly with glycolipids rather than glycoproteins. Additionally, overexpression of human α-1,2FT in the presence of endogenous pig α-1,3FT activity resulted in the formation of cell surface Lewis^Y^.

The observed effects of human α-1,2FT expression on the PLECT cells are best described within the framework of the terminal glycosylation pathways in these cells (Fig. 1). Effective competition between α-1,2FT and α-1,3GT requires that they both glycosylate the same acceptor substrates, be they glycoproteins or glycolipids. Both utilize low molecular weight type 2 substrates *in vitro*, but *in vivo* their activity may be determined by much finer substrate specificity. For instance, on human erythrocyte cell membrane proteins, the H epitope is not uniformly expressed on all membrane proteins but is predominantly associated with polylactosamine residues on band 3 and 4.5 membrane glycoproteins (48–50). Similarly, the distribution of the α-Gal epitope on porcine platelets and endothelial cell glycoproteins is not uniform but appears to be associated primarily with integrins and von Willebrand factor (14, 51). Consistent with this is our observation that Western blots of mock-transfected and human α-1,2-FT-expressing PLECT cell membrane extracts probed with α-Gal-specific *B. simplicifolia* I/B4 and H-specific *U. europaeus* lectins are not identical (Fig. 8), suggesting that the glycoprotein substrates of these two enzymes may be different. The absence of any change in the glycoprotein profiles introduced by overexpression of α-1,2FT in PLECT cells was a surprising finding in view of the results obtained by Sharma *et al.* (60), who transfected Chinese hamster ovary cells with pig α-1,3GT and human α-1,2FT cDNAs and demonstrated effective competition between these two enzymes for the same glycoprotein substrate. It is of interest, however, that the other *B. simplicifolia* I/B4-reactive glycoproteins on their Western blots did not show such an effect. Whether those bands represented genuine α-Gal epitopes synthesized by endogenous Chinese hamster ovary cell α-1,3GT or
merely nonspecific binding of the lectin is unclear, since there are reports both ruling out (61) and supporting (62) the expression of endogenous α-1,3GT in Chinese hamster ovary cells. Our data suggest that the natural acceptor substrates for α-1,3GT in porcine endothelial cells may not function as substrates for human α-1,2FT. Therefore, any reduction of α-Gal expression that occurs through competition with α-1,2FT is likely to be cell type-specific and determined by the individual protein repertoires of cells in different tissues. This may also explain why expression of human α-1,2FT resulted in an approximately 70% decrease in the α-Gal expression in pig epithelial PLL-K1 cells observed by Sandrin et al. (33) but only a 40% decrease in the PLECT cells in this study. A greater than 200-fold increase in α-1,2FT activity in PLECT cells resulted in at most a 9-fold increase in cell surface expression of H and only a 40–50% decrease in the expression of α-Gal. Alternatively, these differences could be explained by a higher level of endogenous α-1,3GT activity in PLECT cells compared with PLL-K1 cells.

Sequential activity of endogenous α-1,3GT on H structures in transfected cells resulted in the formation of LewisY neo-epitopes. LewisX is an oncodevelopmental antigen expressed on the surface of cancer (52–55) and embryonic cells (56–58). It is new and previously unexpressed xenoreactive epitopes associated with sialyl LewisX in response to cytokine stimulation is an important characteristic of endothelial cells. The results of our experiments suggest that this may be altered in α-1,2FT-expressing porcine endothelial cells. Substantially decreased levels of α-2,3-sialylated structures may affect the synthesis of sialyl LewisX and hence, for example, normal lymphocyte adhesion and trafficking. Therefore, alternative, more targeted approaches, such as gene knockout to inhibit expression of the α-Gal epitope, may be more effective in the context of pig to human xenotransplantation.

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