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TRANSGENIC APPROACHES FOR THE REDUCTION IN EXPRESSION OF GALα(1,3)GAL FOR XENOTRANSPLANTATION

Mauro S. Sandrin[1], Narin Osman, Ian F.C. McKenzie

Molecular Immunogenetics Laboratory, Austin Research Institute, Austin & Repatriation Medical Center, Heidelberg Vic. 3084, Australia

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1. ABSTRACT

The major barrier to clinically successful xenotransplantation is the lack of effective therapies aimed at eliminating antibody and complement - dependent hyperacute rejection. This review examines transgenic strategies to eliminate or reduce expression of the major pig to human xenoantigen Galα(1,3)Gal such that the epitope is no longer recognized by natural human antibodies, by the use of glycosidases and/or glycosyltransferases that can competitively and effectively inhibit the activity of the α1,3galactosyl-transferase gene and thereby eliminate the xenoantigen Galα(1,3)Gal.

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[1] To whom correspondence should be addressed, at Molecular Immunogenetics Laboratory, Austin Research Institute, Austin & Repatriation Medical Center, Heidelberg Vic. 3084, Australia. Tel #: 61-3-92870666, Fax #: 61-3-92870600. Email: ms_sandrin @muwayf.unimelb.edu.au

2. INTRODUCTION

A solution to the world-wide problem of transplant organ supply and demand that is gaining increasing attention is the use of xenogeneic organs *i.e.* tissues obtained from animal species other than humans. Based on physiological, biological and ethical considerations, the pig would appear to be the most suitable donor for xenotransplantation (1, 2). However, there is a major problem in that all humans have large amounts of natural antibodies to pig tissues that could bind to Galα(1,3)Gal found in all pig tissues, activate complement and cause hyperacute rejection of transplanted organs. Pigs and humans last shared a common ancestor 64 million years ago, and there are many antigenic differences of importance in the pig-to-human immune response. However, with respect to hyperacute rejection the majority, if not all of the natural antibody is directed to a single carbohydrate epitope Galα(1,3)Gal (3-5).

3. HYPERACUTE REJECTION

Hyperacute rejection is due to the binding of circulating antibodies to cellular antigens on endothelial cells. This in the presence of complement gives rise to a phenomenon characterized by interstitial hemorrhage and thrombosis, and as the name suggests, rapidly leads to graft destruction (within minutes to hours) (3, 4). Originally described in renal allotransplantation, it is now rarely seen in allotransplantation as cross-matching excludes grafting in the presence of preformed anti-graft antibodies. There are three major components to hyperacute rejection: (a) antigen, (b) antibody, and (c) complement. It is clear that if any of the these components were absent or removed, hyperacute rejection would not occur. Strategies aimed at blocking hyperacute rejection by either inhibiting the complement cascade or inhibiting natural antibody deposition have been discussed elsewhere (4, 6, 7), here, we will concentrate on strategies to modify the antigen as a means of preventing hyperacute rejection.

4. THE MAJOR XENOEPITOPE: GALα(1,3)GAL

Many studies concluded that Galα(1,3)Gal is the major xenoepitope recognized by natural human and primate antibodies (3-5, 8, 9). The original observations that humans have substantial amounts of anti-Galα(1,3)Gal IgG antibodies were made by Landsteiner (10) and more recently by Galili (11-13). Subsequently, antibodies of the IgM and IgA classes have also been identified (8, 14). Evidence that Galα(1,3)Gal is the major xenoepitope comes from a number of studies outlined below:

4.1 Carbohydrate inhibition

Although carbohydrates had been implicated as determinants of xenoepitopes detected by naturally occurring human anti-pig antibodies, direct evidence came from inhibition studies, where monosaccharides, disaccharides and complex sugars were shown to specifically inhibit the binding of natural human antibodies to pig cells (8, 15-17); inhibition was observed only with galactose or oligosaccharides having a terminal α-linked but not a β-linked galactose, and in particular Galα(1,3)Gal (Fig 1).

4.2 Absorption studies

The inhibition studies outlined above have been complemented by absorption studies in which complete removal of cytotoxic antibodies was achieved using either melibiose (Galα(1,6)Glu) or Galα(1,3)Gal coupled to a matrix (16, 18, 19). Furthermore, these antibodies could be eluted and reconstitute the absorbed serum.

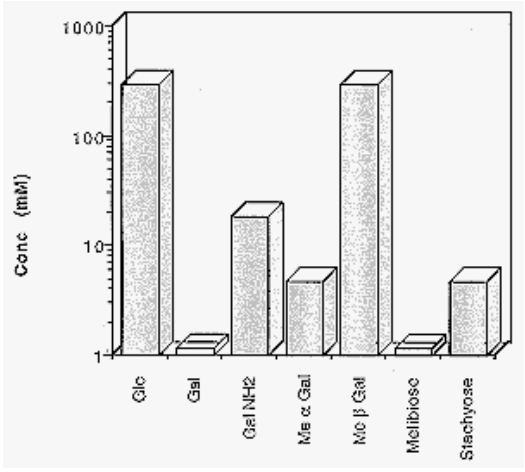

Figure 1. Carbohydrate inhibition of hemagglutination of pig erythrocytes by normal human serum. Concentrations in mM are required to give 50% inhibition in titer. Glc, glucose; Gal, galactose; GalNH2, N-acetylgalactosamine; MeαGal, αmethyl galactoside; MeβGal, βmethyl galactoside. Modified from Sandrin *et al.* (8).

4.3 Transfection studies

Direct molecular evidence of the importance of Galα(1,3)Gal as the major xenoepitope came from transfection studies, where COS cells (which are of Old World monkey origin and do not express Galα(1,3)Gal) could bind natural human antibodies after expression of the mouse or pig α1,3galactosyltransferase cDNA (8, 20). In addition, human serum was able to lyse COS cells after they were transfected with the α1,3galactosyltransferase cDNA (16). Furthermore, these Galα(1,3)Gal$^+$ COS cells could remove all anti-pig antibodies in absorption experiments (19).

4.4 *In vivo* studies

While all the studies described above showed that inhibiting or absorbing anti-Galα(1,3)Gal antibodies depleted reactivity for pig cells *in vitro*, the most convincing evidence that the anti-Galα(1,3)Gal antibodies were responsible for the hyperacute rejection of pig organs transplanted to primates, was the demonstration that infusion of baboons with carbohydrates prevented hyperacute rejection (21). Furthermore, *ex vivo* absorption of baboon blood over a melibiose or Galα(1,3)Gal column removed all cytotoxic anti-pig antibodies, and prevented the hyperacute rejection of a transplanted pig heart (7, 22).

4.5 Are all the human anti-pig antibodies directed to Galα(1,3)Gal?

Based on a number of different studies, it is

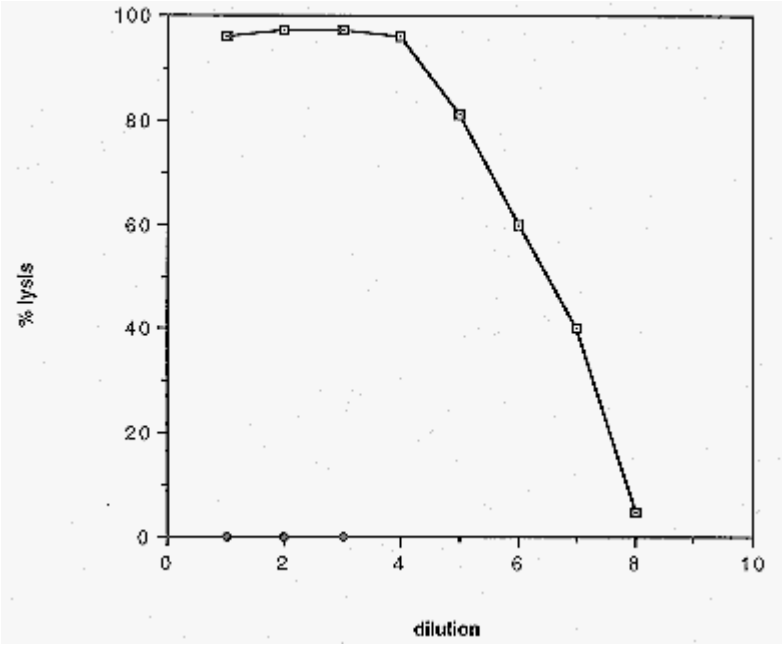

Figure 2. Absorption of anti-pig antibodies by Galα(1,3)Gal$^+$ COS cells. Lysis of pig endothelial cells using non-absorbed normal human serum (☒) and normal human serum absorbed with Galα(1,3)Gal$^+$ COS cells (◆). Modified from McKenzie *et al.* (19).

now clear that >95% of all xenoreactive antibodies are directed to Galα(1,3)Gal (8, 23). From our own work (Fig 2), all cytotoxic anti-pig antibodies can be absorbed from human serum with either Galα(1,3)Gal immunoabsorbant columns or Galα(1,3)Gal$^+$ COS cells (19).

4.6 Cellular and molecular distribution of Galα(1,3)Gal

The distribution of Galα(1,3)Gal on different tissues is of relevance to xenotransplantation, and has been examined histologically (24, 25). These studies clearly demonstrated that virtually all endothelial cells lining arterioles, capillaries and venules carry Galα(1,3)Gal in large amounts. Thus, in vascularized transplants, the antigen would meet the incoming antibody and cause immediate rejection. A very high concentration of Galα(1,3)Gal was observed in all hepatic cells. In the kidney, largest amounts were found in the proximal convoluted tubules, Galα(1,3)Gal was present in less amounts in the distal tubules, little in the glomeruli and none in the collecting ducts. In the heart, the muscle fibres were non-reactive, although the capillaries in the muscles were reactive. At this time, on the basis of these findings one would predict that hyperacute rejection of liver, kidney and heart would occur if they were transplanted to humans from the pig. By contrast, other than some expression in blood vessels and in pancreatic ducts, the pancreas was virtually non-reactive and Galα(1,3)Gal was absent from adult islet cells (24, 26). At present, isolated islet cell transplants are being considered - particularly from the foetal pig.

Galα(1,3)Gal is expressed on many molecules as a normal component of glycosylation, including O-linked and N-linked oligosaccharide chains and glycolipids. On the cell surface, Galα(1,3)Gal is expressed on many molecules (27), *e.g.*, on platelets, Galα(1,3)Gal is predominantly found on fibrinogen, von Willebrand's factor, α2 integrin and β3 integrin (28, 29); on endothelial cells, more than 20 glycoproteins carry Galα(1,3)Gal (27), some of which have been identified as von Willebrand's factor, DM-GRASP, and the α1, α v, α 3/ α 5, β1 and β3 integrins (28, 29).

5. A1,3GALACTOSYLTRANSFERASE

Galα(1,3)Gal is synthesized by the α1,3galactosyltransferase enzyme, which catalyses the addition of a terminal α-linked galactose to N-acetyl lactosamine. cDNA clones encoding the α1,3galac tosyltransferase have been isolated from the mouse (30, 31), ox (32), pig (20, 33) and New World monkeys (34), all encoding a type II integral membrane protein, typical of all glycosyltransferases thus far isolated. Sequence homology of pig α(1,3)galactosyltransferase shows 78% identity at the amino acid level with mouse α(1,3)galactosyl-

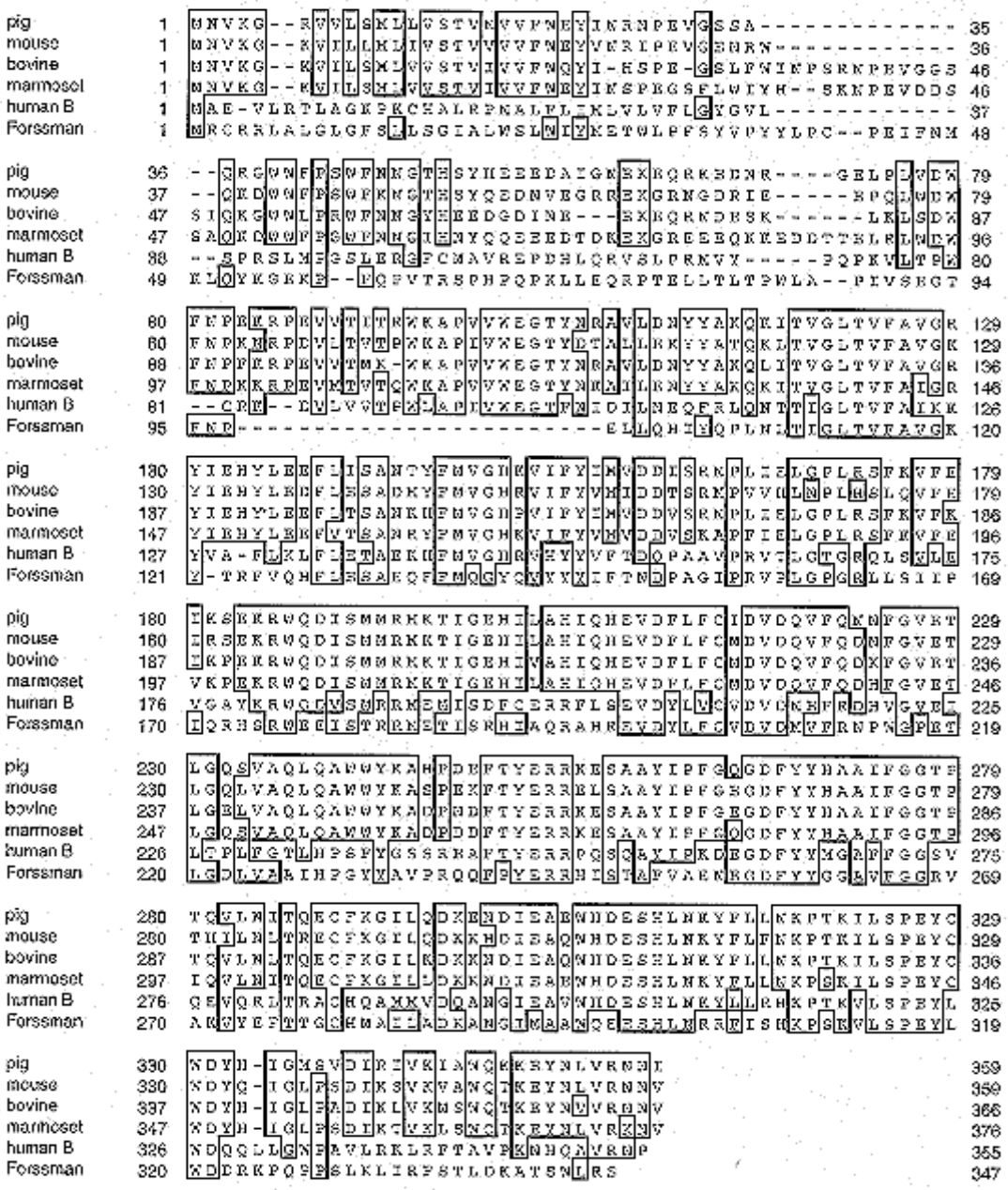

Figure 3. Alignment of amino acid sequences of cloned α1,3galactosyltransferases. See text for references.

transferase and sequence homology with the human ABO blood group transferases (35), and the Forssman transferase (36) (which transfers an α-linked N-acetyl galactosamine) (Fig 3). This suggests a common evolutionary origin of these molecules. In contrast to these species, New World monkeys and primates do not have a functional α1,3galactosyltransferase, as the gene has several nucleotide deletions and substitutions leading to a pseudogene (37, 38). Therefore, these animals have high levels of circulating natural antibodies that react with Galα(1,3)Gal. It has been suggested that the antibodies are induced to normal gut micro-organisms (13).

6. ELIMINATION OF GALα(1,3)GAL BY GENE INACTIVATION STRATEGIES

As it is known that anti-Galα(1,3)Gal antibody removal or blocking can inhibit hyperacute

Reduction of Galα(1,3)Gal by transgenesis.

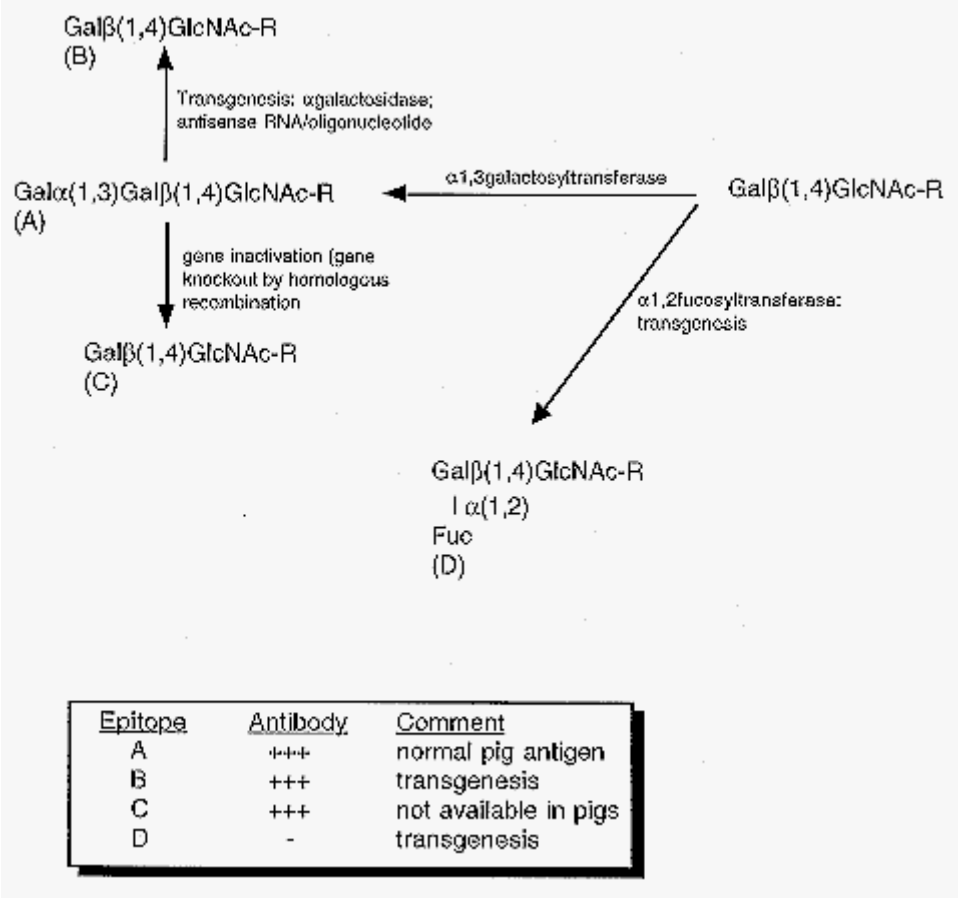

Figure 4. Biosynthetic pathway, and approaches to prevent Galα(1,3)Gal synthesis. Pathway begins with N-acetyl-lactosamine (Galβ(1,4)GlcNAc), and the α1,3galactosyltransferase enzyme adds galactose to generate Galα(1,3)Gal (A). Both gene inactivation by homologous recombination (B) and transgenic approaches to inhibit/breakdown RNA (C) would prevent production of α1,3galactosyltransferase, and eliminate Galα(1,3)Gal. Potentially specific α1,3galactosyltransferase inhibitors could act upon the enzyme (D). Also shown is an alternative transgenic approach to utilize the substrate of α1,3galactosyltransferase by α1,2fucosyltransferase (E). Galα(1,3)Gal could be eliminated by αgalactosidase (F). Modified from Sandrin *et al.* (59).

rejection, it follows that antigen removal would also inhibit hyperacute rejection. The most obvious way of doing this would be to destroy the gene encoding Galα(1,3)Gal - the α1,3galactosyltransferase (Fig 4). However while transgenic technology has been usedin pigs, homologous recombination technology is limited to small species such as mouse and rat. Homologous recombination has been used to inactivate the α1,3galactosyltransferase gene in mice ($gal^{-/-}$ mice) (39, 40), targeting exon 9 (the exon encoding the catalytic domain) for disruption. Several important observations arose from the studies of these mice:

(a) as predicted (8), inactivation of the α1,3galactosyltransferase gene is not lethal.

(b) $gal^{-/-}$ mice lack the expression of Galα(1,3)Gal in all tissues (40).

(c) the $gal^{-/-}$ mice produce natural anti-Galα(1,3)Gal antibodies (39).

It is not clear if these are induced by bacterial infection. The occurrence of these antibodies in these $gal^{-/-}$ mice parallels the observations in humans and Old World monkeys. d) although $gal^{-/-}$ mice do not bind purified human anti-Galα(1,3)Gal antibodies, when examined with whole human serum, some antibody binding was still (observed (39). This reaction is probably due to the binding of natural human anti-N-acetyl lactosamine antibodies, which are present in substantial amounts in all humans tested (41), but are not observed in normal mice as N-acetyl lactosamine is not a terminal carbohydrate.

Other means of preventing the expression of the Galα(1,3)Gal gene have been suggested and

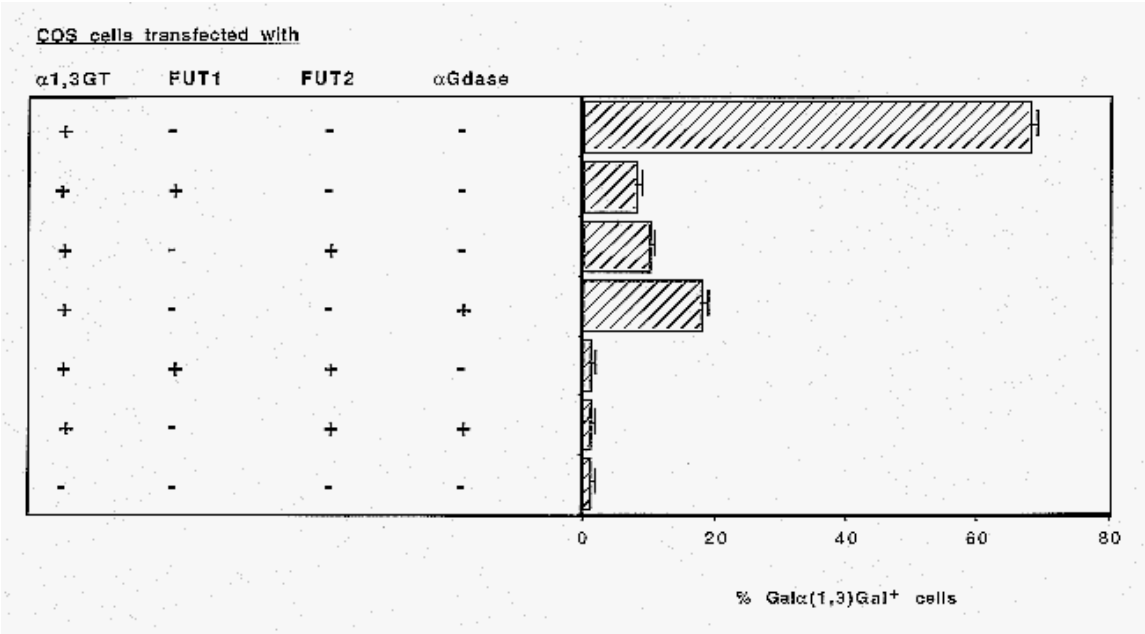

Figure 5. Expression of Galα(1,3)Gal on COS cells after transfection. The cDNA clones used: α1,3GT, α1,3galactosyltransferase; FUT1 and FUT2, pig α1,2fucosyltransferases; αGdase, human α-galactosidase. Modified from Sandrin *et al.* (59), Osman *et al.* (60) and unpublished results.

include the use of anti-sense constructs (4) - either as oligonucleotides or as cDNA, but these have met with disappointing results (42).

7. ELIMINATION OF GALα(1,3)GAL BY TRANSGENIC STRATEGIES

An alternative strategy to the α1,3galactosyltransferase gene inactivation would be to use the transgenic approach to add another transferase which would "deviate" the glycosylation pathway from Galα(1,3)Gal expression (Fig 4) and lead to the production of another carbohydrate not recognized by natural antibodies (4). It is known that different glycosyltransferases can compete for the same substrate and this information can be applied to develop a strategy aimed at blocking a specific carbohydrate epitope.

7.1 The α1,2fucosyltransferase enzyme

The α1,2fucosyltransferase or H transferase could be an appropriate enzyme to decrease the expression of Galα(1,3)Gal, as both the α1,2fucosyltransferase and the α1,3galactosyl-transferase use N-acetyl lactosamine as an acceptor substrate, transferring fucose or galactose to generate fucosylated N-acetyl lactosamine (H substance) or Galα(1,3)Gal, respectively. Furthermore, the α1,3galactosyltransferase of most animals cannot use the fucosylated N-acetyl lactosamine as an acceptor to transfer the terminal galactose, but will only transfer to N-acetyl lactosamine residues.

Our data shows that there is indeed a hierarchy of these glycosyltransferases that are simultaneously expressed within the same cell, and that the α1,2fucosyltransferase takes precedence over the α1,3galactosyltransferase (43, 44).

7.1.1. Expression in COS cells

COS cells simultaneously transfected with cDNA clones encoding α1,2fucosyltransferase and the α1,3galactosyltransferase show preferential expression of the H substance (synthesised by the α1,2fucosyltransferase) rather than Galα(1,3)Gal (synthesised by the α1,3galactosyltransferase) (Fig 5), even though α1,3galactosyltransferase mRNA and functional enzyme was present (43). Furthermore, human antibody binding by the doubly transfected COS cells was decreased (43).

7.1.2. Expression in porcine cells.

In a pig kidney cell line, which expresses both the Galα(1,3)Gal and H, the increased expression of H induced by the transfection and stable expression of α1,2fucosyltransferase cDNA, resulted in decreased expression of Galα(1,3)Gal, decreased human antibody binding and decreased complement mediated cell lysis (43).

7.1.3. Expression in transgenic mice.

Transgenic mice have been produced and they ubiquitously express the α1,2fucosyltransferase enzyme (43, 45). Spleen cells of these mice show a major decrease in Galα(1,3)Gal expression and decreased natural human antibody binding. A major decrease in Galα(1,3)Gal expression was also observed in all tissues, including endothelial cells (45). Similar results have been confirmed in mice by others (46, 47)

7.1.4 . Expression in transgenic pigs.

Two groups have reported the production of transgenic pigs expressing the α1,2fucosyltransferase enzyme (47, 48), and the results parallel those found in transgenic mice, *i.e.* there is a marked reduction in Galα(1,3)Gal expression and decreased natural human antibody binding.

7.1.5 Molecular mechanisms for suppression of Galα(1,3)Gal production.

To address the mechanism of exclusion of Galα(1,3)Gal by the presence of α1,2fucosyl-transferase, we found that the topology of the two glycosyltransferases (α1,2fucosyltransferase and α1,3galactosyltransferase) is likely to play a central role in the temporal order of action of these enzymes (49). Two chimeric transferase proteins were constructed which consisted of the NH_2 terminal cytoplasmic tail of one transferase coupled to the transmembrane and catalytic domain of the other transferase. A complete reversal in the staining pattern of transfected COS cells was seen with the chimeric transferase compared with the wild-type transferases, demonstrating that the cytoplasmic domains of α(1,3)galactosyltransferase and α1,2fucosyl-transferase are sufficient for the retention, localization and activity of these two enzymes. This finding can be used to target an α1,2fucosyltransferase to the same compartment as the α1,3galactosyltransferase, and in conjunction with an α(1,2)fucosyltransferase transgene would lead to greater reduction in Galα(1,3)Gal expression.

7.1.6 Secretor type α1,2fucosyltransferase

The α1,2fucosyltransferase strategy outline in the preceeding sections give significant but not absolute reduction of Galα(1,3)Gal expression. Can other glycosyltransferases be used to give improved Galα(1,3)Gal reduction? It is known that the α1,3galactosyltransferase enzyme can galactosylate two types of precursor chains: Type 1: Galβ(1,3)GlcNAc and Type 2: Galβ(1,4)GlcNAc (50, 51). Similarly α1,2fucosyltransferases can transform both Type 1 and Type 2 precursor chains into H substance (52, 53). At present two α1,2fucosyl-transferases, H-transferase or FUT1 (54) and secretor (Se) transferase or FUT2 (55), have been described, and while both enzymes can use both types of precursors, FUT1 preferentially utilizes Type 2 precursor chains and FUT2 utilizes both Type 1 and Type 2. We have isolated a second α1,2fucosyl-transferases from the pig, FUT2 the homologue of the Secretor gene product, and have shown that this functions in a similar manner to FUT1 (Cohney *et al.* manuscript in preparation), and moreover, using a combination of FUT1 and FUT2, there is a >99% reduction of Galα(1,3)Gal in COS cells (Fig 5).

7.2. aGalactosidase

An alternative approach to the removal of Galα(1,3)Gal is using αgalactosidase, a lysosomal exoglycosidase, to cleave the terminal α-D-galactosyl residue from Galα(1,3)Gal (Fig 4). Several groups have shown that treatment of Galα(1,3)Gal^+ erythrocytes, lymphocytes and endothelial cells with α-galactosidase (coffee bean or bacterial) removes the epitope and eradicates their reaction with human serum (25, 56, 57). Other studies have shown that perfusion of tissue prior to transplantation with bacterial αgalactosidase delayed the onset of hyperacute rejection (56, 58). However, an *ex vivo* approach does not directly address the problem of continual resynthesis and replacement of the epitope by enzyme-treated cells and it would be difficult to ensure total eradication of the epitope. Our studies have examined the efficacy of using α-galactosidase in a transgenic approach to the removal of Galα(1,3)Gal (59).

7.2.1 Expression of αGalactosidase cDNA in COS cells

Co-expression of α-galactosidase cDNA and α(1,3)galactosyltransferase cDNA in COS cells resulted in a 75% reduction in Galα(1,3)Gal expression (Fig 5), with increased levels of α-galactosidase activity (60). Taken together these results demonstrate that expression of αgalactosidase enzyme within a cell, as seen after transfection of cDNA, can significantly reduce surface levels of Galα(1,3)Gal.

7.2.2 Expression of αGalactosidase cDNA in a pig cell line

A pig endothelial cell line PIEC expressing the αgalactosidase cDNA was generated and tested for the ability to bind natural human anti-Galα(1,3)Gal antibody (60) These experiments demonstrated a ten-fold decrease in antibody binding than control PIEC cells demonstrating a significant reduction in cell surface Galα(1,3)Gal.

7.2.3 *In vivo* expression of αGalactosidase

To test the ability of αgalactosidase to reduce Galα(1,3)Gal *in vivo*, transgenic mice expressing αgalactosidase under an H2-K^b promoter were produced. Preliminary results from mice

heterozygous for the human αgalactosidase gene demonstrate that the transgene has been incorporated into the genome and is transmitted, and that there is a small but significant reduction in the surface expression of Galα(1,3)Gal on lymphocytes (60). Further characterisation is currently being performed.

7.3 Combination of α1,2fucosyltransferase and αGalactosidase

As the αgalactosidase did not lead to complete reduction of Galα(1,3)Gal expression, we therefore combined the two approaches: COS cells co-transfected with α(1,3)galactosyltransferase, α1,2fucosyltransferase and αgalactosidase cDNAs showed essentially no cell surface expression of Galα(1,3)Gal, and background lysis observed in the cytotoxicity assay (60). Clearly, the α1,2fucosyl-transferase and αgalactosidase have an additive effect in their ability to reduce the expression of Galα(1,3)Gal on the cell surface (Fig 5).

8. CONCLUSIONS

Transgenic approaches using α1,2fucosyl-transferases or αgalactosidase to reduce Galα(1,3)Gal are both viable and effective. However, it is clear that there is residual Galα(1,3)Gal by using either of these approaches alone. To totally reduce Galα(1,3)Gal to levels such that no deleterious reaction of human antibodies occurs with the transplanted pig tissue, it may be necessary to combine the approaches outlined here, *i.e.* the αgalactosidase transgene (for removal of Galα(1,3)Gal) and at least one α1,2fucosyltransferase (to convert any free N-acetyl lactosamine groups to H substance), together with human complement regulators in a composite transgenic animal. Accordingly, future efforts must be directed towards both optimizing transgene expression and the use of a combination of transgenes to obtain total elimination of Galα(1,3)Gal.

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