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PIG α1,3GALACTOSYLTRANSFERASE: A MAJOR TARGET FOR GENETIC MANIPULATION IN XENOTRANSPLANTATION

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1. ABSTRACT

Terminal carbohydrate residues of glycolipids and glycoproteins display polymorphism among as well as within various species. With the exception of Old World monkeys, great apes and man, the Galα1,3Gal structure is widely expressed in all mammals examined so far. The lack of expression of the glycosyltransferase responsible for the synthesis of Galα1,3Gal leads to the production of high titers of natural antibodies (NAb) against the Galα1,3Gal of other species. The inactivation of this gene occurred during early evolution of primates. Neutralization of viruses (*e.g.* retroviruses) carrying the epitope, by the pre-formed human NAb, indicates one possible evolutionary reason for the polymorphism of terminal carbohydrates among as well as within species. It has been shown that this epitope constitutes the major target, on pig endothelial cells (EC), for the pre-formed human NAb resulting in a hyperacute rejection (HAR) response. This currently makes transplantation of *e.g.* pig organs to humans impossible. Efforts are currently underway to prevent or to eradicate the expression of this epitope in transgenic pigs. Such pigs are likely to display a greatly increased resistance to the HAR.

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2. INTRODUCTION

The major limiting factor in clinical transplantation is the shortage of suitable human donors. If problems regarding ethical, physiological, and immunological issues can be solved, pigs have been suggested to be a future alternative source for at least some organs (1-4). The obstacles in this species combination may appear overwhelming. However, recent technological progress have suggested that overcoming the first immunological hurdle, the 'hyperacute rejection' (HAR), may be possible (5-10).

As defined by the ubiquitous presence of high titers of pre-formed natural antibodies (NAb) binding to the foreign tissue (11), pig to man organ transplantation is an example of a discordant species combination. The binding of these NAb to target epitopes on the donor organ endothelium is believed to be the initiating event in discordant xenogeneic HAR. This binding, within minutes of perfusion of the donor organ with the recipient blood, is followed by complement activation, platelet and fibrin deposition, and ultimately by interstitial edema and hemorrhage in the donor organ (*e.g.* 12).

The efforts to avoid HAR in this species combination have been based on three lines of work:

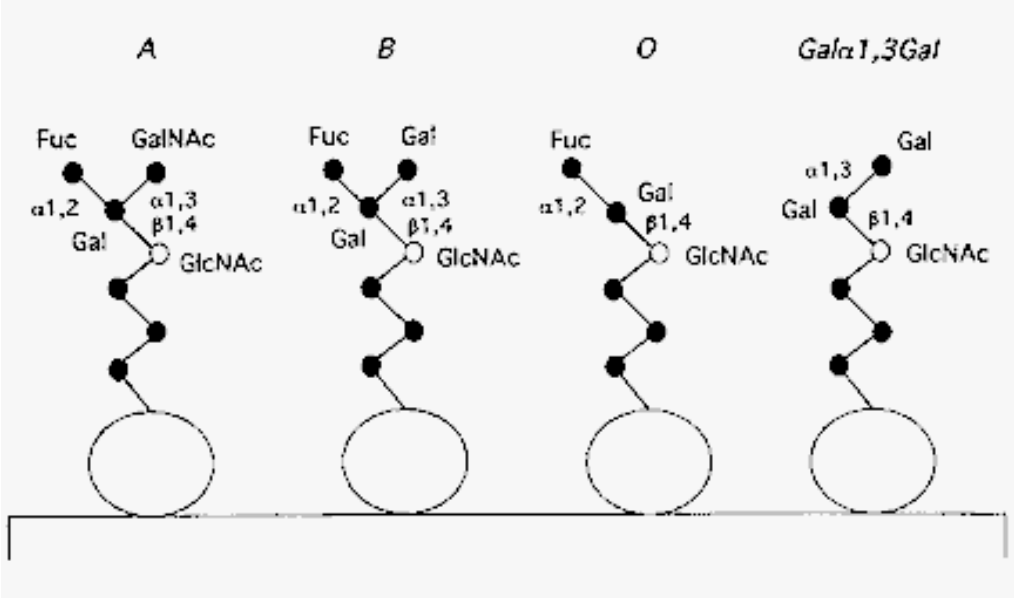

Fig. 1 Structure of ABO and Galα1,3Gal terminal carbohydrates. The individual carbohydrate components are: Fuc, fucosyl, lined horizontally; GalNAc, acetylgalactosaminyl, lined vertically; Gal, galactosyl, black filled; GlcNAc, acetylglucosaminyl, white filled.

1. depletion of anti-pig NAb (5-7); 2. provision of human complement inhibitors in the transplanted organ by transgenic means (8, 9); and 3. inhibition or eradication of the target epitopes for the NAb (10, 13). In this review we describe the background to and the on-going work aimed at down-regulating the main target epitope for the human anti-pig NAb in pig cells.

3. GALA1,3GAL: THE MAJOR TARGET FOR HUMAN ANTI-PIG NAB

The galactosyl (α1,3)galactosyl(β1,4)acetyl-glucosaminyl carbohydrate epitope, hereafter referred to as Galα1,3Gal, is expressed in many mammals, but not in humans, Old World monkeys and the great apes (14-16). Instead, the latter species ubiquitously express high levels of pre-formed 'natural' antibodies (NAb) against this epitope (17, 18). In pigs, as well as in mice, the carbohydrate is present on glycoproteins and glycolipids on a variety of cell types, including endothelial cells (EC)(19). It was therefore a reasonable assumption that the Galα1,3Gal structure would constitute one of the important targets for human NAb in pig-to-primate xenotransplantation. The Galα1,3Gal structure is reminiscent of the ABO-type of carbohydrates in humans, a terminal carbohydrate structure which is polymorphic among individuals of one species (Fig. 1). It is well known that A- or B-type grafts, due to the presence of pre-formed NAb in non-matched recipients, can lead to HAR in human allotransplantation (20).

Complex carbohydrates are synthesized in the Golgi via the action of a number of glycosyltransferases (21). The number of glycosyl-transferase genes with different specificity has been estimated to be as many as one hundred or more. The human ABO carbohydrate terminus is synthesized by two alternative enzymes encoded for by an allelic gene locus. The two A- and B- enzymes differ by only a few amino acids, resulting in the addition of a terminal GalNAc and Gal residue, respectively, whereas the O-type allele does not result in the expression of an active enzyme (22). The Galα1,3Gal structure is identical to the (ABO) B-type with the important exception that this structure lacks the fucosylation in an α1,2-linkage (Fig. 1).

We, as well as others, have cloned the cDNA coding for the pig α1-3galactosyl-transferase (α1-3GT) responsible for synthesizing the Galα1,3Gal epitope in pigs (23, 24). We have also mapped (23) and cloned the corresponding gene (GGTA1)(manuscript in preparation). α1,3GT cDNAs and the genes encoding them have previously been cloned both in mouse and cattle (25, 26). An alignment of α1,3GT amino acid sequences with ABO-transferases reveals considerable similarity consistent with the findings of a close functional relationship (27, 28). Using a cDNA clone coding for mouse α1,3GT, Sandrin and colleagues, by transfection of Galα1,3Gal-negative COS cells, have shown that these cells are able to absorb most of the human anti-pig NAb, confirming the previous

suspicion that this is the major target for such antibodies (29).

4. EVOLUTION AND POSSIBLE FUNCTION OF THE GALα1,3GAL EPITOPE

4.1. Evolution of the gene encoding the α1,3GT:

The α1,3GT gene was inactivated in the primate ancestors preceding the diversification of the great apes and led to the loss of this epitope (for review see 28). However, two non-expressed pseudo-genes remain on human chromosomes 9 and 12, respectively (30, 31). It is most likely that the copy on chromosome 9 represents the corresponding human gene, *vis-à-vis*, the expressed mammalian GGTA1 genes since the copy on chromosome 12 (HGT-2) appears to be a processed pseudogene (31). In addition, we have shown that the pig GGTA1 gene is situated in a region of pig chromosome 1 that is syntenic, *vis-à-vis*, the region harboring the human chromosome 9 copy (23). An interesting possibility, proposed by Galili and colleagues, is that the gene was inactivated in this primate lineage as the result of an intense evolutionary pressure in the form of selection for the presence of antibodies against the Galα1,3Gal structure. This would have occurred at the inevitable expense of the loss of expression of the α1,3GT gene (16). In addition, these authors suggested that the GGTA1 gene was independently inactivated in the respective lineages leading to, on the one hand, apes and man, and on the other hand, Old World monkeys (32). We find the latter interpretation unlikely since both lineages appear to share the same mutation in the form of a stop codon. Although this codon does not necessarily represent the original inactivating mutation, it precedes what has been shown to be the active domain in this enzyme. In addition, Joziasse and colleagues have found that the copy on human chromosome 12 (HGT-2) lacks the different single-base deletions present in the Old World monkey and ape/man lineages of GGTA1 (on chromosome 9 in man). More importantly, the HGT-2 copy does contain the same stop codon as the GGTA1 copy (31, D. Joziasse, personal communication). This indicates that the mutation producing a stop codon preceded the lineage specific mutations. Thus, we favor the interpretation that inactivation of the GGTA1 gene in the common ancestors of both Old World monkeys and apes/man, occurred following the separation of New World monkeys expressing a functional gene product. A shortcoming of this hypothesis is that our own previously constructed phylogenetic tree places the branching point of the human HGT-2 gene before the divergence of all primates in the tree, including New World monkeys expressing the gene (Fig. 2a)(28). However, it must be remembered that when the genetic distances involved are very small, as in this case, mistakes are common in phylogenetic trees. Consequently, based on the observations described above proposing the evolution of the GGTA1 and HGT-2 genes, we have drawn an alternative phylogenetic tree (Fig. 2b). Whatever the exact evolution of the genes involved is, none we can imagine would preclude the explanation that there was a strong selection involved which favored the Galα1,3Gal-negative phenotype. Nevertheless, it is also conceivable that the inactivation was merely an 'evolutionary accident'.

4.2. The possible functional significance of the Galα1,3Gal epitope:

If the inactivation of the gene in one primate lineage was brought about by a strong evolutionary pressure, what might the selective agent have been? Many bacteria (33, 34) and some parasites (35-37) commonly carry epitopes to which anti-Galα1,3Gal NAb can bind and assist in opsonization and/or phagocytosis. In addition, it has been suggested that viruses can 'dress up' in this epitope as they are produced from cells expressing the α1,3GT enzyme (38, 39). Takeuchi and colleagues, in collaboration with us, recently showed that *e.g.* C-type retroviruses produced from murine or canine cells could be readily inactivated by human anti-Galα1-3Gal antibodies (40). Furthermore, expression of the porcine α1,3GT cDNA clone in human cells rendered both these cells as well as the retroviruses produced from them sensitive to human serum. The specificity of these Ab was ascertained following: 1. purification of anti-Galα1,3Gal Ab on a Gal(α1,3)Gal(β1,4)GlcNAc column, and 2. blocking of Ab reactivity using specific disaccharides. We speculated that this mechanism of increased immune reactivity may have given an increased ability of primates carrying high titres of anti-Galα1,3Gal NAb to resist *e.g.* C-type retroviral infections (40). Incidentally, despite the fact that they appear to be readily transmitted between other mammals, no C-type retroviruses have been found in humans. Since it has been previously shown that other viruses can be adorned with the Galα1,3Gal epitope one may generalize from this finding that other viruses can also be inactivated by human anti-Galα1,3Gal NAb. Thus it becomes apparent that the silencing of the gene in our early ancestors could have been influenced by selective evolutionary forces linked to trans-species viral infections. Alternatively, during primate evolution, some individuals may have enjoyed a selective advantage over others following viral transmissions within the same species. The clinical importance of the Galα1,3Gal epitope in eliciting a human anti-viral response is unknown. The human immune system appears to be able to

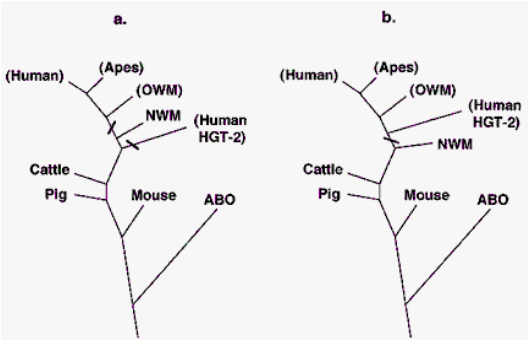

Fig. 2 Predicted phylogenetic trees of Galα1,3Gal evolution (not representing the correct genetic distances). **a**. Phylogenetic tree obtained from partial sequences of α1,3GT's as previously described (28, and references therein), predicting that the HGT-2 gene branched prior to the divergence of New World Monkeys and other primates. **b**. An abbreviated phylogenetic tree based on a synthesis of the tree in Fig. 2a and actual observations of shared inactivating mutations in the respective genes. In this tree the predicted time point for HGT-2 divergence, involving a very short genetic distance to the nearest branching point, has been disregarded (see 28). Possible wrong order of local gene divergences lead to an alternative suggestion for the evolution of α1,3GT genes. Potential gene inactivation events are depicted by short crossing lines. Species names in parantheses indicate non-expressed genes. OWM: Old World Monkeys, NWM: New World Monkeys (16). HGT-2 represents the human processed α1,3GT pseudogene copy on chromosome 12 (31). Other sequences were from ref. 16, 23, 25, 26, 27, and 30.

effectively deal with most intra-species viral infections and, therefore, to eliminate viruses lacking the Galα1,3Gal epitope. Since any viruses produced in Galα1,3Gal-negative pigs are likely to be more resistant to human antibody inactivation, the use of 'clean', *i.e.* zoonotic-free, pigs as organ donors, should be strongly considered (41, 42).

5. DOWN-REGULATION OR OBLITERATION OF THE GALα1,3GAL EPITOPE

As alluded to above, the importance of the Galα1,3Gal epitope in the pig-to-man xeno-transplantation context has been well established. Despite the possibility that inhibition or even total eradication of the epitope in pigs may not be the only action needed, it would most certainly go a long way towards overcoming the HAR. To this end, attempts to inhibit or silence the gene are currently being carried out in several laboratories.

5.1. Knock-out of the gene encoding α1,3GT:

The most straightforward and seemingly logical approach for eradicating the action of the pig α1,3GT would be to knock-out the gene. A gene knock-out (43) involves the cloning of a gene and subsequently making a knock-out construct containing an insertion of an antibiotic resistance gene (*e.g.* neomycin). This insertion serves a dual purpose, in that it interrupts the targeted gene as well as provides a tool for selection. In addition, a second selection mechanism involving the Herpes Simplex Virus-thymidine kinase (HSV-TK) gene flanking the gene of interest is commonly used to provide selective killing of constructs that have not undergone gene targeting by homologous recombination. The construct is subsequently transfected into embryonic stem cells in culture, and the two forms of selection are applied. This is followed by analysis of surviving colonies of cells. ES cells displaying the desired knock-out genotype are subsequently introduced into pseudo-pregnant recipient females by either blastocyst injection or morula aggregation. This type of 'knock-out' approach has become almost a routine procedure in at least one mouse strain (*i.e.* 129 strain). Recently, the corresponding GGTA1 gene in mouse was 'knocked out' by Thall *et al.* (44). Interestingly, the resulting Galα1,3Gal-negative mice appear to secrete NAb against the epitope re-enacting the event in our primate ancestors. These mice also appear healthy and fertile indicating that a similar approach in pigs would not be harmful to the animals. However, despite intensive efforts, ES cells from pigs are not yet available and consequently this approach is not likely to be used in the immediate future.

5.2. α1,2FT competition with the substrate for α1,3GT:

An elegant strategy makes use of the fact that the action of α1,2fucosyltransferase (α1,2FT) adds a fucosyl residue in the position used as the substrate for addition of the terminal galactosyl residue in pigs (see Fig. 2). This strategy is being pursued by Sandrin and colleagues. They recently showed that transfection of a pig kidney epithelial cell line with human α1,2FT (45), the H-transferase synthesizing the substrate for human ABO-transferases, successfully competes with the pig α1,3GT (10). This competition leads to a substantial decrease in human NAb binding as well as in cytotoxic sensitivity of the cells. It should be possible to apply this strategy systemically, by making transgenic pigs expressing the human a1,2FT gene on pig EC. However, it is still unclear if the level of reduction achievable in this setting is enough to avoid the HAR.

5.3. Oligonucleotide antisense inhibition of α1,3GT expression:

By using oligonucleotide antisense technology (46, 47), we have attempted to down-regulate the expression of the Galα1,3Gal epitope. Oligonucleotide antisense DNA consists of short stretches of DNA complementary to a given mRNA molecule. Our experimental system consisted of a cultured pig EC line (PIEC) that was subjected to differing amounts of several antisense oligo-nucleotides corresponding mainly to the region for initiation of translation in the pig α1,3GT mRNA. One such oligonucleotide, when added to the medium every other day for 7 days at a concentration of 20μM, was found to inhibit expression of the epitope by approximately 40-50% (13). Since the binding of affinity-purified Ab paralleled the binding of epitope-specific lectin, these experiments also confirmed that the Galα1,3Gal epitope is the major target for human anti-pig NAb. The data also excluded the possibility that human anti-Galα1,3Gal NAb can cross-react with other pig molecules. One difficulty with such a system is that the turn-over rate of such epitopes on several glycoproteins and glycolipids may be considerable. In addition, it is hard to see how it would be possible to use such oligonucleotides *in vivo*, for at least two reasons. Firstly, administration of oligonucleotides would be a problem *in vivo* as well as *ex vivo* in an organ, and secondly, the effect of the oligonucleotides would be merely confined to one or two days.

5.4. Ribozymes as tools for downregulation of the α1,3GT expression:

A current strategy under development involves, by stable integration, the delivery of ribozymes to target cells. Ribozymes were found more than ten years ago to down-regulate in several species the expression of specific mRNA's by digesting their target in a specific location (48, 49). The hammerhead ribozyme consists of a loop structure interrupting two flanking antisense sequences carrying specificity for the target (50, 51). A number of studies have shown that it is possible to design ribozyme constructs for a desired target specificity in mammalian cells. Design and synthesis of oligonucleotides is followed by their ligation into a eukaryotic expression vector (52-55). This construct is subsequently transfected into the cells of interest leading to expression of a ribozyme RNA molecule. It has been previously shown that ribozymes expressed in a transgenic mouse were able to drastically downregulate expression of β_2-microglobulin (52) and pancreatic β-cell glucokinase (55). Adoption of a similar strategy in pig may allow development of constructs that lead to ribozyme RNA that is capable of down-regulating the α1,3GT mRNA.

6. CONCLUDING REMARKS

From several recent studies, it seems clear that the Galα1,3Gal epitope is the major target for human anti-pig NAb leading to the events that precipitate the HAR. Therefore, attempts are being made to produce transgenic pigs with reduced levels of expression of the Galα1,3Gal epitope. One attractive option will be to breed such animals with other transgenic pigs, notably pigs expressing human complement inhibitors. The offsprings of such animals are likely to provide organs for human transplantation that evade the HAR, as well as lessen the likelihood of production of high titers of non-pre-formed anti-Galα1,3Gal Ab (*e.g.* IgG).

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