

Characteristics of α -Gal epitope, anti-Gal antibody, α 1,3 galactosyltransferase and its clinical exploitation (Review)

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Abstract. The α -Gal epitope (Gal α 1,3Gal α 1,4GlcNAc-R) is ubiquitously presented in non-primate mammals, marsupials and New World Monkeys, but it is absent in humans, apes and Old World monkeys. However, the anti-Gal antibody (~1% of immunoglobulins) is naturally generated in human, and is found as the immunoglobulin G (IgG), IgM and IgA isotypes. Owing to the specific binding of the anti-Gal antibody with the α -Gal epitope, humans have a distinct anti- α -gal reactivity, which is responsible for hyperacute rejection of organs transplanted from α -gal donors. In addition, the α 1,3 galactosyltransferases (α 1,3GT) can catalyze the synthesis of the α -Gal epitope. Therefore, the α 1,3GT gene, which encodes the α 1,3GT, is developed profoundly. The distributions of the α -Gal epitope and anti-Gal antibody, and the activation of α 1,3GT, reveal that the enzyme of α 1,3GT in ancestral primates is ineffective. Comparison of the nucleotide sequence of the human α 1,3-GT pseudogene to the corresponding different species sequence, and according to the evolutionary tree of different species, the results of evolutionary inactivation of the α 1,3GT gene in ancestral primates attribute to the mutations under a stronger selective pressure. However, on the basis of the structure, the mechanism and the specificity of the α -Gal epitope and anti-Gal antibody, they can be applied to clinical exploitation. Knocking out the α 1,3GT gene will eliminate the xenoantigen, Gal(α 1,3)Gal, so

that the transplantation of α 1,3GT gene knockout pig organ into human becomes a potential clinically acceptable treatment for solving the problem of organ shortage. By contrast, the α -Gal epitope expressed through the application of chemical, biochemical and genetic engineering can be exploited for the clinical use. Targeting anti-Gal-mediated autologous tumor vaccines, which express α -Gal epitope to antigen-presenting cells, would increase their immunogenicity and elicit an immune response, which will be potent enough to eradicate the residual tumor cells. For tumor vaccines, the way of increasing immunogenicity of certain viral vaccines, including flu vaccines and human immunodeficiency virus vaccines, can also be used in the elderly. Recently, α -Gal epitope nanoparticles have been applied to accelerate wound healing and further directions on regeneration of internally injured tissues.

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1. Introduction

The α -Gal epitope (Gal α 1,3Gal α 1,4GlcNAc-R) is a unique carbohydrate, which is naturally produced on glycolipids and glycoprotein (1). Based on the carbohydrate chains on glycol-conjugates, α -Gal epitope synthesis is different in various species (2). In rabbits and cows, it is abundantly expressed on red cell membrane glycolipids, on cell surface glycoproteins of mouse Ehrlich ascites cells, lymphoma cells, and on thyroglobulin of bovine, canine and porcine (3). In addition, the glycosylation enzyme α 1,3 galactosyltransferases (α 1,3GT) can transfer galactose from uridine diphosphate (UDP)-gal to N-acetyllactosamine, producing oligosaccharide Gal α 1,3Gal α 1,4GlcNAc-R (termed α -Gal epitope) (4). However, the α 1,3GT was once thought to be a single α 1,3GT

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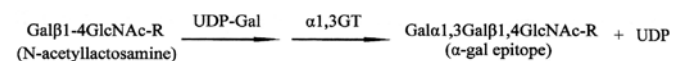
that exclusively synthesized the important xeno-epitope Gal(1,3)Gal. However, the iGb3 synthase and α 1,3GT in rats demonstrated two separate glycosylation pathways to the synthesis of Gal(1,3)Gal, challenging what is known regarding α 1,3GT (5,6). As a matter of fact, the enzyme α 1,3GT is as universal as the α 1,3 galactosyltransferase, and it is encoded by the α 1,3GT gene. In different species, the α 1,3GT gene is on a different locus, for example it resides on chromosome 2 of *Mus musculus* (house mouse) (7), chromosome 1 of *Sus scrofa* (pig) (8), chromosome 11 of *Bos taurus* (cattle) (9), chromosome 9 of *Canis lupus familiaris* (dog) (10), and chromosome 9 of the *Homo sapiens* (human) pseudogene, as the specified gene sites for the locus of the α 1,3GT gene (11). Comparing with the nucleotide sequence of the human α 1,3GT pseudogene with the corresponding different species sequences, and considering the evolutionary tree of different species, inactivation of α 1,3GT genes in ancestral primates is caused by several deletions on DNA sequences, which generates premature stop codons and the truncation of the enzyme molecule (12). The expression of the α -Gal epitope and the activity of α 1,3GT demonstrate a striking difference regarding their distribution in various species. Therefore, although the α -Gal epitope is absent in humans, apes and Old World monkeys, it is profusely generated in non-primate mammals, prosimians and New World monkeys (13). A large quantity of the natural anti-Gal antibody is produced in all humans. Since humans and Old World primates lack the α -Gal epitope, they are not immunotolerant to it, and therefore will produce anti-Gal antibodies (14,15). The anti-Gal antibody in humans is encoded by several heavy-chain genes primarily of the VH3 immunoglobulin gene family (16).

Xenotransplantation is the transplantation from animals, such as pigs, to humans. The α -Gal epitope on the xenografts will be specifically bound by the anti-Gal antibody, and therefore the combination of the anti-Gal antibody with α -Gal epitope contributes to the complement cascade (17,18). In detail, the complement cascade would lead to the collapse of the xenograft vascular bed and hyperacute rejection, which is the major obstacle in xenotransplantation. In order to overcome xenografts rejection, α 1,3GT knockout mice (α 1,3GT KO), lacking the ability to synthesize α -Gal epitope, were generated, and immunotolerance of xenotransplantation was induced (19). According to the success of α 1,3GT KO mouse experiment, the pig, as the major xenografts donor to humans, has been applied to the same experiment. Currently, the α 1,3GT KO pigs, which proved to possess no hyperacute rejection, are used in organs transplantation (20). In addition, regarding its application in xenotransplantation, the α -Gal epitope can also be used to increase the immunogenicity of the tumor cells, and it can be developed for clinical use in cancer immunotherapy as well. Furthermore, the immunogenicity of certain viral vaccines (21), including the flu vaccine (used in the elderly), and human immunodeficiency virus (HIV) vaccine, is deemed as suboptimal. The α -Gal epitope nanoparticles bind with the anti-Gal, which will activate the complement system and will recruit macrophages to induce tissue regeneration (22). Therefore, the application of α -gal nanoparticles could accelerate wound healing (23,24). This therapy may be of further significance in the regeneration of injured tissues, such as ischemic myocardium and injured nerves.

2. α -Gal epitope and anti-Gal antibody

α -Gal epitope. The α -Gal epitope has a special terminal carbohydrate structure in the form of Gal α 1, 3Gal β 1-4GlcNAc-R, which is confirmed by the study of two structures of the major glycolipids in rabbit red cell membranes: Ceramide trihexoside (Gal α 1-4Gal α 1-4Glc-Cer) and ceramide pentahexoside (Gal α 1-3Gal α 1-4GlcNAc α 1-3Gal α 1-4Glc-Cer) (3). The amount of α -Gal epitope exhibits significant differences in different organs. High concentrations of the α -Gal epitope were observed in all the hepatic cells. The largest quantity of α -Gal epitope was observed in the proximal convoluted tubules in the kidney and the α -Gal epitope were presented in smaller quantities in the distal tubules and in the glomeruli. No α -Gal epitope was identified in the collecting ducts. In addition, the α -Gal epitope is expressed on numerous molecules on platelets and it is predominantly found on fibrinogen, von Willebrand's factor, α 2 integrin and β 3 integrin. On endothelial cells, >20 glycoproteins carry α -Gal epitope, some of which have been identified as von Willebrand's factor, DM-GRASP, and the α 1, α v, α 3/ α 5, β 1, β 3 integrins (25). The expression of the α -Gal epitope varies in different species; for example, it is expressed on the red cell membrane glycolipids of rabbits and cows, the cell surface glycoproteins of mouse Ehrlich ascites cells, lymphoma cells and fibroblasts, on mouse laminin and on bovine, canine and porcine thyroglobulin.

The α -Gal epitope is synthesized by the transferase α 1,3GT, and similar to numerous other glycosylating enzymes, the α 1,3GT resides within the Golgi of the cell (5). The α 1,3GT is believed to be the only enzyme to synthesize Gal(1,3)Gal. However, the rat iGb3 synthase is also capable of synthesizing Gal(1,3)Gal as the glycolipid structure iGb3, showing that the rat expresses two distinct α 1,3GT (26), α 1,3GT and iGb3 synthase. Therefore, in rats there exists two separate glycosylation pathways to synthesize Gal(1,3)Gal. The α 1,3GT is one of the α 1,3GTs, and this enzyme is active in the Golgi apparatus of cells and transfers galactose from the sugar-donor UDP-gal to the acceptor N-acetyllactosamine residue (Gal α 1-4GlcNAc-R) on carbohydrate chains of glycolipids and glycoproteins, to form the α -Gal epitope (27).



Additionally, the distributions of the α 1,3GT gene have a significant difference in various species (28). The α 1,3GT gene of murine is composed of nine exon sequences, and only six exons: Exons 4-9 (based on the numbering of mouse α 1,3GT), are translated to α 1,3GT mRNA, which is ~1,500 base pairs (bp) (GenBank: M26925.1) (5). In *Sus scrofa* (pig), the α 1,3GT gene resides on chromosome 1, and α 1,3-GT mRNA is 1,269 bp (GenBank: L36152.1) (8). The α 1,3GT mRNA completed cds for bovine are 1,828 bp (GenBank: J04989.1) (29). The coding sequence of bovine α 1,3GT predicates a membrane-bound protein with three distinct structural features: A large, potentially glycosylated COOH-terminal domain (346 amino acids), a single transmembrane domain (16 amino acids), and a short NH₂-terminal domain (6 amino acids) (29). By the analysis of the panel of human-rodent somatic cell hybrids, the nonfunctional, processed pseudogene and the human homologue gene represented by HGT-10 have been established to be located on human chromosomes 12 and 9 (GenBank: J05421.1) (6,30).

Table I. Distributions of α -Gal epitope, anti-Gal antibody and the α 1,3GT activity demonstrating significant differences in various mammalian species.

Mammals	α -Gal epitope expression	α 1,3-GT activity	Anti-Gal antibody expression
Non-primate mammals	O	O	X
New world monkeys	O	O	X
Prosimians	O	O	X
Old world monkeys	X	X	O
Humans	X	X	O
Apes	X	X	O

O, detected; X, not detected.

The locus of α 1,3GT mRNA for certain primates are about the same locus as those of *Gorilla gorilla* (GenBank: M73304.1), *Pongo pygmaeus* (GenBank: M73305.1) and *Macaca mulatta* (GenBank: M73306.1) (31).

Anti-Gal antibody. The expression levels of the α -Gal epitope were assessed by measuring the binding of the anti-Gal antibody with the α -Gal epitope-specific lectin *Bandeiraea simplicifolia* IB4 in various cells. The distributions of α -Gal epitope, anti-Gal antibody and the activities of α 1,3GT in various species have a significant difference. As shown in Table I, the α -Gal epitope is abundant on red blood cells and nucleated cells of non-primate mammals, prosimians and New World monkeys (such as marmoset, squirrel monkey and spider monkey); however, it is absent in ancestral primates such as humans, apes and Old World monkeys. Due to the continual exposure of α -Gal epitope on the surface of enteric bacteria and other pathogens, and the lack of α -Gal epitope on humans and Old World primates, they are not immunotolerant to the α -Gal epitope, and therefore produce anti- α -Gal epitope antibody throughout the whole life (32,33). As many as 1% of circulating B cells are capable of generating the anti- α -Gal epitope antibody, which is analogous to the mechanism by which the human ABO blood group antibodies are produced. Anti-Gal antibody is not generated at birth [except maternal-derived immunoglobulin G (IgG)]; however, it is developed during the first few months of life. The anti-Gal antibody isotypes of IgM, IgG, and IgA have been identified (34-37), and the anti-Gal antibody in humans is encoded by several heavy-chain genes, which are primarily within the VH3 immunoglobulin gene family (38). When anti-Gal antibody specifically binds with the α -Gal epitope, the binding site of anti-Gal antibody resembles a 'pocket' for the α -Gal epitope with a size corresponding with the free trisaccharide Gal α 1-3Gal β 1-4GlcNAc. The affinity of the anti-Gal antibody to the free trisaccharide Gal α 1-3Gal β 1-4GlcNAc is ~7-fold higher compared to the disaccharide Gal α 1,3Gal. The similarity in binding of anti-Gal antibody to α -Gal epitope on carbohydrate chains of glycoproteins (mannose in the fourth position) and glycolipids (no mannose in the carbohydrate chains) further implies that the pocket size is limited to a trisaccharide length of the antigen.

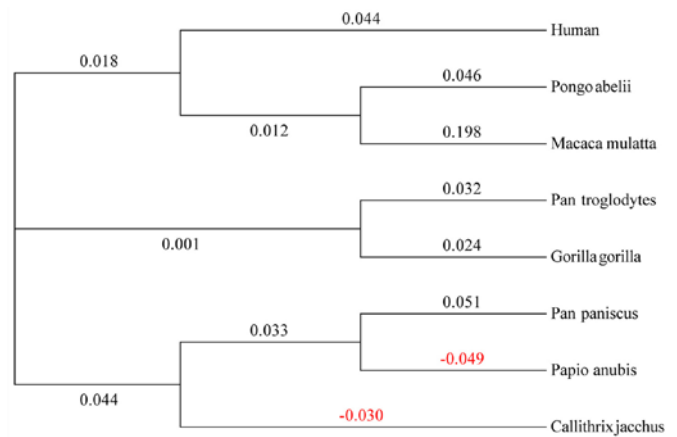


Figure 1. According to the evolutionary tree of mammalian species, the association of the mammalian species can be detected by the time they separated, and the sum of two numbers between different species shows the affinity of them. The smaller the total is, the closer the association is.

Evolution by the inactivation of α 1,3GT and anti-Gal antibody. According to the evolutionary tree of mammalian species (Fig. 1), it is clear that α 1,3GT and α -Gal epitope have been conserved in New World monkeys (evolving on the South American continent, which was separated from the African continent for >35 million years) and in lemurs (prosimians residing in the Madagascar, a land mass isolated from the African continent for >60 million years), which suggests that α 1,3GT appeared early in mammalian evolution, and prior to the divergence of marsupials and placental mammals (>125 million years ago) (39). However, following the geographical separation between the South American and African continents, ancestral Old World primates were subjected to a selective evolutionary pressure, which resulted in the inactivation of the α 1,3GT gene. Northern blot results reveal that no transcripts of the α 1,3GT gene were identified in the higher primate species. Notably, a variety of full-length transcripts were determined by sensitive polymerase chain reaction in the tissues of rhesus monkeys, orangutans and humans. Five crucial mutations were delineated in the coding region of the human and rhesus, and three crucial mutations were identified in the orangutan, any one of which could be responsible for inactivation of the α 1,3GT gene. Therefore, the α 1,3GT gene exhibits a unique evolutionary characteristics.

Starting from the Old World primates, the α -Gal epitope has been interrupted during the course of evolution. Partial sequences of the α 1,3GT gene, which governs the synthesis of the α -Gal epitope, were detected in the human genome and were found to be the corresponding pseudogenes. An experiment was performed using the bovine α 1,3GT cDNA as a probe to isolate two non-overlapping clones (HGT-2 and HGT-10) from a human genomic DNA library (40). HGT-2 contains a putative coding region, containing multiple frame shift mutations and nonsense codons in all three reading frames, which precludes the synthesis of a functional enzyme. The coding sequences of HGT-10, which is located on human chromosomes 12 and 9, contain nonfunctional and processed pseudogene. Comparison of the nucleotide sequence of the human α 1,3GT pseudogene with the corresponding different species (Fig. 2), the evolutionary loss of α -Gal epitope attributes to point mutations in the coding region

Human	CAT CAGCAT GAT GCGTAT GAAGAT CACTGGGGAGCACATCTTGGCCACATCCAACACGA
Macaca	CAT CAGCAT GAT GCGT GT GAAGAT CAT CAGGGAGCACATCTTGGCCACATCCAACACGA
Gorilla	CAT CAGCAT GAT GCGTAT GAAGAT CACTGGGGAGCACATCTTGGCCACATCCAACACGA
Pongo	CAT CAGCAT GAT GCGTAT GAA AAT CACTGGGGAGCACATCTTGGCCACATCCAACACGA
Human	GGTGGACTTCTCTTCTGCATCGATGTCAGCCAGGTCTTCCAAGACCATTGGGGTGGG
Macaca	GGTAGACTTCCTCTTCTGCATCGATGTAGCCAGGTCTTCCAAGACAATTGGGGTGAA
Gorilla	GGTGGACTTCTCTTCTGCATCGATGTGGCCAGGTCTTCCAAGACCATTGGGGTGGG
Pongo	GGTGGACTTCTCTTCTGCATCGATGTCAGCCAGGTCTTCCAAGACCATTGGGGTGGG
Human	GACCCTAGGCCAGTCAAGTGGCTCAGCTACAGCCCTGGTGTACAAGGCAGATCCCTATGA
Macaca	GACCCTAGGCCAGTCAAGTGGCTTAGCTACAGCCCTGGTGTACAAGGCAGATCCCTATGA
Gorilla	CACCCTAGGCCAGTCAAGTGGCTCAGCTACAGCCCTGGTGTACAAGGCAGATCCCTATGA
Pongo	CACCCTAGGCCAGTCAAGTGGCTCAGCTACAGCCCTGGTGTACAAGGCAGATCCCTATGA
Human	CTTTACCTAGGAGAGGCTGGAAGAAGT CAGCAGGATACATTCAATTTGGCCAGG-GGATTT
Macaca	CTTTACCTAGGAGAGGCCAGAAGAAGT CAGCAGCATGCATTCAATTTGGCCAGGAGGATTT
Gorilla	CTTTACCTAGGAGAGGCCGGAAGAAGT CAGCAGGATACATTCAATTTGGCCAGG-GGATTT
Pongo	CTTTACCTAGGAGAGGCCGGAAGAAGT CAGCAGCATATATTCAATTTGGCCAGG-GGATTT
Human	TTATTACCATGAGCCATTTCTGGAGGAACACCCATT CAGGTTCTCAACATCAACCAGGA
Macaca	TTATTACCATGAGCCATTTTGGAGGAACACCCATT CAGGTTCTGACATCCCCAGGA
Gorilla	TTATTACCATGAGCCATTTTGGAGGAACACCCATT CAGGTTCTCAACATCAACCAGGA
Pongo	TTATTACCATGAGCCATTTTGGAGGAACACCCATT CAGGTTCTCAACATCAACCAGGA
Human	GTGCTTTAAGGAATCCTCCTGGACAAGAAAATGACATAGAAGCCAAGTGGCATGATGA
Macaca	GTGCTTTAAGAGAATCCTCCTGGAAAAGAAAATGACAT-----
Gorilla	GTGCTTTAAGGAATCCTCCTGGACAAGAAAATGACAC-----
Pongo	GTGCTTTAAGGAATCCTCCTGGACAAGAAAATGACGT-----

Figure 2. Comparison of the nucleotide sequence of the human α 1,3 galactosyltransferases (α 1,3GT) pseudogene to the corresponding different species sequence, as listed.

of the α 1,3GT gene. The human α 1,3GT pseudogene resides on chromosome 9, and its sequence was found to contain two point mutations, which results in a frame shift mutation and a premature stop codon (13). Sequencing of this exon in the chimpanzee shows the same two mutations, whereas the sequence from gorilla only includes the first frame-shift mutation, which generates a premature stop codon. The common mutations are identified among the α 1,3GT sequences of Old World monkey. These findings in various primates suggest that the evolutionary inactivation in ancestral Old World primates following the divergence of apes and monkeys is estimated to have occurred ~20-25 million years ago. The evolutionary inactivation of the α 1,3GT gene resulted in the loss of immune tolerance to the α -Gal epitope and the appearance of the anti-Gal antibody. Once the α -Gal epitope was eliminated, primates could produce the anti-Gal antibody, possibly by means of defending against pathogens, which express the α -Gal epitope. Therefore, the inactivation of α 1,3GT gene is generated by several deletions, which ultimately encodes premature stop codons. The observed inactivation of primate α 1,3GT, along with distinct geographical boundaries, suggests that it was due to an infectious disease, which is endemic to the connected continents of the Old World.

3. Attempts to overcome xenograft rejection through the knockout of α 1,3GT genes

A worldwide organs shortage for clinical implantation causes 20-35% of patients, who need organ transplantation, to succumb whilst on the waiting list. Therefore, accompanied

with increasing attention, the supply and demand for the transplanted organs are the focuses of clinical transplantation. With the application of xenotransplantation, organs obtained from animal species provide a cure for the organ shortage. Since non-human primates are the closest relatives to humans, they were first considered as a potential organ source for xenotransplantation (25). Although chimpanzees were originally considered the best option, as their organs are of similar size and they have good blood type compatibility with humans, their organs were not widely used as they are listed as an endangered species. Therefore, based on the physiological, biological and ethical considerations, pigs appear to be the most suitable donor for xenotransplantation. However, when the pig organs were transplanted into Old World monkey, the α -Gal epitope activates the complement system, creating C5a and C3b and causing a cascade of further cleavage and activation events. C3b binds to the surface of pathogens, leading to greater internalization by phagocytic cells through opsonization. C5a is an important chemotactic protein, helping the recruitment of inflammatory cells, such as macrophages and neutrophils (41). Therefore, when the α -Gal epitope, expressed on endothelial cells of the blood vessel walls in the xenograft, binds with the anti-Gal antibody, it will result in the activation and induction of platelet aggregation, and leads to the collapse of the xenograft vascular bed and to the rapid hyperacute rejection (42).

There are three major components to hyperacute rejection: i) Antigen, ii) antibody and iii) complement (25). In order to overcome the hyperacute rejection, actions can be taken to relieve the hyperacute rejection by the inactivation of the

α 1,3GT gene. There are two commonly used methods to relieve the hyperacute rejection. One strategy to the inactivation of the α 1,3GT gene would be using the transgenic approach to add another transferase, which would deviate the glycosylation pathway from α -Gal epitope expression and lead to the production of another carbohydrate not recognized by natural antibodies. The α (1,2) fucosyltransferase and α (2,3) sialyltransferase or α (2,6) sialyltransferase are known to be different glycosyltransferases, which compete for the same substrate with each other. They can be applied to develop a strategy aimed at blocking a specific carbohydrate epitope, an appropriate enzyme to decrease the expression of Gal(1,3)Gal. According to a previous study, the results of this method demonstrated that the amount of α -Gal epitope is lesser than normal, but cannot be eliminated completely (25). Another strategy is accomplished by the disruption of the pig α 1,3GT gene locus, mediated by a pPL657 vector, targeting exon 9 (the location encoding the catalytic domain of the α 1,3GT gene) (38). Homozygous inactivation of these two alleles of the α 1,3GT genes results in an inactive enzyme. It is demonstrated that the natural antibodies of the α 1,3GT KO pig would appear to be similar to those antibodies in other species. Therefore, α 1,3GT cannot be encoded without the α 1,3GT gene, and the α -Gal epitope would not be synthesized. The strategy of knocking out the gene of α 1,3GT successfully avoids the hyperacute rejection. In the same way, by studying the anti-Gal positive B-cell in α 1,3GT KO mice responding to α -Gal epitope of xenografts, these mice are indicated to lack the α -Gal epitope due to the disruption of the α 1,3GT gene (43). The same experiment was carried out on pigs, and organ transplantation from α 1,3GT KO transgenic pigs to humans did not result in complement-mediated hyperacute rejection. Therefore in this way, rapid hyperacute rejection can be eliminated, and the organ transplantation from pig to humans becomes a clinically acceptable treatment (Fig. 3).

4. Clinical exploitation

Anti-Gal antibody, which is universal in human, can effectively bind with the α -Gal epitope, and therefore activates the complement cascade, which generates chemotactic cleavage complement peptides, such as C5a and C3b. These chemotactic peptides induce rapid and extensive migration of macrophages. Based on the structure, mechanism and specificity of the anti-Gal antibody, it can be exploited for a variety of clinical uses. Firstly, the α 1,3GT KO pigs can be used as organ donors from pig to human in xenotransplantation. Secondly, the immunogenicity of tumor vaccines and certain viral vaccines, including flu and HIV vaccine, can be increased by the binding of anti-Gal antibody with α -Gal epitope. In addition, wound healing, skin burns and tissue regeneration can be induced by α -gal nanoparticles. The regeneration of internal injuries, such as ischemic myocardium and injured nerves, will be one of its applications in the future as well.

Xenotransplantation. Xenotransplantation is one of the most important methods to solve the problem of the organ shortage in humans. Those cells, tissues or organs from different species are known as xenografts, which are widely used for xenotransplantation (44). For example, the heart valve substitutes and the patch materials are applied to a variety of cardiovascular diseases (45).

However, the major barrier to the clinically successful xenotransplantation is the lack of effective therapies aimed at eliminating antibody and complement. Therefore, subsequent to knocking out the α 1,3GT gene, the α -Gal epitope cannot be produced, and hyperacute rejection can be overcome (46).

As the whole organs of the heart is not the ideal choice for heart xenotransplantation, heart valve substitutes and patch materials are generally applied to a variety of cardiovascular disease (45), and the major application is the bioprosthetic heart valves (BHV) (47,48). Currently, the most commercially available BHVs are made from porcine aortic valve or bovine pericardium (49). They are conventionally cross-linked with glutaraldehyde (GA) to impart tissue stability, to reduce antigenicity and to maintain tissue sterility (50).

However, GA-fixed xenografts are prone to dystrophic calcification after long-term implantation in humans, which is one of the limiting factors affecting the longevity of bioprostheses (47). The mechanism of the calcification of GA-fixed xenografts is complex and is not completely understood. It is supposed that the degeneration of bioprostheses may begin with the penetration of immunoglobulines into the valve-matrix. Subsequently, macrophages are deposited on the valve surface and erythrocytes penetrate into the valve. Finally, collagen breakdown and calcification occur. Thus, the deposition of immunoglobulines represents the initial immunological trigger for calcification in bioprostheses (50). Another proposed mechanism responsible for the calcification of xenografts is an immune response to the xenografts (51). According to the method of anti-calcification treatments, bovine pericardial tissues were divided into eight groups. Prepared tissues were subcutaneously implanted into the α -gal KO mice and wild-type mice. The result demonstrated that except the high concentration GA-treated group, calcium contents of anticalcification-treated groups were significantly lower compared to the control group (45). These findings suggest a possible role of immune response in the calcification of xenografts, and α -Gal epitope on the BHV contributes to the immune response (52-54). The α -Gal epitope on BHV may be removed completely using recombinant α -galactosidase and by performing a decellularization procedure without modifying the mechanical properties of the tissues (25,55,56). There is no difference in the mechanical properties of BHV following the application of the methods (57,58). In addition, a series of 7 heterotopic heart transplantations were performed from cluster of differentiation 46-positive transgenic pigs to baboons using a combination of therapeutic agents mainly targeted at controlling the synthesis of anti-pig antibodies. This study reported that the longest median survival of pig hearts transplanted into baboons was 96 days (59). However, there also are numerous problems regarding BHV transplantation, in pig-to-primate or human BHV. Although hyperacute rejection can be prevented, the next rejection phenomenon unique to xenotransplantation, known as delayed xenograft rejection or acute vascular rejection, will occur (60). In addition, new immunological problems arise, for example, except the α -Gal epitope, there exist non-gal carbohydrate antigens and the identification of their elusive antigens as neutrophils, macrophages, natural killer cells and T cells (61).

Studies on the xenotransplantation of heart and kidney from α 1,3GT KO pigs to monkeys demonstrate that pig hearts could live for \leq 6 months, pig kidneys for 8-32 days, and pig kidneys containing vascularized thymic tissue for up to

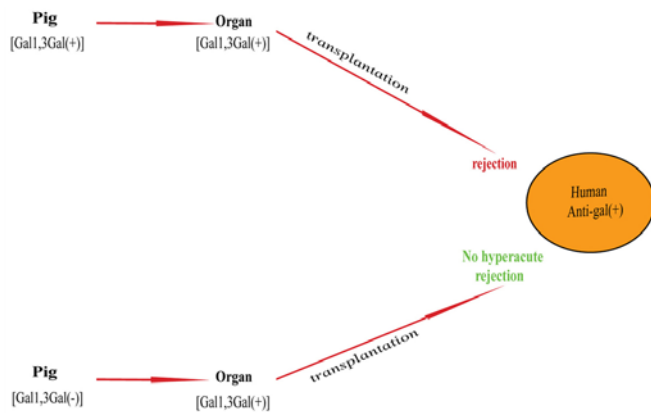


Figure 3. Transplanting organs from α 1,3 galactosyltransferases (α 1,3GT) gene knockout pigs to humans. There is no hyperacute rejection in transplantation following the knock-out of the α 1,3GT gene.

83 days (62). Therefore, transplantation of organs from α 1,3GT KO pigs to humans becomes clinically acceptable. However, in addition to the α -Gal epitope, the non-gal carbohydrate antigen is another obstacle in kidney transplantation. A study of total N-glycans from membrane glycoproteins, derived from specific pathogen-free miniature pig kidneys, was carried out, and the result showed that α -gal was the most abundant compound among the neutral pig N-glycans. However, the various N-glycan structures of Neu5Gc and Gala1-3Lex epitope as new non-gal carbohydrate antigens were identified by mass spectrometry (46,63). Therefore, non-gal carbohydrate antigens may be the next complication in the transplantation from animals to humans.

Cancer immunotherapy. Tumor cells express a variety of tumor-specific antigens in animals and in humans. Some of the tumor antigens are common to numerous types of tumors. Additionally, as certain antigens may also be expressed on normal cells, the immune response caused by these common tumor antigens is usually extremely low (64). However, tumor antigens are specific to the tumor type and to the patient. Therefore, the autologous tumor cells could be considered a suitable source for the production of the vaccine. In order to induce the expression of α -Gal epitope by the tumor cells, chemical, biochemical and genetic engineering can be applied (8). As an example of the chemical and biochemical methods, autologous intact tumor cells from hematological malignancies, or autologous tumor cell membranes from solid tumors are processed to express α -Gal epitope by the incubation with neuraminidase, recombinant α 1,3GT and uridine diphosphate galactose (9). Additionally, the expression of the α -Gal epitope can also be achieved by the transduction of recombinant adenovirus pAd-sGT, expressing pig α 1,3GT genes, into the tumor cells (65).

The adenoviral vector-mediated transduction of the pig α 1,3GT gene into human tumor cells, such as malignant melanoma A375, stomach cancer SGC-7901, and lung cancer SPC-A-1, has been reported. These studies demonstrated that α -Gal epitope did not increase the sensitivity of human tumor cells to human complement-mediated lysis (66). Studies on the immune response to tumor vaccines have indicated that, to achieve effective activation of tumor-specific naive T cells, the vaccinating antigens must be effectively taken up

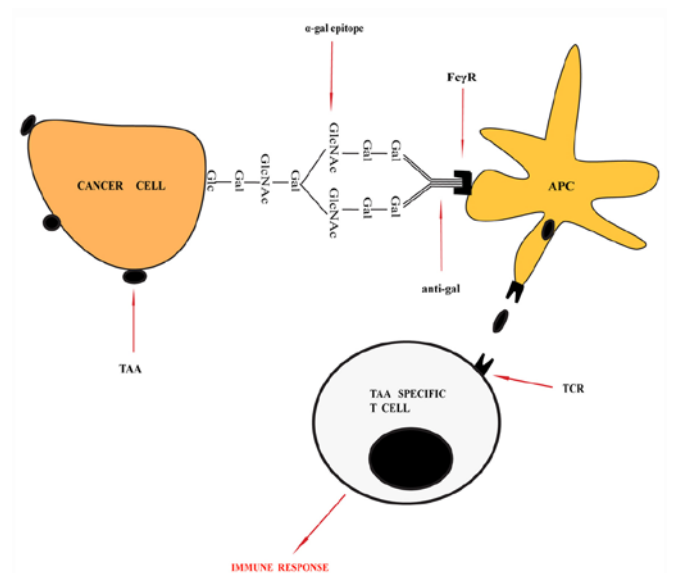


Figure 4. Targeting the anti-Gal-mediated complex to antigen-presenting cells (APCs). Targeting of autologous tumor cells to APCs by the natural anti-Gal antibody is carried out. Subsequently, the anti-Gal antibody binds with α -Gal epitopes on tumor cells, its Fc portion interacts with Fc γ receptor (Fc γ R) on APCs. This interaction stimulates APC processing and presenting of the tumor-associated antigen (TAA) peptides. TAA are transported by the APCs to regional lymph nodes, activating the tumor-specific T cells and generating an immune response. Thus, the immunogenicity of autologous tumor cells is increased.

by antigen-presenting cells (APCs). Subsequently, the vaccinating antigens should be transported from the vaccinating tumor membranes to the regional lymph nodes, and the tumor-associated antigen (TAA) peptides will be processed and presented by APCs (67-69). The autologous TAA peptides activate tumor-specific T cells within the lymph nodes, eliciting an immune response, which would eradicate the residual tumor cells following completion of standard therapy (70).

In addition, the tumor cells can also express the α -Gal epitope by the transfection of α 1,3GT genes through gene engineering. Following genetic manipulation, the binding ability of the tumor cells with human IgG and IgM is increased. However, the sensitivity of human tumor cells to human complement-mediated lysis is not enhanced. In a cancer-related study, studies on the immune response of tumor vaccines have indicated that a major prerequisite for the formation of tumor vaccines is their effective uptake by APCs and the transportation of these APCs to the regional lymph nodes where TAAs can be processed and presented (21,71). In detail, TAAs activate the immune response, and the anti-Gal antibody would specifically bind to α -Gal epitope. The Fc portion of the anti-Gal antibody binds with the Fc α receptors on APCs and induce the effective uptake of the vaccine by the APCs (Fig. 4) (72). Subsequent to transporting the vaccinating tumor membranes to the regional lymph nodes, TAA peptides can be processed and presented. The autologous TAA peptides activate tumor-specific T cells within the lymph nodes, which causes an immune response and it will eradicate the residual tumor cells that remain following the completion of standard therapy (73).

With regard to the application of tumor vaccines, MUC1 was overexpressed and aberrantly glycosylated in the majority

of human ductal pancreatic carcinoma (74). The α 1,3GT KO mice used as the model were pre-immunized with pig kidney and transplanted with B16F10 melanoma cells, and were transfected with the MUC1 expressing vector. Compared with the mice vaccinated with MUC1 alone, the tumor growth was significantly inhibited and the survival time was significantly improved in mice vaccinated with pancreatic cancer cells expressing α -Gal epitope. Based on this study, it will be a novel strategy for the treatment of pancreatic cancer (75,76). Another study reported the treatment of 11 patients with advanced solid tumors with intratumoral injection of 0.1, 1 or 10 mg α -gal glycolipids. There was no dose-limiting toxicity and no clinical or laboratory evidence of autoimmunity, or any other toxicity. Few patients had an unexpectedly long survival. Therefore, it is feasible and safe for inducing a protective antitumor immune response (77). The mice vaccinated with irradiated melanoma cells, which express α -Gal epitope, were followed by a challenge with live parental melanoma cells, and resulted in protection with vaccines for ≥ 2 months in one-third of the mice. The proportion of survival mice protected with the vaccines doubled when they were immunized twice with irradiated melanoma cells expressing α -Gal epitope, and challenged with the life span of those of the control mice. A further histological study on the developing tumors in the challenged mice, which were immunized with melanoma cells expressing α -Gal epitope, demonstrated the extensive infiltration of T lymphocytes and macrophages (73). Chiefly, anti-gal can be applied to the cancer immunotherapy, and breast cancer and brain tumors may be treated by autologous tumor vaccines in the future.

Viral vaccines. Viral vaccines include the flu and HIV vaccines. Similar to the tumor vaccine, targeting the anti-gal-mediated complex to the APCs is essential (78). The Flu vaccine is mainly comprised of the viral envelope glycoprotein 'hemagglutinin' (HA), which may be served as a useful example for the application of anti-Gal into vaccines. In the majority of strains of flu virus, HA has 6-8 N-linked carbohydrate chains, the majority of which are of the complex type. This study demonstrates a method for the expression of α -Gal epitope on the influenza virus HA by recombinant α 1,3GT (79). To generate the flu vaccine expressing α -Gal epitope, sialic acid is removed from viral glycoproteins following its digestion by viral neuraminidase, and the terminal of the carbohydrate chains of HA was residual N-acetylglucosamine. Subsequently, α 1,3GT transfers sugar from the sugar donor UDP-gal to the residual N-acetylglucosamine (64). Therefore, α -Gal epitope could be added to all carbohydrate chains of HA. In the study of the α 1,3GT KO mice, the control group of the KO mice were immunized with the original inactivated flu virus lacking α -Gal epitope, and another group of the KO mice were immunized with 1 μ g of inactivated flu virus vaccine expressing the α -Gal epitope. The result demonstrated that the anti-flu virus antibody and T cell response in the group that was immunized with the virus vaccine expressing α -Gal epitope was ~ 100 -fold higher compared to the control group. Furthermore, the survival time of KO mice with the vaccine expressing α -Gal epitope infected with live virus was ~ 9 -fold longer compared to the KO mice immunized with the vaccine lacking this epitope (21). Therefore, the immunogenicity of flu

vaccines is increased by targeting anti-Gal antibody mediated complex into APCs.

In addition, the HIV vaccine is another notable viral vaccine. The main glycoprotein on the surface of HIV is gp120, which has 24 N-linked glycosylation sites of the carbohydrate chains, and the terminal structure of the gp120 carbohydrate chains is SA-Gal α 1-4GlcNAc-R. Following the incubation with neuraminidase, UDP-gal and α 1,3GT, gp120 can be capped with the α -Gal epitope. Studying the HIV vaccine on KO mice, it demonstrated that the anti-gp120 antibody and T cell responses were increased ~ 100 -fold following the immunization of gp120 with α -Gal epitope (80).

Chiefly, the immunogenicity of the flu and HIV vaccines is increased by expressing the α -Gal epitope. This method can be used for any microbial, soluble or particulate vaccine. However, the recombination does not affect the immunogenic epitopes of the vaccine.

Wound healing, skin burns and tissue regeneration by α -gal nanoparticles. The interaction between the anti-Gal antibody and α -Gal epitope activates complement system, which induces rapid recruitment of macrophages. Wound healing requires the help of the local recruitment and the activation of macrophages. The activated macrophages generate cytokines, such as vascular endothelial growth factor, interleukin-1, platelet-derived growth factor and colony-stimulating factor. These cytokines promote the healing of injuries. However, if the α -Gal epitope was expressed on cells with inserted α -gal glycolipids, the binding of anti-Gal antibody with α -Gal epitope may lead to cell lysis and a pro-inflammatory response. To avoid this pro-inflammatory response, the α -Gal epitope is expressed on cells in the form of nanoparticles and subsequently there will be no insertion into the cells (38). These nanoparticles were generated from phospholipids, cholesterol and α -gal glycolipids. An experiment regarding full-thickness wounds (20x20 mm) with tattooed borders was carried out on the back of α 1,3GT KO pigs. Treated wounds with α -gal nanoparticles exhibited more macrophages compared to the control wounds (saline-treated) in the same pig and at the same time, and it was observed that angiogenesis was increased in the wounds with α -gal nanoparticles. No keloid formation or increase in scar formation was observed in pigs treated by α -gal nanoparticles (81,82). Another study demonstrated that in contrast to the wild-type mice, the healing time of excisional skin wounds treated with α -gal liposomes in α 1,3GT KO mice was twice as fast as that of control wounds. Additionally, the time of scar formation in α -gal liposome-treated wounds is much less compared to those wounds with physiological healing (83).

In addition, the anti-Gal antibody can be used on the skin burns healing as well. Efficacy of treatment in accelerating burn healing is demonstrated in pairs of burns in α 1,3GT KO mice. It has been demonstrated that 3 days later, the α -gal liposomes treated burns contained 5-fold as many neutrophils as those of the control burns, and macrophages were found only in α -gal liposome-treated burns. Six days later, 50-100% of the surface area of the α -gal liposome-treated burns were covered with regenerating epidermis (re-epithelialization), and almost no epidermis was identified in the control burns (84).

Therefore, the extensive recruitment of macrophages by α -gal nanoparticles that have interacted with anti-Gal

antibody will effectively accelerate wound healing, skin burns and tissue regeneration.

Regeneration on ischemic myocardium and injured nerves. In the same way of accelerating wound healing, skin burns healing and tissue regeneration, α -gal nanoparticles may also be applied to the regeneration on ischemic myocardium and injured nerves. Combined with the regeneration on ischemic myocardium and injured nerves, the mechanisms of these treatments have numerous similarities. Firstly, the mechanism of regeneration of ischemic myocardium and injured nerves requires the recruitment of macrophages, which will secrete cytokines/growth factors. One of the differences between them is that the regeneration on ischemic myocardium requires the recruitment of stem cells, which received cues from the adjacent healthy cells, the microenvironment and the extracellular matrix to differentiate into cardiomyocytes, which regenerate the tissue and restore its physiological activity. However, the regeneration of nerves requires re-growth of multiple sprouts from injured axons. These sprouts will reconnect across the lesion and grow into the distal axonal tube of the damaged neurons. In addition, they have a default mechanism for tissue repair simultaneously. When the recruitment of macrophages is delayed, leading to no important cytokines/growth factors regeneration, the useful technique would be removal, which is irreversible.

The treatments for regeneration of ischemic myocardium and injured nerves by α -gal nanoparticles are theoretical possibilities at present, and the experiment is expectantly being carried out on mammalian animal models (38).

5. Conclusion

The α -Gal epitope has a unique carbohydrate structure, and the distribution of the α -Gal epitope, anti-Gal antibody and the α 1,3GT activity has a big difference in various mammalian species. Previous studies showed that the inactivation of the α 1,3GT in ancestral primates can explain the inactivation of the primate α 1,3GT along distinct geographical boundaries. The divergence is ascribed to the pressure coming from natural selective. With regards to the notable differences in the distribution of α -Gal epitope expression, the activity of α 1,3GT and the expression of anti-Gal antibody among ancestral primates and other species, the anti-Gal antibody is regarded as a barrier in the transplantation from animals, such as pigs to humans, which causes the immune response to xenografts. However, the α 1,3GT KO pig can aid with this problem of rejection. The anti-Gal antibody can be exploited for clinical use by increasing the immunogenicity of viral vaccines and autologous tumor vaccines, which can be processed to express the α -Gal epitope. In addition, the α -Gal epitope, expressed by the tumor cells, can be generated by certain methods. *In vivo*, it can be produced by catalysis with α 1,3GT. *In vitro*, its synthesis can be completed through chemical, biochemical and genetic engineering methods. By targeting those vaccines to APCs at the vaccination site, the immune responses induced will be potent to clear up the residual tumor cells following standard chemical therapy. Therefore, this method can be applied to the cancer immunotherapy. In addition, in the same way as the formation of tumor vaccines, the formation of viral vaccines, such as flu and HIV vaccines, will be feasible in the

future. In another way, accelerating wound healing is achieved by the application of α -gal nanoparticles, which would bind with the anti-Gal antibody by activating complement system, recruiting and activating macrophages and therefore inducing tissue regeneration. Thus, with the help of α -gal nanoparticles, the healing time of wounds and injury can be reduced efficiently. The regeneration on ischemic myocardium and injured nerves is a direction for the future.

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