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The Anti-Non-Gal Xenoantibody Response to Galactosyltransferase Gene Knockout (GalTKO) Pig Xenografts

Ivan Harnden, Kathleen Kiernan, and Mary Kearns-Jonker

¹ Department of Cardiothoracic Surgery, Saban Research Institute of the Children's Hospital of Los Angeles, University of Southern California Keck School of Medicine, 4650 Sunset Blvd, Mailstop #137, Los Angeles, CA 90027

Abstract

Purpose of Review—Anti-non-gal xenoantibodies are a major barrier to the survival of genetically modified porcine xenografts. This review summarizes new information on the contribution of anti-non-gal xenoantibodies to the activation of porcine endothelial cells and the pathology of rejection and further provides an update on recent advancements in defining the unique features of anti-non-gal xenoantibody structure.

Recent Findings—Anti-non-gal xenoantibodies pre-exist at low levels in humans and non-human primates, and are notably absent in neonates. Exposure of non-human primates to $\alpha 1,3$ galactosyltransferase gene knockout (GalTKO) endothelial cells initiates an induced xenoantibody response that is restricted and encoded by the germline progenitor IGHV3-21. The target xenoantigen remains undetermined, but several candidate targets have been proposed, including carbohydrate xenoantigens. New advancements in molecular modeling provide insight on the mechanism by which xenoantibodies bind to structurally related carbohydrates.

Summary—Genetic manipulation of porcine donors has significantly prolonged the survival of grafts placed into non-human primate recipients, but anti-non-gal xenoantibodies and thrombosis limit the ability of these grafts to function on a long term basis. Recent developments defining pre-existing anti-non-gal xenoantibody levels, the restriction in the anti-non-gal xenoantibody response and the identification of key sites defining xenoantibody/carbohydrate interactions now provide the information necessary to develop new approaches to preventing xenoantibody-mediated rejection.

Keywords

Gal $\alpha 1,3$ Gal carbohydrate; alpha-1,3 galactosyltransferase gene knockout (GalT-KO); xenotransplantation

Introduction

The development of alpha-1,3 galactosyltransferase gene knockout (GalT-KO) pigs has circumvented the problem of hyperacute rejection, but acute humoral xenograft rejection remains a major obstacle in xenotransplantation [1–4]. Graft survival of 3–6 months can be achieved in primates before antibody-mediated rejection and thrombosis occurs, but the time interval between graft placement and rejection varies, dependent on the type of GalT-KO pig organ transplanted and the immunosuppressive treatment regimen selected [5–10*]. In the

complete absence of immunosuppression, xenografts from GalT-KO donors survive for less than a week [10*].

Surprisingly little is known about the nature of the xenoantibody response to GalTKO xenoantigens. Pre-existing levels of anti-non-gal xenoantibodies have recently been shown to be low in humans and in non-human primates [11*–13], and the induced xenoantibody response following exposure to GalTKO pig endothelial cell xenoantigens is now known to be restricted [11*]. The nature of the target xenoantigen(s), however, still remains elusive and conventional immunosuppressive drugs do not effectively prevent xenoantibody-mediated rejection. This review summarizes the most recent advancements in the understanding of the non-gal xenoantibody response, including potential xenoantibody targets, xenoantibody structure and the role of anti-non-gal xenoantibodies in complement mediated activation of GalT-KO endothelial cells.

Activation and Pathology

GalT-KO pig xenograft rejection has been clearly associated with an increase in anti-non-Gal xenoantibodies and thrombotic microangiopathy [8*–10*]. The immunological reaction to the xenograft and the pathology associated with rejection when immunosuppressed baboons received porcine GalT-KO cardiac xenografts has recently been described in detail [8,9]. The grafts survived for up to 6 months with a median survival time of 78 days prior to undergoing rejection. At rejection, the grafts were characterized by thrombotic microangiopathy, myocardial ischemia, necrosis and focal interstitial hemorrhage. IgM and IgG xenoantibodies as well as complement were deposited onto the graft and the severity of rejection could be correlated with the degree of immunoglobulin (IgM and IgG) and complement (C3, C4d, and C5b-9) deposition. Cell death and endothelial cell activation was associated with antibody and complement deposition which contributed to platelet-fibrin thrombi formation. This finding suggests that thrombotic microangiopathy in GalT-KO xenografts is associated with anti-non-gal antibody-mediated rejection [8*,9]. The pathology of GalTKO heart xenograft rejection included acute humoral xenograft rejection, acute cellular xenograft rejection and chronic rejection [9]. Chronic humoral rejection was likely to be the dominant mechanism by which chronic xenograft vasculopathy developed in this group of animals.

Non-gal antibodies that bound to the graft were primarily directed against endothelial cells [9]. Xenoantigens as well as complement-activating and inhibitory elements that are expressed on the surface of endothelial cells before and after exposure to xenoantibodies are of major interest to investigators seeking to understand and prevent the process of rejection. Saethre et.al have shown that preformed anti-non-Gal xenoantibodies comprise approximately 10% of the total anti-porcine xenoantibodies present in human sera and that they can activate porcine endothelial cells as shown by upregulation of E-selectin expression [14].

Approaches designed to prevent the activation of porcine xenograft endothelium and the resulting intravascular coagulation that occurs *in vivo* include over-expression of complement regulatory factors and anticoagulant proteins on the GalT-KO background [15]. Studies done in small animal models transgenic for human alpha-1,2-fucosyltransferase (HT), hDAF, and/or CD59 support the concept that multiple transgenic modifications are beneficial [16–17*]. Mouse hearts modified to synergistically express more than one genetic modification were perfused *ex-vivo* with human plasma and the survival time and cardiac function were examined [17*]. Deposition of IgM, C3c, or C9 on the cardiac vascular endothelial cells of the HT, HT/CD59, and/or DAF transgenic mice with multiple genetic modifications was markedly decreased compared to controls. Survival time was longer and function was better in co-transgenic mice compared with single HT-positive transgenic mice [17*].

The role of multiple transgenic modifications in delaying the onset of antibody-mediated rejection is currently being addressed in larger animal models in a number of laboratories. Kelishadi et al. transplanted kidneys from GalT-KO pigs expressing hDAF into baboons [18]. Failure of these grafts was not associated with classic acute humoral xenograft rejection, but the xenograft recipients died secondary to the development of consumptive coagulopathy with platelet aggregation, thrombocytopenia, anemia, and bleeding. The grafts remained functional [18]. Investigation into the role of complement regulatory factors and anticoagulant proteins expressed on the endothelium of GalT-KO pigs suggests that complement regulation is not enough to prevent endothelial cell activation and intravascular coagulation. GalT-KO pigs transgenic for a combination of complement regulators and anticoagulants, such as hDAF and CD39, may be more resistant to the effects of acute humoral xenograft rejection and consumptive coagulopathy.

Pre-Formed Anti-Non-Gal Xenoantibodies

There is now evidence from our laboratory and others that GalT-KO xenoantibodies pre-exist in humans and non-human primates at significantly lower levels than anti-Gal xenoantibodies [11^{*}–14]. Since a threshold level of xenoreactive antibodies is necessary to initiate xenograft rejection [19], it is not surprising that hyperacute rejection of GalTKO xenografts has not been reported. Biopsy specimens of GalT-KO cardiac xenografts studied as soon as one hour post-transplant demonstrated clear evidence of IgM deposition on the grafts while IgG was only weakly positive or negative [9]. IgM anti-non-gal xenoantibodies pre-exist in non-human primates as well as in healthy humans. These IgM anti-non-gal xenoantibodies may be responsible for initiating xenograft rejection when they reach sufficient levels after placement of the xenograft. We found that in naive rhesus monkeys, pre-existing IgM xenoantibodies bound to an average of 8% of GalT-KO pig cells, considerably lower than the 78–99% binding of pre-existing IgM xenoantibodies to wild-type pig cells that express the gal carbohydrate [11^{*}]. Interestingly, cytotoxic natural anti-non-Gal xenoantibodies are either absent or present in very low levels in infant baboons and humans [12]. Anti-gal xenoantibodies, in contrast, develop during the first three months after birth and continue to rise thereafter [12]. Transplantation of GalTKO pig cells or organs may therefore be more successful if done shortly after birth when xenoantibodies that represent a major immunological barrier to graft survival are lacking and the recipient may be amenable to the induction of xenograft tolerance.

The Target

The xenoantigen target(s) that initiate anti-non-Gal xenoantibody responses have not been identified. Byrne et al. recently used Western blotting and proteomic analysis to identify a series of potential targets of induced anti-non-gal xenoantibodies in non-human primates [20]. IgG xenoantibodies eluted from GalTKO hearts at rejection or induced by transplantation with CD46 transgenic pig grafts bound to fibronectin, MG-160 (human Golgi apparatus protein 1), various cytoplasmic proteins, several heat shock proteins, annexin, and vimentin. Additional pig endothelial cell antigens were identified but remain to be characterized.

Several other laboratories have data to suggest that a carbohydrate xenoantigen may contribute to anti-non-gal xenoantibody-mediated rejection. The Hanganutziu-Deicher (HD) antigen, N-glycolylneuraminic acid (NeuGc), is a sialic acid present on animal cells but absent on human cells. Humans lack NeuGc due to a mutation in the gene encoding CMP-NeuAc hydroxylase, an enzyme required for its synthesis [21]. Saethre et.al reported that anti-HD antibodies are present in each of 80 human serum samples examined and that human serum induced activation of GalTKO endothelial cells could be correlated with anti-HD IgM and IgG antibody levels [14]. These findings suggest the HD xenoantigen may be a relevant carbohydrate target to which anti-non-Gal xenoantibodies pre-exist in humans.

Other potential xenoantigens include low levels of the gal carbohydrate. Mauro Sandrin's group recently showed that low levels of gal carbohydrate expression remain on galactosyltransferase deficient mouse cells and are encoded by an enzyme called iGb3 synthase [22]. It has been postulated that anti-non-gal xenoantibodies may bind to a residual gal carbohydrate expressed on GalTKO pig cells as a consequence of iGb3 synthase expression. We examined whether iGb3 synthase might be expressed in GalTKO pig cells using primers designed to identify conserved regions in the galactosyltransferase 2 gene (A3galt2 or iGb3 synthase) in humans, rats, and mice [11*]. Our data confirmed that expression of the gene encoding this transferase is detected in GalTKO mice but we did not detect expression of this gene in GalTKO pig cells, GalTKO fetal pig fibroblasts or in minipig kidney cells (MPK) from wild type pigs. These data suggest that iGb3 synthase is not expressed in GalTKO pig cells. Diswall et al. examined small intestine and pancreatic samples from GalT-KO pigs using thin-layer chromatography, and similarly found that Gal α 1,3Gal terminated glycolipids were eliminated by knockout of the porcine α 1,3 galactosyltransferase gene [23,24*]. Interestingly, xenoantibodies induced after immunization do show an increase in binding to purified Gal α 1-3Gal β 1-4GlcNAc, but not to Gal α 1-3Gal β 1-4Glc or to N-Acetyllactosamine [11*]. A substantial increase in binding of anti-non-gal xenoantibodies from post-transplant samples was demonstrated when GalTKO cells were transduced to express the gal carbohydrate [11*]. The data suggests that a substantial proportion of anti-non-gal xenoantibodies can bind to a xenoantigen that is similar in structure to, but distinct from, the gal carbohydrate, supporting the hypothesis that carbohydrate xenoantigens play a key role in the xenoantibody response to GalT-KO cells.

The Structure of Anti-Non-Gal Xenoantibodies

Analysis of xenoantibody structure will allow the molecular basis for xenoantibody/carbohydrate interactions to be understood. This is an important first step in the development of novel ways to prevent xenograft rejection mediated by anti-non-gal xenoantibodies. The structure and sequence of immunoglobulin genes encoding anti-non-gal xenoantibodies in the GalTKO model has recently been reported by our laboratory [11*]. These anti-non-gal xenoantibodies are restricted and are encoded by IGHV3-21. This germline progenitor is not expressed at high levels in the natural antibody repertoire and the most-closely related genes encode antibodies to glycolipids and/or human antibodies to the Gal/GalNAc lectin expressed on the intestinal parasite *Entamoeba histolytica* [25]. Anti-non-gal xenoantibodies differ in D-J usage when compared with antibodies that encode anti-glycolipid responses in humans [26]. IgV_H gene usage in the normal repertoire of primates is not restricted [27,28].

Anti-non-gal xenoantibodies have been studied in other models, for example, after transplantation of hDAF transgenic pig organs into monkeys treated with GAS914. This treatment regimen results in the expanded use of xenoantibodies encoded by IGHV3-11*03 [29]. The germline progenitors IVHG3-21 and IGHV3-11*03 are 91% similar to each other and differ from the IGHV3-11*01 allele encoding anti-gal xenoantibodies by less than 5% [30,31]. This finding suggests to us that the structure of the target xenoantigens is similar. The importance of differences in these antibodies at positions 31, 33, 50 and 58 which are key sites of xenoantibody/carbohydrate interaction identified by computer-simulated models remains to be determined [32], Figure 1. These sites may be important for binding site affinity and/or specificity as predicted by the molecular model. At the present time, our data supports the concept that "signature" antibody repertoires define xenoantibody responses, but the immune response to xenoantigens is initiated by germline progenitors that are evolutionarily and structurally related.

Similar findings were recently reported using a site mapping technique to compare antibody/carbohydrate interactions in the mouse [33,34*]. Agostino et.al. studied antibodies that bind

to carbohydrate xenoantigens and identified four key regions of relevance for antigen interaction [34*]. Specific amino acids within the CDR1, CDR2 and CDR3 of the heavy chain and select amino acids in the light chain rather than entire stretches of the CDR regions are important for xenoantibody/carbohydrate interactions [35]. The amino acids located at these key sites and the configuration of the carbohydrate may impact the manner in which antibodies that are closely-related in structure bind to different carbohydrates [36]. Binding affinity can be further optimized by charged amino acids in the CDR3 region of the immunoglobulin heavy chain gene [37].

Conclusion

Anti-non-gal xenoantibodies do not cause hyperacute rejection of GalT-KO xenografts but do contribute to the acute humoral xenograft rejection of these organs. The combination of transgenic modifications on the GalTKO background appears to be beneficial but insufficient for long-term xenograft survival. Recent advances in xenoantibody structure and the identification of a restriction in Ig gene usage will provide new opportunities to design novel reagents that may be combined with current approaches to minimize the xenoantibody response in a targeted manner.

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	CDR1						CDR2																
VH3-11*01	D	Y	Y	M	S		Y	I	S	S	S	G	S	T	I	Y	Y	A	D	S	V	K	G
Anti-gal	D	Y	Y	M	S		Y	I	S	S	S	G	S	T	I	Y	Y	A	D	S	V	K	G
VH3-11*03	D	Y	Y	M	S		Y	I	S	S	S	S	S	Y	T	N	Y	A	D	S	V	K	G
hDAF Non-gal	S	N	Y	M	N		Y	I	S	S	T	S	T	Y	I	N	Y	A	D	S	V	K	G
VH3-21*01	S	Y	S	M	N		S	I	S	S	S	S	S	Y	I	Y	Y	A	D	S	V	K	G
GalTKO Non-gal	S	Y	G	M	N		S	I	S	S	A	S	S	Y	I	Y	Y	A	D	S	V	K	G

Figure 1. Key contact sites within the CDR1 and CDR2 regions of anti-gal and anti-non-gal xenoantibodies predicted by computer-simulated modeling

The amino acid translation of the complementarity determining regions of the IgV_H genes encoding induced anti-gal and anti-non-gal IgM xenoantibodies in monkeys are aligned with their closest human germline progenitor. Anti-gal xenoantibodies were induced after transplantation of wild type pig grafts [31]. Anti-non-gal xenoantibodies (hDAF non-gal) were induced after transplantation of hDAF transgenic pig grafts in non-human primates treated with GAS914 [29]. The amino acid sequence of the CDR regions of anti-non-gal xenoantibodies induced after immunization of non-human primates with GalTKO endothelial cells are identified as GalTKO non-gal [11]. Amino acids located at contact sites predicted by computer-simulated models to be relevant for xenoantibody/carbohydrate interaction are identified in bold lettering [32].