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REVIEW

Progress in xenotransplantation following the introduction of gene-knockout technology

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Summary

The production of α1,3-galactosyltransferase gene-knockout (GT-KO) pigs has overcome the barrier of preformed anti-Galα1,3Gal (Gal) antibodies that has inhibited progress in pig-to-primate organ xenotransplantation for many years. Survival of GT-KO pig organs in nonhuman primates is currently limited by the development of a thrombotic microangiopathy that results in increasing ischemic injury of the transplanted organ over weeks or months. Potential causative factors include vascular endothelial activation from preformed anti-nonGal antibodies or cells of the innate immune system that recognize nonGal pig antigens directly, and coagulation dysregulation associated with molecular incompatibilities between pig and primate. Carefully isolated pancreatic islets from wild-type (genetically unmodified) adult pigs express minimal Gal epitopes, allowing survival sometimes for weeks or months after transplantation into nonhuman primates receiving immunosuppression directed only at T-cell function. However, there is a considerable immediate loss of islets, probably related to activation of coagulation and complement cascades. Further genetic manipulation of organ-source pigs is therefore required to overcome these problems. GT-KO pigs expressing a human complement-regulatory protein, e.g. decay-accelerating factor, and/or an 'anti-coagulant' gene, e.g. human tissue factor pathway inhibitor, might prevent the change in vascular endothelium from an anti-coagulant to a procoagulant phenotype, and protect the islets from early loss.

Introduction

Survival of pig organ xenografts in primates is initially limited by humoral rejection that can be either hyperacute (defined as occurring within 24 h) or delayed for days or even weeks (variously termed acute humoral xenograft rejection [AHXR], acute vascular rejection, or delayed xenograft rejection). Preformed and/or elicited cytotoxic antibodies against Galα1,3Gal (Gal) epitopes on the pig vascular endothelium are major causative components of the primate anti-pig immune response [1–3], and for many years proved to be a major barrier to achieving the prolonged survival of pig grafts in nonhuman primates [4].

The availability of α1,3-galactosyltransferase gene-knockout (GT-KO) pigs, that do not express Gal antigens [5], produced through nuclear transfer/embryo transfer techniques [6–8], has allowed longer survival with less immune modulation of the nonhuman primate recipient [9–11] (Fig. 1). However, the absence of Gal expression, although clearly an essential advance in addressing hyperacute rejection, has not resulted in a quantum leap in survival. This is not altogether unexpected as the GT-KO pig was expected to primarily target hyperacute rejection mediated by high titers of preformed anti-Gal antibody. The true value of the GT-KO pig has been to eliminate this barrier, and thus allow more subtle immune barriers to be studied.

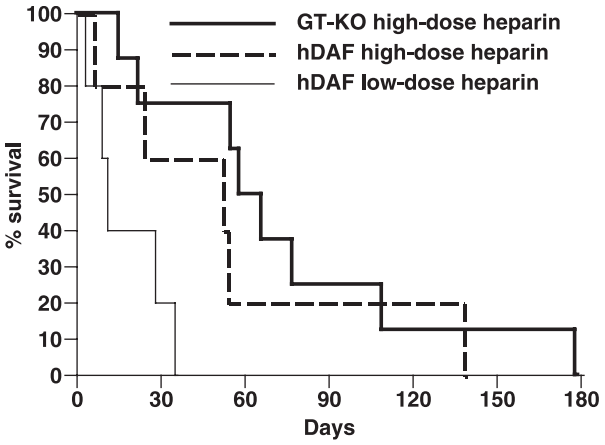

Figure 1 Survival (in baboons) of heterotopic hearts from α1,3-galactosyltransferase gene-knockout (GT-KO) pigs [9,11] compared with that of two groups of hearts from pigs transgenic for human decay-accelerating factor (hDAF) [23]. Recipient baboons of GT-KO hearts ($n = 8$) and of the hDAF hearts that survived longer ($n = 5$) received high-dose heparin anti-coagulation, whereas the remaining baboons with hDAF hearts ($n = 5$) received lower doses of heparin. A soluble Gal-conjugate (to 'neutralize' anti-Gal antibodies) was administered to all baboons that received hDAF hearts, but not to those that received GT-KO hearts. There were no other significant differences in therapy between the three groups. (Reproduced from Cooper DKC. Xenotransplantations: the road ahead. *Curr Opin Organ Transplant* 2006; 11: 151 with kind permission of Lippincott Williams & Wilkins.)

In this brief review, we outline recent progress in research utilizing GT-KO pigs.

Humoral rejection associated with preformed anti-Gal antibodies

The initial immunologic obstacle to pig-to-primate organ transplantation is hyperacute rejection, caused by T cell-independent 'natural' preformed antibodies directed to Gal epitopes on the pig vascular endothelium [1–3]. The binding of these antibodies to Gal activates the complement cascade, resulting in congestion and thrombosis in the small vessels of the graft, disruption of the vascular endothelium, and massive interstitial hemorrhage and edema, leading to irreversible graft damage [12–14].

If hyperacute rejection is avoided, either by manipulation of the primate recipient, e.g. by extracorporeal antibody immunoadsorption [15,16], or by genetic modification of the organ-source pig, e.g. by the transgenic expression of a human complement-regulatory protein, such as decay-accelerating factor (hDAF) [17], then these preformed anti-Gal antibodies [18], or possibly innate immunoresponsive cells, or T cell-dependent elicited anti-pig antibodies [19] can initiate a slower response that also results in graft failure.

The introduction of costimulatory blockade allowed prevention of a T cell-dependent elicited antibody response without the morbidity associated with intensive conventional pharmacologic immunosuppressive therapy. Buhler *et al.* [20] administered an anti-CD154 monoclonal antibody (in combination with mycophenolate mofetil and corticosteroids) in a pig hematopoietic progenitor cell-to-baboon transplantation model, and demonstrated the efficacy of this agent. Confirmation was demonstrated in a solid organ transplant model, but survival was again limited by the continuing effect of preformed antibody [18,21,22].

With effective immunosuppressive therapy that prevents an elicited antibody response, relatively prolonged survival of organs from hDAF transgenic pigs can be obtained, but graft failure still results from what appears to be a humoral mechanism, although the effect of innate immune cells cannot be excluded. The continuous or intermittent administration of a synthetic Gal conjugate, that adsorbs and depletes anti-Gal antibody in the host, prolongs graft survival further, but would be unlikely to be clinically applicable [23–25].

The problems presented by the expression of Gal in the pig have been overcome by the production of GT-KO pigs, allowing survival of five pig heart grafts for periods of 2–6 months (56–179 days) in baboons [9,11] and shorter survival of kidneys (<83 days) [10]. However, graft failure eventually occurs, not with the typical features of humoral rejection, but from the development of a thrombotic microangiopathy (TM) [26] (Fig. 2). If the elicited response is *not* prevented, AHXR occurs within 16 days from the development of antibodies to nonGal antigens [19].

Following life-supporting kidney xenotransplantation, the serum creatinine provides a reliable indicator of graft function. However, apart from direct palpation, it initially proved difficult to determine whether a heterotopic heart was undergoing early AHXR or TM. Careful monitoring of serum troponin, lactate dehydrogenase, and other parameters indicate that these pathologies are developing [21,27].

Since the availability of GT-KO pig organs, attention has largely been concentrated on three key points:

1 How prevalent and how cytotoxic are preformed anti-nonGal antibodies?

2 Are they directed to carbohydrate antigens, and, if so, what is the structure of these antigens?

3 What other factors are important in the development of the TM that occurs in organs even in the absence of both Gal expression on the donor organ and an elicited antibody response in the host?

These topics will be briefly discussed.

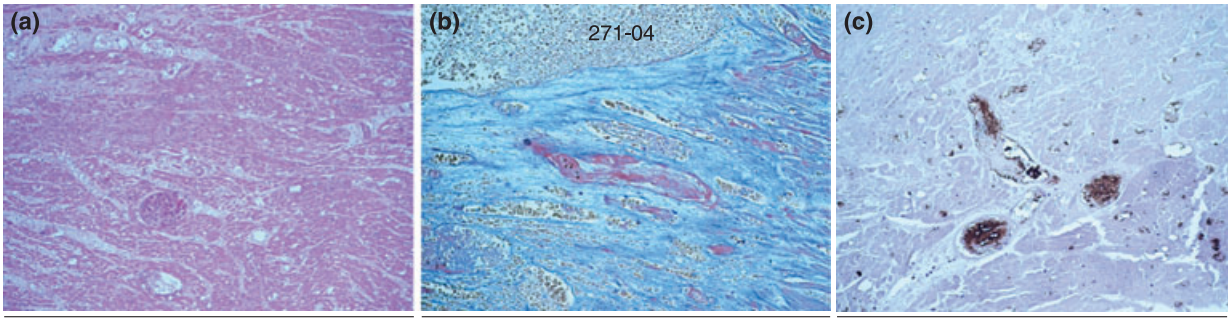

Figure 2 Histopathological sections of (a) a α1,3-galactosyltransferase gene-knockout pig heart showing advanced thrombotic microangiopathy and ischemic injury several weeks after transplantation into an immunosuppressed baboon (hematoxylin and eosin, ×100), demonstrating (b) extensive fibrin deposition (trichrome) and (c) massive platelet accumulation (CD42P) in the thrombosed vessels. (Histopathology by courtesy of Dr Bertha Garcia, unpublished work.)

Incidence and cytotoxicity of anti-nonGal antibodies

Sera from naïve (not intentionally sensitized to pig cells or tissue) humans, baboons, and cynomolgus monkeys have been tested by flow cytometry for binding of IgM and IgG to peripheral blood mononuclear cells (PBMC) from wild-type (WT) and GT-KO pigs. The sera were also tested for complement-dependent cytotoxicity to WT and GT-KO PBMC.

Anti-WT IgM and IgG were found in 100% of the primates tested, but IgM and IgG that bound to GT-KO pig PBMC were identified in only approximately 50% of primate sera, with monkeys demonstrating a rather higher incidence of binding than humans or baboons [28–30] (Fig. 3). Similarly, whereas virtually 100% of sera were cytotoxic to WT PBMC, only approximately 50% were cytotoxic to GT-KO PBMC, and the level of cytotoxicity was significantly less (Fig. 4). These studies indicated that, although the incidence and cytotoxicity of antibodies in primate sera to GT-KO pig PBMC are significantly less than to WT PBMC, approximately half of the primates tested had preformed cytotoxic antibodies to GT-KO PBMC. Anti-nonGal antibodies therefore represent another barrier to successful xenotransplantation.

Following the transplantation of a pig organ into a primate, the target for anti-nonGal antibodies will be vascular endothelial cells, rather than PBMC. When compared with PBMC, these might have different, or possibly more biologically relevant, nonGal epitopes, although there is no definite evidence for this latter assumption. We are currently investigating whether there are differences between anti-pig antibody binding and cytotoxicity to PBMC and vascular endothelial cells; preliminary results suggest that there is actually less binding to, and lysis of, vascular endothelial cells than to PBMC (H. Hara *et al.*, unpublished data).

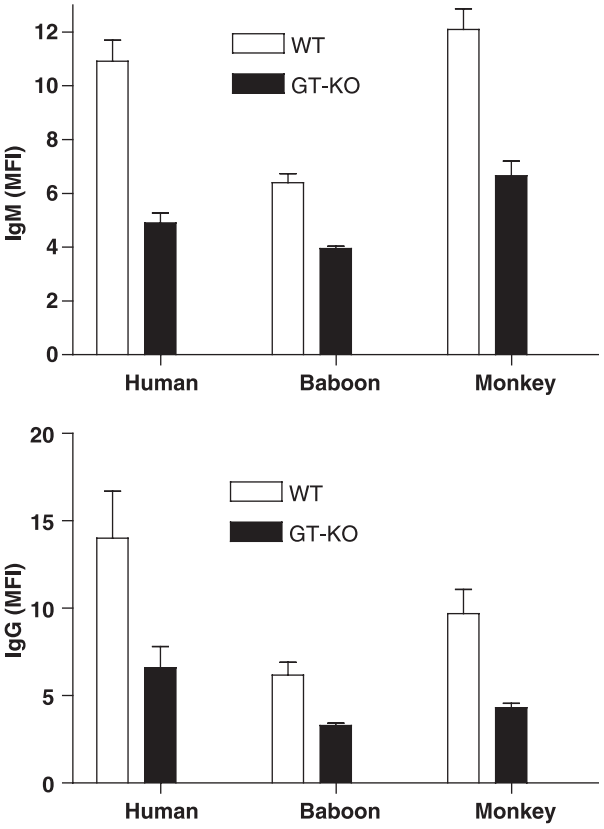

Figure 3 Comparison of binding of human, baboon, and cynomolgus monkey preformed xenoreactive antibodies to wild-type (WT) and α1,3-galactosyltransferase gene-knockout (GT-KO) pig peripheral blood mononuclear cells (PBMC). Mean reactivities of human (n = 21), baboon (n = 56), and monkey (n = 21) sera against WT or GT-KO PBMC are shown, IgM (top) and IgG (bottom). (Reproduced from Road PPM, Hara H, Busch J et al. Incidence and cytotoxicity of antibodies in cynomolgus monkeys directed to nonGal antigens, and their relevance for experimental models. *Transplant Int* 2006; 19: 158 with kind permission of Blackwell publishing Ltd.)

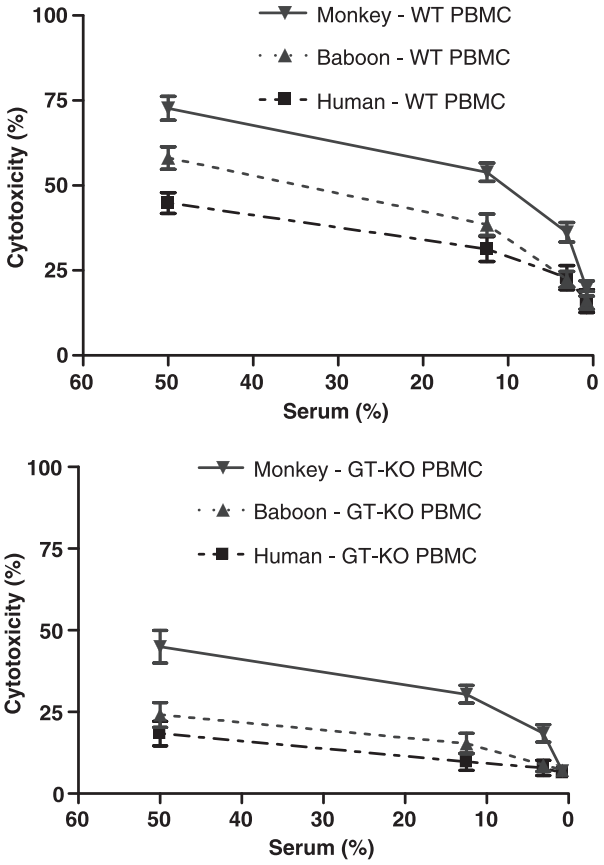

Figure 4 Comparison of mean cytotoxicity of human ($n = 21$), baboon ($n = 39$), and monkey ($n = 19$) serum samples against peripheral blood mononuclear cells from either wild-type (top) or α1,3-galactosyltransferase gene-knockout (bottom) pigs. (Reproduced from Road PPM, Hara H, Busch J et al. Incidence and cytotoxicity of antibodies in cynomolgus monkeys directed to nonGal antigens, and their relevance for experimental models. *Transplant Int* 2006; 19: 158 with kind permission of Blackwell publishing Ltd.)

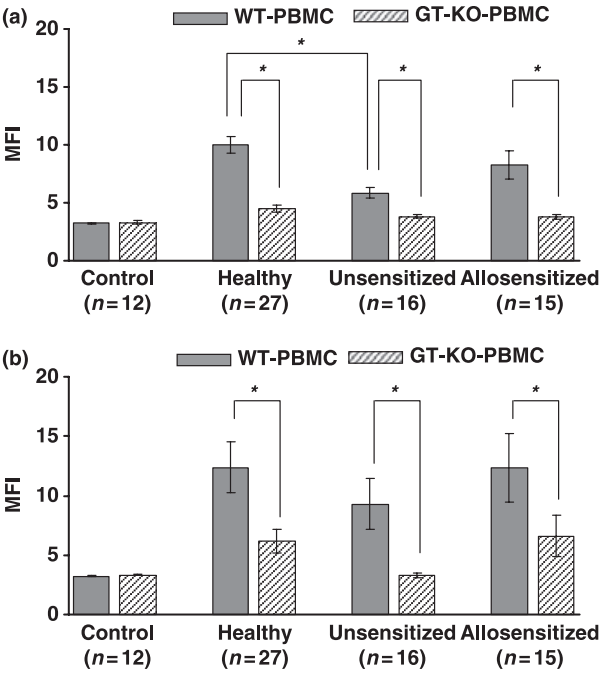

Figure 5 Mean reactivity (MFI levels ± SEM) of sera from 27 healthy humans, 16 unsensitized, and 15 allosensitized patients against wild-type (WT) (gray bar) or α1,3-galactosyltransferase gene-knockout (GT-KO) (shaded bar) peripheral blood mononuclear cells (PBMC) – IgM (a) and IgG (b). Statistically higher ($P < 0.05$) reactivity of both IgM and IgG against WT PBMC versus GT-KO PBMC was observed, irrespective of the type of serum tested (*$P < 0.05$). No increased binding of immunoglobulin from allosensitized sera was observed. The observation that the MFI of IgM binding of unsensitized sera was significantly lower to WT PBMC than that of healthy sera cannot be readily explained. (Reproduced from Hara H, Ezzelarab M, Road PPM et al. Allosensitized humans are at no greater risk of humoral rejection of GT-KO pig organs than other humans. *Xenotransplantation* 2006; 13: 357 with kind permission of Blackwell Publishing Ltd.)

Previous *in vitro* studies involving WT pig cells, and either immunoadsorption of primate sera or treatment of the cells with a galactosidase to remove Gal epitopes, have suggested that antibodies to nonGal antigens represent only 10–30% of anti-pig IgM and 20–50% of IgG antibodies in naïve sera (reviewed in [31]). The data observed using GT-KO PBMC would suggest that anti-nonGal antibodies may represent a greater proportion of anti-pig antibodies in some subjects. This may be associated with greater expression of nonGal antigens in GT-KO pigs. It has been demonstrated that there is some rearrangement of the carbohydrate pattern of the vascular endothelium in mice after GT-KO [32], and neoantigens might be exposed, to which some preformed antibodies may be directed.

Previously, some reports had indicated that sera from allosensitized subjects (resulting from prior blood transfusions, pregnancy, or organ allotransplantation) might be more cytotoxic to pig cells than sera from unsensitized subjects, although other reports did not confirm this observation (reviewed in [33]). It was indicated by some that anti-HLA antibodies might cross-react with pig antigens and preclude an allosensitized patient from undergoing pig organ transplantation because of the risk of hyperacute rejection. A report by Popma *et al.* [34] suggests that allosensitized patients may have an increased cellular response to pig antigens, although this can be suppressed *in vitro* by cyclosporine [35].

An important finding from recent studies by Hara *et al.* [29] and Wong *et al.* [36] is the documentation that sera from allosensitized subjects (panel reactive antibodies >70%) do not show a higher level of binding of preformed anti-pig antibodies (IgM or IgG) to either WT or GT-KO pig PBMC than do sera from healthy subjects

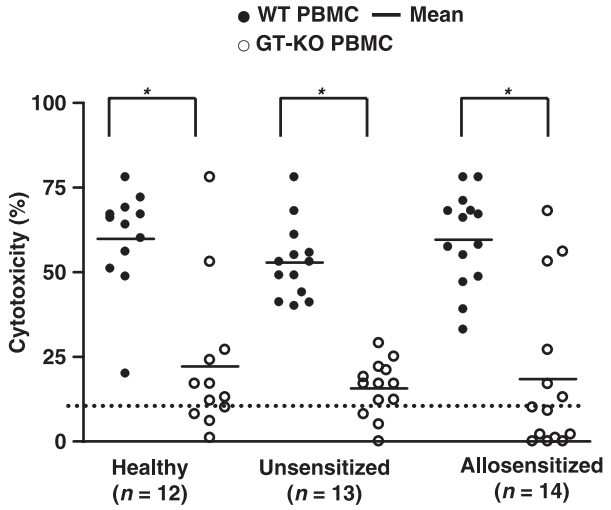

Figure 6 Ability of healthy ($n = 12$), unsensitized ($n = 13$), and allosensitized ($n = 14$) sera (at 25% dilution) to cause lysis of peripheral blood mononuclear cells (PBMC) from wild-type (WT) (●) or α1,3-galactosyltransferase gene-knockout (GT-KO) (○) pigs; mean complement-dependent cytotoxicity is indicated by horizontal lines. Significantly higher lysis of WT PBMC was observed ($P < 0.05$). The horizontal dotted line indicates 10% cytotoxicity, below which lysis of PBMC is considered of doubtful relevance. There was no difference in cytotoxicity to either WT or GT-KO PBMC between the three groups of sera. (A serum dilution of 25% was selected as the volumes of allosensitized sera were limited). (Reproduced Hara H, Ezzelarab M, Road PPM et al. Allosensitized humans are at no greater risk of humoral rejection of GT-KO pig organs than other humans. *Xenotransplantation* 2006; 13: 357 with kind permission of Blackwell Publishing Ltd.)

(Fig. 5), nor increased cytotoxicity (Fig. 6). If these findings are confirmed, namely that humans previously exposed and sensitized to alloantigens are at no greater risk for rejecting a GT-KO pig xenograft by an antibody-mediated mechanism, this will have important clinical implications. Highly-allosensitized patients frequently have difficulty in obtaining a human donor organ against which they do not have anti-HLA antibodies. This group of patients may therefore be among the first to be considered for xenotransplantation.

Furthermore, early *in vitro* and *in vivo* studies demonstrated that sera from baboons that had become sensitized to pig antigens, following the transplantation of a pig organ, did not develop sensitization to baboon antigens (allosensitization) [37]. More recent studies have confirmed that, despite the development of high levels of elicited anti-pig antibodies in baboons exposed to pig antigens, the baboons did not develop antibodies that cross-reacted with allo (baboon) antigens [38]. A patient may therefore undergo an initial pig organ transplant, possibly as a 'bridging' procedure, without the risk of this precluding him or her from receiving a subsequent allograft.

Table 1. Known carbohydrate antigens against which humans can have naturally occurring antibodies*.

1. Blood type A: GalNAcα1,3(Fucα1,2)Galß1,4GlcNAcß-R†
2. Blood type B: Galα1,3(Fucα1,2)Galß1,4GlcNAcß-R
3. Hanganutziu–Deicher: e.g. NeuGcα2,3Galβ1,4Glcβ1-R
4. Thomsen-Friedenreich (T or TF): Galß1,3GalNAcα1-R
5. Tn (TF precursor): GalNAcα-R
6. Sialosyl-Tn: NeuAcα2,6GalNAcα1-R
7. Forssman: e.g. GalNAcα1,3GalNAcß1,3Galα1,4Galß1,4Glcß1-R
8. αRhamnose-containing oligosaccharides
9. Sulfatide I: SO_4-3Gal-R
10. P antigens
11. I or i antigens‡

*Modified from [41] from data collected by several investigators.
†R are glycolipid or glycoprotein carrier molecules anchored in the cell membrane.
‡The core structures of the ABH antigen system, which are fucosylated by H transferase to generate H-substance.

Identification of pig antigenic targets for primate preformed anti-nonGal antibodies and innate immune cells

Continued expression of Gal in the GT-KO pigs, e.g. as a result of iGb3 synthetase activity, has been suggested [39,40], but we and others have not documented evidence to support this hypothesis [9,11,19]. After exposure to GT-KO pig hearts or kidneys, even when sensitization to nonGal antigens has occurred, there has been no increase in the level of anti-Gal antibodies.

Acute humoral xenograft rejection associated with the presence of preformed or elicited antibodies to pig nonGal epitopes could be successfully addressed by further modification of the pig to reduce the expression of nonGal antigens. The identification of nonGal specificities would therefore be an important step forward. However, the nature of the nonGal antigens expressed on GT-KO pig vascular endothelium or PBMC is currently uncertain, although several carbohydrates are candidates (Table 1) [41,42].

N-glycolylneuraminic acid epitopes, so-called Hanganutziu–Deicher antigens, are widely expressed on the endothelial cells of all mammals except humans, and are considered to be potential porcine targets for preformed and elicited anti-nonGal antibodies in humans [43–45], but not in baboons, which express these epitopes [46]. The AHXR and TM seen in GT-KO organ grafts transplanted into baboons, therefore, cannot be associated with the development of anti-NeuGc antibodies. It is very likely, however, that these antibodies will prove important

when clinical organ xenotransplantation becomes a reality.

If there are multiple nonGal epitopes against which primates have preformed antibodies, then gene-knockout may not prove to be a feasible solution. However, the lower level of endothelial cell activation and cytotoxicity associated with anti-nonGal antibodies, compared with anti-pig antibodies (i.e. anti-Gal plus anti-nonGal antibodies), may allow some protection to be achieved by the presence of one or more human complement-regulatory protein. Although transgenesis for hDAF or other complement-regulatory proteins has had only a relatively limited protective effect against anti-Gal antibodies, this approach may be more successful against the weaker anti-nonGal antibodies, when hDAF is expressed in combination with GT-KO.

Several authors have drawn attention to the potential role of cells of the innate immune system in AHXR, some of which can recognize pig antigens directly, in the absence of anti-pig antibodies [47,48]. The specific antigen targets for such cells require investigation and identification (discussed in [49]).

Identification of pig antigens that are targets for natural antibodies, natural killer (NK) cells, and/or macrophages, might allow further genetic modification, either to knock-out those antigens, or to mask them by the introduction of competitive genes, e.g. α1,2-fucosyltransferase (H transferase) [3,50]. NK cell recognition of pig antigens may be prevented by the introduction of a gene for a human leukocyte antigen, e.g. HLA-E or HLA-G [51–54], or it may be that the receptors for NK cells and/or macrophages may need to be knocked out or blocked, or the gene for a specific inhibitor recognized by these cells introduced into the pig [55,56].

Coagulation dysregulation as a potential causative factor in the development of thrombotic microangiopathy

It has long been known that coagulation dysregulation plays a role on the failure of pig grafts in primates (57,58; reviewed in [59,60]). Exposure of porcine vascular endothelial cells to primate anti-pig antibodies, complement, platelets, immune cells, and cytokines results in loss of natural anticoagulant pathways and a change to a procoagulant phenotype. Incompatibilities in the coagulation system between pigs and humans that contribute to this dysregulation have been identified (reviewed in [59,60]) (Fig. 6). In particular, the generation of thrombin, in addition to generating fibrin from fibrinogen, activates platelets and exerts direct effects on the vascular endothelium, serving as a potent inflammatory mediator.

Genetic modification of the organ-source pig may overcome this problem. This approach is likely to be more successful and to have fewer potential complications than systemic drug therapy to the recipient of the organ. Encouraging data have been reported in rodent models of xenotransplantation. For example, Chen *et al.* [61] reported that, whereas control mouse hearts in immunosuppressed rats fail from AHXR after 6 days, hearts from transgenic mice expressing membrane-tethered fusion proteins based on the human tissue factor pathway inhibitor (hTFPI) or hirudin survived indefinitely (>100 days). Although immunofluorescence indicated that antibody and complement were deposited on the vascular endothelium, no features of rejection were identified histologically. This surprising result suggests that coagulation dysregulation may be playing a primary role in graft failure, and not the secondary role hitherto considered likely.

Similar results have been reported by Levy's group, which has explored the role of fibrinogen-like protein 2 [62]. Dwyer *et al.* [63] have demonstrated that transgenic expression of human CD39, a major vascular nucleotidase, rendered the hearts of mice substantially protected from thrombosis, providing further evidence of the important role played by coagulation dysregulation in xenotransplantation. Cross-species incompatibilities that cause dysregulated intravascular coagulation after antibody binding may therefore be critical targets for manipulation in strategies to prevent AHXR and/or TM.

Specific targeting of coagulation pathways alone may therefore inhibit AHXR and/or TM without the need for complete antibody or complement depletion or treatment aimed at immune cells. GT-KO pigs that express one or more 'anti-coagulant' or 'anti-thrombotic' gene (e.g. hTFPI, hirudin, h-thrombomodulin, and CD39) that would maintain the endothelium in an anti-coagulant state and/or inhibit activation of coagulation pathways are therefore a major aim of current research efforts.

Pig islet xenotransplantation

Early studies indicated that adult pig islets expressed minimal or no Gal antigens [64,65], recently confirmed by Dor *et al.* [66]. The careful isolation of adult WT pig islets results in islets that do not express this antigen, although Gal may be present on contaminating cells, such as pancreatic ductule and vascular endothelial cells. Pure islets therefore do not induce an elicited anti-Gal antibody response. This has enabled adult WT pig islet transplantation to be carried out in nonhuman primates with a moderate degree of success when the primate is immunosuppressed only with agents that suppress the T-cell response [67]. Initial experience with the use of *adult* GT-KO pig islets indicates that they do not appear to

provide a major advantage, although they are in no way detrimental in this respect [68]. However, there are increasing data to indicate that adult WT islets express other carbohydrate epitopes that are targets for human natural antibodies [69–71].

Genetic engineering of the pig to possibly knockout these targets may be required, although there are other indications for the use of genetically engineered pigs as sources of islets for clinical transplantation. A number of studies of islet xenotransplantation have suggested that pig islets are not rejected hyperacutely [72,73]. There is, however, a very considerable initial loss of islets, within hours, after their intraportal transplantation into nonhuman primates. This phenomenon, termed the instant blood-mediated inflammatory response (IBMIR), consists predominantly of an acute coagulation response and complement activation [68,74,75]. The administration of a soluble complement inhibitor reduced complement deposition and islet cell lysis after the intraportal transplantation of pig islets in monkeys [76–78]. Furthermore, *in vitro* experiments demonstrated that the expression of a complement regulator, such as membrane cofactor protein, on islet cells protected the cells from complement-mediated lysis [79]. Based on these and other studies, there is a strong rationale for the production of pigs that are transgenic for one of more complement inhibitor genes.

There is also evidence that IBMIR is associated with the over-expression of tissue factor in the islets after transplantation [75,80]. IBMIR may therefore be analogous to the activation of the proinflammatory coagulation pathway observed when porcine vascular endothelial cells are exposed to primate blood. Therefore, transgenic pigs that express hTFPI on their islets may be to some extent protected from this inflammatory response.

There are some advantages in the transplantation of fetal or neonatal pig islets, as these appear to contain primitive cells that can lead to proliferation of insulin-producing beta cells after transplantation [81,82]. Fetal and neonatal pig islets express relatively high levels of Gal and, therefore, the use of GT-KO pigs as sources of these islets is highly likely to prove advantageous.

Can tolerance to a xenograft be achieved?

The induction of T-cell tolerance may be possible if the hurdles provided by the innate immune system and coagulation dysregulation can be overcome.

In allotransplantation, tolerance to an organ can sometimes be induced by the prior or concomitant transplantation of donor-specific hematopoietic progenitor cells [83,84]; chimerism persists either indefinitely or at least for several weeks or months. The transplantation of large numbers of pig hematopoietic progenitor cells, whether WT or GT-KO, into nonhuman primates is followed by their rapid loss (within minutes) from the host primate's circulation, almost certainly a result of macrophage activity (reviewed in [85]). This has prevented the induction of tolerance, even when hDAF transgenic or GT-KO pig cells have been transplanted.

The mechanism underlying the recognition and destruction of human erythrocytes by pig macrophages involves a carbohydrate-lectin interaction [86]. It would therefore seem likely that a similar mechanism underlies the recognition and removal of porcine cells by primate host macrophages. Indeed, recent data demonstrate that human Kupffer cells recognize and phagocytose porcine erythrocytes [87]. It will be necessary to characterize the lectin-carbohydrate molecules involved in this recognition event.

This problem is also of relevance to pig liver xenotransplantation. The *ex vivo* perfusion of a pig liver with human blood indicates that pig macrophages phagocytose human erythrocytes continuously [88]. This phagocytosis is unrelated to antibody binding and complement activation, but is due to direct recognition of erythrocytes by pig Kupffer cells. It has been documented that a pig liver removes approximately one unit of human erythrocytes from the circulation every 24 h. A solution to this problem will therefore be required if extracorporeal pig liver support or pig liver transplantation is to become an effective clinical therapy.

Conclusions

In summary, the production of GT-KO pigs has been an essential and major step towards clinical xenotransplantation, but other barriers remain. The elimination of the Gal barrier by GT-KO pigs provides the opportunity for more thorough evaluation of the secondary immune barriers. These pigs also provide a genetic building block on which further genetic modifications can be introduced, leading towards pigs suitable for clinical trials. In view of the predominance of the TM seen in baboons receiving GT-KO pig hearts, efforts are currently directed largely towards the production of GT-KO pigs that express a human 'anti-coagulant' gene. Control of complement activation by a gene for a complement-regulatory protein is also likely to prove beneficial. Similarly, the risk of activation of the coagulation and complement cascades after pig islet transplantation has directed attention to the prevention of IBMIR by the introduction into the pig islets of anti-coagulant and complement-regulatory genes.

The road towards the introduction of clinical xenotransplantation is proving long and arduous, but progress is steadily being made. Graft survival is now measured in

weeks or months rather than minutes or hours, as it was 20 years ago. But those involved in this field of research have learned the truth and wisdom of the words of dialysis pioneer, Willem Kolff, who is quoted as saying:

> ''Even without hope, you should undertake. Even without success, you should persevere''.

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