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Antibody-mediated Xenograft Injury: Mechanisms and Protective Strategies

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Abstract

The use of porcine organs for clinical transplantation is a promising potential solution to the shortage of human organs. Preformed anti-pig antibody is the primary cause of hyperacute rejection, while elicited antibody can contribute to subsequent “delayed” xenograft rejection. This article will review recent progress to overcome antibody mediated xenograft rejection, through modification of the host immunity and use of genetically engineered pig organs.

Introduction

Organ transplantation is often the best available treatment for irreversible organ failure. However, the shortage of human organs severely constrains their use to prolong and improve the lives of those who might benefit. Porcine organs could solve this problem if challenging immunological obstacles can be overcome [1]. This article will review our current understanding of the mechanisms governing antibody-mediated injury to a porcine organ xenograft in primates.

Anti-pig antibody and hyperacute rejection

Pig organs transplanted into humans or non-human primates rapidly undergo “hyperacute rejection” (HAR, defined as graft failure within hours after transplant) or “early graft failure” (EGF, which we define as non-technical graft loss occurring within 3 days after transplant) [1-4]. Multiple lines of evidence show that in these clinically relevant species combinations HAR and EGF are caused primarily by binding of preformed anti-pig IgM and complement-fixing IgG antibodies, which then trigger activation of the classical complement activation pathway, causing porcine endothelial cell (PEC) injury. PEC injury results in endothelial dysfunction, retraction, and sloughing, local platelet adhesion and activation, coagulation cascade amplification, and loss of intravascular thromboregulatory function. Each of these inter-related pathways contributes to a prothrombotic environment that usually results in intravascular thrombosis and organ necrosis [4-9]. Extensive experimental observations and limited clinical experience confirm that depletion of anti-pig antibodies, complement inhibition, or some combination of these approaches delays or prevents HAR and EGF [10-17].

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Preventing interaction of preformed anti-pig antibody with the graft is therefore an appealing approach to protect a pig organ xenograft from HAR and EGF. Over the past three decades various approaches have been used to accomplish this goal. Antibody levels can be reduced by conventional plasmapheresis, which isolates and discards the plasma fraction containing all the serum proteins, and replaces them with albumin or various colloid and electrolyte solutions. Conventional plasmapheresis perturbs the coagulation and complement cascades, since multiple pro- and anti-coagulant and complement pathway proteins are discarded along with the antibody fractions; and if performed repeatedly has adverse nutritional effects.

Immunoabsorption columns provide a more selective approach to remove specific antibody fractions from the separated plasma. Immunoapheresis using a Protein A column removes primarily IgG antibodies [10], while an anti-mu column will selectively remove IgM [12]. Protein A is more selective and thus safer than pheresis and has been widely used. However, targeting entire classes of IgG isotypes removes immunoglobulins specific for various clinically important non-pig antigens, making the graft recipient more prone to infection, particularly in the context of pharmacologic immunosuppression. Further, antibodies of other isotype classes (particularly complement-fixing IgM, but also IgA and IgE) are not depleted, and could contribute to tissue injury by various well-described mechanisms.

Use of a “sponge” organ has been used to adsorb anti-pig antibodies from plasma or whole blood, including any antibodies that might be organ-specific (binding to antigens only expressed in the graft). Perfusion of pig organs (kidney, liver, lung, spleen) removes a majority of anti-pig natural antibodies [13,18-22]; liver and lung perfusion are associated with relatively more efficient antibody depletion than other organs, perhaps consequent to the relatively large surface area of endothelium available to adsorb anti-pig antibodies. Transient loss of blood volume from the recipient during the pheresis/ adsorption procedure can be attenuated by various technical strategies. Additional hemodynamic perturbations usually respond to transient administration of volume and vasoactive agents, but may increase in severity in direct relation to blood flow through the organ. Collateral depletion of neutrophils and platelets can be minimized by blood separation before adsorption, followed by perfusion of the organ with recipient plasma instead of whole blood.

However no mechanical approach to deplete anti-pig antibodies is without practical limitations and procedural drawbacks. Complete depletion of antibodies *in vivo* is difficult to achieve, and furthermore often results in activation of the complement and coagulation cascades. This is a logical consequence, particularly of *ex vivo* organ perfusion, since the perfused organ is “rejected” unless special precautions are taken. Hypothermia, complement inhibition, or calcium chelation, for example, can attenuate the rejection response, and the procedure can be organized to limit the effect to the *ex vivo* perfusion circuit.

To specifically address the anti-Gal antibodies for Gal-expressing organs, decoy carbohydrate polymers (GAS914; NEX1215) may be administered to overload the recipient’s antigen binding capacity to Gal antigens [4,14,23]. In sufficient concentration these reagents usually prevent the initial graft-specific insult and attenuate subsequent binding of Gal antigens and complement to the graft. Alternatively columns expressing the Gal antigens can be used to adsorb anti-Gal antibodies [4,21,24,25]. More recently, genetic engineering (gene “knock out” technology) has been used to remove the Gal α 1-3Gal epitope that is the target of the preformed antibody attack [26,27]. Since the Gal α 1-3Gal carbohydrate antigen recognized by over 80% of anti-pig antibody found in man is the product of a galactosyl transferase gene not found in humans, organs from genetically modified pigs in which the galactosyl transferase gene is “knocked-out” (GalT KO pig) have been developed. As predicted, most investigators find that endothelium and parenchymal cells from GalT KO animals lack expression of the Gal α 1-3Gal antigen, although this has been challenged. [28] Except where high non-Gal antibody titers

may exist (such as in individuals previously exposed to pig organs or tissues, for example), binding of antibodies and complement cascade activation are greatly diminished, usually limiting endothelial activation sufficiently to protect the organ from HAR and EGF [29-31].

If dominant pig carbohydrate and glycoprotein epitopes *other than* α Gal are shown to be physiologically pivotal in the pig-to-human combination, columns could be designed to selectively remove the relevant anti-pig activity from human- and baboon serum. Alternatively intravenous infusion of specifically designed carbohydrate complexes, peptides mimicking the three-dimensional structure of the antigen [32] or even induction of anti-idiotypic antibodies [33] could be explored to determine the relative importance of that antigen and block binding to the graft. Additional gene knockouts could also be envisioned to manage one or more additional carbohydrate or glycoprotein antigens as they are identified in the course of experiments using GalTKO organs.

Role of antibody in Delayed Xenograft Rejection

When HAR/EGF is avoided, “delayed” xenograft rejection (DXR, also referred to in the literature as Acute Humoral Xenograft Rejection, or AHXR) is the most important obstacle to clinical application of pig organ xenografts. In the baboon recipient of human complement regulatory protein (hCRP) -expressing or GalTKO pig hearts or kidneys, DXR is usually associated with thrombotic microangiopathy (TM) [1,23,34-36]. In its early stages TM is characterized by endothelial activation and patchy infarction associated with thrombosis of small and medium-size veins and arteries [5,7,37]. Infiltration by macrophages and neutrophils, but not lymphocytes, is usually prominent interstitially within graft parenchyma and may also involve vessel walls. If allowed to progress, TM is manifest as diffuse interstitial hemorrhage and widespread thrombosis in graft vessels, and systemic consumptive coagulopathy, with thrombocytopenia, elevated LDH, and petechiae [38]. This constellation of findings resembling idiopathic thrombotic thrombocytopenic purpura, suggesting a similar mechanism, perhaps involving antibody binding procoagulant porcine antigens (such as tissue factor and von Willebrand’s factor) complexed to recipient platelets.

DXR is widely presumed to be caused primarily by immunologic mechanisms, including antibody. Anti-pig antibody is typically detectable while the hCRP or GalTKO graft is in place when less aggressive conventional immunosuppression [36] or antiCD154 monotherapy strategies were tested [35]. When conventional drug treatment is intensified [23], or with high-dose antiCD154, ATG induction, and MMF, anti-pig antibody is rarely detected while the graft is retained [23,30]. However antibody binding and complement activation are readily detectable within explanted hearts from these animals, and when recipients survive graft explant, anti-pig antibody often is detectable [35-38]. We infer that onset of DXR is closely associated with and probably caused at least in part by anti-pig antibody. The majority of antibody is directed against Gal α 1,3Gal when the graft expresses it; or against non-Gal antigens for GalTKO grafts. The extent to which this elicited antibody response is regulated by T-cell help (T-cell dependent versus T-cell-independent) has important implications for selection of an approach to reliably prevent it [39].

For GalTKO organs two general categories of antibodies are relevant to the etiology of delayed antibody-mediated graft injury. Induced primary antibody to novel non-Gal antigens such as pig MHC are readily demonstrated in a variety of species combinations, demonstrating that MHC is particularly immunogenic across species [40-44]. Preformed “secondary” antibody reactive against GalTKO pig cells (recognizing “non-Gal” pig antigens) is readily detected in most humans and baboons, presumably reflecting either prior exposure to pig antigens from dietary or other environmental sources, or incidental “heterotypic” cross-reactivity, perhaps triggered by prior infectious antigens that are similar to pig non-Gal antigens. Byrne et al have

identified about 20 pig carbohydrate and protein non-Gal antigens that are commonly precipitated by serum from naïve humans or baboons (G Byrne, personal communication, and 45). For both protein and carbohydrate antigens recognized by preformed “natural antibody, a secondary memory “recall” response is likely to be elicited against these particular GalTKO pig antigens. As these antigens are identified it will become feasible for the first time to determine how immunity to each of them is regulated, both in general and in individual recipients.

Induced T-helper dependent antibodies may instigate graft injury by multiple related mechanisms in addition to complement-dependent cytotoxicity. Complement-independent mechanisms include antibody-dependent cellular cytotoxicity (ADCC), Fc-mediated neutrophil and platelet sequestration, and NK and macrophage activation, which are not typically efficiently mediated by “natural” IgM antibody. Indeed, Robson et al found that elicited antibodies, strongly induce endothelial cell activation and tissue factor expression with far greater potency than natural antibodies, and without the requirement for complement activation [46]. In vivo, a consumptive coagulopathy occurred when induction of xenoreactive antibody was not inhibited, and was accompanied by an increase in tissue factor [38,47]. These observations strongly support the notion that T-cell dependent adaptive immune responses to pig antigens (presumably including non-Gal antigens) play a prominent role in DXR and TM.

Controlling elicited anti-pig antibody

Costimulation pathway blockade has been utilized in two recent studies that reported dramatic improvements in pig islet survival in primates. Larsen’s group found clinically important synergy using CTLA4-Ig (LEA29Y) with CD154/CD40 blockade, sufficient to achieve reproducible 4-6 month survival of fetal pig islets in rhesus monkeys [48]. Similarly, Hering’s group reported that CD154 was an important element of a similar multi-drug regimen that yielded 6 month survival of adult porcine islets in cynomolgus monkeys [49]. In addition Cooper’s team have pilot data showing that anti-CD154 and MMF alone are sufficient to protect adult islets that express high levels of hCD46, with survival beyond 1 year in occasional recipients (D.K.C. Cooper, personal communication). Anti-pig antibodies were not reported in any of these studies. In aggregate, this experience suggests that inhibition of costimulation is a very promising approach to prevent an induced antibody response to porcine antigens, at least for cellular grafts that are not primarily vascularized.

Over the past decade, we have evaluated the role of the CD40-CD154 costimulation interaction to modulate the adaptive immune response to Gal and non-Gal xenogeneic antigens. Our studies and the work of others [29,30] have confirmed our original prediction that blockade of CD154 modulates anti-pig antibody elaboration and abrogates key facets of DXR:

1. de novo antibody responses to non-Gal antigens are CD154-dependent, and lymphocyte responses to pig antigens remain weak or undetectable both in vivo and in vitro during treatment;
2. CD154-based therapy is significantly safer than conventional immunosuppression, at least in baboons.
3. when only CD154 is blocked (without MMF or ATG, eg), an induced antibody response to both Gal and non-Gal antigens is often detectable during therapy, with intact antibody class-switching, demonstrating that costimulation is incompletely blocked.
4. Adding CTLA4-Fc (blockade of CD28/B7 interactions) to anti-CD154 usually prevents induction of non-Gal antibodies during treatment.

5. ICOS, CD80, and CD86 expression are all upregulated in pig hearts undergoing DXR; CD80 (B7.1) and CD86 (B7.2) are ligands for CD28, and ICOS expression is substantially dependent on CD28 activation in several models.

Together, these observations strongly implicate the CD28/B7 costimulation pathway in the anti-xeno response during CD154 blockade, and suggest that limitations to the efficacy of anti-CD154 monotherapy could be overcome by additionally targeting the CD28 costimulation pathway [50,51].

Using anti-CD154-based immunomodulation and GalTKO pig organs, Cooper, Sachs and their colleagues successfully blocked induction of high anti-donor antibody titers in baboon pig GalTKO heart and kidney recipients [29,30]. Their work revealed that DXR of GalTKO heart xenografts typically becomes manifest at 4-26 weeks in the form of thrombotic microangiopathy (TM). Although no rise in anti-pig antibody titer was detected while the graft survived, and cellular infiltration was sparse, detection of immunoglobulin and complement in some heart and kidney grafts leaves open the possibility that TM is triggered by costimulation-driven adaptive immunity that escapes control by the regimen used.

If costimulation-dependent antibody responses prove to be *unnecessary* for induction of DXR, our leading alternative hypothesis is that TM is caused by previously characterized incompatibilities between pig and primate molecules that regulate coagulation pathway activation (thrombomodulin, tissue factor pathway inhibitor), leading to dysregulated coagulation and subacute vascular injury [38,47,52-56]. While it is conceivable that low levels of antibody contribute to its initiation, well described molecular incompatibilities between pig endothelial anticoagulant defenses and primate coagulation factors may primarily drive this pathology. In either case, preliminary data suggest that expression of human thrombomodulin, tissue factor pathway inhibitor, CD39, or CD73 (or some combination of these) by a GalTKO pig organ may be effective to prevent DXR and TM.

Managing residual preformed or elicited complement-fixing antibody

To manage complement-mediated injury triggered by complement-fixing antibodies, regardless of the target antigen, complement activation can either be aborted through complement inactivation (by administering an inhibitor, such as soluble complement receptor type 1 or C1 esterase inhibitor), complement removal (by consumption, with cobra venom factor), or through enhancement of intrinsic complement defenses in the organ itself [57-68]. Increased expression of molecules that regulate complement activation on graft PECs is particularly attractive since the effect is localized to the graft, and complement-dependent immune defenses elsewhere in the graft recipient (to infectious pathogens, for example) are not likely to be impaired. Enhance local complement control can be accomplished either by over-expression of pig complement regulators or by additional expression of human complement regulatory proteins. The latter approach has theoretic advantages in that human complement is probably more efficiently down-regulated by human complement regulators than by their pig analogues [15].

In addition to its well-established role in HAR, complement may contribute to DXR through complement-fixing antibody. To the extent that complement participates in DXR, additional expression of complement regulatory proteins by GalTKO organs may attenuate DXR. The leading alternative hypothesis is that dysregulated endothelial anticoagulant function is the primary cause of DXR. This could occur if antibody binding triggered endothelial expression of tissue factor or von Willebrand's factor, either of which readily triggers platelet adhesion and activation, as well as amplifying activation of the coagulation cascade. To test this hypothesis, enhanced endothelial expression of anticoagulant proteins, either constitutively or

conditional upon endothelial injury, is being assessed as a strategy to protect the graft from DXR.

Alternative mechanisms of antibody-mediated xenograft injury

Antibody can contribute to xenograft injury by a variety of mechanisms that do not primarily involve complement activation. We among others have shown that when complement activation is inhibited both in plasma (using complement depletion with cobra venom factor, or inhibition with either C1 esterase inhibitor or soluble complement receptor type 1) and on graft surface membranes (by expression of human membrane cofactor protein or decay accelerating factor), pig heart or lung injury still occurs [4,22]. Binding of high-affinity antibodies can cross-link surface glycoproteins, interfering with cell homeostasis via triggering internalization or otherwise disabling functions of critical surface proteins. In addition, NK cells, platelets and monocytes express Fc receptors, and may become activated consequent to antibody-mediated sequestration within the graft, contributing to graft injury, as discussed briefly above. These mechanisms may prove pivotal not only to HAR and EGF but also to DXR and TM.

Conclusion

Efficient control of antibody-mediated injury is likely to be necessary and may prove sufficient to control TM and DXR of pig organ xenografts. Determining how preformed and elicited antibodies contribute to DXR and dysregulated coagulation (TM) is an important objective of current research in the field of xenotransplantation. A costimulation-based approach to protect a xenograft from elicited antibody responses and subsequent immune injury is promising, and incompletely explored. In our estimation it is likely that effective costimulation pathway blockade can be adapted further to safely inhibit both humoral and cellular responses to GalTKO xenografts, and -- alone or with other mechanism-directed pig modifications -- enable their therapeutic application.

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Abbreviations

AbAbs

antibody absorption

ATG

anti-thymocyte globulin

C1-Inh	complement esterase type 1 inhibitor
DXR	delayed xenograft rejection
GalT KO	galactosyl transferase knock-out
HAR	hyperacute rejection
IgA	immunoglobulin type A
IgE	immunoglobulin type E
IgG	immunoglobulin type G
IgM	immunoglobulin type M
MAC	membrane attack complex
MMF	mycophenolate mofetil
PAEC	porcine aortic endothelial cells
PECs	porcine endothelial cells
sCR1	soluble complement receptor blocker type 1 (sCD35)
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TM	thrombotic microangiopathy
vWF	von Willebrand factor
XNA	xenoreactive natural antibodies