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COAGULATION DYSREGULATION AS A BARRIER TO XENOTRANSPLANTATION IN THE PRIMATE

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Abstract

Purpose of review—The ability to generate pigs expressing a human complement regulatory protein (hCRP) and/or pigs in which the $\alpha 1,3$ -galactosyltransferase gene has been knocked out (GT-KO) has largely overcome the barrier of hyperacute rejection of a pig organ transplanted into a primate. However, acute humoral xenograft rejection (AHXR), presenting as microvascular thrombosis and/or consumptive coagulopathy, remains a major hurdle to successful xenotransplantation. This review summarizes recent studies of the coagulation problems associated with xenotransplantation, and discusses potential strategies to overcome them.

Recent progress—Organ transplantation into nonhuman primates from GT-KO pigs that express a hCRP are not susceptible to hyperacute rejection. Nevertheless, most recipients of GT-KO and/or hCRP transgenic pig organs develop a consumptive coagulopathy, even when the graft remains functioning. This is associated with platelet aggregation, thrombocytopenia, anemia, and a tendency to bleed. Whilst this may reflect an ongoing immune response against the graft, (as exposure to anti-nonGal antibodies *in vitro* induces procoagulant changes in porcine ECs, even in the absence of complement), histological examination of the graft often shows only minimal features of immune injury, unlike grafts undergoing typical AHXR. Importantly, recent *in vitro* studies have indicated that the cocubation of porcine endothelial cells (ECs) with human platelets activates the platelets to express tissue factor, independent of a humoral immune response. These observations suggest that the use of organs from GT-KO pigs that express a hCRP may not be sufficient to prevent the development of a coagulation disorder following xenotransplantation, even if complete immunological tolerance can be achieved.

Summary—Both thrombotic microangiopathy and systemic consumptive coagulopathy are increasingly recognized as barriers to successful xenotransplantation. The breeding of transgenic pigs with one or more human anticoagulant genes, such as CD39 or tissue factor pathway inhibitor, is anticipated to inhibit the procoagulant changes that take place on the graft ECs, and thus may prevent or reduce platelet activation that arises as a result of immune-mediated injury. The identification of the molecular mechanisms that develop between porcine ECs and human platelets may allow pharmacological approaches to be determined that inhibit the development of thrombotic

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microangiopathy and consumptive coagulopathy. Hopefully, further genetic modification of the organ-source pigs, combined with systemic drug therapy to the recipient, will prolong graft survival further.

Keywords

Xenotransplantation; Coagulation; Platelets; Pig; Primate

1. INTRODUCTION

Xenotransplantation promises an unlimited supply of organs for clinical use. Pigs are thought to be the most suitable source of xenografts [1,2]. Although several strategies have been developed to overcome hyperacute rejection (HAR) and thus prolong graft survival [2], acute humoral xenograft rejection (AHXR) ensues with manifestations of fibrin deposition within the vasculature of grafts, platelet sequestration, and monocyte infiltration, which leads to intravascular thrombosis, which has proven very difficult to prevent or treat.

Extensive evidence has supported the view that thrombosis leads to the loss of both whole organ and islet xenografts in the pig-to-primate model. However, the exact mechanism by which coagulation disorders are induced after xenotransplantation remain uncertain. Coagulation is initiated by damage to blood vessel endothelium and the expression of tissue factor (TF). TF binds factor VII (FVII) to trigger the formation of thrombin. A trickle of thrombin, which can activate many cells, such as monocytes, platelets, neutrophils and ECs independently of thrombosis, also amplifies multiple feedback loops, propagating the generation of large amounts of thrombin which cleaves fibrinogen to form a fibrin thrombus [3].

Until now, it has been considered that thrombosis results from antibody- and complement-mediated EC activation, initiating AHXR. Exposure of porcine ECs to xenoantibodies, complement, and cells of the innate immune system results in EC activation and loss of anticoagulant regulators on their surface, with a subsequent change to a procoagulant phenotype. These same processes are also active in the humoral rejection of allogeneic organs after HLA-mismatched or ABO-incompatible transplantation. As in allogeneic organ transplantation, humoral rejection of xenografts can also cause a systemic coagulopathy associated with the consumption of coagulation proteases, platelets and fibrinogen within the graft.

Importantly, in xeno- but not allo-transplantation, there are distinct, immune-independent factors that contribute to the development of coagulation disorders, e.g., molecular incompatibilities between pigs and primates that promote, or fail to regulate, pathological clotting. For example, in the initiation stage of thrombosis, porcine von Willebrand Factor (vWF) spontaneously aggregates human platelets, in the absence of shear stress, through aberrant interaction between the O-glycosylated A1 domain and platelet glycoprotein Ib [4]. In addition, during propagation, human thrombomodulin inefficiently regulates the primate coagulation cascade [5–7]. Traditionally, porcine tissue factor pathway inhibitor (TFPI) has been thought inefficient at regulating human TF clotting initiation [8], but this view has been challenged recently [9]. We have just described a novel immune-independent mechanism, whereby human platelets are spontaneously activated to express procoagulant TF after physical interaction with porcine EC, independently of the presence of xenoreactive antibodies or complement (Lin et al. Transplantation, 2008, in press).

The precise importance of thrombin generation during the humoral rejection process has been highlighted recently by studies in small animal models. In a mouse-to-rat model, Chen et al.

reported that hearts from transgenic mice expressing human TFPI or hirudin demonstrated prolonged survival in the absence of immunosuppressive therapy, compared to wild-type mouse hearts. More importantly, hTFPI-transgenic mouse hearts survived indefinitely in immunosuppressed rats [10]. No features of AHXR were identified histologically in spite of antibody and complement deposition on the ECs. Similarly, Mendicino et al., working in the same model, reported that wild-type grafts developed typical features of AHXR within 3 days, associated with increased levels of donor fgl-2 mRNA. In contrast, grafts from fgl-2-knockout mice had remarkably reduced thrombosis and fibrin deposition, but developed a cellular response that was inhibited by treatment by immunosuppressants [11]. These observations, although differing in the emphasis they place on TF-dependent initiation of clotting, nevertheless highlight that in the absence of thrombin generation, humoral rejection mechanisms are significantly impaired, despite antibody and complement deposition on graft ECs. Recent work from our group has indicated that this may relate to the critical role of thrombin, through protease activated receptor signaling, for leukocyte recruitment and activation [12].

Several articles have recently reviewed coagulation issues in xenotransplantation [13–16]. In the present review, we shall 1) review the coagulation disorders associated with solid organ xenotransplantation, 2) describe recent progress in *in vitro* and animal studies, 3) review the potential role of recipient platelets, which may contribute to coagulation dysregulation, and 4) propose some strategies to inhibit coagulation disorder during xenotransplantation.

2. COAGULATION DISORDERS IN PIG-TO-PRIMATE SOLID ORGAN MODELS OF XENOTRANSPLANTATION

In the absence of immunosuppressive therapy, baboons rejected kidneys from triple transgenic pigs expressing human complement-regulatory proteins (hCRPs) within about 5 days, and developed a consumptive coagulopathy (CC) with the features of profound thrombocytopenia, fibrinogen depletion, and consequently increased clotting times [17,18]. Pharmacologic immunosuppression, depletion of anti-Gal antibodies, and combined kidney/thymus transplantation prolonged renal xenograft survival, but did not prevent the development of a thrombotic microangiopathy in the glomeruli [19]. The use of organs from $\alpha 1,3$ -galactosyltransferase gene knock-out (GT-KO) pigs in a pig kidney-to-primate model had little benefit in preventing coagulation when elicited antibodies to nonGal antigens developed; AHXR occurred with graft failure and associated thrombocytopenia [20]. However, vigorous control of the induced humoral response prolonged GTKO pig kidney graft survival and reduced the extent of thrombosis, although at the expense of increased infectious complications [21].

These observations suggest that in these studies the development of coagulation disorders occurred mainly as a consequence of the rejection response, probably as a result of antibody-mediated or immune cell-mediated activation of porcine ECs, with loss of anticoagulant characteristics that promoted the local graft thrombosis and systemic consumptive coagulopathy (CC) (see below).

Transplanting hearts from GT-KO pigs [22] into baboons prolonged median graft survival to 78 days, but eventually all grafts succumbed to ischemic necrosis associated with the development of TM [23,24]. The histopathology in these grafts was different from typical AHXR. There was focal immunoglobulin and complement deposition, but neither preformed antibody against nonGal epitopes nor an elicited antibody response could be detected. Additionally, the grafts revealed microvascular thrombosis in arterioles, capillaries, and venules, with only rare interstitial mononuclear cells. The mixed lymphocyte reaction remained unresponsive, suggesting that the changes seen resulted from low-grade humoral rejection.

The concept that regulatory mechanisms on ECs play a critical role in determining the sensitivity of organs to humoral rejection has been demonstrated in a mouse model of complement-dependent rejection by Shimizu et al [25], so we postulate that the same applies in this setting for coagulation regulatory mechanisms; i.e. aspects of porcine ECs that promote or fail to regulate primate coagulation are responsible for sensitizing the graft to rejection by an otherwise 'innocuous' immune response.

3. RECENT PROGRESS

3.1 GT-KO with CD46 transgenic pigs

More recently, pigs expressing a hCRP gene, e.g., CD46 or hDAF, on a GT-KO pig background have been bred. The transplantation of kidneys from these pigs into nonhuman primates has recently been reported [26]. Failure of these grafts was not associated with the classical features of AHXR, but the recipient succumbed to the development of CC, with platelet aggregation, thrombocytopenia, anemia, and a bleeding tendency, but with a functioning graft.

The results of our own group are similar; histopathologic features show only minimal IgM deposition without deposition of IgG and complement, and no infiltration of macrophages, B, or T cells. However, fibrin is indeed deposited in the grafts. This observation suggests that GT-KO pigs expressing a hCRP gene provide some protection against the humoral-mediated immune response, but do not overcome the problems of coagulation. Moreover, the development of CC does not necessarily correlate with the severity of humoral rejection.

3.2 Anti-nonGal antibodies

Since GT-KO pigs were introduced to eliminate the antibody response to Gal, interest has been directed towards anti-nonGal antibodies. A recent *in vitro* study showed that ECs from GT-KO pigs additionally transgenic for CD46 were more resistant to primate complement-dependent cytotoxicity than those from GT-KO pigs (Hara et al, Transpl Int, 2008, in press). It was therefore anticipated that GT-KO pigs expressing a hCRP protected against a lower level of cytotoxicity associated with anti-nonGal antibodies. However, when considering EC activation rather than cytotoxicity, recent evidence indicates that anti-nonGal antibodies of both human and nonhuman primates [27,28] act through complement-independent pathways. Our *in vitro* results demonstrate that porcine ECs from CD46-transgenic pigs are resistant to activation by naïve baboon serum, but not to sera from baboons sensitized to nonGal antigens or to sera from baboons or humans with high levels of anti-nonGal antibodies. This implies that hCRP transgenic pigs alone may be insufficient to completely prevent the development of procoagulant changes on porcine ECs induced by primate serum.

One solution would be to identify one or more significant nonGal antigen against which natural antibodies are present in primates. However, it is a formidable task to identify significant nonGal antigens, and proceed to genetically modify pigs by KO of the gene for the antigen. A more reasonable approach is to generate GT-KO pigs that express anti-coagulant genes to prevent the development of a procoagulant phenotype when porcine ECs are activated, regardless of the presence of nonGal antigens.

3.3 Role of platelets

The interactions between porcine ECs, platelets, and other blood cells are at the nexus of a complex network that contributes to coagulation during AHXR (Figure 1). According to the standard paradigm, the process is initiated by the immune response against the graft, such that activation of porcine ECs caused by antibodies with/without complement leads to expression of TF that triggers the coagulation cascade [29,30]. Together with loss of anticoagulant regulators, such as TFPI, thrombomodulin, or heparan sulfate, or through fgl-2 conversion

[8,31], thrombin and fibrin are deposited in the graft. This process intrinsically involves potent platelet activation. At the same time, the loss of ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5'-nucleotidases (CD73), which catalyzes the degradation of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and monophosphate (AMP) to adenosine has been documented on activated ECs [32,33]. Failure of enzymatic degradation of ADP aggravates the activation and aggregation of platelets.

Administration of CD39 substitutes inhibits platelet activation and aggregation, thereby significantly prolonging graft survival [34,35]. The clotting time of human blood when pre-incubated with islets from CD39-transgenic mice is significantly prolonged, when compared to pre-incubation with wild-type pig islets [36]. Hearts from CD39-transgenic mice are resistant to thrombosis in a mouse-to-rat xenotransplantation model [37].

However, immune-independent factors, such as molecular incompatibilities, also contribute to the activation of platelets directly. The effect of porcine vWF has been mentioned already (see above). This effect probably has *in vivo* significance, particularly after lung xenotransplantation. For instance, in an *ex vivo* pig-to-human pulmonary xenotransplantation model [38], pre-treatment of porcine lungs with desmopressin, which can reduce the content of vWF, attenuated platelet activation and systemic intravascular coagulation. Furthermore, if pulmonary intravascular macrophages were depleted in the recipients, baboons survived longer after receiving a lung transplant from a vWF-deficient pig than from a CD46-transgenic pig [39].

Our recent data indicate that, when incubated with porcine ECs, primate platelets are activated to express TF without the involvement of antibodies and complement; the molecular mechanisms remain under investigation, but it is contact-dependent, suggesting that it is distinct from the described effects of porcine vWF (Lin et al. Transplantation, 2008, in press).

Once activated, platelets contain many pro-inflammatory mediators and surface ligands that are expressed and/or released [40]. For example, activated platelets express glycoprotein IIb/IIIa (GPIIb/IIIa) that increase platelet aggregation in response to certain stimuli. The secretion of ADP from dense granules recruits other platelets to the site of injury and enhances platelet aggregation in response to agonists through the receptor P2Y₁₂ [41]. Additionally, activated platelets bind avidly to leukocytes, forming platelet-leukocyte complexes through P-selectin and its ligand on the leukocyte [42]. Reciprocally, activated platelets also exert a direct procoagulant effect on porcine ECs, thus contributing to an auto-amplified self-perpetuating vicious cycle. As well as potentiating the damage inside the graft, activated platelets are seen circulating peripherally, and these may be a significant component driving the CC.

3.4 Apoptosis

AHXR is mainly associated with ECs activation and fibrin deposition on the wall of blood vessels, rather than apoptosis in the endothelium [43]. In recent studies using kidney and heart xenografts, however, numerous TUNEL-positive ECs were detected and concomitant with the development of TM [19,44]. By detection of annexin V expression, our own *in vitro* studies demonstrated that both human platelets and monocytes can induce apoptosis of porcine ECs. The disruption of integrity of the ECs and exposure of phosphatidylserine lead to the ECs becoming prothrombotic [45]; any degree of apoptosis of luminal ECs *in vivo* might contribute to the development of thrombosis.

4. FUTURE DIRECTIONS

Based on recent progress, the resolution of coagulation dysregulation is critical to allow xenotransplantation to advance sufficiently for clinical trials. Thrombosis manifests in the

graft, but there is also a systemic CC, which may have more than one cause. We therefore propose that, in addition to genetic modification of the organ-source pig, systemic medication may be necessary (Table 1). Hopefully, this combined approach will enable the adverse effects of coagulation disorders to be overcome.

4.1 Multiple-gene transgenic pigs

Although the introduction of GT-KO pigs as a source of organs has provided a significant advance, many unresolved problems have stimulated the drive for additional genetic modifications of the organ-source pig. GT-KO pigs that express a hCRP and one or more 'anticoagulant' or 'anti-thrombotic' genes are anticipated to inhibit the generation of thrombosis and subsequent platelet activation. With new cloning techniques (F2A system), mice transgenic for CD55/hTFPI/hTM/CTLA4-Ig/hCD39 have been generated [46]. The same technology is expected to be successfully applied to generate pigs with multiple gene modifications and will enable these pigs to become available in a shorter period of time than through conventional breeding.

4.2 Systemic medication

Statins—The statins, inhibitors of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase, significantly reduce synthesis of cholesterol [47,48]. Statins have other independent effects, such as immuno-regulatory, anti-inflammatory, and anticoagulant actions [49,50]. Of relevance to xenotransplantation, atorvastatin reduces porcine EC activation induced by interferon- γ [51]. In mixed lymphocyte reaction, atorvastatin inhibits the proliferative response of primate PBMC and CD4⁺T cells when stimulated with porcine ECs (Ezzelarab et al. Transplantation, 2008, in press). By preventing TF expression and activity, statins have anticoagulant effects, an effect first observed in monocytes and macrophages [52,53]. Subsequently, simvastatin was shown to prevent the induction of TF on human aortic ECs by thrombin, at least in part through inhibition of rho-kinase-dependent Akt dephosphorylation [54].

Furthermore, lovastatin enhanced ecto-5'-nucleotidase activity and membrane expression in ECs, consequently inhibiting platelet aggregation through the action of adenosine [55]. As mentioned above, the activity of ecto-5'-nucleotidase plays an important role in platelet aggregation during AHXR. These promising *in vitro* results encourage investigation of the effects of statins in pig-to-primate transplantation models.

Anti-platelet agents—Anti-platelet agents have been extensively used clinically to prevent or reduce the development of cardiovascular diseases [56,57]. Anti-platelet agents, such as antagonists of P2Y₁₂ or GPIIb/IIIa receptors, would be expected to prevent aggregation when platelets are activated during AHXR. Anticoagulant and anti-platelet agents have been tested with the aim of attenuating coagulation disorders. In a hCD46-transgenic pig-to-baboon cardiac transplantation model, warfarin and low-molecular weight heparin had no impact on graft survival, with no reduction in fibrin deposition and platelet thrombi. The combination of aspirin and clopidogrel neither prolonged graft survival nor inhibited thrombosis in cardiac xenografts [58]. However, clopidogrel exerted some systemic inhibition of platelet aggregation. A high dosage of a GPIIb/IIIa antagonist prolonged graft survival and decreased platelet aggregations in a rodent model [59]. Eptifibatide also effectively prevented platelet aggregation in a primate study [60]. Although the impact on graft survival has hitherto been disappointing, these observations suggest that anti-platelet agents are sufficient to prevent platelet aggregation in the circulation. If thrombosis in the graft is prevented by anti-thrombotic gene modification, this systemic approach may offer survival benefits by the prevention of systemic CC.

5. CONCLUSIONS

Coagulation dysregulation has increasingly been recognized as a critical barrier to successful xenotransplantation. Current studies have revealed that coagulation dysregulation involves not only activation in the graft, which may be related to AHXR, but also systemic changes that take place in the circulation; these may be initiated by factors unrelated to the immune response such as activation of platelets by porcine endothelium. A clearer understanding of the molecular interactions between porcine EC and primate platelets may provide new targets for intervention. Ideally, genetic modification of the organ-source pig offers the optimal approach to avoid adverse systemic effects. The generation of GT-KO pigs transgenic for one or more 'anticoagulant' or 'anti-thrombotic' genes is likely to confer additional benefit. However, the documented systemic coagulation disorders suggest the necessity of an additional pharmacological approach. Multiple genetic modifications of the organ-donor pig combined with medication to inhibit systemic procoagulant changes in the primate recipient are therefore expected to be necessary to overcome this barrier.

ABBREVIATIONS

AHXR, acute humoral xenograft rejection
 CC, consumptive coagulopathy
 Ecs, endothelial cells
 fgl-2, fibrinogen-like protein 2
 Gal, Gal a 1,3 Gal
 GT-KO, α 1,3-galactosyltransferase gene knocked out
 HAR, hyperacute rejection
 hCRP, human complement regulatory protein
 TF, tissue factor 2
 TFPI, tissue factor pathway inhibitor
 vWF, von Willebrand Factor

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Figure 1. Diagrammatic representation of procoagulant change of porcine endothelial cells and activation of platelets in pig-to-primate organ xenotransplantation

(1) Porcine ECs are activated by xenoreactive antibodies with/without the involvement of complement to express TF and trigger the formation of thrombin, which is a potent activator of platelets. Fgl-2 cleaves prothrombin to generate the formation of thrombin. (2) The ligands (vWF or others, designated by ?) on the porcine EC activate platelet receptors probably independent of the humoral immune response. (3) Activated ECs lose expression of CD39 and CD73 and are unable to adequately catalyse ADP to adenosine, and therefore contribute to the activation of platelets. Ag: antigen, Ab: antibody.

TABLE 1

Potential strategies to inhibit the coagulation dysregulation that occurs following pig-to-primate organ xenotransplantation

STRATEGY	MECHANISM	REFERENCES
Genetic modification of the organ-source pig		
GT-KO	Depletion of Gal antigen	22–24
CD46 (+/+), hDAF (+/+)	Complement regulation	17–19
vWF (–/–)	Inhibit platelet activation	38–39
Fgl-2 (–/–)	Inhibit thrombin generation	11
TFPI (+/+)	Antagonize the function of TF	10
CD39 (+/+)	Conversion of ADP to AMP	32–35
Systemic medication of the primate recipient		
Statins	Inhibit TF expression on EC Inhibit platelets aggregation	52–56
Anti-platelet agents		
ADP receptor inhibitors	Inhibit platelet activation	59
GPIIb/IIIa inhibitors	Inhibit platelet activation	60–61