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CONTROLLING COAGULATION DYSREGULATION IN XENOTRANSPLANTATION

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Abstract

Purpose of review—Deletion of the $\alpha 1,3$ -galactosyltransferase (GalT) gene in pigs has removed a major xenoantigen but has not eliminated the problem of dysregulated coagulation and vascular injury. Rejecting GalT KO organ xenografts almost invariably show evidence of thrombosis and platelet sequestration, and primate recipients frequently develop consumptive coagulopathy (CC). This review examines recent findings that illuminate potential mechanisms of this current barrier to successful xenotransplantation.

Recent findings—The coagulation response to xenotransplantation differs depending on the type of organ and quite likely the distinct vasculatures. Renal xenografts appear more likely to initiate CC than cardiac xenografts, possibly reflecting differential transcriptional responses. Liver xenografts induce rapid and profound thrombocytopenia resulting in recipient death within days due to bleeding; ex vivo data suggest that liver endothelial cells and hepatocytes are responsible for platelet consumption by a coagulation-independent process.

It has been proposed that expression of recipient tissue factor on platelets and monocytes is an important trigger of CC. Finally, pigs transgenic for human anticoagulants and antithrombotics are slowly but surely coming on line, but have not yet been rigorously tested to date.

Summary—Successful control of coagulation dysregulation in xenotransplantation may require different combinatorial pharmacological and genetic strategies for different organs.

Keywords

Coagulation; inflammation; thrombosis; xenograft

Introduction

While deletion of the GalT gene represented a significant advance in the field of xenotransplantation, it did not provide a complete solution to the problem of thrombotic vascular injury to xenografts and the development of coagulopathy in recipients. In this review, we will present an overview of coagulation and particularly its relationship to inflammation and its role in transplant rejection; outline several factors that may predispose xenografts to thrombosis; describe recent data from the pig-to-primate preclinical model that

provide insights into the mechanisms of coagulation dysregulation and discuss progress on potential pharmacological and genetic strategies.

Coagulation: activation, regulation, and links to innate immunity and inflammation

Coagulation is integral to the normal hemostatic response to vascular injury (1, 2). Exposure of tissue factor (TF) to the circulation triggers initiation and propagation of highly coordinated processes that seal the endothelial breach with a fibrin-enmeshed platelet plug (Fig. 1). Three major anticoagulant mechanisms cooperate to tightly regulate and localize clot formation. *Tissue factor pathway inhibitor-1* is the primary regulator of the initiation phase (3). Membrane-bound (TFPI β) and circulating (TFPI α) isoforms contain two Kunitz-type domains that bind and neutralize factor Xa (FXa) and TF/FVIIa. TFPI α has a third Kunitz domain that interacts with protein S to enhance the inactivation of FXa (4). *Antithrombin* inhibits coagulation at multiple levels by targeting several serine proteases including FXa and thrombin; it is present in the circulation but is far more active when associated with the endothelial cell glycocalyx (5). The *protein C pathway* comprises several membrane-bound and circulating proteins that inhibit the propagation phase (6). The endothelial protein thrombomodulin (TBM) binds thrombin and alters its substrate specificity, blocking its procoagulant activities and acting as a cofactor for activation of protein C by thrombin. Activated protein C (APC), with its cofactor protein S, inactivates FVa and FVIIIa to arrest further production of thrombin. Endothelial protein C receptor (EPCR) enhances APC generation by the thrombin/TBM complex (7).

Coagulation is intricately connected to innate immunity and inflammation (8–10)•. Thrombin not only activates PARs to cause wide-ranging procoagulant and proinflammatory effects, but also promotes further complement activation by cleaving C3 (11) and C5 (12). Reciprocally, complement activation amplifies coagulation by several mechanisms including C5a-mediated induction of TF on neutrophils (13). While TF is recognized as a key player in inflammation-induced coagulation, recent evidence suggests that TF-independent mechanisms also play a role. Inorganic polyphosphate (polyP) released by activated platelets directly activates the intrinsic coagulation pathway by cleaving FXII (Fig. 1), resulting in thrombin generation and fibrin formation (14)••.

Coagulation is exacerbated during inflammation by the down regulation and degradation of critical endothelial anticoagulant and anti-platelet systems. This is best illustrated by the impact of inflammatory mediators on components of the protein C pathway, with reduced TBM gene expression and mRNA stability, shedding of TBM and EPCR from the endothelial surface, and proteolytic inactivation of EPCR (15–19). Circulating anticoagulants are also affected by inflammation. Activated neutrophils release nucleosomes (DNA/histone complexes), which act as scaffolds for the degradation of TFPI α by neutrophil elastase (20)•. In acute inflammatory states such as sepsis or with liver failure, widespread expression of proinflammatory cytokines and TF can overwhelm regulatory mechanisms, resulting in disseminated intravascular coagulation (DIC). Paradoxically, the systemic consumption of clotting factors and platelets in DIC leaves patients susceptible to life-threatening bleeding.

Coagulation in transplantation

Transplantation of vascularized organs is by its very nature a procedure that generates ischemia, varying degrees of inflammation and, consequently, thrombosis. Graft endothelial cells can be activated by numerous interconnected immune and non-immune factors including ischemia-reperfusion injury (IRI), antibody binding, and complement activation

(21). The procoagulant phenotype adopted by activated endothelial cells can in some circumstances precipitate graft loss. Thrombotic microangiopathy (TMA) is a complication of renal allotransplantation that can be triggered by IRI, immunosuppressive drugs, viral infections, or acute antibody-mediated rejection (22). Recipients with mutations affecting particular complement regulatory proteins are at greater risk of TMA (22), reflecting the interplay between the coagulation and complement systems.

Coagulation is a major problem in intraportal islet allo (and xeno) transplantation. Isolated islets contain TF and collagen, and their infusion into the portal vein triggers a powerful innate response known as the instant blood-mediated inflammatory reaction (IBMIR) (23, 24). The features of IBMIR include platelet binding, complement activation, thrombosis and infiltration by neutrophils.

Special coagulation problems presented by xenotransplantation

The tendency for graft intravascular coagulation in xenotransplantation is exacerbated by pre-existing antibodies and by a number of cross-species differences, as discussed below.

Pre-existing antibodies

Pre-existing human anti-pig antibodies pose a significant challenge to solid organ xenotransplantation. Although anti-Gal antibodies predominate and are the primary drivers of hyperacute rejection, they are no longer considered a problem because their target has been eliminated by deletion of the GalT gene. The remaining antibodies are collectively referred to as anti-non-Gal, but their specificities are still mostly unknown and their titers (both IgM and IgG) show considerable variability between individuals (25). A recent study found that some human sera samples had high levels of pre-existing anti-non-Gal IgG antibodies that activated porcine aortic endothelial cells (PAEC) to express TF in a complement-independent manner (26)••. This finding emphasizes that careful screening of recipients will be important in xenotransplantation, just as it is in allotransplantation.

Molecular incompatibilities affecting the control of coagulation and inflammation

As described at the start of this review, coagulation is a rapid and powerful process that requires multiple levels of regulation. Defects in anticoagulant mechanisms can disrupt this fine balance and result in an increased risk of thrombosis (viz. thrombophilia). Most mutations associated with thrombophilia in humans have been found to affect the protein C system (27). The importance of APC in controlling coagulation is demonstrated by the Factor V Leiden mutation, which encodes an APC-resistant FV and predisposes to venous thrombosis. Recent findings have also highlighted APC's expanding anti-inflammatory role; for example, extracellular histones released by activated macrophages are toxic to endothelial cells, but can be proteolytically inactivated by endogenously generated APC (28)•.

In vitro and ex vivo studies in the 1990s suggested that APC generation in porcine solid organ xenografts may be significantly compromised by cross-species incompatibility in the protein C pathway (29, 30). This was confirmed by a molecular analysis showing that pig TBM binds human thrombin but is a poor cofactor for activation of human protein C (31). The APC-generating capacity of pig TBM in the xenogeneic setting has been estimated at only 1–10% that of human TBM (29–32)•. This defect makes it conceivable that even a relatively small stimulus might give rise to a self-perpetuating cycle of inflammation and coagulation in xenografts.

A second pig-human molecular incompatibility, between von Willebrand Factor (vWF) and platelet glycoprotein Ib (GPIb), has also been documented. Contact with pig vWF

spontaneously aggregates human platelets, in the absence of shear stress, due to an aberrant interaction between the *O*-glycosylated A1 domain and human GPIb (33).

A third potential element relates to porcine endothelial expression of tissue factor pathway inhibitor which does not effectively neutralize human FXa (34, 35), albeit the recombinant forms expressed in primate cells have full anticoagulant activity (36). Several other factors may account for the thromboregulatory failure of pig endothelium and aberrant tissue factor activity in xenograft rejection.

Expression of recipient tissue factor (TF)

Monocyte-associated TF may be induced by both allo- or xenogeneic interactions with endothelium, and is in turn regulated by cell-associated TFPI. Kopp and Robson et al proposed that monocytes may modulate the thrombotic process at sites of vascular injury in association with both allo- and xenograft rejection (35). Dorling and colleagues reported that human platelets and monocytes are likewise activated to express TF by incubation with resting wild-type PAEC, even in the absence of human plasma (37). They proposed this as a procoagulant mechanism generating blood-borne TF that may contribute to the systemic coagulopathy frequently observed in solid organ xenotransplantation.

Direct prothrombinase activity

Pig endothelial cells inducibly express an enzyme that directly activates human prothrombin to thrombin in the absence of other coagulation factors (38). Immunohistochemical analysis of rejected pig-to-baboon renal xenografts revealed upregulation of this enzyme, fgl2, in small vessels and glomerular capillaries, and the intensity of fgl2 staining corresponded to the degree of vascular injury (38). Treatment of PAEC with 20 ng/ml human TNF α has been shown to upregulate fgl2 at the mRNA and protein level (38) and to increase direct prothrombinase activity more than 20-fold (32). Together these data point to an inflammation-mediated mechanism for TF-independent clotting in xenografts.

Coagulation dysregulation in the pig-to-nonhuman primate (NHP) model

The pig-to-NHP model is recognized as the gold standard for preclinical xenotransplantation, but like all models it has limitations, one of which may be exaggeration of the tendency for coagulation specifically in the baboon. A comparison of primate and human whole blood by thromboelastometry, which accurately measures the dynamic process of clot formation and lysis, indicated that primates have a more hypercoagulable profile than humans; in particular, clotting times were shorter, and clot firmness was higher despite lower fibrinogen levels (39). However, this species difference at least gives confidence that any solution to coagulation dysregulation identified in the NHP model is likely to be effective in the clinic.

Coagulation dysregulation in GalT KO pig-to-NHP xenotransplantation has been addressed in a number of recent reviews (40–44). What is generally agreed is that failure to completely control the anti-non-Gal response is associated with unregulated coagulation, manifested by TMA within the graft and (frequently) the development of consumptive coagulopathy (CC) in recipients.

It seems likely that the molecular incompatibilities described earlier contribute to this disproportionate reaction, although this has not yet been formally proven given the multiple other pathways that might activate coagulation within the xenograft e.g. humoral immunity. What is less clear is whether refinements in immunosuppression or tolerance induction will provide a solution to the problem. In this section, we will review recent data from pig-to-NHP solid organ xenotransplantation using GalT KO donors.

Kidney

The Pittsburgh group reported that GalT KO pig kidneys transplanted into baboons under suboptimal immunosuppression were rejected at days 2 and 5, with TMA and infiltration by innate immune cells (45). CC was diagnosed in both recipients on the basis of laboratory tests and/or clinical bleeding. The same group subsequently transplanted Gal KO/CD46 pig kidneys into baboons treated with a more effective immunosuppressive regimen and anticoagulation with heparin (46)••. Four recipients died or were euthanased between days 9 and 16 because of the development of CC. The creatinine level was relatively stable in two animals at the time of death; most of the grafts showed minimal evidence of an immune response, and there was no increase in serum anti-pig antibodies, at least by day 10. Interestingly, however, TF expression was detected on platelets within 2 days, on peripheral blood mononuclear cells (PBMCs) from day 4–6 onwards, and in recipient heart and liver at euthanasia. The authors hypothesized that platelets are activated to express TF early after transplantation either in response to inflammatory mediators or simply by contact with pig endothelial cells (37). PBMCs are subsequently activated by thrombin or by adhesion to activated platelets, and the resulting systemic elaboration of TF causes CC. If substantiated, this implies that recipient-derived TF may be a major significant contributor to CC.

The Boston group reported a substantially different outcome following transplantation of seven composite thymus-kidney (thymokidney) grafts from GalT KO miniature swine into immunosuppressed baboons (47)••. The tolerance-directed protocol appeared to block the T cell-mediated xenogeneic response, and graft function remained relatively stable until the death of recipients at 18 to 83 days from causes unrelated to rejection. Although glomerulopathy and TMA was observed in the grafts, none of the recipients developed frank CC. It is difficult to explain why CC was so pronounced in one study (46)•• but not in the other (47)••; potential contributing factors include differences in donor characteristics (domestic outbred GalT KO/CD46 versus miniature inbred GalT KO), treatment protocols inclusive of approaches to achieve tolerance, and surgical techniques.

Heart

Coagulation disturbances have been reported as a feature of cardiac xenotransplantation, although again CC is not always reported. GalT KO miniature swine hearts transplanted into immunosuppressed baboons were rejected after 59 to 179 days by a slowly progressing humoral process involving the development of TMA, but none of the recipients developed CC (48). In a more recent study, hearts from GalT KO outbred pigs were rejected at 5 and 8 weeks, with evidence of TMA and innate immune cell infiltration, and both recipients developed CC (45). However, to further complicate matters, CC was not exhibited in an immunosuppressed baboon that maintained a cardiac xenograft from an outbred GalT KO/CD46 pig for 7 weeks (49), although coagulation parameters were not reported.

Transcriptional analysis has suggested that recipients of cardiac xenografts may be less prone to developing CC and platelet sequestration than renal xenograft recipients due to differential gene responses in the vasculature of the two organs (50)•. The solution to the problem of coagulation dysregulation may therefore need to account for organ-specific differences.

Liver

Coagulopathy and thrombocytopenia are particularly severe problems in liver xenotransplantation. Four immunosuppressed baboons receiving GalT KO/CD46 pig livers developed profound thrombocytopenia within 5 hrs and were euthanased or died at day 5–7 due to CC, with “functional” grafts (51)••4. A study in which wild-type pig livers were perfused ex vivo with purified human platelets has provided insights into the mechanism of

thrombocytopenia (52)••. The authors reported that 93% of the platelets disappeared from the perfusate within 15 min, and early biopsies showed phagocytosis of intact platelets by liver sinusoidal endothelial cells (LSECs), a process confirmed in vitro. Later biopsies showed degraded platelets within hepatocytes, suggesting that platelets were transferred from LSECs to hepatocytes via transcytosis. This process appears unrelated to antibody binding and/or complement activation since neither was present in the system. While the exact mechanism is unclear, it was suggested to involve an aberrant interaction between a porcine lectin receptor and a human platelet glycoprotein (52)••.

Treatment options to control coagulation dysregulation

Systemic therapy with traditional anticoagulant and anti-platelet agents including heparin and aspirin appears to be largely ineffective against the development of graft TMA and CC in recipients of GalT KO solid organ xenografts (45, 46, 48, 51)••.

Studies on sepsis-associated DIC, which shares some features with xenotransplantation-associated CC, suggest that manipulation of the protein C pathway may be a more fruitful approach. Indeed, APC infusion reduced mortality in severely septic patients (53) and is now the recommended treatment for this condition (54); benefits of TFPI have been less pronounced (55).

APC produced promising results in an ex vivo perfusion model of renal xenotransplantation, blocking the activation of coagulation in wild type kidneys (56). However, the appeal of APC treatment is reduced by links to increased bleeding risk (53), the likely need for continuous administration, and the fact that it has more potent protective effects when generated endogenously on endothelial cells than when provided exogenously (57). A better alternative may be soluble TBM, which acts 'on-demand' as a facilitator of APC production. In rodent models, treatment with recombinant human TBM has been shown to protect against sepsis (58, 59) and ischemia-induced renal dysfunction (60), and liposomal rabbit TBM protected islet grafts from IBMIR (61)•. In the long run, though, genetic overexpression of human TBM on the xenograft seems preferable (see following section).

Statins are a relatively safe class of cholesterol-lowering drugs that have shown promise in small animal and in vitro models of thrombosis, due to their effects on TF expression. Pravastatin was shown to downregulate glomerular TF expression and stabilize renal function in a mouse model of antiphospholipid antibody-mediated TMA (62). Atorvastatin at high concentrations almost completely blocked upregulation of TF transcription in PAEC induced by incubation with sensitized baboon serum, although the corresponding increase in TF activity was only partly prevented (26)••.

For islets, several approaches hold promise. Low molecular weight dextran sulphate protected pig islets from IBMIR in vitro (63) and has been used as an anticoagulant in pig-to-monkey islet xenotransplantation (64). Surface heparinization protected islets from IBMIR in an allogeneic setting (65). Encapsulation offers a means of protecting the xenograft from direct exposure to recipient blood and thus avoiding IBMIR altogether (66, 67), as does the use of alternative transplant sites (68–70).

Further genetic modification of the donor to control coagulation dysregulation

The GalT KO background, preferably bolstered by transgenic expression of one or more complement regulatory proteins, has become the platform for additional genetic

modifications to inhibit coagulation and inflammation, several of which are discussed below.

TBM

Overexpression of human TBM in xenografts has obvious appeal: to compensate for the molecular incompatibility and inflammation-mediated downregulation of pig TBM, to neutralize thrombin generated by TF-dependent and -independent mechanisms, and because TBM has potent anti-inflammatory effects distinct from its capacity to promote generation of APC (71, 72). Wild type PAEC transfected to express human TBM significantly suppressed classical and direct prothrombinase activity and delayed clotting of human plasma compared to vector-transfected cells (32), consistent with earlier transduction studies (73).

Expression of human TBM from the mouse H-2K^b promoter in transgenic mice resulted in an anticoagulant phenotype but importantly without any overt bleeding tendency, and modestly prolonged cardiac xenograft survival in a mouse-to-rat model (74)••. Human TBM has also been expressed from the pig TBM promoter in transgenic pigs, resulting in strong endothelial-specific expression in various organs including heart and kidney (75), but functional consequences have not yet been reported.

CD39

The ectonucleotidase CD39 has anti-platelet activity by degrading the platelet agonist ADP (76). Transgenic mice overexpressing human CD39 are healthy and possess an anticoagulant phenotype similar to that of human TBM transgenic mice (77). CD39 overexpression was shown to protect transgenic mouse islets in an ex vivo model of IBMIR (78), hearts in a model of antibody-mediated rejection (77), and kidneys from IRI and transplant-related vascular injury (79)••.

TFPI

Overexpression of TFPI has great potential to control TF-dependent initiation of coagulation in xenotransplantation. Cardiac xenografts from transgenic mice expressing a membrane-anchored form of human TFPI α survived long-term in immunosuppressed rats (80). Transgenic pigs generated using the same construct showed more modest expression of TFPI, but this was sufficient to partially block anti-non-Gal antibody-mediated upregulation of TF transcription and activity in PAEC (26)••. Another group has recently reported the generation of transgenic pigs expressing a modified human TFPI β attached to the cell surface with a transmembrane linkage (81).

Conclusions

Solving the problem of coagulation dysregulation in xenotransplantation requires careful consideration of the tightly integrated and regulated processes of blood clotting and inflammation. Many natural anticoagulants are also anti-inflammatory, so deficiencies in anticoagulant function resulting from immune activation and/or molecular incompatibility may provoke an excessive self-perpetuating response to even modest proinflammatory signals. Controlling these signals by more effective immunosuppression and/or tolerance induction, and strengthening the xenograft anticoagulant protective defenses, would seem to be the logical and desired approach.

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Interaction of tissue factor (TF) with traces of circulating FVIIa triggers the initiation phase (top), resulting in the generation of a ‘trickle’ of thrombin. Thrombin activates components of the intrinsic pathway and signals via protease-activated receptors (PARs) on platelets, leukocytes and endothelial cells, resulting in propagation of coagulation and formation of a stable fibrin clot (bottom). Dashed arrows signify thrombin-mediated reactions. PolyP; inorganic polyphosphate released by activated platelets.