Genetically Engineering a Pig that Minimizes Coagulation Incompatibilities

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Little progress has been made in genetically engineering a pig that minimizes coagulation incompatibilities, primarily because attention has been focused thus far on dealing with the factors that initiate discordant xenograft rejection, i.e., xenoreactive natural antibodies (XNA) and complement. As a consequence, much has been learned about the mechanisms of xenograft rejection. Simultaneously, hyperacute rejection (HAR) has been overcome. However, the medium- and long-term survival of xenogeneic organs remains an elusive goal and overcoming delayed xenograft rejection (DXR) is now the primary aim of current research.

The factors initiating and mediating this form of late vascular rejection have only just become clear. Extensive intravascular fibrin deposition has been described in all the major models of DXR and is widely assumed to have pathological significance. More recently, disseminated intravascular coagulation (DIC) has also been described in primate recipients of porcine organs. This brief article will review the evidence for the pathogenic role of coagulopathy in DXR and illustrate our view that inhibiting thrombosis may be a necessary adjunct to targeting XNA and complement in order to achieve long-term xenograft survival.

Graft Thrombosis is Associated with Late Xenograft Rejection

Surprisingly, the pathogenic role of thrombosis in DXR has been relatively under-investigated. One recent study in the cobra venom factor (CVF)-treated guinea pig heart-to-rat model of DXR demonstrated that endothelial cell (EC) expression of tissue factor (TF) appears to closely mirror the development of intravascular thrombosis and rejection, implying a pathogenic role for EC-expressed TF in the rejection process.[1] In the same model, apyrase also prolonged xenograft survival,[2] implying a role for platelets in the pathogenesis of DXR.

In primate models, thrombosis is clearly associated with the development of DXR. An early study by Leventhal remains relevant;[3] despite plasma exchange, administration of CVF and immunosuppression with steroids, cyclosporin, deoxyspergualin, cyclophosphamide and antilymphocyte globulin, one of the baboons transplanted with a pig heart nevertheless rejected the organ by a process dominated by widespread intravascular fibrin plugging on histological examination, apparently caused by an increase in anti-graft antibody titers immediately prior to rejection. Histological evidence from recent studies using organs from CD55 and CD59 transgenic pigs also support a role for widespread intravascular thrombosis in the rejection process.[4-6]

Inhibition of DXR by Manipulating XNA and Complement May be Unacceptably Toxic

There is now strong evidence that DXR is initiated by circulating XNA.[6] One implication of this is that strategies to eliminate XNA completely, alongside those to fully inhibit complement activation, should efficiently suppress DXR thereby preventing the thrombosis that normally results. However, in practice this approach seems excessively toxic. Leventhal et al,[3] using the intensive immunosuppressive regimen described above, managed to prevent DXR in the majority of animals, but most died due to technical problems associated with the induction regimen. More recently, Lin et al reported studies in baboon recipients of CD55/CD59 double

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transgenic porcine heart grafts.[6] Their animals were depleted of XNA by immunoadsorption, and received steroids, cyclosporine and cyclophosphamide. DXR was inhibited, but two thirds of the animals died due to complications of treatment. This experience mirrors that gained from the early studies using CD55 transgenic pig organs.

It appears, therefore, that, using present techniques, the ability to suppress DXR is associated with unacceptable toxicity. There are several potential ways to overcome this problem. One is to induce tolerance in the B cell compartment, following the example of Lin et al.[7] Another would be to target, alongside XNA and complement, the effector mechanisms of DXR, including thrombosis.

Inhibiting Thrombosis and Platelet Activation Prevents Xenograft Rejection

Inhibiting thrombosis can prevent HAR. In an ex vivo perfusion study with porcine hearts and human blood, Robson et al demonstrated that a thrombin inhibitor significantly prolonged cardiac work, cardiac blood flow, prevented fibrin deposition, and prolonged the life of the graft to that seen in alloperfused control hearts.[8] Similarly, using porcine kidneys perfused ex vivo with human blood, Cruzado et al showed that a platelet activating factor receptor antagonist improved renal blood flow, allowed the production of urine (whereas control kidneys produced no urine), and prevented fibrin deposition.[9] Both these studies, limited as they are by the constraints of all ex vivo studies, are important because they were performed without manipulation of either complement or XNA levels. They support the conclusions of other studies in small animal models showing that inhibition of thrombosis or platelet activation can prevent HAR and prolong graft survival.

There are no published trials of anticoagulant or antiplatelet therapy as a treatment for DXR in clinically-relevant in vivo transplantation models. However, in the CVF-treated guinea pig heart-to-rat model, administration of apyrase, to inhibit platelet activation, has been shown to prevent platelet thrombus formation and prolong xenograft survival.[2]

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Porcine Xenografts May Have Intrinsic Pro-Thrombotic Activity

The factors promoting irreversible clotting after xenotransplantation are listed in Table 1. The consequences of activating clotting mechanisms are to amplify the inflammatory response (through the actions of activated platelets and several clotting factors, including factor Xa and thrombin) and to promote widespread intravascular fibrin deposition. These occur in part because of the directly prothrombotic stimuli listed in Table 1 and in part because of the failure of normal regulatory processes in the xenograft to limit intravascular thrombosis. This breakdown in regulation has two elements. First, several molecules with anticoagulant or anti-platelet activity are lost from the surface of EC upon activation and, second, several porcine regulatory molecules interact inefficiently with human clotting factors. These include porcine tissue factor pathway inhibitor (TFPI) and porcine thrombomodulin.

Even assuming that XNA binding, complement activation, and EC activation could all be successfully prevented after xenotransplantation, several of the factors listed in Table 1 would still promote a prothrombotic environment within the porcine xenograft. For instance, porcine von Willebrand factor (vWF) spontaneously aggregates and activates human platelets through GP1b.[10] Porcine EC can also spontaneously convert human prothrombin to thrombin,[11] partly because of the failure of porcine thrombomodulin to interact with human thrombin and protein C.[12] Similarly, porcine TFPI fails to inhibit human Xa activity.[13]

Recently, several groups have reported DIC after transplantation of porcine organs into baboons.[14, 15] This appears to be driven by activation of clotting factors and consumption of platelets within the graft[14] and although not completely understood, there is evidence to suggest that this form of co-agulopathy may arise independently of XNA and complement.[14]

Potential Strategies for Graft-Specific Inhibition of Thrombosis.

We and others have previously made a case for graft-specific strategies to prevent xenograft rejection,[16] manipulation of donor tissue has many potential advantages over systemic administration of therapeutic drugs. In vitro studies have established the feasibility of several potential approaches to inhibiting

Table 1 | **Factors Initiating Clotting after Xenotransplantation of Vascularized Organs**

Independent of EC activation
Activation of platelets by binding to C1q-XNA on porcine EC
Molecular incompatibilities between porcine regulators of coagulation and recipient clotting factors
p.TFPI
p.thrombomodulin
p.vWF
Intrinsic ability of porcine EC membrane to intiate clotting of human plasma
Dependent on EC activation
Exposure of tissue factor on subendothelial tissues
Procoagulant vesiculation of EC membrane
EC expression of platelet activating factor, vWF and tissue factor
Loss of regulators of coagulation/platelet activation
Anti-thrombin III
Tissue factor pathway inhibitor
Thrombomodulin
ATPD'ase *

*Loss dependent on oxidative damage rather than EC activation.

The consequences of activating clotting mechanisms are to amplify the inflammatory response . . . and to promote widespread intravascular fibrin deposition.

thrombosis by manipulation of porcine EC. These include the expression of human thrombomodulin[17] and apyrase.[18] Our own work has focused on using membrane-tethered human TFPI and hirudin to inhibit the prothrombotic phenotype of resting and activated porcine EC, as shown in Figure 1.[19, 20] More recently, we have shown that expression of these anticoagulant constructs can inhibit the EC activation normally caused by Xa and thrombin.[21] We are currently developing models using transgenic mice expressing hTFPI-CD4 and hirudin-CD4 to determine whether organs from such mice are resistant to HAR and DXR.

Comment

There are four reasons for trying to prevent localized intra-graft thrombosis after xenotransplantation. First, intravascular thrombosis damages the graft. Second, although effective, current methods to inhibit completely the effects of XNA and complement appear unacceptably toxic. Third, inhibiting thrombosis and platelet activation is effective at preventing xenograft rejection. Fourth, porcine grafts are intrinsically pro-thrombotic and promote DIC. The implication is that targeting coagulation mechanisms may be crucial for long-term xenograft survival.

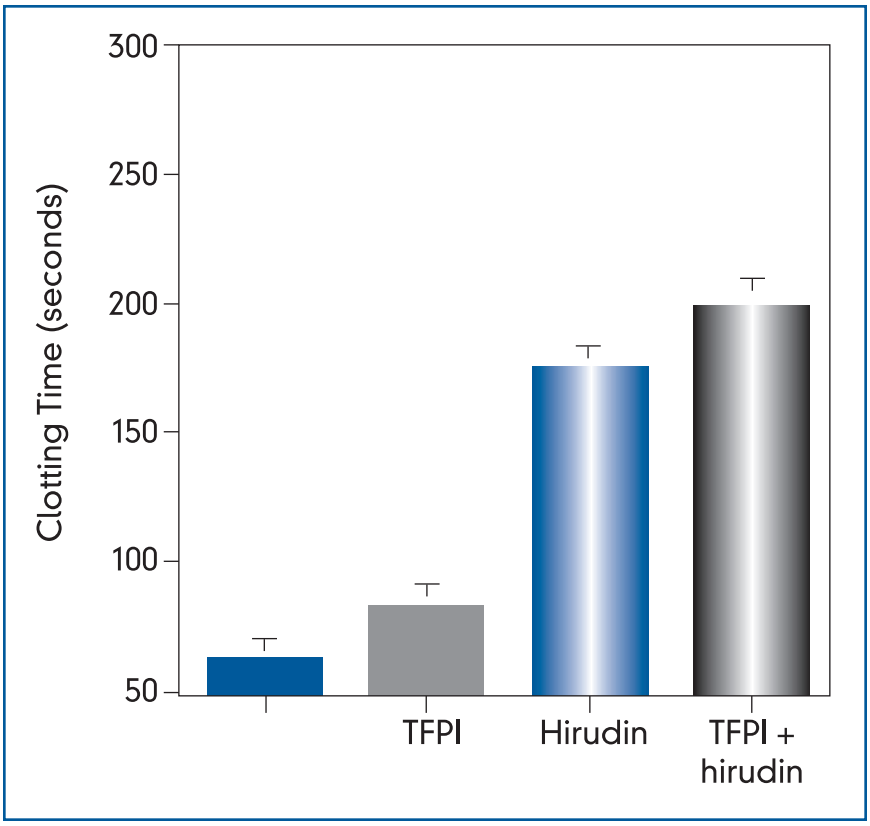

Figure 1. Clotting time of recalcified human plasma in the presence of activated control porcine EC (blue bar) or EC transiently transfected with the CD4-anchored fusion protein constructs as indicated. Clotting time in the absence of any cells was 370 seconds, and in the presence of resting, unactivated cells was 160 seconds (data not shown). For full details refer to refs. 19 and 20.

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